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SURVIVAL OF SPOILAGE AND PATHOGENIC MICROORGANISMS ASSOCIATED
WITH THE PRODUCTION OF PICKLED SAUSAGE USING A COLD FILL PROCESS

A Thesis in

Animal Science

by

Nelson J. Gaydos

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The thesis of Nelson J. Gaydos was reviewed and approved* by the following:

Jonathan A. Campbell
Assistant Professor of Animal Science
Extension Meat Specialist
Thesis Advisor

Catherine N. Cutter
Professor of Food Science
Food Safety Extension Specialist – Muscle Foods

Hassan Gourama
Associate Professor of Food Science

Terry D. Etherton
Distinguished Professor of Animal Nutrition
Head of the Department of Animal Science

*Signatures are on file in the Graduate School

ABSTRACT

Pickling has been used for centuries to preserve and extend the shelf life of different foods, ranging from fruits and vegetables, to eggs and meat. The pickling process inhibits microorganism growth by reducing product pH with organic acids, as well as the addition of salt to the product being pickled. Food items are suspended in a brine solution containing an organic acid, such as citric, lactic or acetic (vinegar) acid and combined with salt, spices and other preservatives, such as potassium sorbate. The majority of pickled foods undergo what is known as a hot fill process, where the brine solution is heated before being introduced to the food item. When the hot brine is applied to pre-cooked, ready-to-eat (RTE) meat sausages, the brine solution can change from clear to cloudy, becoming turbid and unappealing to consumers. To avoid these quality defects, pickled sausage manufacturers may use a cold fill pickling process. Cold fill pickling utilizes a room-temperature brine instead of a heated pickling solution and does not cause the aforementioned quality defects.

Since very little is known about the safety of the cold fill pickling processes, a series of separate experiments was performed using a brine solution (5% acetic acid and 5% salt at ~22-23°C) to pickle pre-cooked, RTE beef and pork smoked sausages inoculated with various spoilage and pathogenic microorganisms. In two separate experiments, spoilage microorganisms consisting of lactic acid bacteria (LAB) (*Lactobacillus curvatus* and *L. sakei*); yeasts (*Debaryomyces hansenii* and *Candida zeylanoides*); molds (*Penicillium nalgiovense* and *Cladosporium cladosporioides*) and pathogenic bacteria (*Salmonella* Typhimurium, *S. Senftenberg*, *S. Montevideo*, *Listeria monocytogenes*, and *Staphylococcus aureus*) were inoculated onto RTE sausages prior to pickling. Microbial populations were evaluated initially (day 0) and up to 28 days after pickling and storage at room temperature. Other parameters, such as pH of the sausage, pH of the brine, water activity of the sausages, and percent salt of both the sausage and the brine, were measured over time.

Results demonstrated that all microorganisms experienced a significant ($p \leq 0.05$) reduction within the first 24 hours of pickling. LAB populations were reduced ca. 6.58 log₁₀ CFU/g in 7 days, while yeasts were reduced ca. 3.89 log₁₀ CFU/g in 48 hours and molds were reduced ca. 4.09 log₁₀ CFU/g in 24 hours. Pathogenic bacteria were reduced ca. 6.80 log₁₀ CFU/g in 72 hours when plated on non-selective media and >6.33 log₁₀ CFU/g when plated on selective media. Likewise, pH of sausages decreased significantly ($p \leq 0.05$) throughout the length of the

study. No significant differences were observed for water activity ($p=0.2062$; $p=0.1291$), or % salt of the sausages ($p=0.0640$; $p=0.1445$) or brine ($p=0.1046$; $p=0.3180$) for all experiments.

It was concluded from the observed data that the cold fill pickling process is capable of inhibiting and destroying contamination from both spoilage and pathogenic microorganisms within 24 hours after initial pickling. Parameters, such as the diameter of the sausages, casing type, and brine concentration were chosen in order to implement a worst case scenario. These results suggest that processors may experience an even more effective inhibition of spoilage and pathogenic microorganisms by using a higher concentrations of salt or acetic acid, pickle smaller diameter sausages or use natural or skinless casings, which will result in a faster penetration of the brine into the sausages and decrease in product pH. Results of these studies may provide scientific validation to manufacturers that cold fill pickled sausages in order to meet requirements for their HACCP systems and for regulatory compliance with the USDA-FSIS.

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Chapter 1

Literature Review

Introduction

For centuries, mankind has used herbs, spices, minerals, and acids as natural preservation methods to protect against food spoilage without knowing the exact mechanism of how the food additives work. These additives and preservation techniques make the food environment less ideal for microorganism growth (decreased pH, decreased water activity [a_w], absence of oxygen, addition of salt, alcohol, carbon dioxide, etc.), and in doing so, contribute to the texture, smell, and taste of the resulting food products. All processes performed with the purpose of inhibiting the growth of one microorganism may unintentionally select for the growth of another (Dillon and Board, 1991). The meat industry is involved in a tenacious arms race with causes of foodborne illness, because microorganisms are constantly evolving, adapting and exploiting opportunities to grow and persist (Newell et al., 2010). With an optimum pH (5.8-6.8), high a_w (0.95-0.99) and a rich supply of nitrogen, carbohydrates, vitamins and minerals, meat is an ideal medium for microbial growth.

Hurdle Technology

Introduction

Meat spoilage is a subjective judgement by consumers that is strongly influenced by cultural background, eating habits, food preparation practices and sensory acuity of the individual (Nychas et al., 2008). Spoilage is not always clear or evident, but most consumers would agree that qualitative factors like abnormal discoloration, development of off-odors, or the formation of slime on the product, would justify rejection (Nychas et al., 2008). Food preservation is done to control sensory and quality deterioration that may occur over the shelf life of the food product. The overriding priority is to control and minimize the growth of spoilage and pathogenic microorganisms and to ensure that the product is safe for consumption. Preservation techniques, such as heating, drying, or reducing pH are based on the idea of inactivation or delaying the growth of microorganisms that can lead to product quality deterioration or growth of unwanted microorganisms (Lee, 2004). This is achieved by causing the microorganism stress, disturbing its homeostasis and forcing the cell to use resources in an effort to survive (Leistner and Gorris, 1995).

Microbial Inhibition

The idea of combining several preservative factors (sometimes at sub-lethal levels) together in an effort to increase the microbial safety of food products is called hurdle technology

(Lee, 2004). It is theorized that the combination of several factors will inhibit the growth of microorganisms better than any single factor alone. Synergism between preservation techniques or compounds often occur, which can lessen the amount of “hurdle” necessary to achieve microbial inhibition, while decreasing possible negative effects on the quality of the food (Leistner and Gorris, 1995; Lee, 2004). “Multi-target preservation,” utilizes different preservation methods that target a number of systems and functions of microbial cells necessary for normal functions simultaneously. These systems include: cell membranes, protein synthesis, metabolic enzymes, DNA, ATP levels, internal pH, a_w , redox potential, substrate transportation, and oxidative phosphorylation (Warth, 1985; Leistner and Gorris, 1995).

Water activity (a_w) is the ratio of partial pressure of water in the test material (p) to the saturation vapor pressure of pure water under the same conditions (p_0); it is numerically equal to the decimal form of equilibrium relative humidity (ERH). Generally, Gram-negative bacteria have a higher a_w demand than Gram-positive (Jay et al., 2005). Figure 1.1 shows the influence of pH and a_w on microbial growth. By using multiple hurdles simultaneously, several systems can be disturbed at once causing the microorganism to expend large amounts of energy, rendering it inactive (lag phase) and thus, increasing the inhibition of the microorganism (Leistner, 2000; Lee, 2004). The factors (or hurdles) that humans have used for centuries are classified as either process-related (high temperature, drying, salting/brining) or additive (acid production during fermentation, addition of organic acids, salts of organic acids, nitrates/ nitrites, or phosphates). Which hurdle to use will depend upon the food product type, the amount of nutrients available for microbial growth, the type of microorganisms most likely to grow in that food, and the shelf life of the product (Lee, 2004).

Using preservation techniques or barriers has been fueled by the increasing consumer demand for more “natural” and “less processed” food products (Leistner and Gorris, 1995). The majority of changes in the food system have stemmed from consumer demand and responses to outbreaks of disease and recalls (Newell et al., 2010). The consumer demand for less processed food with fewer additives, cleaner labels, and less technological interventions contradicts the expectations of products with guaranteed safety against pathogens, longer shelf life and higher convenience (Nychas et al., 2008).

Effect of Temperature on Preservatives

A phenomenon known as auto-sterilization is a positive result of hurdle technology that has been observed in shelf stable meat products. In these products, *Clostridium* species and *Bacillus* species spores (a dormant, non-reproductive, survival phase of a cell) that have survived heat treatment processes can germinate and form vegetative cells (an active and reproducing cell) during storage; however, these cells do not appear to be viable and can quickly die off. Exposure to higher temperatures and other hurdles, such as low pH or low a_w , stresses the vegetative cells, resulting in metabolic exhaustion, and ultimately, cell death. Therefore, it can be argued that foods, such as shelf stable meat products, actually become safer to microbial contamination during storage at ambient temperatures because of this auto-sterilization effect (Leistner and Gorris, 1995; Leistner, 2000).

The temperature of the food product not only affects the growth of microorganisms present in food, but also affects the efficacy of preservatives present in the food. Preservatives have been shown to be more active and more effective against pathogens stored at ambient temperatures (21°C) than at refrigeration temperatures (4°C) (Ihnot et al., 1998). Storing acidified food products at ambient temperatures has been shown to decrease the survival of microorganisms, when compared to food stored at refrigeration temperatures (Skandamis and Nychas, 2000). Both *Salmonella* species and *Listeria monocytogenes* have been shown to persist longer in fermented meat sausages stored at refrigeration temperatures than at ambient temperatures (Leistner, 2000). Breidt et al. (2004) concluded that the lower the temperature, the higher the rate of pathogen (*Escherichia coli*) survival, while increasing temperatures resulted in greater inhibition. Data from a study by Breidt et al. (2007) demonstrated that *E. coli* O157:H7, *Salmonella* species and *L. monocytogenes* were more resistant to acidic conditions at 10°C than at 25°C. A similar study by Lee et al. (2010) demonstrated a greater reduction of *E. coli* O157:H7 cells at 22°C versus 5°C in simulated pickled cucumber samples treated with acetic acid and salt. A study by Porto-Fett et al. (2008) with Soudjouk-style fermented, semi-dry sausages inoculated (in batter and on surface) with a mixture of *L. monocytogenes*, *S. Typhimurium* or *E. coli* O157:H7 resulted in greater inactivation of all pathogens with decreased pH levels (4.8>5.3), decreased a_w and higher storage temperatures (4<10<21<30°C) .

Pickled Foods

From a historical standpoint, the preservation of vegetables and other foods by pickling was a requirement to ensure adequate amounts of food in the colder, harsher seasons of the year. As the preservation technique became more common, people began to favor the taste of acidified foods and expanded upon by the addition of spices and other seasonings (Nabais and Malcata, 1997). According to Etchells and Moore (1971), the commercial pickling process of fruits and vegetables has remained relatively unchanged over the years. The food item intended to be pickled (for vegetables, is initially blanched to inactivate native enzymes) and then immersed in a brine solution containing an organic acid (most notably acetic acid [vinegar]), salt, spices and often food coloring or other preservatives, like sodium benzoate or potassium sorbate. The pickled food is then thermally processed and immediately cooled (Nabais and Malcata, 1997; Lee, 2004). A procedure was established in the 1940s that recommends an internal pasteurization temperature of 74°C for 15 minutes, but the majority of manufacturing companies have developed their own specific procedures (Lee, 2004). In regard to pickled food products and the implementation of hurdle technology, heat, salt and acetic acid are considered major factors for increasing shelf life and microbial stability (Ricke, 2003). Though heated, acidified salt solutions have been shown to be very effective against *S. Typhimurium* (Milillo et al., 2011), the use of heated brine solution has led to product damage and quality defects (Lee, 2004). Manufacturers of pickled food items that demonstrate quality defects from heating often develop processes that are absent of any thermal treatments (Lee, 2004). The majority of the microbial safety of pickled foods is maintained not by thermal processing, but by organic acids, in combination with salt and other preservatives such as sodium benzoate and potassium sorbate (Lee et al., 2010). The concentration of the acid, as well as the type of acid, are important factors in decreasing the pH of the pickled food products (Fischer et al., 1985). Because of the rapid shock of low pH from the brine solution, surface contamination on pickled food items and/or the development of acid tolerance is considered only a marginal concern (Sullivan et al., 2013).

Acetic Acid

Introduction

Organic acids have a very long history as food preservatives and flavor additives, either by direct addition to a product, or development over time due to fermentation by lactic acid bacteria or added starter cultures (Ricke, 2003). Weak acids are classified by the Food and Drug

Administration (FDA) as a Generally Regarded as Safe (GRAS) food additives. See Figure 1.2 for chemical structures of organic acids (Gardner, 1972) and Table 1.1 for a list of organic acids commonly used in the food industry (Barron and Fraser, 2013). Despite the difference in chemical structure, all weak acids (acetic, sorbic, benzoic) appear to have a similar mechanism of inhibition (Freese et al., 1973). Both the type of acid used and the concentration have shown independent and interacting effects on bacterial cells (Krebs et al., 1983; Presser, et al., 1998). The majority of bacteria require a pH range between 4.0 and 8.0 to survive, while yeast and molds are able to grow between 2.0 and 11.0 (Wheeler et al., 1991). Due to the wider pH range, yeasts and molds are more commonly associated with the spoilage of pickled and acidified food products (Beales, 2004). So, weak acid preservatives target and prevent the growth of yeast and mold in pickled food products. Low pH levels alone (<4.5) have been shown to prevent spore germination and bacterial growth. Besides control of pH, acidulants also contribute to texture and flavor, and work synergistically with antioxidants to prevent rancidity. This approach is accomplished by using very small quantities in food products, such as salad dressings, mayonnaise, sauces and pickled foods (Lambert and Stratford, 1999).

Action of Acetic Acid

Acetic acid (CH_3COOH), otherwise known as vinegar, and its sodium, potassium and calcium salts, are some of the oldest known food antimicrobials. It is rarely used by itself as a preservative and often combined with other preservatives, such as salt or preservation methods, such as heating or drying (Lee, 2004). Bacterial cells tend to have a residual negative charge, preventing ionized compounds from entering the cell (Russell et al., 1995). Non-ionized compounds, such as undissociated acids, are able to penetrate the outer cell wall and enter bacterial cells when the surrounding pH has decreased (Freese et al., 1973; Jay et al., 2005). This finding explains why weak acids, like acetic acid, increase in antimicrobial effect when the pH of the environment is lowered. The acid exists primarily in its undissociated state, because it has a relatively low acid dissociation constant (pK_a) value (4.75) (Brul and Coote, 1999; Ricke, 2003; Lee, 2004). The term pK_a represent the pH value where equal proportions of positively charged protons (H^+) and negatively charged anions (A^-) exist in equilibrium (Freese et al., 1973). Proportions of dissociated and undissociated forms of weak acid preservatives can be calculated using the Henderson-Hasselbalch equation: $\text{pH} = \text{pK}_a + \log [\text{A}^-]/[\text{HA}]$ (Lambert and Stratford, 1999). Acetic acid has been shown as being more effective against yeast and molds than lactic

acid or citric acid since, acetic acid has more acid present in the undissociated state when the pH level is 4.0-4.6 (Lindgren and Dobrogosz, 1990).

At low pH, acetic acid will shift towards the uncharged, undissociated state (HA), permeate through the cell wall where it encounters the higher, near neutral pH environment of the cytosol (open area of the cytoplasm compromised of the organelles of the cell). The acid will then dissociate into cation and anion ($HA \rightleftharpoons H^+ + A^-$). Since the ions are now separated, they cannot leave the cell the same way they entered (Lambert and Stratford, 1999; Brul and Coote, 1999). The protons begin to accumulate and cause the pH of the cytosol to drop (Lambert and Stratford, 1999; Ricke, 2003). This diffusion of weak acid into the cell will continue until an equilibrium is reached (Krebs et al., 1983; Brul and Coote, 1999; Lambert and Stratford, 1999; Ricke, 2003; Lee, 2004). The accumulation of protons and subsequent decline in pH will cause severe problems within the cell including: damage to cell membranes, denaturation of proteins, enzymes and DNA, disruption of substrate transportation and oxidative phosphorylation (Warth, 1985; Leistner and Gorris 1995). This acidification takes the cell out of its normal physiological range, putting it in the lag phase of growth, which leads to inhibition of glycolysis, increased metabolic stress, denaturation of important proteins and enzymes, and other effects detrimental to the cell (Lambert and Stratford, 1999; Rowbury, 1995). This cascade of events with weak acids can prevent microbial growth and can extend the lag phase for long periods of time. Cell growth and reproduction can only resume once the internal pH of the cell is raised to near neutrality (Freese et al., 1973; Jay et al., 2005).

The investigation of the effects of pH and organic acid on the growth and death of pathogenic bacteria (for the most part *E. coli*) in meat products has been investigated by numerous authors (Duffy et al., 2000; Pond et al., 2001; Riordan et al., 1998). However, this research is limited to fermented meats and does not included pickled or acidified meat products.

Microbial Defenses Against Acid

Microbial cells are not defenseless to acidification. ATPase proton pump and electron transport system within the cell work to maintain the internal pH level and homeostasis (Beales, 2004). Cation transport ATPase pumps can contribute to pH homeostasis through the exchange of potassium (K^+) ions for protons (Cotter and Hill, 2003). Cells increase the levels of cytoplasmic protein and glutamates, which act as buffers when the cells are exposed to low pH levels (Beales, 2004). Other factors such as the energy (adenosine triphosphate; ATP) capacity of

bacterial cells, internal pH, and buffering capacity can affect an organic acid's ability to inhibit pathogens (Alakomi et al., 2000; Ricke, 2003). Efflux pumps are activated with the intent of removing the protons from the cytosol and pumping them out of the cell until the pH of the cytosol is returned to normal (Figure 1.3; Lambert and Stratford, 1999). The re-alkalizing (increasing pH) process via pumping protons through the cell membrane and out of the cell costs the cell large amounts of valuable ATP. This energy expenditure depends heavily on the concentration and type of weak acid (Krebs et al., 1983; Lambert and Stratford, 1999; Ricke, 2003). Physiologically, for every proton pumped out of the cell, one ATP is expended (Lambert and Stratford, 1999). Because cells are expending large amounts of ATP to pump protons out of the cytosol, the energy requirements of microorganisms in low pH environments is greater than an environment of neutral pH (Ricke, 2003). This energy is taken away from other functions, such as protein synthesis and growth, and leads to severely decreased ATP levels, metabolic exhaustion and death of the cell (Leistner and Gorris 1995; Leistner, 2000; Ricke, 2003).

Anion Accumulation

While the cell works to combat the decrease in pH by pumping the protons out of the cell and prevent acidification, the anion portion accumulates intracellularly, since the negatively-charged molecule cannot pass back through the cell membrane after it has entered. The accumulation of negatively-charged anions is thought to contribute significantly to the inhibition of bacterial cells (Russell, 1992; Brul and Coote, 1999; Alakomi et al., 2000). It is believed that the buildup of the negatively charged anions from this process also plays a role in microbial inhibition. *E. coli* and other bacteria have shown relatively little changes in internal pH, despite being exposed to extremely acidic environments. This finding suggests that ATPase and electron transport system within the cell can withstand the flood of protons, yet their growth is still repressed. Species of *Lactobacillus* can within acidic conditions and allow the internal pH to drop in order to avoid the accumulation of toxic substances, demonstrating that anion accumulation is, in part, responsible for cell death (Russell, 1992). Anion buildup causes a variety of problems for the cell, including high turgor pressure, potential leakage from the cell, production of free radicals, and oxidative stress (Krebs et al., 1983; Brul and Coote, 1999; Ricke, 2003).

Salt and Osmotic Stress

Introduction

Salt was originally used as a preservation technique by itself (chemical drying), and although it has still retained its importance in the food industry, it is used more in combination with other preservation techniques and ingredients. Salt can be added to food products directly with dry salt or by the use of brine solutions (Lee, 2004). The addition of salt with acetic acid has been shown to increase the overall microbial inhibition, due to the lowering of a_w of the product (Yamamoto et al, 1984a). Salt binds up free water and decreases a_w making the food system less hospitable for microbial growth. The osmotic removal of water from a food system is directly correlated with the amount of salt added. Increased salt concentrations affect the amount of water removed in a system by increasing osmotic pressure and decreasing a_w . Salt also reduces the solubility of oxygen in water, which interferes with important actions of enzymes within microorganism cells (Lee, 2004). Maintenance of osmolarity by microorganisms is achieved by increasing inward pressure from moving solutes into the cell keeping its volume constant and keeping outward pressure (turgor) from causing damage to the cell (Foster and Spector, 1995; Archer, 1996).

Function in Meat and in Combination with Acid

In cured meat systems, the addition of salt helps to inhibit microbial growth during the curing process (Jay et al., 2005). Salt also increases the water binding capabilities of meat (Lee, 2004). The addition of nitrite/nitrate prevents the germination of *Clostridium* species spores, slows down the process of oxidative rancidity, serves to stabilize color, and contributes to taste. Sodium erythorbate catalyzes the curing process (increases yield of nitric oxide), as well as stabilizes the color and the distribution of the cure. Phosphates (e.g. sodium tripolyphosphate) are antioxidative agents added to increase water holding capacity and contribute to flavor (Jay et al., 2005).

A study by Lee et al. (2010) examined the effects of different concentrations (0-3%) (v/v) of acetic acid and salt (alone or in combination) on *E. coli* O157:H7. Results demonstrated acetic acid alone was significantly ($p < 0.01$) more effective at reducing *E. coli* O157:H7 populations than acetic acid combined with salt. These data suggest that the synergistic effect between acetic acid and salt may not apply at low concentrations.

Growth Stimulation

The addition of salt, in food or in agar, may stimulate the growth of pathogenic bacteria like *Listeria monocytogenes* (Cole et al. 1990) and *Salmonella* species (Radford and Board, 1995) at decreased pH as well as *Staphylococcus aureus* (O Clements and Foster, 1999). Salt has also been linked to increasing the heat resistance of molds (Doyle and Marth, 1975) and bacteria (Bean and Roberts, 1975). Conflicting results between the synergistic or antagonistic effect between weak acids and salt on bacterial populations has emerged when comparing laboratory media versus food samples. These conflicting results could be due to the differences in acids/acidulants and laboratory media used, strains or species chosen to test, growth phase of the microorganisms, or differences in how the microorganisms behave in laboratory media (under ideal conditions) versus actual food samples (Lee et al., 2010).

Developed Resistance to Preservatives

Introduction

Over the years, spoilage and pathogenic microorganisms have developed physiological and genetic mechanisms, enabling them to tolerate methods of preservation commonly used by the food industry. Controlling or changing temperature, pH, a_w or oxygen levels, along with the addition of preservatives such as weak acids (and their salts), are known to inhibit or destroy microorganisms.

Different microorganisms have different vulnerabilities and resistance when it comes to preservation techniques (Beales, 2004). The nature of the preservative (organic acid, salt, etc.), the type of microorganism (Gram-positive or -negative), pH of the environment, and temperature, may determine how a microorganism responds (inhibition or resistance) to a stressor. In situations where a bacterial population is exposed to an inhibitory preservative or process, there is the potential for the bacterial population to be divided into two extremes: cells unaffected by the process and cells inactivated or killed. In the middle of this range are injured cells. Injured cells have the potential to revive if placed into an environment ideal for the growth of that bacterium (Russell, 1991). pH and temperature are two important factors that can modify the function of the preservative against cells (Russell, 1991). It has been argued that increasing the use of organic acids as antimicrobials in food systems could lead to increased development of acid-tolerant pathogens that normally would be killed off by the acidic environment of the gastrointestinal tract of both humans and animals (Ricke, 2003).

Most microorganisms can survive small changes in their environment by either resisting the stress itself by developing a tolerance or yielding to the stress conditions and adapting to it (Beales, 2004). These adaptations are more concerned with the survival of the cell by maintaining physiological structure and normal internal operations, and not necessarily growth. Preservation techniques, designed to disrupt the internal environment of cells and prevent growth, may not necessarily kill them. This approach can leave microorganisms still metabolically active and, if transferred to more favorable environmental conditions, become active and grow. More intense treatments that would reduce microbial contamination, also may reduce quality, rendering the food undesirable for human consumption (Beales, 2004).

Resistance can be considered as either intrinsic or acquired. Intrinsic resistance is defined as "a natural [innate] chromosomally-controlled property of an organism," while acquired resistance results from "genetic changes in a bacterial cell and arise either by mutation or by the acquisition of genetic material from another cell" (Russell, 1991). Another way bacteria can become resistant and tolerate preservatives is by possessing specialized enzymes that can degrade preservatives, thereby inactivating them and allowing the organism to use them as a nutrient source. This type of resistance can be acquired by bacteria through genetic mutations or by the acquisition of genetic material from other microorganisms found in its surroundings (Russell, 1991).

Reaction to Stresses

Both *S. Typhimurium* and *L. monocytogenes* have evolved and adapted to endure stresses and unfavorable conditions in both the environment and in hosts. Exposure to less favorable conditions (ex. lowered pH) promotes significant changes in the internal protein profiles of both *S. Typhimurium* and *L. monocytogenes*, demonstrating that these bacteria have a keen sense of their environment and have a wide array of proteins intended to aid the cell in the survival against specific stresses (Cormac and Colin, 1999). After exposure low temperature or low pH, microbial cells can alter the fluidity of their membranes by changing the fatty acid composition, allowing normal cell activity to continue (Russell et al. et al., 1995). These changes in fatty acid composition affect and change the fluidity and permeability of plasma membranes, leading to a potential increase in acid tolerance (Beales, 2004). Increasing the levels of saturated, long and straight chain fatty acids make cell membranes more rigid, making it more difficult for

undissociated weak acids to pass through the cell membranes and enter the cytosol (Juneja and Davidson, 1993).

Russell et al. (1991) demonstrated that Gram-negative bacteria are generally more resistant to weak acid preservatives than Gram-positive bacteria. This finding may be due to the physiological difference in cell wall structure between the two types of cells. The outer cell wall of Gram-negative bacteria resist the entrance of some preservatives into the cell, when compared to Gram-positive bacteria (Juneja and Davidson, 1993). Lipopolysaccharides (LPS) found in Gram-negative bacteria can prevent entry of long chain acids. However, the LPS is ineffective against short chain acids, like acetic acid (a saturated straight-chain monocarboxylic) (Russell, 1991). The LPS consists of molecules with a hydrophobic head positioned inward, with a hydrophilic tail protruding outward, giving the cell a negatively charged hydrophilic surface. Preservatives, chemical agents and other bacteria target the LPS layer, causing it to degrade and shed away from the cell with the intentions of weakening and compromising the integrity of the bacterial cell wall surface. This damage to the cell wall structures of bacteria can lead to leakage, as well as interaction and inhibition by molecules too large or lipophilic to initially penetrate into the bacterial cells (Alakomi et al., 2000).

Acid shock is a phenomenon experienced by bacteria when exposed to acidic conditions. It is well documented that bacteria possess the metabolic capabilities of surviving low pH levels and adapting to this stress through an acid tolerance response (ATR). The syntheses of acid shock proteins can protect bacteria and allow them to survive in lethal acidic environments until conditions become favorable for growth. Syntheses of acid shock proteins also have been linked to an increase in virulence, when pathogens are stressed further (Leistner, 2000). This phenomenon has been demonstrated in *Salmonella* species and *L. monocytogenes* and appears to be dependent on the growth phase of the bacterium (lag, log, stationary, death), but also atmospheric conditions, salt form, acid concentration, and genetics of the bacterium (Russell, 1991; Kroll and Patchett, 1992; Ricke, 2003).

The same preservative and preservation techniques that inhibit and induce stress in bacteria can also 'arm' bacteria with the ability to survive. Increasing the virulence of pathogens like *E. coli* O157:H7 and *Salmonella* species, with a low infectious dose, with current preservation practices is particularly disconcerting (Archer, 1996). It has been shown in some pathogenic bacteria that resistance to a particular hurdle can lead to a developed tolerance of

other unrelated hurdles in a process known as “cross-tolerance” (Leyer and Jonson, 1993; Leistner, 2000). For example, acid-adapted cells become more tolerant of mild heat treatments and conversely, heat-tolerant cells can develop a tolerance to acid (Rowbury, 1995). The effects of cross-tolerance can be reduced if the stresses are applied at the same time, not giving the microorganisms time to adapt and survive (Leistner, 2000).

Government Agencies and Food Safety

Background Information

Modern food quality and safety assurance programs focus on risk analysis and prevention through constant monitoring and recording of critical parameters that determine the safety and susceptibility of food products (Nychas et al., 2008). The Food Safety and Inspection Service (FSIS) is an agency within the United States Department of Agriculture (USDA) that is responsible for “ensuring the safety, wholesomeness, and accurate labeling of meat, poultry, and egg products” (Levine et al., 2001). The presence of any microbial hazard, such as pathogenic bacteria in ready-to-eat meat or poultry products, is considered the basis for declaring a product “adulterated.” Products found to have a microbial hazard are subject to retention, seizure, or voluntary recall. Special emphasis is placed on ready-to-eat products, since consumers are unlikely to reheat them. Pathogens, such as *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7 and *Staphylococcus aureus* are of particular concern to FSIS, because of the potential for illness and death associated with these organisms (Levine et al., 2001).

The Food and Drug Administration (FDA) is a federal agency of the United States Department of Health and Human Services responsible for protecting and promoting public health through the regulation and supervision of several departments and types of products including food, medications and vaccines, animal foods/feed and veterinary products. Additionally, the Foodborne Diseases Active Surveillance Network (FoodNet) was established in 1996 to determine the burden of foodborne disease and monitor food safety trends over time (Flint et al., 2005).

Besides preventative measures like Good Manufacturing Practices (GMPs), Standard Operating Procedures (SOPs) Sanitation Standard Operating Procedures (SSOPs), and Hazard Analysis Critical Control Point (HACCP) principles, knowledge of past foodborne outbreaks and what microorganisms are strongly associated with particular food items is important to the prevention of future outbreaks (Reij and Aantrekker, 2004).

Regulations of Meat and Acidified Foods

The term “acidified foods” describes any food product to which acid is added to decrease the pH of the food. Acidification has been shown to be an effective way to control and inhibit the growth of *Clostridium botulinum* (Breidt et al., 2004). The use of preservatives, such as benzoate or sorbate as primary factors for microbial inhibition, are not permitted (Acidified Foods, 2015). All acidified foods must have a $a_w > 0.85$ and the final equilibrium pH of the product must be ≤ 4.6 and achieved within a designated period of time as outlined in 21 Code of Federal Regulations (CFR) 114.80(a) authority (Acidified Foods, 2015; Barron and Fraser, 2013). Because of this lack of regulatory detail, pickled food manufacturing companies have developed their own specific processes to achieve the pH level ≤ 4.6 (Breidt et al., 2004). Equilibrium is achieved when “acid is fully diffused throughout the food (especially solid particles) and any successive measurements produce the same results at 25°C.” FDA regulations also state that the pH must be checked, controlled, and well documented to two decimal places if above 4.0 with an accurate and calibrated pH meter or measured to one decimal place if below 4.0 with pH paper or pH meter. After proper acidification, a heating process must be applied to the product in order to destroy vegetative spoilage and pathogenic microorganisms and to inactivate enzymes that could negatively affect the product. The time-temperature combination of this heating process is based on the size, density, amount of salt, and acidity of the product. Regulations leave the development of the process up to the food manufacturer, but it must also be reviewed and approved by a designated FDA authority (Barron and Fraser, 2013; Acidified Foods, 2015). These regulations do not take into account the amount or the type of organic acid, just the final pH (Breidt et al., 2004; Barron and Fraser, 2013).

The FDA Acidified Food Guidelines state that, “the following items are not considered to be acidified foods or low-acid foods: any food prepared under the continuous inspection of the Meat and Poultry Inspection Program of the Animal and Plant Health Inspection Service of the Department of Agriculture under the Federal Meat Inspection Act and the Poultry Products Inspection Act” (FDA Acidified Food Guidelines, 2010). “Because these foods are not recognized as acidified foods, commercial processors do not have to file and register their processing information for these products with the Food and Drug Administration” (Barron and Fraser, 2013).

This regulation was created because pathogens, such as *E. coli* O157:H7 and *Salmonella* species have been shown to survive and grow in acidic environments. A pH level of <4.6 was chosen, based on the value necessary to inhibit the growth of *C. botulinum* and the production of toxin associated with the bacterium and its spores. Growth of spoilage bacteria, yeast, and mold in acidified foods will raise the pH of that food system (>4.6), which in turn can lead to the activation and growth of *C. botulinum* spores. Scientific literature states that *C. botulinum* spores do not germinate at pH \leq 4.8, but the FDA has added a safety factor of 0.2 pH units for consumer safety (FDA Acidified Food Guidelines, 2010).

Due to recent outbreaks of *E. coli* O157:H7 in apple juice and apple cider, 21 CFR Part 120 was publicized in 2001 in addition to 21 CFR Part 114. This regulation mandates a 5-log reduction in acid-resistant bacterial pathogens that may be present in juices. Acidified vegetables that have a pH of 3.3 or less must ensure a 5-log reduction in bacterial pathogens without the use of a heat treatment of the product (Breidt et al., 2007). Prior to pathogenic outbreaks associated with acidified juices (pH \leq 4.0), lactic acid bacteria were the primary microorganisms that manufacturing regulations (ex. time and temperature treatments) were based on (Breidt et al., 2005).

Food processes, like pickling, requires the conduction of a validation study under laboratory controlled condition in order to be accepted by the USDA and used as verification documentation in HACCP plans (Richard and Cutter, 2011). Any changes to previously approved processes must be reevaluated to ensure its validity (Scheinberg et al., 2013). Small meat processors often lack adequate resources to validate the lethality of their processes against appropriate pathogenic bacteria and are unable to provide proper documentation for their HACCP programs to meet USDA-FSIS requirements (Porto-Fett et al., 2008).

Studies of Pickled Foods

Acton and Johnson (1973), along with several others, (Ball and Saffores, 1973; Fischer et al., 1985; Breidt et al., 2004; Lee, 2004; Breidt et al., 2005; Breidt et al., 2007; Lee et al., 2010; Milillo et al., 2011; Richard and Cutter, 2011; Barron and Fraser, 2013; Scheinberg et al., 2013; Sullivan et al., 2013) cited studies examining the presence of pathogenic bacteria in acidified food products, such as pickled eggs, apple cider, and pickled vegetables with no mention of pickled meat products. It is important to note that most research around pickled and acidified

foods focuses mostly on homogenous mixtures like salad dressings, mustard, ketchup or vegetable purees, and not on heterogeneous products such as meat.

In a study by Breidt et al. (2005), pickling brines (containing 4% sodium chloride and 0.2% calcium chloride, with a target concentration of 200 mM acetic acid) with an equilibrium pH of 4.1 were inoculated with a cocktail of *E. coli* O157:H7, *Salmonella* species (Braenderup, Cerro, Enteritidis, Newport and Typhimurium) and *L. monocytogenes* (15 strains total, 5 of each pathogen) with initial cells counts of 10^8 CFU/mL and heated to temperatures ranging from 50 to 60°C to achieve a 5-log reduction. Results demonstrated that there was no significant difference in reduction time between *E. coli* O157:H7 and *L. monocytogenes* and that the reduction time of *Salmonella* species was significantly less than the previous two pathogens (Breidt et al., 2005).

Acidification of fruits and vegetables should not be compared to high protein products, such as eggs or meat, since they have a much higher buffering capacity (Sullivan et al., 2013). The studies focusing on pickled eggs are most relevant to the topic of pickled meat products, because of similar protein content and susceptibility to microorganism contamination. Two studies in 1973 examined the acidification of pickled eggs. Acton and Johnson (1973) realized a final pH of 4.2-4.3 achieved in the center yolk after acidification for 3-6 days at room temperature with a 3% acetic acid solution containing sucrose and pickling spices. A second study by Ball and Saffores (1973) reported that an equilibrium pH (4.0-4.4) was achieved in 6-7 days between pickled eggs and brine with solutions ranging from 1 to 5% acetic acid and with or without 2.5 or 4.7% sodium chloride at room temperature.

In a study by Richard and Cutter (2011), pre-cooked and pre-peeled eggs were inoculated with a cocktail of *Salmonella* species (Enteritidis and Typhimurium), *Staphylococcus aureus*, *L. monocytogenes*, and *E. coli* O157:H7 at levels of $\geq 8.40 \log_{10}$ CFU/mL. Results also demonstrated that the pickling process (pickling solution containing 0.99-0.11 mol/L acidity of acetic acid and salt content of 1.2-1.4 mol/L) achieved $>4.85 \log_{10}$ CFU/mL reduction of the inoculated hard cooked eggs for all pathogens in 24 hours at 22°C; populations were reduced to $<1.70 \log_{10}$ CFU/mL after 48 hours. Even with enrichment procedures, *S. Enteritidis*, *S. Typhimurium*, *L. monocytogenes*, and *E. coli* O157:H7 were not detected in samples following acidification for 48 hours, while *S. aureus* was still viable up to 96 hours post acidification. Reduction of pathogenic populations were significantly higher ($p < 0.05$) in the hard cooked eggs at room temperature (22°C) than at refrigeration temperatures (4°C), confirming that temperature

plays an important factor in the acidification process. The pH of the brine solution increased, while the pH of the egg (yolk and albumen) increased, eventually coming to equilibrium between 2.0-2.5.

A follow-up study by Scheinberg et al. (2013) found that inoculation ($8.8 \log_{10}$ CFU/mL) of pre-peeled, hard cooked eggs (HCE) with a cocktail of *S. Enteritidis* and *S. Typhimurium*, *S. aureus*, *L. monocytogenes*, and *E. coli* O157:H7 and treatment with a pickling solution (0.99 to 0.11 mol/L acidity of acetic acid and salt content of 1.2-1.4 mol/L with 0.008 mol/L sodium benzoate and 0.005 mol/L potassium sorbate), reduced pathogen populations $>5.0 \log_{10}$ CFU/mL after 24 hours. Decreases in the pathogens within the first 24 hours coincided with a rapid decline in the pH of the HCEs, suggesting that the kill was pH driven. No significant changes in a_w of the HCEs occurred as a result of pickling in this manner (Scheinberg et al., 2013).

Results from a study investigating pickled eggs by Sullivan et al. (2013) indicate that both *L. monocytogenes* and *S. aureus* can survive up to 14 days when pickled with only a 1% (w/v) acetic acid solution. Populations only decreased when the pickled eggs were moved from refrigerated temperatures to ambient temperature (25°C). Despite the low pH and temperatures, these bacteria were able to survive and adapt to the high protein environment of the eggs, but not when exposed to ambient temperatures (Sullivan et al., 2013).

Spoilage Microorganisms

The spoilage of food by bacteria, yeast, and/or mold is a complex system that is greatly affected by the composition of the food and other conditions, such as pH, a_w , temperature and presence or lack of oxygen. In addition to these environmental factors, strain-to-strain variations, formation of tolerance or resistance to antimicrobials, cell detoxification mechanisms, or possession of special enzymes able to breakdown antimicrobials, can dictate which microorganisms out compete others (Ricke, 2003). If such conditions are ideal for all 3 types of microorganisms, then bacteria will most often grow more quickly than yeast and yeast will grow more quickly than mold (Jay et al., 2005). The microflora of food products changes and the preservation increases along the following path: non-fermentative, psychrotrophic, Gram-negative bacteria → fermentative, Gram-negative bacteria → LAB → yeasts → filamentous fungi (mold) (Gram et al., 2002).

Compared to pathogenic bacteria, research on microflora categorized as spoilage microorganisms for meat and poultry, is limited. Furthermore, little to no research has been

performed on the survival and growth of spoilage microorganisms in acidified meat and poultry products, such as pickled sausage. Although studies on microorganism survival in acidified conditions have been conducted, these studies have focused on pathogenic bacteria associated with pickled vegetables and purees (Lee, 2004; Breidt et al., 2004; Breidt et al., 2005; Breidt et al., 2007; Lee et al., 2010) or hard cooked eggs (Richard and Cutter, 2011; Scheinberg et al., 2013; Sullivan et al., 2013).

Lactic Acid Bacteria

Background Information

Lactic acid bacteria (LAB) are a group of Gram-positive, rod and cocci-shaped, homo- and hetero-fermentative bacteria. Most have their optimum growth values between 30 and 37°C and pH 4.0 to 4.5, with some species able to grow outside of those ranges (Jay et al., 2005). LAB are one of the dominant bacterial groups in both fresh (Borch et al., 1996; Doulgeraki et al., 2010) and processed meat products (Samelis et al., 2000; Rimaux et al., 2012), as well as being found in and isolated from meat plant environments (Andrighetto et al., 2001). LAB grow well at neutral pH and in the high moisture environment of meat products. Similar to other bacteria like *Salmonella* species or *L. monocytogenes*, LAB have demonstrated the potential to become acid-tolerant if exposed to sub-lethal acidic conditions for long periods of time (Noonpakdee et al., 2003). Contamination of food products preserved with weak acids is most often caused by lactic acid bacteria, yeast, or mold since the environmental conditions effectively inhibit the growth of pathogenic bacteria (Beales, 2004; Lee, 2004).

Effects of Atmosphere, Acid and Salt

LAB can survive and flourish in a variety of storage conditions utilized in the meat industry, including modified atmosphere packaging (MAP), high carbon dioxide, under vacuum (anaerobic), and refrigeration temperatures (Borch et al. 1996; Doulgeraki et al., 2010; Doulgeraki et al., 2012). Their survival is due, in part, to the fact that changing the atmosphere of the product via vacuum packing, MAP, or other treatments, causes a shift in the microflora towards LAB (Dainty and Mackey, 1992). The addition of salt, decrease in pH, the reduction of a_w , brine injection, and smoking has been shown to cause similar shifts in microflora (Borch et al., 1996; Samelis et al., 2000; Doulgeraki et al., 2012). Korkeala et al. (1992) reported that lactic acid bacteria growth is enhanced by 1-2% sodium chloride (w/v), while concentrations at greater than 3% demonstrated an inhibitory effect. Samelis et al. (2000) demonstrated a correlation

between LAB growth and intrinsic factors of certain meat products. LAB grew faster in cooked/smoked products, such as bacon, ham, pork loin, frankfurters and turkey filet with low salt and high a_w . Additionally, the lower the brine concentration of the product, the higher the amount of LAB growth observed. In low salt, high a_w meat products, LAB populations are often below detection limits (<10 CFU/g) in the initial stages of processing, but populations increase during time of distribution and storage (Chenoll et al., 2007). Growth as high as $10^9 \log_{10}$ CFU/g has been reported in some meat products (Kelly et al., 1996).

Any change via visual, taste or smell in a food product that renders it unacceptable to the consumer is known as spoilage (Chenoll et al., 2007). Spoilage of meat products by LAB also is evident by off-flavors (sour or tangy) or off-odors due to the production of lactic acid and other metabolites, changes in texture (soft or slimy), and visual changes (ropy slime, milky exudates), discoloration (gray or green), or swelling of the packaging (gas production) (Samelis et al., 2000; Vermeiren et al., 2004; Jay et al., 2005).

Lactobacillus curvatus* and *Lactobacillus sakei

LAB, such as *Lactobacillus curvatus* and *Lactobacillus sakei*, have a consistent presence in ready-to-eat (RTE) meat products. *L. curvatus* is an obligate, homofermentative bacterium, meaning it produces lactic acid as the primary end product of glucose metabolism, while *L. sakei* is classified as facultative heterofermentative, implying that it produces other end products besides lactic acid, such as carbon dioxide. Both *L. curvatus* and *L. sakei* are considered streptobacteria because they produce up to 1.5% lactic acid, with an optimal growth temperature of 30°C (Jay et al., 2005).

In a study by Samelis et al. (2000), *L. sakei* was the dominant bacterium isolated from several cooked and smoked products: bacon, ham, pork loin, frankfurters, and turkey filet. *L. curvatus* also was found in all products evaluated. The authors observed that these two strains of LAB were particularly well adapted to stresses designed to extend the shelf life of meat products, including reduced a_w , increased salt, refrigeration and various atmospheric storage conditions. In similar studies examining both fresh and processed meat products, authors Borch et al. (1996), Andrighetto et al. (2001), Hu et al. (2009), Audenaert et al. (2010), Doulgeraki et al. (2012), and Rimaux et al. (2012) noted similar results with regard to the presence of *L. sakei* and *L. curvatus*. *L. sakei* was the dominant species isolated from chorizo, a dry fermented, Spanish sausage,

followed by *L. curvatus*; both grew in the presence in as much as 10% sodium chloride (w/v) (Santos et al. 1998).

A study done by Doulgeraki et al. (2010) demonstrated that *L. sakei* populations increase and outcompete other LAB species minced beef under long term refrigerated storage and various atmospheric conditions. Storage temperature and the atmosphere that the meat product is stored can affect the spoilage potential of that product (Doulgeraki et al., 2010). *L. curvatus* and *L. sakei* have been shown to be some of the most cold temperature (psychrotrophic) and high-salt tolerant strains of LAB (Vermeiren et al., 2004).

Both *Lactobacillus curvatus* and *Lactobacillus sakei* have been identified as the main spoilage bacteria from various cooked meat products, including refrigerated ham and turkey breast deli meats, smoked pork loin, and bacon (Samelis et al., 2000), sliced cooked hams (Hu et al., 2009), minced beef (Doulgeraki et al., 2010), as well as chicken breast and turkey (Audenaert et al., 2010).

Inhibition of Other Microorganisms

Because of their ability to grow well and flourish in meat matrices, *L. sakei* and *L. curvatus* are used in starter cultures (Ravyts et al., 2008); both species are commonly associated with fermented sausages (Andrighetto et al., 2001). The use of LAB in meat products demonstrates that these organisms are able to adapt to and grow in meat products, as well as produce metabolites that effectively inhibit the growth of other microorganisms.

The antimicrobial efficiency of LAB is encompassed in three different mechanisms: the production of organic acids (decrease of environmental pH), competition for nutrients (competitive exclusion), and the production of secondary antagonistic compounds (Schnürer and Magnusson, 2005).

Lactic acid is the primary metabolite produced by LAB; the undissociated form of the acid easily penetrates cell membranes, where it dissociates and releases H⁺ ions, which in turn causes acidification of the cytosol of the cells (Lindgren and Dobrogosz, 1990). In meat products with LAB contamination, the pH of the product can be reduced as much as 1 pH unit (6.0-6.5 to pH 5.0-5.3) (Borch et al., 1996). Organic acids also may neutralize the electrochemical potential of a cell's plasma membrane, causing an increase in permeability of the membranes, eventually leading to the acidification and death of the cell (Schnürer and Magnusson, 2005). LAB have been shown to outcompete other bacteria for important nutrients, specifically iron. Iron is an

important element for bacterial growth as it serves a vital role in bacterial respiration and in redox reactions within the cell (Gram et al., 2002). Lastly, LAB may produce a wide variety of secondary antimicrobial compounds such as: acetic acid, hydrogen peroxide, formic acid, propionic acid, or diacetyl. With the exception of the weak acids, the precise mechanism of many of these compounds is complex and difficult to study independently because they appear to work in synergistically (Lindgren and Dobrogosz, 1990; Schnürer and Magnusson, 2005). Hydrogen peroxide (H₂O₂) demonstrates a strong oxidizing effect on the cell walls of bacteria and cellular proteins (Lindgren and Dobrogosz, 1990).

LAB also are greatly affected by their surrounding environment. Subsequently, their production of antifungal compounds also affected. Factors like incubation period, temperature, environmental pH, availability of nutrients, and presence of specific yeast, mold or other competing bacteria can alter the production of inhibitory metabolites (Gourama and Bullerman, 1995; Schnürer and Magnusson, 2005; Dalié et al., 2010).

In addition to antifungal compounds, LAB produce antimicrobial peptides, known as bacteriocins, which are capable of inhibiting the growth of taxonomically-related Gram-positive bacteria (Dalié et al., 2009). However bacteriocins are essentially inactive against Gram-negative bacteria and eukaryotic microorganisms, such as yeast and mold (Gourama and Bullerman, 1995). *Lactobacillus sakei* is known for the production of several of these compounds, which can inhibit *L. monocytogenes*, but is less effective against Gram-negative bacteria like *Salmonella* species (Marceau et al. 2004; Ravyts et al., 2008). Dalié et al. (2010) even mention research investigating the ability of some LAB strains to interact with and neutralize mycotoxins produced from mold contamination in foods.

Yeast

Background Information

Yeasts are eukaryotic microorganisms that reproduce by means of budding. Due to their slow rate of reproduction, yeast often do not compete with bacteria. However, if something is done to change the product and in turn inhibits the growth of bacteria, the microflora of the product will shift from a bacterial dominated system to one that favors the growth of yeast (Jay et al., 2005). Meat products that are cured, fermented, pickled, or stored at low temperatures for long periods of time, often experience these changes in microbial domination (Dillon and Board, 1991). Yeast are important spoilage microorganisms, especially in foods of low pH, high salt or

sugar content, low a_w ; foods stored in liquids (brines); and foods containing weak acid preservatives, where bacterial growth could be inhibited (Tokuoka, 1993; Praphailong and Fleet 1997; Osei et al., 2000). Yeast can utilize both carbon and nitrogen from food products, and in doing so, they generate various volatile and non-volatile metabolites, causing signs of food spoilage, including bitter and off-flavors, changes in color and texture of the food product, and occasionally deformed or blown out packaging material (Asefa et al., 2009). The ability of yeast to break down poultry components for its own nutrients and make those nutrients more readily available for bacterial growth, suggests yeast play a larger role in the spoilage of meat products than previously thought (Ismail et al., 2000). Yeast species also are opportunistic and have been shown to thrive in high fat meat products, primarily due to their high lipolytic activity and ability to compete against other microorganisms for nutrients (Dillon and Board, 1991).

Effects of Acid and Salt

Betts et al. (1999) demonstrated a synergistic effect between decreased pH and increased sodium chloride concentration that resulted in decreased yeast growth. These effects were further enhanced by lowering the temperature from 22°C to 8°C. The tolerance of yeast to salt and sugar is decreased at and below pH 5.0, where the majority of the acid is in its dissociated state and more effective at microbial inhibition (Praphailong and Fleet 1997). Lowering the cytoplasmic pH can slow down and inhibit important enzyme activities necessary for the yeast to function and reproduce (Piper et al., 2001; Brul and Coote, 1999). Yeast growth can be decreased by the addition of as little as 1% acetic acid, and entirely inhibited at concentrations of 3.5 to 4% (Yamamoto et al., 1984b).

Adaptation and Growth

Though links have been made between the addition of weak acid preservatives and decreased cell yield of yeast species (Warth et al., 1985), yeast are becoming increasingly resistant (Brul and Coote, 1999) and adaptable since they can break down preservatives, such as potassium sorbate, and use the products as nutrient sources (Schnürer and Magnusson, 2005). Yeasts also have been shown to consume and utilize lactic acid in fermented meat products and in doing so, raise the pH of the products (Walker, 1977). Yeast species that display resistance to benzoic acid have shown tolerance to sorbic and lactic acid as well. This shared resistance suggests that there is a common mechanism of inhibition between weak acid preservatives and how they inhibit microorganism growth (Warth et al., 1985; Krebs et al., 1983).

The adaptation of microorganisms to weak acid preservatives to aid in growth in low pH environments poses problems for the food industry. As such, there may be a need to increase the amount of preservatives in millimolar, instead of micromolar amounts (Piper et al., 2001). It is presumed that yeasts that display a tolerance to acidic pH values have an adapted plasma membrane that aid in the export of protons and anions from within the cell (Piper et al., 2001; Brul and Coote, 1999). This system that regulates intercellular pH is called the ATPase system (Brul and Coote, 1999; Praphailong and Fleet 1997; Tokuoka 1993). Additionally, altered cell wall composition and the production of intracellular polyols (compatible solutes), such as glycerol, arabitol, mannitol and erythritol, have been described as contributing to acid and salt tolerance in yeasts (Nishi and Yagi 1995). For instance, the accumulation of glycerol within the yeast cell acts as both an osmoregulator and an osmoprotector of protein and enzyme functionality under osmotic stress (Nishi and Yagi 1995). A study by Stratford et al. (2013) proposed that weak acid resistance of yeast populations is due to the presence of sub-populations of cells with lower internal cytoplasmic pH (by 0.4-0.8 pH units). Having a lower internal pH will result in less weak acid being taken up by the yeast cells, thus reducing the effectiveness of the acid (Piper et al., 2001; Brul and Coote, 1999).

Debaryomyces hansenii* and *Candida zeylanoides

Yeast contribute a significant part to the contamination and spoilage of meat when efforts are taken to prevent the growth of bacteria. Published research on the occurrence of yeasts in meat products focuses generally on fresh and fermented products. Two genera of yeast in particular, *Debaryomyces* and *Candida*, have isolates that are prevalent throughout the various phases of meat harvesting and processing (Deak and Beuchat, 1996). The organisms *Debaryomyces hansenii* and *Candida zeylanoides* are the subject of dozens of published articles and are consistently associated with and isolated from RTE and processed meat products.

D. hansenii is a cryo- and osmo-tolerant species of yeast that is one of the most frequently isolated species of yeast from food (especially meat) products (Encinas et al., 2000; Nielsen et al., 2008; Asefa et al., 2009). On agar, *D. hansenii* produces colonies between 2 and 4mm in diameter with irregular budding, sometimes simultaneously at more than one location (Pitt and Hocking, 2009). Optimum growth temperature for *D. hansenii* is 24-25°C, and the organism rarely grows at temperatures $\geq 37^\circ\text{C}$ (Jermini and Schmidt Lorenz, 1987b). It has a pH range for growth between 2.0-2.5 and 8.0 at 25°C (Praphailong and Fleet, 1997). *D. hansenii* is

considered a major spoilage yeast species due to its ability to grow in low and intermediate a_w foods preserved with salt, including foods preserved with pickling brines, fermented milk, soft drinks, and fresh, fermented, and dry-cured meats (Betts et al., 1999; Osei et al., 2000; Andrade et al., 2006). In a study by Praphailong and Fleet (1997), *D. hansenii* grew in salt concentrations as high as 15% salt (w/v) and at pH as low as 3.0. The organism can grow in salt concentrations as high as 24% (w/v) (Mrak and Bonar, 1939) and is capable of utilizing a wider range of carbon sources, when compared to other yeasts. Studies by Praphailong and Fleet (1997) and Betts et al. (1999) have shown the synergistic inhibitory effects of increased acidity and increased salt on the growth of *D. hansenii*. Yeasts like *D. hansenii*, have been shown to commonly disperse and travel through the air, greatly increasing their range of possible contamination (Dillon and Board, 1991).

C. zeylanoides is a diverse yeast species found in humans, meat, fish, soil and water (Asefa et al., 2009) that has emerged as a pathogen preying on immuno-compromised individuals, causing fungemia (Candidemia) and septic arthritis (Levenson et al., 1991; Dorko et al., 2000). On media, colonies typically appear as glossy white or cream-colored. *C. zeylanoides* has been shown to grow at refrigeration temperatures (4°C) up to 32-34°C and a minimum a_w of 0.90 (Deak and Beuchat, 1996; Jay et al., 2005). It has been frequently isolated from fresh beef and lamb, sausage, and ham and is particularly prominent in fresh chicken and turkey (Dillon and Board, 1991; Deak and Beuchat, 1996).

Studies Involving Meat Spoilage

Several studies examining fresh and processed meat products and meat manufacturing facilities have found *D. hansenii* and *C. zeylanoides* as major components of the yeast microflora. The majority of samples (58%) taken from raw, marinated, smoked and roasted chickens and turkeys (152) and plated on dichloran rose bengal chloramphenicol (DRBC) agar plates were *Candida* species, with *C. zeylanoides* being the most common strain (26%) (Ismail et al., 2000). Yeasts isolated from fermented Spanish sausages were evaluated in a study by Encinas et al., 2000. The authors identified *D. hansenii* as the most common yeast found in the cooked and raw product, with *C. zeylanoides* also found with a high prevalence. The psychrotrophic yeast, *C. zeylanoides*, was only detected at the initial mixing and filling stages of the manufacture of fermented Spanish sausages, while *D. hansenii* was isolated from the later stages of the process (Encinas et al., 2000). In a study done by Nielsen et al. (2008), *D. hansenii*

and *C. zeylanoides* were the 2 principal yeast species isolated during processing, in the final product and at the end of the product's shelf life from four fully cooked meat products: bacon, ham, salami, and liver paté. *C. zeylanoides* often is found in fresh meat products, while *D. hansenii* is associated with salted, dry, semi-dry, and fermented meat products. Because of its high lipolytic activity and ability to grow at refrigeration temperatures, *C. zeylanoides* has been classified as a notable spoilage microorganism in meat products. In low % salt bacon, *C. zeylanoides* was the predominant microorganism. Once the % salt of the product increased to >4%, *D. hansenii* took over and outcompeted *C. zeylanoides*. In the production of fermented salami, *C. zeylanoides* predominated during the initial processing steps; however, *D. hansenii* was predominately isolated during later fermenting and drying steps (Nielsen et al., 2008). In a study done by Asefa et al. (2009), *D. hansenii* and *C. zeylanoides* represented 63.0% and 26.4%, respectively of the total 401 isolates obtained from both smoked and unsmoked dry-cured meat products. The unpredictable and irregular isolation of *C. zeylanoides* from the environment of meat processing facilities in the early stages of processing, suggests the contamination of the product is coming from external sources, when compared to the more consistent isolation of *D. hansenii* populations (Asefa et al., 2009). There also appears to be a trend where *C. zeylanoides* dominates the early stages of dry-cured meat production, while (usually after salting step) *D. hansenii* takes over during the later ripening stages. This finding suggests *D. hansenii* has greater tolerance and adaptability to lower pH, higher salt, and lower a_w environments than *C. zeylanoides* (Osei et al., 2000; Nielsen et al., 2008; Asefa et al., 2009). Authors Asefa et al. (2009) made note that this shift in yeast microbiota was a positive change for the quality and safety of both the product and the process, since *C. zeylanoides* has been noted as an emerging problem and pathogen in the meat industry.

Mold

Background Information

Meat products that are dry aged, fermented, cured, stored at low temperatures, or held for long periods of time, decrease bacterial growth, and allow slower growing molds to flourish (Mizakova et al., 2002). Dry cured meats favor the growth of xerotolerant (organisms that can tolerate dry environments) and xerophilic (organisms that prefer to grow in dry environment) fungi (Sonjak et al., 2011). Mold spores are ubiquitous, spreading all over the environment. Important reservoirs for mold spores can be humans, animals (fur, fleece, etc.), soil, dust, raw

product, floor drains, windows, shoes/boots ventilation ducts and fans (Leistner and Ayres, 1967; Sorensen et al., 2008; Sonjak et al., 2011). Mold growth can not only be a visible quality defect of meat products, but also can produce off flavors, mycotoxins, penicillin, and other potentially dangerous substrates (Papagianni et al., 2007). *Penicillium* and *Cladosporium* species, specifically *Penicillium nalgiovense* and *Cladosporium cladosporioides*, have been shown to be among the most highly detected molds in meat products and meat processing environments.

Penicillium nalgiovense

Penicillium is an extremely diverse genus, in terms of number of species and range of habitats. The organisms are ubiquitous, opportunistic, and nutritiously undemanding fungi responsible for a significant amount of food spoilage (Hocking and Pitt 1979). *Penicillium nalgiovense* colonies usually grow between 28 and 35mm in diameter, with heavy sporulation, irregularly-branched hyphae. Colors range from white to green depending on the food or media it is growing on. Germination is activated at 5°C, and growth is completely retarded at 37°C. *Penicillium* species have been shown to grow at temperatures as low as 10°C, although at a very slow rate. The rate of growth of mold increases as temperature increases to 15°C and 25°C. Increasing a_w demonstrated similar results in regard to growth rate, until it maxed out at 0.92. The ideal a_w for *Penicillium* species mold growth is between 0.97 and 0.98 (Hocking and Pitt 1979; Lopez-Diaz et al., 2002).

The optimum growth rate of most fungi occurs at about pH 5.0 (Pitt and Hocking, 2009). Reduction of growth (reduction in colony diameter), but not necessarily inhibition, occurred for several strains of *Penicillium* below pH 5.0 (Thompson et al., 1993). No significant changes in the growth of *P. nalgiovense* occurred when testing the effect of different environmental pH values (range of 4.5-6.0) with the addition of 3% sodium chloride (w/w) (Lopez-Diaz et al., 2002) and are in agreement with a study done by Thompson et al. (1993). Acetic acid also has been shown to be more effective against yeasts and bacteria than against molds (Ingram et al., 1956).

In Europe, *P. nalgiovense* is commonly associated with contamination of starter cultures used for fermented meat products, as well as cured meat products (Castellari et al., 2010) (Pitt and Hocking, 2009). Mold species also have adapted to chemical treatments and preservatives. Some *Penicillium* species are able to grow in the presence of potassium sorbate, a commonly

used food preservative (Davidson, 2001), or even degrade sorbate and use it as a source of nutrients (Nielsen and Boer, 2000).

Mycotoxins

Some species of mold have been linked to the production of harmful metabolites. *Penicillium* species are a mycotoxinogenic mold species, meaning that they can produce mycotoxins depending on intrinsic and extrinsic factors. These harmful metabolites can significantly impact public health and pose economic problems for food companies (Dalié et al., 2009). *Penicillium* species are known to have the widest range of mycotoxins of any genus of mold (Mizakova et al., 2002). The toxic effects of mycotoxin can be organized into six categories: carcinogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic, and hepatotoxic (Dalié et al., 2010). Once the production of a mycotoxin has been detected, all strains of mold of that species are considered to be potentially toxicogenic (possess the ability to produce mycotoxins under ideal growth conditions) mold (Mizakova et al., 2002). Those conditions include: presence of oxygen, temperature between 4°C and 40°C, pH values between 2.5 and 8 (with an optimum pH of 5 to 8), minimum a_w of 0.80, and maximum salt concentration of 14% (Ostry et al., 2001).

The majority of *P. nalgioense* strains have tested positive for the production of the antibiotic penicillin, which limits its uses as a starter culture in the production of salami and fermented sausage (Andersen and Frisvad, 1994; Lopez-Diaz et al., 2001). Papagianni et al. (2007) investigated mold growth on traditional Greek sausages. *Penicillium* species was isolated from 90.8% of samples, with 19.5% of those identified as *P. nalgioense*. All samples tested positive for penicillin production.

Reaction to Nitrates and Salt

Nitrates and nitrites are incorporated into meat products with the intent to stabilize cured meat color, contribute to flavor development, and inhibit spoilage and pathogenic microorganisms. However, the addition of potassium nitrate and sodium nitrite to chorizo sausage at concentrations as high as 180 ppm demonstrated no significant effect on inhibiting the growth of *P. nalgioense* (Lopez-Diaz et al., 2002). On the contrary, a stimulating effect on *P. nalgioense* growth also was observed when sodium chloride, sodium nitrite, and potassium nitrate were mixed together at 15°C and 25°C (Lopez-Diaz et al., 2002). In the same study, the addition of sodium chloride to Spanish chorizo demonstrated a stimulating effect on the growth

of *P. nalgiovense* with optimum growth at sodium chloride concentrations at 2.5% (w/w) (Lopez-Diaz et al., 2002).

Cladosporium cladosporioides

Cladosporium species are small, non-mycotoxin-producing molds and common to airborne microflora. Colonies grow between 15 and 40mm in diameter (depending on the media) and are low, dense, velvety, and often an olive green color. Species like *C. cladosporioides* can grow at temperatures at or near 0°C, making them a common agent in the spoilage of refrigerated meat, cheese, and other food products. *C. cladosporioides* can generate growth at a minimum of -5°C (Gill and Lowry, 1982), a maximum near 32°C (Domsch et al., 1980) and are capable of growing at a_w of 0.86 (at 25°C) (Pitt and Hocking, 2009). The presence of this species is considered a contaminant rather than a pathogen in most foods, including meat products, with the exception being fresh fruits and vegetables. *C. cladosporioides* have been shown to cause what is known as “black spot” mold, where the fungal colonies feature uniformly black mycelium. The hyphae penetrate into the top layers of meat products, like dry-cured hams and fermented sausages held at refrigerated and freezing temperatures (Leistner and Ayres, 1967; Gill and Lowry, 1981). Though the outer fungal growth can be removed by trimming, the product could still be considered aesthetically unacceptable, thus creating financial problems for businesses (Gill and Lowry, 1981). Hyphae were shown to penetrate as far as 7mm into the lean and fatty tissue, which could be due to the higher moisture content (a_w) of the inner tissue. The outer surface of meat will dry out as a result of storage at refrigeration and freezing temperatures (Gill and Lowry, 1981). Since molds grow at a slower rate than bacteria or yeast, a study by Gill and Lowry (1981) took 16 weeks to see substantial black spot growth. Storage at low temperatures also contributes to the slow growth rate of the molds.

Meat and Meat Processing Contamination

In a study done by Sorensen et al. (2008), *C. cladosporioides* was frequently isolated from air samples taken in meat processing plants. Interestingly, the number of colonies isolated from air samples increased significantly from spring to autumn, suggesting that both seasonality and air coming from outside of the processing plant plays an important role in product contamination. This finding agrees with a study by Mizakova et al., 2002, who concluded that mold contamination on both the product and in the processing plant depended on location and season of the year. *C. cladosporioides* was more heavily isolated from air samples and

equipment surfaces than from raw product. Castellari et al. (2010) reported similar results in regard to *C. cladosporioides* contamination in air samples.

A study by Tabib et al., (1984) examined the inhibition of mold by organic acids (propionic, acetic, sorbic, and benzoic) in cornmeal and revealed that the pH of the environment greatly affected the ability of the acid to inhibit mold growth. The lower the pH, the greater inhibition of mold growth. Results also demonstrated that only total mold counts differed between treatments, *Aspergillus candidus* and *Penicillium* species were found in all samples. Other variations in the cornmeal, such as the concentration of limestone, fat, and proteinaceous material high in basic amino acids, as well as by the particle sizes of the ingredients, was shown to influence the efficacy of the organic acids.

Pathogenic Bacteria

Salmonella species

Background Information

Salmonella is a Gram-negative, facultative anaerobic bacterium belonging to the family *Enterobacteriaceae*. The organism is rod-shaped, with most strains being very motile with peritrichous flagella. The name was given in honor of Dr. D.E. Salmon who discovered the bacterium in a pig intestine (Agbaje et al., 2011). The classification of the genus *Salmonella* is based on somatic (O), flagellar (H) and capsular (Vi) antigens (Xu et al., 2008). From a nomenclature standpoint, the genus *Salmonellae* is complicated, with over 2,500 serovars (serotypes). For example, the two most commonly isolated foodborne *Salmonellae* are *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) (Agbaje et al., 2011).

Growth Parameters

Salmonella has a temperature range from 6 to 46°C, with an optimum growth temperature of 37°C. *Salmonella* grow at a pH range of 4.1-9.0, with optimum level being 6.5 to 7.5. The pH range is debatable, since many *Salmonella* isolates possess or acquire acid resistance. By nature, *Salmonella* species are neutrophilic, meaning the organisms prefers to live in environments of near neutral pH. Yet, it can survive extremely acidic environments, such as the gastrointestinal tract of animals and humans (Foster and Spector, 1995). Casadei et al. (2001) concluded that the optimum pH for *S. Enteritidis* growth was between pH 5.9 and 6.5. The minimum pH for growth varies between pH 4.05 (hydrochloric acid) and 5.4 (acetic acid), as the

acidulant used greatly effects the growth of the bacterium (Chung and Goepfert, 1970). The lowest reported a_w level for *Salmonella* is 0.93. This finding also is debatable, because the bacteria is prominent in spices, dried herbs and other foods or ingredients with a_w levels <0.93 (Jay et al., 2005). *Salmonella* species has exhibited an ability to survive in food products with low a_w such as potato chips (Lehmacher et al., 1995), peanut butter (CDC, 2009) and black and red pepper (Zweifel, et al., 2012).

Infection and Symptoms

Incubation period of the bacterium is usually between 6 and 72 hours. Blaser and Newman (1982) reported an inverse relationship between the initial infectious dose and the incubation period before symptoms arise in people. They also discussed *Salmonella* species serovars, host-adapted specifically for humans, such as *S. Typhi*, have lower infectious doses than nonadapted serovars like *S. Enteritidis*. The infectious dose in humans ranges from 100-1000 (10^2 - 10^3) cells from human adapted serovars. Both *S. Typhimurium* and *S. Enteritidis* are not host-adapted, enabling them to cause infection in both humans and a wide range of animals (Agbaje et al., 2011). The vehicle of transmission, whether it be oral (food, water, person-to-person contact) or even aerosol, can vary the infectious dose needed to cause illness (Blaser and Newman, 1982). Symptoms of salmonellosis include the following: fever, diarrhea (sometimes bloody) and cramps. In rare cases, bacteremia (presence of bacteria in the blood) and focal infection throughout the body (meningitis, arthritis, cholangitis, and pneumonia) can occur (Hohmann, 2001). Characteristics of the human host, like age, immuno-compromised individuals (ex. kidney stones, HIV/AIDS, gallstones, diabetes), taking antibiotics or antacids, being pregnant, or strength of the immune system (previous exposures to *Salmonella* infection) can increase a person's susceptibility to infection (Blaser and Newman, 1982; Hohmann, 2001). The ability of salmonellae to invade epithelial cells in the mucosal lining of the intestines is essential to the bacterium's virulence (Durant et al., 1999).

Salmonella species colonize a wide array of hosts, including all of the major livestock species (poultry, cattle, and swine) asymptotically, leading to direct contamination of product from those animals (Newell et al., 2010). Jay et al. (2005) indicated that the holding pens during the slaughtering process and hatcheries were significant sources of *S. Enterica*. Food workers boots were also found to be an abundant niche Jay et al. (2005).

Additional Factors

The moisture content of a food has been shown to inversely affect the thermal resistance of bacterial contamination in food. Data from Liu et al. (1969) demonstrated higher D values (minutes required to kill 90% of a population) with samples of bone meal and a chicken starter feedstuff with higher moisture content (4.5-5.2 versus 9.0 and 9.8% moisture) inoculated with *S. Senftenberg* (775W). The heat resistance of *S. Senftenberg* also has been demonstrated in studies by Ng et al. (1969) and Orta-Ramirez et al. (1997).

Bacteria present on the surface of meat products can migrate deeper into the product with or without the aid of a vacuum. Factors that affect bacterial migration include proteolytic activity and a_w . Increasing the water content (a_w) of food products can increase bacterial penetration into food products. This finding is a concern, especially since *Salmonella* are motile bacteria (Warsow et al., 2008).

Outbreaks

Majowicz et al. (2010) estimated the global burden of non-typhoidal *Salmonella* by synthesizing data from literature, special studies, and laboratory-based surveillance, and extrapolated the information to countries where surveillance was lacking/non-existent. The authors concluded that an estimated 93.8 million cases of infections occur per year, with 80.3 million (86%) of those cases being foodborne related, resulting in 155,000 deaths. These estimations indicated that *Salmonella* species infections are responsible for ~3% of total illness worldwide. In the United States, foodborne illness is responsible for an estimated 9.4 million illnesses, 55,961 hospitalizations and 1,351 deaths in a given year (Scallan et al., 2011). Non-typhoidal *Salmonella* species led all bacteria, with 11% of total cases, 35% of all hospitalizations and 28% of deaths (Scallan et al., 2011). Of all the cases of salmonellosis in a typical year, 95% are foodborne related; salmonellosis also accounts for nearly 30% of foodborne related deaths yearly (Hohmann, 2001).

In 2013, the Foodborne Diseases Active Surveillance Network (FoodNet) reported 7,277 cases of salmonellosis, resulting in 2,003 (28%) hospitalizations and 27 (0.4%) deaths. Of the 6,520 (90%) serotyped *Salmonella* isolates, the top two were Enteritidis (1,237; 19%) and Typhimurium (917; 14%). Compared with the 2010-2012 data, the estimated number of incidences for *Salmonella* was significantly lower in 2013, but similar to 2006-2008 estimates (Crim et al., 2014). The number of salmonellosis incidences, for the most part, has not changed

over the past decade; but the serovars responsible and the food sources are constantly changing (Jackson et al., 2013). In 2009, 20 serovars encompassed >82% of all the ~36,000 serotyped, human-derived *Salmonella* isolates reported to the Center for Disease Control (CDC) (Jackson et al., 2013). Between 1998 and 2008, 1,491 outbreaks of *Salmonella* were reported, with 1,193 cases (80%) caused by single serovar. *S. Typhimurium* was one of the top serovars, comprising 14% of the total cases. Of all the food commodities identified as the sources of those outbreaks, chicken, pork, and beef led with 64 outbreaks (16%), 37 outbreaks (9%) and 33 outbreaks (8%), respectively (Jackson et al., 2013). Even though *S. Enteritidis* is strongly associated with eggs and broiler chickens, a study by White et al. (2007) isolated the bacterium from 3.13% (2 of 64) of ready-to-eat meat and poultry products and 0.47% (18 of 3,839) of ground beef samples between 1998 and 2003. The main serovars identified in this study were *Enteritidis*, *Typhimurium*, and *Heidelberg*. Scharff (2012) used data from 2010 and estimated the cost of *Salmonella* (non-typhoidal) illnesses in the U.S., based on cost of medical care, productivity lost, quality of life and several other factors. It was estimated that each case of salmonellosis cost \$11,086, with a total cost of \$11.4 million nationwide.

S. Typhimurium, *Montevideo* and *Senftenberg* are significant pathogens, with respect to foodborne illness. An outbreak in England of small German salami sticks was linked to *S. Typhimurium* DT 104 (Cowden et al., 1989). *S. Typhimurium* was found responsible for 26% of all chicken, 18% of beef, 14% of turkey, 56% of dairy, and 16% of tomato *Salmonella* species outbreaks in a review by Jackson et al. (2013), between the years 1998 to 2003.

Outbreaks have been linked to contaminated spices and herbs, emphasizing the organism's ability to survive in low a_w conditions (Zweifel, et al., 2012). In 2007, *S. Senftenberg* was linked to an outbreak of contaminated fresh basil, resulting in 51 cases in the United Kingdom, Denmark, and the United States. The source of the contamination was linked to a producer in Israel (Pezzoli et al., 2008). In 2010, a nationwide outbreak of *S. Montevideo* in a ready-to-eat salami product, identified black and red pepper as the source of the contamination, resulting in 272 cases, in 44 states and over 640,000 kg of product recalled. *S. Senftenberg* was also linked to this 2010 outbreak (Liu et al., 2011; Zweifel, et al., 2012). *S. Montevideo* has been attributed to >80% of cases associated with animal-related products, while *S. Senftenberg* was found primarily in turkey and chicken, as well as in plant-related outbreaks (Jackson et al., 2013).

It is important to recognize that reported cases represent only a fraction of the total cases, since the majority of people who get sick do not seek medical attention. This finding makes estimation of foodborne illness extremely difficult (Majowicz et al., 2010). In order for an illness to be ascertained and reported, the ill person must seek medical attention, a specimen must be taken and submitted to a laboratory for testing, the specimen must be identified and the illness and cause must both be reported to health authorities. If even one of these steps is not accomplished, then the case goes undiagnosed (Scallan et al., 2011).

Acid Tolerance Response

The study of *S. Typhimurium* and its resistance/ tolerance to antimicrobials, such as organic acids and salt, has been reported over the past 25 years. The literature uses three terms, almost interchangeably, to describe an organism's response to highly acidic environments: acid resistance, acid tolerance, and acid habituation. It is difficult to determine if the terms are truly different or synonymous (Bearson et al., 1997). Rowbury et al. (1995) defines the terms separately. "Tolerance and resistance are used to indicate survival ability plus potentially lethal acidity, and acid habituation is used to describe induction of tolerance." Acid stress itself is defined as "the combined biological effect of low pH and weak (organic) acids present in the environment" (Bearson et al., 1997). In order to survive encounters with stressful conditions, bacteria sense disturbances in their surrounding environment, such as a change in pH or a lack of nutrients, and undergo a pre-programmed molecular response where specific proteins are synthesized in order to prevent or repair damage to the cell. Some stress response proteins are very general, while others are created in order to aid in the survival against specific stresses. *S. Typhimurium* is a bacterium that adapts very well (Bearson et al., 1997). Even though *S. Typhimurium* is considered a neutralophilic bacterium, it can maintain its internal pH over a wide range of pH levels, suggesting a very complex system at work to maintain the internal pH of the cells at lethal acidic levels (Figure 1.4) (Foster, 1991).

A specific pattern of gene expression, known as 'acid tolerance response' (ATR), is a way for microorganisms to survive weak acid preservatives and low pH levels. This system has been demonstrated in *S. Typhimurium* and has enabled this bacterium to survive encounters with pH levels as low as 3.0 (Foster, 1991; Foster and Spector, 1995). *S. Typhimurium* possesses several low pH-induced survival systems in both the stationary and exponential phases of growth (Foster and Spector, 1995; Bearson et al., 1997). The ATR is thought to not only protect

against general low pH environments, but also to amend the effects of weak acids on the cell. It is speculated that the ATR is closely related to the starvation stress response system, since acid resistance is linked to resistance against heat and osmotic stresses (Foster and Hall, 1991; Foster and Spector, 1995).

The acid tolerance response is composed of several complex systems that overlap between the stationary and logarithmic phases of growth. The first stage of the ATR is a pre-shock adaptation at mildly acidic pH (5.5–5.8), where one or two generations of cells are created. This step is followed by a post-shock adaptation at a more acidic pH (≤ 4.4). Each stage separately can protect against acidic stresses, but joining them is far more effective (see Figure 1.5). This acid adaptation step alters the metabolism, enhances the internal pH homeostasis, and maintains viability of the cell (Foster, 1991; Foster and Hall, 1991; Leyer and Johnson, 1993).

S. Typhimurium grown in media with a pH of 7.7 experienced rapid cell death when suddenly exposed to media with a pH of 4.0; however, if one or two generations is first exposed to a mildly acidic (pH 5.8) medium, a tolerance is developed. This exposure to a sub-lethal, mild level of acidity (pre-acid shock), gives *S. Typhimurium* a chance to alkalize its cytoplasm, synthesize acid shock proteins, and better prepare itself for more acidic environments (post-acid shock) (Foster and Spector, 1995; Bearson et al., 1997). Both steps in the ATR, though they work in conjunction, are thought to be very different, since dramatic effects on one only produces a mild effect on the other (Foster and Spector, 1995). Foster (1991) concluded that both the synthesis of acid shock proteins and the pre-shock ATR were necessary for *S. Typhimurium* to generate viable cells with full acid tolerance at pH levels ≤ 4.0 . Strains grown in non-lethal levels of organic acid to induce the ATR did not show enhanced resistance when exposed to lethal levels of those same organic acids (Baik et al., 1996). Additionally, Chung and Goepfert (1970) also concluded that subsequent exposure to acidic pH levels will not give *Salmonella* the ability to grow and flourish in that environment. The ATR also appear to be dependent on the presence of several amino acids (glutamate, arginine and lysine) and decarboxylase enzymes (Bearson et al., 1997; Cotter and Hill, 2003), which work to consume excess protons in the system, as well as the ferric uptake system (FUR). The FUR does not appear to be dependent on the presence or absence of iron, but rather the pH of the environment (Foster and Hall, 1990).

Research has also shown a connection to the Mg^{2+} -dependent proton-translocating ATPase system which helps contribute to the acid tolerance of the cell (Foster, 1991; Foster and Hall, 1991).

Studies of Acid Tolerance Response

Numerous studies have been published testing how the ATR manifests itself, how different acids affect its development and how the development of the ATR at different phases of growth contributes to its efficacy. Foster and Hall (1991) demonstrated differences in internal pH values of *Salmonella* cells 0.2-0.9 pH units between acid adapted and non-adapted cells in pH levels 7.5 to 3.3. Their study also determined that non-adapted *Salmonella* cells lose their viability when internal pH reaches 5.5. Baik et al. (1996) looked at the ATR system of ten *S. Typhimurium* strains and how they responded to organic acids (benzoic, propionic, acetic and a mixture of all three) at the sub-lethal pH level of 4.4. The authors compared acid-adapted (exposed to pH 4.4 for 1 hour) and non-adapted cells at both the exponential and stationary phases of growth. Results demonstrated that all exponential phase acid-adapted strains resisted all three organic acids (propionic, acetic, benzoic) while the stationary phase acid-adapted cells were resistant to propionic and acetic acid but not benzoic. This finding suggests that different mechanisms in acid tolerance are present between exponential and stationary phases of growth. The results also imply that inhibition by organic acids goes beyond just the acidification of the cell's cytoplasm and that variances in inhibition exist between the anion activities of organic acids (Baik et al., 1996). Durant et al. (1999) demonstrated *S. Typhimurium* grown in the presence of propionate and butyrate short-chain fatty acids (SCFA) (25-100 mM) at pH 6.0 and 7.0 resulted in decreased invasion of epithelial cells, while growth in acetate increased cell invasion. Growth of *S. Typhimurium* was decreased when comparing the cells grown with SCFA and those that weren't. Populations also were lower at pH 6.0 than 7.0, highlighting the fact that the pH level of the system directly affects the lethality of organic acids (Durant et al., 1999). A study by Casadei et al. (2001) aided in the confirmation that low pH is more damaging at higher temperatures. A 4- \log_{10} reduction in *S. Typhimurium* was achieved at both 4.7 hours at 37°C and approximately 22 hours at 25°C at pH 3.0 (Casadei et al., 2001). This finding contrasts data from a study by Álvarez-Ordóñez et al. (2010) where *S. Typhimurium* cells grown at higher incubation temperatures (25-45°) survived longer than cells grown at lower temperatures (10°C).

Greenacre et al. (2003) examined the development of the ATR in *S. Typhimurium* and *L. monocytogenes* using acetic and lactic acidulants and then exposed the bacteria to HCl (pH 3.0) at 20°C. Differences were observed in the time (hours) required to reach an optimum ATR level between bacteria, pH of pretreatment (5.8, 5.5, 5.0) and acid used in the pretreatment step. Overall, both bacteria demonstrated increased viability to HCl (pH 3.0) when pretreated with acetic acid at pH of 5.5, as compared to non-acid pretreated (control) cells. *S. Typhimurium* experienced optimal ATR when pretreated with acetic acid for two hours and three hours with lactic acid. *L. monocytogenes* had best results with a three hour pretreatment of acetic acid and two hours with lactic acid. This study validated the idea that organic acid adaption provides bacteria protection against inorganic acid stress and that link between a bacteria's adaptation time and how well it can maintain its internal pH is present. It also brings attention to the fact that differences in the ability of an organic acid to inhibit bacteria are dictated by the membrane of the bacteria (Gram positive or negative) (Greenacre et al., 2003).

Koutsoumanis et al. (2004) tested five strains of both *L. monocytogenes* and *S. Typhimurium* by exposing the bacteria (at stationary phase) to acid pretreatment, ranging from pH 6.0 to 4.0 at 30°C for 90 minutes, then exposing them to tryptic soy broth (TSB) at pH 3.5 and assessing cell survival. Non-acid adapted *S. Typhimurium* cells were significantly ($p < 0.05$) more sensitive to the acidic treatment than non-adapted *L. monocytogenes* with cells being undetectable after two hours. Exposure to pH levels 4.0 to 5.5 resulted in increased acid resistance, while no significant ($p > 0.05$) protection was provided by exposure to pH 6.0. *L. monocytogenes* experienced the greatest acid resistance with pretreatment at pH 5.5, while pH 4.5 yielded the best survival for *S. Typhimurium*. Results of the study reveal that different pH levels of acid pretreatment directly affect the acid tolerance of bacteria (Koutsoumanis et al., 2004).

Xu et al. (2008) took *S. Enterica* cells, adapted cells to pH 5.0 with acetic acid at various times, exposed them to HCl at pH 4.0 and measured viability at various temperatures, ranging from 0 to 20°C. The estimated recovery of sub-lethally injured cells was done by comparing the counts between a non-selective tryptic soy agar (TSA) and a selective xylose lysine desoxycholate (XLD). Cell injury is identified by the inability of a microorganism to proliferate under certain conditions. One way of measuring this phenomena is to observe the lack of growth on a selective media and compare it to growth of the same culture on a nonselective media (like

TSA). The difference between the two is the population of injured cells (Ahamad and Marth, 1990; Jay et al., 2005). Both non-adapted and adapted cells were more sensitive to the acidic environment at higher temperatures and demonstrated less recovery when later enriched. In general, cells allowed to adapt to the pH 5.0 environment longer exhibited increased viability and recovery (Xu et al., 2008). Sub-lethally injured cells, if provided more favorable growth conditions, may repair sub-lethal damage and regain viability and pathogenicity. Alternatively, some injured cells do not appear to grow, resulting in a viable, non-culturable (VBNC) state. This observation makes *S. Enterica* a dangerous and unpredictable bacterium (Xu et al., 2008). VBNC cells take on a coccoidal shape and despite their reduced levels and the inability to display growth, they retain their virulence and have been shown to recover (Jay et al., 2005). Álvarez-Ordóñez et al. (2009) used several different organic and inorganic acids (pH 6.4, 5.4 and 4.5) to induce the ATR in *S. Typhimurium*, and they concluded that pretreatment with citric acid > acetic > lactic > malic ≥ hydrochloric > ascorbic for ATR.

Though spraying with organic acids (lactic, acetic, etc.) of carcasses immediately following the slaughtering process results in a 1 to 3-log₁₀ reduction in surface bacteria, it also may contribute to the rise in acid tolerant strains of pathogenic bacteria. It has also been shown that the pH of a carcass surface over time will rise due to the buffering capacity of the tissue. The increase in favorable conditions could result in a resurgence of bacteria that tolerated the acidic spray treatment (Álvarez-Ordóñez et al., 2009). Álvarez-Ordóñez et al. (2012) noted changes in the fatty acid content of membranes between non-acid adapted and acid adapted *S. Typhimurium* cells (see Figure 1.6).

Listeria monocytogenes

Background Information

L. monocytogenes is a Gram-positive, non-spore forming aerobic bacterium that was first described in a detailed publication by Murray et al. (1926). *L. monocytogenes* is considered psychrotrophic because it can grow at refrigeration temperatures (Juneja and Davidson, 1993). *L. monocytogenes* is also ubiquitous and can be found almost everywhere including plants, soil, sewage, water, air samples, livestock, animal carcasses and food manufacturing facilities (see Figure 1.7) (Gray and Killinger, 1966; Audurier and Martin, 1989). While *Salmonella* and *E. coli* are most notably associated with pre-mortem and post-mortem animal carcasses, *Listeria monocytogenes* is known to thrive in the processing room on equipment, counter and table

surfaces, in ventilation systems and in floor drains, even at refrigeration temperatures (Nel et al., 2003).

Infection and Symptoms

L. monocytogenes is one of six species in the genus *Listeria*, but it is the only one that is considered to be pathogenic (Todd and Notermans, 2011). Within *L. monocytogenes* resides 13 serovars with 1/2a and 4b being the most relevant regarding foodborne illness. The vast majority of human infections are caused by the same serotypes of *L. monocytogenes* (4b, 1/2a and 1/2b) (Lianou and Sofos, 2007). 1/2a and 1/2b are most commonly isolated from meat and dairy products, while 4b is more closely linked to human cases of listeriosis (Jay et al., 2005). Listeriosis was first seen in livestock, specifically sheep, in the early 1920s. It was given the name “circling disease” because sickened animals would continuously walk in circles (Gray and Killinger, 1966). There are two variants recognized today. The first is the invasive form that targets susceptible populations and causes infection of the central nervous system, sepsis, and still birth. The second, non-invasive form, mainly causes febrile gastroenteritis. This illness is characterized by fever, sore throat, diarrhea and in some cases muscle/ joint pain, with an incubation period between 11 hours to seven days after exposure (Borch and Arinder, 2002; Sim et al., 2002; Todd and Notemans, 2011). Although *L. monocytogenes* is considered a ‘minor pathogen’ compared to *Salmonella* species and *Campylobacter* species, infection of this bacteria comes with high hospitalization and mortality rates (20-30%). It is an opportunistic bacterium, meaning it most often affects the very young, the elderly, pregnant or immuno-compromised (HIV/AIDS) patients. Listeriosis is notorious for causing meningitis in newborn infants and even abortions and stillbirths (Cole et al., 1990; Newell et al., 2010; Todd and Notemans, 2011). It is unique to other Gram-positive bacteria, because it is an intracellular pathogen in that the bacterium enters epithelial cells of its host where it resides in the cytosol and replicates (Jay et al., 2005). *L. monocytogenes* utilizes a unique set of virulence factors that enable the bacteria to aid in cell invasion, escape, and avoid host cell phagosomes and spread to other host cells (Cormac and Colin, 1999). *Listeria monocytogenes* has a long incubation period, which makes it difficult to identify the pathogen and its source in outbreak situations (Gandhi and Chikindas, 2007). Though the risk of listeriosis is higher with increased levels of contamination, the infectious dose of the bacterium is dependent on the food product, how much is consumed, the virulence of the strain, and the susceptibility of the host (Lianou and Sofos, 2007).

Growth Parameters

Studies accessing the growth parameters of *L. monocytogenes* all conclude that temperature, pH, a_w , and salt concentration all greatly effect each other. *L. monocytogenes* grows at a range of temperatures from 0.5 to 45°C (optimum of 37°C), growth between pH 4.4 and 8.0 (Jay et al., 2005), and when grown with sodium chloride, the lowest a_w growth of *L. monocytogenes* was 0.92. Tolerance to low a_w was decreased at lower temperatures (4°C) versus higher (30°C) (Tapia de Daza et al., 1991). The term osmoadaptation describes both the physiological and genetic manifestations that occur within a microorganism when exposed to a low a_w environment (Hill et al., 2002).

Presence in Meat Products

In terms of meat products, *L. monocytogenes* is most commonly associated with post-lethality contamination. Low initial levels of contamination in meat products can increase significantly during transportation and storage (Juneja and Davidson, 1993). *L. monocytogenes* can persist and linger in processing environments, despite routine cleaning and disinfecting procedures (Borch and Arinder, 2002). Though *L. monocytogenes* is well known for its presence in RTE meat products, it is also commonly isolated in raw beef, pork and poultry (Jay et al., 2005). A study by Levine et al. (2001) focused on testing for the presence of pathogens in ready-to-eat meat products at 1,800 federally inspected meat establishments throughout the U.S. between 1990 and 1999. An apparent trend between ready-to-eat products that required substantial amounts of post-lethality handling (peeling, slicing, repackaging, etc.) or addition of ingredients and high counts of *L. monocytogenes* were observed (Levine et al., 2001). Despite the many hurdles, like low pH, low temperature, low a_w , and high salt concentration utilized in the production of fermented sausages, *L. monocytogenes* is still a very real problem in the U.S. and other countries (Ravyts et al., 2008). Both *Salmonella* and *L. monocytogenes* counts in all ready-to-eat meat products tested (large-diameter cooked sausages; small-diameter cooked sausages; cooked beef, roast beef, and cooked corned beef; sliced ham and luncheon meats; small and large cooked sausages; jerky; fermented sausages; cooked poultry products and salads/spreads/pates) displayed a downward trend from 1991 to 1999, suggesting that the meat industry has made improvements in both plant sanitation procedures and control of product contamination (Levine et al., 2001).

Zero Tolerance Policy

Currently, USDA-FSIS has instituted a “Zero Tolerance Policy” which requires companies to recall any product in which *L. monocytogenes* is detected, because the bacterium is considered an adulterant (Gombas et al., 2003). Additionally, if *L. monocytogenes* is detected on a packaging line, then USDA-FSIS assumes that all product on that line is adulterated (Tompkin et al., 2002; Gombas et al., 2003). *L. monocytogenes* is considered by the USDA-FSIS as a pathogen that is “reasonably likely to occur” and therefore must be addressed in Hazard Analysis Critical Control Point (HACCP) plans (Tompkin et al., 2002).

Outbreaks

In 2013, FoodNet reported 123 cases involving *L. monocytogenes* with 112 (91%) hospital visits and 24 (19.5%) deaths. These numbers do not differ from 2006-2008 or 2008-2010 estimates. Incidences were highest among people ≥ 65 years old (Crim et al., 2014). *L. monocytogenes* was the third leading cause of death (19%) in a study by Scallan et al. (2011) behind *Salmonella* species (28%) and *Toxoplasma gondii* (24%). Several large outbreaks in the early 1980s associated with *L. monocytogenes* compelled the FDA and the USDA-FSIS to establish policies that address the presence of *L. monocytogenes* in RTE foods (Gombas et al., 2003). Listeriosis carries with it an estimated cost of \$1,282,069 per case and a total cost of \$2 million (Scharff, 2012).

In a review by Lianou and Sofos (2007), several *L. monocytogenes* outbreaks associated with meat and poultry products were mentioned. Products included: hotdogs, delicatessen meats, minced meat, raw beef, raw pork, raw poultry, pate and other meat spreads, corned beef, pastrami, ham, fermented sausages, liver sausages, and salami (CDC, 1989). From June 1997 to July 2002, *L. monocytogenes* was cultured and identified in over 100 different RTE meat, dairy and seafood products throughout the state of New York and compared to isolates from over 30 clinical cases of listeriosis from August 1998 to August 2002. Results demonstrated that nearly 20 *L. monocytogenes* ribotypes isolated from the RTE products matched human isolates and that virulent strains are present on a number of products and have the potential to cause more outbreaks. Over half of the ribotypes found in both categories were labeled as persistent strains. Identical strains from the same or different products were often isolated months, even years apart (Sauders et al., 2004).

Sim et al. (2002) highlighted several outbreaks involving non-invasive *L. monocytogenes*. Products included leftover corned beef, luncheon ham, and corned silverside. All cases reported *L. monocytogenes* populations as high as 5-log₁₀ CFU/g of product, indicating that although the initial contamination levels were unknown, the levels most likely grew during storage. In a survey by Gombas et al. (2003), over 31,000 samples of deli salad and luncheon meat throughout Maryland and California were taken. The authors found a prevalence rate of 1.82% for *L. monocytogenes* in those products. Gianfranceschi et al. (2006) found the prevalence of *L. monocytogenes* at 22.7% in over 1000 samples of RTE salami, despite the products low pH, low a_w, and addition of competitive LAB. The same *L. monocytogenes* strains were isolated year round, regardless of the season. Angelidis and Koutsoumanis (2006) collected over 200 samples, representing 16 different types of meat products from various markets in the city of Thessaloniki, Greece. Of these, 8.1% (17) of the samples tested positive for *L. monocytogenes*, with the majority of samples containing <10 CFU/g.

Two publications (Olsen et al., 2005 and Gottlieb et al., 2006) discuss multistate outbreaks of *L. monocytogenes* linked to turkey deli meat. In those two publications, the USDA-FSIS reported that 83% of foodborne listeriosis cases derived from the consumption of deli meat sliced at retail (estimated 919.6 cases with 166.9 deaths). The combination of *L. monocytogenes* and luncheon meats was also reported as the number one cause of foodborne-related death in the U.S. (Todd and Notemans, 2011). Endrikat et al. (2010) wrote that “retail-sliced RTE meat and poultry products are almost five times more likely to cause listeriosis than are prepackaged products on a per [cent] basis, and over four times more likely on a per serving basis.” Additionally, the FDA ranked deli meats, frankfurters and pates and meat spreads as the top three highest risk per serving of RTE foods for listeriosis (Todd and Notemans, 2011).

Post-Process Contamination, Persistence and Biofilms

Post-process contamination by *L. monocytogenes* is one of the leading causes of foodborne outbreaks, followed by insufficient hygiene, cross-contamination, processing or storage in inadequate rooms, contaminated equipment and contamination by personnel/ food handlers (Reij and Aantrekker, 2004). Recontamination can occur through various routes in a food processing facility, including but not limited to raw materials/ ingredients, food contact surfaces, food processing equipment, cross contamination, personnel, pests (insects, birds and rodents), distribution/ handling (by workers or consumers), defective equipment or packaging,

and ventilation or drain systems (Reij and Aantrekker, 2004). Low levels of contamination present on fingers, clothes, knives, and utensils or food contact surfaces have been shown to result in levels of bacterial transfer, sufficient for illness (Lianou and Sofos, 2007). This finding indicates that food handlers play key roles in the prevention, as well as the contamination of food products.

One of the major sources of food contamination is food handlers. Poor personal hygiene of food handlers, such as forgetting to wash hands after using the bathroom or not wearing gloves, can result in as much as 10^7 pathogens (*Salmonella* species, *Shigella* species, *E. coli* O157:H7, *S. aureus*, and *Bacillus cereus*) on the hands and under the fingernails. Proper education, communication and management practices are vital to ensure public safety (Nel et al., 2003). This problem most definitely applies to the meat industry. Both fresh and processed meat products are extremely susceptible to contamination and can harbor many species and strains of microorganisms (Nel et al., 2003).

Persistence of *L. monocytogenes* on equipment and in the processing environment of meat plants was demonstrated by numerous researchers (Senczek et al., 2000; Lunden et al., 2002; Tompkin et al., 2002; Lunden et al., 2003; Martin et al., 2014). In these studies, similar pulsed-field gel electrophoresis (PFGE) patterns of *L. monocytogenes* were found in the same areas of the meat processing plant or on the same equipment, despite continuous dismantling, cleaning and sanitizing procedures. The design and compartmentalization of the facility and the separation of raw and processed product also plays an extremely important role in the prevention of cross contamination (Lunden et al., 2003). Sheen et al. (2008) demonstrated that during the slicing process, high levels of contamination are capable of being transferred from contaminated knives or slicers to product or vice versa.

It is no wonder that RTE foods can become contaminated with *L. monocytogenes*, when one considers all of the further processing and exposure that occurs after cooking. Tompkin et al. (2002) determined there were three scenarios that could lead to foodborne listeriosis. Scenario one involves isolated cases of illness where the information about the origin of the food is not readily available. Symptoms do not arise in individuals until weeks after consumption, making it difficult to pinpoint which food item was the source. Scenario two consists of an outbreak or a cluster of related cases from which a single source or lot as the cause of the illness. Typically, these events are the cause of an error during food manufacturing, handling, or transporting.

Significant periods of time may elapse before the problem is detected, thus increasing the number of contaminated products sold to consumers. Once a specific lot is recalled and disposed of, illness ceases. Scenario three entails outbreaks that contain hundreds of reported cases of illness that are scattered, regardless of location or time. These types of outbreak involve particularly virulent strains that have become well established in the processing environment and have contaminated several lots of product over a long period of time. In all of these scenarios, the food is contaminated before it is eaten (Tompkin et al., 2002).

Environmental testing has proven to be a more effective and less expensive practice than product testing for determining the source of *L. monocytogenes* contamination, though both should be done in conjunction with one another. Continuous testing will allow manufacturers to narrow down the location of the contamination (niches), as well as identify when it arose. Short-term and long-term assessments are vital in detecting trends or increases in contamination (Tompkin et al., 2002).

A specific area or site within a processing facility where reservoirs of microorganisms establish themselves and persist is called a 'niche'. These sites are typically hard to reach during cleaning and sanitizing procedures or in places that never receive attention during sanitation. Examples of niches include on-off valves and switches for equipment, worn or cracked rubber seals around doors, spaces between close-fitting metal-to-metal or metal-to-plastic parts, piping and hoses, fans or blowers and floor drains. (Tompkin et al., 2002). The ability of *L. monocytogenes* to persist and linger in food processing environments is attributed to its ability to attach to a variety of surfaces (Blackman and Frank, 1996; Sheen et al., 2008). The bacterium is able to position itself in grooves and crevices of otherwise smooth-appearing surfaces and attach and colonize. Mafu et al. (1990) studied the attachment time and growth of *L. monocytogenes* on stainless steel, glass, polypropylene, and rubber. The researchers found that increased contact time (20 minutes vs. 1 hour) resulted in increased attachment (Mafu et al., 1990). Biofilms vary greatly, depending on the types of microorganisms, the surrounding environment, and how long it has been present. In general, biofilms are composed of water, exopolysaccharides (EPS) synthesized by the microorganism, components from lysed and dead cells, microorganism waste products, and other components from the environment. Biofilms have been shown to not only aid in the attachment of microorganisms to surfaces, but also provide protection from cleaners and

sanitizers by preventing access of the compounds to the cells, and diluting them to sub-lethal concentrations (Sutherland, 2001).

Effects of Acid, Salt and Temperature

L. monocytogenes has demonstrated the ability to grow at various pH levels, depending on the acidulant. Studies have shown the pathogen can grow in the presence of hydrochloric acid (HCl) at pH of 4.39 (at 30°C) (George et al. 1988), while growing at a minimum pH of 4.8 with lactic acid (Conner et al. 1986). Growth of *L. monocytogenes* at pH 4.4 also has occurred for multiple acids at multiple temperatures (Sorrells et al., 1989). Similar results were observed with regard to the efficacy of acetic acid and increased antimicrobial activity at lower pH levels and higher temperatures (Ahamad and Marth. 1990; Conner et al., 1990; George et al., 1996 and Vasseur et al., 1999).

Cole et al. (1990) reported that the growth of *L. monocytogenes* depended heavily on pH, salt concentration, and temperature. Low concentrations of salt also appear to have a stimulatory effect on the bacteria. A study by Buchanan et al. (1994) displayed a similar stimulatory effect with low levels of sodium chloride. Cole et al. (1990) also found that organic acids in general are more inhibitory to microorganisms than inorganic acids, and that each organic acid (acetic, propionic, lactic, etc.) can affect the growth of different microorganisms in different ways. Inorganic HCl was less effective than both lactic and acetic acid, suggesting the anions of organic acids have more antimicrobial action than inorganic anions (O'Driscoll et al., 1996). A study by Sorrells et al. (1989) tested various acids at different pH levels, incubation times, and temperature combinations. Results demonstrated that acetic acid had a higher antimicrobial activity than lactic, citric, malic, and HCl at temperatures of 10, 25 and 35°C, when pH was constant. This finding indicates that the low pK_a of acetic acid (4.75) increases its antimicrobial activity by having more acid present in the disassociated form at lower pH levels. In general, the lower the temperature (30, 10, 5°C), the more viable cells were recovered at lower pH and higher salt concentrations. Additionally, as temperature increased, so did the antimicrobial activity of all of the acids (Sorrells et al., 1989). *L. monocytogenes* cultures survived low pH and high salt growth conditions at 5°C than at 30°C, suggesting lower temperatures may provide the bacteria some protection from stresses (Conner et al., 1986).

Enrichments of *L. monocytogenes* also revealed that pH-injured cells in the presence of 4-8% salt recovered more rapidly than pH-injured cells in the absence of salt (Cole et al., 1990). *L.*

monocytogenes possess the ability to survive osmotic stress. The pathogen has even been detected after 150 days in pure salt stored at 22°C (Gnanou Besse et al., 2000). In this survival state, the bacterium shuts down most of its metabolic activity and focuses on the synthesis of stress proteins. According to Duche et al. (2002) synthesis of stress proteins typically begins between 30 and 90 minutes after exposure to osmotic stress.

Increasing concentrations of salt has been shown to increase the lag phase in *L. monocytogenes* (Vasseur et al., 1999). Sorrells et al. (1990) demonstrated that *L. monocytogenes* was capable of growing in TSB supplemented with 10% (w/v) sodium chloride at 35°C and in 12% sodium chloride at 25 and 10°C. A 4 to 5-log₁₀ decrease was observed between *L. monocytogenes* populations at 35-25°C and 10°C. Results again point to a protective effect that lower temperatures may have on *L. monocytogenes*. Buchanan et al. (1994) concluded that a temperature range between 10 and 12°C offered the best protection to *L. monocytogenes* when subjected to acid and salt stresses.

Response to Stresses

ATPase proton-translocation membrane systems, well known in *Salmonella* species, have been reported in Gram-positive bacteria like *L. monocytogenes*. This approach gives the pathogens the potential to survive acidic conditions by the removal of H⁺ out of the cell or in exchange for K⁺ (Davis et al., 1996; Cotter and Hill, 2003). Additionally, the presence of a lysine, arginine, and glutamate decarboxylases (GAD) system has been noted in *L. monocytogenes* strains. The GAD system consumes excess H⁺ by combining them with amino acids and negates the acidification process (Cotter and Hill, 2003).

Differences in stress response often vary from strain to strain. Dykes and Moorhead (2000) found that clinical isolates of *L. monocytogenes* were more tolerant to both acid and osmotic stresses than isolates from meat sources, indicating differences in virulence between the strains. Despite these differences, several of the same acid stress proteins appear when the bacteria are exposed to cold stress again linking the stress response systems (Lou and Yousef, 1997).

Osmotic stress promotes the uptake of carnitine and other compatible solutes (small highly soluble molecules which possess no net charge at physiological pH) like glycine, betaine, and proline with the intention of increasing inward pressure and preventing water loss to offset turgor pressure from the outside environment (Dykes and Moorhead, 2000; Duche et al., 2002;

Hill et al., 2002). A decrease in both the size of cells, as well as the internal pH (7.6 decreased to ~6.8-6.0) was observed by Fang et al. (2004), following exposure to high sodium chloride concentrations (1.5-2.5 M).

Changes in Cell Membranes

Intact and healthy membranes are vital to bacterial cells in that they perform important functions, like proton-motive force, nutrient acquisition and in general, acting as a barrier to the outside environment (Russell et al., 1995). Bacterial membranes, particularly the lipids, are strongly affected by changes in a_w (osmolarity), temperature, and pH (Russell et al., 1995). By changing the lipid composition (increasing saturation) in its membranes, *L. monocytogenes* increases resistance to salt and other antimicrobial preservatives (Juneja and Davidson, 1993; Gandhi and Chikindas, 2007). In response to decreased a_w in the external environment, *L. monocytogenes* increases the amount of anionic phospholipid and/or glycolipid in its membranes to combat the increase in charged and uncharged solutes. These changes are only observed when the salt concentration of the system is >2%; below 2%, the membrane lipid composition is relatively unaltered (Russell et al., 1995).

Acid Tolerance Response

L. monocytogenes, though a neutrophilic bacterium, can develop acid tolerance and survive acidic conditions (pH <4.0) (Kroll and Patchett, 1992). O'Driscoll et al. (1996) concluded that protein synthesis was a key component in the activation of the acid tolerance response (ATR) in *L. monocytogenes*. *L. monocytogenes*, when exposed to sub-lethal stress and its resistance to acid, heat treatments, and low pH is particularly disconcerting since consumer demand is shifting towards foods with less preservatives and more minimally processed, which may result in an increase in foodborne illnesses (Lou and Yousef, 1997; Hill et al., 2002). Cormac and Colin (1999) published data that suggest that an increase in acid tolerance of *L. monocytogenes* cells does not increase the virulence (the ability of an organism to invade and infect a host) of the bacterium. Development of the ATR in *L. monocytogenes* may confer resistance to other factors, like heat and osmotic stress, implying that these strategies of survival are all linked (O'Driscoll et al., 1996; Phan-Thanh et al., 2000; Hill et al., 2002).

L. monocytogenes subjected to acid shock at pH 5 also expressed increased survival capabilities in media at pH 3.0, when compared to cultures grown at pH 7 (O'Driscoll et al., 1996). The same authors tested the development of an acid tolerance response in mid-

exponential phase cultures of *L. monocytogenes* by pretreating the pathogen with sub-lethal acid shocks at pH of 4 to 7 (HCl) and then exposed them to pH 3 (HCl). The authors determined a 3- \log_{10} decrease within 60 minutes in *L. monocytogenes* grown at pH 7 then exposed to pH 3.5 with HCl, lactic and acetic acid. A reduction of less than 1-log-unit was observed in *L. monocytogenes* cells pre-treated with pH 5.5 acid under the same conditions. An inverse relationship was detected between the acidity of the pretreatment and growth rates of the bacterium; as the pH of the acid shock decreased, so did the growth rates, suggesting while under stress, the bacteria are focused on survival not growth. All levels gave *L. monocytogenes* an increased acid tolerance; however pH 5.0, acid shock was shown to provide the strongest. A difference in acid tolerance between phases of growth was also noted (O'Driscoll et al., 1996). While exponential-phase *L. monocytogenes* required adaptation to survive lethal levels of acidity, stationary phase cultures were naturally tolerant, suggesting ATR depended on the growth phase of the bacteria. The acid tolerance expressed by cells pre-treated with pH 5.0 was shown to be stronger than other pre-treatment levels in a similar study by Lou and Yousef (1997). In addition, Koutsoumanis et al. (2003) found that a similar acid shock at 30°C induced a stronger ATR than at 15 and 4°C, concluding that the ATR is affected by temperature. The presence of sodium chloride >10% (w/v) also resulted in decreased ATR. Davis et al. (1996) also determined that the ATR system in *L. monocytogenes* is influenced by exposure time (0 to 60 minutes) to acid shock since increased viability of cells with increased exposure time was observed.

Phan-Thanh et al. (2000) found that maximum acid tolerance was achieved when cells were pre-treated with pH 4.8 or 5.5 (HCl) for 2 to 3 hours. A longer adaptation time resulted in a weakened acid tolerance. The tolerance also was conserved for several weeks if the cells were maintained at 4°C (Phan-Thanh et al., 2000). Following a 30 minute treatment of various acids at pH 3.8, *L. monocytogenes* displayed a survival rate of 96% in HCl (intracytoplasmic pH 4.46) and only 31% in acetic acid (intracytoplasmic pH 3.58) (Phan-Thanh et al., 2000).

Staphylococcus aureus

Background Information

The *Staphylococcus* genus consists of over 50 species, with *Staphylococcus aureus* being the most notable for causing human illness and disease (Hennekinne et al., 2012). *S. aureus* is a spherical, nonsporulating, non-motile bacterium that, when observed under the microscope,

occurs in pairs, short chains or grape-like clusters. These Gram-positive, halotolerant, facultative-anaerobic bacteria that typically grow more rapidly under aerobic conditions (Smith et al., 1983; Hennekinne et al., 2012). *S. aureus* is a member of the Micrococcaceae family that is distinguished from other staphylococcal species by its gold pigmentation of its cocci colonies and positive results of coagulase, mannitol-fermentation, and deoxyribonuclease tests (Lowy, 1998). Classification of staphylococci is determined based on the ability to produce coagulase, an enzyme that clots blood plasma (Chapman et al., 1934; Cruickshank, 1937). Coagulase-positive staphylococci (CPS) are recognized for their involvement in staphylococcal food poisoning (SFP) (or food-intoxication syndrome) than the noncoagulase-producing strains, called coagulase-negative staphylococci (CNS) (Ho, 1989; Hennekinne et al., 2012).

S. aureus was the first foodborne pathogen in which its mode of pathogenicity was fully recognized and established. Research on the pathogen dates back to the late 1800s, but was not proven conclusive until the 1930s (Dack et al., 1930). *S. aureus* strains can be classified into biotypes according to their human or animal origin. Based on biochemical characteristics, six different biotypes were developed. Human, non- β -hemolytic human, avian, bovine, ovine, and nonspecific, which are associated with multiple hosts (Devriese, 1984). Later, a poultry-like “slaughterhouse” biotype, strongly associated with meat workers, was added. There are strong similarities between the *S. aureus* biotypes of poultry and humans (Isigidi et al., 1990).

S. aureus is host-adapted to domestic animals, as well as humans and is commonly harbored in the nasal passages and on the skin of large portions of the human population, cattle, and swine. It is versatile in that it can cause a wide assortment of diseases in varying degrees of severity ranging from light skin infections to pneumonia and septicemia (Lowy 1998). The largest numbers are found on the body surface and near openings such as nares, axillae, and areas of moisture, in addition to the hands/arms. As far as food contamination goes, *S. aureus* is strongly associated with protein-rich foods that experience inadequate handling or temperature abuse (Jay et al., 2005). The presence of staphylococci in raw meat and the known heat resistance of enterotoxins present problems in heat-processed meat products (Palumbo et al., 1977).

Generally, *S. aureus* does not compete well with other microorganisms in most food products and environments, though when conditions, such as decreased a_w or increased salt concentration inhibit the growth of other microorganisms, *S. aureus* can flourish. Moreover, it is

capable of producing high-affinity iron binding siderophores that can scavenge for free iron or remove iron from other microorganisms and its host organism (O Clements and Foster, 1999).

Growth Parameters

In order for *S. aureus* growth and enterotoxin production to occur, nutritional requirements, such as an organic source of nitrogen (amino acids), source of energy (carbohydrates), and sources of vitamins (thiamine and niacin), and minerals need to be met, along with adequate environmental conditions (Tatini et al., 1973). Table 1.2 summarizes all of the growth parameters. *S. aureus* is classified as a mesophilic bacterium, meaning it grows best at moderate temperatures, typically between 20 and 45°C (Jay et al., 2005). Some strains have grown as low as 6.7°C and as high as 114°C (Angelotti et al., 1961). Enterotoxin production occurs between 10 and 46°C (optimum between 40 and 45°C) (Smith et al., 1983). *S. aureus* has a growth range between pH 4.0 to 9.8, with an optimum pH between 6.0 and 7.0. With a general minimum a_w level of 0.86, it grows at a_w levels far lower than all other non-halophilic bacteria (Scott, 1953). The bacterium was shown to grow at lower a_w levels (0.86) under aerobic conditions than anaerobic (0.91) (Scott, 1953). It is commonly found growing in environments between 7 and 10% sodium chloride, with some strains growing in 20% salt environments (Jay et al., 2005).

Intoxication and Symptoms

Illness from *S. aureus* is unique from *Salmonella* species and *L. monocytogenes*, because it is an intoxication rather than an infection. SFP is caused by the ingestion of enterotoxins, not strictly the bacterium alone (Jay et al., 2005). *S. aureus* produces a heat stable, pepsin-resistant enterotoxin with an infectious dose as low as <1µg (Bergdoll, 1989). The symptoms of SFP include nausea, (projectile) vomiting, severe abdominal cramps, diarrhea, sweating, headache, body aches and weakness, and often a decrease in body temperature. These symptoms develop within 4 hours of ingestion, with a reported range of 1 to 6 hours. Symptoms are usually self-limiting, lasting between 24 and 48 hours, and the mortality rate is extremely low (Le Loir et al. 2003; Normanno et al., 2006). Wieneke et al. (1993) found only five deaths that were recorded as a result of SFP. Treatment of sick individuals consists of bed rest and maintenance of body fluids. Despite its seemingly mild course, this pathogenic bacterium causes considerable social and economic burdens worldwide every year (Normanno et al., 2006).

Hennekinne et al. (2012) surmised that there were five conditions that are required to induce SFP: “(1) a source containing enterotoxin-producing staphylococci: raw materials, healthy, or infected carrier; (2) transfer of staphylococci from source to food, e.g., unclean food preparation tools because of poor hygiene practices; (3) food composition with favorable physicochemical characteristics for *S. aureus* growth and toxinogenesis; (4) favorable temperature and sufficient time for bacterial growth and toxin production; and (5) ingestion of food containing sufficient amounts of toxin to provoke symptoms.”

Staphylococcal Enterotoxin

Only staphylococcal enterotoxins (SEs) that induce vomiting after oral administration in a primate model are classified as such. Related toxins that do not meet this requirement are classified as staphylococcal enterotoxin-like (SEIs). The SEs and (SEIs of *S. aureus* consist of 22 members (see Table 1.4). SEC (staphylococcal enterotoxin C) is comprised of several variants: SEC₁, SEC₂ and SEC₃, SEC ovine and SEC bovine variants. TSST-1, the toxic shock staphylococcal toxin, initially designated as SEF, lacks emetic activity (Argudin et al., 2010). All SEs are proteins composed of 18 amino acids with aspartic, glutamic, lysine, and tyrosine being the most abundant (Jay et al., 2005). SEs that demonstrate emetic activity have similar potency, with doses ranging from 5 to 30 µg/kg by intravenous (IV) route. Production of SEs in food products ranges from 50 to 800 µg/kg (Jay et al., 2005). Domestic animals have shown resistance to enterotoxin after subsequent oral doses, but this phenomenon does not appear to occur in humans (Bergdoll, 1972).

SEs are notorious for their resistance to heat and enzyme activity. SEC is reported as having the greatest heat resistance, followed by SEB, then SEA (Smith et al., 1983; Tibana et al., 1987). Conditions, such as decreased temperature or pH, that sometimes inhibit enterotoxin production, appear to not affect staphylococcal growth (Rienamm et al., 1972). In their active states, the enterotoxins are resistant to a wide variety of proteolytic enzymes, but sensitive to pepsin at pH 2.0 (Bergdoll, 1967).

Czop and Bergdoll (1974) concluded that production of enterotoxins A and B occurred during all phases of growth, with the highest yields evident in the stationary phase. Those authors also indicated that the synthesis of enterotoxin B coincided with the synthesis of cellular proteins. This finding agrees with research conducted by Siems et al. (1971) who indicated possible links to SE production and de novo protein synthesis. High staphylococcal numbers

cannot be used as a reliable indicator of enterotoxin formation, as the two actions are somewhat independent of one another (McCoy and Faber, 1966). The lowest amount of *S. aureus* cells required to produce the minimum amount of enterotoxin necessary to cause SFP in humans differs greatly, depending on the food product and particular type of enterotoxin (Jay et al., 2005).

SE production was observed as early as 4 hours when cultures of *S. aureus* were introduced to fresh media (McLean et al., 1968). Enterotoxin B production was first detectable in aerobically grown cultures (37°C) after 5 hours of incubation near the end of the exponential growth phase and continued to steadily increase for 16 to 20 hours even after growth of the *S. aureus* cultures had ceased. This finding suggests that enterotoxin production is not bound to cell growth (McLean et al., 1968; Czop and Bergdoll, 1974). Injured *S. aureus* cells do not show synthesis of SEs, yet when they are removed from a stressful environment, the cells undergo repair and are able to reinitiate growth and SE synthesis (Collins-Thompson et al., 1973).

SEA production occurs under nearly all conditions that allow growth of *S. aureus*. SEB production is sensitive to a_w and does not grow below 0.93; SEC has similar sensitivity to a_w as SEB and is also sensitive to temperature; SED is very similar to SEA in that it will not be produced under a_w level 0.86. Moreover, SEA appears to be less sensitive to pH change than SEB (Notermans and van Otterdijk, 1985; Ewald and Notermans, 1988). SEA has been detected from as few as $\sim 10^4$ CFU/g of *S. aureus* (Hirooka et al., 1987); other studies have reported production of SEs in meat products when counts ranged between 10^6 to 10^8 CFU/g (Tompkin et al., 1973; Notermans and van Otterdijk, 1985; Jay et al., 2005). The highest level of staphylococcal growth in meat samples was reported between 10^7 and 10^8 cells, with a yield of enterotoxin B and C being 70 and 20 μg , respectively by Genigeorgis et al. (1971b).

Of all the known enterotoxins, type A (SEA) is responsible for most staphylococcal food poisoning (Tompkin et al., 1973). Wieneke (1974) found enterotoxins B and C to also be strongly associated with cooked food products and strains of *S. aureus* from human beings. Conversely, enterotoxin D had stronger links to raw products and *S. aureus* strain of animal origin. Terayama et al. (1972) tested for enterotoxin production (A-D) in food samples and found that 83% of the *S. aureus* strains were positive. The authors also tested strains of staphylococci of human origin from nasal and finger swabs and feces, resulting in 95% positive for enterotoxin production.

Mode of Action

All SEs are considered bacterial super-antigens (SAgs). These are most likely produced by pathogenic bacteria and viruses as a defense mechanism against the immune system of their host (Jay et al., 2005). Unlike conventional antigens, SAgs are able to bypass antigen-presenting-cells recognition and interact with T-cell receptors directly. This finding leads to the activation of large numbers of T-cells, followed by proliferation and release of chemokines and pro-inflammatory cytokines that leads to potentially lethal toxic shock syndrome (Argudin et al., 2010; Hennekinne et al., 2012). The ability of SEA and other SEs to interfere with the functioning of the immune system indicates that there may be a suppression of immune response occurring after an episode of food poisoning. This occurrence would further suggest that ingestion of *S. aureus* enterotoxins may have negative health consequences beyond acute food poisoning (Smith et al., 1983). It has been proposed that SEs stimulate the vagus nerve in the abdominal viscera, which in turn transmits the signal to the brain that induces a vomiting reaction. In addition to this, SEs are capable of penetrating the lining of the gut and activate local and systemic inflammation and immune responses (Argudin et al., 2010; Hennekinne et al., 2012). The diarrhea associated with SE intoxication is thought to be attributed to the inhibition of water and electrolyte reabsorption in the small intestine (Hennekinne et al., 2012). Mossel et al. (1995) cited an emetic dose 50 value of about 0.2 µg SE per kg of human body weight. They concluded that an adult would need to ingest about 10 to 20 µg of SE to suffer symptoms. Although the super-antigenic activity of SEs has been studied and characterized, there is little information on the mechanisms leading to the emetic activity (Hennekinne et al., 2012).

Factors Effecting Growth and Enterotoxin Production

The levels of salt, pH, and nitrite normally found in most non-fermented, non-pickled, cured meats, that are not extensively dried, are found to not prevent growth or enterotoxin formation by *S. aureus* under aerobic conditions (Tompkin et al., 1973). *S. aureus* is a difficult bacterium to eradicate, due to its ubiquitous presence in the environment and consistent colonization of human and animal hosts (O Clements and Foster, 1999).

pH

As with other microorganisms, the type of acidulant used in pH experiments has a profound effect on inhibition. Similar to *Salmonella* species and *L. monocytogenes*, *S. aureus* can develop resistance to lethal pH levels if first exposed to a sub-lethal level of acid (O Clements

and Foster, 1999). A study by Cebrian et al. (2010) investigated the stress response of *S. aureus* to acid. Exponential-phase cultures of *S. aureus* were exposed to pH values between 5.5 and 3.5 (HCl) at 25°C, and adaptation times varied from 5 minutes to 2 hours. Following the sub-lethal acid adaptation times, cultures were exposed to pH 2.5, where time for the first decimal reduction (TFDR, min) was measured. Results demonstrated increased acid resistance was attained by the cultures exposed to pH levels 4.0 to 5.5 with 4.5 providing the greatest resistance. Cultures exposed to pH level 3.5 displayed increased sensitivity to pH 2.5. Increased sub-lethal exposure time resulted in increased acid resistance, with 2 hour cultures demonstrating 1.6 fold increased acid resistance compared to the control (unadapted cells). Acid shock of *S. aureus* cultures resulted in cross protection effects with respect to hydrogen peroxide and heat inactivation. As with *Salmonella* species and *L. monocytogenes*, protein synthesis is a necessary component to the development of stress resistance in *S. aureus* (Cebrian et al., 2010).

Brown and Booth (1991) reported the formation of aminophospholipids (such as alanyl- and lysyl-phosphatidylglycerol) on the outer membrane of *S. aureus*, which is believed to give the surface of the cell a net positive charge. This charge blocks the influx of protons and aids in the survival of *S. aureus* in acidic conditions. Additionally, *S. aureus* has been shown to possess ATPase proton pumps, which helps it to regulate its internal pH and survive acidic environments (Cotter and Hill, 2003). Bore et al. (2007) noted increase in gene transcription involved with proton excretion and cellular repair mechanisms after just 10 minutes of exposure to HCl at pH 4.5. Additionally, Rode et al. (2010) noted the production of ammonia (NH₃) and the metabolism of pyruvate through pathways other than the tricarboxylic acid (TCA) cycle, as a means to avoid the production of organic compounds in the already acidic cells.

Tatini et al. (1971) found that growth and enterotoxin A production by *S. aureus* occurred at pH ≥ 5.0 , with either lactic acid or HCl, in reconstituted nonfat dry milk, whereas neither growth nor enterotoxin production took place at pH 4.5 when obtained with only lactic acid. SEC production occurred between pH 4.0 and 9.83 with no sodium chloride while the addition of 4% sodium chloride restricted the production range of SEC to 4.4 to 9.43; toxin was produced at 10% sodium chloride at pH 5.45 and higher, but not at 12% sodium chloride (Genigeorgis et al., 1971a). Interestingly, meat products treated with wood smoke experience a drop in surface pH and a notable shift in microflora from catalase-positive (like staphylococci) to catalase-negative (most notably LAB) (Rienamm et al., 1972). Genigeorgis (1976) surmised that the lowest pH

levels that permitted growth of *S. aureus* and SE production in anaerobic cultures was 4.6 and 5.3, respectively; pH 4.0 is the lowest level under aerobic condition that permitted both.

In the medical community, acetic acid has been reported to completely eradicate colonization of *S. aureus* from superficial wounds, skin lesions, and venous leg ulcers (Nihei et al., 1993; Hansson and Faergemann, 1995). In sausage production, *S. aureus* is typically eliminated in the fermentation process, due to its pH sensitivity, but the bacteria may produce enterotoxin prior to this step, still making the product a possible health hazard (Ravyts et al., 2008). Both lactic and acetic acid have been shown to significantly reduce ($p < 0.05$) *S. aureus* populations within 72 hours in meat broth (Oliveria et al., 2010).

Rode et al. (2010) investigated the ability of *S. aureus* growth at pH level 4.5 with organic (lactic and acetic) and inorganic (HCl) acids. The authors noted strong differences in growth patterns between the organic and inorganic acids. Acetic acid-treated cultures experienced no notable growth, while lactic acid-treated cultures experienced $>1\text{-log}_{10}$ CFU/mL growth after a lag time of 3 hours. Similar to the control group, HCl-treated cultures experienced no lag time and growth similar to lactic acid-treated cultures ($>1\text{-log}_{10}$ CFU/mL). All broth cultures experienced decreases in glucose levels. This finding is attributed to *S. aureus* requirement of nutrients to combat acidification and for repair. pH of the HCl and control cultures increased over the 24 hour period from 4.5 to 7.5-8.0, while both organic acid cultures did not deviate from pH 4.5. Having the pH of the system lower than the pK_a value of acetic acid ensured the majority of ions were in the undissociated state, thus increasing bacterial inhibition. (Rode et al., 2010).

In another study, *S. aureus* was grown in TSB acidified with 3 different acids (acetic, lactic and HCl) to pH 5.0 and 6.0 and then biofilm formation was evaluated by Nostro et al. (2013). Results demonstrated that acetic acid inhibited the initial adhesion of *S. aureus* by 92% and overall growth, significantly more ($p < 0.05$) than lactic acid or HCl at pH 5.0; again demonstrating the increased inhibitory ability of acetic acid at pH levels close to its pK_a value. Minimal inhibition occurred for all three acids at pH 6.0 (Nostro et al., 2013). Sullivan et al. (2013) demonstrated that *S. aureus* populations were able to survive up to 72 hours on the inside of pickling container lids and were only decreased to undetectable levels when the pickled eggs were moved from refrigerated temperature to ambient. The author stipulated that producers may

want to tilt and invert the pickling containers as to expose the inside surface of the lid to the lethal brine solution.

a_w , Salt and Osmotic Stress

The minimum a_w reported associated with SEA production is 0.87 in raw pork. This a_w was achieved with cell counts of 10^7 per mL (Lotter and Leistner, 1978). In beef, *S. aureus* grew at a_w of 0.88, but not 0.86 (Tatini, 1973). These studies have demonstrated that a_w has been shown to have profound effect on the growth of *S. aureus* (see Table 1.3).

Manipulation of salt content alone within the levels acceptable to consumer taste will not control staphylococcal enterotoxin production (Genigeorgis et al., 1969). Salt concentrations between 5.5% and 9.5% (w/v) have been shown to stimulate the growth of *S. aureus* at temperatures between 20-37°C and simultaneously inhibit the growth of competing bacteria (Peterson et al., 1964). Genigeorgis and Sadler (1966) detected the production of enterotoxin B in Brain Heart Infusion (BHI) agar at pH 6.9 containing up to 10% sodium chloride. Mclean et al. (1968) also was able to detect enterotoxin production when the pathogen was grown at 10% sodium chloride. Additionally, the authors found levels as low as 3% sodium chloride decreased toxin production, while neither 1000 ppm of NaNO₃ nor 200 ppm of NaNO₂ affected enterotoxin production. Hojvat and Jackson (1969) and Tompkin et al. (1973) observed similar negative effects of salt on enterotoxin production, along with inhibitory effects of decreased incubation temperature.

Like *L. monocytogenes*, *S. aureus* accumulates small molecules such as choline, glycine, betaine, and proline that serve as osmoprotectants in situations of osmotic stress. Accumulation of these molecules also appears to stimulate synthesis of SEs (O Clements and Foster, 1999; Qi and Miller, 2000). The phospholipid cardiolipin, which is found in the cell membrane of *S. aureus*, is thought to contribute considerably to its osmotolerance. Levels of cardiolipin were found to increase as *S. aureus* enters the stationary phase and in high salt environments (Tsai et al., 2011). Expression of genes related to osmotic stress resistance also are expressed during other stresses, such as increased and reduced temperature and decreased pH, suggesting that separate stress systems are related (O Clements and Foster, 1999).

Temperature

S. aureus does not grow well at refrigeration temperatures, but has shown the ability to grow and produce SEs in temperature-abused heat-processed foods, especially those that contain

salt or decreased a_w (Smith et al., 1983). Furthermore, decreased temperatures appear to have a more pronounced negative effect on enterotoxin production than on growth. When put into a cold temperature situation, *S. aureus* tends to focus on survival and not on enterotoxin production (Mclean et al., 1968).

A study by Palumbo et al. (1977) examined the heating of frankfurters inoculated with counts $>10^8$ cells/g of *S. aureus*. Heat injury was best demonstrated at 58.9 to 61.7°C. There was little heat injury or killing below 58.9°C and above 61.7°C, the cells died too rapidly to demonstrate cell injury. Tibana et al. (1987) tested the thermal stability of SEA, SEB and SEC in phosphate buffered saline solution with temperatures ranging from 80 to 120°C. The greatest thermal inactivation of all three toxins occurred during the first 5 to 10 minutes of heating. Overall, SEC demonstrated the greatest thermal stability, followed by SEB the SEA (Tibana et al., 1987).

Combination of Multiple Factors

The combined effects of pH, a_w , atmosphere, and/or temperature have been examined in numerous publications. Growth of *S. aureus* has been inhibited in media broth at pH 4.8 and 5% sodium chloride. In the same study, SEB production occurred in 10% sodium chloride at neutral pH, but not with 4% sodium chloride at the lower pH of 5.1 (Genigeorgis et al., 1966). Growth and SEB production occurred under anaerobic conditions in cured ham, with a brine concentration of 9.2%, but not below pH 5.30 and 30°C or below pH 5.58 at 10°C (Genigeorgis et al., 1969). Genigeorgis et al. (1969) similarly concluded that enterotoxin appears to occur sooner under aerobic conditions than anaerobic and in higher amounts at 30°C than 25°C. The optimum pH for the production of enterotoxin C was shown to be between 5.5 and 6.5, but was detected as low as pH 4.0 with no salt present. (Genigeorgis et al., 1971a). Tompkin et al. (1973) noted that the inhibitory effects of nitrite (with sodium chloride 0-3%) became more evident as pH decreased (levels 5.0-4.5). Notermans and Heuvelman (1983) demonstrated inhibition of SEB production at pH 7.0 (at 37°C) with $\geq 6\%$ sodium chloride. Salt concentration appears to shift the pH range of enterotoxin production. Increased salt concentration inhibits enterotoxin production at acidic pH levels (<4.0) and shifts the range to more alkaline levels. No growth of *S. aureus* occurred at pH <5.5 , 12°C and a_w of 0.90 or 0.93, nor at pH <4.9 , 12°C and a_w of 0.96. Increasing sodium chloride concentration raises the minimum pH of growth of *S. aureus* (Jay et al., 2005).

Outbreaks

In many countries throughout the world, *S. aureus* is considered the third most common pathogen associated with foodborne illness, behind *Salmonella* species and *Campylobacter* species (Atanassova et al., 2001). Staphylococcal food poisoning represents a considerable burden in terms of loss of working days and general productivity, hospital expenses, and economic losses in food manufacturing companies, catering companies and restaurants (Argudin et al., 2010).

The first known investigation of staphylococcal food poisoning was performed in Michigan in 1884 by Vaughan and Sternberg in response to the consumption of contaminated cheese (Hennekinne et al., 2012). The 1900s saw several reports of staphylococcal food poisoning from soldiers in WWI and WWII, to school children and large gatherings of people, like weddings or church festivals. SFP occurs in a variety of food items including meat (ham, chicken, turkey, sausages and hamburgers), cheese, milk, rice, desserts and pastries (Hennekinne et al., 2012).

Of the estimated 9.4 million foodborne illnesses caused by known pathogens that occur every year in the United States, 1.3 million (13%) are caused by food intoxication (Scallan et al., 2011). From 1998 to 2008, *Bacillus cereus*, *Clostridium perfringens*, and *S. aureus* were responsible for 1229 reported cases of foodborne intoxication in the United States (Bennett et al., 2013), with an estimated total cost of \$168 per case. The estimated cost of *S. aureus* alone was \$695 per case (Scharff, 2012). In that period of time, *S. aureus* was confirmed as the cause of 167 outbreaks causing 4818 illnesses and three deaths. Additionally *S. aureus* was suspected as the cause of another unconfirmed 291 outbreaks and 1923 illnesses, making *S. aureus* responsible for 37% of total food intoxication outbreaks in that decade (Bennett et al., 2013).

A study by Wieneke et al. (1993) investigated staphylococcal food poisoning in the United Kingdom from 1969 to 1990. Of the 359 outbreaks of *S. aureus* reported, meat, poultry or their products were implicated as the vehicle of intoxication in 75% of the outbreaks. Ham and chicken were the two most frequently implicated items. The average contamination level present in food products from those outbreaks was 3.0×10^7 CFU/g. Over the 22 year period in the study, a shift in the type of SE causing illness was noted. SEA alone was the cause of 49% of outbreaks between 1969 and 1977 which increased to 64% between 1978 and 1990. This finding is attributed to a decrease in SED related intoxications (Wieneke et al., 1993).

A study by Levine et al. (1996) investigated contaminated canned mushrooms from China that caused a number of SEPs in the United States. Food workers shedding *S. aureus* from the skin or nasopharynx most likely handled the mushrooms that were stored in brine at room temperature prior to the canning process. This step allowed the bacteria to grow and produce enterotoxin, which was able to withstand the canning process and cause intoxication, even though the bacterium was destroyed (Levine et al., 1996).

Confirmation of *Staphylococcal* food poisoning can be done one of three ways: bacteria of the same phage-type must be isolated from the stool or vomit of two or more sick individuals; enterotoxin must be detected in epidemiologically implicated food; or 10^5 organisms per gram must be isolated from the epidemiologically-implicated food (Bennett et al., 2013). Though phage-typing patterns do not indicate if the strain of *S. aureus* produces enterotoxin or not, it is still a useful technique when trying to match *S. aureus* strains isolated from clinical patients, food items and food handlers in outbreaks (Wieneke, 1974). In some cases, confirmation of SFP is difficult, because *S. aureus* is heat sensitive, whereas SEs are not. Thus, in heat-treated food matrices, *S. aureus* may be eliminated without inactivating SEs. In such cases, “it is not possible to characterize a food poisoning outbreak by enumerating CPS in food remnants or detecting SE genes in isolated strains.” (Hennekinne et al., 2012). Cases of staphylococcal food poisoning occur often with no viable bacteria able to be cultured and identified from suspected food or patients (Atanassova et al., 2001). Since cases of SFP are sporadic and the intoxication is self-limiting, illnesses are rarely reported, making the estimated annual number of illnesses far less than the actual number of cases that occur (Wieneke et al., 1993; Bennett et al., 2013).

Presence in Meat Products and Processing Environments

Meat is regarded as one of the leading vehicles of transmission for *S. aureus* and SEs (Blaiotta et al., 2004). Meat (specifically pork) and poultry dishes have been associated with *S. aureus* intoxication, compromising 55% of the total outbreaks between 1998 and 2008 (Bennett et al., 2013). Ham is the food most often implicated in staphylococcal food poisoning, but other meat dishes containing beef, pork and chicken also serve as vehicles for the bacterium and its enterotoxins (Atanassova et al., 2001). Cured meats are often implicated in outbreaks of staphylococcal food poisoning, because the bacteria are capable of growing and producing enterotoxins in the presence of curing salts (McClean et al., 1968). Though pickling salt and brine injection are effective at inhibiting microorganisms, including pathogenic bacteria in cured meat

products, the high salt concentrations selects and gives an advantage to the growth of *S. aureus* (Atanassova et al., 2001).

McCoy and Faber (1966) observed the inhibition of staphylococcal growth and inhibition of enterotoxin formation with no apparent effect on growth in meat slurries (prepared from cooked and minced beef and pork). Inhibition was also more apparent at 25°C than at 35°C. LAB were shown to be the most consistent microorganism in inhibiting staphylococcal growth (McCoy and Faber, 1966).

A study by Atanassova et al. (2001) examined the prevalence of *S. aureus* and SEs (A, B, C and D) in pork at three different stages of production: raw pork, salted meat, and ready-for-sale, uncooked ham. Using polymerase chain reaction (PCR), results demonstrated that of the 135 samples taken, *S. aureus* was found in 51.1% of samples and 57.7% were positive using classical microbiological techniques. SE was detected in 34.8% of samples, with SEA detected 50% of the time. Though differences were present in the sampling techniques, both methods concluded that the majority of *S. aureus* contamination and SEs were present in the raw pork stage (Atanassova et al., 2001).

Normanno et al. (2006) analyzed 993 meat products and found that 100 (10%) of the samples were contaminated with *S. aureus*. SED was the most frequently detected SE, followed by SEA, SEC and SEB. The *S. aureus* strains isolated from beef, roast pork, fresh pork sausage, and poultry were all biotypes that strongly resemble those isolated from the nasal passages and hands of food handlers, suggesting that human contamination is the primary source of staphylococcal food poisoning.

Wallin-Carlquist et al. (2010) studied the prevalence of *S. aureus* and SEA in four processed pork products (boiled ham, hot-smoked ham, Serrano ham, and black pepper salami) for one week. The boiled ham product demonstrated the greatest increases in *S. aureus* counts ($\sim 10^4$ CFU/cm² increase) and SEA production (4400 ng/cm³), followed by the smoked ham ($< \sim 10^4$ CFU/cm² increase; 1300 ng/cm³) and the Serrano ham ($\sim 10^1$ CFU/cm² increase; 5.3 ng/cm³). The black pepper salami experienced no significant growth from initial the inoculum level, and no SEA production was detected. The greatest increases in *S. aureus* growth occurred in the first 48 hours, while SEA production was observed throughout the entire seven days (Wallin-Carlquist et al., 2010). Similar results were observed regarding SED production in boiled ham, hot-smoked ham, and Serrano ham for the same amount of time (Marta et al., 2011).

Though the study of biofilms has focused almost exclusively on *Salmonella* species and *L. monocytogenes*, it is important to note that *S. aureus* is capable of biofilm formation and can persist in food processing environments, carcasses, raw meat, and cooked RTE products (Rode et al., 2007). High osmolarity, high temperature, and sub-lethal levels of acid and antibiotics have been shown to enhance biofilm formation (Rode et al., 2007). *S. aureus* was shown to form the most biofilm at 25 and 46°C when salt and glucose concentrations were at 2.5% (w/v), regardless of aerobic or anaerobic conditions. It is known to persist in food processing environments in the form of biofilms, while being easily transferred through cross-contamination by food handlers and equipment (Shale et al., 2004). Sampling of South African abattoirs by Shale et al. (2004) found total viable counts (TVC) of staphylococci to be between 10^3 and 10^9 CFU/g, which surpassed their country's National Guideline of 10^2 CFU/g regulation for abattoirs.

Contributors to Staphylococcal Food Poisoning

Presence or absence of *S. aureus* is a good indicator of poor personal hygiene practices of food handlers (Shale et al., 2004). The contribution of the infected food worker (whether symptomatic or not) to outbreaks is difficult to establish. The CDC estimates that at least 20% of foodborne illnesses caused by bacterial agents are the result of transmission from infected workers (FDA, 2000). A study by Greig et al. (2007) examined 816 reports totaling 80,682 cases of worker-associated food outbreaks between 1927 and March 2006. The majority of reports were collected from the United States, Canada, Europe, and Australia. *Salmonella* was the number two most cited agent with 151 outbreaks and 9,136 cases, followed by *S. aureus* at number four with 53 outbreaks and 6,423 cases. Though *S. aureus* was trumped in terms of number of outbreak and number of cases by *Salmonella* species, *S. aureus* has a higher number of reported cases per outbreak (121.2 versus 60.5). Notable factors pertaining to the cases of *S. aureus* were “bare-hand and glove-hand contact by food handlers or preparers” and “failure to properly wash hands when necessary.” Meat or food containing meat was reported as the number one food source for these outbreaks (Greig et al., 2007). Errors in processing or preparation, such as: insufficient time or temperature for initial cooking and reheating; too slow at cooling leftovers; inadequate storage temperatures; inadequate cleaning of equipment or utensils; contamination from the storage environment; and contamination from the food handler were cited in 93% of outbreaks (Bennett et al., 2013). Foods subjected to temperature abuse ($>10^\circ\text{C}$)

are considered strong potential candidates for staphylococcal enterotoxin production and potential food poisoning outbreaks (Smith et al., 1983).

Carriage of Staphylococcal Species in Humans

Staphylococci are most frequently found in the anterior nares of humans, with numbers far greater than those found on the skin. Staphylococcal bacteria flourish here where they are able to colonize, resist defense of the immune system, establish strong interactions with epithelial cells, and spread to the parts of the body without causing symptoms (Kluytmans et al., 1997; Acco et al., 2003). The application of penicillin to the nose of staphylococcal carriers has shown a significant reduction in the number of *S. aureus* recovered from other areas, suggesting that the maintenance of Staphylococci on the skin is dependent on the colonies in the nose (Moss et al., 1948). The hands and forearms are the next two locations that yielded the highest number of Staphylococci (Williams, 1963).

Williams (1963) concluded there was three main classes of staphylococci carriers: the persistent carriers ($\pm 20\%$), the persistent non-carriers ($\pm 20\%$), and the intermittent (occasional) carriers ($\pm 60\%$). It has also been proposed that staphylococci carriers, particularly on the hands, fall into two classes: those in whom the cocci are carried superficially on the skin and those whom the bacteria live in the depths of the skin glands (Williams, 1963). The highest carrier rates of staphylococci are reported in infants. By the age of 5 to 6 years old, numbers of staphylococci decrease and better represent carrier rates observed in adults (Williams, 1963). Rates of staphylococcal colonization are high among hospital patients, especially individuals with type 1 diabetes, those who use intravenous drug, those with defects in leukocyte function, those undergoing hemodialysis, those who have recently undergone surgery, and those with the acquired immunodeficiency syndrome (Lowy, 1998).

Food handlers play an important role in the prevention of foodborne illness; however, they also contribute to the genesis and transmission of food poisoning, since they may introduce pathogens in the food during production, processing, distribution and handling (Acco et al., 2003). Unsanitary practices of food handlers in meat processing facilities have been reported by Little et al. (1998) and linked to product contamination of *S. aureus*. Strains of *S. aureus* isolated from various meat products (poultry carcasses, shrimp, minced meat [beef + pork], ham and salami) were compared to strains collected from the anterior nares of slaughterhouse and meat plant workers. Results demonstrated strong similarities between strains of these several sources

pointing to obvious contamination of meat products by food handlers (Isigidi et al., 1990). Acco et al. (2003) sampled the anterior nares of 47 food handlers and found that 14 (30%) of them harbored *S. aureus* with 11 of the 14 harboring multiple strains.

Statement of the Problem

Typical acidified foods, including pickled meat sausages, are processed with the use of a heated brine solution, known as hot fill processing. However, the use of a heated brine solution in the pickling of sausages has been shown to cause the brine solution to become turbid and unappealing to consumers. To avoid this problem, some processors may use a cold fill process in which the brine solution is kept at room temperature. Any deviation in processing requires revalidation and scientific data to prove the ability of the deviation in processing to control microorganisms that are most likely to contaminate the sausages. No published scientific data specifically addresses the efficacy of a pickling process to inhibit microbial contamination in ready-to-eat meats. Therefore, a challenge study can validate the efficacy of the cold fill pickling process in its ability to inhibit the growth of undesirable microorganisms.

Statement of Objectives

The objective of this project is to test the ability of a room temperature brine solution (of specific vinegar and salt concentrations) to inhibit the growth of and/or kill both spoilage and pathogenic microorganisms experimentally inoculated onto the surface of ready-to-eat meat sausages. Microbial populations and other parameters, such as pH, a_w , and percent salt will be monitored from the initial inoculation time and up to 28 days. Results from the study could provide the appropriate scientific support for pickled sausage processors to comply with current food safety regulations. Additionally, these data could aid regulatory authorities in reevaluating and possibly changing regulations in regard to pickled meat products.

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Tables

Compound(s)	Microbial Target	Primary Food Application	Title 21 CFR Designation
Acetic acid acetates diacetates dehydroacetic acid	Yeast, bacteria	Dairy products, meat	184.1005, 182.6197, 184.1754, 184.1185, 184.1721, 172.130
Lactic acid, lactates	Bacteria	Meats, fermented foods	184.1061, 184.1207, 184.1639, 184.1768
Sodium propionate	Molds	Meat products	184.1784

Table 1.1 Organic acids commonly used in meat products as antimicrobials (Mani-López et al., 2012).

Condition	For Growth		For Toxin Production	
	Optimum	Range	Optimum	Range
Water Activity	>0.99	0.83->0.99	>0.99	0.86->0.99
pH	6-7	4-10	6-7	4-9.8
Temperature (°C)	37	7-47.8	40-45	10-46
% Sodium chloride	0	0-20	0	0-10
Availability of O ₂	Aerobic	Aerobic-anaerobic	Aerobic	Aerobic-anaerobic

Table 1.2 Ranges of environmental conditions for growth and enterotoxin production by *Staphylococcus aureus* (Tatini et al., 1973).

Beef ^b				Pork ^b			
<i>S. aureus</i> /g	Final pH	a_w^c	Toxin A	<i>S. aureus</i> /g	Final pH	a_w^c	Toxin A
1.6×10^8	4.7	0.96	—	2.4×10^8	5.0	0.97	+
3×10^9	5.3	0.94	+	2.7×10^9	5.3	0.94	+
1.2×10^9	5.5	0.88	+	1.7×10^9	5.4	0.90	+
1.0×10^9	5.5	0.86	—	1.1×10^9	5.5	0.86	+
4×10^4	—	0.82	—	3.1×10^7	5.8	0.83	—

Table 1.3 Influence of water activity on growth and enterotoxin production by *S. aureus* 265-1 at 35°C (Tatini et al., 1973).

Toxin	Molecular Mass (kDa)	Emetic Activity	Crystal Structure Solved	Gene	Accessory genetic element
SEA	27.1	yes	yes	<i>sea</i>	ΦSa3ms, ΦSa3mw, Φ252B, ΦNM3, ΦMu50a
SEB	28.4	yes	yes	<i>seb</i>	pZA10, SaPI3
SEC	27.5–27.6	yes	yes	<i>sec</i>	SaPI _n 1, SaPI _m 1, SaPI _m w2, SaPI _{bov} 1
SED	26.9	yes	yes	<i>sed</i>	pIB485-like
SEE	26.4	yes	no	<i>see</i>	ΦSa ^b
SEG	27.0	yes	yes	<i>seg</i>	<i>egc1</i> (vSaβ I); <i>egc2</i> (vSaβ III); <i>egc3</i> ; <i>egc4</i>
SEH	25.1	yes	yes	<i>seh</i>	MGE _{mw2} /mssa476 <i>seh/Δseo</i>
SEI	24.9	weak	yes	<i>sei</i>	<i>egc1</i> (vSaβ I); <i>egc2</i> (vSaβ III); <i>egc3</i>
SEJ	28.5	nd	no	<i>selj</i>	pIB485-like; pF5
SE/K	26.0	nd	yes	<i>selk</i>	ΦSa3ms, ΦSa3mw, SaPI1, SaPI3, SaPI _{bov} 1, SaPI5
SE/L	26.0	no ^a	no	<i>sell</i>	SaPI _n 1, SaPI _m 1, SaPI _m w2, SaPI _{bov} 1
SE/M	24.8	nd	no	<i>selm</i>	<i>egc1</i> (vSaβ I); <i>egc2</i> (vSaβ III)
SE/N	26.1	nd	no	<i>seln</i>	<i>egc1</i> (vSaβ I); <i>egc2</i> (vSaβ III); <i>egc3</i> ; <i>egc4</i>
SE/O	26.7	nd	no	<i>selo</i>	<i>egc1</i> (vSaβ I); <i>egc2</i> (vSaβ III); <i>egc3</i> ; <i>egc4</i> ; MGE _{mw2} /mssa476 <i>seh/Δseo</i>
SE/P	27.0	nd ^a	no	<i>selp</i>	ΦN315, ΦMu3A
SE/Q	25.0	no	no	<i>selq</i>	ΦSa3ms, ΦSa3mw, SaPI1, SaPI3, SaPI5
SER	27.0	yes	no	<i>ser</i>	pIB485-like; pF5
SES	26.2	yes	no	<i>ses</i>	pF5
SET	22.6	weak	no	<i>set</i>	pF5
SE/U	27.1	nd	no	<i>selu</i>	<i>egc2</i> (vSaβ III); <i>egc3</i>
SE/U2 (SEW)	nd	nd	no	<i>selu2</i>	<i>egc4</i>
SE/V	nd	nd	no	<i>selv</i>	<i>egc4</i>

Table 1.4 General properties of SEs and SEIs and genomic location of the encoding genes (Argudin et al., 2010).

Figures

Figure 1.1 Schematic diagram showing the combined influences of water activity and pH on microbial growth (Pitt and Hocking, 2009).

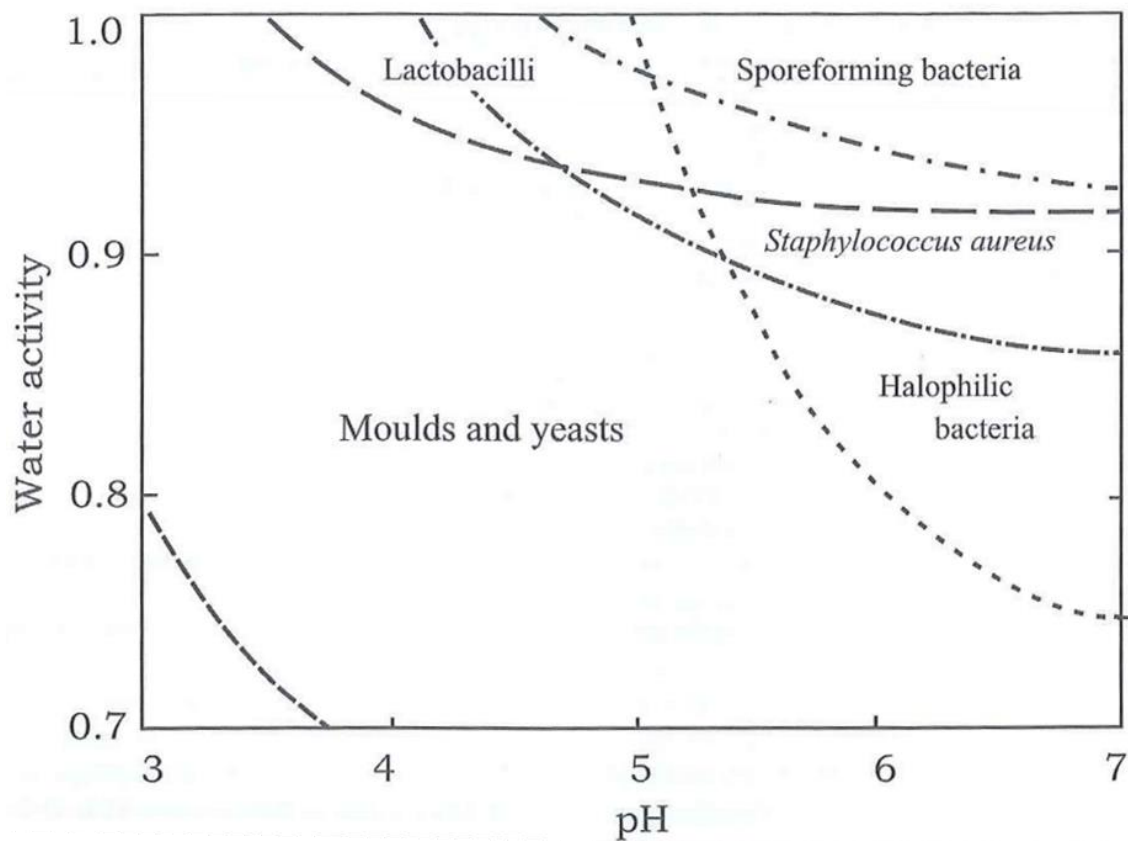


Figure 1.2 Chemical structure of organic acids commonly used in meat and poultry products (Mani-López et al., 2012).

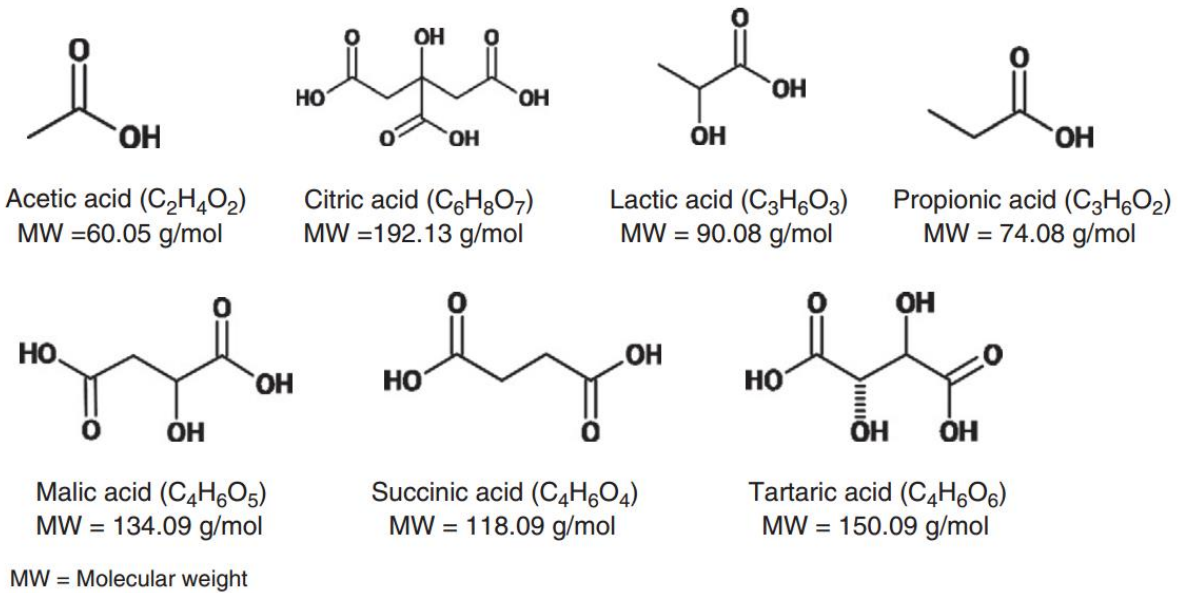


Figure 1.3 Predicted medium and cytoplasmic weak-acid/ anion equilibria. Only uncharged weak-acid molecules (HA) can diffuse freely across the plasma membrane. Charged anions (A^-) and protons (H^+) are retained within the cell; cytoplasmic protons are expelled by the membrane-bound H^+ -ATPase (Lambert and Stratford, 1999).

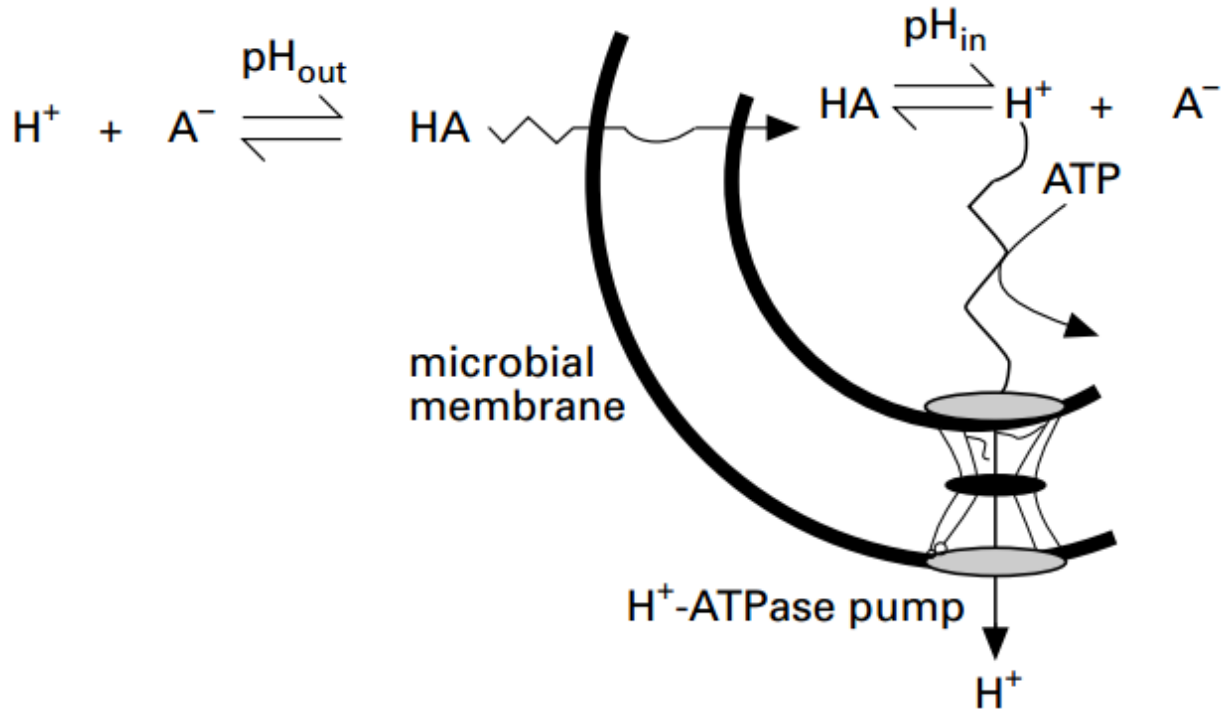


Figure 1.4 Effects of pH_i on the pH_o of unadapted cells. Cells were grown to 2×10^8 cells per mL in E medium plus glucose (pH 7.7), and the medium pH was adjusted to the values indicated. After 20 minutes of equilibration, the pH_i values were measured as described in Materials and Methods. The accuracy of each measurement, done in triplicate, was ± 0.1 unit (Foster and Hall, 1991).

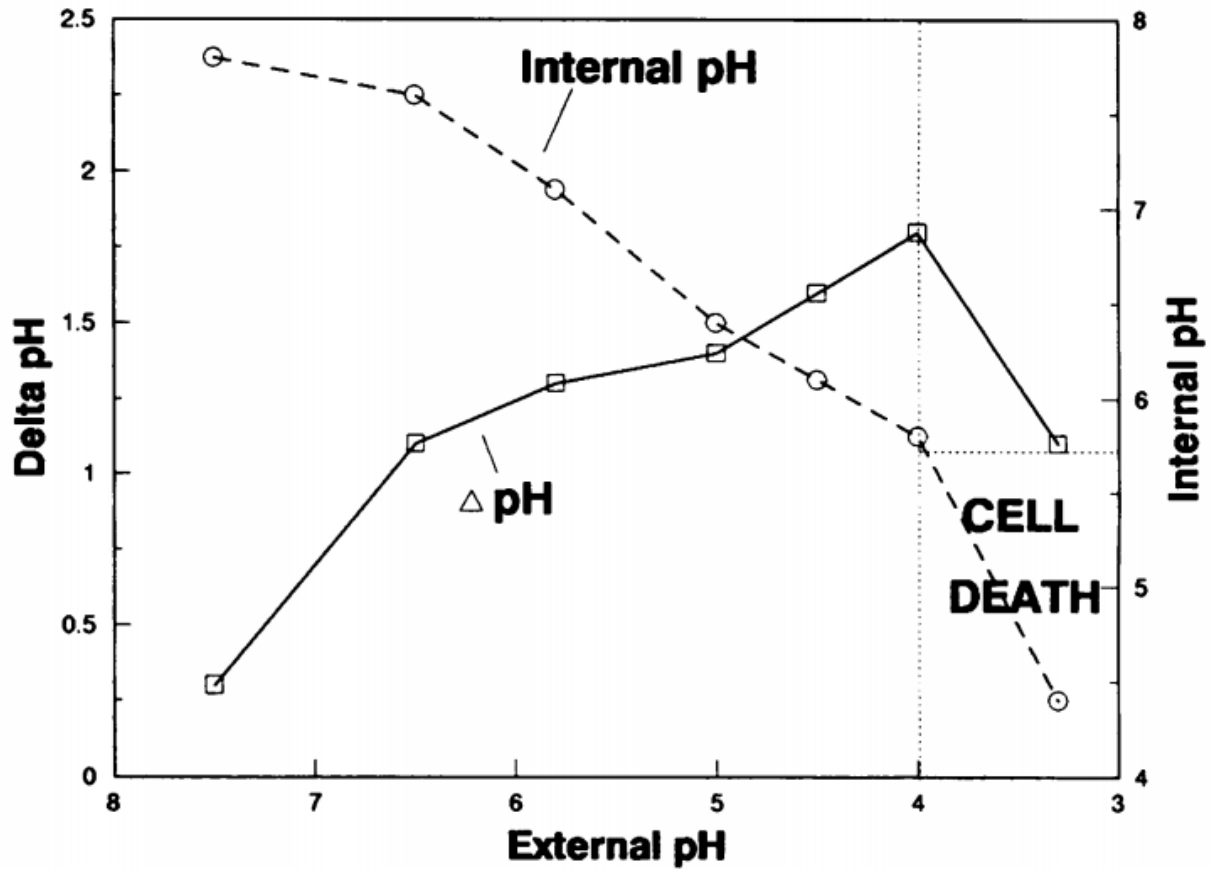


Figure 1.5 Schematic representation depicting the two stages of the ATR (Foster, 1991).

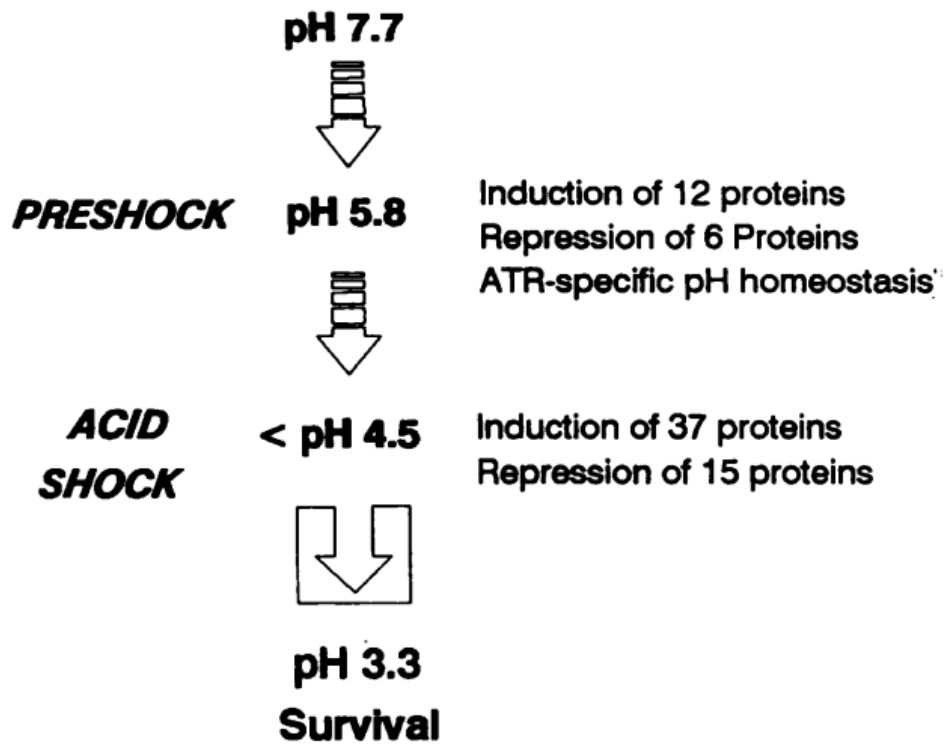


Figure 1.6 Membrane content of *Salmonella* Typhimurium non-acid adapted and acid adapted cells in saturated fatty acids (blue), cyclopropane fatty acids (red) and unsaturated fatty acids (yellow) (Álvarez-Ordóñez et al., 2012).

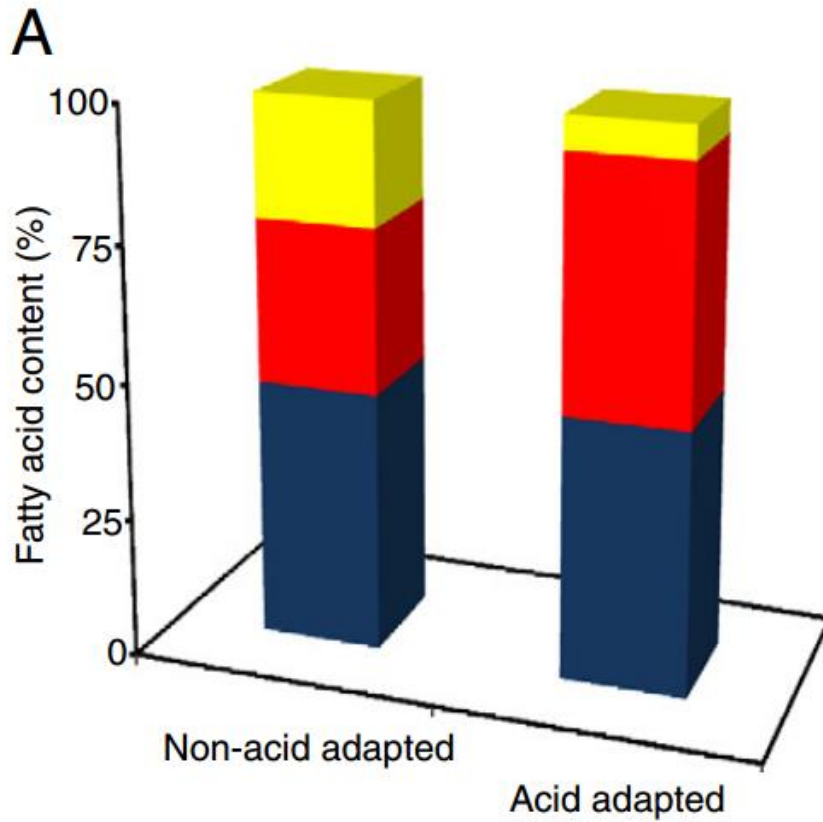
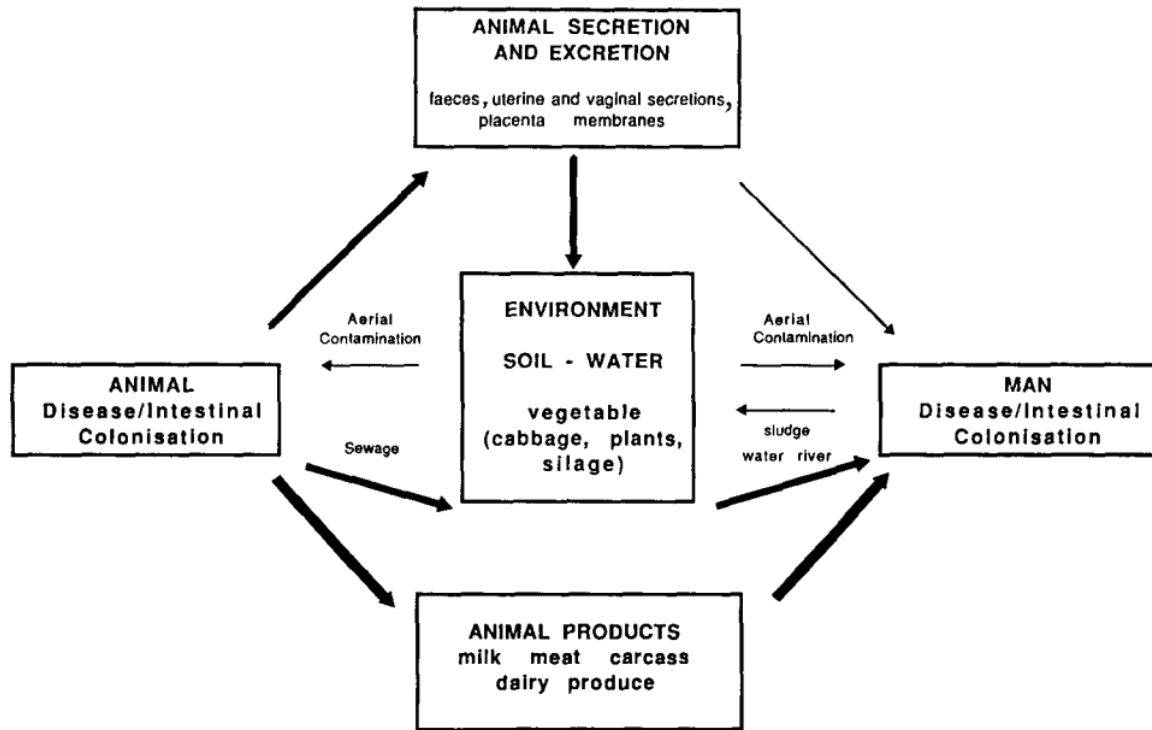


Figure 1.7 The epidemiology of listeriosis (Audurier and Martin, 1989).



Chapter 2

Acid Penetration of Sausages made from Various Casings

Introduction

To have a better understanding of how the brine solution penetrates sausages during pickling, a series of preliminary experiments were conducted to determine how different sausage casings affect the pickling process. Three different casing types were chosen (skinless (no casing), natural and collagen), along with two different size diameters (small and large). By determining which size diameter sausage and casing type impeded brine penetration (yielded the slowest pH decline) the most, parameters for a worst case scenario were established and utilized in future experiments.

Materials and Methods

Sausage Preparation

Pre-cooked, ready-to-eat (RTE) sausages of the three casing types and two sizes were purchased from local supermarkets and stored at refrigeration temperature (4°C). Initial measurements were taken and can be seen in Table 2.1. Sausages were removed from their packaging and cut into four pieces, ranging from 12 to 14 cm long. Skinless, natural casing and collagen casing sausages, both small (20-24 mm) and large (30-34 mm) diameter, were placed into six separate one liter food grade plastic screw top containers (Pretium Packaging, Hazleton, PA).

Initial Temperature and pH Measurements

Initial temperature of one of the sausages was taken (Cooper Pocket Test Plus Thermometer Model TTM59). Internal pH of the sausages also was measured using a Thermo Scientific Orion 4 Star pH ISE Benchtop meter with a connected Orion 9103BNWP Semi-Micro Combination pH Probe (Beverly, MA). pH was measured in three of the four sausages (not including the temperature measurement sausage) in three separate locations: top, middle and bottom. pH measurements were taken from these same exact spots on each of the same sausages throughout the remainder of the experiment.

Brine Preparation

Food-grade, distilled white vinegar (5% acidity) (H.J. Heinz Company, Pittsburgh, PA) and plain, food grade salt (Morton, Chicago, IL) were purchased from local supermarkets. Pickling brine (5% salt and 5% acetic acid) was mixed 24 hour prior to pickling of the sausages, covered with aluminum foil, and allowed to fully equilibrate to room temperature (~22-23°C).

Sausage Pickling and Sampling

A room temperature (~22-23°C) brine solution (5% salt and 5% acetic acid) was poured into each of the three, one liter containers (Pretium Packaging, Hazleton, PA) covering the sausages and allowing a headspace of 0.5 inches. The containers were then capped and sealed, simulating a cold fill commercial pickling and packaging process. Temperature and pH measurements of sausages made with the skinless, natural casing and collagen casing were taken at the following time points: 3, 6, 9, 12, 18, 24, 48, 72, 96, 120 and 144 hours, until equilibrium (time point where the pH of the sausage and pH of the brine are the same value or fail to move after two time points) was reached. Three separate replicates (N=3) were performed for each casing type and size combination.

Results and Discussion

pH results of all six types of sausages are presented in Table 2.2 and Figure 2.1. Overall, small diameter sausages (20-24 mm) appeared to have a faster drop in pH than their large diameter counterparts (30-34 mm). Natural casing sausages experienced the fastest decline in pH and overall, the lowest pH values (3.32-3.33) of any of the sausages evaluated. Skinless sausages generated similar, but higher pH results. Collagen casing sausages demonstrated the most resistance to acidification, yielding the slowest rate of pH decline and the highest final pH values (3.50-3.53). Temperature (data not shown) rose steadily from ~4°C to 23°C within 6 hours in all products evaluated.

Natural sausage casings come from the intestinal tract of farm animals like swine, cattle, or sheep and consists of naturally occurring collagen. The epithelial lining is removed, along with the surrounding smooth muscle layer and packed in a salt or a saturated brine solution before use. Natural casings are very permeable to both moisture and smoke. In contrast, collagen casings are generated from collagen extracted from the skin and hides of cattle. Collagen is the principle structural protein in connective tissue. Collagen casings share similar characteristics to

natural casings, but with increased thickness, strength and uniformity, while easily degrading upon chewing (Aberle et al., 2001). Prior to pickling, during the cooking process of the sausages, the structural proteins of the collagen casings denature, shrink in size, and form a dense network of cross-linked proteins. Given these properties, it was hypothesized that the collagen casing sausages impeded the penetration of the brine solution from entering the sausage, resulting in the lower rate of pH decline and higher overall pH values. In a similar manner, an outer skin formed during the cooking process of the skinless sausage that demonstrated some resistance to the influx of brine solution. Because moisture can readily pass through natural casings, those sausages exhibited the least resistance to brine penetration, as is evident by the fastest decline in pH and lowest final pH values.

Conclusions

These results demonstrate that the large diameter sausages made with an edible collagen casing had the slowest rate of pH decline during pickling. Thus, future challenge studies in which a “worst case scenario” occurs, should utilize sausages made with an edible collagen casing and exhibiting a diameter >30mm.

References

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Tables

	Casing type					
	Skinless		Natural		Collagen	
	Small	Large	Small	Large	Small	Large
Length (mm)	130	255	165	220	140	400
Diameter (mm)	24.2	29.9	20.01	33.7	22.5	33.8

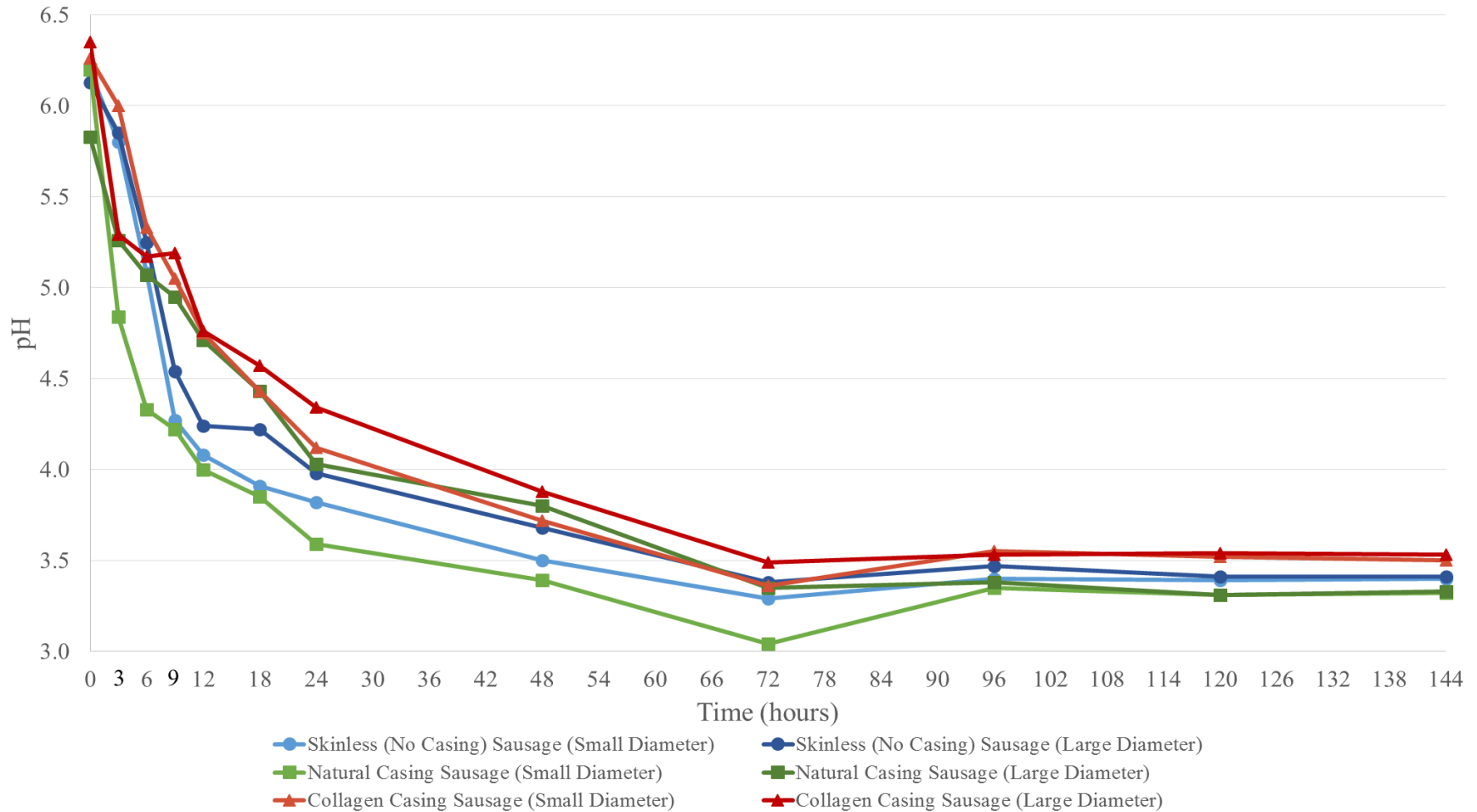
Table 2.1 Initial measurements of pre-cooked, RTE sausages with varying casing types and dimensions, prior to pickling.

Time (hours)	Casing Type					
	Skinless		Natural Casing		Collagen Casing	
	Sausage (Small Diameter)	Sausage (Large Diameter)	Sausage (Small Diameter)	Sausage (Large Diameter)	Sausage (Small Diameter)	Sausage (Large Diameter)
0	6.19	6.13	6.20	5.83	6.26	6.35
3	5.80	5.85	4.84	5.26	6.00	5.29
6	5.08	5.25	4.33	5.07	5.33	5.17
9	4.27	4.54	4.22	4.95	5.05	5.19
12	4.08	4.24	4.00	4.71	4.75	4.76
18	3.91	4.22	3.85	4.43	4.43	4.57
24	3.82	3.98	3.59	4.03	4.12	4.34
48	3.50	3.68	3.39	3.80	3.72	3.88
72	3.29	3.38	3.04	3.35	3.36	3.49
96	3.40	3.47	3.35	3.38	3.55	3.53
120	3.39	3.41	3.31	3.31	3.52	3.54
144	3.40	3.41	3.32	3.33	3.50	3.53

Table 2.2 pH results from pickling of both small and large diameter sausages with skinless, natural casing, and collagen casing from time 0 to 144 hours. Sausage measurements are an average of three measurements (top, middle, and bottom sections) of three separate sausages from multiple replicates (N=3).

Figures

Figure 2.1 pH results from pickling of both small and large diameter skinless, natural casing and collagen casing sausages from time 0 to 144 hours.



Sausage measurements are an average of three measurements (top, middle, and bottom sections) of three separate sausages of three separate sausages from multiple replicates (N=3).

Chapter 3

Various Factors Effecting the Pickling Process of Sausages

Introduction

To our knowledge, no published literature address how changes in temperature or density affects the pickling of meat products. To better understand the differences between hot and cold fill pickling, a series of preliminary tests varying temperature and density were conducted to observe how these differences impacted the processes. Parameters evaluated in these experiments included the storage temperatures of the sausages, temperature of the brine solutions (including the parameters of the cold fill process), and amounts of sausage (pounds) in a container to determine the effect on the acidification process. Three different temperatures (4, 22, and 85°C) and three different amounts of sausage (two, three, and four pounds) were utilized in two separate experiments. The temperatures 4°C and 22°C represent refrigerator and room temperature, respectively, while 85°C was chosen as a representative temperature for the “hot fill” process (personal communication, PA pickled sausage processors). Eight different pickling scenarios were tested and presented (Table 3.1).

Materials and Methods

Sausage Preparation

Sausages were manufactured at The Pennsylvania State University Meat Laboratory and formulated with the following ingredients: beef trim (80% lean; 22% w/w), pork trim (80% lean; 44% w/w and 50% lean; 22% w/w), water (7.26% w/w), salt (2.21% w/w), dextrose (0.44% w/w), ground black pepper (0.28% w/w), sodium tripolyphosphate (2500 ppm), ground mustard (0.55% w/w), granulated garlic (0.55% w/w), ground ginger (0.01% w/w), sodium erythorbate (540 ppm) and curing salt (6.25% NaNO₂; 156 ppm). Sausages were stuffed to a weight of ca. 130.4 grams into a 35 mm curved, edible collagen casings (Devro Ltd, Moodiesburn, Scotland, UK) using a Handtmann VF608 Plus with twist linker (Albert Handtmann Maschinenfabrik GmbH and Co, Biberach, DE). The sausages were smoked and cooked in a Kerres Smoke Air® JS 1950 (Kerres Anlagensysteme GmbH, Backnang, DE) to a core temperature of 70°C and

chilled to 4°C in accordance with Appendix B (USDA, FSIS 1999). The average measurements of the cooked and chilled sausages were as follows: length 144 mm ± 2, width 31 ± 2 mm, weight 127 ± 2 g. A separate batch of sausage was created for each repetition of the study. Sausages were then placed in 14 in x 24 in food grade, 3mil gauge pouches (PCS Supplies Inc., Penns Groove, NJ) and vacuum sealed (Smith SuperVac Digimat, Clifton, NJ). Pre-cooked, ready-to-eat (RTE), packaged sausages were stored in a walk-in cooler at 4°C (<21 days) until they could be transported to The Penn State University Muscle Foods Microbiology Laboratory for subsequent pickling.

Brine Preparation

Food-grade distilled white vinegar (5% acidity) and plain, food grade salt was purchased from local supermarkets. Pickling brine (5% salt and 5% acetic acid) was mixed 24 hour prior to pickling of the sausages, covered with aluminum foil and allowed to fully equilibrate to room temperature (22°C). A portion of the brine solution was refrigerated (4°C) overnight. On the day of the experiments, additional brine solution was transferred to a one liter glass beaker (VWR, USA), microwaved (Sanyo model EM-Z2001S, Chatsworth, CA) and heated in 15 second increments until reaching 85°C (Cooper Pocket Test Plus Thermometer Model TTM59).

Pickling and Sausage Sampling: Temperature Experiments

For each treatment group, pre-cooked, RTE sausages were taken directly from refrigerated storage and removed from the vacuum packaging prior to use. Room temperature sausages were allowed to equilibrate on the laboratory bench top to 22°C. To heat, sausages were placed on a microwave safe tray and heated in 15 second increments in the microwave until reaching 85°C, as described above.

Internal pH measurements (Thermo Scientific Orion 4 Star pH ISE Benchtop meter, Orion 9103BNWP Semi-Micro Combination pH Probe; Beverly, MA) were evaluated in three separate sausage locations: top, middle, and bottom throughout the experiment. Cold, room temperature, or heated sausages were transferred to 1L food-grade, screw-top containers (Pretium Packaging, Hazleton, PA) and brine solutions (5% salt and 5% acetic acid; 4°C, 22-23°C, 85°C) added to each of eight separate containers (A-H), allowing for a headspace of 0.5 inches. The containers were then capped. Treatments C, D, E, and F were placed in a refrigerator. Treatments A, B, G, and H were stored at 22°C on a laboratory bench top. Internal

temperature and pH measurements were made at the following time points: 3, 6, 9, 12, 18, 24, 48, 72, 96, 120 and 144 hours (N=3) after pickling.

Pickling and Sausage Sampling: Density Experiments

Pre-cooked, RTE sausages (made as described above; 4°C) were transferred from the vacuum packaging to 3.79 liter plastic food-grade, screw-top containers. Individual sausages were placed vertically in a single layer at the bottom of the container. Four pound containers were filled with 16 sausages, three pound containers with 12, and two pound containers with eight. A sausage placed in the center of the container was marked with food-grade dye (Bunzl Processor Division, North Kansas City, MO). Initial temperature and pH measurements were taken from the center of the dyed sausage, as well as a random sausage along the outside edge of the container. pH measurements were only taken from the center of each sausage. The containers were placed on a bench top to simulate a cold fill pickling process. pH and temperature measurements were taken from the sausages at the following time points: 3, 6, 9, 12, 18, 24, 48, 72, 96, 120 and 144 for (N=3) replications.

Results and Discussion

Table 3.1 details the initial and final temperature and pH values of the eight different pickling treatments. Time to reach equilibrium (defined as the time point where the pH of the sausage and pH of the brine are the same value or fail to move after two time points) was reached, as well as the time the sausage pH reached ≤ 4.6 also was determined. A pH of ≤ 4.6 was chosen as a reference point for shelf stability and as a way to compare the different conditions. Treatment A follows the parameters of the cold fill process; more detailed results are displayed in Figure 3.1. Overall, Treatment G decreased the internal pH of the sausage the fastest, reaching 4.47 within 6 hours, followed by Treatment H and A; the cold fill processed sausages (Treatment A) reached below 4.53 within 24 hours. The final pH of Treatment D only reached 4.65, resulting in the slowest pickling scenario. Equilibrium pH between the sausage and brine solution was only achieved by Treatments F, G, and H and was not applicable to the other five scenarios, including the cold fill process (Treatment A).

Overall, room temperature (22°C) storage increased the rate of pH decline in the sausages. Treatments A, B, G, and H had internal pH values below 4.6 within 48 hours. In contrast, sausages pickled and stored at 4°C were not acidified as quickly. Sausages subjected to

Treatments C, D, and E did not reach a pH below 4.6 until the last time point, and no sausages in these treatments groups reached equilibrium. Heating the sausages prior to pickling did demonstrate an increase in pH penetration and acidification of the sausages (Treatments E, F, G, and H), when compared to refrigerated sausages (Treatments A, B, C, D). Fat particles were observed in all of the sausages pickled with heated (85°C) brine, but more so with the Treatments F and G, especially when the sausage also was heated.

Table 3.2 summarizes the initial and final temperature and pH results of Treatments I, J and K with various amounts of sausages per container. For all scenarios, the pH of the center sausage decreased at a slower rate and resulted in a higher pH than sausages along the outside of the storage containers. Treatment I (2 lbs of sausage) displayed the fastest decline in pH, reaching a pH of 4.31-4.34 within 48 hours and the lowest overall pH values (3.64-3.66) observed. In contrast, Treatment K (4 lbs of sausage) took the longest time to reach a pH 4.6 for the center sausage (4.40 at 72 hours) and had the highest sausage pH values (3.99-4.06) at the end of the experiment.

As the amount of sausage pickled increased in the containers, so did the time to drop below pH 4.6, as indicated by the variation between pH of outer and center sausages and the final pH values of the sausages. The extra available space in the Treatment I containers allowed for more brine to be added (since all the containers were filled to the same volume) and for more complete coverage by the brine solution. Additionally, increasing the amount of sausage in the container increased the buffering capacity of the system from the greater amount of meat in the container. Sausages subjected to Treatment K were tightly packed, allowing less brine to fill in between the sausages. Treatment K also presented the greatest contrast in pH between the outer and center sausages. These findings demonstrate that the amount of sausage in a container affects the pH penetration by the brine.

Conclusions

Room temperature storage had a very positive effect on the pickling process by increasing the rate of pH decline in the sausages. These experiments demonstrated the ability of the cold fill pickling process to effectively decrease the pH of sausage. Though other scenarios that featured heated sausages and brine solutions yielded a more rapid decline in pH, fat leeching and turbid brine solutions were observed.

Container fill volume was shown to greatly affect the ability of the brine solution to decrease the pH of sausages. Lower rates of pH decline and higher overall pH values were observed in the densely packed sausages containers (Treatment K), when compared to more loosely packed (Treatments I and J) containers. Greater differences also were observed between outer and center sausages of the densely packed containers, which indicates that the location of the sausage within the container also impacts the degree of acidification. Sausages located in the middle of the pickling containers should be utilized when measuring internal pH of the sausages.

Tables

Table 3.1 Temperature specifications of treatments A through H with initial and final temperature and pH values.

Trt*	Sausage Temperature (°C)	Brine Temperature (°C)	Storage Temperature (°C)		Initial pH	Final pH	Initial Temperature (°C)	Final Temperature (°C)	Equilibrium (hours)	Time to pH ≤4.6
A	4	22	22	Sausage	5.78	3.86	3.6	22.1	N/A	4.53 at 24h
				Brine	2.13	3.71	21.2	22.1		
B	4	85	22	Sausage	5.75	3.75	3.4	22.0	N/A	4.12 at 48h
				Brine	2.13	3.62	81.1	22.1		
C	4	4	4	Sausage	6.55	4.53	2.6	0.6	N/A	4.53 at 144h
				Brine	2.63	4.18	3.0	1.9		
D	4	85	4	Sausage	6.34	4.65	2.6	1.3	N/A	4.65 at 144h
				Brine	2.63	4.21	85.7	1.6		
E	85	4	4	Sausage	6.55	4.59	86.1	2.1	N/A	4.59 at 144h
				Brine	2.63	4.20	3.0	1.9		
F	85	85	4	Sausage	6.55	4.21	86.2	1.6	144	4.33 at 72h
				Brine	1.97	4.12	85.8	1.7		
G	85	85	22	Sausage	6.55	3.92	84.8	22.1	72	4.47 at 6h
				Brine	1.97	4.00	86.2	22.1		
H	85	4	22	Sausage	6.55	3.75	84.1	22.1	72	4.46 at 24h
				Brine	1.97	3.90	1.8	22.0		

*Treatment

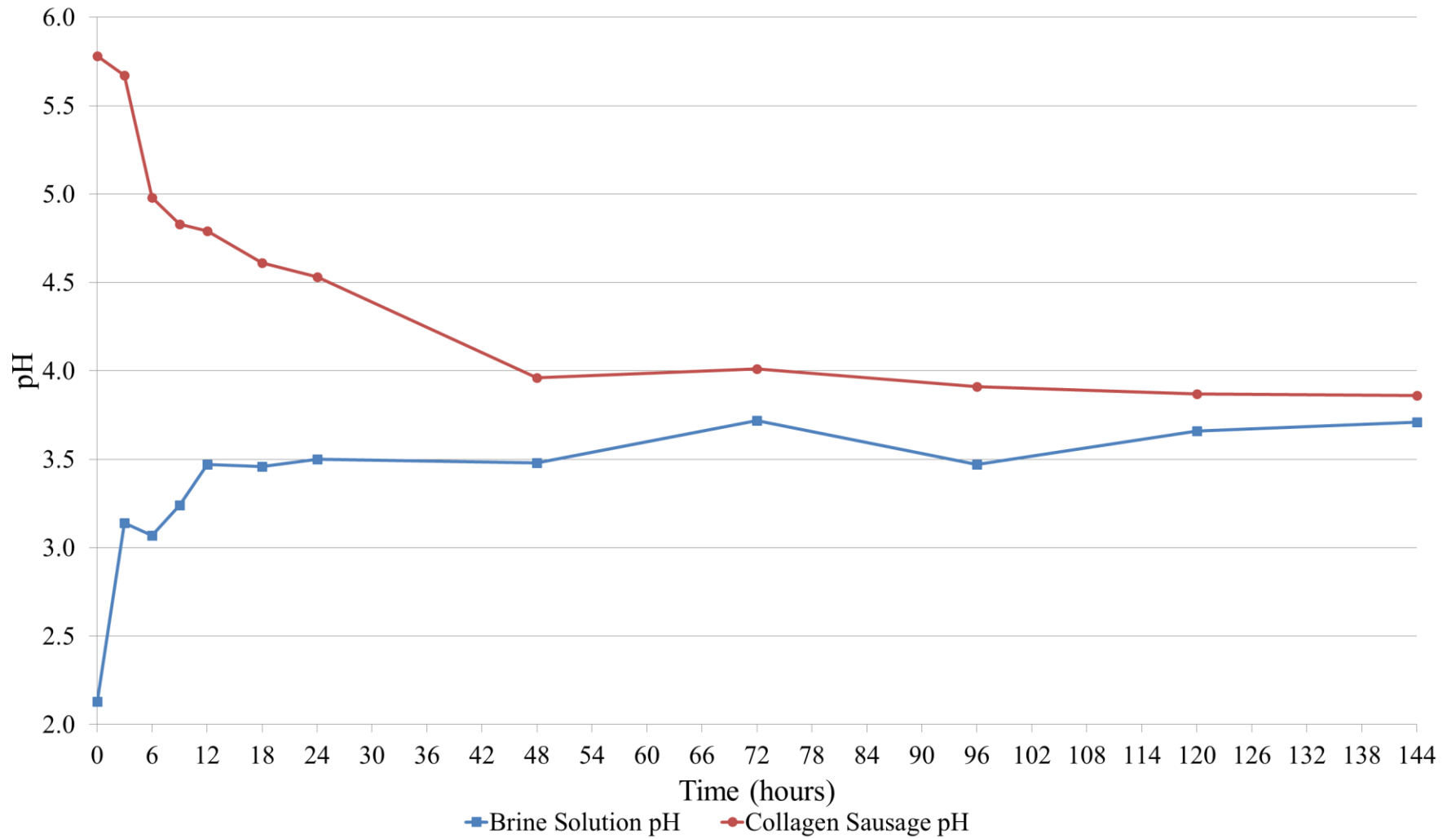
Table 3.2 Specifications of treatments I, J, and K with initial and final temperature and pH values.

Trt*	Amount of Sausage in Container (lbs)		Initial pH	Final pH	Initial Temperature (°C)	Final Temperature (°C)	Equilibrium (hours)	Time to pH ≤4.6
I	2	Center Sausage	5.46	3.64	8.9	22.3	144	4.31 at 48h
		Outer Sausage	5.79	3.66				4.34 at 48h
		Brine	2.08	3.58	22.2	22.2		
J	3	Center Sausage	5.46	3.93	9.7	22.1	N/A	4.43 at 48h
		Outer Sausage	5.79	3.86				4.40 at 48h
		Brine	2.08	3.73	22.2	22.1		
K	4	Center Sausage	5.46	4.06	8.6	22.1	N/A	4.40 at 72h
		Outer Sausage	5.79	3.99				4.54 at 24h
		Brine	2.08	3.78	22.2	22.1		

*Treatment

Figures

Figure 3.1 Sausage and brine pH data from the cold fill process (Treatment A) throughout the length of the study.



Chapter 4

Spoilage Microorganisms Antagonism Study

Introduction

It is important to understand how lactic acid bacteria, (LAB), yeasts, and molds grow and interact with one another. LAB in particular, have been known to produce organic acids and other secondary metabolites, such as hydrogen peroxide and diacetyl that may inhibit the growth of certain strains of yeast and mold (Lindgren and Dobrogosz, 1990; Gourama and Bullerman, 1995; Schnürer and Magnusson, 2005; Dalié et al., 2010). In order to evaluate how each of the microorganisms affect one another, a series of antagonism experiments were conducted.

Materials and Methods

Lactic Acid Bacteria Culture Preparation

LAB cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Lyophilized cultures were transferred into fresh de Man, Rogosa and Sharpe (MRS) broth (Difco, Sparks, MD). Both *L. curvatus* cultures were incubated aerobically at 30°C for 48 h, while *L. sakei* was incubated at 30°C in a microaerophilic environment (5.0% O₂, 10% CO₂, 85% N₂) using a CO₂ incubator (VWR International, West Chester, PA) following ATCC recommendations. Colonies were then re-suspended in fresh MRS broth containing 10% glycerol (VWR, Radnor, PA) and stored at -80°C until future use. Working stocks were maintained on MRS agar (Difco) and stored at 4°C.

Yeast Culture Preparation

Yeast cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Upon receipt, yeast cultures were transferred into fresh yeast mold (YM) broth (Difco, Sparks, MD) and incubated aerobically at 25°C for 72 h following ATCC recommendations. Yeast cultures were then re-suspended in YM broth containing 10% glycerol (VWR, Radnor, PA), and stored at -80°C. To create working stocks, the yeast freezer stock cultures were streaked onto Malt Extract Agar (MEA; Difco, Santa Maria, CA) slants in sterile, screw top test tubes, incubated at 25°C for 72 h, and stored at 4°C until use.

Mold Culture Preparation

Mold cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Each mold culture was transferred to fresh YM broth and incubated aerobically at 25°C for 96 h following ATCC recommendations. Molds were then re-suspended in YM broth containing 10% glycerol (VWR, Radnor, PA), and stored at -80°C for future use. Cultures were transferred from freezer stocks using a sterile 10 µl loop to fresh MEA slants prepared in 125 ml glass bottles, by spreading the inoculated loop across the agar slant surface. Agar slants were then incubated aerobically at 25°C for 96 h and stored at 4°C until use.

Antagonism Experiments Overview

In total, 112 individual tests were conducted to determine how three strains of LAB (*Lactobacillus curvatus* ATCC # 51436, *Lactobacillus curvatus* ATCC # PTA5150 and *Lactobacillus sakei* ATCC # 15521), four strains of yeast (*Debaryomyces hansenii* ATCC # 90624, *Debaryomyces hansenii* ATCC # 10619, *Candida zeylanoides* ATCC # 26318, *Candida zeylanoides* ATCC # 24745) and four strains of mold (*Penicillium nalgiovense* ATCC # 96457, *Penicillium nalgiovense* ATCC # 96460, *Cladosporium cladosporioides* ATCC # 60549, *Cladosporium cladosporioides* ATCC # 11275) interacted with one another on agar plates.

Antagonism Experiments - LAB vs. LAB

To test if there was any antagonism between the three strains of LAB, sterile 1µL inoculation loops were used to collect the LAB strains from MRS broth and streak them onto fresh MRS agar plates. The distribution of the bacteria was carried out in two ways, both of which had the strains forming intersecting lines on the agar surface. Illustrations of both techniques can be seen in Figure 4.1(A) and 4.1(B). The first technique was a simple cross shape made by taking a loop of the first LAB (blue colored line) and making a complete line from one side of the plate to the opposite side. Next, a loop of the second LAB (red colored line) was made perpendicularly from the edge of the middle of the plate and brought to the middle of the plate, intersecting with the line created by the first LAB. Another loop of the second LAB was spread in the same way from the opposite side of the first line of the second LAB, thus forming a cross shape, and where the two strains interact in the middle of the plate. The second technique was made by taking a loop of the first LAB (blue colored line) and making a complete line from one side of the plate to the opposite side. Next, a loop of the second LAB (red colored line) was taken perpendicularly from the edge of the upper portion of the plate and brought to the middle

of the plate, intersecting with the line creating by the first LAB and continuing the line till reaching the edge of the plate. This technique was done again with another loop of the second LAB in a lower portion of the plate, thus having two lines of the second LAB intersecting and mixing with the single line of the first LAB. Eight different scenarios in total were tested. All MRS antagonism spread plates were done in duplicate and incubated aerobically at 30°C for 48 hours. Because the growth parameters of *L. sakei* require the bacteria be incubated in an anaerobic environment, all tests scenarios in that strain of LAB were performed twice; once storing the plates aerobically at 30°C and the second time storing the plates in 10% CO₂.

Antagonism Experiments - Yeast vs. Yeast

To test if there was any antagonism between the four strains of yeast, sterile 1µL inoculation loops were used to collect the yeast strains from YM broth and streak them onto fresh MEA and Dichloran-Rose Bengal Chloramphenicol (DRBC; Difco, Sparks, MD) plates. Although DRBC is a selective medium and there is a possibility that some sub-lethally spores or fungal cells may not recover and grow on it, it was chosen over Malt Extract Agar (MEA) because both mediums resulted in similar yeast and mold counts when plated together. Additionally, yeast and mold colonies were easier to discern and count on DRBC compared to MEA because DRBC has the advantage of restricting the size of mold colonies and allows for a more accurate count of fungal colonies. Fungal counts on MEA overlapped with one another making counting and differentiating yeasts and molds more difficult. The same techniques shown in Figure 4.1(A) and 4.1(B) were conducted for the four strains of yeast and as described above. Twenty-four different scenarios in total were tested. All MEA and DRBC antagonism spread plates were done in duplicate and incubated aerobically at 25°C for 72 hours.

Antagonism Experiments - Mold vs. Mold

To test if there was any antagonism between the four strains of mold, sterile 1µL inoculation loops were used to collect the mold strains from BPW spore solutions and struck onto fresh MEA and DRBC plates. The same techniques shown in Figure 4.1(A) and 4.1(B) were conducted for the four strains of mold as described above. Twenty-four different scenarios in total were tested. All MEA and DRBC antagonism spread plates were done in duplicate and incubated aerobically at 25°C for 96 hours.

Antagonism Experiments - LAB vs. Yeast

Antagonism between the three LAB and four yeast strains was assessed by growing the LAB on MRS plates and pour-plating yeast inoculated into molten 1% MEA in an overlay. The three LAB were streaked onto MRS in the center of the plate in two 2.0 cm lines, 3.0 cm apart, as shown in Figure 4.1 (C), inverted and incubated aerobically at 30°C for 72 hours to fully establish the colonies and their production of possible antagonistic metabolites; *L. sakei* was again grown under 10% CO₂. Yeast strains were transferred to 10 ml of fresh YM broth and incubated aerobically at 25°C for 24 hours. Dilutions were made to achieve an inoculum level between 10³ and 10⁴ CFU/mL for all 4 yeast strains by adding 1 ml of YM broth to 9 ml of BPW. Counts were verified prior to LAB vs. yeast antagonism experiments by spread plating the dilution on MEA and incubating aerobically at 25°C for 72 hours. After the proper dilution was achieved, 1 mL of an individual yeast strain was added to 12.5 mL of molten 1% (w/v) MEA in a 15 ml falcon test tube (VWR, Radnor, PA) and held at a constant 50°C in a water bath (Precision, Jouan Inc. Winchester, VA). This mixture was vortexed for 10 s at a low level as to avoid air bubbles being formed. This mixture was poured over the top of an MRS plate containing LAB colonies, rotated on the bench top surface four times clockwise, four times counter-clockwise, and four times up and down to properly spread and mix the molten MEA around the petri dish, allowed to settle at room temperature, and refrigerated (4°C) for two hours to aid in the diffusion of any potential inhibitory LAB metabolites into the newly poured MEA. The plates were then placed in an incubator suited to the growth conditions of the yeast (25°C aerobically for 72 hours). Twelve different scenarios in total were tested and repeated in duplicate.

Antagonism Experiments - LAB vs. Mold

Antagonism between the three LAB and four mold strains was assessed by growing the LAB on MRS plates and pour plating yeast inoculated into a molten 1% MEA overlay. The three LAB were streaked onto MRS in the center of the plate in two 2.0 cm lines 3.0 cm apart shown in Figure 4.1 (C), inverted and incubated aerobically at 30°C for 72 hours to fully establish the colonies and their production of possible antagonistic metabolites; *L. sakei* was again grown under 10% CO₂. Spores of each individual mold were collected from slants of MEA by pouring sterile BPW in the test tubes and a 10 µL loop was used to loosen the colonies from the agar slant. One mL of the BPW spore solution was pipetted out of the test tube and serially diluted

into 9 ml of fresh sterile BPW to achieve an inoculum level between 10^3 and 10^4 CFU/mL. Counts were verified prior to LAB vs. mold antagonism experiments by spread plating the dilution on MEA and incubating aerobically at 25°C for 96 hours. After the proper dilution was achieved, 1 mL of an individual mold spore solution was added to 12.5 mL of molten 1% MEA in a 15 ml falcon test tube (VWR, Radnor, PA) and held in a 50°C in a water bath. This mixture was vortexed for 10 s at a low level as to avoid air bubbles being formed. This mixture was poured onto an MRS plate containing LAB streaks in an overlay, rotated on the bench top surfaces four times clockwise, four times counter-clockwise, and four times up and down to properly spread and mix the molten MEA around the petri dish. It was allowed to settle at room temperature then placed in a refrigerator at 4°C for 2 hours to aid in the diffusion of any potential inhibitory LAB metabolites into the newly poured MEA. The plates were placed in an incubator suited to the growth conditions of the mold (25°C aerobically for 96 hours). Twelve different scenarios in total were tested and repeated in duplicate.

Antagonism Experiments - Yeast vs. Mold

To test if there was any antagonism between the four strains of yeast and mold, sterile 1µL inoculation loops were used to collect yeast strains from YM broth and mold strains from BPW spore solutions and streaked them onto fresh MEA and DRBC plates. The same techniques shown in Figure 4.1(A) and 4.1(B) were conducted for the four strains of yeast and mold as previously described. 32 different scenarios in total were tested. All MEA and DRBC antagonism spread plates were done in duplicate and incubated aerobically at 25°C for 96 hours.

Results and Discussion

Results of all 112 antagonism experiments are shown in Table 4.1. No antagonism was observed within each of the microorganisms (LAB, yeast, or mold). Likewise, no antagonism was observed between LAB and yeasts, nor between yeast and molds. However, inhibition was observed between the LAB strains and the molds in nine of the twelve experiments. *L. curvatus* 51436 inhibited the growth of both *P. nalgiovensis* strains and *C. cladosporioides* 11275. Similarly, *L. curvatus* PTA5150 inhibited the growth of both *P. nalgiovensis* strains. *L. sakei* 15521 inhibited the growth of all four molds. Inhibition was observed in Figures 4.2 (A) and (B).

Of the 4 mold species, both *P. nalgiovensis* strains were inhibited by all three LAB strains. *C. cladosporioides* 60549 appeared to be the most tolerant mold in the presence of the

LAB strains, showing reduced growth only with *L. sakei* 15521. *C. cladosporioides* was inhibited when grown alongside *L. curvatus* 51436 and *L. sakei* 15521. The results of these antagonism experiments conclude that combining all 11 spoilage microorganism together with the intent of inoculating pre-cooked, ready-to-eat sausages, would not be prudent, due to the potential for inhibition by species. Therefore, it is recommended that the microorganisms should be separated (yeast and molds; LAB) during future challenge studies.

Conclusions

The results from the antagonism study indicated that the three LAB chosen for the inoculation of pre-cooked, ready-to-eat sausages possess the ability to inhibit the growth of *P. nalgiovense* and *C. cladosporioides* strains. In order to accurately assess the survival of spoilage microorganisms in a cold fill pickling process, it is recommended that future challenge studies with LAB should be conducted alone, while yeast and mold strains can be combined.

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Tables

Table 4.1 Results of all antagonism experiments showing whether or not the microorganism (left) inhibited the growth of the microorganisms (right).

	<i>L. curvatus</i> 51436	<i>L. curvatus</i> PTA5150	<i>L. sakei</i> 15521	<i>D. hansenii</i> 90624	<i>D. hansenii</i> 10619	<i>C. zeylanoides</i> 26318	<i>C. zeylanoides</i> 24745	<i>P. nalgiovense</i> 96457	<i>P. nalgiovense</i> 96460	<i>C. cladosporiodies</i> 60549	<i>C. cladosporiodies</i> 11275
<i>L. curvatus</i> 51436	-	No	No	No	No	No	No	Yes	Yes	No	Yes
<i>L. curvatus</i> PTA5150	No	-	No	No	No	No	No	Yes	Yes	No	No
<i>L. sakei</i> 15521	No ¹	No ¹	-	No	No	No	No	Yes	Yes	Yes	Yes
<i>D. hansenii</i> 90624	-	-	-	-	No ²	No ²	No ²	No ²	No ²	No ²	No ²
<i>D. hansenii</i> 10619	-	-	-	No ²	-	No ²	No ²	No ²	No ²	No ²	No ²
<i>C. zeylanoides</i> 26318	-	-	-	No ²	No ²	-	No ²	No ²	No ²	No ²	No ²
<i>C. zeylanoides</i> 24745	-	-	-	No ²	No ²	No ²	-	No ²	No ²	No ²	No ²
<i>P. nalgiovense</i> 96457	-	-	-	-	-	-	-	-	No ²	No ²	No ²
<i>P. nalgiovense</i> 96460	-	-	-	-	-	-	-	No ²	-	No ²	No ²
<i>C. cladosporiodies</i> 60549	-	-	-	-	-	-	-	No ²	No ²	-	No ²
<i>C. cladosporiodies</i> 11275	-	-	-	-	-	-	-	No ²	No ²	No ²	-

“No” represents no visible antagonism. “Yes” represents visible antagonism. All 112 separate tests were done in duplicate.

¹Tests including *L. sakei* were conducted at 30°C with and without 10% CO₂.

²Tests were conducted on both MEA and DRBC plates.

Figures

Figure 4.1 Surface streaking patterns utilized for antagonism experiments. (A) Cross pattern with the blue line streaked first to the first microorganism followed by the two separate red lines of the second microorganism. (B) The second pattern followed the same format where the first microorganism was streaked (blue line) followed by the 2 separate lines of the second microorganism (red lines). (C) The two black lines represent LAB strain that were streaked onto MRS agar prior to introduction of yeast or mold inoculated molten 1% MEA.

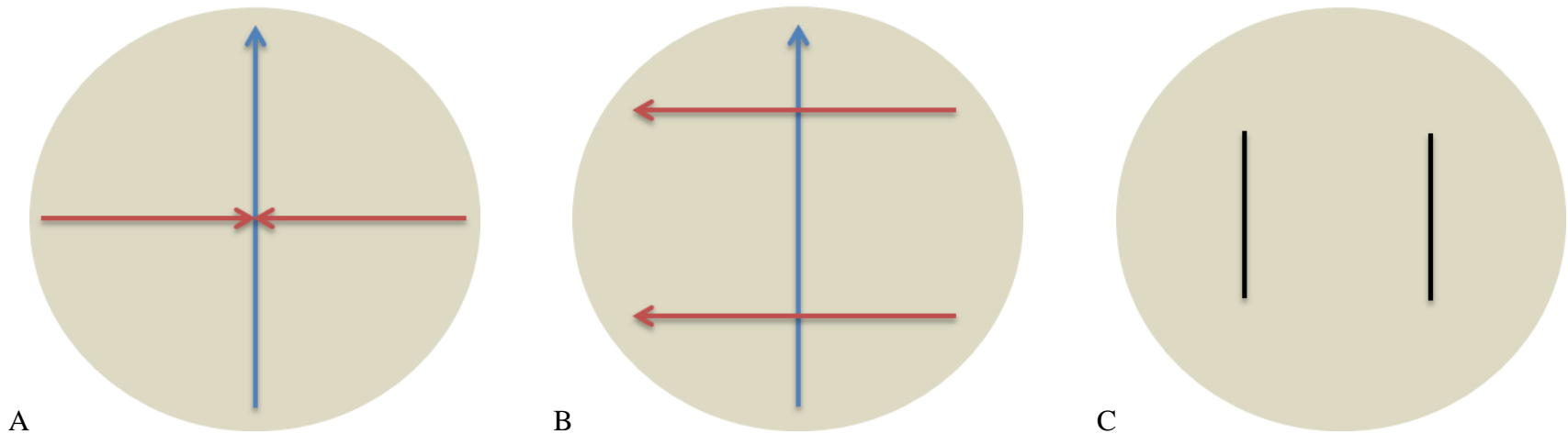
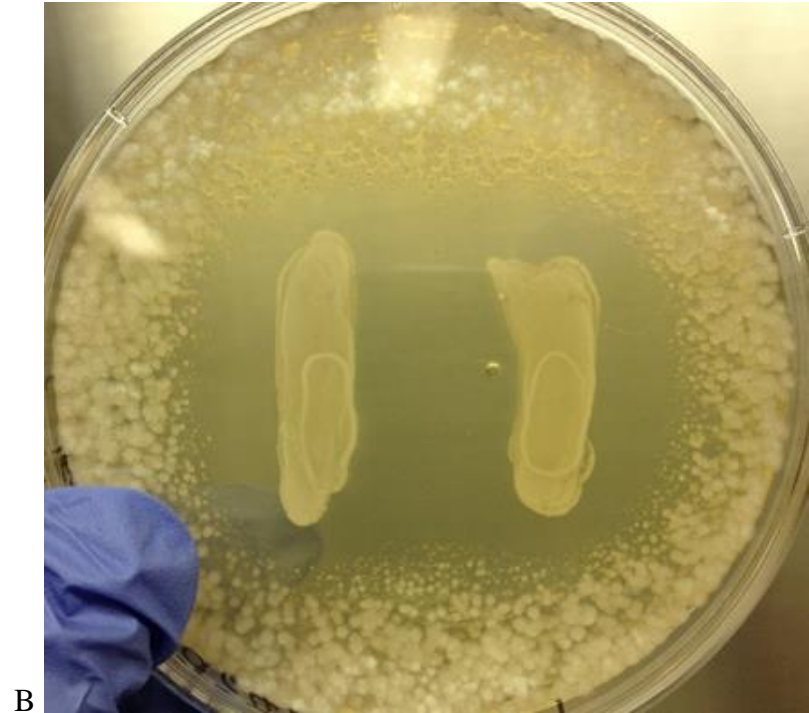


Figure 4.2 Inhibition of molds by LAB. (A) *L. curvatus* PTA5150 inhibiting the growth *P. nalgiovense* 96460. (B) *L. sakei* 15521 inhibiting the growth *C. cladosporioides* 60549.



Chapter 5

SURVIVAL OF SPOILAGE MICROORGANISMS ASSOCIATED WITH THE PRODUCTION OF PICKLED SAUSAGE USING A COLD FILL PROCESS

**Survival of Spoilage Microorganisms Associated with the Production of Pickled Sausage
Using a Cold Fill Process**

Nelson J. Gaydos¹, Hassan Gourama², Joshua A. Scheinberg², Catherine N. Cutter² and Jonathan A. Campbell^{1*}

¹Department of Animal Science; ²Department of Food Science
Pennsylvania State University; University Park, PA 16802

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*Author for correspondence. Tel: (814) 867-2880; Fax; 814-863-6042

Email: meatscience@psu.edu

ABSTRACT

Pickling is a means to preserve and extend the shelf life of a variety of foods, including meat products. The pickling of sausage has traditionally been performed using a heated solution of vinegar, salt, and spices in a process known as hot filling. Hot fill pickling of sausages can result in undesirable quality defects. Alternatively, room temperature brine does not cause the various quality defects resulting from heated brine. To date, no study has determined the efficacy of a cold fill pickling process to inhibit the growth of lactic acid bacteria (LAB), yeasts, and molds associated with acidified meat products. Therefore, the efficacy of cold fill pickling of sausage, using a brine solution (5% acetic acid and 5% salt at 22-23°C), was validated through the inoculation and enumeration of sausages with select LAB, yeasts, and molds over 28 days of pickling. The results revealed a reduction of LAB populations by 5.51 log₁₀ CFU/g in 24 h. Yeast was reduced 3.89 log₁₀ CFU/g in 48 h, while mold was reduced 4.09 log₁₀ CFU/g in 24 h. To our knowledge, the results of this experiment are the first to demonstrate that a cold fill pickling process can effectively reduce and inhibit spoilage microorganisms.

Pickling is a means of preservation that has been used for many years to extend and maintain the shelf life of various types of food products, such as fruits, vegetables, eggs, and meat. In particular, pickled sausage is a common acidified meat product which is generally prepared by curing and cooking sausages, which are then covered with a heated brine solution containing an organic acid (typically acetic or citric acid), salt, spices, food coloring, and sometimes preservatives. The sausages are acidified or pickled in containers which are sealed, letting the brine solution penetrate into the sausages, and allowing for the acidification of the meat product. The pickling process utilizes a combination of microbiological inhibitory effects, which together prevent the further growth and survival of microorganisms. By placing meat products in an acidic environment (reducing the pH), using salt to decrease the water activity (a_w), and sealing the storage container to decrease the oxygen level, the shelf life of the product can be greatly extended. Leistner and Gorris referred to the use of multiple processing factors to inhibit microorganisms in food as “hurdle technology” (16). The use of multiple, sub-lethal factors to kill and inhibit unwanted microorganisms works by disturbing the homeostasis of microorganisms on multiple fronts simultaneously, resulting in the destruction of microorganisms and allowing for the preservation of the food product (15). Preventing the spoilage of such products is critical to manufacturers of pickled sausage and other acidified meat products, to ensure their products remain of high microbiological quality and safety.

The preparation of pickled sausage has traditionally used either a cold fill or hot fill process, in which sausages are covered with either a cold or hot brine solution to begin the pickling process. However, hot filling has generally been found to result in significant quality defects, including cloudiness, turbidity, or discoloration of the brine, which makes the product less appealing to consumers. To avoid these quality defects, many commercial pickled sausage

processors may utilize a cold fill process, in which the brine solution is kept at room temperature prior to pickling.

The pickling of sausages has been regarded as a safe method of meat preservation by federal regulatory authorities. However, recent incidences of spoilage contamination involving pickled meat products have prompted the United States Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) to reconsider the microbiological stability of pickled sausages subjected to a cold fill pickling process (19). Current U.S. regulations addressing acidified foods (21 CFR parts 114 and 120) (1, 13) are mainly focused on canned food products intended to ensure processors properly address the potential growth of *Clostridium botulinum*. Title 21 CFR part 114 (1) states that "...the pH must be maintained at or below 4.6..." with no specification to the type or concentration of the acid, while title 21 CFR part 120 (13) states that "...manufacturers of acidified fruit and vegetable products must demonstrate that their process achieves a 5-log reduction in appropriate acid-tolerant pathogenic bacteria, as validation of product safety." It does not appear that either of these policies has been applied to pickled meat products or used to address issues with spoilage, producing a gap in current regulation of pickled meat products produced in the U.S. Based on current USDA-FSIS policies, processors of pickled meat products must demonstrate stability and safety through the monitoring and recording of several quantitative parameters, including pH, a_w , % salt, temperature and overall formulations. To date, no specific scientific validation has been performed to demonstrate which intrinsic and extrinsic factors are important to ensure the stability and safety of pickled meat products. The lack of scientific validation has left processors of pickled sausage and other acidified meat products without sufficient supporting documentation of their Hazard Analysis Critical Control Point (HACCP) systems. As such, many processors have been found to be in non-compliance

with current USDA-FSIS policies. Furthermore, the use of a cold fill, rather than a hot fill pickling process, has generated additional post-processing contamination concerns by federal regulators, since it is unknown whether a cold fill process might allow for the survival and growth of spoilage and pathogenic microorganisms.

Research on the microflora categorized as spoilage microorganisms associated with acidified meat products is limited. To our knowledge, no scientific literature exists that addresses: 1) the microbial stability of pickled meat products or 2) the growth or survival of spoilage microorganisms associated with acidified meat and sausage products using a cold fill pickling process. Recent studies have demonstrated the effectiveness of pickling processes using various organic acids to reduce the growth and survival of pathogenic bacteria in fruits and vegetables (2, 15), cucumber purees and pickles (6, 7, 8) and hard cooked, pickled eggs (24, 28, 31). Research on pickled eggs may be most relevant to pickled meat products, because of their similar protein content and susceptibility to microbial contamination, although these studies did not address spoilage microorganisms such as LAB, yeasts, and molds. Therefore, the purpose of this study was to validate the efficacy of a cold fill pickling process of sausage to prevent the growth and survival of LAB, yeasts, and molds associated with ready-to-eat (RTE) sausage products.

MATERIALS AND METHODS

In this study, a cooked, smoked, and cured sausage product was placed into an acidified brine (5% acetic acid; 5% salt; 22-23°C) solution and held at room temperature for 28 days. Table 1 highlights the spoilage microorganisms chosen for the challenge study, based on their prominence in fresh and RTE meat products, relevant isolation sources, and availability.

Antagonism of Select Lactic Acid Bacteria and Fungi. Preliminary studies (data not shown) indicated that the lactic acid bacteria (LAB) cultures (Table 5.1) identified for use in this challenge study inhibited select mold cultures. All three LAB cultures inhibited both *Penicillium nalgiovense* strains; *Lactobacillus sakei* ATCC 15521 inhibited both *Cladosporium cladosporioides* strains; and *L. curvatus* 51436 inhibited *C. cladosporioides* 11275. No inhibition occurred between the select yeast and mold cultures. Therefore, the inoculation and spoilage challenge study of sausages by fungi and LAB cultures were performed separately.

Preparation of Select Lactic Acid Bacteria Cultures and Challenge Study Inoculum.

LAB cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Lyophilized cultures were transferred into fresh de Man, Rogosa and Sharpe (MRS) broth (Difco, Sparks, MD). Both *L. curvatus* cultures were incubated aerobically at 30°C for 48 h, while *L. sakei* was incubated at 30°C in a microaerophilic environment (5.0% O₂, 10% CO₂, 85% N₂) using a CO₂ incubator (VWR International, West Chester, PA) following ATCC recommendations. Colonies were then re-suspended in fresh MRS broth containing 10% glycerol (VWR, Radnor, PA) and stored at -80°C until future use. Working stocks were maintained on MRS agar (Difco) and stored at 4°C.

To prepare the separate LAB inoculum, frozen stocks were transferred twice into fresh MRS broth and streaked onto MRS agar using the incubation conditions described above. Single colonies of each LAB were isolated and transferred into 10 ml of fresh MRS broth. Following incubation, 10 ml of each culture were transferred to 990 ml of fresh MRS broth and incubated for an additional 24 h at the same temperature and atmospheric conditions as previously described to obtain a cell concentration of ~8log₁₀ CFU/ml. Each single LAB inoculum was prepared in duplicate, providing a total inoculum volume of 6 liters. Two liters of each LAB

culture were mixed in a sterilized metal container under a biological safety hood to produce a homogenous LAB cocktail (6 liters total) for the sausage inoculation immersion bath as described previously (24, 28). This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Yeast Cultures. Yeast cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Upon receipt, yeast cultures were transferred into fresh yeast mold (YM) broth (Difco, Sparks, MD) and incubated aerobically at 25°C for 72 h following ATCC recommendations. Yeast cultures were then re-suspended in YM broth containing 10% glycerol (VWR, Radnor, PA), and stored at -80°C. To create working stocks, the yeast freezer stock cultures were streaked onto Malt Extract Agar (MEA; Difco, Santa Maria, CA) slants in sterile, screw top test tubes, incubated at 25°C for 72 h, and stored at 4°C until use.

To prepare the yeast inoculum, single colonies of each yeast strain were individually transferred using a sterile 1 µl loop from MEA slants to 10 ml of fresh YM broth and allowed to grow aerobically at 25°C for 24 h. Incubated yeast cultures (10 ml) were transferred to 490 ml of fresh YM broth and incubated aerobically at 25°C for an additional 72 h to obtain a cell concentration of $\sim 6 \log_{10}$ CFU/ml for each of the yeast cultures. Five hundred ml of each of the four yeast cultures were mixed in a sterilized metal container under a biological safety hood to produce a homogenous yeast cocktail (2 liters total) for the sausage inoculation immersion bath. This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Mold Cultures. Mold cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Each mold culture was transferred to fresh YM broth and incubated aerobically at 25°C for 96 h following ATCC recommendations. Molds were then re-

suspended in YM broth containing 10% glycerol (VWR, Radnor, PA), and stored at -80°C for future use. Cultures were transferred from freezer stocks using a sterile 10 µl loop to fresh MEA slants prepared in 125 ml glass bottles, by spreading the inoculated loop across the agar slant surface. Agar slants were then incubated aerobically at 25°C for 96 h and stored at 4°C until use.

To prepare the mold inoculum, 50 ml of sterile 2% (w/v) buffered peptone water (BPW; HiMedia, Mumbai, India) with Tween 80 at 0.03% (w/v) was poured into each MEA bottle slant, and a sterile 10 µl loop was used to scrape off and collect the mold spores for transfer into the BPW. Tween 80 was added to the BPW to prevent mold spores from clumping while keeping spores in suspension. The BPW/Tween 80 spore solution was filtered through sterile cheese cloth to separate mold spores from other fungal debris and transferred to a sterile, 500 ml glass bottle. This process was repeated five times for each individual mold culture to ensure complete removal of spores and produce a spore solution of 500 ml. Through this technique, spore concentrations of $\sim 6 \log_{10}$ spores/ml were produced for each mold culture based on plate counts performed using a pour plating technique. Briefly, 1 ml of BPW spore solution was added to molten 1% (w/v) MEA in a 15 ml Falcon test tube (VWR, Radnor, PA) and held in a 50°C water bath (Precision, Winchester, VA). The 15 ml Falcon tubes were then vortexed, pour-plated, and incubated at 25°C for 72 to 96 hours before enumeration.

To prepare the final inoculum, equal volumes of each of the four mold cultures (500 ml) were mixed in a sterilized metal container under a biological safety hood to produce a homogenous mold cocktail (2 liters total) for the sausage inoculation immersion bath. The 2 liter yeast cocktail was then mixed with the 2 liter mold spore cocktail to produce homogeneous yeast and mold cocktail inoculum (4 liters total) for the sausage inoculation immersion bath. This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Preparation of Sausages. Sausages used in this study were manufactured at The Pennsylvania State University Meat Laboratory and formulated with the following ingredients: beef trim (80% lean; 22% wt/wt), pork trim (80% lean; 44% wt/wt and 50% lean; 22% wt/wt), water (7% wt/wt), salt (2.2% wt/wt), dextrose (0.44% wt/wt), ground black pepper (0.28% wt/wt), sodium tripolyphosphate (2500 ppm), ground mustard (0.55% wt/wt), granulated garlic (0.55% wt/wt), ground ginger (0.013% wt/wt), sodium erythorbate (540 ppm) and curing salt (6.25% NaNO₂; 156 ppm). Sausages were stuffed to a weight of 130.4 g into a 35 mm curved, edible collagen casing (Devro Ltd, Moodiesburn, Scotland, UK) using a Handtmann VF608 Plus with twist linker (Albert Handtmann Maschinenfabrik GmbH & Co, Biberach, DE). A large diameter (>30 mm), collagen type casing was chosen because it demonstrated the most resistance to brine penetration and acidification, as compared to skinless and natural type casings in preliminary experiments (data not shown). The sausages were smoked and cooked in a Kerres Smoke Air[®] JS 1950 (Kerres Anlagensysteme GmbH, Backnang, DE) to a core temperature of 70°C, transferred to a walk-in cooler (1-2°C) and chilled to 4°C in accordance with Appendix B (32). Table 5.2 illustrates the thermal processing schedule used to smoke and cook the sausages. The average measurements of the cooked and chilled sausages were: length 144 mm ± 2, width 31 ± 2 mm, wt 127 ± 2 g. Sausages were placed in 14 in x24 in (5.5 cm x 9.5 cm) food grade, 3mil gauge pouches (PCS Supplies Inc., Penns Groove, NJ) and vacuum sealed (Smith SuperVac Digimat, Clifton, NJ). Packaged sausages were stored in a walk-in cooler at 2°C (<21 days) until they could be transported to The Pennsylvania State University Muscle Foods Microbiology Laboratory for the microbiological challenge study. Three separate batches of sausages were made for each of the 3 inoculation and sampling replications. Fat analysis was conducted in

duplicate for each of the 3 batches of sausages following a modified Babcock Fat Analysis procedure (12).

Preparation of Brine Solution. Food-grade distilled white vinegar (5% acidity; H. J. Heinz Company, Pittsburgh, PA) and non-iodized salt (NaCl; Morton, Chicago, IL) were purchased from local supermarkets. Pickling brine (5% salt and 5% acetic acid) was mixed 24 h prior to the inoculation and subsequent pickling of sausages, covered with aluminum foil and allowed to fully equilibrate to room temperature (~22-23°C). This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Inoculation of Sausages with Select LAB. Precooked and chilled (~2°C) sausages (n=144) were aseptically removed from the vacuum packaged bags and transferred to sterilized metal bins under a biological safety hood. The LAB cocktail (6 liters total) was poured over the sausages (n=48 per replication) and allowed to sit for 30 minutes with gentle mixing periodically following the methods described previously (24, 28). The LAB inoculation was repeated in the same manner for each of the 3 replications of the challenge study.

Inoculation of Sausages with Select Yeast and Mold. Precooked and chilled (~2°C) sausages (n=144) were removed aseptically from the vacuum packaged bags and transferred to sterilized metal bins under a biological safety hood. The yeast and mold cocktail (4 liters total) was poured over the sausages (n=48 per replication) and allowed to sit for 30 minutes with gentle mixing periodically, following the methods described previously (24, 28). The yeast/mold inoculation was repeated in the same manner for each of the 3 replications of the challenge study.

Negative Control Sausages. In addition to the inoculated sausages, negative control sausages also were utilized. Precooked and chilled (~2°C) sausages (n=36) were aseptically

removed from the vacuum packaged bags, transferred to sterilized metal bins under a biological safety hood and covered with 3 liters of sterile BPW. The sausages (n=12 per replication) were allowed to sit for 30 minutes in the sterile BPW with gentle, periodic mixing as described above. Three replications of the negative control sausage group were performed for the challenge study.

Pickling of Sausages. Following the inoculation of sausages through the immersion bath technique, sausages were removed aseptically from the inoculum and placed into sanitized 1 gal (3.8 L) plastic food grade screw top containers (Pretium Packaging, Hazleton, PA). All sausages inoculated with LAB, separate from the fungal inoculation, were evenly distributed into 4 separate containers (n=11 per container), with the exception of 4 sausages that were withdrawn for initial sampling. Yeast and mold-inoculated sausages and the negative control sausages also were aseptically transferred to containers as previously described. Room temperature (~22-23°C) pickling brine was poured into each container, allowing for a headspace of 0.5 inches (0.19cm), the containers capped and sealed, simulating a cold fill commercial pickling and packaging process. The pickled sausages were stored at room temperature (~22-23°C) for the remainder of the challenge study (28 days) for each of the 3 replications. A total of 24 containers were utilized for each of the LAB and fungal treatment groups for the duration of the study, while 3 containers were used for the negative control group.

Sampling and Microbial Analysis. Immediately following the inoculation of the sausages and prior to the pickling step, 4 sausages per replication (for both the LAB and fungal treatment groups) and 2 sausages from the negative control group were selected randomly and removed from the inoculum immersion bath. Sausages were sampled and analyzed to determine initial inoculation concentrations, pH, a_w , and % salt of both the individual sausages and pickling brine. Three sausages were removed at the initial sampling (time 0) for each of the LAB- and

fungal-inoculated samples, while one sausage from the negative control was sampled and evaluated for microbial analyses. Additional sausages (n=1 per treatment per time point) were used to analyze pH, a_w , and % salt. This sampling protocol was repeated for all treatment and control groups at various time points (2h, 4h, 6h, 12h, 1d, 2d, 3d, 7d, 14d, 21d, and 28d) for each of the three replications.

To enumerate surviving LAB, yeast, and mold cultures on sampled sausages at each time point, pickled sausages were aseptically removed from their respective containers, individually placed into 3500 ml filtered stomacher bags (Interscience, Rockland, MA), weighed, diluted with five times the vol of the sausage with sterile BPW, and stomached for 1.5 minutes at 360 rpm (Interscience Jumbo Mix Stomacher, Rockland, MA). The stomachate (~35 ml) of each pickled sausage was serially diluted in 9 ml of BPW and aliquots of 1 ml and 0.1 ml were spread plated in duplicate onto selective agars to enumerate both LAB and fungal cultures. MRS agar (Difco) was used for the enumeration of LAB cultures, while Dichloran-Rose Bengal Chloramphenicol agar (DRBC; Difco) was used for enumeration of yeast and mold cultures. DRBC spread plates were incubated (upright) aerobically at 25°C for 3 to 5 days, while MRS spread plates were incubated aerobically (inverted) at 30°C for 48 h. For the negative control sausages, any naturally occurring microflora was monitored using Aerobic Count Plate (AC) and Yeast Mold (YM) Petrifilm™ (3M, St. Paul, MN). AC Petrifilm™ were incubated both aerobically and in microaerophilic conditions (5.0% O₂, 10% CO₂, 85% N₂) at 30°C for 48 to 72 h while YM Petrifilm™ was incubated aerobically at 25°C for 96 h.

Enrichment Procedures. During each sampling of inoculated pickled sausage, 1 ml aliquots of stomachate and associated brine solution were transferred to 9 ml of YM broth and MRS broth for enrichments of fungi and LAB respectively. Yeast and mold enrichments were

incubated aerobically at 25°C for 3 d before plating onto DRBC plates, which were incubated at 25°C for 3 to 5 d. LAB enrichments were incubated aerobically at 30°C for 48 h before plating onto MRS agar plates, which were incubated at 30°C for 48 to 72 h.

Sausage and Brine Chemical Analysis. Brine pH was measured using a Thermo Scientific Orion 4 Star pH ISE Benchtop meter with a connected Orion 9103BNWP Semi-Micro Combination pH Probe (Beverly, MA). Sausage pH was measured using a Thermo Scientific Orion 4 Star pH ISE Benchtop meter with an Orion 8163BNWP Ross Combination Spear Tip pH Electrode (Beverly, MA). The sausage designated for pH measurements were first cut in half using a cross sectional cut and then the tip of the pH probe was inserted into the center of the cut half. The other portion of the cut sausage was used for water activity (a_w) analysis. A_w was analyzed using an Aqua Lab 4TE (Decagon Devices, Pullman, WA) water activity meter. Percent salt concentrations for sampled sausages and pickling brine were determined using Chloride Quantabs (Hach, Loveland, CO) according to a method previously described.(30).

Statistical Analysis. Means of the 3 replications of LAB, yeast, and mold plate counts were calculated and converted to \log_{10} CFU/g for statistical analysis. The limit of detection for the MRS and DRBC spread plates was 0.40 \log_{10} CFU/g, while the limit of detection for the Aerobic Count and Yeast and Mold Petrifilm™ was 0.70 \log_{10} CFU/g. Comparison of spoilage microorganism populations was determined at each time point, within and between groups, using one way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test at $\alpha = 0.05$. Comparison of pH, a_w , % salt of sausages and brine values also were determined using a one way analysis of variance (ANOVA) with Tukey's HSD test at $\alpha = 0.05$. Analysis of Variance (ANOVA) tests were conducted using SAS (SAS software version 9.3, SAS Institute Inc., Cary, NC.).

RESULTS

Inoculated pickled sausages, using a cold fill process and stored at room temperature (~22-23°C), experienced rapid and significant decreases ($p \leq 0.05$) in microbial populations measured in this study (Fig. 5.1 and 5.2; Table 5.3). Within the first 24 h of pickling, LAB populations were reduced ~5.51 log₁₀ CFU/g, with a total reduction of 6.58 log₁₀ CFU/g at the conclusion of the 28-day study. After 7 days, LAB populations were reduced to undetectable levels (0.40 log₁₀ CFU/g) (Fig. 5.1; Table 5.3). Enrichments of LAB-inoculated sausages and brine were found to be negative after 21 days (Table 5.3). Similarly, all mold and yeast populations were reduced to undetectable levels (0.40 log₁₀ CFU/g) within the first 24 and 48 h of the study, respectively (Fig. 5.2; Table 5.3). Yeast and mold populations were reduced 3.89 log₁₀ CFU/g and 4.09 log₁₀ CFU/g respectively by day 28 of the study. Enrichments of yeast and mold populations were negative after 48 h, while brine enrichments were not positive for the organisms at any sampling period (Table 5.3). Significant changes ($p \leq 0.05$) in pH of the inoculated sausages and brine were observed throughout the length of the study (Table 5.4). The majority of the changes in pH occurred within the first 72 h. Within this time period, the pH of inoculated sausages and brine equilibrated to 3.92 ± 0.04 and 3.94 ± 0.06 , respectively. Similarly, negative control sausages experienced a significant ($p \leq 0.05$) and rapid decline in sausage pH, coupled with an increase in brine pH (Table 5.4). There was no significant difference observed in a_w throughout the study, with values ranging between 0.95 and 0.97. The % salt concentration of cold fill pickled sausages rose slightly from 2.26% to 2.80%, while the % salt concentrations of the brine decreased from 4.86% to 4.30% over the course of the study. Average % fat assessed through the modified Babcock fat analysis procedure for all 3 reps was 26.4%, with a range of 24.5 to 29.5% fat (data not shown).

DISCUSSION

The idea of combining several antimicrobial processes (often at sub-lethal levels), in an effort to increase the microbial safety of food products, is known as hurdle technology (15). It is theorized that the combination of antimicrobial processes works better than a single intervention to inhibit the growth of potential spoilage or pathogenic microorganisms in foods. Typically, the process of pickling sausage utilizes heat, acetic acid, and salt, which in combination, can increase the shelf life and microbial stability of the acidified meat product (25).

Weak acids like acetic ($pK_a = 4.76$) exert antimicrobial effects through the action of their undissociated form, which can diffuse through bacterial and fungi cell membranes, altering the homeostasis of the cell. In addition, the dissociated form of weak acids can concentrate within the cell, cause the buildup of protons (H^+), resulting in the acidification of the cytoplasm and cell interior (14, 15). The accumulation of protons and subsequent decline in pH can cause damage to cell membranes, denaturation of proteins, enzymes and DNA, and metabolic exhaustion (16, 33). The accumulation of anions also is thought to contribute significantly to the inhibition of microorganisms, causing high turgor pressure, potential leakage from the cell, production of free radicals, and oxidative stresses (9, 25, 26). It is also important to note that weak acids exhibit greater antimicrobial effects in lower pH environments (3, 14). Therefore, the low pH of the brine used in this study (pH 2.72), likely aided in the reduction of LAB and fungal populations. In addition, since the pH of the brine solution used in this study was below the pK_a value of acetic acid, this step ensured that the majority of the weak acid was in the undissociated form and able to penetrate into cell membranes.

The addition of salt in acidified food products has been shown to increase the antimicrobial activity of acetic acid, by lowering the a_w and increasing osmotic pressure (15).

Although the addition of salt has inhibitory effects on bacteria and fungi, this process also can result in an environment which favors LAB (5, 27). Although salt was used in the formulation of the sausages and brine used in this study, a_w was not observed to change dramatically throughout the length of the challenge study. Therefore, any observed antimicrobial effects were not due to decreasing a_w . These results are similar to those observed by Richard and Cutter (24) and Scheinberg et al. (28) who also noted no significant changes in a_w while investigating pickled eggs.

The results of this study demonstrated that the decrease in spoilage microorganism populations coincided with the rapid decrease in pH of inoculated sausages within the first 24 h. This result suggests that the total reduction and inhibition of these microorganisms was driven mainly by the rapid decline in pH. This result is in agreement with Richard and Cutter (24) who demonstrated a $>4.85 \log_{10}$ CFU/ml reduction in hard cooked eggs inoculated with a cocktail of *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in 24 h at 22°C, with a similar associated drop in pH. The authors also noted that the reduction of pathogenic populations were significantly higher ($p < 0.05$) in the hard cooked eggs at room temperature (22°C) than at refrigeration temperatures (4°C), suggesting that temperature is also an important factor in the acidification process. Studies by Leistner and Gorris (16) and Leistner (17) also have reported evidence supporting the hypothesis that increasing temperatures lead to an increase in the ability of acids and preservatives to produce antimicrobial effects. Similar results were observed in a follow-up pickled egg study by Scheinberg et al. (28).

Among the three groups of spoilage microorganisms utilized in the current study, select LAB cultures demonstrated the greatest acid tolerance, exhibiting higher populations throughout the study, when compared to select yeast or mold cultures (Fig. 5.1 and 5.2; Table 5.3). The

addition of salt, decrease in pH, the reduction of a_w , and brine injection have caused shifts in the microflora of meat products to favor LAB (5, 11). The pickling process in this study may have created an environment more suitable for LAB populations, which initially, allowed LAB to persist longer than both the yeast or mold cultures. Moreover, LAB populations have demonstrated the potential to become more acid-tolerant if exposed to sub-lethal acidic conditions for long periods of time (21).

In other studies, synergistic effects between decreased pH and increased sodium chloride concentrations also resulted in decreased yeast populations(4). The 5% salt and 5% acetic acid brine solution utilized in this study achieved significant reductions in yeast populations that suggest a synergistic inhibitory effect of the brine. Other studies have found that yeast populations can be decreased by the addition of as little as 1% acetic acid, and yeast growth can be entirely inhibited at concentrations of 3.5 to 4% (34). In contrast, molds have a wider pH growth range and lower optimum pH level than LAB or yeast, and can grow in the presence of salt (2-3% wt/wt; 18, 23). These factors did not appear to aid in the survival or growth of select molds (*P. nalgiovense*, *C. cladosporioides*) used in this study, since all four mold cultures were reduced to undetectable levels in 24 h. While previous studies have noted the abilities of LAB, yeasts, and molds to adapt, resist, and breakdown food preservatives, the acidity of the brine solution used in this study was likely too high for the microorganisms to adapt to or counteract its lethality (10, 20, 21, 22, 29). To our knowledge, this is the first study which has measured the survival of spoilage microorganisms in a pickled meat product.

Furthermore, this study demonstrated the ability of a cold fill pickling process to inhibit the growth and survival of LAB, yeast, and mold on a pre-cooked RTE pickled sausage product. The use of industry standard sausage production parameters (casing type, diam, formulation) and

LAB, yeast, and mold strains which were most associated with the spoilage of meat products, also greatly enhance the applicability of this study. The findings of this study will be most useful as Hazard Analysis and Critical Control Point (HACCP) validation for manufacturers of pickled sausage who utilize a cold fill process. Future work by these authors will examine the efficacy of the same cold fill pickling process on sausages inoculated with relevant strains of *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus*.

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FIGURE LEGENDS

Fig. 5.1: Average spread plate counts (\log_{10} CFU/g) of LAB (*Lactobacillus curvatus* and *L. sakei*) from time points 0 to 168 h (7 days) with standard deviation error bars shown. 0.40 \log_{10} CFU/g detection limit is shown by the dotted blue line. ^{a-e} Different letters represent significant difference between counts at different time points ($p < 0.05$).

Fig. 5.2: Average spread plate counts (\log_{10} CFU/g) of Yeast (*Debaryomyces hansenii* and *Candida zeylanoides*) and Mold (*Penicillium nalgiovense* and *Cladosporium cladosporioides*) from time points 0 to 48 h with standard deviation error bars shown. 0.40 \log_{10} CFU/g detection limit is shown by the dotted blue line.

^{a-e} Different letters represent significant difference between counts at different time points ($p < 0.05$). \log_{10} CFU/g counts of yeast and mold counts are compared to themselves between the different time points, not to each other.

Table 5.1: Spoilage microorganisms inoculated onto sausages prior to cold fill pickling.

Spoilage Microorganism	Genus/Species	ATCC* /ID	Isolation Source
Lactic Acid Bacteria (LAB)	<i>Lactobacillus curvatus</i>	51436	fermented sausage
	<i>Lactobacillus curvatus</i>	PTA5150	Kimchi (fermented Korean dish)
	<i>Lactobacillus sakei</i>	15521	moto (sake starter culture)
Yeast	<i>Debaryomyces hansenii</i>	90624	salty pickle
	<i>Debaryomyces hansenii</i>	10619	horse meat sausage
	<i>Candida zeylanoides</i>	26318	Spanish sausage
Mold	<i>Candida zeylanoides</i>	24745	grape must
	<i>Cladosporium cladosporioides</i>	60549	frozen lamb carcass
	<i>Cladosporium cladosporioides</i>	11275	wheat bread
	<i>Penicillium nalgiovense</i>	96457	mold ripened sausage
	<i>Penicillium nalgiovense</i>	96460	mold ripened salami

* American Type Culture Collection (ATCC); Manassas, VA.

Table 5.2: Thermal processing schedule of sausages used in the spoilage challenge study.

Function	House Temperature (°F)	% Relative Humidity	Core Temperature (°F)	Time (minutes)
Steam Cook	120	90	0	00:15
Dry Cook	135	0	0	00:15
Steam Cook	163	90	0	00:10
Dry Cook	135	0	0	00:15
Heissrauch	140	0	0	00:15
Dry Cook	140	0	0	00:20
Heissrauch	140	0	0	00:15
Dry Cook	140	0	0	00:20
Heissrauch	140	0	0	00:10
Dry Cook	140	0	0	00:10
Steam Cook	154	90	0	00:10
Dry Cook	140	0	0	00:10
Steam Cook	165	90	158	00:00
Dry Cook	140	0	0	00:10
Shower	0	0	0	00:15

Note: “Heissrauch” – hot smoking step

Table 5.3: Average counts (\log_{10} CFU/g) of LAB (*Lactobacillus curvatus* and *L. sakei*), Yeast (*Debaryomyces hansenii* and *Candida zeylanoides*) and Mold (*Penicillium nalgiovense* and *Cladosporium cladosporioides*) cultures inoculated onto sausages, negative control Petrifilm™ results and enrichment results of the inoculated sausages over the course of the 28 day challenge study.

Time Points	LAB		Yeast		Mold		Negative Control	
	\log_{10} (CFU/g)	Enrichment	\log_{10} (CFU/g)	Enrichment	\log_{10} (CFU/g)	Enrichment	AC Petrifilm™ \log_{10} (CFU/g)	YM Petrifilm™ \log_{10} (CFU/g)
0 h	6.98 ^a	+	4.29 ^a	+	4.49 ^a	+	1.36 ^a	≤0.70 ^a
2 h	3.76 ^b	+	0.85 ^b	+	2.24 ^b	+	1.36 ^a	≤0.70 ^a
4 h	3.46 ^b	+	0.61 ^b	+	1.45 ^{bc}	+	1.36 ^a	≤0.70 ^a
6 h	3.16 ^{bc}	+	≤0.40 ^b	+	1.10 ^{cd}	+	1.36 ^a	≤0.70 ^a
12 h	2.30 ^{cd}	+	≤0.40 ^b	+	0.53 ^d	-	0.80 ^a	≤0.70 ^a
24 h	1.63 ^{de}	+	0.62 ^b	-	≤0.40 ^d	-	0.86 ^a	≤0.70 ^a
2 d (48 h)	0.43 ^e	+	≤0.40 ^b	-	≤0.40 ^d	-	0.86 ^a	≤0.70 ^a
3 d (72 h)	1.15 ^e	+	≤0.40 ^b	-	≤0.40 ^d	-	0.86 ^a	≤0.70 ^a
7 d (168 h)	≤0.40 ^e	+	≤0.40 ^b	-	≤0.40 ^d	-	≤0.70 ^a	≤0.70 ^a
14 d (336 h)	0.45 ^e	+	≤0.40 ^b	-	≤0.40 ^d	-	≤0.70 ^a	≤0.70 ^a
21 d (504 h)	0.55 ^e	+	≤0.40 ^b	-	≤0.40 ^d	-	≤0.70 ^a	≤0.70 ^a
28 d (672 h)	≤0.40 ^e	-	≤0.40 ^b	-	≤0.40 ^d	-	≤0.70 ^a	≤0.70 ^a

^{a-e} Different letters represent significant difference between values within each column ($p=0.3690$). \log_{10} CFU/g values at each time point are the mean of (n=9) 3 sausages/replication. Enrichment results are the sum of 3 sausage and 4 brine samples/replication.

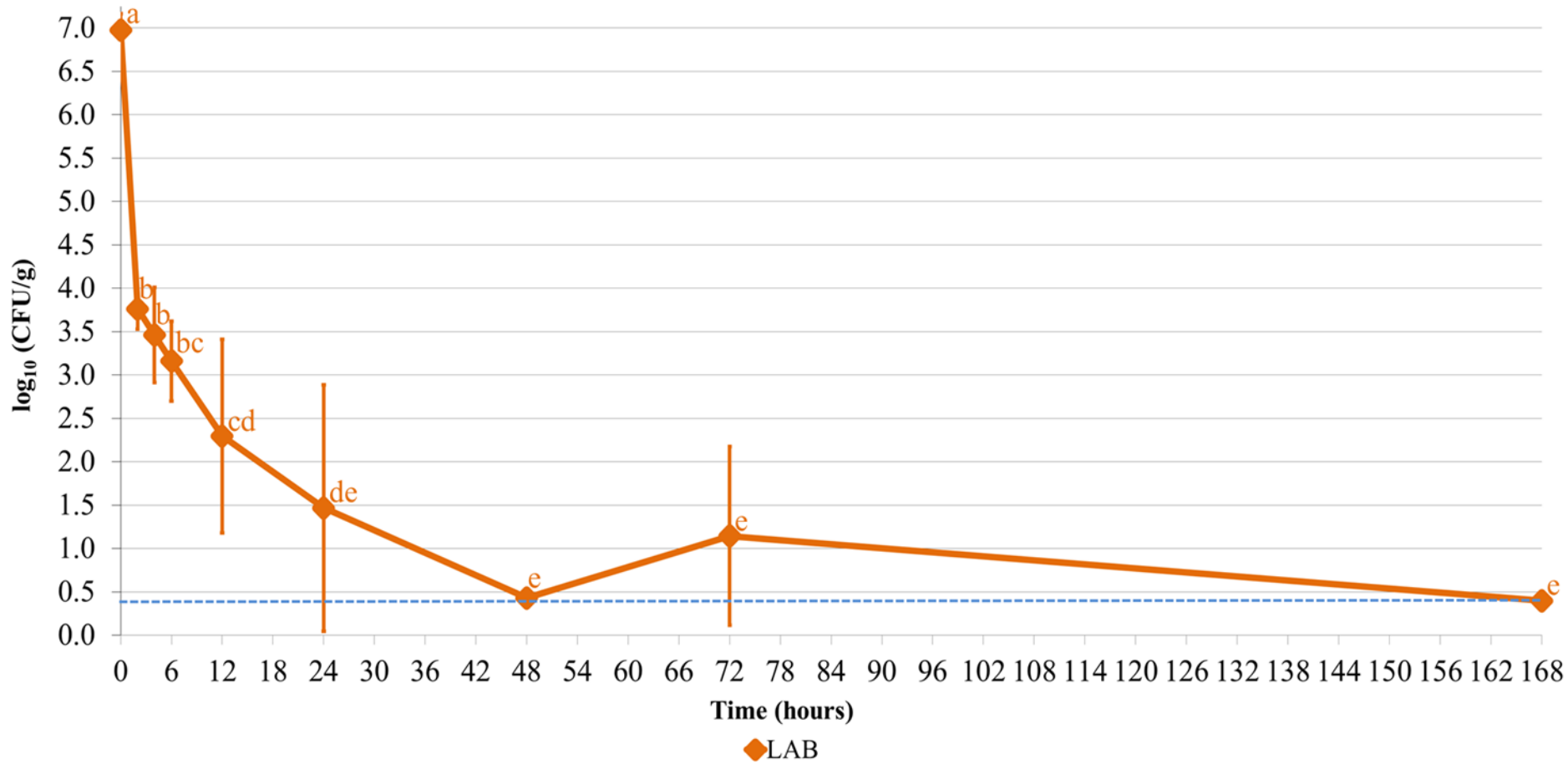
Note: “+” = positive enrichment result and “-” = negative enrichment result.

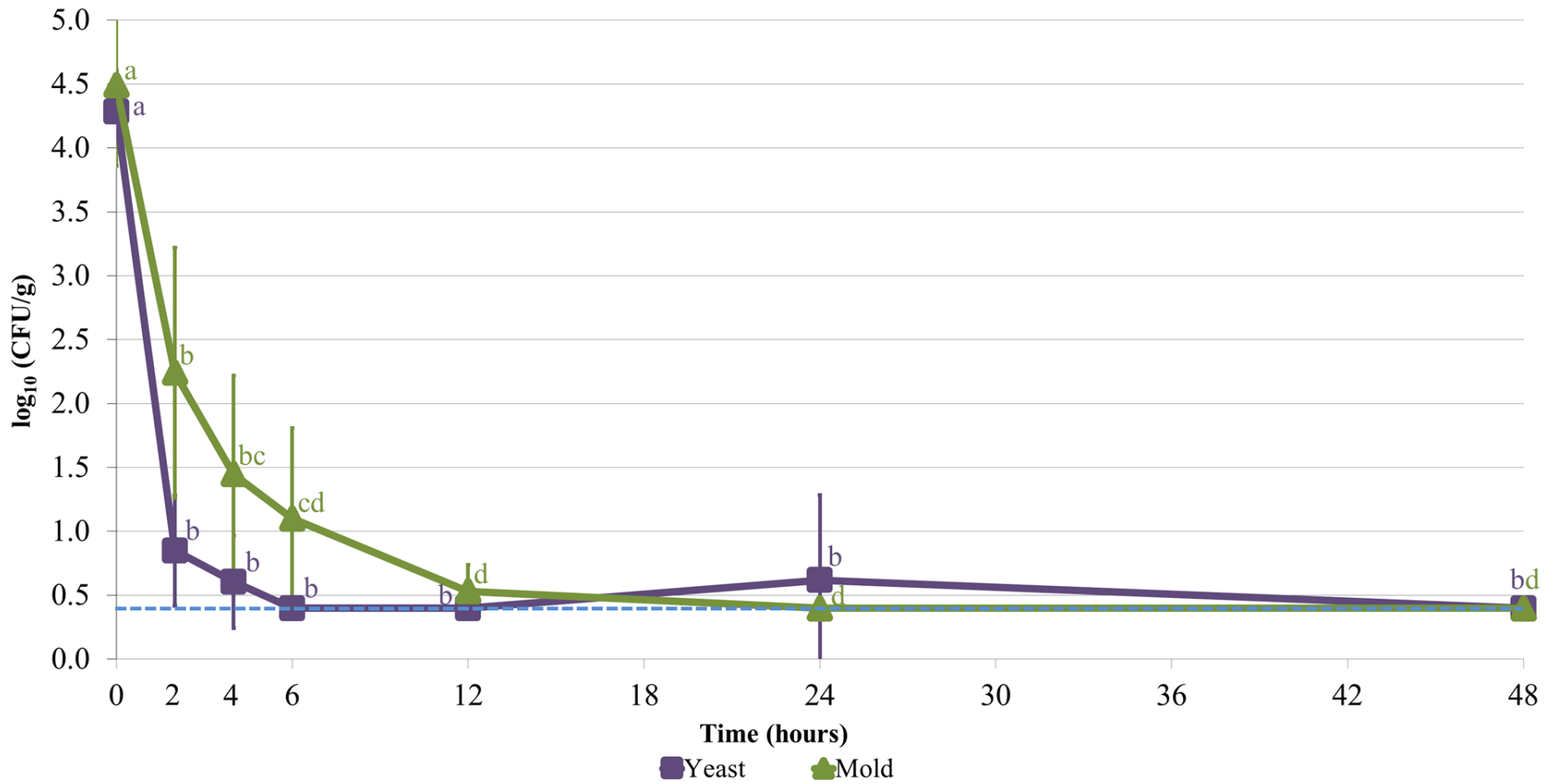
Note: The greater \log_{10} CFU/g counts shown in the negative control group compared to the inoculated sausages is an artifact from the differences in the detection limits of the spread plates (0.40 \log_{10} CFU/g) and Petrifilm™ (0.70 \log_{10} CFU/g) sampling procedures.

Table 5.4: pH of inoculated sausage and brine and pH, a_w , and % salt of negative control sausages throughout the 28 day challenge study.

Time Points	LAB		Yeast + Mold		Negative Control				
	pH Sausage	pH Brine	pH Sausage	pH Brine	pH Sausage	pH Brine	a_w	% Salt Sausage	% Salt Brine
0 h	6.54 ^{ab}	2.72 ^d	6.52 ^a	2.72 ^e	6.44 ^a	2.61 ^c	0.97 ^a	2.26 ^a	4.86 ^a
2 h	6.80 ^a	3.40 ^{abcd}	6.62 ^a	3.03 ^{de}	-	-	-	-	-
4 h	6.54 ^{ab}	3.26 ^{bcd}	6.54 ^a	3.12 ^{cde}	-	-	-	-	-
6 h	6.32 ^{ab}	2.98 ^{cd}	6.55 ^a	3.23 ^{cde}	-	-	-	-	-
12 h	6.23 ^{ab}	3.12 ^{bcd}	6.33 ^a	3.03 ^{de}	6.26 ^a	3.08 ^{bc}	0.96 ^a	2.19 ^a	4.64 ^a
24 h	5.81 ^b	3.26 ^{bcd}	6.08 ^a	3.19 ^{cde}	6.09 ^a	3.17 ^{abc}	0.97 ^a	2.29 ^a	4.71 ^a
2 d (48 h)	4.59 ^c	3.33 ^{bcd}	4.67 ^b	3.30 ^{bcde}	-	-	-	-	-
3 d (72 h)	4.26 ^c	3.61 ^{abc}	4.21 ^b	3.39 ^{abcd}	-	-	-	-	-
7 d (168 h)	4.13 ^c	3.93 ^{ab}	4.05 ^b	3.76 ^{abc}	3.97 ^b	3.64 ^{ab}	0.96 ^a	2.69 ^a	4.73 ^a
14 d (336 h)	4.26 ^c	4.20 ^a	4.11 ^b	3.93 ^{ab}	-	-	-	-	-
21 d (504 h)	3.89 ^c	3.95 ^{ab}	3.92 ^b	3.91 ^{ab}	3.88 ^b	3.82 ^{ab}	0.95 ^a	2.53 ^a	4.44 ^a
28 d (672 h)	3.88 ^c	3.89 ^{ab}	3.96 ^b	4.00 ^a	3.96 ^b	2.61 ^a	0.96 ^a	2.80 ^a	4.30 ^a

^{a-e} Different letters represent significant difference in values within each column ($p=0.2062$). All numerical values are the mean of (n=3) 1 sausage/replication).





Chapter 6

**SURVIVAL OF PATHOGENIC BACTERIA ASSOCIATED
WITH THE PRODUCTION OF PICKLED SAUSAGE USING A
COLD FILL PROCESS**

Survival of Pathogens in Cold Fill Pickled Sausage

Survival of Pathogenic Bacteria Associated with the Production of Pickled Sausage Using a cold fill Process

Nelson J. Gaydos¹, Catherine N. Cutter², Hassan Gourama² and Jonathan A. Campbell^{1*}

¹Department of Animal Science; ²Department of Food Science

Pennsylvania State University; University Park, PA 16802

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*Author for correspondence. Tel: (814) 867-2880; Fax; 814-863-6042

Email: meatscience@psu.edu

Abstract

Preservation by pickling has been used for many years to extend the shelf life of various types of food products. By storing various meat products in a brine solution containing acetic acid (vinegar), salt, spices and sometimes other preservatives like potassium sorbate, the pH of the product is reduced, thus increasing to the safety and shelf life of the product. Pickling may involve the use of heated brines to further add to the safety of the food product. When pre-cooked, ready-to-eat (RTE) sausages are pickled with a heated brine solution, the process is referred to as hot filling. Hot filling may cause fat to leach out of the sausages and into the brine, making it turbid and unappealing to consumers. Due to the potential quality defects caused by higher temperatures in hot fill pickling, cold fill pickling using room temperature brine, is preferred by many pickled sausage manufacturers. Since very little information exists on the safety of cold fill pickled sausages, a challenge study was designed using a brine solution (5% acetic acid and 5% salt at 25°C) to pickle pre-cooked, RTE sausages inoculated with a cocktail of three strains of each of the following pathogenic bacteria: *Salmonella* spp. (Typhimurium, Senftenberg and Montevideo), *Listeria monocytogenes* and *Staphylococcus aureus*. All pathogens experienced a total reduction of 6.80 log₁₀ CFU/g in 72 h on general media. On selective media, *Salmonella* spp. and *L. monocytogenes* decreased 6.33 and 6.35 log₁₀ CFU/g in 12 h, respectively while *S. aureus* declined 6.80 log₁₀ CFU/g in 24 h. Sausages experienced significant ($p \leq 0.05$) decreases in pH over the 28 days of the study while no significant differences were observed in water activity ($p = 0.1291$) or % salt of the sausages ($p = 0.1445$) or brine ($p = 0.3180$). The results of this experiment demonstrate that cold fill pickling can effectively reduce and inhibit pathogenic bacteria contamination.

Pickling is a preservation technique that has been used for centuries to extend and maintain the shelf life of food products. In the case of pickled sausages, cured and cooked sausages are placed into containers then a heated brine solution is pouring over them containing acid (typically acetic or citric acid), salt, spices, food coloring, and sometimes other preservatives like sodium benzoate. The containers are then sealed and while in storage, the brine solution penetrates into the sausages and decreases the pH in a process called acidification. The pickling process utilizes reduced pH levels and reduced oxygen levels to inhibit the growth of microorganisms. Utilizing multiple factors to preserve foods and inhibit microorganism growth at the same time synergistically is known as hurdle technology (19). By simultaneously targeting multiple systems, microorganisms' homeostasis is disturbed and not given the opportunity to adapt resulting in its inhibition or destruction (17).

The pickling process highlighted above utilizes what is known as a “hot fill” process where the brine solution is heated before introducing to the sausages. However, numerous quality defects can result from hot fill pickling sausages including cloudy/ turbid brine solution which, in turn, makes the product less appealing to consumers. Though heated, acidified salt solutions have been shown to be very effective against pathogens (21), the use of heated brine solution has led to numerous product damage and quality defects (17). To avoid these problems, processors use a cold fill process where the brine solution is kept at room temperature (~22-23°C). The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) requires manufacturing processes to be validated for its efficacy to inhibit the growth of appropriate pathogenic bacteria (37). Therefore, a challenge study is necessary to access the efficacy of a cold fill pickling process and fulfill the validation requirement. Current regulations that address acidified food products do not specifically address pickled meat

products. The Code of Federal Regulations (21 CFR part 114) states that “only that acid or acid ingredients must be added so that the pH is maintained at or below 4.6 and does not take into account the type of acid used (1). This regulation was designed to control the growth and toxin production of *Clostridium botulinum* (3). According to 21 CFR part 120, manufacturers of fruit and vegetable products that utilize an acidification (pickling) step must ensure that their processes demonstrates a 5-log reduction in pathogenic bacteria relevant to the particular product (12).

The current validation enforcement by the USDA-FSIS for these products is based on the measurement of pH, a_w , % salt of the brine and sausages, temperature of the brine solution at filling, and the amount of antimicrobial ingredients in the sausage and/or brine formulations. Control of these intrinsic and extrinsic factors have been linked to the inhibition of unwanted microorganisms in other meat products. Hazzard Analysis Critical Control Point (HACCP) plans require validation of manufacturing processes against foodborne contamination and any such changes in processing techniques, such as switching from hot fill pickling to cold fill, must be confirmed through scientific research (30). Specifically in the case of the cold fill pickling, the USDA-FSIS was concerned with the use of a room temperature brine solution (cold fill) instead of one that is heated (hot fill) to pickle pre-cooked, RTE sausages as a processing step to control and eliminate pathogenic bacteria.

To our knowledge, no scientific literature exists addressing the growth or prevention of pathogenic microorganisms in pre-cooked, RTE pickled sausages. Research concerning pickled and acidified foods focuses mostly on homogenous mixtures like salad dressings, mustard, ketchup or vegetable purees and not on heterogeneous products. Scenarios of acidified fruits and vegetables do not compare well to high protein products such as eggs or meat that have a much

higher buffering capacity (34). Previous studies involving the inoculation of pickled eggs with pathogenic bacteria (25, 30, 34) are most relevant to the topic of pickled meat products because of similar protein content and susceptibility to microorganisms contamination.

Due to the lack of evidence supporting the safety of pickled sausages using a cold fill process, a challenge study was conducted to demonstrate the inhibition of relevant pathogenic bacteria.

MATERIALS AND METHODS

In this study, a cooked, smoked and cured sausage product was inoculated with a cocktail of pathogenic bacteria, placed into an acidified brine (5% acetic acid; 5% salt; 22-23°C) solution and held at room temperature for 28 days. Table 6.1 includes the spp., subtype, identification code and isolation source of all of the pathogens used to inoculate sausages prior to the cold fill pickling process. All strains listed were specifically chosen because of their prominence in fresh and RTE meat products, strong associations with post-lethality contamination, relevant isolation sources and availability from reputable sources. The bacteria used in this study were not pretreated in acidic conditions to induce a resistance.

Preparation of Select Pathogens and Challenge Study Inoculum. Cultures of *Salmonella* spp. (serovars Typhimurium, Montevideo and Senftenberg), *Listeria monocytogenes* and *Staphylococcus aureus* were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and from the Food Microbiology Culture Collection at The Pennsylvania State University Food Science Department. Lyophilized cultures were transferred to fresh Tryptic Soy broth (TSB) (Hardy Diagnostics, Santa Maria, CA) and incubated aerobically at 37°C for 24 h following ATCC guidance. Colonies were then re-suspended in fresh TSB broth containing 10%

glycerol (VWR, Radnor, PA) and stored at -80°C until future use. Working stocks were maintained on Tryptic Soy Agar (TSA) (Hardy Diagnostics, Santa Maria, CA) and stored at 4°C. To prepare the pathogen cocktail inoculum, frozen stocks of each of the 9 pathogens were transferred twice into fresh TSB broth and streaked onto TSA using the incubation conditions described above. Single colonies of each pathogen were isolated and transferred into 10 ml of fresh TSB. Following incubation, 10 ml of each culture were transferred to 690 ml of fresh TSB and incubated for an additional 24 h at the same temperature and atmospheric conditions as previously described to obtain a cell concentration of $\sim 8.0 \log_{10}$ CFU/ml. Equal vol of each pathogen (700 ml) were mixed in a sterilized metal container under a biological safety hood to produce a homogenous pathogen cocktail (6.3 liters in total vol) for the sausage inoculation immersion bath as previously described by Richard and Cutter (25) and Scheinberg et al. (30). This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Preparation of Sausages. Sausages used in this study were manufactured at The Pennsylvania State University Meat Laboratory and formulated with the following ingredients: beef trim (80% lean; 22% wt/wt), pork trim (80% lean; 44% wt/wt and 50% lean; 22% wt/wt), water (7.26% wt/wt), salt (2.21% wt/wt), dextrose (0.44% wt/wt), ground black pepper (0.28% wt/wt), sodium tripolyphosphate (2500 ppm), ground mustard (0.55% wt/wt), granulated garlic (0.55% wt/wt), ground ginger (0.01% wt/wt), sodium erythorbate (540 ppm) and curing salt (6.25% NaNO₂; 156 ppm). Sausages were stuffed to a wt of 130.4 g into a 35 mm curved, edible collagen casing (Devro Ltd, Moodiesburn, Scotland, UK) using a Handtmann VF608 Plus with twist linker (Albert Handtmann Maschinenfabrik GmbH & Co, Biberach, DE). Large diam (>30 mm), collagen type casing was chosen because it demonstrated the most resistance to brine

penetration and acidification compared to skinless and natural type casings in preliminary experiments (data not shown). The sausages were smoked and cooked in a Kerres Smoke Air[®] JS 1950 (Kerres Anlagensysteme GmbH, Backnang, DE) to a core temperature of 70°C, transferred to a walk-in cooler (1-2°C) and chilled to 4°C in accordance with Appendix B (35). Table 6.2 illustrates the thermal processing schedule used to smoke and cook the sausages. The average measurements of the cooked and chilled sausages were as follows: length 144 mm ± 2, width 31 ± 2 mm, wt 127 ± 2 g. Sausages were then placed in 14 in x 24 in (5.5 cm x 9.5 cm) food grade, 3mil gauge pouches (PCS Supplies Inc., Penns Groove, NJ) and vacuum sealed (Smith SuperVac Digimat, Clifton, NJ). Packaged sausages were stored in a walk-in cooler at 2°C (<21 days) until they could be transported to The Pennsylvania State University Muscle Foods Microbiology Laboratory for the microbiological challenge study. Three separate batches of sausages in total were made for each of the 3 inoculation and sampling replications. Fat analysis was conducted in duplicate for each of the 3 batches of sausages following a modified Babcock Fat Analysis procedure (11).

Preparation of Brine Solution. Food-grade distilled white vinegar (5% acidity) (H.J. Heinz Company, Pittsburgh, PA) and non-iodized salt (NaCl) (Morton, Chicago, IL) were purchased from local supermarkets. Pickling brine (5% salt and 5% acetic acid) was mixed 24 h prior to the inoculation and subsequent pickling of sausages, covered with aluminum foil and allowed to fully equilibrate to room temperature (~22-23°C). This was repeated in the same manner for each of the 3 replications of the challenge study.

Inoculation of Sausages with Select Pathogens. Precooked and chilled (~2°C) sausages (n=144 total) were aseptically removed from the vacuum packaged bags and transferred to sterilized metal bins under a biological safety hood. The pre-prepared pathogen cocktail (6.3

liters total vol) was poured over the sausages (n=48 per rep) and allowed to sit for 30 minutes with gentle mixing periodically following the methods described by Richard and Cutter (25) and Scheinberg et al. (30). The pathogen inoculation was repeated in the same manner for each of the 3 replications of the challenge study.

Negative Control Sausages. In addition to the inoculated sausages, a negative control sausage group was also utilized. Precooked and chilled (~2°C) sausages (n=36 total) were aseptically removed from the vacuum packaged bags, transferred to sterilized metal bins under a biological safety hood and covered with 3 liters of sterile Buffered Peptone Water (BPW; HiMedia, Mumbai, India). The sausages (n=12 per rep) were allowed to sit for 30 minutes in the sterile BPW with gentle, periodic mixing, following the methods described by Richard and Cutter (25) and Scheinberg et al. (30). Three replications of the negative control sausage group were performed for the challenge study.

Pickling and Storage of Inoculated and Negative Control Sausages. Following the inoculation of sausages through the immersion bath technique, sausages were aseptically removed from the inoculum and placed into sanitized 1 gal (3.8 liter) plastic food grade screw top containers (Pretium Packaging, Hazleton, PA). All pathogen inoculated sausages were evenly distributed into 4 separate containers (n=11 per container), with the exception of 4 sausages that were withdrawn for initial sampling. The negative control sausages were also aseptically transferred to a separate container as previously described. Room temperature (~22-23°C) pickling brine was then poured into each container, allowing for a headspace of 0.5 in, and the containers were capped and sealed, simulating a cold fill commercial pickling and packaging process. The pickled sausages were stored at room temperature (~22-23°C) for the remainder of the challenge study (28 days) for each of the 3 replications. A total of 12 different containers

were utilized for the pathogen inoculated sausages for all 3 replications for the duration of the study, while 3 containers were used for the negative control group.

Sampling and Microbial Analysis. Immediately following the inoculation of the sausages and prior to the pickling step, 4 sausages per replication of the pathogen inoculated sausages and 2 sausages from the negative control group, were randomly selected and removed from the inoculum immersion bath. Sausages were sampled and analyzed to determine initial inoculation concentrations, pH, a_w , and % salt of both the sausage and pickling brine. For sausages removed for initial sampling (time 0) for the inoculated group and the negative control group, three sausages (n=3 per replication per time point) were used for microbial analysis for each of the treatment groups and one sausage (n=1 per treatment per time point) was used for the negative control group. One sausage (n=1 per treatment per time point) from each of the treatment and control groups was used to analyze pH, a_w , and % salt. This sampling protocol was repeated for all treatment and control groups at various time points (2 h, 4 h, 6 h, 12 h, 1 d, 2 d, 3 d, 7 d, 14 d, 21 d and 28 d) for each of the three replications.

To enumerate surviving pathogens on sampled sausages, at each time point, pickled sausages were aseptically removed from their respective containers, individually placed into 3500 ml filtered stomacher bags (Interscience, Rockland, MA), weighed, diluted with 5 times the vol of the sausage with sterile BPW, and stomached for 1.5 minutes at 360 rpm (Interscience Jumbo Mix Stomacher, Rockland, MA). The stomachate (~35 ml) of each pickled sausage was serially diluted in 9.0 ml of BPW and aliquots of 1.0 ml and 0.1 ml were spread plated in duplicate onto selective agars. 4 different media agars were utilized to enumerate the cocktail of pathogens; Modified Oxford (MOX; Hardy Diagnostics, Santa Maria, CA) for *L. monocytogenes*; xylose lysine deoxycholate (XLD; HiMedia, Mumbai, India) for *Salmonella*

spp.; Baird Parker (BP; Hardy Diagnostics, Santa Maria, CA) for *S. aureus* and TSA, with the intention of enumerating injured pathogen cells that otherwise may not grow on the selective media. All plates were incubated aerobically at 37°C for 24 h. For the negative control group, the same 4 agar plates were used to enumerate the stomachates of the uninoculated sausages at all sampling time points.

Enrichment Procedures. Enrichment procedures for all 3 pathogens were carried out as detailed in the USDA-FSIS Microbiology Laboratory Guidebook (36) with minor modifications. For *L. monocytogenes* enrichment, 1.0 ml of stomachate was transferred to 9 ml of University of Vermont Medium (UVM; Difco, Sparks, MD), grown for 24 h at 37°C, followed by transferring 1.0 ml to 9 ml of Fraser broth (EMD, Gibbstown, NJ), grown for 24 h at 37°C, streaked onto MOX plates, incubated at 37°C, and examined for colonies 24 h later. For *Salmonella* spp. enrichment, 1.0 ml of stomachate was transferred to 9 ml of Lactose broth (HiMedia, Mumbai, India), grown for 24 h at 37°C, followed by transferring 1.0 ml to 9 ml Rappaport-Vassiliadis (RV) broth (EMD, Gibbstown, NJ), grown for 24 h in duplicate at both 37 and 42°C, streaked onto XLD plates, incubated at 37 and 42°C, and examined for colonies 24 h later. For *S. aureus* enrichment, 1.0 ml of stomachate was transferred to 9 ml of TSB supplemented with 5% sodium chloride (w/v) (BDH, West Chester, PA), grown for 24 h at 37°C, streaked onto BP plates, incubated at 37°C, and examined for colonies 48-72 h later. Similarly, 1.0 ml aliquots of brine solution were extracted from the jars and handled in the same manner for each separate pathogen as the stomachate enrichments. Single colonies of the pathogens were taken from the enrichment plates and confirmed using rapid latex test kits for *Salmonella* (Oxoid, Ltd. Hants, U.K.), *Listeria* (Microgen Bioproducts, Camberley, U.K.) and *Staphylococcus* (Lenexa, KS).

Sausage and Brine Analysis. Brine pH was measured using a Thermo Scientific Orion 4 Star pH ISE Benchtop meter with a connected Orion 9103BNWP Semi-Micro Combination pH Probe (Beverly, MA). Sausage pH was measured using a Thermo Scientific Orion 4 Star pH ISE Benchtop meter with an Orion 8163BNWP Ross Combination Spear Tip pH Electrode (Beverly, MA). The sausage designated for pH measurements were first cut in half using a cross sectional cut and then the tip of the pH probe was inserted into the center of the cut half. The other portion of the cut sausage was used for a_w analysis. A_w was analyzed using an Aqua Lab 4TE (Decagon Devices, Pullman, WA) water activity meter. Percent salt concentrations for sampled sausages and pickling brine were determined using Chloride Quantabs (Hack, Loveland, CO) according to the procedure outlined by Sebranek *et al.* (31).

Statistical Analysis. Means of the 3 replications of pathogen plate counts were calculated and converted to \log_{10} CFU/g for statistical analysis. The limit of detection for all inoculated and negative control spread plates was 0.40 \log_{10} CFU/g. Comparison of pathogenic bacteria populations were determined at each time point within and between groups using one way analysis of variance (ANOVA) and Tukey's honesty significant difference test at $\alpha = 0.05$. Comparison of pH, a_w , % salt of sausages and brine values also were determined using a one way analysis of variance (ANOVA) with Tukey's honesty significant difference test at $\alpha = 0.05$. Analysis of Variance (ANOVA) tests were conducted using SAS (SAS software version 9.3, SAS Institute Inc., Cary, NC.).

RESULTS

Pre-cooked, RTE sausages inoculated with a cocktail of pathogens, pickled using a cold fill process, and stored at 25°C experienced quick and significant decreases ($p \leq 0.0001$) (Fig 6.1;

Table 6.3) in bacterial populations. Within 24 h, all *Salmonella* spp., *L. monocytogenes* and *S. aureus* plate counts were undetectable on the selective medias: XLD, MOX and BP, respectively. TSA plate counts were undetectable after 72 h. Within the first 12 h, *Salmonella* spp. experienced a total reduction of 6.33 log₁₀ CFU/g; *L. monocytogenes* dropped 6.35 log₁₀ CFU/g and *S. aureus* declined 6.158 log₁₀ CFU/g; *S. aureus* was reduced ca. 6.57 log₁₀ CFU/g in 24 h, before becoming undetectable by plating alone. Bacterial population on TSA were reduced 6.04 log₁₀ CFU/g within the first 12 h and a total reduction of 6.80 log₁₀ CFU/g after 72 h. Enrichments for *Salmonella* spp. (incubated at both 37 and 42°C) and *L. monocytogenes* were negative after 48 h and *S. aureus* was negative after 72 h (Table 6.3). Negative control plates were done on all three selective medias (XLD, MOX, BP), but their counts were insignificant, when compared to the TSA negative control plates, so comparisons were not included (data not shown).

Significant changes occurred in the pH of the sausages ($p \leq 0.0001$) and the brine ($p = 0.0002$) throughout the length of the study (Table 6.4), with the majority of the changes occurring within the first 72 h. Both the pH of the sausage and brine moved towards equilibrium, ending at 3.94 and 3.63, respectively. pH of the sausage stomachate also was measured (Table 6.4) to determine if the pH of the stomachate itself would have an inhibitory effect on the pathogen cultures after plating and were not found to occur; pH values experienced significant decreases over time ($p \leq 0.0001$).

Likewise, the negative control sausages experienced significant drops and in pH ($p < 0.0001$), accompanied by significant increases in brine pH ($p = 0.0008$), resulting in a product with a pH < 4.6 in under 168 h (7 d) (Table 6.4). A significant pH drop ($p < 0.05$) occurred between time points 0 and 12 h of the negative control sausages, then remained unchanged

throughout the length of the study (Table 6.4). There was no significant difference ($p=0.1291$) in a_w throughout the study, with values ranging between 0.95 and 0.97 (Table 6.4). The % salt of the sausages rose slightly from 2.32% to 2.79% ($p=0.1445$), while the % salt of the brine decreased from 4.86% to 4.30% ($p=0.3180$); neither change was significant (Table 6.4). The results of the Modified Babcock Fat analysis demonstrated that the average % fat of the sausages for all 3 reps was 23.2%, with a range of 20.0 to 26.0% fat (data not shown).

DISCUSSION

The microbial inhibition of weak acids like acetic acid (acid dissociation constant (pK_a) 4.76) involves the diffusion of the undissociated form of the acid through the cell wall of bacteria and into the cytoplasm (16). The acid dissociates into cation and anion ($HA \rightleftharpoons H^+ + A^-$) because of the near neutral pH of the cytoplasm. The buildup of protons (H^+) causes the acidification of the cytoplasm and the interior of the cell (16, 17) and other problems within the cell including: damage to cell membranes, denaturation of proteins, enzymes and DNA and metabolic exhaustion (19, 39). Anion accumulation adds considerably to the inhibition of bacteria resulting in high turgor pressure, potential leakage from the cell, production of free radicals and oxidative stress (8, 26, 29). Weak acids have displayed increased antimicrobial effect when the pH of their environment is decreased (4, 16), as was seen in this study with the pH of the brine beginning at 2.76 and reaching 3.63 by 28 d. It is hypothesized that having the pH of the brine solution below the pK_a value of acetic acid ensured that the majority of the weak acid was in the undissociated form, thus increasing its ability to penetrate into the bacterial cells.

Destruction of pathogens occurred within the first 12 h and coincided with a rapid and significant decrease in pH of sausages, suggesting the kill was driven by the declining pH.

Similar reductions of pathogenic bacteria in pickled, hard cooked eggs have been reported. Richard and Cutter (25) achieved $>4.85 \log_{10}$ CFU/ml reduction of *Salmonella* spp., *L. monocytogenes*, *Escherichia coli* O157:H7 and *S. aureus* in 24 hours at 22°C. A subsequent study by Scheinberg et al. (30) achieved $>5.0 \log_{10}$ CFU/ml reduction of similar pathogens in 24 h using a similar pickling procedure of hard cooked eggs.

Gram-negative bacteria (*Salmonella* spp.) are generally more resistant to weak acid preservatives than Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) due to differences in cell wall structure between the two types of cells (28). The outer membrane of Gram-negative bacteria has been shown to play a more active role in resisting the entrance of preservatives into the cell than Gram-positive bacteria (15). Lipopolysaccharides (LPS) on the outer surface of Gram-negative bacteria prevent entry of long chain fatty acids; LPS is however ineffective against short chain acids like acetic (a saturated straight-chain monocarboxylic) acid (40). The cold fill pickling process appeared to affect *Salmonella* spp. and *L. monocytogenes* in the same manner and significantly inhibited them both (Fig. 6.1; Table 6.3).

The high salt environment appears to provide an advantage to *S. aureus* over the other pathogens, as evidenced by the consistently higher counts on BP, the longer times until counts were determined to be undetectable and the longer lasting positive enrichment results (Fig. 6.1; Table 6.3). Richard and Cutter (25) noted similar enrichment results: *S. Enteritidis*, *S. Typhimurium*, *L. monocytogenes*, and *E. coli* O157:H7 enrichments were undetectable after 48 hours, while *S. aureus* was still viable up to 96 hours.

Several studies have reported the increased inhibitory effects of acetic acid compared to other acids. A study by Sorrells et al. (33) found that acetic acid had a higher antimicrobial activity against *L. monocytogenes* than lactic, citric, malic and hydrochloric acid at temperatures

10, 25 and 35°C. Rode et al. (27) noted strong differences in growth patterns of *S. aureus* in organic (lactic and acetic) and inorganic hydrochloric acid (HCl) at pH 4.5. Cultures exposed to acetic acid cultures experienced less growth, while cultures exposed to lactic and HCl cultures were reduced >1 log₁₀ CFU/ml growth after ~3 h. In another study by Nostro et al. (22), *S. aureus* was grown in TSB acidified with 3 different acids (acetic, lactic and HCl) to pH of 5.0 and 6.0 and biofilm formation evaluated. Results demonstrated that acetic acid inhibited *S. aureus* initial adhesion and overall growth significantly more ($p < 0.05$) than lactic acid or HCl at pH 5.0.

Additionally, the increased effect of acid at room temperature on bacterial populations in meat products has been demonstrated in numerous published studies (14, 24), pickled cucumbers (6, 7) and other foods (20, 32). Richard and Cutter (25) noted a reduction of pathogenic populations were significantly higher ($p < 0.05$) in hard cooked, pickled eggs at room temperature (22°C) than at refrigeration temperatures (4°C), confirming that temperature plays an important factor in the acidification process. Sullivan et al. (34) found that both *L. monocytogenes* and *S. aureus* survived up to 14 days when pickled with only a 1% (w/v) acetic acid solution and populations only decreased when the pickled eggs were moved from refrigerated to ambient temperature. A study by Lee et al. (18) demonstrated acetic acid alone was significantly ($p < 0.01$) more effective at reducing *E. coli* O157:H7 populations than acetic acid combined with salt at concentrations 0-3% in cucumber puree. These data suggest that the synergistic effect between acetic acid and salt may not apply at low concentrations. Our findings suggest that inhibition of pathogens is possible with the 2% salt of the sausages and 5% salt of the pickling brine.

The ability of pathogenic bacteria to develop resistance when exposed to sub-lethal stresses like acid is particularly disconcerting since consumer demand is shifting towards

products with less preservatives and minimally processed foods, implying that foodborne illnesses with such products may result (13). *S. Typhimurium* (5), *L. monocytogenes* (38) and *S. aureus* (23) have developed resistance to lethal pH levels if first exposed to a sub-lethal level of acid. This resistance known as acid tolerance response (ATR) is an adaptation and survival mechanism that bacteria employ to overcome lethal levels of acidity. ATR is based on the expression of certain genes and the synthesis of specialized bacterial stress proteins (9). All 3 pathogens in this study have demonstrated increased tolerance to low pH when exposed to acetic acid, as compared to lactic and hydrochloric acids (2, 10). While ATR was not evaluated in this study, the results demonstrate that the acid treatments inhibited the pathogens sufficiently. Because of the shock of low pH from the brine solution, surface contamination on pickled food items and/or the development of acid tolerance is considered only a marginal concern (34). In the cold fill process, the bacteria were not exposed to a sub-lethal level of acid (4.5-5.5) and given time to adapt. Rather, the pathogens were exposed to lethal levels of acidity (pH 2.76) immediately after inoculation.

This study demonstrates the ability of the cold fill pickling process to inhibit pathogenic bacteria: *Salmonella* spp., *L. monocytogenes*, and *S. aureus* in a pre-cooked, RTE, pickled sausage. The strains of bacteria were chosen based on their appropriate meat isolation sources and outbreaks in order to be applicable to pickled, ready-to-eat sausage. The pH of the sausages in this study were reduced to ≤ 4.6 within 72 h (Table 6.4), affording a $>5 \log_{10}$ CFU/g reduction of appropriate pathogen populations (Fig. 6.1; Table 6.3). Moreover, parameters such as the diam of the sausages, casing type, and brine concentration were chosen in order to implement a “worst case scenario” in this challenge study. Processors may effectively inhibit pathogenic bacteria by using higher concentrations of salt or acetic acid, smaller diameter sausages, or use natural or

skinless casings, which would result in a faster penetration of the brine into the sausages and decrease the internal pH.

Based on these findings, meat processors who utilize a cold fill process can use this research to validate their process for their HACCP plans, and therefore, comply with USDA-FSIS regulations. By establishing a baseline amount of acetic and salt for the brine solution and a reference sausage formulation, results of this study could be used as a best practices for pickled sausage. Additionally, results from the study could also provide the appropriate scientific support for regulatory authorities to re-evaluate and possibly modify regulations in regard to pickled meat products.

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FIGURE LEGEND

Figure 6.1: Average plate counts (\log_{10} CFU/g) of *Salmonella* spp. (Typhimurium, Senftenberg and Montevideo), *Listeria monocytogenes*, and *Staphylococcus aureus* from time points 0 to 72 h with standard deviation error bars shown. 0.40 \log_{10} CFU/g detection limit is shown by the dotted blue line. ^{a-d} Different letters represent significant difference between \log_{10} (CFU/g) plate counts of points of the same color ($p < 0.05$).

Table 6.1: Pathogenic bacteria used to inoculate sausages prior to cold fill pickling.

Species	Subtypes	Identification Code	Isolation Source
<i>Salmonella enterica</i>	subspecies <i>enterica</i> serovar Typhimurium	ATCC# 14028	Chicken Tissue (Heart and Liver)
<i>Salmonella enterica</i>	subspecies <i>enterica</i> serovar Senftenberg	ATCC# 43845	Meat Pie (England)
<i>Salmonella enterica</i>	subspecies <i>enterica</i> serovar Montevideo	SMvo13	Salami Outbreak, 2010
<i>Listeria monocytogenes</i>	serotype 4b	H3396	Hotdog Outbreak, 1998
<i>Listeria monocytogenes</i>	serotype 4b	FSL J1-129	Pate Outbreak, 1989
<i>Listeria monocytogenes</i>	serotype ½ a	FSL R2-603	Deli Meat Outbreak, 2000
<i>Staphylococcus aureus</i>	subspecies <i>aureus</i> Rosenbach	ATCC# 13565	Ham
<i>Staphylococcus aureus</i>	subspecies <i>aureus</i> Rosenbach	ATCC# 27154	Sliced Turkey
<i>Staphylococcus aureus</i>	subspecies <i>aureus</i> Rosenbach	ATCC# 27664	Chicken Tetrazzini

Note: American Type Culture Collection (ATCC).

Table 6.2: Thermal processing schedule of sausages used in the pathogen challenge study.

Function	House Temperature (°F)	% Relative Humidity	Core Temperature (°F)	Time (min)
Steam Cook	120	90	0	00:15
Dry Cook	135	0	0	00:15
Steam Cook	163	90	0	00:10
Dry Cook	135	0	0	00:15
Heissrauch	140	0	0	00:15
Dry Cook	140	0	0	00:20
Heissrauch	140	0	0	00:15
Dry Cook	140	0	0	00:20
Heissrauch	140	0	0	00:10
Dry Cook	140	0	0	00:10
Steam Cook	154	90	0	00:10
Dry Cook	140	0	0	00:10
Steam Cook	165	90	158	00:00
Dry Cook	140	0	0	00:10
Shower	0	0	0	00:15

“Heissrauch” – hot smoking step

Table 6.3: Average plate counts (log₁₀ CFU/g) of *Salmonella* (Typhimurium, Senftenberg and Montevideo), *Listeria monocytogenes*, and *Staphylococcus aureus* cultures inoculated onto sausages, enrichment results of the inoculated sausages and negative control TSA results over the course of the 28 day challenge study.

Time Points	TSA	<i>Salmonella</i> spp.		<i>L. monocytogenes</i>		<i>S. aureus</i>		TSA Negative Control
	Log ₁₀ (CFU/g)	Log ₁₀ (CFU/g)	Enrichment	Log ₁₀ (CFU/g)	Enrichment	Log ₁₀ (CFU/g)	Enrichment	Log ₁₀ (CFU/g)
0 h	7.20 ^a	6.73 ^a	+	6.74 ^a	+	6.97 ^a	+	3.91 ^c
2 h	4.99 ^b	3.66 ^b	+	3.91 ^b	+	4.36 ^b	+	3.91 ^c
4 h	3.40 ^c	2.40 ^c	+	2.73 ^c	+	3.46 ^{bc}	+	3.91 ^a
6 h	2.75 ^c	2.24 ^c	+	2.11 ^c	+	2.87 ^c	+	3.91 ^a
12 h	1.16 ^d	≤0.40 ^d	+	≤0.40 ^d	+	0.81 ^d	+	≤0.40 ^a
24 h	0.76 ^d	≤0.40 ^d	+	≤0.40 ^d	+	≤0.40 ^d	+	≤0.40 ^a
2 days (48 h)	1.03 ^d	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^d	+	≤0.40 ^a
3 days (72 h)	≤0.40 ^d	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^a
7 days (168 h)	≤0.40 ^d	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^a
14 days (336 h)	≤0.40 ^d	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^a
21 days (504 h)	≤0.40 ^d	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^a
28 days (672 h)	≤0.40 ^d	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^d	-	≤0.40 ^a

^{a-d} Different letters represent significant difference between values within each column ($p < 0.05$). Log₁₀ (CFU/g) values at each time point are the mean of (n=9) 3 sausages/replication. Enrichment results are the sum of (n=7) 3 sausage and 4 brine samples/ replication.

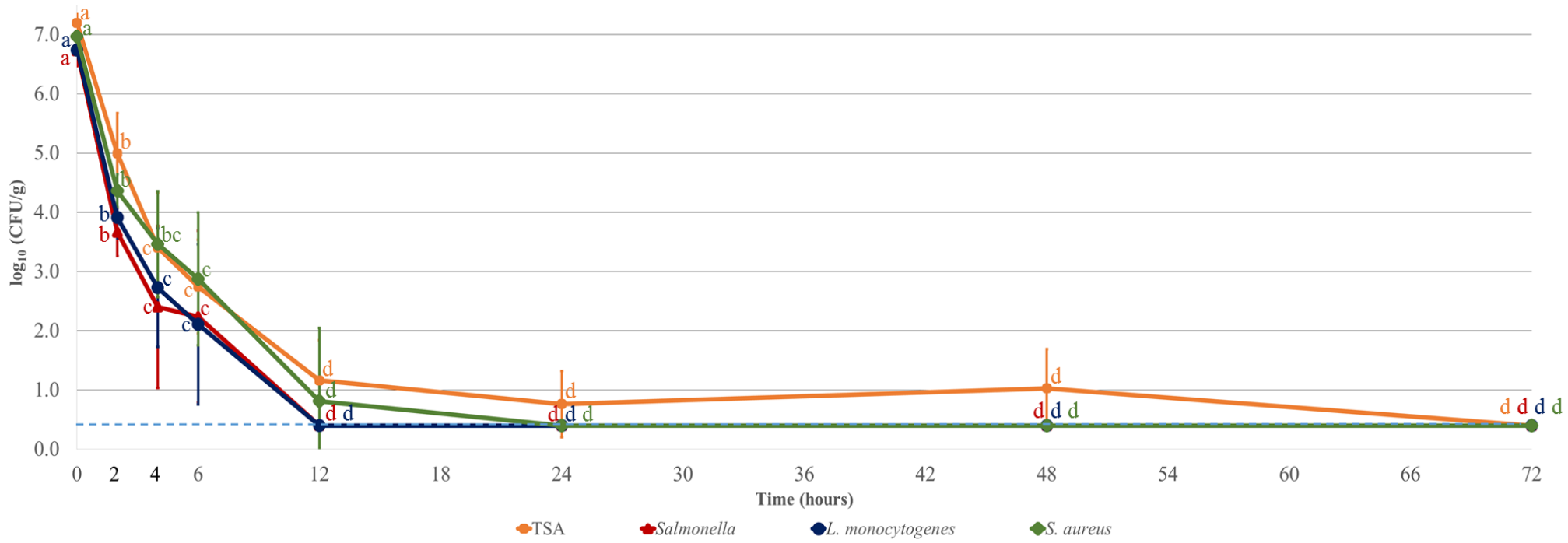
Note: “+” = positive enrichment result and “-” = negative enrichment result.

Enrichments for *Salmonella* spp. (incubated at 37 and 42°C) and *L. monocytogenes* were negative after 48 h; *S. aureus* was negative after 72 h (data not shown).

Table 6.4: pH of inoculated sausage, brine and stomachate and pH, a_w , and % salt of negative control sausages throughout the 28 day challenge study.

Time Points	Pathogen			Negative Control				
	pH Sausage	pH Brine	pH Stomachate	pH Sausage	pH Brine	a_w	% Salt Sausage	% Salt Brine
0 h	6.41 ^a	2.76 ^d	6.82 ^a	6.41 ^a	2.60 ^c	0.97 ^a	2.32 ^a	4.86 ^a
2 h	6.32 ^{ab}	2.59 ^d	6.43 ^{ab}	-	-	-	-	-
4 h	6.20 ^{ab}	2.84 ^{bcd}	6.18 ^{ab}	-	-	-	-	-
6 h	6.33 ^{ab}	2.91 ^{abcd}	6.06 ^{ab}	-	-	-	-	-
12 h	6.27 ^{ab}	3.02 ^{abcd}	5.66 ^{bc}	6.28 ^a	3.05 ^{bc}	0.95 ^a	2.53 ^a	4.43 ^a
24 h	5.68 ^b	3.17 ^{abcd}	5.33 ^{bcd}	5.72 ^a	3.21 ^{abc}	0.95 ^a	2.72 ^a	4.56 ^a
2 days (48 h)	4.76 ^c	3.17 ^{abcd}	4.64 ^{cd}	-	-	-	-	-
3 days (72 h)	4.60 ^{cd}	3.51 ^{abc}	4.74 ^{cd}	-	-	-	-	-
7 days (168 h)	4.39 ^{cde}	3.49 ^{abc}	4.66 ^{cd}	4.15 ^b	3.34 ^{ab}	0.95 ^a	3.01 ^a	4.29 ^a
14 days (336 h)	4.15 ^{cde}	3.60 ^{ab}	4.60 ^{cd}	-	-	-	-	-
21 days (504 h)	4.06 ^{de}	3.67 ^a	4.59 ^{cd}	4.19 ^b	3.58 ^{ab}	0.95 ^a	2.79 ^a	4.58 ^a
28 days (672 h)	3.94 ^e	3.63 ^{ab}	4.44 ^d	3.96 ^b	3.71 ^a	0.94 ^a	2.79 ^a	4.30 ^a

^{a-e} Different letters represent significant difference in values within each column. ($p=0.3108$). All numerical values are the mean of (n=3) 1 sausage/replication.



Chapter 7
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

This project determined the ability of the cold fill pickling process, utilizing a brine solution containing 5% salt and 5% acetic acid, to significantly reduce populations of lactic acid bacteria, yeast, mold, *Salmonella* spp. (serotypes: Typhimurium, Senftenberg, and Montevideo), *Listeria monocytogenes* and *Staphylococcus aureus* inoculated experimentally onto the surface of cured, ready-to-eat sausages. All lactic acid and pathogenic bacteria experienced $>6.0 \log_{10}$ (CFU/g) decreases within seven days and 72 hours, respectively. Spoilage yeast and mold were reduced $>3.8 \log_{10}$ (CFU/g) within 48 hours.

The parameters of this project, such as the formulation of the sausage and brine solution, type of casing and size of sausages, were chosen in order to make these challenge studies as general and applicable across the meat industry as possible. By establishing a baseline formulation, results of this study could be used as best practices for pickled sausage manufacturing, based on the monitoring of the quantitative parameters previously mentioned for the storage of pickled sausages at ambient temperatures. Processors may effectively inhibit spoilage and pathogenic microorganisms by using higher concentrations of salt or acid in the brine solution than what was utilized in the study. Processors also may increase the ratio of brine solution to amount of sausage, manufacture smaller diameter sausages, or use natural or skinless casings to affect a faster pickling process and acidification of the sausage. The results of this project can be used by pickled sausage processors as scientific supporting documentation of their HACCP plans. In doing so, citing the results of these challenge studies will aid processors in complying with USDA-FSIS regulations and in producing safe and wholesome foods products.

Future Directions

Additional challenge studies focusing the use of other organic acids such as lactic or citric could be conducted with the intention of comparing the effectiveness of the different organic acids to one another. Changing the formulation of the sausages to all beef, all pork or implementing a poultry-type sausage would further expanded the validation of the cold fill pickling process. Furthermore, it would be interesting to examine the survival of both spoilage and pathogenic microorganisms in cold fill pickling processes where the salt and acid concentration are $<5\%$.