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THE EVALUATION OF PATHOGEN SURVIVAL IN
DRY CURED CHARCUTERIE STYLE SAUSAGES

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in the College of Agriculture,
Food and Environment at the University of Kentucky

By

Jennifer Michelle McNeil

Lexington, KY

Director: Dr. Gregg Rentfrow, Associate Extension Professor – Meat Science

Lexington, KY

2019

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ABSTRACT OF THESIS

THE EVALUATION OF PATHOGEN SURVIVAL IN DRY CURED CHARCUTERIE STYLE SAUSAGES

The objective of this study was to evaluate the survival of non-O157:H7 STEC, *Salmonella* spp., and *S. aureus* in dry fermented sausages. Chorizo and Landjager sausages were inoculated with individual bacterial cocktails and stuffed into natural casings. Temperature, relative humidity, pH, and water activity were monitored through fermentation, drying, and storage. Bacterial counts were determined by serial dilution and plated in triplicates on selective media. Plates were incubated at 37°C for 24 hours and colony forming units per gram (CFU/g) were observed.

Results of the first study validate that contaminated raw materials contribute to pathogen survival and background bacteria outcompeted the starter culture. The pH critical limit of <5.3 was met but there was no pathogen inhibition. Results from the second study confirm that pH and water activity are not enough to eliminate pathogens when post processing interventions are not used. Critical pH (<5.3) and water activity (<0.85) limits were met, but pathogens still survived.

In chorizo, non-O157:H7 was recovered through enrichments until the end of the study. In landjager, non-O157:H7 STEC and *Salmonella* were recovered through enrichments until the end of the study. The studies suggest that sausages produced without post processing interventions are a health risk to consumers.

KEYWORDS: Dry Cured Fermented Sausages, Fermentation, Lactic Acid Bacteria Starter Cultures, Non O157:H7 STEC

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04/24/2019

THE EVALUATION OF PATHOGEN SURVIVAL IN
DRY CURED CHARCUTERIE STYLE SAUSAGES

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CHAPTER 1

INTRODUCTION

Dry cured sausages represent some of the oldest forms of meat preservation. Thousands of years ago our ancestors discovered that salting meat was an effective method of preservation, and the use of salt and drying of meat led to the production and preservation of many dried meat products. As a result, sausage techniques evolved. Sausage makers started adding spices to their homemade sausage and developing different recipes and flavor profiles. The spices also consequently contributed to a longer shelf life (Marianski, 2009).

The production of fermented sausages in the past relied heavily on the microbiological flora that was found in the processing facilities. Sanitation methods were not as advanced as they are in the present, so microorganisms remained even after cleaning and sanitizing. Also, because the bacterial flora was unique to the production facility, producers had a hard time replicating the exact same flavor profile when produced elsewhere. The manufacture of fermented meat products evolved, so has knowledge of the importance of producing a microbiologically safe product. Therefore, the concept of Hurdle Technology was developed to combat pathogens. As a result, producers acknowledged the important of controlling pH, water activity (a_w), temperature, and humidity during the production of sausages.

Dry cured sausages generally have a high salt content (3-4.5%) and low moisture content ($a_w < 0.90$), which is not enough to inhibit the growth of pathogens like *Escherichia coli* O157:H7, *Salmonella spp.*, and *Staphylococcus aureus*. Hurdle

technology is an effective combination of preservative factors, called hurdles, which are used to secure the microbiological safety of our food supply. There are more than 60 that are recognized, the most important hurdles include temperature, water activity (aw), acidity (pH), redox potential, preservatives, and competitive microorganisms (Leistner, 2000).

Studies have shown that the most effective way to control pathogens in food products is through the combination of more than one hurdle. When producing fermented sausages, it is important that the meat has virtually no pathogens present to reduce the risk of microbes in the finished product. Meat that is completely free of microbes isn't plausible in a production facility setting, however, through Hurdle Technology; the risk of pathogens in finished product is greatly reduced.

The objective of this study was to examine the effectiveness of the fermenting, drying, and storage of dry cured fermented sausages against pathogens that could potentially be present. Non-O157:H7 shiga toxin producing *Escherichia coli*, *Salmonella spp.*, and *Staphylococcus aureus* were among the pathogens that were evaluated. The effectiveness of *Pediococcus pentosaceus* + *S. xylosus*, a starter culture, against the growth of pathogens was also examined. The objectives of this research were:

1. To understand the inhibitory effect of starter cultures against non-O157:H7 *E. coli*, *Salmonella*, and *Staphylococcus aureus*.
2. To understand the importance of controlling pH, water activity, and relative humidity against pathogens in the production of fermented sausages.

3. To understand if the methods used to produce fermented sausages are effective against pathogenic bacteria if they are present in high numbers initially.
4. To recognize the threat of pathogen presence in sausages that do not have post processing interventions prior to consumption.

CHAPTER 2

LITERATURE REVIEW

2.1 Red Meat and Poultry

Red meat and poultry consumption has increased in the past several years. The USDA predicted that in 2018, consumers will eat 222.2 lbs of meat, reaching a record high (USDA ERS, 2017). Past food trends like fermented food products, smoked foods, and artisan style sausages are becoming popular again because of the millennial generation. They prefer a wide variety of foods with bold flavors and new twists on old recipes. Innovative food trends over the past few years have shown that millennials want flavorful food that encompasses old traditional food manufacturing as well as being minimally processed. To meet that need, millennials are expanding their palates and looking into more foods outside of the United States.

The millennial generation has also adopted food diet trends such as the keto diet, a low carb, high protein, and high fat diet that encourages the consumption of fatty meats and cheeses. Red meats, sausage, and bacon are explicitly consumed on this diet because of their higher fat content. Charcuterie boards have also become a staple item on many restaurant menus in the past few years. They have become increasingly popular for social gatherings and parties with the millennial generation.

Charcuterie comes from the French word for dry cured, smoked, and cook meats. Charcuterie plates are comprised primarily of smoked and dry cured bacons, hams, sausages, pates, cheeses with my different texture and flavors, sauces, and breads. The smoked and cured meats used for charcuterie boards are shelf stable and ready to eat. They are shelf stable due to low pH and water activity, inhibiting the growth of microorganisms. Today, fermentation chambers are used to cure shelf stable sausages. Traditionally, however, salt curing was the method used to preserve meat for extended periods of time.

There are many methods of preserving meat and other foods have been practiced throughout history. Salt preservation was a popular method to preserve meat, fish, and vegetables for many months at a time before refrigeration was developed. Salt curing and smoking were the most popular forms of meat preservation and date back to ancient times. This trend has evolved in the present to make specialty meat products with high value.

Meat curing functions to control microbial growth, extend shelf life, and develop unique sensory properties for the product. The control of microbial growth is the most important part of curing meat to decrease the risk for foodborne illnesses and to extend shelf life. The synergistic effect of salt, sugar, nitrite/nitrate, polyphosphates, low pH, low water activity, and a decreased redox potential collectively contribute to microbial safety as well as a longer shelf life (Clavero and Beuchat, 1996).

The safety of meat and fermented meat products has been an increasing concern in society for the past several years, particularly with newly identified and evolving

microorganisms. The pathogens of most concern have been *E. coli* O157:H7, *Listeria monocytogenes*, *Campylobacter jejuni*, *Salmonella*, and many others (Sofos, 2008).

Changes in animal production, product processing and distribution practices, changing consumer needs for minimally processed products, increasing foreign food trade, and worldwide higher meat consumption are all potential reasons for increasing concerns about food safety (Sofos, 2008). Therefore, advances in food safety production practices and education for producers and consumers are vital to combat meat safety issues.

2.2 Food Safety

2.2.1 Hurdle Technology

Bacteria can survive and grow in various conditions in food products and the environment. There are obstacles that can be used to combat the survival of these pathogens in the food industry, referred to as “Hurdle Technology.” Hurdle technology is a term that refers to an effective combination of preservative factors, called hurdles, which are used to secure the microbiological safety of our food supply. The most important hurdles include temperature, water activity (a_w), acidity (pH), redox potential, preservatives, and competitive microorganisms, although more than 60 are recognized (Leistner, 2000). The objective of hurdle technology is to expose bacteria into an environment that will inhibit their growth or survival without compromising the integrity of the food product (e.g., color, taste, texture). The drop of pH, water activity (a_w), temperature, and other factors will influence the survival of bacteria, which can be confirmed by the bacterial growth curve.

The bacterial growth curve demonstrates the activity of pathogens during their 4 stages of growth: lag phase, log (exponential phase), stationary phase, and the death phase. The growth curve allows for monitoring bacteria growth under different chemical and physical conditions and is important in developing specific hurdles for each pathogen or food product (Zwietering, Jongenburger et al., 1990). The bacterial growth curve model is also a good representation of product shelf life, or longer lag phase, so better production and distribution plans can be implemented. (Kreyenschmidt, Hübner et al., 2010)

Hurdle technology can affect bacteria in multiple ways such as the disruption of homeostasis, metabolic exhaustion, stress reactions, and multitarget preservation (Leistner, 2000). The disruption of homeostasis is a key phenomenon that causes the bacteria to stop multiplying so they remain in the lag phase or die before it is reestablished. The most effective way of disturbing homeostasis is by using several hurdles at a time (Leistner and Gould, 2012).

Metabolic exhaustion occurs when available energy is restricted so that concentrations of osmoregulation solutes are low and there is not enough energy for bacteria to have a stress reaction (Lee, 2004). Stress reactions are a mechanism bacteria employ to recover from the stressful conditions in its surrounding environment. By restricting energy available for stress reactions, the bacteria will not have enough energy to grow and they will become metabolically exhausted and die (Leistner, 2000; Lee, 2004). Multitarget preservation is a concept where multiple hurdles are used to cause a synergistic effect inside the bacteria cells. Multiple cell components (e.g., DNA, cell membrane, pH) are targeted at the same time and homeostasis is interrupted, making

recovery more difficult (Leistner, 2000). For example, in fermented sausages, using a combination of hurdles (preservatives, redox potential, competitive flora, pH, and a_w), will now allow the initial bacteria present at the start of fermentation to overcome all of the hurdles (Leistner and Gould, 2012).

2.2.2 pH

Most pathogens prefer to grow in pH neutral environments (6.5-7.0). If the pathogen encounters an acidic environment it will react to the stress using an acid tolerance response (ATR). Acid stress is defined as the effect of low pH and weak organic acids present in the environment. The weak organic acids are volatile fatty acids (VFAs) like are butyric, propionic, and acetate which are produced as the result of fermentation (Bearson, Bearson et al., 1997).

The microbial cell is directly affected by pH values. The function of enzymes and the transport of nutrients into cell are inhibited at a low pH. When microorganisms are placed in an unfavorable environment, their ability to multiply depends on their ability to bring the pH back to a more neutral value (Jay, 2000). When bacterial cells are in acidic environments, balancing H^+ becomes extremely important for key cellular components ATP and DNA to function. Amino acid decarboxylases function best at pH 4.0 and lose functionality at pH 5.5 which causes the cells to spend ATP to reestablish intracellular pH (Jay, 2000). To maintain a neutral pH depends on amino acid decarboxylation antiporter reactions, requiring the presence of many decarboxylases (Bearson, Bearson et al., 1997).

A study from Labas and Krin et al. in 2002 showed that modification of the membrane protein composition of a gram-negative pathogen is linked to the acid tolerance response to low pH. Furthermore, Labas found that the macromolecules located outside of the cytoplasmic membranes were also subject to damage from acidic conditions. The study concluded that environmental challenges have a significant effect on motility, adherence, and the coordinate control of barrier permeability (Labas, Krin et al., 2002).

2.2.3 Water Homeostasis

Water activity is one of the most important vehicles for microbial growth and survival in fermented meat products. Dry and semidry fermented are considered “ready to eat” so they do not technically need a heat treatment before consumption. Producers must make sure the production process is enough to achieve a low pH and water activity product. If fermentation temperatures, drying temperatures, and relative humidity aren’t controlled properly, it may not be adequate to inhibit existing pathogens. Only a few cells (<10) of *Salmonella* or shiga-toxin producing *E. coli* can be enough to cause illness. These pathogens can survive and grow in low water activity, low pH, and high salt environments. *E. coli* O157:H7 uses its many acid tolerance mechanisms to survive lower pH environments better than neutral pH environments (Foster, 2004). Studies have also shown that *E. coli* O157:H7 can survive pH 2.0 for hours (Gorden and Small 1993, Small, Blankenhorn et al. 1994). *Salmonella* has been shown to survive pH 3.0-4.0 through its acid tolerance response (Álvarez-Ordóñez, Prieto et al. 2012). *Salmonella* has also been shown to survive low water activity values for extended periods of time, making it harder to thermally inactivate (Scott, Chen et al., 2009).

Controlling moisture of fermented sausages is the most important way to prevent microbial growth because if moisture is present pathogens can grow. If low water activity products can rehydrate before consumption, there is the possibility that pathogens will grow if the product isn't stored properly (Beuchat, Komitopoulou et al., 2013). Most bacteria cannot grow below 0.87 water activity, although some pathogen bacteria can grow below 0.85 (Beuchat, Komitopoulou et al., 2013). Physiological activities necessary for cell division are impaired below water activity of 0.87. The International Commission on Microbiological Specifications for Food reported that *Staphylococcus aureus* can survive, but not produce toxins, at water activity as low as 0.83 (ICMSF, 1996).

Bacterial cell membranes are easily penetrated by water, so cellular hydration is easily altered by an osmotic shift. Bacteria respond to osmotic stress by storing or releasing electrolytes and small organic solutes (Wood, 2011). If the osmolarity of a certain food is high and solutes are unable to penetrate the cell membrane, microorganisms use osmosis to reestablish equilibrium.

The microbial cell membrane and walls have differentiating solute permeabilities depending on physical and chemical properties of the solute. It is also affected by the cell membrane and cell wall structure and chemical composition (Harris, 1981). The response of a microorganism to water potential stress is due to its biophysical need to attain equilibrium in its environment.

The materials entering a bacterial cell must be in a soluble form to penetrate the bacterial wall because the bacterial cell can only transfer nutrients into the cell and waste materials out of the cell. When a bacterial cell is exposed to low water activity, it may

synthesize osmoprotectants from the environment. Examples of osmoprotectants are proline, glutamine, betain, and sugar trehalose for survival (Gibbs and Gekas, 1998). These compatible solutes are used to help the organism survive extreme osmotic stress. Metabolic exhaustion occurs from the loss of water because energy is restricted so that concentrations of osmoregulation solutes are low and there is not enough energy for the bacteria to have a stress reaction (Lee, 2004), therefore halting growth. This leads to successful preservation.

2.3 Intrinsic and Extrinsic Factors Affecting Fermentation in Dry Cured Sausages

Intrinsic factors are those that are inherent to a food product such as pH, water activity, redox potential, starter culture, and processing methods. Extrinsic factors are the environmental factors that affect the food product such as oxygen present, carbon monoxide present, temperature, and relative humidity. Both types of factors are considered when producing a dry cured sausage because they will determine how pathogens will respond if they are present.

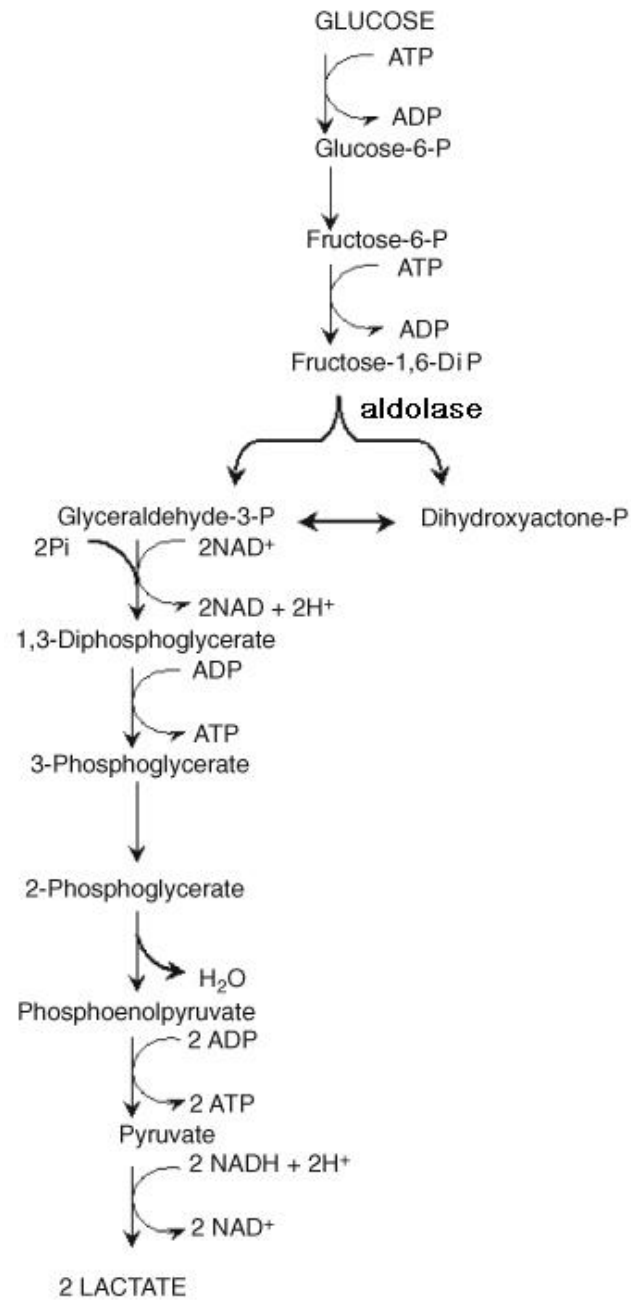
2.3.1 Extrinsic Factors

2.3.1.1 Lactic Acid Bacteria Starter Cultures and Effects on Fermentation

The ability of lactic acid bacteria (LAB) starter cultures to produce lactic acid is dependent on proper temperatures, storage, and humidity. Most homofermentative LAB starter cultures include some lactobacilli and other species of pediococci, enterococci, and streptococci. These LAB strains produce lactic acid primarily from hexose via the Embden-Meyerhof (E-M) pathway (McDonald, McFeeters et al., 1987). During homolactic fermentation, one molecule of glucose is catabolized to yield 2 molecules of

pyruvate. Through the oxidation of NADH and reduction of pyruvate to lactic acid, intracellular redox balance is maintained. During the formation of glyceraldehyde-3-phosphate (G3P), two molecules of NAD are reduced and then reoxidized to favor the reduction of pyruvate to lactic acid by lactic dehydrogenase (LDH) (Piard and Desmazeaud 1991). The result is the two molecules of lactic acid. The homolactic fermentation pathway is illustrated below in Figure 2.1.

Figure 2.1 The homolactic fermentation pathway of Lactic Acid Bacteria. Adapted from Todar 2012.



There are many advantages to using LAB starter cultures in fermented meat products. The most important antimicrobial properties LAB have are the production of organic acids and bacteriocins, lowering of redox potential, and nutrient competition (Holzapfel, Geisen, and Schillinger, 1995). The function of LAB starter cultures can be inhibited if not held at proper fermentation temperatures, therefore, posing the risk of the growth of pathogenic bacteria.

Homofermentative LAB produce 1.8 moles of lactic acid per mole of metabolized hexose and 10% of byproducts in dry fermented sausages leading to a pH decrease to around 5.0 (Gottschalk, 1986). The decrease in pH values leads to changes within the sausage conformation. The water holding capacity of the meat is reduced, stimulating drying and gelation of myofibrillar proteins, undesirable spoilage bacteria are inhibited, and reduction reactions for color formation are favored as well as enzymatic reactions for flavor and odor (Ordóñez, Hierro et al., 1999).

2.3.2 The effect of humidity and temperature on fermentation.

Starter cultures are a key element of producing fermented processed meats. Lactic acid bacteria starter cultures work by converting glucides to lactic acid and other metabolites to ensure extended stability against pathogenic bacteria (Ceylan and Fung, 2000). LAB starter cultures reduce pH through their physiological activities leading to the breakdown of carbohydrates, fats, and proteins to produce desirable flavor and color (Ray and Bhunia, 2013). However, if not held at optimum relative humidity and temperature, the starter culture will not start producing lactic acid enough to drop pH and inhibit pathogenic bacteria that may be present.

The speed of fermentation is directly dependent upon temperature. At temperatures below 12°C, starter cultures stop metabolizing sugar. Fermentation is also hindered if there is no more sugar available for utilization for LAB when the water activity drops below 0.95 (Marianski, 2008). Aroma development in sausages is temperature dependent for appropriate chemical and enzymatic reactions to take place (Stahnke, 1995). Therefore, if the temperature is not optimal, proper fermentation will not occur.

The main role of LAB starter cultures in dry sausages are to inhibit pathogens, shorten fermentation time, produce a uniform aroma and texture, drop the pH, and reduce spoilage (Leroy and De Vuyst, 2004). LAB starter cultures yield many natural antimicrobials such as organic acids, bacteriocins, reuterin, and reutericyclin (Messens, Neysens et al., 2002). The production of these antimicrobials works against microbial contamination. Bacteriocins are proteinaceous toxins that combat against pathogenic bacterial strains and food spoilage organisms.

Reuterin is inhibitory toward many types of bacteria, yeasts, and molds by producing glycerol, a cell growth inhibitor (Gänzle, 2004). Reutericyclin is effective against a broad spectrum of bacterial strains but doesn't have a great effect on gram negative bacteria (Leroy and De Vuyst, 2004). Reutericyclin is effective against gram positive bacteria due to its proton ionophore activity (Gänzle, 2004).

The optimum relative humidity for *Pediococcus pentosaceus* and *Staphylococcus xylosus* when producing chorizo or landjager sausage is 90% and the optimum temperature for fermentation is 16-20°C (Marianski, 2009). If the humidity is not high

enough at the beginning of fermentation, starter cultures will not perform appropriately. Low relative humidity will cause the rate of acid production to slow and prevent pH from declining. It can also cause casing hardening, preventing moisture loss. Sausages should be held at the proper temperature to enhance starter culture growth, while suppressing the growth of spoilage organisms (Heinz and Hautzinger, 2007).

2.3.3. Specific USDA regulations for Ready to Eat (RTE Foods)

The USDA has regulations for the manufacture of dry fermented sausages because they have been linked to foodborne illness outbreaks in the past. There is a zero-tolerance policy for *E. coli* O157:H7, non-O157:H7 STEC, *Salmonella*, *Listeria monocytogenes*, and coagulase positive *Staphylococcus aureus* for ready to eat (RTE) foods (USDA-FSIS, 2016). In RTE shelf stable meat products, producers must show a 5-log reduction of *Salmonella*, and *E. coli* and sufficient reduction of *Listeria monocytogenes* in their lethality treatment (USDA-FSIS, 2017).

The control of pH is important initially to inhibit the growth of *Staphylococcus aureus* because it can survive in high salt and sugar foods and lower water activities. The USDA requires that dry fermented sausages attain a final pH of 5.3 or less to control the growth of pathogenic organisms with moisture loss of 25-50%. However, research has shown that Shiga toxin producing *E. coli*, specifically *E. coli* O157:H7 can survive very low pH values for an extended period so the combination of low pH (<3.8), a_w (<0.85), temperature, and in some cases a heat treatment is needed to keep the product microbiologically safe. The USDA FSIS requires manufacturers to either demonstrate the ability to reduce a 7-log inoculum of *E. coli* O157:H7 by greater than 5 logs or

implement a statistically based sampling program to affirm its absence in the final product (USDA-FSIS, 1995).

2.3.4 The survival of pathogenic bacteria in low moisture environments.

Studies have shown that vegetative cells of some pathogenic bacteria can persist and survive in low moisture foods ($a_w < 0.85$) and ingredients for months and even years, although their metabolism is greatly reduced. The ability for pathogenic bacteria to survive drying processes is concerning, particularly because processors assume low water activity foods to be sterile.

Recent outbreaks of foodborne illnesses in dry fermented meats have led to more research into their survival mechanisms. A study of *E. coli* 0157:H7 in fermented, dried, vacuum packed sausage showed that it can survive, but not grow, for up to 8 weeks (with a 2 log₁₀ reduction at 4°C) (Glass, Loeffelholz et al., 1992). Another study showed that at an initial population of 6.3 log CFU/g, it was able to survive, but not grow, in infant rice cereal (a_w 0.35-0.73) for 16-22 weeks at 5-25°C (Deng, Ryu et al., 1998). Deng et al (1998) also showed that at an initial population of 5.5-5.7 log CFU/g, it was able to survive, but not grow, in apple juice powder (a_w 0.16-0.23), buttermilk powder (a_w 0.21-0.38), cheddar cheese seasoning (a_w 0.21-0.36), and powdered chicken (a_w 0.34-0.38) stored at 5°C for 19 weeks and 21°C for 16 weeks. The resilience of some pathogenic bacteria to survive in such low moisture environments like fermented sausages shows why the use of starter cultures and acidification is so important for their control.

2.4. Starter cultures and fermented meat

Hurdle technology is important in the production of fermented meat products as most bacteria cannot grow at low water activity values (<0.85) combined with acidic conditions ($\text{pH} < 3.8$) (USDA-FSIS, 2011). Fermentation is a preservation method of biological acidulation of a meat product that enhances flavor, tenderness, microbiological safety, and color that is desirable for a consumer. A fermented meat product is defined as “comminuted meat and fat mixed with salt, nitrite and/or nitrate, sugar, and spices that are put into a casing and go through a fermentation period” (Hugas and Monfort, 1997). Fermented meat has a relatively long shelf life (up to 3 months or longer if frozen) due to the addition of salt, nitrite and/or nitrate, and a low pH from acid production, with the end product being a shelf stable, microbiologically presumed safe item (Ockerman and Basu, 2007).

Meat has a relatively high-water activity (a_w) of 0.99 which promotes bacterial survival. The USDA considers any food with a water activity higher than 0.86 a potentially hazardous product (Marianski, 2009). Meat fermentation requires a starter culture to produce lactic acid which results in reduced fermentation times and consistent organoleptic characteristics (Hugas and Monfort, 1997). Important starter cultures that are used in meat fermentation are *Lactobacillus* and *Pediococcus* species for acid production, often in combination with other curing bacteria such as *Micrococcus* and *Staphylococcus* for flavor and color development (Marianski, 2009).

Staphylococcus starter cultures are non-pathogenic, food grade organisms. They typically include strains such as: *Staphylococcus xylosum*, *Staphylococcus carnosus*, and

Staphylococcus saprophyticus. Pathogenic *Staphylococcus* strains can cause infectious diseases because of their virulence factors that the non-pathogenic strains do not contain. The virulence factors include evasion and adhesion to host cell tissues and colonization abilities (Rosenstein and Götz 2012). *Staphylococcus* starter culture strains are used during ripening (active fermentation phase) of fermented sausages to stabilize color, develop aromas, decomposition of peroxides, and improve flavor through proteolysis. (Leroy, Verluyten et al. 2006). Meat proteases contribute to peptide formation and proteolysis during fermentation (Hierro et al., 1999; Molly et al., 1997).

All starter cultures can be divided into 2 categories: first generation cultures and second-generation cultures. First generation starter cultures are lactic acid producing bacteria that have a plant origin while second generation starter cultures originate from meat (Hugas and Monfort, 1997). First generation starter cultures use lactic acid production to control the fermentation and ripening process, though, spontaneous lactic acid bacteria can grow and compromise the sensory characteristics of the meat. Most Lactobacilli are also capable of producing hydrogen peroxide, leading to discoloration. The discoloration is typically a green color (Hugas and Monfort, 1997). To avoid this problem, second generation starter cultures, can be used in combination with other catalase positive cocci. Catalase can combat hydrogen peroxide by catalyzing the decomposition of hydrogen peroxide to water and oxygen (Hugas and Monfort 1997; Chelikani et al., 2004).

Main traits of second-generation starter cultures are bacteriocin production, flavor production, and genetic manipulation (Hugas and Monfort, 1997). Lactic acid bacteria restrict microbial growth through the production of acid, nutrient competition,

attachment/adhesion site competition, and the production of bacteriocins (Hugas 1998). Bacteriocins are ribosomal synthesized antimicrobial polypeptides that work to inhibit bacteria and have a proteinaceous nature that can cause them to become inactivated in the gastrointestinal tract (Hugas, 1998).

Lactic acid bacteriocins are divided into 3 major classes based on molecular weights, primary structure, genetic features, and post translational modifications: class I is lantibiotics, class II is non-lantibiotics, and class III is bacteriocins (Zacharof and Lovitt, 2012). Class I bacteriocins are post translationally modified and create unusual amino acids like lanthionin and methyllanthionine. Class II bacteriocins are smaller, heat stable, hydrophobic, cationic, non-modified peptides. Class III bacteriocins are large, heat labile, lytic peptides, with some having the ability to cause degradation of the cell wall, causing cell lysis (De Vuyst and Leroy 2007).

Lactic acid bacteria starter cultures can produce bacteriocins in fermented meats, fermented dairy products, and fermented vegetables and displays inhibitory effects against pathogenic bacteria and spoilage organisms (De Vuyst and Leroy, 2007). In dry cured fermented sausages, starter culture *Pediococcus pentosaceus* + *Staphylococcus xylosus* is commonly used. This starter culture is used for fast fermentation as well as the development of color and flavor. Fast fermentation is used in the United States to rapidly drop pH values and can take as little as 48 hours with a finished pH value less than 5.0 (Dalmis and Soyer, 2008). The starter culture chosen depends on the type of sausage being produced, but the result will be a microbiologically safe product.

2.5. Foodborne pathogens

The Center for Disease Control (CDC) estimates that each year 1 in 6 Americans (or 48 million people) will become ill from contaminated food or beverages, 128,000 are hospitalized, and 3,000 will die of foodborne illnesses. In 2017, FoodNet received reports of 24,484 illnesses reported with 5,677 hospitalizations, and 122 fatalities in its surveillance area. The surveillance area covered 15% of the U.S. population. Of these foodborne illnesses, *Campylobacter* and *Salmonella* represented the highest incidence of foodborne illness in 100,000 people. Culture-independent diagnostic tests (CIDTs) revealed more illnesses from non O157 STEC, *Cyclospora*, *Yersinia*, and *Vibrio*, that were previously not identified due to limited testing abilities. (CDC, 2017).

Meat can become contaminated while it is being processed, cut, handled, and packaged. To inhibit the growth of pathogens it is important to control temperature, salt, acidity, moisture, and time (Marianski, 2009). Most pathogens prefer to grow in ambient temperatures and need oxygen to be present to grow. The “Danger Zone” for pathogen growth is 40-140°F.

There are 3 recognized types of bacteria found in meat: food spoilage, pathogenic, and beneficial (Marianski, 2009). Food spoilage bacteria include bacteria, molds, and yeast that produce metabolites that cause food spoilage. Food spoilage can be seen visually through the formation of slime, mold, off colors, and the production of off odors (Gram, Ravn et al., 2002). Pathogenic bacteria, however, cause foodborne disease although the meat does not look compromised or have an off odor. Consumers are not aware that they have consumed a foodborne pathogen until they display foodborne illness

symptoms. The most dangerous and recognized pathogens associated with fermented meats are *Salmonella spp.*, *Listeria monocytogenes*, *Campylobacter*, and *E. coli* O157:H7 (Marianski, 2009). *Staphylococcus aureus* is also associated with fermented meats particularly because it can form toxins in improperly handled and stored food.

Research has shown that comminuted and ground meat products contain higher numbers of pathogens compared to whole pieces of meat like steaks and roasts. Ground meat products are often trimmings from various cuts of a carcass and handled frequently during carcass breakdown. This can contribute to high numbers of microbial contamination. Ground meat products also have a larger surface area compared to whole muscle cuts and contributes to an overall higher microflora level because there is more exposure to harmful bacteria (Samelis and Metaxopoulos, 1999).

Pathogens are more prevalent in these types of products because processed meats go through extensive processes such as grinding, cutting, and slicing and if bacteria are present then they will be present on the meat. If there is a piece of meat that has high microbial numbers it is very easy to contaminate other pieces of meat, especially if they are being processed on the same table and stored in the same place (Sofos, Geornaras et al. 2008). Another source of pathogen contamination in ground meats is the lymphatic system.

The lymphatic system is responsible for immune function and works by filtering and inhibiting bacteria, viruses, and other foreign invaders. Meat that is heavily contaminated is often due to the lymph nodes embedded in fat and studies have shown that those organs contain high numbers of microbes (Samuel, Eccles et al., 1981). In cattle,

lymph nodes can be found in within the fatty tissue of the carcass. Therefore, the lean and fat trimmings destined for ground beef could then contain contaminated lymph nodes and contribute to microbial contamination (Arthur, Brichta-Harhay et al., 2008).

Ground meat that is used for fermented sausages has additional hazards because of the non-meat components. Spices that are used often have high microbial counts as well as other condiments unless they have been irradiated. *Salmonella* is a common concern in spices due to multiple recalls (Vij, Ailes et al., 2006). Sausage casings also pose a threat for microbial contamination, mainly for natural casings which are made from the intestines of pigs, sheep, and cattle. A study of the microflora of fresh pork sausage casings looked specifically at salt-packed and wet-packed casings. The study found that contamination levels on wet casings are much higher than collagen casings and the casings contributed to the more contamination than ingredients (Riha and Solberg, 1970).

2.6. *Salmonella*

Salmonella is a gram negative, facultative, rod-shaped bacterium that is in the proteobacterial family *Enterobacteriaceae*. *Salmonella* serotyping is used to identify homologous antigens using specific antibodies (Jackson, Griffin et al., 2013). *Salmonella* has three major antigens and they can be identified based on their structure, recognized as somatic (O), surface (envelope), and flagellar (H) (Lüderitz, Staub et al., 1966). The O antigens have O polysaccharide side chains exposed on the outermost portion of the bacterial layer and envelope antigens may mask these. The H antigens are identified based on flagellar proteins (Luk, Kongmuang et al., 1993).

The antigens are distinguished from each other by the chemical makeup of the somatic antigen and the protein content on the flagella of the flagellar antigen. Somatic (O) antigens are alcohol resistant and generally heat stable, while flagellar (H) antigens are heat-labile. Surface (envelope) antigens are found in other genera of *Enteric* bacteria but can also be found in some *Salmonella* serovars. Surface antigens may also mask somatic antigens (Luk, Kongmuang et al., 1993).

Salmonella can be found in eggs, poultry, raw meats, unpasteurized milk, and raw vegetables like alfalfa sprouts and spinach. It can be present in processed meat products as the result of contamination during the eviscerations, washing, or transporting of carcasses. *Salmonella* is commonly found in the intestinal tracts of animal hosts such as cattle, reptiles, pigs, poultry, and humans. *Salmonella* is heat sensitive, so it is destroyed with cooking products to an internal temperature of at least 165°F for poultry items. The USDA-FSIS requires a 6.5log₁₀ reduction of *Salmonella* to be safe for human consumption (USDA-FSIS, 2001) although there is a zero-tolerance policy in RTE items.

Controlling the pH and water activity in fermented sausages is the best way to control the growth of *Salmonella*. A study of the control of *Listeria monocytogenes* and *Salmonella enterica* in Italian fermented sausages showed that pH and water activity were crucial to eliminating the pathogens. The study also showed that if fermentation temperatures are at or below 20°C, water activity becomes more important for eliminating the pathogen than pH value, and if water activity reduction is slow, the pathogen can survive longer.

They studied also revealed that at fermentation temperature $>20^{\circ}\text{C}$, the critical factor to reduce pathogens was pH decline (Mataragas, Bellio et al., 2015). Initial meat contamination has also been associated with the survival of *Salmonella* in fermented sausages. Studies have also shown that temperature abuse during processing, storage, and distribution also contribute to the survival and growth of pathogens if they are present initially (EFSA and ECDC, 2012).

The USDA estimates that *Salmonella* causes 1.2 million cases of foodborne illness a year with 360,000 illnesses related to FSIS regulated products (USDA, 2016). In humans, *Salmonella* causes two kinds of Salmonellosis diseases, enteritis and gastroenteritis. The infective dose to cause illness is normally 10^7 - 10^9 cells, although outbreaks with <10 cells has been observed (Jay, 2000). Symptoms occur within 12-72 hours after infection and include: diarrhea, fever, abdominal cramps, and vomiting (CDC, 2015).

Salmonella enterica serovars possess 2 pathogenicity islands (SP-1 and SP-2) (Morgan 2007). Both pathogenicity islands encode separate type III secretions that introduce effectors into the host environment (Hapfelmeier et al., 2005). Pathogenic species of *Salmonella* use two type III secretion (TTSS) systems that allow the delivery of effector proteins into the cytoplasm of host cells. After invasion, cell signaling, and vesicular trafficking are manipulated (Chopra et al. 1999; Hapfelmeier et al., 2005). SPI-1 TTSS effects early host cell cytoskeletal and membrane rearrangements involved in bacterial uptake into cells. SPI-2 plays a main role in intracellular infection by allowing the formation of vacuoles and the evasion of host cell defenses (Coombes et al., 2005).

A study in vitro of *Salmonella typhimurium* showed that effector proteins were translocated by the pathogenicity island 1 TTSS. This resulted in the reorganization of host cell structure, invasion of epithelial cells, expression of cytokines and chemokines (Hernandez et al., 2004; Hardt et al., 1998), and eventually cell death (Hapfelmeier et al., 2005). The pathogenicity island 2 TTSS from *S. typhimurium* is shown to be expressed after it has entered a macrophage or epithelial cell. The exact reason *S. typhimurium* triggers gastrointestinal inflammation is still unknown, despite in vitro evidence.

In non-phagocytic cells, virulent *Salmonella enterica* strains can attach the intestinal mucosa with the aid of fimbrial adhesions encoded by a gene on SPI-1 (Jay, 2000; Van Der Velden, Bäumlner et al., 1998). After the intestinal mucosa is penetrated, infection occurs at the ileum site of the small intestine (Jay, 2000; Jones and Falkow, 1994). The lysosomes are then penetrated and protein (SPiC) is secreted into the cytoplasm, preventing the fusion of lysosomes with vesicles (Morgan 2007). This pathogen can perform phagocytic functions to allow them to reach deeply into tissues and avoid host defense mechanisms (Galán, 1996). The exchange of biochemical signals between cells leads to cytoskeletal arrangement, membrane disruption, and bacterial uptake by micropinocytosis. This causes enlargement of membrane-bound phagosomes (Galán, 1996).

Salmonella typhimurium has the ability to enter a large variety of nonphagocytic cells (Ginocchio, Olmsted et al., 1994). A study from Ginocchio and Olmsted of *S. typhimurium* showed that contact with cultured epithelial host cells resulted in the assembly of appendages on the surface of the microorganism. When the host cell response is triggered, the appendages retract or are shed from the bacterial surface,

leading to membrane ruffling and bacterial uptake. The study also found that the appendages on the bacterial surface is required for *S. typhimurium* entry into cells. The study also found that protein synthesis is not required for the formation of the appendages (Ginocchio, Olmsted et al., 1994).

Salmonella enterica serovar Typhimurium is capable of virulence using 60 genes (Groisman and Ochman, 1997). It is the leading cause of gastroenteritis in humans (McClelland, 2001 #40) and research suggests that toxins play a role in the diarrheal part of salmonellosis syndrome because of the 29-kDa polypeptide enterotoxin it possesses. This enterotoxin is a thermolabile protein that is released into the cytoplasm of the infected host and cross-reacts with the cholera toxin, activating adenylate cyclase. Concentrations of cyclic AMP then increase and there is fluid exsorption in the intestinal lumen, causing illness. (Jay, 2000; O'brien and Holmes, 1996). *S. typhimurium* definitive type 104 (DT104) is a serious foodborne threat because it is resistant to 5 antimicrobials: sulfa drugs, penicillin, streptomycin, chloramphenicol, and ampicillin. This resistance could be attributed to the use of antimicrobial drugs used in livestock (Helms, Ethelberg et al., 2005).

2.6.1 The survival of *Salmonella* in fermented meat

Poultry, eggs, livestock, fresh produce, non-irradiated spices, water, pets, seafood, and wildlife are common transmission routes for *Salmonella* infection in humans (Pires, Evers et al. 2009). Fermented meat products are also a source of *Salmonella spp.* because some strains can survive low pH levels of 3.8 and water activity levels of 0.94

(Marianski, 2009). *Salmonella*, like other enteric pathogens, can survive in the acidic conditions of fermented meat because of its acid tolerance response.

S. typhimurium, in particular, has multiple low pH survival strategies depending on whether the cells are in stationary or exponential phase (Bearson, Bearson et al. 1997). Stationary phase cells are more 1000 fold more acid tolerant than exponential phase cells. (Lee, Slonczewski et al. 1994). The stationary phase acid tolerance response is dependent on pH. A study of the survival of the growth potential of *S. typhimurium* in fermented sausages proved that if acidification is delayed or inhibited initially, higher fermentation temperatures will support the growth of the pathogen (Birk, Henriksen et al. 2016).

Another study of the survival of *Salmonella enterica* in Italian fermented sausages Cacciatore and Felino revealed that it can survive the duration of fermentation in rapid fermentation and slow fermentation sausages (Mataragas, Bellio et al., 2015). The study concluded that pH and water activity are the most important factors for inhibiting pathogen growth. At fermentation temperatures below 20°C, water activity reduction was more important than pH reduction. In contrast, at higher fermentation temperatures (>20°C), pH was vital in eliminating pathogens.

The acid tolerance response (ATR) is a two-stage process that is activated at pH 5.0-6.6 to protect cells from severe acid stress although the ATR can protect against pH of 3.0-4.0 if pH homeostasis fails (Foster and Hall, 1991). Emergency pH homeostasis is triggered by mild acid function to keep the pH above 5.0 as the cells encounter a severe acid pH (3.0 or less). Many inducible amino acid decarboxylases contribute to emergency pH homeostasis in *S. typhimurium*. The use of pH homeostasis alone will not protect

cells from acid tolerance so acid shock protein (ASP) synthesis is required in the second stage of ATR for the exponential phase *Salmonellae* to survive the acidic conditions (Bearson, Bearson et al., 1997).

Acid tolerance response systems are regulated by three regulatory proteins: RpoS, Fur, and PhoP (Bearson, Bearson et al., 1997). The alternative sigma factor σ^s or RpoS is an important regulator of stationary phase physiology. Evidence shows that RpoS-dependent systems are required for the survival of *Salmonella* in extremely acidic conditions (Baik, Bearson et al., 1996). A regulator of σ^s -dependent system, PhoP, is the regulatory constituent of the PhoP/PhoQ 2-component system. The PhoP/Q region of that system is important for macrophage survival and protection against antimicrobial peptides and virulence (Mahan, Schlauch et al., 1996).

The ferric uptake regulator (Fur) is another regulator of acid tolerance in *Salmonella*. When Fe^{2+} is present, Fur suppresses the communication of iron-controlled genes. This protein is also managing the expression of many acid shock proteins (Bearson, Bearson et al., 1997).

2.7 Non- O157:H7 *Escherichia coli*

Escherichia coli is a gram negative, facultatively anaerobic rod that is in the proteobacterial family *Enterobacteriaceae*. It is the predominant facultative microflora of intestinal tracts in humans and warm-blooded animals. *E. coli* isolates are differentiated by disease they cause through serotyping and are separated into somatic antigen (O), flagellar antigen (H), and capsule antigen (K). *E. coli* is primarily found in ground beef, unpasteurized dairy products, apple cider, and vegetables. The infective dose is less than

a few hundred cells for pathogenic *E. coli* O157:H7 according to foodborne outbreak data (USDA-FSIS, 2010).

Although some strains of *E. coli* are harmless, there are other strains that can cause illness. Pathogenic *Escherichia coli* are divided up by virotypes that cause diarrheal diseases (Todar, 2012). The five classes of pathogenic *E. coli* are: enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC) (González, 2002).

Enterohemorrhagic *E. coli* is responsible for severe foodborne illness and was first identified as a human pathogen in 1982 after an outbreak of hemorrhagic colitis in the United States (Law, 2000). These strains of *E. coli* produce verotoxins or shiga-like toxins which are similar to the toxins produced by *Shigella dysenteriae* (Griffin and Tauxe, 1991). Shiga-toxins alone are not sufficient to cause disease, but they rely on a combination of other virulence factors such as virulence and toxin genes (Lindqvist and Lindblad, 2011). Shiga-toxin producing *E. coli* are referred to as STEC.

The most common disease causing EHEC is serotype *E. coli* O157:H7, which is the leading *E. coli* that cause most of the serious illnesses and deaths. *E. coli* O157:H7 is responsible for 96,534 infections each year. Other serogroups of shiga-toxin producing *E. coli*, non-O157:H7 STECs are responsible for 168,698 cases of infections each year (CDC, 2012). The non-O157 serogroups include O145, O45, O26, O111, O121, and O103. They are recognized as the “big-6” STECs because of their emerging health concern (Conrad et al., 2014)

The numbers of infections each year are estimated as not all illnesses are reported or laboratory identified. Non-O157:H7 STEC has been generally under recognized in the past due to research limitations and insufficient investigation (Brooks, Sowers et al. 2005). STEC infection mechanisms are difficult to categorize because of many hindrances to identify them (Law, 2000). There are no suitable animal models to study the mechanism of infection of STEC because animals generally do not show symptoms of infection and they do not develop HC or HUS. Moreover, because of the potentially lethal complications of STEC infection volunteer studies cannot be carried out (Law, 2000).

STEC infections cause symptoms such as bloody diarrhea, vomiting, and stomach cramps. In more serious cases it can cause Hemolytic Uremic Syndrome (HUS) that causes a tired feeling, loss of pink color to the face, and decreased urination and can lead to renal failure (CDC, 2014). Those most at risk for HUS are young children, elderly, and people that are immunocompromised.

It is difficult to accurately identify the reasons that contribute to infection in human because it is not known whether virulence factors are dependent on certain circumstances such as infective dose (Law, 2000; Tuttle et al., 1999). It was not until 2000 that non-O157:H7 was considered an important disease and the Council of State and Territorial Epidemiologists requested that health departments report infection of this pathogen to the National Notifiable Diseases Surveillance System (Brooks, Sowers et al. 2005).

STEC strains produce 2 cytotoxins called Shiga toxins (Stx1 and Stx2) (Blanco, Blanco et al., 2004). Stx1 is a part of a homogenous family of toxins while Stx2 toxins are more heterogeneous. The production of Stx2 and the ability to adhere to the bowel mucosa are very important factors to cause disease in humans (Law, 2000). STEC express the protein, intimin, a virulence-associated factor that is responsible for the attachment of STEC to the intestinal epithelial wall and causes attaching and effacing (A/E) lesions in the intestinal mucosae (Wang, Clark et al., 2002; Law, 2000). Stx2 toxins have also been shown to be more frequently present in cases of Hemolytic Uremic Syndrome (HUS) than Stx1 (Wang, Clark et al., 2002).

The A/E lesions are categorized by the degradation and effacement of epithelial cell microvilli, adherence of bacteria to epithelial cells, and the formation of cytoskeletal structures in the cells (Law, 2000; Knutton et al., 1989). Intimin can attach and efface lesions is because it is encoded by chromosomal gene *eae*. The *eae* gene is a part of the pathogenicity island called the locus for enterocyte effacement (LEE) (Kaper, Elliott et al., 1998).

2.7.1 The survival of STECs in fermented meat

E. coli O157:H7 and non-O157:H7 STEC can be found in a variety of products including ground meats, raw unpasteurized milk, and vegetables. In the past decade there have been increasing numbers of incidences of STEC foodborne outbreaks in fermented sausages in many countries. Therefore, some countries have imposed firm regulations for the production of fermented sausages (Holck, Axelsson et al., 2011). Fermented meat was not seen as a microbial threat due to its low pH, low water activity, and high salt content,

but several studies have shown that shiga-toxin producing *E. coli* can survive fermentation, drying, and storage if the pathogen is present in high numbers (Glass, et al., 1992). Foodborne outbreaks from non-O57:H7 *E. coli* are concerning because not much is known about them, although their mechanisms of infection are assumed to be very similar to *E. coli* O157:H7.

Enteric bacteria, such as *E. coli*, are naturally found in the digestive tracts of humans and mammals. To withstand the acidic of gastric fluid, bacteria must be able to survive the acidic environment of the stomach (pH 1.5-3.0) (Foster, 2000). Some laboratory studies have shown that *E. coli* is able to survive a pH of 2.0 or less for several hours (Lin, et al., 1996). Non-O157:H7 STEC can survive in fermented sausages because of their acid resistant response that allows its survival in the presence of lactic acid and other low pH food products (Leyer, et al., 1995).

Intrinsic factors of fermented sausages alone are not enough to inhibit pathogens if they are present in the raw materials. In 2006, there was an outbreak of hemolytic uremic syndrome in raw cured mutton sausages from *E. coli* O103:H25 in Norway (Nørrung and Buncic, 2008). Further investigation into the outbreak concluded that the production process was not sufficient to inactivate the *E. coli* (VTEC) that was present in the raw meats used for the sausages.

In 1994, 23 individuals in Washington and California fell ill to *E. coli* O157:H7 after consuming dry-cured salami (Chacon, Muthukumarasamy et al., 2006). This outbreak led USDA-FSIS to require meat processors to validate that processing dry and

semi-dry sausages would result in at least a 5-log unit reduction in *E. coli* O157:H7 cells (Chacon, Muthukumarasamy et al., 2006).

In 2002, there was a large outbreak of *E. coli* O157:H7 from the consumption of fermented sausages (Sartz, De Jong et al., 2008). The contamination was attributed to multiple factors. The factors were lack of heat-treatment, addition of the starter culture while in a dormant state, inhibiting the start of fermentation, and short curing (Sartz, DeJong et al. 2008). The starter culture was in an inactivated state when introduced into the sausage batter, slowing fermentation, and allowing *E. coli* O157:H7 to survive. A starter culture added to a sausage in an inactivated state will not begin producing lactic acid to drop the pH of the sausage.

The bacteria were able survive the short curing (5-7 days) at 2-4°C because the temperature was too cold. Ambient temperatures are optimal for starter cultures to begin fermentation. *E. coli* O157:H7 has been also shown to survive short curing fermentation because of its acid resistant properties (Duffy et al., 2000). The ability for shiga toxin producing *E. coli* to adapt to extremely acid environments makes it a dangerous and concerning pathogen in fermented sausages. More recent studies have found that the acid resistance of non O157:H7 STEC serogroups O45, O103, and O26 during fermentation is higher than O157:H7 (Balamurugan, Ahmed et al., 2017). Therefore, post processing interventions have been implemented after fermentation and drying to aid in the elimination of pathogens.

A study of the control of non- O157:H7 STEC and O157:H7 STEC in pepperoni revealed that non-O157 strains have the same ability as O157 to withstand the

manufacture of pepperoni (Glass, Kaspar et al., 2012). The inoculated pepperoni was fermented to a pH <4.8, heated for 1 hour at 53.3°C, and dried. The study revealed that serotypes O103 and O157 had the greatest survival rate. Serotypes O111 and O121 were unable to be recovered during the drying phase, but all other serotypes were recovered in the drying phase through enrichments. The study also concluded that storing dried sausages at -20°C for 2-3 weeks produced a 1.0-1.5 log reduction.

Another study of post fermentation heat times and temperatures to control Shiga toxin-producing *E. coli* showed that fermentation to pH 4.6-5.0 produced a >5 log reduction in pathogen levels, after heating for 1-8 hours at 43.3°-54.4°C (Shane, Porto-Fett et al., 2018). However, studies have revealed that thermal treatment post processing on sausages could yield undesirable sensory characteristics (Dalmış and Soyer, 2008).

Research studies using non-thermal interventions have only been able to achieve a 2-3 log₁₀ reduction (Graumann and Holley, 2008; Balamurugan et al., 2017). Non-thermal processing interventions such as high-pressure processing and irradiation have also been utilized to reduce pathogens, but they can produce undesirable sensory attributes as well. Non-O157:H7 STEC are still not completely understood, but further investigation is needed to determine the most effective way to achieve a 5-log reduction of these pathogens while still maintaining the quality of the product.

2.7.2. Acid resistance systems of *E. coli*

The acid resistance (AR) systems allow bacteria to counteract acid stresses through active and passive acid resistance mechanisms (Kanjee and Houry, 2013). Active acid resistance use physiological, metabolic, and proton-consuming systems to survive,

while passive acid resistance uses buffering capacity of amino acids, polyamines, proteins, polyphosphates, and other inorganic phosphates present in the cytoplasm (Kanjee and Houry, 2013).

There are 3 known inducible systems that allow *E. coli* to have stationary phase acid tolerance: Acid-resistance system 1, acid-resistance system 2, and acid-resistance system 3 (Richard and Foster, 2003). Acid-resistance system 1 (AR1) is the acid induced oxidative system and is dependent on sigma factor RpoS that is responsible for encoding sigma factor 38 sigma and regulating stationary phase transcription genes (Law, 2000). This system provides the least amount of protection against a pH of 2.5 (Richard and Foster, 2003).

Acid-resistance system 2 (AR 2) is glutamate dependent and is also prompted in the stationary phase. The glutamic acid-dependent acid resistance system (GDAR) system requires homologous inducible glutamic decarboxylases (GadA and GadB) enzymes and glutamate/ γ -aminobutyric acid (GABA) antiporter GadC (Kanjee and Houry 2013). This system provides the highest level of protection against extreme pH (Richard and Foster, 2003).

Acid-resistance system 3 (AR3) is the arginine-dependent acid resistance system (ADAR). This system requires inducible arginine decarboxylase AdiA and arginine/agmatine antiporter AdiC. It provides a moderate level of protection and is induced at pH lower than 5.0 in anaerobic conditions (Blethen, Boeker, and Snell, 1968). Glutamic acid decarboxylase and arginine decarboxylase are the most important enzymes that *E. coli* uses to survive extreme pH 2.0 environments (Lin, et al., 1996). These 3

systems along with log-phase acid tolerance protect cells from low pH strains (Richard and Foster, 2003).

2.8 *Staphylococcus aureus*

Staphylococcus aureus is a gram positive, facultatively anaerobic, coccal bacterium that occurs in grape-like clusters. It is a part of the bacterial family Staphylococcaceae and it is considered to be a serious pathogen due to toxin-facilitated virulence, antibiotic resistance, and invasiveness (Le Loir, Baron et al., 2003). The most significant sources of *Staphylococcus aureus* in foods are individuals whose hands and arms have open wounds and through nasal carriers (Jay, 2000). *Staphylococcus aureus* is a part of the normal body microflora and around 30% of humans are carriers of it in their nasal passages (CDC, 2016). Carriers of *S. aureus* have a higher risk for subsequent infections because they are already carrying the bacteria.

Staphylococcus aureus is found in foods that have human hand contact with no further heat treatment such as bakery products, meat salads, and dairy products, although it can also be found in other meat and poultry items. *S. aureus* is considered a major foodborne disease because it can contaminate food during preparation and processing. The bacteria can be introduced to food products through human carrier cross contamination, especially if there is temperature abuse. Temperature abuse allows the bacteria to replicate and produce toxins (Le Loir, Baron et al., 2003). *Staphylococcus aureus* most commonly causes staph infection and is responsible for numerous illnesses including skin infections, foodborne illnesses, and wound infections.

Staphylococcal food poisoning has an onset range of 30 minutes to 6 hours with symptoms including diarrhea, sweating, nausea, vomiting, abdominal cramps, and headache (Hennekinne, De Buyser et al., 2012). The mortality rate for *Staphylococcus aureus* is low and most infected people recover with rest and fluids in 24-48 hours, although immunocompromised, elderly, and infants are susceptible to severe dehydration (Hennekinne, De Buyser et al., 2012).

Staphylococcal food poisoning occurs when food containing toxins produced by *Staphylococcus aureus* are consumed. Specifically, a food source containing the enterotoxin must be consumed and the food must contain enough amounts of toxins to provoke foodborne illness symptoms (Hennekinne, De Buyser et al., 2012). The severity of the illness depends on the amount of toxin-containing food consumed, the susceptibility of the individual to the toxin, the overall health of the victim, and the type of toxin consumed.

The infective doses of Staphylococcal enterotoxin A can be as low as 0.5 µg to cause symptoms. Factors affecting infective dose are food composition, temperature, and other chemical and physiological properties. The type of toxin, amount produced, and multiple enterotoxin types also contribute to infective dose (Bergdoll, 1989). *S. aureus* can grow and produce enterotoxins over a variety of temperatures, water activity, pH, and sodium chloride concentrations. Optimal conditions for Staphylococcal enterotoxin production are temperatures 34-40°C, a_w 0.99, and pH values 7-8 (Schelin, Wallin-Carlquist et al., 2011).

Although this organism can easily be eliminated through cooking, the enterotoxins are very heat resistant and can persist in food products even when the bacteria are eliminated, causing Staphylococcal food poisoning (SFP) (Schelin, Wallin-Carlquist et al., 2011). There are 5 major classes of enterotoxins produced by Staphylococcal species: SEA, SEB, SEC, SED, and SEE (Johler, Sihto et al., 2016). However, SEA and SED are responsible for the most frequent cause of foodborne illness (Le Loir, Baron et al., 2003). Staphylococcal enterotoxins are very resistant to environment conditions and can withstand and survive freezing, drying, heat treatment and low pH, unlike the non-enterotoxin producing strains (Hennekinne, De Buyser et al., 2012).

S. aureus strains are separated into 6 different biotypes based on their biochemical characteristics: human, non-b-hemolytic, avian, ovine, bovine, and nonspecific. *S. aureus* strains producing Staphylococcal enterotoxins are the main cause of food poisoning (Le Loir, Baron et al. 2003). Other strains of Staphylococcus can produce staphylococcal enterotoxins although it is *S. intermedius* causing a foodborne outbreak of 265 cases in 1991 from blended margarine and butter (Khambaty et al., 1994).

There is currently 14 SE identified which share similar characteristics. Staphylococcal enterotoxins are small proteins that are released by the pathogen and target the intestines. The SEs are water soluble and generally heat stable (Schelin, Wallin-Carlquist et al., 2011). Most SEs possess a cystine loop which is required for proper conformation and this probably contributes to emetic activity of the toxins (Le Loir, Baron et al., 2003). They are classified as superantigens because they can activate contact between T-cell antigen receptors and major histocompatibility complex class II

molecules. This causes a large release of cytokines from the immune system and in turn, a substantial inflammation of the intestinal tract (Jay, 2000). Among other superantigens, SEs are the only ones able to cause emetic activity. Superantigen activity and emetic activity are located on different domains of the protein and have separate functions (Le Loir, Baron et al., 2003).

Staphylococcus aureus is a very demanding bacteria in terms of what it needs to thrive. It requires amino acids valine, arginine, and cystine for growth and SE production in the strains of *S. aureus* that produce enterotoxins SEA, SEB, and SEC. This pathogen does not compete well with other bacteria (Sameshima et al., 1998). In fermented products, *S. aureus* does not grow well due to lactic acid production, acidic pH, oxygen peroxide production, and competition for nutrients (Le Loir, Baron et al., 2003).

2.8.1. The survival of *Staphylococcus aureus* in fermented food products

Staphylococcal food poisoning occurs when SE producing *S. aureus* strains are present and food is not promptly refrigerated after cooking. Fermented meat is an excellent medium for the growth of *S. aureus* as well as cheeses, canned meats, milk, and previously cooked foods.

S. aureus contamination is caused by food handlers and the contamination of the food after heat treatments. However, in raw meat products, the contamination can be from animal origin because livestock carry *S. aureus* in their nasal cavity, skin, and hair (Le Loir, Baron et al., 2003).

The frequency of enterotoxigenic strains of *S. aureus* in food samples has been estimated to be 25% (Bergdoll, 1989). Another estimation of enterotoxigenic strains was

evaluated in raw milk cheese in France and showed that of 61 samples, 15.9% were enterotoxigenic (Rosec et al., 1997).

Literature has shown a variety of cases of staphylococcal poisoning, in one case cheese was the source of an outbreak due to the milk becoming contaminated after pasteurization and before the addition of the lactic acid starter culture (Bergdoll, 1989). Because of the contamination, the starter culture was unable to grow properly, hindering fermentation, resulting in the growth and development of *S. aureus* enterotoxins.

Another incidence of *S. aureus* contamination was in 2013 in California. Lee Bros. Foodservice Incorporated recalled 740 pounds of dried sausage products due to contamination from *S. aureus* enterotoxins. The water activity level of the sausages was too high, allowing for the growth of the pathogen (USDA-FSIS, 2013). Controlling water activity levels in fermented sausages is the most important safety measure to reduce microbial contamination, followed by controlling pH. Genoa salami produced by two different companies was the source of several *S. aureus* outbreaks with some samples containing more than 1 million coagulase positive staphylococci per gram (NCDC, 1971a, b).

The use of starter cultures for chemical acidulation is the most effective method of controlling *S. aureus*. *Lactobacillus* and *Pediococcus* species are commonly used as lactic acid producing starter cultures in sausages to jumpstart fermentation through acidulation. Chemical acidulation can be performed using glucono-delta-lactone alone or in combination with citric acid to drop the pH of the meat. Glucono-delta-lactone works to drop the pH of the meat by producing gluconic acid in the presence of water. Citric

acid chelates metal ions and that causes inhibitory effects against *S. aureus* (Daly et al., 1973).

A study of the control of *S. aureus* by Daly et al. (1973) showed that if there are high numbers of *S. aureus* present in a meat product before adding a starter culture, there is less inhibition of the pathogen initially. The ability of starter cultures to inhibit *S. aureus* growth when it is present in a raw meat product is much greater with low populations of *S. aureus*. The study demonstrated that fermentation temperatures of 21°C, 30°C, and 37°C, combined with starter culture *Lactobacillus plantarum* was the most effective method of reducing microbial numbers after 25 hours. The starting microbial numbers for this study were 10^7 CFU per gram. It was also demonstrated that the best way to inhibit *S. aureus* is the starter culture combined with chemical acidulation which enabled the pH to continue to drop because the starter cultures were able to initiate fermentation in the extremely acidic conditions. (Daly et al., 1973).

Bacteria can survive and grow in a variety of conditions in food products and the environment. However, obstacles put into place through Hurdle Technology combat pathogen growth in food items. The control of pH and water activity during fermentation and drying will prevent the growth of pathogens like non-O157:H7 STEC, *Salmonella*, and *S. aureus*. Bacteria prefer to grow in high water activity, pH neutral environments. The microbial cell is directly affected by pH values. The function of enzymes and the transport of nutrients into the cell are inhibited at a low pH (Beales, 2004).

Water activity is one of the most important vehicles for microbial growth and survival in fermented meat products, so during fermented sausage production, the water

activity values must decrease. Closely monitoring temperature and relative humidity during fermentation and drying while producing fermented sausages will also inhibit pathogen growth. Starter cultures also play an important role in acidification of fermented sausages. The ability of lactic acid bacteria (LAB) starter cultures to produce lactic acid is dependent on proper temperatures, storage, and humidity.

CHAPTER 3

PATHOGEN SURVIVAL IN FERMENTED SAUSAGES WITH INITIAL MICROBIAL CONTAMINATION

3.1 Introduction

History has shown the evolution of dry cured fermented meats dating back to Mediterranean and European countries. Climates that were cool with a mild humidity were ideal for sausage production. The type of sausage produced varied by region (Marianski, 2009). Back slopping, the practice of reusing part of a sausage batch previously produced to “inoculate” the next batch of sausages, was a common practice during this time. Back slopping helped preserve a uniform sausage quality. During this time, sausage production was solely dependent on temperature, humidity, and air flow outside. The temperature and relative humidity couldn't be controlled using this production method, so the requirements were not as strict as they are today. As sausage production became more modernized, technologies were developed to control outside factors to produce a higher quality safe product (Gul, Singh et al., 2016).

Although the production of dry cured fermented sausages was used as a way of preservation in ancient times, the focus was on a microbiologically safe product (Incze

1998). Future research would highlight the risks that were associated with producing dry cured meats, especially pathogenic bacteria that could be present in the final product.

Dry cured sausages are produced from a combination of ground meats, spices, starter culture, and curing ingredients that are stuffed into a casing and undergo fermentation. Foodborne illness can occur from these sausages if the initial meat and spices are not free of contamination or if cross contamination from outside sources occurs during production. Dry cured sausages are particularly a concern because they are considered “ready to eat” and therefore do not receive a heat treatment before consumption to kill pathogens if they are present.

Pathogens that can be found in dry cured sausages include non-O157:H7 shiga toxin-producing *E. coli*, *Salmonella spp.*, and *Staphylococcus aureus*, and *Listeria monocytogenes*. Although these pathogens cannot grow at low pH and water activity (a_w), if given the opportunity they can recover and thrive (Otero, Garcí'a-Lo'pez et al., 1998, Talon, Lebert et al., 2007). If pathogens are present in raw materials in high numbers at the beginning of sausage fermentation, the starter culture might not work as effectively.

The purpose of this study was to evaluate the survival of non-O157:H7 Shiga toxin-producing *E. coli*, *Salmonella spp.*, and *Staphylococcus aureus* during the production of dry cured charcuterie style sausages. There were three objectives of this study. The first objective was to observe the effects of relative humidity and temperature on the final pH and a_w values of dry cured sausages. The second objective was to observe the antimicrobial effects of starter cultures on raw material with high microbial contamination initially during sausage production. The third objective was to recognize

the threat of the presence of pathogens in sausages that do not receive a heat treatment before consumption. Chorizo and landjager were chosen for this research study because they have a small diameter and can undergo fast fermentation.

3.2 THE SURVIVAL OF PATHOGENS IN DRY CURED FERMENTED SAUSAGES

3.2.1 Materials and Methods

3.2.1.1. Bacterial Strains and Culture Conditions.

Strains of pathogenic non-O157:H7 *E. coli*, *Salmonella spp.*, and *Staphylococcus aureus* were supplied from the American Type Culture Collection. Non-O157:H7 strains used were ATCC BAA- 2440, strain 0111, ATCC BAA-2219, strain 2002-3211, ATCC BAA- 2215, strain 2006-3008, ATCC BAA- 2196, strain 2003-3414, ATCC BAA- 2193, strain 2000- 3039, and ATCC BAA-2192, strain 99-3311. *Salmonella* strains used were ATCC 13312, Biocontrol (S-67), UK Micro, and ATCC 13311. *Staphylococcus aureus* strains used were ATCC 29740, ATCC 12600, ATCC 43300, and ATCC 27543.

Bacterial cultures were obtained from frozen stock cultures stored on brain-heart infusion (BHI) agar slants (Difco Laboratories, Becton, Dickinson and Company, USA). Cultures were grown and maintained in BHI broth (Difco Laboratories, Becton, Dickinson and Company, USA) and stored at 4°C until needed.

3.2.1.2 Preparation of inoculum

Individual strains were inoculated in BHI broth (Difco Laboratories, Becton, Dickinson and Company, USA) before the study and transferred three times to ensure growth. After transfer into BHI broth, cultures were incubated at 37°C for 24 hours.

Individual bacterial cocktails were mixed together in sterile 50 mL polypropylene conical tubes (Fisher Scientific, USA) and vortexed to ensure they were mixed thoroughly. One ml of inoculum was taken from the bacterial cocktails and dispensed into 9 ml sterile phosphate buffer dilution blank tubes which were then vortexed. Two ml of inoculum from dilution blanks was used to inoculate sausage batter to achieve a concentration target of $7.0 \log_{10}$.CFU/g. Two ml of phosphate buffer from dilution blanks was used in control to mimic the other treatments.

3.2.1.3 Preparation of meat

Beef and pork trimmings were obtained from the University of Kentucky Meat Laboratory. All equipment and utensils were sterilized prior to use. Meat was ground through a 8.0 mm plate prior to creating the sausage batter. The traditional recipe for chorizo and landjager was followed for production, referencing the book “The art of making fermented sausages” by Stanley and Adam Marianski (Marianski, 2009). Ingredient amounts in Tables 3.1 and 3.2 were adjusted to the size of the sausage batches. The Spanish chorizo sausage recipe used 2.49 kg of pork trim. The Landjager recipe used 1.74 kg of pork trim and 0.75 kg of beef trim and the fat percentage target was 40%.

Table 3.1. Traditional Spanish Chorizo Sausage Recipe

Ingredients	Salt, 3% (sodium nitrite accounted for)	70g
	Sodium Nitrite	6g
	Dextrose (glucose), 0.2%	5g
	Sugar, 0.2%	5g
	Black Pepper	15g
	Smoked Paprika	50g
	Oregano	5g
	Garlic	22.5g
	Bactoferm T-SPX Culture (<i>P. pentosaceus</i> and <i>S. xylosus</i>) (Chr. Hansen)	0.3g

Table 3.2. Traditional Landjager Sausage Recipe

Ingredients	Salt, 3% (sodium nitrite accounted for)	70g
	Sodium Nitrite	6g
	Dextrose (glucose), 0.3%	7.5g
	Black Pepper	7.5g
	Cumin	5g
	Nutmeg	5g
	Bactoferm T-SPX Culture (<i>P. pentosaceus</i> and <i>S. xylosus</i>) (Chr. Hansen)	0.3g

Ground meat, spices, and commercial starter culture Bactoferm T-SPX (*Pediococcus pentosaceus* and *Staphylococcus xylosus*; Chr. Hansen Holding A/S, Roskilde, Denmark) were mixed to create the sausage batter. Sausage batter was divided into four batches for each sausage treatment, weighing approximately 0.68 kg each.

3.2.1.4 Production and inoculation of sausages

Meat was inoculated and stuffed in University of Kentucky Food Microbiology Lab. Sausage batches were prepared individually as four treatments: control, STEC, *Salmonella*, and *Staphylococcus aureus*. Each sausage batch was divided into sterile self-

sealing 3.78L bags (SC Johnson Company, Racine, WI) to be inoculated. Two ml of inoculum was taken from previously prepared phosphate buffer dilution blank tubes and dispensed into respective treatments. Control treatments only contained meat, spices, starter culture, and 2ml of phosphate buffer.

Sausages were mixed thoroughly to distribute inoculum. When thoroughly mixed, sausages were stuffed into natural sheep casings (Quality Casing Company, Inc., Cincinnati, OH) to a length of 76.2 mm. Sausages were stuffed by treatment to prevent cross contamination. Between each treatment, the sausage stuffer and materials being used were sanitized in a quaternary sanitizer and then autoclaved to ensure they were sterile, and no pathogen was present on equipment. This step was repeated for all treatments. The initial fat content of the raw meat was 18.40%, and the initial pH was 5.90.

The treatments in this study for chorizo were control, *Salmonella*, STEC, and *S. aureus*. The treatments in this study for landjager were control, *Salmonella*, STEC, and *S. aureus*. Individual sausages were stored in sterile test tube racks and spaced evenly apart for proper airflow. Each treatment was stored on a separate shelf in the fermentation chamber to prevent cross contamination.

3.2.1.5 Storage conditions of sausages

Sausages were stored in a Lunaire Environmental Chamber (Lunaire, New Columbia, PA, USA) initially at 20°C and 90% relative humidity for fermentation. After 72 hours, the sausages were dried at 20°C and 70% relative humidity. After drying, sausages were stored at 15°C and 80% relative humidity. Humidity and temperature

during fermentation, drying, and storage was monitored by a Thermadata™ Temperature and Humidity Logger (ThermoWorks Inc., USA). The humidity and temperature levels were chosen based on recommendations from book “The art of making fermented sausages” by Stanley and Adam Marianski (Marianski, 2009).

3.2.1.6 Microbiological analysis

The control samples for chorizo and landjager were analyzed for initial data. A 1:10 dilution with peptone water (Difco Laboratories, Becton, Dickinson and Company, USA) was performed and plated using a spiral plater on Plate Count Agar (Difco Laboratories, Becton, Dickinson and Company, USA) to get initial bacterial counts. The media used to plate the bacteria was CHROMagar™ STEC base (DRG International, US), XLD agar (Difco Laboratories, Becton, Dickinson and Company, USA), and 3M™ Petrifilm™ Staph Express Count Plates (3M, USA).

For microbiological analysis, the total number of bacteria was determined by serial dilution in a phosphate buffer, homogenized for 1 minute to uniformly mixed samples, and plated in triplicates on the selective media using a spiral plater. All plates were incubated at 37 degrees Celsius for 24 hours and colony forming units per gram (CFU/g) were observed using a Flash & Go Automatic colony counter (Neutec Group Inc, USA).

3.2.1.7 Chemical analysis

The pH was measured using a Hanna pH meter (Hanna Instruments, USA). Equipment was calibrated before each use. The initial control fat and moisture percentage was measured using a fat analyzer (Data Support Company, USA) at the beginning of

this study on a random sample of each sausage batter. A sample of each control sausage was used on sampling days to determine the pH value.

3.2.1.8 Statistical analysis

Data was analyzed in JMP software using a simple linear regression model. A linear regression line was used to determine the correlation between multiple variables (pH, a_w , bacteria strain, and sausage type). A significance level $P < 0.05$ of the null hypothesis was used to determine significance.

3.3 Results and Discussion

Initial counts on meat used for sausages were 5.979 log CFU/g, plated on PCA (plate count agar) and too numerous to count (TNTC) on petrifilm. Control sausage (no pathogen inoculum) had bacterial counts for the duration of the study on XLD agar. Inhibition of *Salmonella*, STEC, and *S. aureus* varied among each sausage and treatment. No pathogen was eliminated in either sausage by the end of the study and bacterial counts did not decrease. The bacterial counts for *Salmonella* and non O157:H7 steadily increased throughout the study. The increase in CFU/g was probably due either to sample variation or contamination levels of the initial meat block competing against the starter culture and/or inoculum.

Table 3.3. The average growth of *Salmonella* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of chorizo sausage and pH value of chorizo control, p=0.746.

Time (Days)	Log CFU/g <i>Salmonella</i>	pH
0	6.41	5.91
1	6.56	5.47
2	6.55	5.59
3	6.53	4.88
4	5.35	4.91
5	6.52	6.47

Table 3.4. The average growth of non-0157:H7 STEC (CFU/g) during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of chorizo sausage and pH value of chorizo control, p=0.501.

Time (Days)	Log CFU/g STEC	pH
0	6.2781	5.91
1	5.6975	5.47
2	5.7063	5.59
3	6.1893	4.88
4	5.9663	4.91
5	5.341	6.47

Table 3.5. The average growth of *S. aureus* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of chorizo sausage and pH value of chorizo control, p=0.996.

Time (Days)	Log CFU/g <i>S. aureus</i>	pH
0	6.2781	5.91
1	5.6975	5.47
2	5.7063	5.59
3	6.1893	4.88
4	5.9663	4.91
5	5.341	6.47

Table 3.6. The average growth of *Salmonella* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of landjager sausage and pH value of landjager control, p=0.042.

Time (Days)	Log CFU/g <i>Salmonella</i>	pH
0	2.36	5.62
1	5.47	5.67
2	6.54	5.17
3	6.46	5.31
4	6.15	6.18
5	6.70	6.70

Table 3.7. The average growth of non-0157:H7 STEC (CFU/g) during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of landjager sausage and pH value of landjager control, p=0.031.

Time (Days)	Log CFU/g STEC	pH
0	4.04	5.62
1	5.05	5.67
2	5.44	5.17
3	6.33	5.31
4	6.40	6.18
5	5.57	6.70

Table 3.8. The average growth of *S. aureus* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of landjager sausage and pH value of landjager control, p=0.022.

Time (Days)	Log CFU/g <i>S. aureus</i>	pH
0	0.61	5.62
1	0.35	5.67
2	1.87	5.17
3	2.62	5.31
4	1.03	6.18
5	0.68	6.70

Table 3.9. The average log CFU/g in chorizo control during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) and pH value, p=0.098.

Time (Days)	Log CFU/g Chorizo Control	pH
0	2.37	5.91
1	1.99	5.47
2	1.94	5.59
3	2.30	4.88
4	2.28	4.91
5	0.77	6.47

Table 3.10. The average log CFU/g in landjager control during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) and pH value, p=0.134.

Time (Days)	Log CFU/g Landjager Control	pH
0	3.27	5.62
1	2.60	5.67
2	3.68	5.17
3	3.86	5.31
4	3.88	6.18
5	3.32	6.70

Table 3.11. The average CFU/g growth of *Salmonella*, non-0157:H7 STEC, and *S. aureus* during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of chorizo sausage, p= 0.743.

Time (Days)	Log CFU/g <i>Salmonella</i>	Log CFU/g STEC	Log CFU/g <i>S. aureus</i>	pH
0	6.41	6.28	1.15	5.90
1	6.56	5.69	0.76	5.47
2	6.55	5.71	2.36	5.59
3	6.53	6.19	2.93	4.88
4	5.35	5.96	1.13	4.91
5	6.52	5.34	0.82	6.47

Table 3.12. The average CFU/g growth of *Salmonella*, non-0157:H7 STEC, and *S. aureus* during fermentation (90% relative humidity and 20°C), drying (70% relative humidity and 20°C), and storage (80% relative humidity and 15°C) of landjager sausage, p= 0.115.

Time (Days)	Log CFU/g <i>Salmonella</i>	Log CFU/g STEC	Log CFU/g <i>S. aureus</i>	pH
0	2.36	4.04	0.61	5.62
1	5.47	5.04	0.35	5.67
2	6.53	5.44	1.87	5.17
3	6.46	6.33	2.62	5.31
4	6.15	6.41	1.03	6.18
5	6.71	5.57	0.67	6.70

This study revealed that pathogenic bacteria can survive fermentation, drying, and storage, especially when the raw material has a high bacterial load initially. The starter culture should have caused a significant decrease in pH values; however, due to the competition between growth of background bacteria and pathogens, the starter culture may have been out competed. Without starter culture activity, rapid pH drop is inhibited in the initial stages of fermentation, leading to pathogen survival if present.

There was so significant change in bacterial counts in the control sausages or any treatments. Chorizo control had a large drop in numbers from day 4 to day 5, but that was probably due to sample variation since numbers remained constant up until that point. There were no changes in bacterial counts in either sausage in the first study. The fluctuating bacterial counts day to day was probably due to sample variation, since there was no inhibition.

The pH did not decrease significantly for either sausage, however, the pH for both sausages met the critical limit of 5.3. The initial pH value for chorizo was 5.9, increasing 6.47 at the end of the study (Table 3.3-3.5). The initial pH of landjager was 5.62, increasing to 6.70 by the end of the study (Table 3.6-3.8). Bacterial counts varied throughout the study, so the pH drop had no influence on numbers. Pathogenic bacteria can grow and thrive in neutral pH conditions, explaining why the bacterial counts did not significantly decrease during the study.

Water activity wasn't recorded until the final day of the study on chorizo and landjager control sausages. The ending water activity was 0.71 and 0.88. A water activity value of 0.71 is enough to inhibit the growth of *Salmonella*, non O157:H7 STEC, and *S.*

aureus, however, there were no other hurdles in place to prevent microbial growth (i.e. low pH value, heat treatment, etc.). The final sausage conditions were able to support bacterial growth, even after fermentation, drying, and storage (Beuchat, Komitopoulou et al., 2013, Jay 2000).

Further studies would be beneficial as to see what could be done to inhibit pathogens and get the true fermentation action in dry cured fermented sausages if the initial meat microbial count is very high. High contamination of initial raw meat materials only contributed to the persistence of bacteria survival.

CHAPTER 4

PATHOGEN SURVIVAL IN FERMENTED SAUSAGES

The purpose of this study was to evaluate the survival of non-O157:H7 Shiga toxin-producing *E. coli*, *Salmonella spp.*, and *Staphylococcus aureus* during the production of dry cured charcuterie style sausages. There were three objectives of this study. The first objective was to observe the effects of relative humidity and temperature on the final pH and a_w values of dry cured sausages. The second objective was to understand if we can control the grow of pathogens through the control of pH, water activity, relative humidity, and temperature. The third objective was to recognize the threat of the presence of pathogens in sausages that do not receive a heat treatment before consumption. Chorizo and landjager were chosen for this research study because they have a small diameter and can undergo fast fermentation.

4.1 Materials and Methods

4.1.1 Bacterial Strains and Culture Conditions

Strains of pathogenic non-O157:H7 *E. coli*, *Salmonella spp.*, and *Staphylococcus aureus* were supplied from the American Type Culture Collection. Non-O157:H7 strains used were ATCC BAA- 2440, strain 0111, ATCC BAA-2219, strain 2002-3211, ATCC BAA- 2215, strain 2006-3008, ATCC BAA- 2196, strain 2003-3414, ATCC BAA- 2193, strain 2000- 3039, and ATCC BAA-2192, strain 99-3311. *Salmonella* strains used were ATCC 13312, Biocontrol (S-67), UK Micro, and ATCC 13311. *Staphylococcus aureus* strains used were ATCC 29740, ATCC 12600, ATCC 43300, and ATCC 27543.

In this study they were evaluated to determine their survival during the production of charcuterie style sausages. Bacterial cultures were obtained from frozen stock cultures stored on brain-heart infusion (BHI) agar slants (Difco Laboratories, Becton, Dickinson and Company, USA). Cultures were grown and maintained in BHI broth (Difco Laboratories, Becton, Dickinson and Company, USA) and stored at 4°C until needed.

4.1.2 Preparation of inoculum

Individual strains were inoculated in BHI broth (Difco Laboratories, Becton, Dickinson and Company, USA) before the study and transferred three times to ensure growth. After transfer into BHI broth, cultures were incubated at 37°C for 24 hours.

Individual bacterial cocktails were mixed together in sterile 50 mL polypropylene conical tubes (Fisher Scientific, USA) and vortexed to ensure they were mixed thoroughly. One ml of inoculum was taken from the bacterial cocktails and dispensed

into 9 ml sterile phosphate buffer dilution blank tubes which were then vortexed. Two ml of inoculum from dilution blanks was used to inoculate sausage batter to achieve a concentration target of 7.0 log₁₀.CFU/g. Two ml of phosphate buffer from dilution blanks was used in control to mimic the other treatments.

4.1.3 Preparation of meat.

Beef and pork trimmings were obtained from the University of Kentucky Meat Laboratory. All equipment and utensils were sterilized prior to use. Meat was ground through a 8.0 mm plate prior to creating the sausage batter. The traditional recipe for chorizo and landjager was followed for production, referencing the book “The art of making fermented sausages” by Stanley and Adam Marianski (Marianski, 2009). Ingredient amounts in Table 4.1 and 4.2 were adjusted to the size of the sausage batches. The Spanish chorizo sausage recipe used 2.49 kg of pork trim. The Landjager recipe used 1.74 kg of pork trim and 0.75 kg of beef trim and the fat percentage target was 40%.

Table 4.1. Traditional Spanish Chorizo Sausage Recipe

Ingredients	Salt, 3% (sodium nitrite accounted for)	70g
	Sodium Nitrite	6g
	Dextrose (glucose), 0.2%	5g
	Sugar, 0.2%	5g
	Black Pepper	15g
	Smoked Paprika	50g
	Oregano	5g
	Garlic	22.5g
	Bactoferm T-SPX Culture (<i>P. pentosaceus</i> and <i>S. xylosus</i>) (Chr. Hansen)	0.3g

Table 4.2. Traditional Landjager Sausage Recipe

Ingredients	Salt, 3% (sodium nitrite accounted for)	70g
	Sodium Nitrite	6g
	Dextrose (glucose), 0.3%	7.5g
	Black Pepper	7.5g
	Cumin	5g
	Nutmeg	5g
	Bactoferm T-SPX Culture (<i>P. pentosaceus</i> and <i>S. xylosus</i>) (Chr. Hansen)	0.3g

Ground meat, spices, and commercial starter culture Bactoferm T-SPX (*Pediococcus pentosaceus* and *Staphylococcus xylosus*; Chr. Hansen Holding A/S, Roskilde, Denmark) were mixed to create the sausage batter. Sausage batter was divided into four batches for each sausage treatment, weighing approximately 0.68 kg each.

4.1.4 Production and inoculation of sausages

Meat was inoculated and stuffed in University of Kentucky Food Microbiology Lab. Sausage batches were prepared individually as four treatments: control, STEC, *Salmonella*, and *Staphylococcus aureus*. Each sausage batch was divided into sterile self-sealing 3.78L bags (SC Johnson Company, Racine, WI) to be inoculated. Two ml of inoculum was taken from previously prepared phosphate buffer dilution blank tubes and dispensed into respective treatments. Control treatments only contained meat, spices, starter culture, and 2ml of phosphate buffer.

Sausages were mixed thoroughly to distribute inoculum. When thoroughly mixed, sausages were stuffed into natural sheep casings (Quality Casing Company, Inc., Cincinnati, OH) to a length of 76.2 mm. Sausages were stuffed by treatment to prevent cross contamination. Between each treatment, the sausage stuffer and materials being

used were sanitized in a quaternary sanitizer and then autoclaved to ensure they were sterile, and no pathogen was present on equipment. This step was repeated for all treatments. The initial fat content of the raw meat was 13.23%. The initial pH for Spanish chorizo was 5.49 and water activity was 0.93. The initial pH for Landjager was 5.46 and water activity was 0.91.

The treatments in this study for chorizo were control, *Salmonella*, STEC, and *S. aureus*. The treatments in this study for landjager were control, *Salmonella*, STEC, and *S. aureus*. Individual sausages were stored in sterile test tube racks and spaced evenly apart for proper airflow. Each treatment was stored on a separate shelf in the fermentation chamber to prevent cross contamination.

4.1.5 Storage conditions of sausages

Sausages were stored in a locked Lunaire Environmental Chamber (Lunaire, New Columbia, PA, USA) initially at 20°C and 90% relative humidity for fermentation. After 72 hours, the sausages were dried at 16°C and 85% relative humidity. After drying, sausages were stored at 15°C and 75% relative humidity. Humidity and temperature during fermentation, drying, and storage was monitored by a Thermadata™ Temperature and Humidity Logger (ThermoWorks Inc., USA). The humidity and temperature levels were chosen based on recommendations from book “The art of making fermented sausages” by Stanley and Adam Marianski (Marianski, 2009).

4.1.6 Microbiological analysis

The control samples for chorizo and landjager were analyzed for initial data. A 1:10 dilution with peptone water (Difco Laboratories, Becton, Dickinson and Company,

USA) was performed and plated using a spiral plater on Plate Count Agar (Difco Laboratories, Becton, Dickinson and Company, USA) to get initial bacterial counts. The media used to plate the bacteria was CHROMagar™ STEC base (DRG International, US), XLD agar (Difco Laboratories, Becton, Dickinson and Company, USA), and 3M™ Petrifilm™ Staph Express Count Plates (3M, USA).

For microbiological analysis, the total number of bacteria was determined by serial dilution in a phosphate buffer, homogenized for 1 minute to uniformly mixed samples, and plated in triplicates on the selective media using a spiral plater. All plates were incubated at 37 degrees Celsius for 24 hours and colony forming units per gram (CFU/g) were observed using a Flash & Go Automatic colony counter (Neutec Group Inc, USA).

When bacterial growth was not observed on selective media, enrichments were performed. The enrichment procedure used lactose broth (Difco Laboratories, Becton, Dickinson and Company, USA) for *Salmonella spp.* and non-O157:H7 STEC and brain-heart infusion (BHI) broth (Difco Laboratories, Becton, Dickinson and Company, USA) for *S. aureus*. A 10-gram sample of each sausage was submerged in 100mL of lactose broth in a sterile stomacher bag, homogenized for 1 minute to uniformly mixed samples, and incubated at 37 degrees Celsius for 24 hours. Serial dilutions were taken from the bags after 24 hours and plated in triplicates on selective media using a spiral plater. Plates were counted using a Flash & Go Automatic colony counter (Neutec Group Inc, USA).

4.1.7 Chemical analysis

The pH and water activity (a_w) were measured using a Hanna pH meter (Hanna Instruments, USA) and an AquaLab Pawkit water activity meter (AquaLab, USA). Equipment was calibrated before use. A sample of each control sausage was used on sampling days to determine the pH and water activity values. The initial control fat and moisture percentage was measured using a fat analyzer (Data Support Company, USA) at the beginning of this study on a random sample of each sausage batter.

4.1.8 Statistical analysis

Data was analyzed in JMP software using a simple linear regression model. A linear regression line was used to determine the correlation between multiple variables (pH, a_w , bacteria strain, and sausage type). A significance level $P < 0.05$ of the null hypothesis was used to determine significance.

4.2. Results and Discussion

Table 4.3. The average growth of *Salmonella* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) of chorizo sausage and pH and a_w of chorizo control, p=0.025.

Time (Days)	Log CFU/g <i>Salmonella</i>	pH	A _w
0	5.193	5.49	0.93
1	5.355	5.48	0.94
2	4.868	5.08	0.97
3	4.49	4.79	0.89
4	0.551	5.14	0.78
5	0	5.16	0.69
6	0	5.39	0.61
7	0	5.13	0.78

Table 4.4. The average growth of STEC (CFU/g) during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) of chorizo sausage and pH and a_w of chorizo control, p=0.002.

Time (Days)	Log CFU/g STEC	pH	A _w
0	5.004	5.49	0.93
1	5.268	5.48	0.94
2	3.948	5.08	0.97
3	4.584	4.79	0.89
4	3.659	5.14	0.78
5	3.116	5.16	0.69
6	1.471	5.39	0.61
7	0.773	5.13	0.78

Table 4.5. The average growth of *S. aureus* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) of chorizo sausage and pH and a_w of chorizo control, p=0.029

Time (Days)	Log CFU/g <i>S. aureus</i>	pH	A _w
0	4.526	5.49	0.93
1	3.813	5.48	0.94
2	3.591	5.08	0.97
3	4.06	4.79	0.89
4	0	5.14	0.78
5	0	5.16	0.69
6	0	5.39	0.61
7	0	5.13	0.78

Table 4.6. The average growth of *Salmonella* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) of landjager sausage and pH and a_w of landjager control, $p=0.005$.

Time (Days)	Log CFU/g <i>Salmonella</i>	pH	A_w
0	5.369	5.46	0.91
1	5.026	5.54	0.90
2	5.157	5.28	0.90
3	4.565	5.24	0.87
4	2.937	5.66	0.81
5	2.764	5.69	0.61
6	1.204	5.56	0.64
7	0.769	5.91	0.71

Table 4.7. The average growth of non O157 STEC (CFU/g) during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) of landjager sausage and pH and a_w of landjager control, $p=0.006$.

Time (Days)	Log CFU/g STEC	pH	A_w
0	5.28	5.46	0.91
1	4.975	5.54	0.90
2	3.864	5.28	0.90
3	4.34	5.24	0.87
4	3.879	5.66	0.81
5	3.286	5.69	0.61
6	2.916	5.56	0.64
7	0.819	5.91	0.71

Table 4.8. The average growth of *S. aureus* (CFU/g) during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) of landjager sausage and pH and a_w of landjager control, $p=0.071$.

Time (Days)	Log CFU/g <i>S. aureus</i>	pH	A_w
0	4.079	5.46	0.91
1	3.377	5.54	0.90
2	3.757	5.28	0.90
3	3.976	5.24	0.87
4	0	5.66	0.81
5	0	5.69	0.61
6	0	5.56	0.64
7	0	5.91	0.71

Table 4.9. The average CFU/g growth of *Salmonella*, non-O157:H7 *STEC*, and *S. aureus* during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) in chorizo sausage, p=0.053.

Time (Days)	Log CFU/g <i>Salmonella</i>	Log CFU/g <i>STEC</i>	Log CFU/g <i>S. aureus</i>	A _w	pH
0	5.19	5.00	4.53	0.93	5.49
1	5.35	5.27	3.81	0.94	5.48
2	4.87	3.95	3.59	0.97	5.08
3	4.49	4.58	4.06	0.89	4.79
4	0.55	3.66	0	0.78	5.14
5	0	3.12	0	0.69	5.16
6	0	1.47	0	0.61	5.39
7	0	0.77	0	0.78	5.13

Table 4.10. The average CFU/g growth of *Salmonella*, non-O157:H7 STEC, and *S. aureus* during fermentation (90% relative humidity and 20°C), drying (80% relative humidity and 16°C), and storage (75% relative humidity and 15°C) in landjager sausage, p=0.046.

Time (Days)	Log CFU/g <i>Salmonella</i>	Log CFU/g STEC	Log CFU/g <i>S. aureus</i>	A _w	pH
0	5.37	5.28	4.08	0.91	5.46
1	5.03	4.98	3.38	0.90	5.54
2	5.16	3.86	3.76	0.90	5.28
3	4.57	4.34	3.98	0.87	5.24
4	2.93	3.88	0	0.81	5.66
5	2.76	3.29	0	0.61	5.69
6	1.20	2.92	0	0.64	5.56
7	0.77	0.82	0	0.71	5.91

Inhibition of *Salmonella*, STEC, and *S. aureus* varied among each sausage and treatment. Chorizo sausage showed greater overall pathogen inhibition. Chorizo sausage contains garlic and oregano, which are proven to have antimicrobials effects against bacterial growth (Marques, Encarnaç o et al., 2008). No pathogen was eliminated in either sausage by the end of the study, but all microbial counts were significantly affected by fermentation, drying, and storage. If microbial counts were <10 CFU/g, enrichments were used to recover any bacterial counts still present. If bacterial counts were recovered, it indicated the pathogen was still present and not eliminated.

Results throughout the study shows that ideal pH and water activity values might not be enough alone to eliminate pathogens. *S. aureus* was present at <10 CFU/g from

Day 4 of this study until the end in both sausages. *S. aureus* is very demanding of water and nutrients which declined while water activity dropped (Le Loir, Baron et al., 2003). On days 4-7 in chorizo, *Salmonella* and *S. aureus* counts were <10 CFU/g on selective media but recovered through enrichments in chorizo sausage. STEC remained present until the end of the study (Tables 4.3-4.5). In landjager, *S. aureus* counts were <10 CFU/g on selective media on days 4-7, but were recovered in enrichments (Tables 4.6-4.8)

STEC was not eliminated in either sausage through the duration of the study. The ability of non-O157:H7 shiga-toxin producing *E. coli* to survive in low pH and water activity environments probably contributed to its persistence (Benjamin and Datta, 1995; Buchanan and Edelson, 1996).

Chorizo and landjager both exhibited a rapid drop in pH in the first three days of the study. The pH value in chorizo decreased from 5.49 to 4.79. The pH value in landjager decreased from 5.46 to 5.24. Bacterial counts for all treatments were reduced. The initial pH drops during fermentation confirmed that the starter culture was producing acid to reduce the pH of the sausages. The pH values during drying and storage were inconsistent. This was probably due to sample variation.

The water activity of chorizo and landjager both met the critical limit of <0.87. Chorizo sausage had an initial water activity value of 0.93, ending the study at 0.71. Landjager had an initial water activity value of 0.91, ending the study at 0.78. Water activity values varied towards the end of the study and this was probably due to sample variation and size of the sausages.

CHAPTER 5

OVERALL CONCLUSIONS

In conclusion, the objective of the validation study in chapter 3 was to evaluate the survival of pathogens during the production of dry cured charcuterie style sausages. Results demonstrate that if raw materials are contaminated initially and pathogens are present in high numbers, pathogen growth will not be inhibited.

In chapter 3, the results in Tables 3.11 and 3.12 show that contaminated raw materials contribute to the survival of pathogens during the fermentation process. The starter culture did cause a pH drop during the initial stages of fermentation to the critical limit of <5.3, but due to the competition between background bacteria and pathogens, the starter culture was out competed and bacteria present survived.

The results in Tables 3.11 and 3.12 show that a 5-log reduction was not achieved in either sausage. No pathogen was eliminated, and bacterial counts remained steady in *S. aureus*, *Salmonella* and non-O157:H7 STEC persisted until the end of the study. Studies have shown that *S. aureus* does not compete well with other bacteria because of the requirement for amino acids valine, arginine, and cystine for growth and SE production (Sameshima et al., 1998).

The outcome of this validation study raises a concern for at home sausage manufacturing and small production facilities. If proper equipment and a controlled atmosphere are not available, there is a potential for consumers to be exposed to dangerous bacteria. It is important to recognize the threat of the presence of pathogens in

sausages that do not receive a heat treatment before consumption. It is important to adhere to critical control points during production for food safety.

In chapter 4, the objective of the validation study was to evaluate the survival of pathogens during the production of dry cured charcuterie style sausages that are produced with clean raw materials. The results of Table 4.9 and 4.10 show that critical limits for water activity (<0.85) and pH (<5.3) were met during fermentation, drying, and storage. However, *S. aureus* was the only pathogen eliminated. *Salmonella* and non-O157:H7 STEC were able to be recovered through enrichments and survived until the end of the study.

The perseverance of non-O157:H7 SEC and *Salmonella* through lethal pH and water activity levels raises a question of relying on pH and water activity only to control pathogenic bacteria in fermented sausages. The results of the study confirm that pH and water activity are not enough to eliminate these pathogens. The results conflict with results of other studies concluding that pH and water activity are the most important factors for inhibiting pathogen growth (Mataragas, Bellio et al. 2015).

The persistence of non-O157:H7 STEC is particularly concerning because of outbreaks in various products in the recent years. The ability for shiga toxin producing *E. coli* to adapt to extremely acid environments makes it a dangerous and concerning pathogen. Studies show that serogroups O45, O103, and O26 have a higher acid resistance during fermentation than O157:H7 (Balamurugan, Ahmed et al., 2017).

Post processing interventions should be implemented after fermentation and drying to ensure product safety, especially if sausages are being produced in a non-

commercial setting. Some research studies using non-thermal interventions have only been able to achieve a 2-3 log₁₀ reduction (Graumann and Holley 2008; Balamurugan et al., 2017). Some examples of post-processing interventions are a heat treatment, smoking, and if available high-pressure processing. Post processing intervention might be the only way for small sausage producers to ensure the product is microbiologically safe.

Further studies would be beneficial to explain why *Salmonella*, STEC, and *S. aureus* are able to survive fermentation, drying, storage when critical pH and water activity limits are met. The results should also raise awareness to small producers about the potential dangers of making dry cured charcuterie style sausages in non-commercial settings. It would also be beneficial to explore the variability with pathogen survival between various types of sausages.

CHAPTER 6

FERMENTED SAUSAGE PRODUCTION IN RESTAURANTS

6.1 Summary

Dry cured sausages represent some of the oldest forms of meat preservation. Thousands of years ago our ancestors discovered that salting meat was an effective method of preservation. Over time, this led to the production of many dried meat products. As a result, the manufacture of sausage techniques has evolved over time.

Dry fermented sausages are made from a combination of ground meats, spices, and starter cultures that are blended together and stuffed into a casing. The meat, spices and starter cultures used depends on the type of sausage you are making. Some examples of fermented sausages are chorizo, landjager, salami, and pepperoni.

Dry and semidry fermented sausages are considered “ready to eat” so they do not technically need a heat treatment before consumption. However, fermented sausage production requires controlling physical, chemical, and environmental conditions to ensure the safety of the product. The physical and chemical factors are referred to as intrinsic. Intrinsic factors are inherent to food products, such as pH, water activity, starter culture, and processing methods. Extrinsic factors are environmental such as temperature and relative humidity.

The right equipment must be used when making sausages to ensure the product is microbiologically safe. For chemical analysis, a pH meter and water activity meter should be used. The pH and water activity levels should be monitored every day during the

initial fermentation phase to monitor progress. A controlled humidity chamber should be used for fermentation, drying, and storage. The chamber temperature and relative humidity can be adjusted based on the stage of processing. If the temperature and relative humidity are not appropriate for the production phase, the fermentation process will not work.

6.2 Equipment and Materials

The following equipment and materials are recommended:

- Meat (type and fat % target will be determined based on recipe)
- Meat grinder with an 3/8-inch plate
- Knives
- Mixing Containers
- Cutting Boards
- Sausage Stuffer
- Natural or artificial casing
- Spices (based on recipe)
- Sanitizer
- Controlled Humidity Chamber
- pH meter
- Water activity meter
-

6.3 Meat

Meat should be kept below 40°F. The Danger Zone for rapid bacterial growth is between 40°F and 140°F. If meat is held in an improper temperature range, bacteria will rapidly multiply. Meat should also be kept as clean as possible. Ground meat products also have a larger surface area compared to whole muscle cuts and contribute to an overall higher microflora level because there is more exposure to harmful bacteria

(Samelis and Metaxopoulos 1999). The process of slicing and grinding can introduce bacteria to the meat.

Raw materials with high contamination levels initially will compete with and inhibit the starter culture performance. All equipment surfaces should be sanitized prior to processing. Sanitizers should meet minimum parts per million requirements. Parts per million describes the concentration of the sanitizer. The requirement varies based on the type of sanitizer. There are three primary sanitizers that can be used: quaternary ammonium, chlorine based, and iodine sanitizers.

6.4 Casings

Natural casings are typically used for fermented sausages. Sheep or hog casings are appropriate in this scenario because of their small diameter. Sheep casings must be pre-soaked in fresh water prior to use.

6.5 Starter Cultures

Meat fermentation requires a starter culture to produce lactic acid. When lactic acid is produced, it will reduce the pH and develop consistent sensory characteristics (Hugas and Monfort, 1997). Bacteria strains *Lactobacillus* and *Pediococcus* species are used for acid production, along with curing bacteria such as *Micrococcus* and *Staphylococcus* for flavor and color development (Leroy, Verluyten et al. 2006).

Dry fermented sausages require specific starter cultures for fermentation and the development of organoleptic characteristics. The starter culture *Pediococcus pentosaceus* + *Staphylococcus xylosum* is commonly used for smaller diameter sausages. This starter

culture is used for fast fermentation as well as the development of color and flavor. Fast fermentation is used to rapidly drop pH values and can take as little as 48 hours to achieve a finished pH value <5.0 (Dalmis and Soyer 2008). The starter culture chosen depends on the type of sausage being produced, but the result will be a microbiologically safe product.

6.6 Hurdle Technology

Bacteria can survive and grow in various conditions in food products and the environment. There are obstacles that can be used to combat the survival of these pathogens in the food industry, referred to as “Hurdle Technology.” Hurdle technology is a term that refers to an effective combination of preservative factors, called hurdles, which are used to protect the microbiological safety of our food supply.

The most important hurdles include temperature, water activity (a_w), acidity (pH), redox potential, preservatives, and competitive microorganisms (Leistner 2000). Hurdle technology is most effective when multiple hurdles are used at a time. In fermented sausages, a combination of temperature, water activity, pH, and relative humidity are the most effective combination to combat pathogen survival.

6.7 Temperature

Temperature control during the fermentation process is critical for starter cultures to perform properly. The function of lactic acid bacteria (LAB) starter cultures, most commonly used in sausage production, can be inhibited if not held at proper relative humidity and temperature after the addition of the starter culture. If conditions are not

right, the starter culture will not start producing enough lactic acid to drop the pH of the sausage.

Fermentation temperatures should be around 20°C. The fermentation phase of sausage production happens in the first 48-72 hours. After the fermentation is complete, the starter culture should have produced enough lactic acid to significantly drop the pH of the sausage. The water activity of the sausage will start to drop once relative humidity is decreased and the drying phase begins.

6.8 Water Activity (a_w)

Water activity is defined as the amount of unbound water in the product. Water activity is one of the most important vehicles for microbial growth and survival in fermented meat products. Meat has a relatively high-water activity (a_w) of 0.99 which promotes bacterial survival. Studies have shown that vegetative (dormant) cells of some pathogenic bacteria can persist and survive in low moisture foods and ingredients for months and even years, although their metabolic process is greatly reduced.

The ability for pathogenic bacteria to survive the drying process is concerning, particularly because low water activity foods are assumed to be sterile. Therefore, it is important to reduce the water activity level to inhibit the potential growth of pathogens. The minimum water activity value for most bacteria to grow is 0.87. At a water activity of 0.87, physiological activities necessary for cell division are impaired (Beuchat, Komitopoulou et al. 2013). However, the USDA recommends a final water activity of <0.85 (USDA-FSIS, 2011).

6.9 pH

Most pathogens prefer to grow in pH neutral environments (6.5-7.0), so a pH drop during fermentation is crucial to inhibit the ability for pathogens to thrive (Bearson, Bearson et al. 1997). During the fermentation period, starter cultures produce lactic acid that drop the pH of the meat. The optimal fermentation temperature for the starter culture to perform depends on the strain but can be between 20-24°C (Marianski, 2009). The USDA requires that dry fermented sausages attain a final pH of 5.3 or less to control the growth of pathogenic organisms with moisture loss of 25-50% (USDA-FSIS, 2017).

6.10 Relative Humidity

Humidity control is critical during each step of fermentation, drying, and storage. During fermentation, humidity is very high (90%) to prevent hardening of the sausage casing. During the drying stage, humidity levels are reduced to aid in the removal of moisture from the sausage and lower the water activity (Beuchat, Komitopoulou et al. 2013).

Controlling moisture of fermented sausages is the most important way to prevent microbial growth because if moisture is present pathogens can grow. During the drying period, the humidity level should be 75% or less. If the humidity is higher than that during storage it could promote the growth of mold on the outside of the casing.

6.11 Conclusion

The manufacture of fermented sausages outside of a commercial production facility can be accomplished safely if the right process is followed. The pH, water

activity, temperature, and relative humidity must be controlled to reduce the risk of pathogen survival. It is critical in sausages that will not receive a post processing intervention like heat treatment. A deviation in processing could lead to an unsafe product. Therefore, the appropriate equipment must be used to ensure that fermentation, drying, and storage conditions are optimal.

The manufacture of dry fermented sausages in restaurants by chefs is becoming increasingly popular, so it is critical that processors understand the risk associated with manufacturing outside of a typical processing facility. Food safety training focusing specifically on dry fermented sausages would be greatly beneficial to individuals producing these. It is important that the restaurant chefs understand the potential presence of pathogens in dry cured sausages and the possible exposure to consumers.

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