INVESTIGATION OF FOOD SAFETY PARAMETERS FOR FERMENTED SEMI-DRY
AND DRY SAUSAGE PRODUCTS

A Thesis in
Animal Science
by
Samantha R. McKinney

© 2017 Samantha R. McKinney

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

May 2017
The thesis of Samantha R. McKinney was reviewed and approved* by the following:

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

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

Nancy M. Ostiguy
Associate Professor of Entomology

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

*Signatures are on file in the Graduate School
ABSTRACT

Fermentation and drying are two methods utilized by humans for thousands of years to preserve food. Fermented semi-dry and dry sausages are safe, ready-to-eat (RTE) meat items produced using strict government regulations. One of these regulations requires meat processing establishments to create and have a scientifically-validated Hazard Analysis Critical Control Point (HACCP) plan. HACCP plans are validated utilizing a combination of data collected in the plant and scientific literature to ensure that process controls exist for identified food safety hazards. When little or incomplete data exists for very specific products or processes, challenge studies may be conducted to investigate the safety of the processes used to produce the food item.

Three experiments were conducted to determine the effects of varying fermented semi-dry and dry sausage production parameters on the reduction of three pathogenic bacteria: *E. coli* O157:H7 (EC), *Salmonella* spp. (*S. Typhimurium, S. Montevideo, and S. Panama*), and *L. monocytogenes* (LM). These experiments will serve as challenge studies for semi-dry and dry sausages under various conditions.

The first experiment evaluated the effects of fat content and grind size on the attachment and survival of pathogenic bacteria in fully-cooked, semi-dry sausages. Lean and fat trim consisting of beef and pork were analyzed for fat content and mixed to create meat blocks consisting of 10%, 20%, and 30% fat. Meat blocks were ground a second time to create meat particles that were 3.00 mm, 4.75 mm, or 12.50 mm in size. Neither fat content nor grind size significantly impacted pathogen populations for any of the bacteria examined (*p*>0.05).
A second experiment demonstrated the efficacy of a low-heat treatment for an extended time to reduce pathogenic bacteria in a fermented, dry sausage. Ground pork (20% fat) was stuffed into 38-mm, 64-mm, and 120-mm diameter casings, fermented, heat treated, dried, and vacuum-packaged up to 28 d. A 5 log$_{10}$ reduction was achieved by day 9 (D9) of drying for S and LM regardless of diameter; by D16 of drying, EC was reduced by 5 log$_{10}$ CFU/g for all diameters. Significant differences were observed among casing diameters for S.

The third experiment demonstrated no difference among casing type or pork trim sprayed with tap water or an antimicrobial prior to grinding in a non-heat treated, dry salami ($p>0.05$). Pork trim was treated with either tap water or a 2.5% Beefxide spray prior to grinding. The ground pork was stuffed into three casings (natural, collagen, and fibrous), fermented and dried. There was no significant difference between the control and treated sausages for the reduction of EC ($p=0.1645$), S ($p=0.3746$), or LM ($p=0.1762$). There was also no significant difference between casing types within each sausage treatment. A 5 log$_{10}$ reduction was achieved for S and LM by the end of vacuum packaging (up to 28 d), but EC was not reduced by a 5 log$_{10}$ reduction by the end of vacuum packaging. EC was reduced by a maximum 4.02 log$_{10}$ CFU/g in the collagen sausage when treated with the antimicrobial.

These experiments may be utilized by processors as supporting documentation in HACCP plans, as well as be accepted by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) personnel as validation and scientific evidence for use in HACCP plans.
TABLE OF CONTENTS

List of Figures ............................................................................................................. viii
List of Tables ................................................................................................................ ix
Acknowledgements ........................................................................................................ xi

Chapter 1 Introduction ................................................................................................. 1
  Fermented Meat Products ......................................................................................... 2
    History ..................................................................................................................... 2
    Current Trends ...................................................................................................... 3
    Semi-dry and Dry Fermented Sausages ................................................................. 4
    Casings .................................................................................................................... 5
  The Hurdle Effect ..................................................................................................... 8
    Hurdles in Foods .................................................................................................. 8
    Fermentation and pH ............................................................................................ 9
    Bacteriocins and Antimicrobials ......................................................................... 10
    Water Activity ..................................................................................................... 12
    Preservatives .................................................................................................... 13
    Temperature ......................................................................................................... 13
  Bacteria Attachment to Meat Surfaces .................................................................. 14
    Biofilms ................................................................................................................ 14
    Mechanism .......................................................................................................... 15
    Attachment of E. coli O157:H7, Salmonella spp. and L. monocytogenes .......... 18
  Escherichia coli O157:H7 ....................................................................................... 18
    History ................................................................................................................... 18
    Characteristics ..................................................................................................... 19
    E. coli O157:H7 as a Cause of Hemorrhagic Colitis and HUS ......................... 21
    Outbreaks ........................................................................................................... 22
  Salmonella spp. ....................................................................................................... 22
    History .................................................................................................................. 22
    Characteristics .................................................................................................... 23
    Salmonellosis ....................................................................................................... 24
    Outbreaks ............................................................................................................ 25
  Listeria monocytogenes .......................................................................................... 25
    History ................................................................................................................... 25
    Characteristics .................................................................................................... 26
    Listeriosis .............................................................................................................. 27
    Outbreaks ............................................................................................................ 28
  Zero Tolerance Policy ............................................................................................. 28
  Cross-Protection of Bacteria .................................................................................. 29
  Regulatory Statutes ................................................................................................. 30
  Statement of the Problem ....................................................................................... 33
  Objectives ............................................................................................................... 34
  References .............................................................................................................. 35
Chapter 2 Investigating the Effect of Fat Content and Grind Size on Pathogen Attachment and Survival in Fermented Semi-Dry Sausages

Abstract ................................................................. 47
Introduction ............................................................. 47
Materials and Methods ............................................... 49
Preparation of Inoculum ............................................. 49
Manufacture and Inoculation of Sausages ......................... 50
Microbial Analysis .................................................... 52
Statistical Analysis .................................................... 53
Results ................................................................. 54
Fat Content ............................................................ 54
Sausage pH .............................................................. 54
Bacteria Populations Based on Fat Content ....................... 55
Bacteria Populations Based on Grind Size ......................... 56
Sausage Temperature .................................................. 60
Discussion .................................................................. 60
References .................................................................. 65

Chapter 3 Pathogen Reductions in Fermented Dry Sausages Using a Low-Temperature Heat Treatment

Abstract ................................................................. 68
Introduction ............................................................. 68
Materials and Methods ............................................... 72
Preparation of Inoculum ............................................. 72
Manufacture and Inoculation of Sausages ......................... 72
Microbial Analysis .................................................... 74
Statistical Analysis .................................................... 76
Results ................................................................. 76
pH and aw .............................................................. 76
Bacteria ................................................................. 77
Discussion .................................................................. 83
References .................................................................. 87

Chapter 4 Pathogen Reductions During Traditional Fermentation and Drying of Pork Salamis ................................................................. 90

Abstract ................................................................. 91
Introduction ............................................................. 92
Materials and Methods ............................................... 94
Preparation of Inoculum ............................................. 94
Preparation and Inoculation of Sausages ......................... 95
Microbial Analysis .................................................... 98
Weight and aw Correlation ........................................ 99
Statistical Analysis .................................................... 99
Results .................................................................... 100
Discussion .................................................................. 109
References .................................................................. 113
Chapter 5 Conclusions


116
LIST OF FIGURES

Figure 1.1 Image of *E. coli* O157:H7 antigens. .................................................................20

Figure 1.2 Examples of how to calculate degree hours to ensure compliance ..................32

Figure 2.1 Average *E. coli* O157:H7 counts (log$_{10}$ CFU/g) during semi-dry sausage production. ........................................................................................................57

Figure 2.2 Average *S. Typhimurium* counts (log$_{10}$ CFU/g) during semi-dry sausage production. ........................................................................................................58

Figure 2.3 Average *L. monocytogenes* counts (log$_{10}$ CFU/g) during semi-dry sausage production. ........................................................................................................59

Figure 2.4 Smokehouse (dry bulb) and sausage (internal) temperatures during thermal processing. ........................................................................................................60

Figure 3.1 Average *E. coli* O157:H7 counts for 38-, 64-, and 120-mm diameter pork sausages ........................................................................................................78

Figure 3.2 Average *Salmonella* spp. counts for 38-, 64-, and 120-mm diameter pork sausages ........................................................................................................80

Figure 3.3 Average *L. monocytogenes* counts for 38-, 64-, and 120-mm diameter pork sausages ........................................................................................................82

Figure 4.1 Average *E. coli* O157:H7 populations (log$_{10}$ CFU/g). A is results for CTRL and B is results for TRT .........................................................................................................103

Figure 4.2 Average *Salmonella* spp. populations (log$_{10}$ CFU/g). A is results for CTRL and B is results for TRT .........................................................................................................105

Figure 4.3 Average *L. monocytogenes* populations (log$_{10}$ CFU/g). A is results for CTRL and B is results for TRT .........................................................................................................107
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Three alternatives processors can incorporate into the facility to control <em>L. monocytogenes</em> in RTE products.</td>
<td>29</td>
</tr>
<tr>
<td>2.1</td>
<td>Smokehouse parameters</td>
<td>51</td>
</tr>
<tr>
<td>2.2</td>
<td>Average pH of sausages based upon fat content</td>
<td>55</td>
</tr>
<tr>
<td>2.3</td>
<td>Average pH of sausages based upon grind size (mm)</td>
<td>55</td>
</tr>
<tr>
<td>2.4</td>
<td>Average <em>E. coli</em> O157:H7 counts (log_{10} CFU/g + Standard error (SE)) during semi-dry sausage production</td>
<td>57</td>
</tr>
<tr>
<td>2.5</td>
<td>Average <em>S. Typhimurium</em> counts (log_{10} CFU/g + SE) during semi-dry sausage production</td>
<td>58</td>
</tr>
<tr>
<td>2.6</td>
<td>Average <em>L. monocytogenes</em> counts (log_{10} CFU/g + SE) during semi-dry sausage production</td>
<td>59</td>
</tr>
<tr>
<td>3.1</td>
<td>Smokehouse parameters</td>
<td>74</td>
</tr>
<tr>
<td>3.2</td>
<td>Average <em>E. coli</em> O157:H7 counts (log_{10} CFU/g + Standard Error (SE)). All comparisons were done with forward sampling times. Same letters are not significantly different within a column. Same numbers are not significantly within rows (p&lt;0.05).</td>
<td>79</td>
</tr>
<tr>
<td>3.3</td>
<td>Average <em>Salmonella</em> spp. counts (log_{10} CFU/g + SE). All comparisons were done with forward sampling times. Same letters are not significantly different within a column. Same numbers are not significantly within rows (p&lt;0.05).</td>
<td>81</td>
</tr>
<tr>
<td>3.4</td>
<td>Average <em>L. monocytogenes</em> counts (log_{10} CFU/g + SE). All comparisons were done with forward sampling times. Same letters are not significantly different within a column. Same numbers are not significantly different within rows (p&lt;0.05).</td>
<td>83</td>
</tr>
<tr>
<td>4.1</td>
<td>Salami formulation.</td>
<td>97</td>
</tr>
<tr>
<td>4.2</td>
<td>Drying cabinet settings during the manufacture of dry pork salamis. <em>During this time, the drying cabinet had a run-pause cycle of 30 min run time and 45 min pause time for the fan.</em></td>
<td>97</td>
</tr>
<tr>
<td>4.3</td>
<td>Average <em>E. coli</em> O157:H7 populations (log_{10} CFU/g). Statistical differences are noted by different letters within treatments (p&lt;0.05). Pairwise comparisons were completed using forward comparisons. There were no significant differences between casings. TR=total log_{10} reduction.</td>
<td>104</td>
</tr>
<tr>
<td>4.4</td>
<td>Average <em>Salmonella</em> spp. populations (log_{10} CFU/g). Statistical differences are noted by different letters within treatments (p&lt;0.05). Pairwise comparisons were</td>
<td></td>
</tr>
</tbody>
</table>
completed using forward comparisons. There were no significant differences between casings. TR=total log$_{10}$ reduction. .................................................. 106

Table 4.5 Average *L. monocytogenes* populations (log$_{10}$ CFU/g). Statistical differences are noted by different letters within treatments (p<0.05). Pairwise comparisons were completed using forward comparisons. TR=total log$_{10}$ reduction. ............................................. 108
ACKNOWLEDGEMENTS

To Dr. Campbell, thank you for the opportunities the past two years. I could not have made it this far without your help in the lab, knowledge, and support. Thank you for challenging me, for not letting me give up when I wanted to, but most importantly, for instilling a passion in me for the meat industry.

Thank you, Dr. Cutter for your expertise in both meat science and microbiology. Your knowledge has helped me to succeed as both a meat scientist and a microbiologist.

Thank you, Dr. Ostiguy for your statistics expertise. No paper would be complete without your countless hours of help to run statistical programs and create tables.

I would like to thank Minerva Rivera and Nelson Gaydos for helping me with my research projects. Your help and advice over the last two years were much appreciated. Not only were you my lab mates, but also my colleagues and friends.

To the undergraduates who worked in the lab: Emily Cutter, Thomas Pastor, and Christine Kosciewcz. This research would not be complete without your countless hours of cleaning, pouring plates, and making tubes.

Lastly, to my mom, dad, and brother: thank you for enduring me being gone for weeks at a time and managing the cattle without me. Your love and support has gotten me to where I am today, and for that, I am forever grateful.
Chapter 1

Introduction
Fermented Meat Products

History

Fermentation of meat products is a process “in which lactic acid bacteria…convert fermentable carbohydrates...in the meat mixture to lactic acid” which reduces the pH (AMIF, 1997). Dating back at least 6,000 years, fermentation is one of the oldest preservation methods used for food products (Holzapfel, 2002). It is believed that women would use fermentation processes to preserve food in preparation for times of food scarcity, such as drought and extremely cold, wintry weather (Marshall and Mejia, 2012). Fermentation was completed by the naturally occurring microflora in the product (Marshall and Mejia, 2012). In addition to preserving the food being fermented, these same women unknowingly altered the flavor profile of the food and made the food safer for consumption. During the time of Louis Pasteur, it was discovered that fermentation was occurring due to the conversion of carbohydrates to lactic acid by lactic acid bacteria (LAB) (Pasteur, 1857).

Similar to preservation methods over 6,000 years ago, fermentation today is used to increase shelf life, create a desirable flavor profile, and reduce pathogens in the product. Modern fermented products not only include meat and wine, but also encompass vegetable, dairy, and grain products. Items include foods such as sauerkraut, cheese, tofu, and bread. The processing procedures used in the meat industry to make fermented meat products include grinding or chopping of meat, blending of meat and nonmeat ingredients (e.g. salt, spices, and preservatives), addition of commercially available starter cultures, fermentation, smoking, heat processing, and drying (Bell and Kyriakides, 1998). Single or mixed strains of starter cultures are added to various fermented sausages to convert
fermentable carbohydrates to alcohols and carbon dioxide or to organic acids (Holzapfel, 2002). Starter cultures aid in the development of specific flavor profiles, as well as decrease product pH and create an environment inhospitable for pathogenic bacteria survival and growth.

Current Trends

In a 2013 study, 46% of American consumers responded eating fewer processed foods, including processed meat products (Nielsen, 2015). In 2014, total sales from sausage products were an estimated $2.75 billion (Nielsen, 2015). Despite this shift, American consumers still seek fermented meat products for their flavor profile, convenience, and price point.

Ready-to-eat (RTE) products have grown in popularity, because there is no additional preparation required prior to consumption. As a RTE product, fermented sausages are expected to be free of pathogenic bacteria to prevent foodborne illness. In the 2015 incidence trend report, the Center for Disease Control and Prevention (CDC) found foodborne illnesses overall have decreased by 30% since 1996-1998 (CDC, 2016). During the same period, illnesses from L. monocytogenes (LM), Salmonella spp. (S), and Shiga-toxin producing E. coli O157:H7 (EC) have decreased by 45%, 3%, and 44%, respectively (CDC, 2016).

Foodborne illnesses have decreased over the last three decades, but the CDC reports that one in six Americans will still fall ill due to a food-related infection or intoxication (CDC, 2014b). Over 95% of those illnesses will be due to one of 15 known
pathogens, which the United States Department of Agriculture Economic Research Service estimated to cost over $12.6 billion in health care costs.

The fact that there are fewer outbreaks due to pathogenic bacteria demonstrates improved safety of the American food supply. In recent years, the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) put in place guidelines and policies for processors to follow to decrease pathogenic bacteria in food products, thus decreasing the incidences of foodborne illness outbreaks. Some of these guidelines and requirements include implementing Good Manufacturing Practices (GMPs), enactment of a Hazard Analysis Critical Control Point (HAACP) plan, and citing scientific studies to ensure proper validation of processes as stated in 9 CFR §417 (USDA-FSIS, 2016b). Processors also utilize a combination of interventions at sub-lethal levels to combat pathogens that could potentially be found in products.

Semi-dry and Dry Fermented Sausages

Following fermentation, fermented sausages often undergo a drying period. Currently, the Code of Federal Regulations (CFR) does not have regulations or definitions for semi-dry and dry fermented sausages. The characteristic that separates semi-dry sausages from dry sausages is the amount of moisture lost during the drying period. Semi-dry products, such as summer sausage and cervelat, typically lose between 15% to 30% moisture, while dry products, such as Genoa salami and pepperoni, lose between 30% to 50% moisture (USDA-FSIS, 2005; Ricke and Keeton, 1997). Although no standard of identity is defined in the CFR, some products have a moisture-to-protein ratio that must be met in order to label a product as a specific item. For example, a dry
sausage must have a moisture-to-protein ratio of 2.3:1 or less to be labelled as Genoa salami (USDA-FSIS, 2005).

Heat treatments are applied to some U.S.-style semi-dry and dry fermented sausages to reduce microbial populations and increase the safety and shelf-life of the product. In addition to improving food safety, heating a product alters the compounds that make up the product, specifically the meat portion, which may affect the flavor profile and other sensory attributes. Following heating, sausages may undergo a drying stage to reduce moisture, which also decreases water activity (a_w). During the drying stage, free fatty acid formation is increased and lipid oxidation occurs, leading to the aroma consumers are familiar with (Gandemer, 2002).

Casings

Casings play an integral role in determining characteristics of finished fermented sausages. In order to manufacture a product, processors need to choose the casing that will achieve the desired yield and sensory aspects. Casings may be permeable or impermeable to smoke, water vapor, and gas transfer between the environment and product to produce these yield and sensory traits. In addition, casings for fermented sausages must be strong enough to contain the weight of the meat, but retain the ability to expand and shrink during processing and storage (Choi et al., 2008).

Traditionally, natural casings have been used to make sausages. Casings are made from gastrointestinal sections, stomachs, and bladders from cattle, hogs, and sheep (Chawla et al., 2004; Pearson and Dutson, 1988). The parts intended for casing use are carefully removed during evisceration and the fat removed (Pearson and Dutson, 1988).
They are then cleaned, slimed (removal of mucosa layer), and packaged in salt or a salt solution to preserve the casing and prevent microbial growth (Pearson and Dutson, 1988). Natural casings are strong enough to hold the weight of the meat, remain permeable to water and smoke, and expand and shrink during the filling and drying steps, however, they pose a risk of biological hazards including fecal *Streptococci, Enterobacteriaceae*, and *Clostridia* (Chawla et al., 2004). The microbial load of casings depends upon the hygiene of the processing facility, manufacturing procedure, post-processing handling, and storage temperatures (Chawla et al., 2004). To combat microorganisms, natural casings are typically stored highly salted and dried or frozen, although neither storage method has proven completely effective as some microorganisms, such as *Clostridia* spp., have been shown to remain viable and grow following rehydration (Chawla et al., 2004; Pearson and Dutson, 1988).

Natural casings are used to produce a variety of products, depending on the size of the casing and the species from which it originated. Hog casings are used to produce smaller diameter products, such as braunschweiger, Genoa salami, and liver sausage (Pearson and Dutson, 1988). Like hog casings, sheep casings are used for smaller diameter products, such as frankfurters (Pearson and Dutson, 1988). Beef casings are used to produce medium to large diameter products, depending on the casing. Beef bladders and bungs are used to produce mortadella and large bolognas, respectively (Pearson and Dutson, 1988). Beef rounds and beef middles are smaller in diameter and are used to make sausage products like mettwurst and semi-dry and dry sausages (Pearson and Dutson, 1988).
Artificial casings have multiple advantages over natural casings, including uniformity and price (Romans et al., 2001). Artificial casings are made of either natural or synthetic materials. Collagen casings were developed as an alternative to natural casings with the uniformity of artificial casings. (Romans et al., 2001). They are made from split cattle and swine hides by grinding the corium layer of the hide, swelling in acid, sieving, filtering, and extruding (Romans et al., 2001). Collagen casings are of smaller diameters due to less structural strength than cellulose or natural casings (Romans et al., 2001).

In contrast, cellulose casings, including fibrous casings, are made of plant material, such as cotton linters or paper pulp containing cellulose (Romans et al., 2001). Most cellulose casings are permeable to gas, smoke, and water, but can also be impermeable (Romans et al., 2001). Cellulose casings range in casing diameter and length to accommodate the production of a variety of products, such as frankfurters, small diameter sausages, and bolognas. Fibrous casings are commonly utilized to produce large diameter sausage products, such as deli meats, summer sausage, and bologna due to the strength of the casing (Romans et al., 2001). Casings may or may not be permeable depending on the material used to reinforce it.

Other artificial casings seen in the meat industry are made of synthetic thermoplastic materials, such as polyamide, polyethylene, and polyester. Advantages of synthetic casings are their strength and impermeability to smoke, gas, and water for products in which a lot of water loss is not desired. Impermeable casings are not desirable for fermented sausage products due to this lack of gas and water exchange during smoking and/or drying.
The type and diameter of casings chosen to produce fermented sausages can affect pathogen control. After a Lebanon bologna outbreak in 2011, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) suggested that casing type and diameter could affect the rate of reduction in foodborne pathogens (USDA-FSIS, 2013). Casing type affects moisture exchange, leading to changes in water activity ($a_w$), and may also affect the rate of pH drop and heat penetration (USDA-FSIS, 2013). A larger diameter product may take longer to reach the desired pH or temperature, which can lead to bacterial adaptation and resistance and a lower reduction of pathogens (FSIS, 2013). It is because of these variances that the USDA enforces regulations and requires process validation studies to ensure the safety of products.

**The Hurdle Effect**

**Hurdles in Foods**

Since fermented sausages are considered a RTE product, processors utilize the hurdle effect to manufacture safe products. The hurdle effect uses a combination of interventions to decrease microbial levels and increase the safety and shelf-life of foods (Leistner, 2000). Multiple hurdles force bacteria to overcome a challenge in their environment by expending energy to maintain homeostasis in order to survive. The most common hurdles used include: pH reduction, use of antimicrobials, temperature, $a_w$, and preservatives (Leistner, 2000). Fermented meat products use a combination of these hurdles to cause injury to pathogenic bacteria and spoilage organisms to prevent growth. In addition to increasing product safety, product quality is often improved through the alteration of flavor and nutritional profiles and preservation of foods (Leistner, 2000).
Alone, each hurdle may be limited in their ability to fully inhibit microbial growth; but hurdles in combination, provide a synergistic effect, making the food environment more challenging for microorganisms to survive. Low quality ingredients, poor cleaning and sanitizing procedures, improper food or ingredient handling, and the absence of a validated HACCP plan are all factors that can decrease the effectiveness of the hurdle concept and be detrimental to the safety and quality of the product through the attachment and survival of microorganisms (García Díez and Patarata, 2013). It is important that processors adhere to guidelines set forth by the USDA and FDA to produce a product safe for consumption.

Fermentation and pH

Raw meat used in the production of fermented meats typically has a pH around 5.5 to 5.6, depending on species and age of the animal upon slaughtering. At this pH, in addition to other factors, bacteria are able to survive and grow. To decrease pH, processors incorporate acidic ingredients and starter culture into the meat batch, a mixture of lean tissue, fat, carbohydrates, and spices. Sausages are fermented to a pH of 5.3 or less in order to control *Staphylococcus aureus* toxin production and reduce microorganism growth. Acidity level is a delicate balance in fermented foods. The pH must reach a level that imparts stress to microorganisms by disrupting the homeostasis of the cell, but does not achieve a pH so low as to adversely affect the product’s flavor profile (Leistner, 2000).

Bacterial cell pH is dependent upon environmental pH and intracellular pH. Environmental pH determines the intracellular pH by affecting enzymatic activity,
protein stability, and the structure of nucleic acids (Slonczewski et al., 2009). Most bacteria work to maintain an intracellular pH that is close to neutral (pH of 7) and have the ability to maintain homeostasis within a wide range of environmental pH values (Slonczewski et al., 2009). For example, under optimal pH growth conditions, *E. coli* has an intracellular pH of 7.4 to 7.8 and has the ability to maintain homeostasis when the environmental pH ranges from 5.0 to 9.0 (Slonczewski et al., 2009). As the environmental pH becomes more acidic during fermentation, microorganisms expend energy in an attempt to pump hydrogen (H\(^+\)) protons into or out of the cell to maintain homeostasis and intracellular pH. During this process, the cellular membrane is altered and energy is depleted. The cell is not able to transport the H\(^+\) protons out of the cell, leading to an accumulation of acid in the cell (Slonczewski et al., 2009; Wesche et al., 2009). Low pH may also denature proteins that microbes need to survive, including the bacteria’s DNA and RNA. (Jeong et al., 2008)

**Bacteriocins and Antimicrobials**

Lactobacilli and pediococci are two common species of LAB added to meat products at high concentrations to induce fermentation (Leroy et al., 2006). Not only do LAB compete with other microorganisms for resources needed to survive, but certain strains have been found to produce bacteriocins (Cleveland et al., 2001). Bacteriocins are antimicrobial peptides, or proteins, produced in the bacterial ribosome and have been described as “biological food preservatives” (Hurst, 1981). Bacteriocins are produced by both eukaryotic and prokaryotic cells, but have gained much attention in prokaryotes due to the use of LAB in food preservation, such as cheese and meat products. The most
notable characteristic about bacteriocins are the antimicrobial properties against LM, *Enterococci*, and *Clostridium* spp. (Nes et al., 1996).

Bacteriocins are 20 to 60 amino acids in length and are hydrophobic (Héchard and Sahl, 2002). Two of the most commonly used bacteriocin producers are *Lactobacillus plantarum* and *Pediococcus acidilactici* (Cleveland et al., 2001). LAB secrete the majority of bacteriocins via the ATP-binding cassette transporter (Nes et al., 1996; Héchard and Sahl, 2002). Any bacterial cell is susceptible to the effects of bacteriocins; however, the LAB that produce a specific bacteriocin, have immunity proteins to protect themselves (Héchard and Sahl, 2002). Bacteriocins form pores to disrupt the cell membrane in bacterial cells, leading to dissipation of the cell membrane and collapse of the proton motive force, followed by depletion of ATP stores as the cell works to maintain homeostatic balance (Drider et al., 2006).

Bacteriocins have been demonstrated to work in the food industry. Nisin, for example, is a commonly used bacteriocin in the dairy industry for the production of cheese (Cleveland et al., 2001). Cutter and Siragusa (1995) demonstrated a positive effect of nisin on fresh meat products; however, a purified nisin substrate was utilized in the study which may differ from nisin produced within the product by LAB during fermentation. Nonetheless, there is potential for bacteriocins as a natural preservative in processed meats as shown in frankfurters, Belgian-type fermented sausages, and dry fermented sausages (Chen et al., 2004; Verluyten et al., 2004; Aymerich et al., 2000). The level of production and effectiveness of bacteriocins is dependent upon other ingredients added to the product. For example, black pepper is high in manganese and enhances antimicrobial properties against *Listeria* spp. (Hugas, et al., 2002).
Contrastingly, high levels of nitrite have a negative impact on LAB and their ability to produce bacteriocins (Leroy and De Vuyst, 1999).

In addition to bacteriocins, antimicrobials may be applied directly to the product prior to grinding. Antimicrobials commonly used are citric acid, lactic acid, and acetic acid, or a mixture of two or more of these acids. The efficacy of antimicrobials depends on the temperature applied, the buffering capacity of the meat, and length of time on the product. Dickson et al. (1994) found the application of trisodium phosphate (TSP) at 55°C provided a more lethal effect to S. Typhimurium, LM Scott A, and EC, especially the longer the TSP was in contact with the beef tissue. Raftari et al. (2009) found a 2.0% formic acid spray was the best organic acid to reduce EC up to 1.84 log$_{10}$ CFU/g and S. aureus up to 3.16 log$_{10}$ CFU/g.

**Water Activity**

A decrease in $a_w$ is another hurdle that may be incorporated into the processing of fermented sausages to aid in the inhibition of bacterial growth. Many bacteria cannot grow below an $a_w$ of 0.90 (Hospital et al., 2014). By adding salt and other dry ingredients, the $a_w$ is decreased and the environment becomes inhospitable for bacterial survival and growth. The addition of ingredients to the product is not the only method of decreasing $a_w$. Fermented sausages are often dried and allowed to mature, during which the $a_w$ decreases due to moisture evaporation from the product. As less water is available and salt concentration increases, cells attempt to maintain homeostasis by moving water across the cell membrane from the cell to the environment. This process leads to osmotic
stress as the cell begins to desiccate, damaging the cell wall and leading to membrane leakage (Wesche et al., 2009).

**Preservatives**

A variety of preservatives are often incorporated into the meat formulation to improve both safety and quality. Preservatives work in tandem with other hurdles to decrease $a_w$ and/or pH. Salt is added to bind water molecules and decrease the $a_w$ (Työppönen et al., 2003). The second most common preservative, nitrite, is added to develop and retain cure color, but has also been found to inhibit the growth of *Clostridium* spp. and has been found to be an antimicrobial against *Listeria* spp. (Hospital et al., 2014). Nitrite, in the form of nitrous acid, is able to penetrate the cell membrane and disturb bacterial enzyme function (Työppönen et al., 2003).

**Temperature**

Many pathogenic bacteria are considered mesophiles, indicating that they prefer mild temperatures, similar to humans and other organisms. Mesophilic bacteria grow in a range of 25°C to 40°C, with an optimum growth temperature around 37°C. Although bacteria are able to adapt to temperature changes to remain viable, research (Murano and Pierson, 1993; Jones et al., 1996) has shown that sudden shock of high or low temperatures can be used to inhibit the growth of microorganisms by providing sub-lethal or lethal measures.

Heat shock occurs when bacteria are exposed to temperatures above the normal range (Weshce et al., 2008). It can occur as low as 42°C for *E. coli*, 48°C for *S.*
Typhimurium and 45° to 48°C for LM (Weshce et al., 2008). High temperatures denature cell proteins, causing gene expression inactivation, slowed cell proliferation, and changes in cell morphology (Kampinga, 1993). Common methods of applying heat include pasteurization (commonly used in the dairy industry), cooking, and canning.

A heat treatment is typically applied to RTE meat products. The heat treatment can be severe enough to cause lethality to any microorganism present in the product, or can be applied at a sub-lethal level. A sub-lethal level of heat would injure the microorganisms in the product; however, processes following the heat treatment, such as drying and packaging, further injure cells and provide an environment inhospitable for bacterial growth and survival.

**Bacteria Attachment to Meat Surfaces**

Bacteria attachment was first reported by Zobell (1943), who described marine bacteria “sticking” to submerged surfaces. Since then, bacteria attachment has been described in a variety of settings and products, including dentistry, drinking water, and various food processing systems (Hood and Zottola, 1995).

**Biofilms**

According to Ferreira et al. (2014), a biofilm is described as “microbial cells that adhere to each other and/or surfaces and are enclosed in an extracellular polymeric matrix.” The biofilm is comprised of the bacteria, extracellular material, proteins, nucleic acids, sugars, and other materials (Hood and Zottola, 1995). Bacteria gain advantages due to the fact that there is environmental protection, nutrient availability, and acquisition of
new genotypic traits that allow the bacteria to survive in stressful conditions (Ferreira et al., 2014). In the human body, biofilms are beneficial in the gut, but biofilms in the food industry need to be avoided through proper cleaning and sanitation procedures because attached bacteria can become resistant to cleaning agents (Sandine, 1979; Frank and Koffi, 1990). Biofilms become an issue when the biofilm is disturbed, allowing for viable bacteria to break away from the surface and attach to a new surface, such as meat.

**Mechanism**

Prior to slaughter, animal tissue is essentially sterile, but during the slaughtering process, tissue becomes contaminated through the complex attachment of bacteria to the meat surface. There are four stages in which bacteria attach to meat: initial contact, attachment, maturation, and dispersion (Vogeleer et al., 2014). The first stage is dependent upon physiochemical forces, including hydrophobicity of the bacteria, charge of the bacteria and meat surface, and cell structures (Benito et al., 1996). Initial contact is considered a reversible stage in which bacteria quickly attach and are entrapped in a water film in meat crevices or channels (Firstenberg-Eden et al., 1979; Piette and Idziak, 1992). Bacteria attached to surfaces during this stage have been shown to be removed through the use of organic acid sprays and hot water washes (Hood and Zottola, 1995). Attachment and maturation phases are considered irreversible stages due to bacteria creating a more permanent attachment through the secretion of extracellular polysaccharides (Selgas et al., 1993; Vogeleer et al., 2014). Finally, during dispersion, the bacteria are released and transmitted through the air or to another surface (Vogeleer et al., 2014).
The type of surface is one way attachment may be affected. In the meat industry, there is concern of bacteria attachment to both lean and adipose tissue. Reports in the literature are contradictory as to whether the type of tissue (lean v. adipose) affects the number of bacteria that attach. Chung et al. (1989) demonstrated no significant difference in the number of bacteria that attached to lean and adipose beef tissue. However, Dickson (1988) demonstrated a difference in the attachment numbers to beef tissue. It was reported that bacteria are easily removed from adipose tissue, indicating they do not adhere as strongly to adipose tissue as they adhere to lean tissue (Dickson, 1988).

One of the forces effecting bacterial attachment is hydrophobicity, or the concept of being attracted to adipose. Hydrophobicity of bacteria can be measured using several methods: bacterial adherence to hydrocarbons, contact angle measurement, hydrophobic interaction chromatography, and a salting-out aggregation test (Benito et al., 1996; Hood and Zottola, 1995). Bacterial hydrophobicity may be dependent upon protein structures on the cell surface through the production of proteolytic enzymes that decrease the hydrophobicity of bacteria, as shown with Vibrio proteolytica (Paul and Jeffrey, 1985).

Overall, bacteria have a net negative charge that varies between strains of bacteria (Gilbert et al., 1991). The net negative charge is due to the carboxyl and phosphate groups inserted throughout the cell membrane (Lillard, 1985). Dickson and Koohmaraie (1989) found there is a linear correlation between net negative charge and the initial adherence to the beef muscle. This correlation was not as strong for the attachment of bacteria to adipose tissue as seen in experiments conducted by Dickson and Koohmaraie (1989) and Benito et al. (1996).
The structure of bacteria varies from strain-to-strain, but the most common traits between strains that affect attachment strength and rates are flagella, fimbriae, and extracellular polysaccharides (Selgas et al., 1993). Motile bacteria with flagella have been shown to have similar attachment rates as non-motile bacteria, while other studies reported differences based on bacteria motility (Meadows, 1971; Lillard, 1986; Butler et al., 1979; Farber and Idziak, 1984). Motile, Gram-negative bacterial species have been documented to attach at a higher rate than non-motile, Gram-positive organisms due to their ability to move towards a surface (Selgas et al., 1993; Hood and Zottola, 1995). In addition to flagella, Zulfakar et al. (2012) found differences in attachment between strains of *E. coli* and *Salmonella*, indicating there may be surface receptors on some strains of bacteria which could account for differences in attachment.

Although hydrophobicity, cell charge, and cell structures are the major factors affecting bacterial attachment, there are multiple other factors that add to the complexity of the effectiveness of the attachment. Piette and Idziak (1992) reported strain, culturing method, concentration of microorganisms, contact time, temperature, pH, and ions in the meat as other factors that could affect bacterial cell attachment. The quantity of bacteria that attach to a meat surface depends upon the concentration of the bacteria that contaminates the meat, as well as the amount and type of bacteria already on the meat product. Some microorganisms do not compete well with other microorganisms, and therefore do not attach, or attach and do not survive (Selgas et al., 1993). Delaquis and McCurdy (1990) found that lower temperatures demonstrated a higher attachment rate to beef muscle. The pH has been found to affect bacterial attachment to meat surfaces, but Zulfakar et al. (2012) found neutral pH (5 to 9) does not have a large impact on
attachment. With regard to ions, Zulfakar et al. (2012) found salt type and concentration had an effect on bacterial attachment. By increasing the ion concentration, bacteria were able to overcome the electrical repulsion between the cell membranes, get closer to the meat surface, and increase chances of attaching (Zulfakar et al., 2012).

**Attachment of E. coli O157:H7, Salmonella spp. and L. monocytogenes**

As previously described, varying strains of bacteria will attach at different rates and strengths; however, the end goal is to attach to a meat surface so as to not be easily removed (Selgas et al., 1993). The attachment of EC to beef tissue is dependent upon the concentration of the inoculum (Fratamico et al., 1996). A direct correlation was seen between beef tissues and inoculum level in an experiment conducted by Fratamico et al. (1996). Selgas et al. (1993) reported that attachment is inversely related between surface electronegativity and bacterial adherence and hydrophobicity and bacterial adherence of EC. With S, bacteria were found to attach to chicken collagen fibers when muscle was immersed in water for a prolonged period of time, suggesting a slower attachment rate (Thomas and McMeekin, 1981). LM attached at a higher rate to adipose tissue than lean tissue due to its hydrophobic nature (Foong and Dickson, 2003).

**Escherichia coli O157:H7**

**History**

A non-pathogenic strain of E. coli was first described in 1885 by Theodor Escherich (Lim et al., 2010). Pathrogenic strains of E. coli evolved over time by acquiring virulence factors from different bacteria, most notably the Shiga-like toxins from *Shigella*
E. coli O157:H7 (EC) was first identified as a potential pathogen in 1982 when two outbreaks of bloody diarrhea were observed in Oregon and Michigan (Incze, 1998; Lim et al., 2010). The same microorganism was identified in 1983 as the causative agent of hemolytic uremic syndrome (HUS) in children (Incze, 1998). Following a dry sausage outbreak in California and Washington, the U.S. Department of Agriculture (USDA) outlined guidelines for fermented meat processors to utilize an inactivation treatment to reduce EC (Incze, 1998).

Characteristics

EC is Gram-negative, rod-shaped, facultative anaerobic bacteria belonging to the Enterobacteriaceae family (Chauret, 2011). Cells are typically 1 micrometer (µm) in diameter and 3 µm in length (Reshes et al., 2008). It has an optimal growth temperature of 37˚C, but can grow as low as 10˚C (Getty et al., 2000). Multiple species of E. coli are naturally found in the intestinal tract of warm blooded animals, food, and the environment (CDC, 2015a). Commensal strains are coliforms that are used to analyze food and water for contamination. An enterohemorrhagic strain, EC is commonly found in ruminant intestinal tracts, with the most notable source being cattle (Stenutz et al., 2006; Ducic et al, 2016). Of the seven pathogenic strains, EC is currently the strain found most in the literature.

Each serovar of E. coli is identified by a series of antigens. The Kauffmann-White scheme is used to serologically identify strains of E. coli (Stenutz et al., 2006). The O- and H-antigens are the two commonly seen, but K-antigens are used as well (Figure 1.1) (Stenutz et al., 2006). The O-antigen is the somatic antigen found in the cell wall, and the
H-antigen is the flagellar antigen present on an *E. coli* cell (Stenutz et al., 2006). O-antigens define the specific serogroup; together with the H-antigen, they define a specific serotype of an isolate (Stenutz et al., 2006). EC has O-antigen 157 and H-antigen 7 giving it the name *E. coli* O157:H7.

**Figure 1.1** Image of *E. coli* O157:H7 antigens. (EcL, 2004)

In fermented products, such as fermented sausages, LAB may help to reduce the levels of EC by creating an inhospitable, acidic environment for growth (Lücke, 2000). As the pH of the sausage decreases, EC growth will cease, but bacteria can remain viable (Getty et al., 2000). Due to this characteristic, EC is able to survive gastric conditions that include pH levels as low as 2 (Getty et al., 2000). This ability to remain viable in acidic conditions makes EC an issue in fermented sausages, a product that relies on low pH as part of the hurdle effect to inhibit pathogenic bacteria. Other sensitivities of EC include heat and aw. EC is fairly heat sensitive, with temperatures as low as 42°C providing lethal or sub-lethal temperatures (Wesche et al., 2009). Line et al. (1991) reported that heat tolerance of EC is increased as fat is increased in the product. EC cannot grow below an aw of 0.95, but may remain viable (Sperber, 1983). During the production of some
fermented sausages, a drying process occurs, which may further serve to inhibit EC growth by decreasing $a_w$ and injuring cells.

*E. coli* O157:H7 as a Cause of Hemorrhagic Colitis and HUS

Annually, in the United States, 63,000 cases of foodborne illnesses can be attributed to EC (USDA-ERS, 2016). In 2011, EC infections accounted for 2,100 hospitalizations, making it the fifth highest pathogen to cause hospitalizations (CDC, 2014b). In 2014, EC infections had an estimated economic impact over $271 million (USDA-ERS, 2016). The infectious dose of EC is as low as 10 ingested bacterial cells, with intoxication occurring when the bacteria colonize the intestinal tract and produce Shiga-toxin 1 or Shiga-toxin 2 (Lücke, 2000; Getty et al., 2000; Fantelli and Stephan, 2001).

Hemorrhagic colitis is the most common infection caused by EC. Symptoms present themselves one to five days after consuming contaminated food and can include the following sequela: mild non-bloody diarrhea, abdominal pain, fever, and moderate dehydration (Getty et al., 2000; CDC, 2015a).

Of the 63,000 cases of illness caused by EC, approximately 5% to 10% will result in HUS cases (CDC, 2015a). HUS is a life-threatening infection that affects young children and the elderly at a higher rate than healthy individuals due to naïve or weakened immune systems (CDC, 2015a). Symptoms of HUS include bloody diarrhea, abdominal cramps, and vomiting (CDC, 2015a). Infected people who are not medically treated immediately can develop life threatening symptoms such as hemolysis, anemia, and acute renal failure (CDC, 2015a; Noris et al., 2005).
Outbreaks

Contamination of raw meat can occur during the hide removal and evisceration processes at slaughter, and further contamination can occur during boning, cutting, and mincing (Ducic et al., 2016). Having low levels of bacteria is not an issue when proper lethality processes are conducted. Improper heating and mishandling of contaminated raw meat can result in a foodborne outbreak of either fresh meat or processed meat not properly manufactured.

Multiple outbreaks in fermented sausages have been attributed to EC. In 1994, 23 people were infected by consuming dry cured salami in Washington and Northern California (CDC, 1995). In 1998, Genoa salami caused an outbreak in Ontario, Canada that infected 39 people (Williams et al., 2000). The most recent outbreak in the U.S. occurred in 2011 with contaminated Lebanon bologna (CDC, 2011).

Salmonella spp.

History

Salmonella was discovered in 1885 by Daniel Elmer Salmon and Theobald Smith while they were attempting to find the cause of hog cholera (Fábrega and Vila, 2013). Formerly called S. cholera-suis, Salmonella was renamed after Salmon, when later research revealed the organism was not the cause of the cholera in pigs (Fábrega and Vila, 2013). Since the discovery of Salmonella, more than 2500 serovars have been identified between two species: Salmonella bongori and Salmonella enterica (Fábrega and Vila, 2013). Salmonella enterica, which is the most common source of bacterial illness in the U.S., has six subspecies: S. enterica spp. enterica, S. enterica spp. salamae,
S. enterica spp. arizonae, S. enterica spp. diarizonae, S. enterica spp. houtenae, and S. enterica spp. indica (Fábrega and Vila, 2013). Most Salmonella illnesses in the U.S. are caused by S. enterica spp. enterica with Salmonella enterica serovar Typhimurium and Salmonella enterica serovar Enteritidis linked to most foodborne illnesses.

**Characteristics**

Salmonella (S) are Gram-negative, rod-shaped bacteria that belong to the Enterobacteriaceae family (Fábrega and Vila, 2013). The non-spore forming bacteria tend to be 0.7 μm to 1.5 μm in diameter and 2 μm to 5 μm in length (Fábrega and Vila, 2013). S are facultative anaerobes that have the ability to remain viable in a range of temperatures from 2º to 54ºC and grow above a aw of 0.95 (Doyle and Beuchat, 2007; Fábrega and Vila, 2013; Sperber, 1983). The bacterium is found in a range of hosts, including both humans and animals (CDC 2015b).

Nomenclature of S is similar to that of E. coli spp. with the Kauffman-White scheme being used to identify individual serovars. The O-antigen, or somatic antigen, identifies which of the six serogroups the bacterium expresses (Pui et al., 2011). The H-antigen, or flagellar antigen, further identifies the serovar, similar to that of E. coli spp. (Pui et al., 2011). A third antigen, the K-antigen, is used to identify the virulence of the serovar (Pui et al., 2011).

Since the 1990s, S have begun to gain antibiotic resistance to many drugs, such as trimethoprim-sulfamethoxazole, once used to commonly treat Salmonella infections (Fábrega and Vila, 2013). Strains resistant to ampicillin, quinolones, cephalosporins, and aminoglycosides have also been identified (Alban et al., 2001). Multiple antibiotic
resistance has already been determined in S. Typhimurium DT104, which is resistant to ampicillin, streptomycin, tetracycline, sulfonamides, and chloramphenicol (Fábrega and Vila, 2013).

**Salmonellosis**

Non-typhoidal *Salmonella* infections occur in more than 1.2 million cases annually in the U.S., resulting in 450 deaths. (CDC, 2015b). In 2012, of the 800 outbreaks observed in the U.S., 106 were caused by a strain of non-typhoidal *Salmonella* (CDC, 2015b). As of 2014, *Salmonella* was the second highest foodborne pathogen to cause illness, following norovirus, and led the U.S. in the number of hospitalizations with 19,000. (CDC, 2014b). Annually, *Salmonella* infections cost Americans over $3.6 billion in healthcare costs (USDA-ERS, 2016).

The onset of salmonellosis is typically seen within 6 to 72 hours following ingestion of more than 50,000 bacteria in a contaminated food source (Fábrega and Vila, 2013). In healthy individuals, symptoms include diarrhea, abdominal cramps, fever, nausea, vomiting, and headaches, and symptoms typically subside within five to seven days due to the fact the infection is self-limiting (CDC, 2015b; Alban et al., 2001). Salmonellosis poses a higher risk to older adults and young children, causing serious medical conditions such as meningitis, septic arthritis, and osteomyelitis (CDC, 2015b; Dougan et al., 2011).
Outbreaks

Outbreaks in fermented sausages are not commonly seen due to the varying intrinsic factors limiting the growth of S. The first documentation of a Salmonella-contaminated fermented sausage in the U.S. was in 1995 when a Lebanon bologna product was found to be contaminated with S. Typhimurium (Sauer et al., 1997). In 2010, an outbreak of salami infected 272 individuals due to black and red pepper contaminated by Salmonella enterica serovar Montevideo that was used to manufacture the product (CDC, 2010).

Listeria monocytogenes

History

*L. monocytogenes* (LM) was first isolated in 1924 by E.G.D Murray in lab animals (Murray, et al., 1926). Murray initially named the microorganism Bacterium monocytogenes, but Pirie renamed it Listeria in 1940 (Hof, 2003). The role of LM as a potential pathogen was not detected until 1949 when an outbreak in human newborns in Germany occurred (Hof, 2003). It was also found that “circling disease” in ruminants was due to a strain of Listeria: *L. monocytogenes*. The outbreak that solidified LM as a pathogen was a high-case fatality outbreak in Canada in 1982 due to contaminated coleslaw (Swaminathan, 2007; Schlech, 2000). Today there are multiple known subspecies of Listeria, with two being pathogenic: *L. monocytogenes* and *L. ivanovii*. More commonly seen in human infections, *L. monocytogenes* has 13 serovars, with 1/2a, 1/2b, and 4b causing 95% of listeriosis cases (Chaturongakul et al., 2008; Fantelli and Stephan, 2001).
Characteristics

LM is a Gram-positive, facultative aerobe belonging to the *Listeriaceae* family (Ferreira et al., 2014; Low and Donachie, 1997). The rod-shaped organism is 0.5 µm in diameter and 1 µm to 2 µm in length (Low and Dinachie, 1997). LM is a hardy organism and is ubiquitous in nature, being found in soil, decaying vegetation, streams, sewage, slaughterhouse waste, milk, and human and animal feces (Ferreira et al., 2014; Farber and Peterkin, 1991). The hurdle effect is effective at decreasing levels of LM, but the bacterium is difficult to completely eradicate due to its ability to adapt to its environment. Characteristics of LM allow it to tolerate high salt concentrations (up to 10% wt/vol) and a$_w$ as low as 0.90 (Ferreira, 2014; Doyle and Beuchat, 2007). It is able to remain viable at refrigeration and freezing temperatures from -0.5°C to 45°C, with the optimum temperature being 30°C to 37°C, and optimum pH being 4.7 to 9.2 (Ferrerira et al., 2014; Doyle and Beuchat, 2007; Low and Donachie, 1997).

LM is most likely to be found on products exposed to the processing environment post-lethality treatment due to contaminated equipment and other contact surfaces (Ducic et al., 2016). Once a biofilm is formed, it is difficult to remove the pathogen and sanitize the equipment (Swaminathan et al., 2007). Due to the ability of LM to create biofilms on equipment and in processing facilities, it can survive in plants from months to years and becomes an issue post-lethality treatment (Ducic et al., 2016; Ferreira et al., 2014; Swaminathan and Gerner-Smidt, 2007).
Listeriosis

Listeriosis is a rare disease that requires an ingestion dose of $10^7$ to $10^9$ cells for healthy individuals and $10^5$ to $10^7$ cells in individuals with underlying health conditions (Farber et al., 1996). The incubation period of LM in the body is one week to several weeks (Farber and Peterkin, 1991). A normal, healthy person has the ability to fight LM cells that are consumed, but the young, elderly, and immunocompromised lack this ability due to naïve or weakened immune systems (Ferreira et al., 2014). Cells invade the host tissue via endocytosis, making it difficult for the immune system to attack and destroy LM cells (Farber and Peterkin, 1991). LM targets the central nervous system and causes symptoms such as fever, headaches, vomiting, osteomyelitis, pneumonia, hepatitis, and bacterial meningitis in adults (Schlech, 2000; CDC, 2014c; Farber and Peterkin, 1991). Pregnant women carry a risk of passing the infection to the fetus through the umbilical cord, leading to abortion, still birth, or newborn death (Ferreira et al., 2014).

Annually, listeriosis causes 1,600 illnesses and has up to a 30% mortality rate (CDC, 2014a). In 2011, LM was listed as the third highest cause of deaths due to pathogenic illness (CDC, 2014b). Listeriosis has a high economic impact with over $2.8 billion in healthcare fees, despite only causing 1,600 cases per year (USDA-ERS, 2016). Untreated invasive listeriosis is fatal, however, the infection is treatable with ampicillin, penicillin, tetracycline, vanomycin and aminoglycosides (Schlech, 2000; Swaminathan and Gerner-Smidt, 2007).
Outbreaks

Listeriosis outbreaks have been reported in several countries, including the United Kingdom, Italy, and the U.S. (Ducic et al., 2016). Most outbreaks that occur due to LM are sporadic, meaning they are infrequent and irregular (Farber and Peterkin, 1991). LM contamination becomes an issue when food processing establishments are having hygienic problems. Hygiene may be due to poorly cleaned and sanitized equipment, trafficking of the outdoor environment into the plant, and microorganism growth near poorly cleaned areas, such as drains.

Due to the hurdle effect, LM is inhibited in fermented sausage products. On average, there are less than 0.1 cases annually due to contaminated fermented sausages (Swaminathan and Gerner-Smidt, 2007). No notable listeriosis outbreaks have occurred in fermented sausages. However, there was one outbreak of listeriosis that occurred in Philadelphia from December 1986 to January 1987 that was caused by multiple food sources, one of which was a salami (Schwartz et al., 1989). Most processed meat related outbreaks due to L. monocytogenes occur in non-fermented deli meats, many of which were linked to deli slicers that were poorly cleaned and sanitized between products (Lücke, 2000; Ferreira et al., 2014).

Zero Tolerance Policy

Past outbreaks have led to the introduction of laws to eliminate pathogenic bacteria from food sources, specifically RTE foods. Currently, the U.S. has a “zero tolerance” policy with regard to the presence of LM in RTE foods (USDA-FSIS, 2010). In two 25 gram samples obtained by inspection personnel, the pathogen must be
undetected or the product must be considered adulterated and recalled (Leroy et al., 2006; Gandhi and Chikindas, 2007). In accordance with the CFR, a product is considered adulterated if it: 1) contains LM or 2) came in contact with a surface contaminated with LM (USDA-FSIS, 2016c). Processors are expected to control for LM through the use of a HACCP plan or prevent LM in a sanitation standard operating procedure (USDA-FSIS, 2016c). Currently, there are three alternatives outlined in the compliance guideline processors can implement to control LM (Table 1.1) (USDA-FSIS, 2014).

<table>
<thead>
<tr>
<th>Alternative 1</th>
<th>Establishment uses a post-lethality treatment to reduce or eliminate <em>L. monocytogenes</em> and an antimicrobial agent or process to limit the growth of <em>L. monocytogenes</em> in the product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative 2</td>
<td>Establishment uses a post-lethality treatment to reduce or eliminate <em>L. monocytogenes</em> or an antimicrobial agent or process to limit the growth of <em>L. monocytogenes</em> in the product</td>
</tr>
<tr>
<td>Alternative 3</td>
<td>Establishment relies on sanitation to control <em>L. monocytogenes</em> in the processing environment and on the product</td>
</tr>
</tbody>
</table>

*Table 1.1* Three alternatives processors can incorporate into the facility to control *L. monocytogenes* in RTE products.

**Cross-Protection of Bacteria**

Upregulating one stress response has the ability to protect against other stress factors by leading to enhanced resistance (Chung et al., 2006; Lou and Yousef, 1997). For example, inducing a starvation response may lead to an increase in acid tolerance in bacteria as seen in an experiment conducted by Cheville et al. (1996). Greenacre and Brocklehurst (2006) found *S. Typhimurium* pre-exposed to one molar of acetic acid provided cross-protection against increased salt concentrations and lower *a_w*. Through
cross-protection, S have the capability to withstand multiple hurdles simultaneously. LM has been demonstrated to have an increased thermotolerance of higher temperatures when heat was increased at less than 5°C/min (Lou and Yousef, 1997). In relation to fermented sausage manufacture, bacteria may undergo the process of cross-protection. The gradual decrease in pH may lead to acid resistance and cells remaining after production (Lou and Yousef, 1997).

**Regulatory Statutes**

Pathogenic bacteria pose a threat to the health of the population if consumed. Several regulations have been signed into law by Congress and are carried out by the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) to ensure the safety of consumers. EC, S, and LM are considered biological hazards that are “most likely to occur” in beef, pork, and RTE products, respectively, and must be addressed in the establishment’s HACCP plan (USDA-FSIS, 2010). The USDA-FSIS has set forth a “zero tolerance” policy for EC and LM (USDA-FSIS, 2010). Due to this policy, it is rare to find pathogenic bacteria in RTE products (Porto-Fett et al, 2010).

USDA-FSIS has issued compliance guidelines to enforce regulations that address processing and manufacturing processes of fermented sausages. Current processes for the manufacture of cooked, RTE, fermented sausages must meet the heating and cooling guidelines as outlined in 9 CFR §318.17 (USDA-FSIS, 2016a). The lethality procedures used must achieve a $6.5 \log_{10}$ reduction of S in beef products, while the stabilization processes must demonstrate no growth of “toxigenic microorganisms,” such as *Clostridium* spp. and may not have more than a $1 \log_{10}$ increase in remaining pathogens.
Appendix A of the USDA Compliance guidelines addresses the lethality process and states products must meet temperature and temperature cooking times to ensure a $6.5 \log_{10}$ or $7.0 \log_{10}$ reduction in $S$ (USDA-FSIS, 1999a). Appendix B of the USDA Compliance Guidelines addresses the stability of the product in terms of growth of spore-form and toxin-producing bacteria. (USDA-FSIS, 1999b).

Other processing controls include pH, as most bacteria cannot survive below a pH of 4.6. A decrease in pH occurs during fermentation due to the addition of a commercial starter culture containing LAB to the meat batch. A rapid drop in pH is important to inhibit the growth of EC, $S. \text{aureus}$, and $S$ (AMIF, 1997; Lücke, 2000). During fermentation, degree-hours are used to ensure the product reaches a pH of 5.3 or less to prevent $S. \text{aureus}$ toxin production (AMIF, 1997). Degree-hours are the product of time (hours) and temperature (degrees Fahrenheit), with degrees measuring the difference between the oven temperature and 60°F (the temperature in which the $S. \text{aureus}$ toxin will begin to be produced) (AMIF, 1997). Products fermented at 90°F (32.2°C) must reach a pH of 5.3 within 1200 degree-hours; 1000 degree-hours for products fermented at 100°F (37.8°C); and 900 degree-hours for products fermented above 100°F (37.8°C) (Figure 1.2) (AMIF, 1997).
When changing processes, unless previous research can be used to scientifically support the process, a validation study must be conducted to ensure lethality of the process (Getty et al., 2000; Porto-Fett et al., 2010). Validation studies can be conducted with a third-party institute or an in-house study can be completed to demonstrate the procedures being used will achieve lethality. After the 1994 EC outbreak in fermented sausages manufacturers of both semi-dry and dry fermented sausages containing beef were required to follow procedures that ensured a $5 \log_{10}$ reduction of the pathogen (Calicioglu et al., 1997). In addition, there are other options to achieving lethality as outlined in the Federal Register, such as conducting a hold-and-test program for finished product in which 15-30 sausages are sampled or instituting a raw batter testing program in HACCP plan and achieving a $2 \log_{10}$ reduction during fermentation and drying (USDA-FSIS, 2001).
An outbreak in 2011 with Lebanon bologna was due to a change in processing without a validation study. The company produced the Lebanon bologna in a larger casing than what was validated for, emphasizing the need for validation studies to ensure safe products and to prevent future outbreaks (USDA-FSIS, 2013). Validation studies assess production of a RTE product by ensuring a $5 \log_{10}$ reduction of $S$ in pork and a $5 \log_{10}$ reduction of EC in beef containing products (USDA-FSIS, 2012). LM is assessed during storage to ensure no more than a $1 \log_{10}$ growth occurs (USDA-FSIS, 2016c). Processors also fall under Alternative 1 (Table 1.1) if a validation study is conducted that “demonstrates both elimination of $L.\ monocytogenes$ before product leaves the establishment and that $L.\ monocytogenes$ growth is not supported during the shelf life” (USDA-FSIS, 2014).

**Statement of the Problem**

Fermented sausages can be made with a variety of production parameters. Fat content, grind size, casing diameter, and casing type are some of these parameters. In addition, the level of heat used to produce the product is another factor to study. European-style, dry, fermented sausages are traditionally made without a heat treatment, whereas U.S.-style, dry, fermented sausages commonly incorporate a thermal lethality treatment in order to reduce pathogenic bacteria in RTE meat products. Due to the combination of fermentation and drying of the product, the pH and water activity drop to levels inhospitable for bacterial growth. Producing products without incorporating a heat treatment may be possible in order to preserve the authentic flavor that heating may take away. However, before processors are able to alter their processing methods, they must
be able to provide validation for the process. Currently, there is little scientific research that demonstrates the safety of RTE meat products using these different safety parameters. A challenge study can be conducted to determine the safety of these parameters in fermented sausages.

**Objectives**

This study will validate the safety of RTE products using different parameters. One objective is to study bacterial attachment differences in sausages produced with different fat content and grind size combinations. The second objective is to study the effects of casing diameter in a fermented dry sausage with a mild heat treatment. The third objective is to study the effects of casing type in a fermented dry salami produced without a heat treatment. Three strains each of EC, S, and LM will be used to determine the safety of the products through a fermentation and drying period. The data collected may be used by the USDA and processors alike as scientific support for the production of semi-dry and dry sausages with these varying parameters.
References


Chapter 2

Investigating the Effect of Fat Content and Grind Size on Pathogen Attachment and Survival in Fermented Semi-Dry Sausages
Abstract

The effect of varying fat content (10%, 20%, 30%) and grind size (3.00 mm, 4.75 mm, and 12.50 mm) on the attachment and survivability of different pathogens was investigated in a fermented, semi-dry sausage. *E. coli* O157:H7 (EC), *S. Typhimurium* (ST), and *L. monocytogenes* (LM) were incorporated into a beef and pork summer sausage and subjected to fermentation and a lethality treatment. Fat content was significant for EC (*p*=0.0086) during the attachment stage and survival following fermentation, while the survival of ST (*p*=0.0127) was affected by fermentation. Fat content did not significantly impact LM attachment or survival (*p*=0.3931). Grind size did not significantly impact attachment of survival for any of the bacteria examined (*p*>0.05). Following fermentation, EC decreased up to 1.74 log_{10} CFU/g, ST decreased up to 2.73 log_{10} CFU/g, and LM decreased up to 3.69 log_{10} CFU/g. EC and ST were below the detection limit following smoking, while LM was still detectable through to chilling. This study provides data for producers to utilize in HACCP plans for a semi-dry sausage.

Introduction

Annually, one in six Americans will become ill due to a foodborne illness (CDC, 2014). Foodborne pathogens are found in a variety of food products including fruits and vegetables, fresh meat, eggs, and ready-to-eat (RTE) products. RTE foods are of utmost concern due to the fact that consumers typically do not perform any additional preparation (i.e. cooking).

Semi-dry sausages, such as summer sausage, Lebanon bologna, and cervelat, are examples of RTE meat products that Americans consume. Semi-dry sausages may be
made with beef, pork, poultry, or a combination, and are typically produced by fermenting, smoking, cooking, and chilling. During this process, the product will lose 15-30% of its original weight (USDA-FSIS, 2005; Ricke and Keeton, 1997).

The ability of bacteria to attach to and survive in meat products depends on environmental conditions during the initial attachment phase, as well as during processing. Factors include tissue type, surface area, pH, water activity (a_w), temperature, and added ingredients. Bacterial strains have been shown to attach differently to lean and adipose tissue. There are multiple forces that may affect the rate and strength of attachment for bacteria to tissue. Once force is hydrophobicity, or the concept of being attracted to fat. Another impact factor is the net negative charge on the bacterial membrane. Dickson and Koohmaraie (1989) found a linear correlation between net negative charge and adherence to beef muscle; however, this correlation was not as strong between net negative charge and adherence to adipose tissue.

By changing the ratio of lean-to-adipose tissue in a product, the attachment rate of pathogens may be affected. Dickson (1988) demonstrated a difference in bacterial attachment numbers on lean and adipose tissue and reported that bacteria are more easily removed from adipose tissue than from lean tissue surfaces. A higher lean-to-fat ratio suggests a higher bacterial attachment due to the increased efficiency rate of attachment to lean tissue. Bacteria attachment is also dependent upon the surface area available for bacteria to attach. Smaller particle size in a ground meat product increases surface area, which may increase the number of bacteria that attach.

Heat treatments are commonly applied to many RTE, processed meat products as a lethality process to reduce pathogens, such as *E. coli* O157:H7 (EC), *S. Typhimurium*
(ST), and L. monocytogenes (LM). The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) issued a compliance guideline that aids meat processors in determining lethality times at various temperatures for a variety of meat products to achieve either a 6.5 log_{10} or a 7.0 log_{10} reduction of Salmonella spp. in RTE beef and poultry products (USDA-FSIS, 1999a). In addition to the lethality of Salmonella spp., Appendix A Compliance Guidelines have also been accepted by the USDA-FSIS to provide lethality measures for other pathogens. USDA-FSIS has also provided a cooling compliance guideline (Appendix B; USDA-FSIS, 1999b) to prevent the growth of spore-forming pathogens, such as Clostridium spp. This study aims to confirm the time and temperature guidelines of Appendix A while determining if there are differences in the attachment and survival of pathogens using different fat content and grind size combinations in a fermented summer sausage made from beef and pork.

Materials and Methods

Preparation of Inoculum

Cultures of E. coli O157:H7 (ATCC 43895), L. monocytogenes (Scott A), and S. Typhimurium (ATCC 14028) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), Center for Disease Control and Prevention (CDC; Atlanta, GA), and the Microbiology Culture Collection at The Pennsylvania State University Food Science Department. Frozen cultures were transferred to fresh tryptic soy broth (TSB; Becton Dickinson and Company; BD, Sparks, MD) and incubated aerobically at 37°C for 24 h. The overnight cultures of EC, ST, and LM were streaked onto Cefixime-tellurite Sorbitol MacConkey agar (CT-SMAC; BD), Xylose Lysine Deoxycholate agar (XLD;
BD), and Modified Oxford agar (MOX; BD), respectively. Plates were incubated at 37°C for 24 h before performing culture confirmation tests (EC: Remel; Lenexa, KS; LM: Microgen Bioproducts; Camberley, UK; ST: Oxoid; Hants, United Kingdom). Individual colonies were used to inoculate bottles of fresh TSB for each bacterium. Bottles were incubated at 37°C for 24 h to obtain cell concentrations of ~8 \log_{10} CFU/ml (adapted from USDA-FSIS, 2012).

**Manufacture and Inoculation of Sausages**

Pork fat (50% lean), lean pork trim (95% lean) and beef fat (50% lean) were supplied by the Pennsylvania State Meat Laboratory, and lean beef trim (95% lean) was received from Nuevo Carnic, S.A. (Managua, Nicaragua). Equal amounts of lean pork trim and lean beef trim were combined and ground through a 12.50 mm plate (Hollymatic Corporation; GMG 180A; Countryside, IL). This process was repeated for fat pork and fat beef. Duplicate samples of lean and adipose were analyzed for fat content by utilizing the modified Babcock analysis method (Griffith Laboratories, 1971). The duplicate samples were used to determine the average fat content, which were used in Pearson square calculations to formulate meat batches with target fat values of 10%, 20%, and 30%. A soxhlet analysis using petroleum ether was conducted using a ANKOM XT15 Extractor (ANKOM Technology, Macedon, NY) to determine if the target fat content was achieved (AOCS, 2005). After desired fat formulation, the meat was ground a second time to achieve the final grind size (3.00 mm, 4.75 mm, 12.50 mm).

Dry ingredients were added on a per weight basis to the meat block as follows (g/kg): salt 0.023, dextrose 0.0075, ground black pepper 0.0034, ground coriander
0.0037, ground mustard 0.0025, garlic powder 0.00031, and curing salt (6.25% sodium nitrite) 0.0025. Lactacel® 115, a lactic acid starter culture (Kerry Ingredients and Flavours, Beloit, WI), was added per the manufacturer’s instructions at the end of the mixing process. Dry ingredients were hand mixed into the batter, followed by the addition of the starter culture. Meat batters were stored at 4°C until ready for inoculation and used within 6 h following the addition of starter culture.

Inoculation was completed by aseptically mixing each pathogen to the sausage batter to create a 1:10 dilution. Batters were hand massaged for ~2 min to ensure thorough distribution and allowed to sit for 10 min to allow for attachment of bacteria to the surface. Batters were stuffed into a 65 mm fibrous casing (Globe Casings; Carlstadt, NJ).

<table>
<thead>
<tr>
<th>Process</th>
<th>Internal Temperature (°C)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation</td>
<td>43</td>
<td>18.0</td>
</tr>
<tr>
<td>Smoking</td>
<td>58</td>
<td>1.5</td>
</tr>
<tr>
<td>Lethality</td>
<td>70</td>
<td>1.0</td>
</tr>
<tr>
<td>Stabilization</td>
<td>7.4</td>
<td>24.0</td>
</tr>
</tbody>
</table>

Table 2.1 Smokehouse parameters.

Table 2.1 shows the procedures used to thermally process the sausages. Sausages were fermented, smoked, and cooked using a Vortron smokehouse (Model TR2-850; Vortron; Beloit, WI). Smoking was completed using Wundersmoke™ sawdust (Frantz Company, Inc., Milwaukee, WI). Relative humidity (RH) was >90% for fermentation, smoking, and lethality. The lethality step followed the guidelines of Compliance Guide Appendix A (USDA-FSIS, 1999a). During stabilization, sausages were chilled in a 1 to
2°C walk-in cooler to an internal temperature of 7.4°C according to Appendix B for cured meat products (USDA-FSIS, 1999b). Both internal and ambient temperatures were monitored using data loggers (HOBO U12 Stainless Temp Logger; Onset Computer Corporation, Bourne, MA).

Microbial Analysis

Samples were collected after inoculation (time 0) and following each thermal process (Table 2.1). Three sausages (n=3) were sampled for each fat content and grind size combination and were used to make a composite sample. Samples were prepared individually by creating a 1:5 dilution in Buffered Peptone Water (BPW; Hardy Diagnostics; HD; Santa Maria, CA) in a filtered stomacher bag (Interscience, St.-Normandy, France). Samples were homogenized for 30 s at 230 RPM (Stomacher® 400 Circulator; Seward Limited; West Sussex, UK), 10 ml from each sample composited, and composite samples serially diluted using 9 ml of BPW.

Aliquots (0.1 ml) of the serial dilutions were plated in duplicate on CT-SMAC, XLD, or MOX to determine survival of EC, ST, and LM, respectively. Tryptic soy agar (TSA; HD) was also plated as a measure of total plate counts. CT-SMAC, XLD, and TSA plates were incubated at 37°C for 24 h, and MOX plates for 48 h at 37°C in accordance with the USDA Microbiological Laboratory Guidebook (USDA-FSIS, 2012).

Samples were enriched (plated simultaneously) for the various sampling times when colonies were below the detection limit following the procedures from the USDA Microbiological Laboratory Guidebook (USDA-FSIS, 2012). The detection limit for colony counts was 0.40 CFU/g. For EC enrichment, 1 ml of the stomachate was
transferred to 9 ml of Gram Negative broth (Hajna; BD) and incubated for 24 h at 37°C. The enrichment was streak-plated onto CT-SMAC, incubated for 24 h at 37°C and examined for colonies. For ST enrichment, 1 ml of the stomachate was transferred to 9 ml of Lactose broth (HiMedia, Mumbai, India), grown for 24 h at 37°C, followed by 1 ml transferred to 9 ml of Rappaport-Vassiliadis R10 broth (HD). After a 24 h incubation at 37°C, the Rappaport-Vassiliadis R10 broth was streak-plated on XLD, incubated at 37 °C for 24 h and examined for colonies. For LM enrichment, 1 ml of the stomachate was transferred to 9 ml of UVM Modified Listeria Enrichment broth (HD), grown for 24 h at 37°C, followed by a transfer of 1 ml to 9 ml of Fraser broth (Oxoid; Basingstoke, UK). The Fraser broth enrichment was grown for 24 h at 37°C, streaked onto MOX agar, incubated at 37 °C and examined for the presence or absence of colonies after 48 h of incubation. Colonies were verified using agglutination latex tests as described earlier (EC: Remel; LM: Microgen Bioproducts; ST: Oxoid).

In addition to microbial analyses, the pH was measured at each sampling time for all sausage types to calculate averages between fat content and grind size (testo 206-pH2 pH Meter; Testo, Inc; Sparta, NJ).

Statistical Analysis

A 3 x 3 factorial design was used for this experiment. Bacterial populations were converted to log<sub>10</sub> CFU/g to complete statistical analyses. For plates with zero populations, a CFU count of 0.01 less than the detection limit (0.39 log<sub>10</sub> CFU/g) was assigned to incorporate in to the analyses. Statistical tests were performed using a general
linear model procedure in Statistical Analysis Software (Version 9.4, SAS Institute Inc., Cary, NC), with a Tukey HSD comparison test. A significance level of 0.05 was used.

**Results**

**Fat Content**

The low-fat (10%) sausage analyzed via soxhlet extraction had a fat content of 9.90%, while the 20% and 30% fat sausages had final calculated fat contents of 18.90%, and 22.98% respectively.

**Sausage pH**

The effect of pH on bacteria counts was significant ($p<0.0001$). On average, the sausages with a fat content of 10% had a higher pH than the sausages with a fat content of 20% and 30%. Sausages with a fat content of 30% had the lowest pH (Table 2.2). All sausages had the same rate of pH decline during fermentation and smoking. The 10% fat sausage increased in pH the most through cooking and chilling. Sausages with a larger grind size had a slightly higher pH through smoking, after which it had the lowest pH (Table 2.3). These are trends observed, however, pH values did not vary greatly between the different fat content and grind sizes at each sample point and overall.
<table>
<thead>
<tr>
<th></th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>5.80</td>
<td>5.81</td>
<td>5.76</td>
</tr>
<tr>
<td>Ferment</td>
<td>4.82</td>
<td>4.80</td>
<td>4.74</td>
</tr>
<tr>
<td>Smoke</td>
<td>4.73</td>
<td>4.75</td>
<td>4.71</td>
</tr>
<tr>
<td>Cook</td>
<td>4.78</td>
<td>4.74</td>
<td>4.72</td>
</tr>
<tr>
<td>Chill</td>
<td>4.84</td>
<td>4.80</td>
<td>4.77</td>
</tr>
</tbody>
</table>

Table 2.2 Average pH of sausages based upon fat content.

<table>
<thead>
<tr>
<th></th>
<th>3.00</th>
<th>4.75</th>
<th>12.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>5.79</td>
<td>5.78</td>
<td>5.80</td>
</tr>
<tr>
<td>Ferment</td>
<td>4.79</td>
<td>4.75</td>
<td>4.82</td>
</tr>
<tr>
<td>Smoke</td>
<td>4.74</td>
<td>4.69</td>
<td>4.77</td>
</tr>
<tr>
<td>Cook</td>
<td>4.77</td>
<td>4.74</td>
<td>4.73</td>
</tr>
<tr>
<td>Chill</td>
<td>4.87</td>
<td>4.77</td>
<td>4.77</td>
</tr>
</tbody>
</table>

Table 2.3 Average pH of sausages based upon grind size (mm).

**Bacteria Populations Based on Fat Content**

Fat content was significant \(p=0.0086\) for EC during attachment and survival following fermentation. The 20% fat sausage had a higher initial attachment than the 10% fat sausage \(p=0.0036\) and a higher attachment level than the 30% fat sausage \(p=0.0068\). Following fermentation, EC decreased by 1.29, 1.72, and 1.52 log\(_{10}\) CFU/g in 10%, 20%, and 30% sausages, respectively (Figure 2.1; Table 2.4). There was a significant difference between the 10% fat sausage when compared to both 20% and 30% fat sausages, with the 10% fat sausage having a greater pathogen survival following fermentation. Following smoking, there were no significant differences between fat content for EC.

There was no significant difference in initial attachment for ST; however, the 20% fat sausage had the highest attachment. Significant differences were observed in ST counts following fermentation \(p=0.0127\), with bacteria survival highest in the 10%...
sausages and lowest in the 30% fat sausage. After fermentation, ST decreased by 2.22, 2.47, and 2.73 log_{10} CFU/g in 10%, 20%, and 30% sausages, respectively (Figure 2.2; Table 2.5). The 10% fat sausage had a higher population survival than the 30% fat sausage (p=0.0003) following fermentation. The 20% fat sausage had a higher population survival than the 30% fat sausage (p=0.0009) following fermentation. There were no significant differences following fermentation for ST populations.

Fat content was not significant for LM for attachment or survival. Following fermentation, LM decreased by 3.69, 3.41, and 3.49 log_{10} CFU/g in 10%, 20%, and 30% sausages, respectively (Figure 2.3; Table 2.6).

**Bacteria Populations Based on Grind Size**

Grind size did not significantly impact bacterial populations for any of the three pathogens (EC, p=0.0602; ST, p=0.8609; LM, p=0.9264). There was a significant difference for the initial attachment of EC between the 3.00 mm and 12.50 mm grind sizes (p=0.0252), with the 12.50 mm having a higher attachment. Following fermentation, EC decreased by 1.40, 1.39, and 1.74 log_{10} CFU/g in the 3.00 mm, 4.75 mm, and 12.50 mm ground sausages, respectively (Figure 2.1; Table 2.4). ST decreased by 2.49, 2.46, and 2.57 log_{10} CFU/g in the 3.00 mm, 4.75 mm, and 12.50 mm ground sausages, respectively (Figure 2.2; Table 2.5). LM decreased by 3.58, 3.45, and 3.56 log_{10} CFU/g in the 3.00 mm, 4.75 mm, and 12.50 mm ground sausages, respectively (Figure 2.3; Table 2.6).
**Figure 2.1** Average *E. coli* O157:H7 counts (log$_{10}$ CFU/g) during semi-dry sausage production.

<table>
<thead>
<tr>
<th>Process Step</th>
<th>10% (3.00, 4.75, 12.5)</th>
<th>20% (3.00, 4.75, 12.5)</th>
<th>30% (3.00, 4.75, 12.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>7.58 ± 0.12</td>
<td>7.90 ± 0.06</td>
<td>7.56 ± 0.15</td>
</tr>
<tr>
<td>Ferment</td>
<td>6.47 ± 0.12</td>
<td>6.09 ± 0.14</td>
<td>6.29 ± 0.05</td>
</tr>
<tr>
<td>Smoke</td>
<td>0.40 ± 0.01</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>Cook</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>Chill</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
</tbody>
</table>

**Table 2.4** Average *E. coli* O157:H7 counts (log$_{10}$ CFU/g ± Standard error (SE)) during semi-dry sausage production with various fat levels (%) and grind sizes (mm).
Figure 2.2 Average S. Typhimurium counts (log₁₀ CFU/g) during semi-dry sausage production.

Table 2.5 Average S. Typhimurium counts (log₁₀ CFU/g ± SE) during semi-dry sausage production with various fat levels (%) and grind sizes (mm).
Figure 2.3 Average *L. monocytogenes* counts (log<sub>10</sub> CFU/g) during semi-dry sausage production.

<table>
<thead>
<tr>
<th>Process Step</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.00</td>
<td>4.75</td>
<td>12.50</td>
</tr>
<tr>
<td>Initial</td>
<td>7.25 ± 0.12</td>
<td>6.96 ± 0.08</td>
<td>7.40 ± 0.03</td>
</tr>
<tr>
<td>Ferment</td>
<td>4.50 ± 0.58</td>
<td>3.66 ± 0.96</td>
<td>3.70 ± 0.30</td>
</tr>
<tr>
<td>Smoke</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>Cook</td>
<td>1.20 ± 0.81</td>
<td>0.90 ± 0.50</td>
<td>0.90 ± 0.51</td>
</tr>
<tr>
<td>Chill</td>
<td>0.55 ± 0.16</td>
<td>0.90 ± 0.50</td>
<td>1.05 ± 0.65</td>
</tr>
</tbody>
</table>

Table 2.6 Average *L. monocytogenes* counts (log<sub>10</sub> CFU/g ± SE) during semi-dry sausage production with various fat levels (%) and grind sizes (mm).
Sausage Temperature

Temperature profiles throughout the process followed that of a local manufacturer, as well as Appendix A (Table 2.4). Internal temperature during the cooking stage targeted 70°C for 0 s; this experiment achieved 71°C for 15 min. To meet stabilization guidelines, the internal temperature was targeted to be below 26.6°C within 5 h after the lethality step and below 7.4°C in an additional 10 h, but was below 26.6°C within 1 h and was below 7.4°C within 3 h following lethality.

![Graph showing temperature profiles](image)

**Figure 2.4** Smokehouse (dry bulb) and sausage (internal) temperatures during thermal processing.

Discussion

The actual fat content of the sausages was similar to the target fat content, with the 30% fat target sausage having the greatest difference between the target and analyzed amounts. While homogenizing the sample, there was fat accumulation on the side of the container, which was not collected and reincorporated into the batter prior to the Soxhlet
analysis. Although collecting the fat may have increased the actual fat percentage obtained following analysis, there is a possibility that hand incorporation would have resulted in uneven mixing.

The trend observed in the rise in pH of the sausages following smoking can be explained by the amount of lean tissue in the product. Lean tissue is composed of nitrogen containing compounds that are broken down to form amines, an alkaline product. With more lean tissue to form amines, the acidity of the 10% fat product was buffered more, thus allowing for a higher end pH when compared to sausages with higher fat content.

Due to humidity being high during the processing steps, sampling for this experiment did not include $a_w$ because it would not have a significant impact on the survival of pathogens, specifically ST. The minimum requirement for lethality of *Salmonella* spp. in the Appendix A Lethality Compliance Guideline is $70^\circ$C for 0 sec with RH $>90\%$. This product had a lethality step that achieved $71^\circ$C for 15 min, indicating ST would have a $6.5 \log_{10}$ reduction. This time-temperature combination also provided a $6.5 \log_{10}$ reduction for EC and $>5 \log_{10}$ reduction for LM.

To be in compliance with Appendix B of the USDA compliance guidelines, the sausages could not remain between $54^\circ$C and $26.6^\circ$C for more than 5 h or between $26.6^\circ$C and $7.4^\circ$C for more than 10 h. The tested product was below $26.6^\circ$C within 50 min and under $7.4^\circ$C within an additional 2 h and 5 min (total 2 h and 55 min to be under $7.4^\circ$C). Based upon the efficiency of heat transfer and cooling rate, an increase in microbial growth was not expected. Of the products that had been inoculated with LM, a
less than a 1 log₁₀ increase was seen between the lethality and stabilization periods.

Tsigarida et al. (2000) demonstrated a decrease in LM in meat vacuum-packaged in a low permeable film and stored at 5°C, therefore, it is possible that vacuum-packaging and storage of the sausages may eliminate the remaining LM.

All bacteria were inhibited following the smoking treatment. This inhibition could be due to one of two factors: heat or smoke. This process involved a hot smoke step which would have been at temperatures high enough and long enough to inhibit bacteria growth and lead to lethality. Smoking can also effect bacterial growth and survival due to antimicrobial compounds (phenols) in the smoke (Suñen, 1998).

Chung et al. (1989) and Dickson and Macneil (1991) found there was no significant difference for initial attachment populations of ST and LM on adipose and lean tissue, which agrees with data reported here. There was a significant difference observed in the attachment of EC, indicating fat content effects the attachment of the bacterium. Previous studies observed the attachment of bacteria to adipose and lean tissue separately, but the current experiment observed the attachment as one system and did not test for bacterial attachment between tissue types.

Fat content affected the survival of EC and ST following fermentation. The 10% fat sausage, which had a higher percentage of lean tissue, had a higher population of bacteria survive following the fermentation process. The product may have provided a protective barrier for the bacteria, leading to a higher survival rate. Bacteria do not attach as well to adipose tissue and are easily removed from adipose tissue surfaces (Dickson, 1988). It is hypothesized that the higher percentage of adipose tissue in the product
provided less of a protective barrier for bacteria to be able to survive the fermentation process.

It was thought a larger grind size would have a negative impact on the attachment of bacteria due to less surface area available. This experiment demonstrated grind size does not impact the attachment of ST and LM. For EC, the 12.50 mm sausage had a higher attachment than the 3.00 mm and 4.75 mm sausages. Literature (Selgas et al., 1993; Hibbing et al., 2010) has shown that bacteria will compete amongst species to access nutrients to survive, which may explain why EC had a significant difference in attachment and S and LM did not.

In previous studies, the lethality effects on LM did not achieve more than a 2.5 log\textsubscript{10} reduction at the end of fermentation (Baccus-Taylor et al., 1993; Berry et al. 1990). In a chicken sausage produced with \textit{P. acidilactici}, a 2 log\textsubscript{10} reduction was observed in LM, however, the fermentation was conducted at a temperature lower (37°C) than that of the current study (42°C), which may explain higher decimal reductions for the majority of treatments examined (Baccus-Taylor et al., 1993). Berry et al. (1990) also achieved 2 log\textsubscript{10} LM reductions in a beef and pork, fermented, semi-dry sausage. The LM counts in the 20% fat and 3.0 mm grind size sausage in the current study was less than that of Berry et al. (1990) and Baccus-Taylor et al. (1993), 1.53 log\textsubscript{10} vs. 2 log\textsubscript{10}. All other sausage types in the current experiment had reductions of LM greater than that of Berry et al. (1990) and Baccus-Taylor et al. (1993), ranging from 2.75 to 3.70 log\textsubscript{10} reductions using similar strains of the \textit{Pediococcus acidilactici} starter culture.

The current experiment confirmed the USDA Appendix A Compliance Guideline for meeting the lethality of \textit{Salmonella} spp. for non-poultry meat products by achieving
greater than a 6.5 log_{10} reduction. In addition, the Appendix A Compliance Guideline also provided a lethality measure for EC and LM by demonstrating at least a 5 log_{10} reduction. Based upon the current research, processors have the ability to produce RTE, fermented semi-dry sausages of varying fat contents and grind sizes while still maintaining a safe product.
References


Chapter 3

Pathogen Reductions in Fermented Dry Sausages Using a Low-Temperature Heat Treatment
Abstract

This study validated the safety of a process that deviates from USDA-FSIS Appendix A by using a lower temperature at a longer time to produce dried salami. Ground pork, starter culture, and non-meat ingredients were mixed, experimentally-inoculated with three strains each of *E. coli* O157:H7 (EC), *Salmonella* spp. (S), and *L. monocytogenes* (LM) and stuffed into 38-, 64- and 120-mm fibrous casings (N=72). The salamis were then fermented for 7 h at 45°C, heated for 10.5 h at 52°C, dried for 23 d, and vacuum-packaged up to 28 d. A 5 log<sub>10</sub> reduction of all three pathogens was achieved for all three diameters by the end of the drying period. A 5 log<sub>10</sub> reduction in EC was achieved by D17 of the process. A 5 log<sub>10</sub> reduction in S was achieved in both the 64- and 120-mm diameter salamis following the heat treatment, while a 5 log<sub>10</sub> reduction occurred in the 38-mm diameter salami after 48 h of drying. A 5 log<sub>10</sub> reduction in LM was achieved by day 10 of the process for all diameter salamis. This study validated the safety of fermented dry sausage produced using alternate thermal processing methods that differ from the suggested regulatory standards.

Introduction

For thousands of years, food has been preserved for times when food would be scarce. Fermentation and drying were two common practices of meat preservation that are still used today to manufacture a variety of fermented dry sausages (FDS). Today’s meat products use fermentation and drying to: i) create a desired flavor profile, ii) increase the shelf life of the product, and iii) increase the safety and quality of the product by reducing spoilage and pathogenic bacteria.
Although not common, FDS have caused global foodborne illness outbreaks. In the United States (U.S.) *E. coli* O157:H7 (EC) was the known agent of a dry salami outbreak in 1994 in Washington and Northern California that resulted in 23 people being infected (CDC, 1995). In 1998, 39 people were diagnosed with an EC infection due to contaminated Genoa salami in southern Ontario (Williams et al., 2000). *Salmonella* spp. (S) has also been an issue in FDS. In the U.S., two notable outbreaks occurred due to *Salmonella* infections. In 1995, an outbreak occurred in Lebanon bologna; in 2010 an outbreak occurred due to sausage manufactured with contaminated pepper (Sauer et al., 1997; CDC 2010). In 2010, an outbreak occurred in France, where 32 individuals became ill due to consumption of dried pork sausage having been found to contain *Salmonella enterica* serotype 4,12:i:- (Bone et al., 2010). One outbreak due to *L. monocytogenes* (LM) occurred in Philadelphia, PA from December 1986 to January 1987 that was caused by multiple food sources, one of which was a salami (Schwartz et al., 1989).

FDS are ready-to-eat (RTE) products made with beef, pork, poultry, or a combination of species. Processors benefit from a food safety perspective by utilizing the hurdle effect, which is a combination of various challenges inherent to the production process that are presented to bacteria at sub-lethal levels to reduce or eliminate microorganisms (Leistner, 2000). Most FDS formulations include the addition of salt, nitrites, nitrates, and flavor ingredients to create a sensory profile appealing to consumers. In addition to desirable sensory qualities, non-meat ingredients are added to inhibit microbial growth, for example salt. Salt is a common preservative and flavor enhancer that has been used for thousands of years (Albarracín et al., 2011). It is used in FDS to increase salt concentration, bind water molecules, and decrease water activity ($a_w$)
Nitrites are incorporated for color development, but have also been found to be antimicrobial against LM (Hospital et al., 2014). Commercial starter cultures are also added to the meat matrix to decrease the pH to 5.3 or less in order to control *Staphylococcus aureus* toxin production. The decrease in pH also prevents the growth of various other microorganisms. Following fermentation, sausages undergo a drying process in which the FDS will lose approximately 30% to 50% of the original product weight (Ricke and Keeton, 1997). Drying also decreases aw which further assists with microbial growth inhibition, as most bacteria cannot grow below an aw of 0.90 (Hospital et al., 2014). Shelf-stability of FDS relies on pH and aw. However, EC and LM can survive low acid environments, presenting challenges to address during the production of FDS.

The use of heat prior to drying is a common process applied to products to reduce or eliminate microorganisms. The U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) Appendix A serves as a reference for time-temperature combinations required to achieve a 6.5 log10 reduction of S (USDA-FSIS, 1999). In addition, the time-temperature combinations are accepted by USDA-FSIS as a measure for lethality of other pathogens. Appendix A has a minimum time-temperature combination of 54.4°C for 112 min to achieve a 6.5 log10 reduction in S. Lower temperatures for longer times may result in a 6.5 log10 reduction in S and 5 log10 reduction of other bacteria. Scientific evidence is required to ensure an appropriate reduction of pathogens is achieved by heating at a time-temperature combination that differs from those listed in Appendix A. The scientific evidence must also be stated in the
plant’s Hazard Analysis Critical Control Point (HACCP) plan (9 CFR §417; USDA-FSIS, 2016).

In addition to Appendix A, USDA-FSIS has issued guidelines and policies to help processors comply with government regulations to produce safe products. To reduce or eliminate EC in RTE products containing beef, there are five options processors can elect to utilize to produce a safe product. One is to conduct a validation study to demonstrate a treatment achieves >5 log\text{10} reduction in EC (USDA-FSIS, 2001). To prevent LM from entering the food supply of RTE products, the “zero tolerance policy” must be adhered to (USDA-FSIS, 2010). Processors can control for LM by using one of the three alternatives as outlined in the Code of Federal Regulations (CFR; USDA-FSIS, 2014).

Smokehouse parameters may affect the efficacy of the process to reduce pathogens, but product diameter may also affect pathogen reduction. Research has demonstrated the effects of casing diameter on product quality of fermented semi-dry sausages and chemical composition in fermented dry sausages (Keller et al., 1974; Komprda et al., 2009). Previous research has also demonstrated the efficacy of heating FDS at lower temperatures to reduce bacterial populations in semi-dry and dry RTE sausages (Ellajosyula et al., 1998; Hinkens et al., 1996). The objective of this study was to validate a similar process using a low-temperature heat treatment for an extended period of time to manufacture FDS of various casing diameters.
Materials and Methods

Preparation of Inoculum

Cultures of EC (ATCC 43895, ATCC BAA-460, and PA-2), S (S. Typhimurium ATCC 14028, S. Montevideo SMvo13, and S. Panama ATCC 7378) and LM (Scott A serotype 4b, H3396 serotype 4b, and FSL J1-129 serotype 4b) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), the Center for Disease Control and Prevention (CDC; Atlanta, GA), and the Microbiology Culture Collection at The Pennsylvania State University Food Science Department. Frozen cultures were transferred to fresh tryptic soy broth (TSB; Becton Dickinson and Company; BD, Sparks, MD) and incubated aerobically at 37°C for 24 h. The overnight cultures of EC, S, and LM were streaked onto Cefixime-tellurite Sorbitol MacConkey agar (CT-SMAC; BD), Xylose Lysine Deoxycholate agar (XLD; BD), and Modified Oxford agar (MOX; BD), respectively. Plates were incubated at 37°C for 24 h before performing culture confirmation tests (EC: Remel; Lenexa KS; S: Oxoid; Hants, United Kingdom; LM: Microgen Bioproducts; Camberley, UK). Individual colonies were used to inoculate fresh TSB for each bacterium. Bottles were incubated at 37°C for 24 h to obtain cell concentrations of ~8 log₁₀ CFU/ml (adapted from USDA-FSIS, 2012).

Manufacture and Inoculation of Sausages

Raw pork and dry ingredients were received in an overnight shipment from a regional pork processor. Individual pathogens were added to the meat block at a 1:10 dilution rate and hand-mixed aseptically prior to the addition of dry ingredients and seasoning. A proprietary dry ingredient mixture containing salt, nitrite, nitrate, garlic
purée, and red wine was added to the meat block and hand-mixed aseptically. SAGA™ 200 starter culture (Kerry Ingredients and Flavours; Beloit, WI) was hand-mixed aseptically into the meat block and allowed to sit for five minutes prior to stuffing. Sausages were stuffed into permeable fibrous casings of varying diameters: 38-mm, 64-mm, and 120-mm (Globe Casings; Carlstadt, NJ).

The 120-mm sausages were placed in the smokehouse (Model TR2-850; Vortron; Beloit, Wisconsin) immediately following stuffing. The 64-mm sausages were held at 4°C until placed in the smokehouse, approximately 1 h after the large diameter sausage was placed in the smokehouse. The 38-mm sausages were held at 4°C until placed in the smokehouse, approximately 0.5 h after the medium diameter sausages had been placed in the smokehouse. This process allowed for the 120-mm sausages to come to temperature so all sausages reached the same internal temperature at approximately the same time to start fermentation.

Table 3.1 displays the duration of each step and the parameters followed. Sausages were targeted to a pH of 5.0 for fermentation before proceeding to the heating step. The product targeted an internal temperature of 43.3°C and 46.2°C during fermentation and heating, respectively. Following the heat treatment, the smokehouse was cooled quickly by opening the smokehouse door and adding ice above and below the product. The smokehouse was monitored multiple times daily for target temperature and relative humidity (RH). The damper was manually adjusted as needed to maintain proper humidity in the smokehouse.
### Table 3.1 Smokehouse parameters.

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration</th>
<th>Ambient Temp. (°C)</th>
<th>RH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation</td>
<td>7 h</td>
<td>45</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Heating</td>
<td>10.5 h</td>
<td>52</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Drying</td>
<td>23 d</td>
<td>16-18</td>
<td>40-80</td>
</tr>
<tr>
<td>Packaging</td>
<td>28 d</td>
<td>20-22</td>
<td>55</td>
</tr>
</tbody>
</table>

Data loggers (HOBO U12 Stainless Temp Logger, Onset Computer Corporation, Bourne, MA) were used to record ambient and core product temperatures. A third data logger was used to measure RH of the smokehouse (HOBO UX100-003 Temperature/Relative Humidity 3.5% Data Logger, Onset Computer Corporation).

**Microbial Analysis**

Samples were taken following inoculation, fermentation, and the heat treatment. Drying began immediately following the heat treatment, with samples taken on days 1 and 2 of drying and weekly until day 23 of drying. Following drying, sausages were vacuum-sealed using an Ultravac UV-250 (UltraSource, Kansas City, MO) in 152.4 mm x 304.8 mm bags of 3 mil thickness (OTR average 52 cc/m²/24 h; PCS Supplies, Inc.; Penns Grove, NJ) and sampled weekly until day 28 of packaging.

Three sausages (n=3) were sampled for each diameter and used to make a composite sample. Samples were individually prepared by creating a 1:5 dilution in Buffered Peptone Water (BPW; Hardy Diagnostics; HD; Santa Maria, CA) in a filtered stomacher bag (Interscience, St.-Normandy, France). Samples were homogenized for 30 s at 230 RPM (Stomacher® 400 Circulator; Seward Limited; West Sussex, UK), 10 ml used...
from each sample for the composite, and the composite sample serially diluted using 9 ml of BPW.

Aliquots (0.1 ml) were plated in duplicate on CT-SMAC, XLD, and MOX to determine survival of EC, S, and LM, respectively. Tryptic soy agar (TSA; HD) was also plated to determine total plate count. CT-SMAC, XLD, and TSA plates were incubated at 37°C for 24 h, and MOX plates for 48 h at 37°C in accordance to the USDA Microbiological Laboratory Guidebook (USDA-FSIS, 2012).

Samples were enriched (plated simultaneously) for the various sampling times when colonies were below the detection limit (0.40 log10 CFU/g). The enrichment procedures were adapted from the USDA Microbiological Laboratory Guidebook (USDA-FSIS, 2012). For EC enrichment, 1 ml of the stomachate was transferred to 9 ml of Gram Negative broth, Hajna (BD) and incubated for 24 h at 37°C. The enrichment was streak-plated onto CT-SMAC, incubated for 24 h at 37°C and examined for colonies. For S enrichment, 1 ml of the stomachate was transferred to 9 ml of Lactose broth (HiMedia, Mumbai, India), grown for 24 h at 37°C, followed by 1 ml transferred to 9 ml of Rappaport-Vassiliadis R10 broth (HD). After a 24 h incubation at 37°C, the Rappaport-Vassiliadis R10 broth was streak-plated on XLD, incubated at 37°C for 24 h and examined for colonies. For LM enrichment, 1 ml of the stomachate was transferred to 9 ml of UVM Modified Listeria Enrichment broth (HD), grown for 24 h at 37°C, followed by a transfer of 1 ml to 9 ml of Fraser broth (Oxoid; Basingstoke, UK). The Fraser broth enrichment was grown for 24 h at 37°C, streaked onto MOX agar, incubated at 37°C and examined for the presence or absence of colonies after 48 h of incubation. Colonies were verified using confirmation tests (EC: Remel; S: Oxoid; LM: Microgen Bioproducts).
In addition to microbial analyses, pH (testo 206-pH2 pH Meter; Testo, Inc; Sparta, NJ) and \( a_w \) (AquaLab Water Activity Meter, Series 4TE; Decagon Devices, Inc.; Pullman, WA) were measured at each sampling time for each casing diameter.

**Statistical Analysis**

Bacterial populations were converted to log_{10} CFU/g to complete statistical analyses. For plates with zero populations, a CFU count of 0.01 less than the detection limit (0.39 log_{10} CFU/g) was assigned to incorporate into the analyses. Statistical tests were performed using a mixed model procedure in Statistical Analysis Software (Version 9.4, SAS Institute Inc., Cary, NC). The model included differences within a sausage diameter by day, differences between the sausage diameters at each day, and a sausage by time interaction. A significance level of 0.05 was used.

**Results**

**pH and \( a_w \)**

Sausages reached a pH of 4.93 ± 0.5 during fermentation. Sausage pH values continued to decline through the heating step, before stabilizing. Overall, pH did not have an effect on bacteria populations (EC: \( p=0.3110; \) S: \( p=0.6822; \) LM: \( p=0.4356 \)).

All three bacteria populations were impacted by \( a_w \) (\( p<0.05 \)). The \( a_w \) remained above the lower \( a_w \) limit for EC and S growth (0.95) until 18 h for the 38- and 64-mm diameter sausages. The 120-mm diameter sausage reached an \( a_w \) of <0.95 by 72 h. The lower limit for LM growth (\( a_w=0.92 \)) was achieved by day (D) 17 for all three sausages. At the end of the drying period, sausages had an \( a_w \) of 0.82, 0.83, and 0.84 for the 38-,
64-, and 120-mm diameter sausages, respectively. During the packaging period, $a_w$ remained 0.86 or lower.

**Bacteria**

A 5 log$_{10}$ reduction was achieved for the three sausage diameters by the end of the drying period for EC (Figure 3.1). Following fermentation, EC decreased by 0.70 to 0.72 log$_{10}$. The heat treatment provided an additional reduction greater than 3 log$_{10}$ for all sausages (Table 3.2). Following the heat treatment, the 64-mm diameter sausage had the largest decrease in EC with a 3.39 log$_{10}$ reduction, while the 120-mm diameter sausage had the smallest decrease in EC with a 3.07 log$_{10}$ reduction. All sausages achieved a 5 log$_{10}$ or greater reduction by D17 of the experiment (D16 of the drying period).

EC populations were below the detection limit upon sampling at packaging. Enrichments indicated EC was able to be detected in samples until D31 for the 38- and 120-mm diameter sausages and D38 for the 64-mm diameter sausage.

The varying sausage diameters did not have a significant impact on EC survival ($p=0.0712$). Table 3.2 shows the differences between sausages for each sampling time. Significant differences were observed at D10 between the 38- and 64-mm diameter sausages ($p=0.0090$); D17 between the 38- and 120-mm diameter sausages ($p=0.0112$) and the 64- and 120-mm diameter sausages ($p=0.0395$); and on D24 between the 38- and 120-mm diameter sausages ($p=0.002$).

Both the 38- and 64-mm diameter sausages had a significant difference between 7h and 18h, D10 and D17, and D17 and D24. The 120 mm-diameter sausage had
significant differences in EC populations between 7h and 18h, D10 and D17, D17 and D24, and D24 and D31 (Table 3.2).

Figure 3.1 Average *E. coli* O157:H7 counts for 38-, 64-, and 120-mm diameter pork sausages.
Table 3.2 Average *E. coli* O157:H7 counts and reductions (log$_{10}$ CFU/g ± Standard Error (SE)). All comparisons were done with forward sampling times. Same lowercase letters are not significantly different within a column. Same uppercase letters are not significantly within rows (p<0.05). Reductions are the differences between the last day of a phase and the last day of the previous phase.

A 5 log$_{10}$ reduction was achieved for S by 72h (D2 of drying) for all diameters (Figure 3.2). Table 3.3 shows the population counts for each sampling time. Following the 7h fermentation, a 0.95 log$_{10}$ to 1.30 log$_{10}$ reduction was observed, with the 120-mm diameter sausage having the largest decrease in S populations. Following the heat treatment, both the 64- and 120-mm diameter sausages achieved greater than a 5 log$_{10}$ reduction with 6.48 log$_{10}$ and 6.08 log$_{10}$ reductions, respectively. The 38-mm diameter
sausage achieved a 5 log_{10} reduction after 72h. Throughout the drying and packaging periods, S populations remained below the detection limit, with the exception of the 120-mm diameter sausage at D10. Enrichments were positive until 72h for the 120-mm diameter sausages and D45 for the 38- and 64-mm diameter sausages.

Sausage diameter was statistically significant for S survival ($p=0.0004$). The 38-mm diameter sausage had a higher S population than the 64- and 120-mm diameter sausages until D10 of the process. Statistical differences were observed between 0h and 7h and 7h and 18h for both the 64- and 120-mm diameter sausages. The 38-mm diameter sausage had statistical differences between 0h and 7h, 7h and 18h, 18h and 48h, and 48h and 72h (Table 3.3).

Figure 3.2 Average *Salmonella* spp. counts for 38-, 64-, and 120-mm diameter pork sausages.
Table 3.3 Average *Salmonella* spp. counts and reductions (log\(_{10}\) CFU/g ± SE). All comparisons were done with forward sampling times. Same lowercase letters are not significantly different within a column. Same uppercase numbers are not significantly within rows (p<0.05). Reductions are the differences between the last day of a phase and the last day of the previous phase.

<table>
<thead>
<tr>
<th>Sample time</th>
<th>38 mm</th>
<th>64 mm</th>
<th>120 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>6.87 ± 0.09(^a,A)</td>
<td>6.87 ± 0.09(^a,A)</td>
<td>6.87 ± 0.09(^a,A)</td>
</tr>
<tr>
<td>7h-Fermentation</td>
<td>5.92 ± 0.01(^b,A)</td>
<td>5.60 ± 0.05(^b,A)</td>
<td>5.57 ± 0.28(^b,A)</td>
</tr>
<tr>
<td>Reduction</td>
<td><strong>0.95</strong></td>
<td><strong>1.27</strong></td>
<td><strong>1.30</strong></td>
</tr>
<tr>
<td>18h-Heat Treatment</td>
<td>2.47 ± 1.37(^c,A)</td>
<td>0.40 ± 0.01(^c,B)</td>
<td>0.79 ± 0.09(^c,B)</td>
</tr>
<tr>
<td>Reduction</td>
<td><strong>3.45</strong></td>
<td><strong>5.20</strong></td>
<td><strong>4.78</strong></td>
</tr>
<tr>
<td>48h-Drying</td>
<td>2.34 ± 0.06(^d,A)</td>
<td>0.39 ± 0.00(^c,B)</td>
<td>0.40 ± 0.01(^c,B)</td>
</tr>
<tr>
<td>72h-Drying</td>
<td>1.15 ± 0.76(^c,A)</td>
<td>0.39 ± 0.00(^c,B)</td>
<td>0.39 ± 0.00(^c,B)</td>
</tr>
<tr>
<td>D10-Drying</td>
<td>0.40 ± 0.01(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.95 ± 0.56(^c,A)</td>
</tr>
<tr>
<td>D17-Drying</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
</tr>
<tr>
<td>D24-Drying</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
</tr>
<tr>
<td>Reduction</td>
<td><strong>2.08</strong></td>
<td><strong>0.01</strong></td>
<td><strong>0.40</strong></td>
</tr>
<tr>
<td>D31-Packaging</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
</tr>
<tr>
<td>D38-packaging</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
</tr>
<tr>
<td>D45-Packaging</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
</tr>
<tr>
<td>D52-Packaging</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
<td>0.39 ± 0.00(^c,A)</td>
</tr>
<tr>
<td>Reduction</td>
<td><strong>0.00</strong></td>
<td><strong>0.00</strong></td>
<td><strong>0.00</strong></td>
</tr>
<tr>
<td>Total Reduction</td>
<td><strong>6.48</strong></td>
<td><strong>6.48</strong></td>
<td><strong>6.48</strong></td>
</tr>
</tbody>
</table>

Table 3.4 shows the LM populations of each sampling time. Following fermentation, a 0.71 log\(_{10}\) to 1.11 log\(_{10}\) reduction was observed, with the 120-mm diameter sausage having the lowest LM reduction and the 38-mm diameter sausage having the highest LM reduction. Heating provided an additional 2.78, 3.28, and 3.39 log\(_{10}\) reduction for the 38-, 64-, and 120-mm diameter sausages, respectively. By D10 (D9 of drying), all sausages achieved a 5 log\(_{10}\) reduction (Figure 3.3). Populations
continued to decline until D24 of the process when LM was below the detection limit. It was also not detected during the enrichment process.

Sausage diameter was not statistically significant for LM ($p=0.1549$). At 72h, the 38-mm and 120-mm diameter sausages ($p=0.0043$) and the 64-mm and 120-mm diameter sausages ($p=0.0414$) were significantly different. The 120-mm diameter sausage had smaller LM populations than both the 38- and 64-diameter sausages. The 38-mm and 64-mm diameter sausages were significantly different ($p=0.0397$) at D10, with the 38-mm diameter sausage having a larger LM population.

For each of the sausage diameters, there was a significant difference between 7h and 18h, demonstrating the effectiveness of the heat treatment to eliminate LM. There was a significant difference observed between D10 and D17 ($p=0.0474$) for the 38-mm diameter sausage, with an additional $1.43 \log_{10}$ reduction. A significant difference was observed between 72h and D17, in the 64- and 120-mm diameter sausages ($p<0.0001$ and $p=0.0194$, respectively), but no significant difference was observed between 72h and D10, nor D10 and D17.

![Figure 3.3 Average L. monocytogenes counts for 38-, 64-, and 120-mm diameter pork sausages.](image)
Table 3.4 Average *L. monocytogenes* counts and reduction (log_{10} CFU/g ± SE). All comparisons were done with forward sampling times. Same lowercase letters are not significantly different within a column. Same uppercase numbers are not significantly different within rows (p<0.05). Reductions are the differences between the last day of a phase and the last day of the previous phase.

<table>
<thead>
<tr>
<th>Sample time</th>
<th>38 mm</th>
<th>64 mm</th>
<th>120 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>7.19 ± 0.21</td>
<td>7.19 ± 0.21</td>
<td>7.19 ± 0.21</td>
</tr>
<tr>
<td>7h-Fermentation</td>
<td>6.08 ± 0.26</td>
<td>6.36 ± 0.04</td>
<td>6.48 ± 0.03</td>
</tr>
<tr>
<td>Reduction</td>
<td>1.11</td>
<td>0.83</td>
<td>0.71</td>
</tr>
<tr>
<td>18h-Heat Treatment</td>
<td>3.30 ± 0.56</td>
<td>3.08 ± 0.55</td>
<td>3.09 ± 1.22</td>
</tr>
<tr>
<td>Reduction</td>
<td>2.78</td>
<td>3.28</td>
<td>3.39</td>
</tr>
<tr>
<td>48h-Drying</td>
<td>3.63 ± 0.35</td>
<td>2.89 ± 0.97</td>
<td>3.76 ± 0.68</td>
</tr>
<tr>
<td>72h-Drying</td>
<td>4.19 ± 0.37</td>
<td>3.42 ± 0.45</td>
<td>2.07 ± 1.68</td>
</tr>
<tr>
<td>D10-Drying</td>
<td>2.06 ± 0.05</td>
<td>0.69 ± 0.3</td>
<td>1.52 ± 1.21</td>
</tr>
<tr>
<td>D17-Drying</td>
<td>0.63 ± 0.24</td>
<td>0.54 ± 0.16</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>D24-Drying</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>Reduction</td>
<td>2.91</td>
<td>2.69</td>
<td>2.70</td>
</tr>
<tr>
<td>D31-Packaging</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>D38-Packaging</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>D45-Packaging</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>D52-Packaging</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
<td>0.39 ± 0.00</td>
</tr>
<tr>
<td>Reduction</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Total Reduction</td>
<td>6.80</td>
<td>6.80</td>
<td>6.80</td>
</tr>
</tbody>
</table>

**Discussion**

Statistical differences were observed between the different stages of the process (i.e. fermentation vs heating). Statistical smaller quantities were observed between 72h and D17 for EC and LM, but not between 72h and D10. The change in the length of time between samplings can be attributed to the statistical differences seen. Sample times were every 24 h until 72h of processing, after which the time between samplings was increased to one week.
Numerical differences were noticed between casing sizes, with the 120-mm diameter sausage having lower populations following most steps in comparison to the smaller diameter sausages. This may be explained by the 120-mm diameter sausage having a longer exposure time to heat than the smaller diameter products. The 120-mm diameter sausages were in the smokehouse for additional 1 h and 1.5 h in comparison to the 64- and 38-mm diameter sausages, respectively.

Currently, the USDA-FSIS suggests that processors incorporate a heat treatment in the production of RTE sausages to reduce or eliminate pathogenic bacteria. USDA-FSIS Appendix A heating guidelines (USDA-FSIS, 1999) offer time-temperature combinations to effectively eliminate S. Typhimurium and EC (Ellajosyula et al., 1998). The experiment also had a gradual temperature increase to 48.9°C over a 10.5 h period, and then held for an additional time. The current experiment had a quicker come up time (10 min) to achieve the desired temperature of 52°C. This product was also held for a total 10.5 h at 52°C.

Other experiments have used casing diameter to study the effects of varying parameters on pathogenic microorganisms in sausages (Foegeding et al., 1992; Porto-Fett et al., 2010). Porto-Fett et al. (2010) used 65 and 105 mm diameter casings to produce
FDS and found no significant difference in EC, S, or LM viability after fermentation and drying between both diameters, but the product did not have a heat treatment. The current experiment found a significant difference between diameter casings with regard to S survival. The difference was observed following the heat treatment between the 38-mm and 64-mm diameter sausages and the 38-mm and 120-mm diameter sausages, until D10 when the S populations were below the detection limit for all sausage diameters. There was no significant difference between the 64-mm and 120-mm diameter sausages, as seen with similar diameters in Porto-Fett (2010).

USDA-FSIS does not impose regulation on EC in all pork products due to lack of an association of EC with pork products and also does not require testing for the pathogen for pork products. The main microorganisms of concern in this experiment were S and LM because of their association with pork and RTE products, respectively. Although EC was not of concern, the bacterium still has the ability to attach to pork, as demonstrated, and may be of importance to address in smaller facilities where beef and pork may be processed in the same area. The procedure of the current experiment achieved a 5 log_{10} reduction by the end of drying phase for all pathogens present. Therefore, it would be up to the discretion of the processor whether to hold the product or to allow immediate distribution. It is recommended to maximize shelf life and increase the profit-cost margin, processors package and distribute the product within the first week following the drying phase.

This experiment provides valid scientific evidence to be included in HACCP plans for processors wanting to use this process for the production of FDS. Heating for 10.5 h at 52°C provided the level of lethality required for pathogenic bacteria and may be
used as an alternative heating method to USDA-FSIS Appendix A. Following fermentation and the heat treatment, it is recommended producers allow sausages to dry for at least 9 days for proper lethality of pathogenic microorganisms. Although 9 days is the minimum required for product safety, the producer can decide to lengthen the drying time to achieve the desired moisture-to-protein ratio and/or quality attributes of the product.
References


Chapter 4

Pathogen Reductions During Traditional Fermentation and Drying of Pork Salamis
Abstract

Traditionally-processed meat products produced without thermal processing are common in European countries and are increasing in popularity in the United States. Processors are met with the challenge of creating these high-quality products while ensuring food safety. The purpose of this study was to validate the safety of a process to produce a traditional fermented and dried salami. This experiment investigated the impact of casing type and an antimicrobial intervention on the survival of foodborne pathogens in a salami product made with minimal ingredients. Pork butts were cubed and experimentally-inoculated with three strains each of *E. coli* O157:H7 (EC), *Salmonella* spp. (S), and *L. monocytogenes* (LM). The cubes were either sprayed with water (CTRL) or a 2.5% antimicrobial solution (TRT) prior to grinding through a 6.0 mm plate. Dry ingredients and starter culture were thoroughly mixed into the ground pork before being stuffed into ~50 mm natural, collagen, and fibrous casings (N=72). The salamis were subjected to fermentation (72h), drying (21d), and packaging (28d). There was no significant difference between the CTRL and TRT sausages for bacteria populations for EC (*p*=0.1645), S (*p*=0.3746), or LM (*p*=0.1762) for the 60 d sampling period. There was also no significant difference in bacteria reductions between casings types within each treatment. A 5 log\(_{10}\) reduction was achieved for S and LM by the second week of packaging, but treatments did not achieve a 5 log\(_{10}\) reduction of EC by the end of packaging. This study validated the safety of a fermented pork salami manufactured without a heat treatment and no additional lethality process following fermentation and drying.
Introduction

Pathogenic bacteria are of concern in ready-to-eat (RTE) meat products due to the lack of further preparation (i.e. cooking) by the consumer, and may impart a health danger to the consumer if ingested. The United States Department of Agriculture Food Safety and Inspection service (USDA-FSIS) has issued guidelines and policies to help processors comply with government regulations to produce safe, RTE meat products. One such regulation requires processors to implement a valid Hazard Analysis Critical Control Point (HACCP) plan. Appendix A has been published to address Salmonella spp. (S), and the “zero tolerance policy” has been issued to address E. coli O157:H7 (EC) and L. monocytogenes (LM) in products containing beef and RTE meats respectively (USDA-FSIS, 1999; USDA-FSIS, 2010). Processors can control for LM, especially in the post-processing environment, by using one of the three alternatives outlined in the Code of Federal Regulations (CFR; USDA-FSIS, 2014). To specifically address EC, USDA-FSIS has stated five practices that processors must utilize to ensure a safe product. One of those practices is to validate a 5 log_{10} bacteria lethality treatment (USDA-FSIS, 2001).

There are multiple definitions of traditional foods found in the literature. Bertozzi (1998) defines a traditional food as “representation of a group, it belongs to a defined space, and it is part of a culture that implies the cooperation of the individuals operating in that territory.” Germany, Hungary, and Italy are associated with the production of salami, with Italian salamis being the focus of this research (Leroy et al., 2013).

Traditionally, Italian salamis are processed without a heat treatment and are a raw, RTE product. Pork is used to produce Italian salamis due to the flavor and appearance pork provides to the finished product (Leistner, 1995). Processing consists of mixing the
meat, spice blend (including 2.5-3.0% salt and nitrite), and starter culture; stuffing into casings; fermenting at low temperatures; and drying at 15°C or less for up to six months to develop the aroma consumers are familiar with (Leistner, 1995). The combination of a high salt content, low pH, nitrite, and low water activity work \( (a_w) \) synergistically to create an appealing sensory profile, as well as inhibit microbial growth (Työppönen et al., 2003; Hospital et al., 2014).

Despite being fermented and dried, non-heat treated sausages have led to global outbreaks. *Salmonella* Goldcoast in Thuringia, Germany led to an outbreak of 44 cases from the consumption of a raw, fermented sausage (Bremer et al., 2004). In 2010, 69 salmonellosis cases were identified in France as the result of the consumption of dried pork sausage contaminated with *Salmonella enterica* serotype 4,12:i:- (Bone et al., 2010).

One of the challenges facing processors of traditional salami is the safety of the product. Today’s traditionally-produced salamis are starting to incorporate the “traditional meets technology” concept. Traditionally, the natural flora of the meat was used for fermentation, which could lead to growth of spoilage or pathogenic organisms. The pH of the product was not controlled and could vary from product-to-product. Today, starter cultures are utilized for controlled acidification and a quicker fermentation period. Starter cultures have also been selected for bacteria that produce bacteriocins. Bacteriocins play an important part in slightly acidified salamis by inhibiting the growth of Gram-positive bacteria, namely LM (Leroy et al., 2006). Artisanal salamis have a unique flavor component due to the natural microflora of the raw meat; however, starter culture development has been able to replicate these flavors and standardize aromas and flavors across salamis.
Though not traditionally done, antimicrobials are applied to raw product as an extra hurdle to prevent bacterial growth, or in the post-processing environment to reduce microorganisms that could reduce product safety or shelf-life. The use of citric acid, lactic acid, acetic acid, or a combination has been accepted as a safe way to inhibit bacterial growth.

A concern with bacteria is the concept of cross-adaptation. Bacteria have been demonstrated to enhance resistance to stress factors upon initiation of stress. For example, Greenacre and Brocklehurst (2006) found S. Typhimurium pre-exposed to one molar of acetic acid provided cross-protection against increased salt concentrations and lower a\textsubscript{w}. Through cross-protection, bacteria have the capability to withstand multiple hurdles simultaneously. In relation to fermented sausage manufacture, bacteria may undergo the process of cross-protection. The gradual decrease in pH may lead to acid resistance and cells remaining after production (Lou and Yousef, 1997).

Previous research has demonstrated the efficacy of traditional processing of landjäger with no thermal treatment (Rivera-Reyes et al., 2017). The objectives of this experiment were to validate a process used to make traditionally-processed salami using modern day technologies and determine if a correlation can be associated with weight loss and a\textsubscript{w} for processor control.

**Materials and Methods**

**Preparation of Inoculum**

Cultures of EC (ATCC 43895, ATCC BAA-460, and PA-2), S (S. Typhimurium ATCC 14028, S. Montevideo SMvo13, and S. Panama ATCC 7378) and LM (Scott
serotype 4b, H3396 serotype 4b, and FSL J1-129 serotype 4b) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), Center for Disease Control and Prevention (CDC; Atlanta, GA), and the Microbiology Culture Collection at The Pennsylvania State University Food Science Department. Frozen cultures were transferred to fresh tryptic soy broth (TSB; Becton Dickinson and Company; BD, Sparks, MD) and incubated aerobically at 37°C for 24 h. The overnight cultures of EC, S, and LM were streaked onto Cefixime-tellurite Sorbitol MacConkey agar (CT-SMAC; BD), Xylose Lysine Deoxycholate agar (XLD; BD), and Modified Oxford agar (MOX; BD), respectively. Plates were incubated at 37°C for 24 h before performing culture confirmation tests (EC: Remel; Lenexa KS; S: Oxoid; Hants, United Kingdom; LM: Microgen Bioproducts; Camberley, UK). Individual colonies were used to inoculate fresh TSB for each bacterium. Bottles were incubated at 37°C for 24 h to obtain cell concentrations of ~8 log<sub>10</sub> CFU/ml (adapted from USDA-FSIS, 2012). The inoculation bath was prepared by mixing the three strains of each bacteria together in a sterile autoclave bin.

**Preparation and Inoculation of Sausages**

Pork shoulder butts (Indiana Kitchen, Delphi, IN) were stored frozen (-5°C) and thawed at 3.3°C prior to cutting into approximately 2.54 cm x 2.54 cm cubes. Cubes were vacuum-packaged and stored at 2-4°C until ready for use (up to 48 h).

Pork cubes were placed in an inoculation bath and stirred every 5 min for a total of 30 min to allow for adequate pathogen attachment. The pork was removed using a
sterilized slotted spoon to drain excess liquid and then placed into a sterile tub. Following a 30 min chilling period (4°C), the pork was sprayed with one of two treatments. Half of the pork was spread into a single layer on trays (2.99 g/cm²) and sprayed with tap water (23.9°C) for 30 s on each side (CTRL). The other half was sprayed with a 2.5% Beefxide solution (23.9°C; Birko, Henderson, CO) for 30 s on each side (TRT). The pork was chilled (4°C) overnight before grinding.

After 24 h, the meat was ground through a 6-mm plate (MG22 #22, Avantco, China). The dry ingredients were added (Table 4.1) followed by the starter culture (SafePro B-LC-007, CHR Hanson, Milwaukee, WI). Three casing types were used: beef middles (Globe Casings, Carlstadt, NJ), collagen (ConYeager Spice Company, Inc., New Castle, PA), and fibrous (Globe Casings, Carlstadt, NJ). Sausages were stuffed to an average weight of 204.4 ± 10.2 g and average diameter of 49.7 ± 1.2 mm. Casings for the CTRL were prepared by placing in 3 L tap water at 41°C and casings for the TRT were prepared in 3 L 2.5% Beefxide solution prepared at 41°C. Each casing contained three sausages, with one casing having a single sausage to serve as the reference sausage. Sausages were sprayed with distilled white vinegar (5% acetic acid; Giant Food Stores, LLC, Carlisle, PA) at days 11 and 25 to control mold growth on the casings.
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork shoulder butt (80/20)</td>
<td>457.2</td>
</tr>
<tr>
<td>Salt</td>
<td>457.2</td>
</tr>
<tr>
<td>Dextrose</td>
<td>133.4</td>
</tr>
<tr>
<td>Potassium nitrate (KNO₃)</td>
<td>16.4</td>
</tr>
<tr>
<td>Sodium erythorbate</td>
<td>10.4</td>
</tr>
<tr>
<td>Curing salt (6.25% NaNO₂)</td>
<td>47.6</td>
</tr>
</tbody>
</table>

Table 4.1 Salami formulation.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Temperature °C</th>
<th>RH %</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static Cooling</td>
<td>6-8</td>
<td>0</td>
<td>5 h</td>
</tr>
<tr>
<td>Hot Dry</td>
<td>24-26</td>
<td>0</td>
<td>36 h</td>
</tr>
<tr>
<td>Drying</td>
<td>24-26</td>
<td>55-65</td>
<td>12 h</td>
</tr>
<tr>
<td>Drying</td>
<td>22-24</td>
<td>60-70</td>
<td>12 h</td>
</tr>
<tr>
<td>Drying</td>
<td>20-22</td>
<td>65-75</td>
<td>12 h</td>
</tr>
<tr>
<td>Drying</td>
<td>18-20</td>
<td>68-78</td>
<td>24 h</td>
</tr>
<tr>
<td>Drying</td>
<td>16-18</td>
<td>72-80</td>
<td>24 h</td>
</tr>
<tr>
<td>Drying</td>
<td>14-16</td>
<td>75-82</td>
<td>24 h</td>
</tr>
<tr>
<td>Seasoning</td>
<td>12-14</td>
<td>75-80</td>
<td>24 h</td>
</tr>
<tr>
<td>Seasoning*</td>
<td>12-14</td>
<td>77-85</td>
<td>21 d</td>
</tr>
</tbody>
</table>

Table 4.2 Drying cabinet settings during the manufacture of dry pork salamis. *During this time, the drying cabinet had a run-pause cycle of 30 min run time and 45 min pause time for the fan.

Table 4.2 shows the drying cabinet (AS50/A; Salumifici Conditioning Sytems Vanni Sprocatti and C snc., Modena, Italy) inputs for temperature, relative humidity (RH) and time for each phase during the process. Sausages were fermented to a pH of 4.9 ± 0.1 and dried to an aw<0.88 and weight loss of 38-40%. Following ripening, salamis were vacuum-sealed (Ultravac UV-250, UltraSource, Kansas City, MO) in 101.6 mm x 254.0 mm bags of 3 mil thickness (OTR average 60 cc/m²/24 h; UltraSource, Kansas City, MO).
Microbial Analysis

Samples were collected every 24 h for the first 96 h, after which they were collected weekly. Three salamis (n=3) were collected per sample time to make a composite sample for each casing type. Samples were individually prepared by creating a 1:5 dilution in Buffered Peptone Water (BPW; Hardy Diagnostics; HD; Santa Maria, CA) into a filtered stomacher bag (Interscience, St.-Normandy, France). Samples were homogenized for 1 min at 230 RPM (Stomacher® 400 Circulator; Seward Limited; West Sussex, UK) and 10 mL of each sample saved for the composite sample. The composite sample was serially diluted using 9 ml of BPW.

Aliquots (0.1ml) were plated in duplicate on CT-SMAC, XLD, and MOX to determine survival of EC, S, and LM, respectively. Tryptic soy agar (TSA; HD) was also plated for total bacteria counts. CT-SMAC, XLD, and TSA plates were incubated at 37°C for 24 h, and MOX plates for 48 h at 37°C in accordance to the USDA Microbiological Laboratory Guidebook (USDA-FSIS, 2012).

Samples were enriched (plated simultaneously) for the various sampling times when colonies were below the detection limit (0.4 CFU/g). For EC enrichment, 1 ml of the stomachate was transferred to 9 ml of Gram Negative broth, Hajna (BD) and incubated for 24 h at 37°C. The enrichment was streak-plated onto CT-SMAC, incubated for 24 h at 37°C and examined for colonies. For S enrichment, 1 ml of the stomachate was transferred to 9 ml of Lactose broth (HiMedia, Mumbai, India), grown for 24 h at 37°C, followed by 1 ml transferred to 9 ml of Rappaport-Vassiliadis R10 broth (HD). After a 24 h incubation at 37°C, the Rappaport-Vassiliadis R10 broth was streak-plated on XLD, incubated at 37 °C for 24 h and examined for colonies. For LM enrichment, 1
ml of the stomachate was transferred to 9 ml of UVM Modified Listeria Enrichment broth (HD), grown for 24 h at 37°C, followed by a transfer of 1 ml to 9 ml of Fraser broth (Oxoid; Basingstoke, UK). The Fraser broth enrichment was grown for 24 h at 37°C, streaked onto MOX agar, incubated at 37 °C and examined for the presence or absence of colonies after 48 h of incubation. Colonies were verified using confirmation tests(EC: Remel; S: Oxoid; LM: Microgen Bioproducts).

In addition to microbial analyses, pH (Testo 206-pH2 pH Meter; Testo, Inc; Sparta, NJ) and \( a_w \) (AquaLab Water Activity Meter, Series 4TE; Decagon Devices, Inc.; Pullman, WA) were measured at each sampling time for each casing diameter. Reference sausages were weighed and product diameter (dialMax; Wiha Quality Tools; Monticello, MN) recorded at each sampling time.

**Weight and \( a_w \) Correlation**

Product weight and water activity for each casing type was graphed using Excel 2016 (Microsoft, Redmond, WA). A line of best fit was established using the program and an R\(^2\) value calculated.

**Statistical Analysis**

Bacterial populations were converted to \( \log_{10} \) CFU/g to complete statistical analyses. For plates with zero populations, a CFU count of 0.01 less than the detection limit (0.39 \( \log_{10} \) CFU/g) was assigned to incorporate into the analyses. Statistical tests were performed using a mixed model procedure in Statistical Analysis Software (Version 9.4, SAS Institute Inc., Cary, NC). The model included differences between the CTRL
and TRT, differences within a casing type by day for CTRL and TRT, differences between the casing types at each day for CTRL and TRT, and a casing type by time interaction for CTRL and TRT. A significance level of \( p < 0.05 \) was used.

**Results**

The correlation between weight and \( a_w \) was found to be \( R^2 = 0.8826 \); however, this correlation is only possible in a product made with this formulation. The type and amount of dry ingredients added impact \( a_w \) by binding water molecules. The correlation should not be the sole source for \( a_w \) in this salami in order to have quality or product safety control.

There was no significant difference between the CTRL and TRT sausages for EC \((p = 0.1645)\), S \((p = 0.3746)\), or LM \((p = 0.1762)\). pH did not differ between the CTRL and TRT sausages; however, the CTRL sausages were observed to have a pH 0.1-0.3 higher than the TRT sausages, especially during packaging. The \( a_w \) did not differ between CTRL and TRT sausages. CTRL sausages had an average final \( a_w \) of 0.86 between the three casings, and the TRT sausages had an average final \( a_w \) of 0.85 between the three casings.

Casing type did not have an effect on EC populations in the CTRL sausages \((p = 0.5315)\) or TRT sausages \((p = 0.9193)\). Figure 4.1 and Table 4.3 show EC populations for both CTRL and TRT sausages. Following treatment sprays, EC decreased by 3.18 \( \log_{10} \) on the trim used for the CTRL sausages and decreased by 2.4 \( \log_{10} \) CFU/g on the trim used for the TRT sausages. An increase in EC populations was seen with both CTRL and TRT sausages between 24 h (grinding) and 48 h (24 h of fermentation).
Within the CTRL sausages, EC populations decreased $1.49$, $3.19$, and $1.49$ log $10$ CFU/g in the natural, collagen, and fibrous casings, respectively, following the fermentation and drying. TRT sausages decreased by $2.15$, $2.16$, and $2.18$ log $10$ CFU/g in the natural, collagen, and fibrous casings, respectively, during fermentation and drying. Following packaging, CTRL sausages had an additional reduction of $2.26$, $0.78$, and $2.04$ log $10$ CFU/g for natural, collagen, and fibrous casings, respectively. Following packaging, TRT sausages had a $1.77$, $1.86$, and $1.73$ log $10$ CFU/g reduction for natural, collagen, and fibrous casings, respectively.

Casing type did not have an effect on S populations in the CTRL sausages ($p=0.6581$) or TRT group ($p=0.9734$). Figure 4.2 and Table 4.4 show S populations for both CTRL and TRT sausages. The treatment sprays provided a $0.38$ log $10$ and $0.34$ log $10$ CFU/g reduction on pork trim used for the CTRL and TRT sausages, respectively. S populations began to decrease with the first $24$ h of fermentation and decreased $0.35$ to $0.51$ log $10$ CFU/g.

S populations decreased $2.21$ log $10$ in the natural casing, $2.46$ log $10$ in the collagen casing, and $2.62$ log $10$ CFU/g in the fibrous casing in the CTRL sausages during fermentation and drying. TRT sausages had S reductions of $2.76$ log $10$ in the natural casing, $2.77$ log $10$ in the collagen casing, and $2.89$ log $10$ CFU/g in the fibrous casing during fermentation and drying. Through packaging and storage, S populations decreased $2.90$, $2.84$, and $2.00$ log $10$ CFU/g in the natural, collagen, and fibrous casings, respectively, in the CTRL sausages. S populations decreased $3.46$, $3.44$, and $3.02$ log $10$ CFU/g in the natural, collagen, and fibrous casings in the TRT sausages, respectively.
Casing did not have an effect on LM populations in the TRT sausages ($p=0.1610$), but did have an effect in the CTRL sausages ($p=0.0192$). Figure 4.3 and Table 4.5 show LM populations for both CTRL and TRT sausages. The treatment sprays provided a $0.12 \log_{10}$ and $0.19 \log_{10}$ CFU/g reduction on pork trim used for the CTRL and TRT sausages, respectively. LM populations decreased $0.31$ to $0.59 \log_{10}$ CFU/g within the first 24 h of fermentation.

LM populations decreased $3.61$, $3.29$, and $3.37 \log_{10}$ CFU/g in the natural, collagen, and fibrous casings, respectively, in the CTRL sausages during fermentation and drying. LM populations in the TRT sausages decreased $2.99$, $2.69$, and $4.56 \log_{10}$ CFU/g in the natural, collagen, and fibrous, respectively, casing in the TRT sausages. An additional $3.24$ to $3.72 \log_{10}$ reduction was achieved during packaging in the CTRL sausages, and an additional $1.92$ to $3.99 \log_{10}$ reduction achieved during packaging in the TRT sausages.

Specific differences in bacterial populations between casings were observed at days 25 and 39 in the CTRL sausages. At D25, bacteria populations in fibrous and natural casings were significantly different ($p=0.0081$) and bacteria populations in fibrous and collagen casings were significantly different ($p=0.0036$). A significant difference was seen in bacteria populations at D39 between fibrous and collagen casings ($p=0.0053$). A significant difference was observed in bacteria populations in sausages in fibrous and natural casings ($p=0.0004$) and fibrous and collagen casings ($p<0.0001$) at D32 for the TRT sausages. However, there were no significant differences in bacteria populations, regardless of casing type, on the last day of the experiment.
Figure 4.1 Average *E. coli* O157:H7 populations (log10 CFU/g). A is results for CTRL and B is results for TRT.
<table>
<thead>
<tr>
<th>Sample time</th>
<th>Natural</th>
<th>Collagen</th>
<th>Fibrous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTRL</td>
<td>TRT</td>
<td>CTRL</td>
</tr>
<tr>
<td>0h</td>
<td>6.88 ± 0.64^a</td>
<td>6.48 ± 0.76^ac</td>
<td>6.88 ± 0.64^ac</td>
</tr>
<tr>
<td>24h</td>
<td>3.70 ± 1.31^b</td>
<td>4.08 ± 0.90^bd</td>
<td>3.70 ± 1.31^b</td>
</tr>
<tr>
<td>Reduction</td>
<td>3.18</td>
<td>2.40</td>
<td>3.18</td>
</tr>
<tr>
<td>48h</td>
<td>6.84 ± 0.04^a</td>
<td>6.79 ± 0.03^ac</td>
<td>6.85 ± 0.04^ac</td>
</tr>
<tr>
<td>96h</td>
<td>6.57 ± 0.17^a</td>
<td>6.30 ± 0.31^af</td>
<td>6.56 ± 0.32^ac</td>
</tr>
<tr>
<td>Reduction</td>
<td>+2.87</td>
<td>+2.22</td>
<td>+2.86</td>
</tr>
<tr>
<td>D11</td>
<td>6.40 ± 0.31^a</td>
<td>6.38 ± 0.01^af</td>
<td>6.35 ± 0.29^ac</td>
</tr>
<tr>
<td>D18</td>
<td>6.19 ± 0.33^ac</td>
<td>6.14 ± 0.15^ab</td>
<td>6.02 ± 0.28^a</td>
</tr>
<tr>
<td>D25</td>
<td>5.78 ± 0.32^acd</td>
<td>5.49 ± 0.22^ab</td>
<td>5.62 ± 0.23^a</td>
</tr>
<tr>
<td>D32</td>
<td>5.39 ± 0.47^acd</td>
<td>4.33 ± 0.33^abe</td>
<td>3.69 ± 0.28^c</td>
</tr>
<tr>
<td>Reduction</td>
<td>1.18</td>
<td>1.97</td>
<td>2.87</td>
</tr>
<tr>
<td>D39</td>
<td>4.39 ± 0.11^ad</td>
<td>3.51 ± 0.36^abe</td>
<td>3.63 ± 0.18^bc</td>
</tr>
<tr>
<td>D45</td>
<td>3.77 ± 0.01^abd</td>
<td>3.60 ± 0.30^cde</td>
<td>3.39 ± 0.19^bc</td>
</tr>
<tr>
<td>D53</td>
<td>3.56 ± 0.34^abd</td>
<td>2.22 ± 0.92^cde</td>
<td>2.79 ± 0.15^bc</td>
</tr>
<tr>
<td>D60</td>
<td>3.13 ± 1.16^b</td>
<td>2.56 ± 0.96^cde</td>
<td>2.91 ± 0.46^bc</td>
</tr>
<tr>
<td>Reduction</td>
<td>2.26</td>
<td>1.77</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 4.3: Average E. coli O157:H7 populations (log_{10} CFU/g). Statistical differences are noted by different letters within treatments (p<0.05). Pairwise comparisons were completed using forward comparisons. There were no significant differences between casings. Reductions are the differences between the last day of a phase and the last day of the previous phase. TR=total log_{10} reduction.
Figure 4.2 Average *Salmonella* spp. populations (log$_{10}$ CFU/g). A is results for CTRL and B is results for TRT.
<table>
<thead>
<tr>
<th>Sample time</th>
<th>Natural CTRL</th>
<th>Natural TRT</th>
<th>Collagen CTRL</th>
<th>Collagen TRT</th>
<th>Fibrous CTRL</th>
<th>Fibrous TRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>7.48 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.28 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.48 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.28 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.48 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.28 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24h</td>
<td>7.10 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.94 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.10 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.94 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.10 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.94 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>0.38</td>
<td>0.34</td>
<td>0.38</td>
<td>0.34</td>
<td>0.38</td>
<td>0.34</td>
</tr>
<tr>
<td>48h</td>
<td>6.60 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.47 ± 0.19&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>6.75 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.53 ± 0.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.69 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.43 ± 0.25&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>96h</td>
<td>6.16 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.92 ± 0.20&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>6.14 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.91 ± 0.25&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>5.95 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.85 ± 0.31&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>0.94</td>
<td>1.02</td>
<td>0.96</td>
<td>1.03</td>
<td>1.15</td>
<td>1.09</td>
</tr>
<tr>
<td>D11</td>
<td>5.79 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.16 ± 0.24&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>5.79 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.12 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.86 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.42 ± 0.24&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>D18</td>
<td>5.58 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.11 ± 0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.48 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.58 ± 1.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.21 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.94 ± 1.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D25</td>
<td>5.03 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.35 ± 1.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.85 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.07 ± 1.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.04 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.20 ± 1.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D32</td>
<td>4.39 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.84 ± 1.94&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.29 ± 1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.83 ± 1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.45 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.71 ± 1.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>1.77</td>
<td>2.08</td>
<td>1.85</td>
<td>2.08</td>
<td>1.50</td>
<td>2.14</td>
</tr>
<tr>
<td>D39</td>
<td>2.29 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.78 ± 0.39&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.48 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.15 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39 ± 0.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>D46</td>
<td>1.97 ± 0.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.78 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.11 ± 0.81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39 ± 0.01&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.31 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02 ± 0.63&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>D53</td>
<td>2.07 ± 1.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.65 ± 1.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.69 ± 0.31&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>1.90 ± 1.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.15 ± 0.76&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>D60</td>
<td>1.49 ± 1.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.45 ± 1.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39 ± 0.01&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.45 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.69 ± 0.31&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>2.90</td>
<td>3.46</td>
<td>2.84</td>
<td>3.44</td>
<td>2.00</td>
<td>3.02</td>
</tr>
<tr>
<td>TR</td>
<td>5.99</td>
<td>6.90</td>
<td>6.03</td>
<td>6.89</td>
<td>5.03</td>
<td>6.59</td>
</tr>
</tbody>
</table>

Table 4.4 Average *Salmonella* spp. populations (log<sub>10</sub> CFU/g). Statistical differences are noted by different letters within treatments (p<0.05). Pairwise comparisons were completed using forward comparisons. There were no significant differences between casings. Reductions are the differences between the last day of a phase and the last day of the previous phase. TR=total log<sub>10</sub> reduction.
**Figure 4.3** Average *L. monocytogenes* populations (log_{10} CFU/g). A is results for CTRL and B is results for TRT.
<table>
<thead>
<tr>
<th>Sample time</th>
<th>Natural</th>
<th>Collagen</th>
<th>Fibrous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTRL</td>
<td>TRT</td>
<td>CTRL</td>
</tr>
<tr>
<td>0h</td>
<td>7.55 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.37 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.55 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24h</td>
<td>7.43 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.18 ± 0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.43 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>0.12</td>
<td>0.19</td>
<td>0.12</td>
</tr>
<tr>
<td>48h</td>
<td>6.87 ± 0.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.71 ± 0.02&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>6.84 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>96h</td>
<td>5.75 ± 0.42&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.93 ± 0.17&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>5.94 ± 0.26&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>1.68</td>
<td>1.25</td>
<td>1.49</td>
</tr>
<tr>
<td>D11</td>
<td>5.52 ± 0.33&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>5.41 ± 0.08&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>5.52 ± 0.26&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>D18</td>
<td>5.00 ± 0.31&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.31 ± 0.07&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>5.27 ± 0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>D25</td>
<td>3.94 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.55 ± 0.02&lt;sup&gt;df&lt;/sup&gt;</td>
<td>4.18 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>D32</td>
<td>3.94 ± 0.15&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>4.38 ± 0.12&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>4.26 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>1.81</td>
<td>1.55</td>
<td>1.68</td>
</tr>
<tr>
<td>D39</td>
<td>2.77 ± 1.07&lt;sup&gt;de&lt;/sup&gt;</td>
<td>1.18 ± 0.78&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.29 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>D45</td>
<td>1.93 ± 0.58&lt;sup&gt;fg&lt;/sup&gt;</td>
<td>2.40 ± 0.14&lt;sup&gt;h&lt;/sup&gt;</td>
<td>2.16 ± 0.53&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>D53</td>
<td>1.35 ± 0.35&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.02 ± 0.13&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1.48 ± 0.24&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>D60</td>
<td>0.70 ± 0.30&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.79 ± 0.09&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.54 ± 0.16&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduction</td>
<td>3.24</td>
<td>3.59</td>
<td>3.72</td>
</tr>
<tr>
<td>TR</td>
<td>6.85</td>
<td>6.58</td>
<td>7.01</td>
</tr>
</tbody>
</table>

Table 4.5 Average *L. monocytogenes* populations (log<sub>10</sub> CFU/g). Statistical differences are noted by different letters within treatments (p<0.05). Pairwise comparisons were completed using forward comparisons. Reductions are the differences between the last day of a phase and the last day of the previous phase. TR=total log<sub>10</sub> reduction.
Discussion

Traditionally, Italian salamis are produced using the natural microflora in the meat for fermentation and then dried. This experiment is one of the first to demonstrate the safety of a fermented and dried salami that does not have thermal processing. Traditional methods met modern technology to achieve product safety. A bio-protective starter culture was used to control pH and the rate of decline. The starter culture also produced bacteriocins that inhibited the growth of S and LM as demonstrated with the first 24 h of fermentation.

Antimicrobials also have proven to be effective against spoilage and pathogenic bacteria (Greer and Dilts, 1995; Stivarius et al., 2002). Laury et al. (2009) tested the efficacy of Beefxide on beef trim and achieved a $1.4 \log_{10} \text{CFU/100 cm}^2$ reduction in EC and $1.1 \log_{10} \text{CFU/100 cm}^2$ reduction for S immediately after spray application. In this study, we achieved a $2.4 \log_{10} \text{CFU/g}$ reduction for EC and $0.34 \log_{10} \text{CFU/g}$ reduction for S after 24 h. Bacterial populations were measured in the first 24 h (data not shown) and demonstrated a small reduction in EC and S on pork trim immediately after application of a 2.5% Beefxide solution. The difference observed may be due to sampling method. Laury et al. (2009) used microbial swabs covering 100 cm$^2$, while this experiment used a thin portion of the top muscle layer for sampling.

Ellebracht et al. (1999) demonstrated the efficacy of a 2% lactic acid spray on beef trimmings proved most effective at reducing EC and S. Typhimurium, compared to a hot water wash. Lactic acid may be used up to 5% in sprays and dips, and may provide a higher level of efficacy to reduce pathogenic microorganisms on pork trim (USDA-FSIS,
The use of a lactic acid spray instead of Beefxide in this product would require further research.

Drying was effective at reducing pathogenic bacteria populations, but did not inhibit bacteria enough to achieve a $5 \log_{10}$ reduction for any of the three bacteria. The first week of packaging provided the additional bacteria reductions required to achieve a $5 \log_{10}$ reduction for S and LM, except for LM in the natural and fibrous casings in the CTRL sausages. The $5 \log_{10}$ reduction was achieved by D46 for the natural casing and D53 for the fibrous casing. Vacuum-packaging inhibited bacteria by reducing the level of oxygen available for bacteria growth.

Holding products may be problematic to producers who want to ship their product as quickly as possible to maximize shelf-life. Holding a product to ensure pathogenic bacteria loads are at an acceptable level also increases the likelihood an employee would ship the product too soon and lead to a potential recall. Conducting a recall, in addition to discarding recalled product, would cost the company both time and money.

Following the spray treatment, the CTRL sausages had higher EC and S reductions compared to the TRT, but had lower reductions for LM. This is opposite of what is expected as the Beefxide reduces Gram-negative microorganisms. The TRT sausages had the largest decrease in most casings following fermentation and drying, and had fewer bacterial populations throughout packaging. This demonstrates application of an antimicrobial early in the process has a long-term effect when followed by fermentation, drying, and packaging.

Additional lethality treatments may be utilized to achieve a $5 \log_{10}$ reduction sooner than the one week of packaging. Ducic et al. (2016) used pasteurization on dry
sausages following a fermentation and drying period of 15 days, but found that pasteurizing at too high of a temperature had a negative impact on sausage quality. Porto-Fett et al. (2010) produced a non-heat treated Genoa salami and followed the drying phase with a high-pressure processing (HPP) treatment, then stored the product for four weeks. HPP at 600 MPa for 5 min was enough to achieve a $5 \log_{10}$ CFU/g reduction of S and LM in most of the salamis made, and 600 MPa for 1 min was enough to achieve the proper lethality level for EC (Porto-Fett et al., 2010). Although HPP achieved a $5 \log_{10}$ reduction, the paper does not state the effects of HPP on product quality.

This experiment used ingredients that would be common among processors and presented a worse-case scenario salami by using minimal ingredients to produce the product. Additional non-meat ingredients added to salamis, such as garlic and other seasonings, may further affect the survival of pathogens. The addition of more ingredients could bind water molecules and decrease $a_w$, making it difficult for microbes to grow and survive. Certain ingredients have also been demonstrated to exhibit antimicrobial properties. Linares et al. (2013) studied the antimicrobial effects in vitro of garlic and red wine in chouriço and found garlic powder and juice were able to inhibit LM and S. Garlic powder and garlic juice worked synergistically with red wine to increase the antimicrobial effect in the meat batter. Essential oils of several herbs and spices have also been found to decrease LM, S, and S. aureus populations in dry sausages (Garcia-Diez et al., 2016).

This study does not validate a process for a pork salami produced in beef middles due to the inability to achieve a $5 \log_{10}$ reduction. USDA-FSIS has 5 alternatives to EC control in beef products (USDA-FSIS, 2001). Another option processors could utilize is
implementing a raw product testing program on incoming beef products and making sure there is no more than $2 \log_{10} \text{CFU/g}$. By adhering to that guideline, processors would be able to produce this product in beef middles, as greater than a $2 \log_{10}$ reduction was achieved through fermentation, drying, and packaging.

In the United States, a heat treatment is commonly applied to RTE meat products to extend shelf life and reduce pathogenic load. Despite not having a heat treatment, the procedures and ingredients used in the current experiment were able to achieve a $5 \log_{10}$ reduction of S and LM. This result is due to the combined effects of obstacles presented to the pathogens, such as by-products produced by lactic acid bacteria, reduced pH and $a_w$, and a reduced oxygen environment.

Although this process should be accepted by the USDA-FSIS as validation for HACCP plans, there may be regulatory issues. In the majority of HACCP plans, biological hazards are addressed using temperature to control growth or reduce or eliminate bacteria. Except for raw material being stored at low temperatures, this product is kept within the “danger zone” (4.4-60°C) and may lead to issues with acceptance by USDA-FSIS personnel.

This experiment provides valid scientific support for meat processors for HACCP plans when producing a non-heat-treated, fermented and dried salami. It is advised slaughter facilities and processing facilities utilize good manufacturing practices and purchase meat from reputable suppliers to minimize pathogenic contamination and start with a low microbial load for raw product. Although there was no significant difference between the CTRL and TRT sausages, it is recommended processors spray trim with an antimicrobial prior to grinding to achieve additional reductions of S and LM.
References


Chapter 5

Conclusions
The research presented demonstrates how changing food safety parameters may affect the survival of pathogenic bacteria. A 5 log<sub>10</sub> reduction was able to be achieved by altering fat content, grind size, and product diameter. These parameters also may include a mild heat treatment during processing. Casing type did not have an effect on pathogen survival, and a 5 log<sub>10</sub> reduction was achieved for *Salmonella* spp. and *L. monocytogenes*, despite not having a heat treatment for salamis vacuum-packaged after drying. The use of antimicrobials is effective at reducing microbial populations on meat products.

This research provides the groundwork for future projects, especially traditionally-made sausages produced without a heat treatment. With regard to raw sausages, future research could be conducted on sausages of larger diameter. Diameter may impact the survival of pathogens, although this was not evident when thermal processing was incorporated into the production of salamis. More work is also needed on all-beef or beef and pork mixed products, since USDA considers *E. coli* O157:H7 a pathogen of concern and must achieve a 5-log reduction to produce a safe product. Should this research be repeated on sausages containing beef, using non-O157:H7 STEC would strengthen the research as those bacteria are becoming problematic in the industry.

The final suggestions for future work is to alter the addition of dry ingredients to eliminate synthetic dry ingredients, such as potassium nitrate and sodium nitrite, and replace those ingredients with natural ingredients, such as celery juice powder and other natural antioxidants. As the move towards clean labeling becomes stronger, this type of research is going to be important for ensuring natural ingredients can be added at rates that produce a safe product without effecting the end flavor of the sausages.
The results of the current research may be used by processors, scientists, and USDA-FSIS personnel. Processors can utilize the results to ensure similarly-processed products achieve a $5 \log_{10}$ reduction and therefore produce a safe, ready-to-eat meat product. The data should be accepted by USDA-FSIS personnel as a means of scientific support for HACCP plans. Scientists may use this research as the foundation for future innovations to continue to strive to create safe ready-to-eat products that meet the quality and shelf-life standards of consumers.