INVESTIGATION OF THE BACTERIAL SAFETY OF FERMENTED AND DRIED DUCK SALAMI

A Thesis in
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by
Samuel C. Watson

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The thesis of Samuel C. Watson was reviewed and approved* by the following:

Jonathan A. Campbell
Associate Professor of Animal Science
Thesis Advisor

Catherine N. Cutter
Professor of Food Science

Edward W. Mills
Associate Professor of Meat Science

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

Creating artisanal, dry salami products is an increasing trend for charcuterie companies in the United States. These raw, ready-to-eat products are required by USDA-FSIS to have a scientifically valid HACCP system addressing relevant biological hazards. To our knowledge, no literature exists for the validation (at least a $5 \log_{10}$ reduction of pathogens per FSIS) of salami products containing duck. Therefore, experiments were designed to validate the safety of duck salami. The objectives of this study were to determine if *Salmonella* spp., *Listeria monocytogenes*, and *Campylobacter* spp. could cohabitate in an inoculation cocktail, to validate the safety of fermented and dried duck salami, and to investigate if a duck salami manufacturing processes could achieve a $5 \log_{10}$ CFU/g reduction of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp.

Prior to conducting the validation study, three separate experiments were performed to determine if three strains each of *Salmonella* spp. and *L. monocytogenes*, two strains of *Campylobacter jejuni*, and one strain of *Campylobacter coli* could cohabitate in an inoculation cocktail. A plate overlay assay demonstrated that there were no direct antagonistic relationships among any of the nine organisms selected. Coinoculation into ground pork demonstrated the selected pathogens could coexist in a meat system for at least 48 hours. Coinoculation on lean pork and pork fat demonstrated that the selected pathogens could successfully attach to meat surfaces in open air, refrigerated environments. Collectively, these three experiments demonstrated that *Campylobacter* spp. can be used in the same validation trials as *Salmonella* spp. and *L. monocytogenes*. 
To begin the validation experiment, duck trim and pork belly (70% duck trim, 30% pork belly) were placed in a mixed culture bath approximating three liters. The culture bath was made by growing individually and then combining three strains each of *Salmonella* spp. and *L. monocytogenes*, two strains of *Campylobacter jejuni*, and one strain of *Campylobacter coli*. After inoculation, meat was air dried (30 min @ 23°C), tumbled with one liter of 2.5% Beefxide® antimicrobial treatment (lactic and citric acid and hydrogen peroxide), and placed in a walk-in cooler (2-4°C) overnight. After inoculation and antimicrobial treatment (~24 h), the meat was ground (6mm grinding plate) and seasoned with salt (2.5%), cure (NaNO₃ & NaNO₂), spices, and starter culture. The ground duck-pork mixture was stuffed into 55mm collagen casings, fermented for 48 hours (23°C, 95% rH), and dried (12°C, 75% rH) to ~45% weight loss (approx. 5 weeks). Salamis were then vacuum packaged and stored at 23°C (approx. 4 weeks). Three independent replications were conducted, and pathogen concentrations, pH, and water activity (a_w) were analyzed at days 0, 1, 2, 3, 5, 10, and weekly until day 66 during each replication. Critical parameters for production included a final pH less than 5.3 and final a_w less than 0.90.

A 5 log₁₀ CFU/g reduction was achieved for all three types of pathogens. *Salmonella* spp. achieved a 5.47 log₁₀ CFU/g (p<0.0001) reduction by day 38, *L. monocytogenes* achieved a 5.20 log₁₀ CFU/g (p<0.0001) reduction by day 59, and *Campylobacter* spp. achieved a 6.85 log₁₀ CFU/g (p<0.0001) reduction by day 45. Final reductions of 7.03 (p<0.0001), 5.90 (p<0.0001), and 7.19 (p<0.0001) log₁₀ CFU/g were achieved for *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp., respectively. During the entire fermentation and drying process, populations of each taxa never increased by more than 1 log₁₀ CFU/g. A final pH of 5.11 and a final a_w of 0.81 were also achieved. The results of this study indicate that the parameters used to
ferment and dry this product are able to achieve a $5 \log_{10}$ CFU/g reduction of each pathogen to validate the safe production of duck salami.
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Chapter 1
Literature Review

Processed Meats

History of Meat Preservation and Salami Production

Salting and drying are some of the oldest methods used to preserve meat, and the consumption of preserved meat was crucial for the growth of ancient civilizations (Zeuthen, 2007a; Hierro et al., 2015b). Curing meat was most likely developed accidentally as a result of nitrate impurities present in salt (Maddock, 2014; Lücke, 2015) that was commonly utilized for meat preservation in ancient civilizations (Hierro et al., 2015b; Majou and Christieans, 2018a). Salt curing as a method of preservation was most commonly used to preserve whole-muscle pieces. Other methods needed to be developed to preserve trim and other parts of the carcass that are commonly made into sausage today and otherwise would have been wasted. Likely developed in ancient Greece around 800 B.C., sausage production was a way to preserve extra trim that was not eaten fresh or salted and dried as whole pieces of muscle. Sausages were made by chopping and mixing meat leftovers with fat and blood, and then cooking to ensure a longer shelf life (Zeuthen, 2007a). Drying as a preservation method for sausages was applied later and was most likely developed in southern Europe during the time of the Roman Empire (Lücke, 1998). Chopped meat was mixed with salt and spices and then stuffed into skins or intestines, and shelf stability was achieved by hanging the sausages to dry in cool, damp rooms for an extended period of time (Zeuthen, 2007a).

Similar to the discovery of salt curing, fermentation of sausages was developed accidentally as butchers and meat processors developed and honed their drying procedures. Raw meat was inoculated during chopping and mixing with lactic acid fermenting bacteria found
naturally on the meat and sausage ingredients, as well as in the environment (Zeuthen, 2007a). Because the dried sausages were not cooked, the bacteria were able to ferment some of the ingredients in the sausage used as flavor components, creating a more acidic final product. As the popularity of dried, fermented sausages grew, butchers and meat processors began to intentionally inoculate sausage batters using “back-slopping”, a technique that involves saving a small portion of a previous batch (aka “Mother Batch”) of sausage batter or a finished sausage and adding it to the next production batch (Zeuthen, 2007a). Back-slopping or back inoculum increased consistency across multiple batches of sausages compared to environmental or ingredient only inoculation and was used by meat processors for centuries and modern salami production facilities (Leroy et al., 2006b).

Although back-slopping is still used, it is not a precise inoculation method. In the 1950’s, lactic acid fermenting starter cultures were isolated from meat and salami products and grown separately as pure cultures (Incze, 1998). Isolating and purifying the culture achieved greater efficiency of fermentation by reducing the time necessary to achieve appropriate lactic acid production and also achieved improved safety by removing any pathogenic bacteria that could have been present in the back inoculum (Lücke, 1998). Adding pure starter cultures directly to meat has modernized salami production and helped to ensure greater repeatability of flavors and textures, as well as overall consistency of final products (Incze, 1998). Today’s large-scale production of salami would not be possible without the improvements that have been made to starter cultures and fermentation techniques.
**Salami**

Salami are a type of sausage that are traditionally made with beef, pork, or a combination of both and are ready-to-eat (RTE) (Hierro *et al*., 2015b; Maddock, 2015; Rust, 2015). Poultry and wild game meat have also been used in variations of popular styles of salami (Arnaud *et al*., 2015). To produce salami, meat containing both lean and fat is ground or chopped to a desired particle size and then mixed with salt, spices, sodium nitrate and/or nitrite, and some form of lactic acid producing starter culture (Maddock, 2015; Rust, 2015). The resulting batter is then stuffed into casings, fermented and dried to a desired moisture level. Depending on the amount of water that is lost during drying, a salami can be classified as a dry or semi-dry sausage. A dry sausage has a final moisture to protein ratio (MPR) < 2.3:1, and a semi-dry sausage has a final MPR > 2.3:1 and <3.7:1 (Rust, 2015). Some salamis can also be cooked to further ensure microbial safety and shelf stability. The pH range of dry and semi-dry sausages depends on the type of sausage that is being produced and can range from 4.5-5.3 (Maddock, 2015). Types and styles of salami vary by region. Many styles of sausage that are common in Europe were brought to the United States by immigrants and have been steadily growing in popularity (Maddock, 2015). Typically, dry and semi-dry salami produced in the United States are drier than those of similar styles produced in Europe. Salamis in the United States also tend to have a lower pH, resulting in a more tangy flavor than their counterparts from Europe (Maddock, 2015). Genoa salami, chorizo, and cacciatore are types of dry sausage commonly produced in Europe (Hierro *et al*., 2015b). Summer sausage, bologna, and snack sticks are common semi-dry sausages produced in both Europe and the United States (Rust, 2015).
Current Trends in Processed Meat Production

There has been a recent increase in the production of old-world, artisanal charcuterie and salumi products in the United States as meat processors are discovering or re-discovering old methods of producing products like salami. In the United States, poultry consumption has been increasing and the presence of poultry sausages on the market has grown with that trend. Poultry meat may also be perceived as ‘healthier’ than beef or pork by some consumers, thus creating the increased potential for interest in processed poultry products (Arnaud et al., 2015). Unlike fermented products made with red meat species, fermented poultry products do not have longstanding traditions associated with their production. Over the last few decades, meat processors have begun to use poultry instead of beef and pork in some of their processed products and have created niche markets for those products. Processed poultry products have not replaced processed products made with beef and pork in popularity, but their popularity is increasing (Alter et al., 2006; Arnaud et al., 2015).

Hurdle Technology

Hurdle technology principles

Hurdle technology relies on implementing the hurdle effect to control the growth and presence of spoilage and pathogenic bacteria in food products (Leistner and Gorris, 1995; Leistner, 2000). The hurdle effect employs the use of multiple preservation methods within a single food product; the limiting of bacterial growth and bacterial death is achieved due to the combined synergistic effect of all of the preservation methods present in the food (Leistner and Gorris, 1995; Khan et al., 2017). Bacterial cell death in foods that rely on hurdle technology for preservation is caused primarily by metabolic exhaustion of the cell due to the cell’s
homeostasis, or ideal internal cellular environment, being disrupted. Cells maintain ideal internal pH, salt ion concentration, and water concentration (osmotic pressure), and changing the external environment around the cell via several preservation methods in combination causes changes to the inside of the cell. In the presence of external cellular stressors, the cell expends energy using various cell membrane pumps in an attempt to rebalance the cell’s ideal internal concentrations (Leistner and Gorris, 1995; Archer, 1996). If a cell is not able to adapt to the environment within the food, it will die. Cell death without thermal processing occurs because cells deplete energy stores while attempting to reestablish an ideal internal cellular environment (Leistner, 2000). Additionally, the combination of multiple preservation methods is able to cause cell death faster than if just one preservation method were applied, because cells will attempt to combat multiple stressors simultaneously, thus exhausting their energy stores faster than if one cellular stressor were being applied (Leistner, 2000).

There are many different hurdles that can be implemented in a food product. Processed meat products like salami rely heavily on hurdle technology to ensure that they are free of pathogenic bacteria. The most effective hurdles to inhibit microbial growth are temperature, pH, water activity, use of preservatives, and the presence of competitive microorganisms (Leistner, 2000; Chawla et al., 2006). The use of temperature in the form of thermal processing, also known as a kill step, is the fastest and easiest hurdle method to kill pathogenic microorganisms. Salami products are fermented using lactic acid bacteria, and this process contributes two major hurdles to the final product. During growth of these fermenting bacteria, available nutrients for all microbial growth are consumed, thereby making it more difficult for pathogens to grow. Fermentation also decreases the pH of the product, making it more difficult for the pathogens to grow. Adding salt, garlic, nitrite, and countless other potential antimicrobial ingredients further
contributes to ensuring that pathogens in a salami are not able to grow and/or survive (Leistner, 2000).

Meat is an excellent growth medium for all types of bacteria including fermenting starter cultures, nonpathogenic spoilage bacteria, and pathogenic bacteria. Meat is composed of water, protein, fat, carbohydrates, and minerals, and its nutrient density is one of the main reasons that it is such an excellent growth medium for bacteria (Dave and Ghaly, 2011; Ghabraie et al., 2016). Favorable pH (5.5-7.0) and high water activity (a_w; 0.98-0.99) of fresh meat also contributes to the suitable environment for microbial growth (Ghabraie et al., 2016).

Temperature

Increasing temperature to lethal levels for bacteria is the most effective method for reducing pathogens in a meat product (Leistner and Gorris, 1995; Leistner, 2000). Cooking poultry to an internal temperature of 165° F (73.9° C) ensures instantaneous death of any pathogen in or on the product. Thermal processing meat products at lower core temperatures for extended time periods can also achieve sufficient lethality of pathogens. According to USDA-FSIS, a 7 log_{10} reduction in *Salmonella* spp. can be achieved in meat products if 130° F (54.4° C) is held for 121 minutes utilizing a cooking process with at least 90% relative humidity (United States Department of Agriculture, 2017). This low temperature-long time treatment is considered to be a thermal lethality treatment by USDA-FSIS and would be sufficient to ensure the safety of meat products.

Traditional salami production methods do not utilize high temperature or low temperature-long time thermal lethality steps to kill pathogens. The traditional salami production steps of fermenting and drying also usually occur at temperatures below thermally lethal levels
(130°F) and above refrigeration levels (40°F) (Maddock, 2014; Hierro et al., 2015; Rust, 2015). The temperatures used for fermenting and drying are ideal for the growth of pathogens, especially *Listeria monocytogenes* (Montville et al., 2012). Thus, temperature of raw meat ingredients, as well as the effectiveness of additional hurdles, must be carefully monitored.

**pH Reduction**

Reducing the pH of raw meat via fermentation has been used as a preservation technique for hundreds of years. Traditional fermentation uses the natural microflora that may be present in the meat or processing environment to slowly decrease the pH of the product, as well as to create aroma and flavor profiles (Leroy et al., 2006). Modern techniques use the direct addition of lactic acid producing starter culture to the meat to achieve a faster decrease in pH and a more reliable fermentation when compared to traditional fermentation. The production of lactic acid from fermentation, when combined with other hurdles, has been shown to inhibit the growth of pathogenic bacteria present in meat. Multiple hurdles in addition to fermentation must be used, because pH reduction alone cannot be relied upon since the pH sufficient to reduce pathogenic loads reliably is often not palatable (Beales, 2004; Leroy et al., 2006).

Pathogenic bacteria function most efficiently with neutral intracellular and extracellular pH; however, most pathogens are able to grow at pH ranging from acidic to slightly basic. Cells grow at acidic pH by maintaining intracellular pH homeostasis thus preventing enzyme activity from being affected. (Archer, 1996; Beales, 2004). Intracellular pH homeostasis is maintained over a small range of pH, and different types of bacteria prefer different intracellular pH ranges. Acidophiles typically prefer an intracellular pH range of 6.5 to 7, neutrophiles typically prefer an intracellular pH range of 7.5 to 8, and alkalophiles typically prefer an intracellular pH range of
8.4 to 9 (Booth, 1985). The membrane bound enzyme H⁺-ATPase is the primary mechanism responsible for maintaining intracellular pH in bacterial cells. A membrane bound rotational motor hydrolyzes ATP, forcing free protons from the cytoplasm through a membrane channel and out of the cell. In very acidic (below 4) environments that are created by the dissociation of strong acids, pH homeostasis can be disrupted by the unregulated flow of protons into the cell, and ATPase is used to actively remove excess protons and keep intracellular pH within the desirable limits (Booth, 1985; Beales, 2004). In moderately acidic environments created by weak acids like lactic acid, dissociated protons do not move freely across the cellular membrane, since weak acids do not dissociate in moderately acidic environments. Instead, weak acids are able to move freely across the cellular membrane and dissociate once they reach the neutral pH environment of the cytoplasm. An excess of intracellular protons is then created by weak acid dissociation inside the cell causing ATPase to pump out the excess protons (Beales, 2004).

During ideal growth under neutral pH, ATPase is typically responsible for using approximately 10% of the cell’s energy, but when the cell is attempting to restore pH homeostasis, ATPase can use upwards of 60% of the cell’s energy (Beales, 2004). In this environment, the cell uses more energy to maintain ATPase activity to continually pump excess protons out of its intracellular environment than if it were in a neutral extracellular environment. Cells may survive without growing in low pH environments if they are able to maintain pH homeostasis, but cell death will occur when the cell requires more energy for maintaining ATPase activity than it is able to produce (Beales, 2004).
**Water activity and osmotic pressure**

Water activity ($a_w$) is defined as the moisture available in a system for microbial growth (Majou and Christeans, 2018). Although decreasing $a_w$ alone is not guaranteed to ensure the death of bacterial pathogens, reducing $a_w$ slows bacterial growth (Sperber, 1983). Lowering the $a_w$ of a meat product achieves greater safety and shelf stability by increasing the osmotic pressure on bacterial cells present in the product (Beales, 2004). Adding solutes like salt or sugar, as well as moisture loss via thermal processing and drying are the most common methods of lowering $a_w$ in meat products. Both adding solutes and removing moisture lower $a_w$ by increasing the amount of dissolved solutes in the environment thereby decreasing the amount of freely available water bacterial cells can use for growth. The resulting stress on the bacterial cells causes them to utilize more energy maintaining favorable internal conditions (Beales, 2004; Majou and Christeans, 2018).

Bacteria require freely available water in both their environment and cytoplasm in order to survive and grow. Since water is able to move freely across bacterial cell membranes, bacteria have developed mechanisms to maintain the intracellular concentration of water. Under ideal growth conditions, cells will have a higher osmotic pressure internally by maintaining a concentration of molecules, called compatible solutes, in the cytoplasm that is slightly more concentrated than the environment external to the cell (Sperber, 1983; Beales, 2004). Compatible solutes are able to be stored in the cytoplasm without impacting other cellular activities, and potassium and sodium ions, as well as sugars like trehalose and sucrose and amino acids like proline and glutamine are commonly used as compatible solutes in the cytoplasm. Due to the semipermeable bacterial membrane, water flows from areas of low ion concentration outside the cell to areas of high ion concentration inside the cell. Outward pressure caused by the inflow of
water is called turgor pressure, and it is responsible for maintaining the shape of bacterial cells. To maintain turgor pressure, cells (Sperber, 1983; Beales, 2004; Montville et al., 2012).

The ionic strength of a cell’s external environment can change depending on multiple factors, including the amount of water present in the environment and the level of dissolved substrates in the environment. Bacterial cells combat changes in external osmolarity and maintain turgor pressure through a process called osmoregulation (Sperber, 1983; Beales, 2004; Montville et al., 2012). When the external environment around a bacterial cell loses water or gains dissolved solutes (salt, sugar, etc.), the change in concentration gradient between the environment and the bacterial cell will cause an excess of water to migrate out of the cell, decreasing the turgor pressure of the cell. The cell responds by increasing the concentration of compatible solutes in the cytoplasm which equilibrates the solute concentration between external and internal environments and allows for some water to flow back into the cell, thus restoring turgor pressure. The cell will resume growth processes once turgor pressure is restored but at a slower rate due to lesser total volume of water present in the cytoplasm (Sperber, 1983; Beales, 2004; Montville et al., 2012).

Bacteria that are able to grow in low $a_w$ meat products do so by maintaining two cellular parameters. The first is maintaining cellular membrane structure in the presence of salt. Membrane bilayer function can be disrupted by the presence of salt in the external environment, and bacteria increase the concentration of anionic phospholipids in the membrane to prevent disruption from occurring. *L. monocytogenes* cells are able to grow in 2% salt in part because the membrane exhibits an increased ratio of anionic lipids to neutral lipids (Beales, 2004). In addition to modifying membrane composition, bacteria that are able to grow in low $a_w$ meat products maintain cytoplasmic enzymatic activity at low $a_w$ using neutral compatible solutes.
Proline, a neutral amino acid, is the preferred osmoprotectant for most bacterial cells, because it can be used at higher concentrations compared to other charged compatible solutes. Glutamate and potassium ions can also be used for osmoregulation, but the two molecules must be used together so that the charges of each molecule do not disrupt cytoplasmic enzyme function. Bacteria that rely solely on charged compatible solutes will increase the internal concentration of those molecules in an attempt to continually maintain turgor. Eventually, the high concentration of charged solutes will negatively impact cytoplasmic enzymatic activity and cause the cell to be unable to continue growth processes (Sperber, 1983). Because proline is a neutral molecule, it does not impact enzymatic activity and can be used at higher concentrations in the cytoplasm. Bacterial cells that utilize proline more than other solutes are able to maintain osmolarity and turgor at water activity values significantly lower than bacteria that rely solely on charged compatible solutes (Sperber, 1983). *L. monocytogenes* is one example of a bacteria that is able to use proline as an osmoprotectant (Beales, 2004).

**Nitrites**

Nitrites or nitrates have been used as preservatives for thousands of years. Originally added to meat as impure saltpeter, nitrates are now added as purified curing salts usually containing sodium chloride and sodium nitrite and/or sodium nitrate (Majou and Christieans, 2018). The direct addition of nitrates or nitrites in combination with sodium chloride to processed meats produces the cured meat color and flavor that consumers prefer. Nitrite also adds an additional antimicrobial hurdle in cured meat products. At the levels allowed in cured meats, nitrite alone is not able to kill pathogenic bacteria, but its use in combination with the other hurdles present in cured meats has a well-documented bactericidal effect on common
pathogens and prevents the germination of *Clostridium botulinum* spores in the product (Hospital *et al.*, 2014; Majou and Christieans, 2018). Although the bactericidal mechanism is not completely understood, nitrite that was either added to the meat directly or converted from nitrate via bacterial reduction enters pathogenic cells as nitrous acid. Nitrous acid is able to enter pathogenic cells and aid in the disruption of normal cellular activity (Hospital *et al.*, 2014; Majou and Christieans, 2018).

**Duck**

**Duck production**

In the United States, domesticated ducks grown for their meat are primarily farmed indoors to prevent contact with wild fowl and predators. Ducks are typically inspected for wholesomeness during slaughter at the federal or state level if the meat is intended to enter commerce. Some producer-growers may operate under one of several federal exemptions to mandatory inspection, but this may limit where the poultry may be sold or what products may be produced with the fresh poultry (9 CFR 381.10). *Anas platyrhynchos* (Pekin) (Figure 1) and *Cairina moschata* (Muscovy) (Figure 2) are the two most common breeds farmed for meat (Chartrin *et al.*, 2006). Although duck is increasing in popularity in the United States, production numbers for duck are still substantially lower than chicken and turkey production numbers. In 2017, over 26.6 million ducks were slaughtered in the United States compared to approximately 9 billion chickens and 240 million turkeys. China lead the 2017 worldwide production of duck with an estimated 2.3 billion ducks produced (Food and Agriculture Organization of the United Nations, 2019).
**Duck Meat**

Ducks are waterfowl, and their carcasses are known for a thick layer of fat covering the breast muscles that serves to maintain buoyancy. Duck meat is also known for its darker, red color compared to other domesticated poultry, especially in the *Pectoralis major* and *minor* muscles (Smith *et al.*, 1993). The difference in muscle color between duck and chicken is due to the differences in muscle fiber composition. The *Pectoralis* muscles in chicken are almost exclusively comprised of white-type muscle fibers, whereas the *Pectoralis* muscles of *Anas platyrhynchos* are composed of greater than 80% red-type muscle fibers and approximately 20% white-type muscle fibers (Smith *et al.*, 1993). Although duck meat is noticeably darker and redder than chicken and turkey meat, the USDA still considers duck meat to be white meat (United States Department of Agriculture, 2013). Compositional differences between duck and chicken meat also exist. According to the National Nutrient Database for Standard Reference (USDA), skinless chicken meat has an approximate MPR of 3.5:1 and approximately 3% total fat (United States Department of Agriculture, 2018a). Skinless, domesticated duck meat has an approximate MPR of 4.0:1 and higher fat content than chicken with approximately 6% total fat (United States Department of Agriculture, 2018b). Both the higher fat content and higher MPR of duck contribute to higher cook loss in duck meat than chicken meat (Smith *et al.*, 1993).

**Processed duck products**

Curing, smoking, or drying have been ways of extending the shelf life and improving the flavor of all types of meat for thousands of years, and duck is no exception to that principle (Zeuthen, 2007; Hierro *et al.*, 2015). It is difficult to know the exact history of processed duck products because they do not maintain storied histories, but markets all over the world today
offer products that range from cured, dried breast slices to more further processed products like prosciutto and fermented salamis. In the United States, companies that sell and distribute duck meat also sell processed products like salami, ham, bacon and sausage that are made with duck. Numerous charcuterie companies have added duck products to their inventory of processed products. Although the duck market in the United States is a niche compared to the chicken and turkey industries, processed duck products have the potential to increase in sales and sustain the duck industry long term (Arnaud et al., 2014).

Salami production using poultry instead of beef or pork does not require any additional processing steps; however, unique characteristics of poultry must be considered when producing processed meats with poultry. Due to its higher unsaturated fatty acid content, poultry fat has a lower melting temperature than beef and pork fat and could become liquid if a processing environment is not chilled appropriately (Arnaud et al., 2014). The high unsaturated fat content also makes a salami that contains poultry fat much more susceptible to lipid oxidation caused by light and oxygen. Meat processors who wish to use poultry fat in a product must take these potential pitfalls into account. Supplementing a duck salami with pork fat rather than poultry fat would create a product that is less likely to contain liquid fat at room temperature, as well as experience negative flavors or aromas produced by products of oxidation that may be present in the final product (Arnaud et al., 2014). There is no known literature on the safety of duck sausages or salami, but there has been some international research on the palatability of duck products, including duck sausages (Bhattacharyya et al., 2007; Yang et al., 2009; Lorenzo et al., 2011), duck hams (Kim et al., 2017), ground duck (Ramadhan et al., 2012), and raw duck meat (Chartrin et al., 2006; Liu et al., 2007; Ali et al., 2008; Wang et al., 2013; Ahmed et al., 2018).
High Pathogenic Avian Influenza in ducks

The biggest threat to the welfare of ducks worldwide and the safety of humans consuming duck is Type A influenza virus which can be divided into low pathogenic and high pathogenic subtypes. Low pathogenic subtypes are rarely able to cause illness in poultry, and any illness that is caused by low pathogenic avian influenza in poultry is even less likely to spread to humans (CDC, 2017). High pathogenic avian influenza (HPAI) is a much more dangerous group of viruses, and subtypes H5 and H7 cause the most illness in commercial birds (Widjaja et al., 2004). The exact biological mechanism by which HPAI functions has yet to be effectively categorized and is a leading area of research in avian immunology and food safety fields around the world (Kuchipudi et al., 2014). HPAI has extremely high mortality rates in chicken and turkey and commonly spreads from wild waterfowl to domesticated poultry. The virus is absorbed in the lungs of the domesticated birds and is able to stop internal organ function within 48 hours of initial infection (CDC, 2017; Kuchipudi et al., 2014). HPAI can be transmitted to humans through the air into the lungs; onto human skin and into the eyes, nose, or mouth; or through contaminated meat (CDC, 2017). Human mortality rate, once infected with HPAI, is very high. Sixty percent of humans infected with HPAI subtype H5N1 die (Kuchipudi et al., 2014). For this reason, HPAI infection is highly monitored by poultry producers worldwide that have the necessary technology, and drastic culling measures are taken when HPAI is found in domesticated birds.

HPAI infection is common in poultry and humans throughout many of the countries in Asia, especially in the countries like Indonesia that raise the majority of its duck in open fields where wild and domestic ducks are easily able to comingle and spread virus (Henning et al., 2010). Thankfully, HPAI is much less of a concern in the United States than in Asia and Europe,
because the USDA Animal and Plant Health Inspection Service has taken a very proactive approach at combating the highly virulent, Asian strains of HPAI. HPAI outbreaks in the US in poultry are extremely rare and do not achieve the same amount of devastation compared to incidents that occur in the rest of the world. Commercial duck flocks are controlled much more compared to the large amounts of free-roaming flocks in countries like Indonesia. Farmers in the United States are able to prevent wild ducks from coming into contact with domesticated ducks much more effectively by indoor production methods (United States Department of Agriculture, 2019). In addition to protective measures taken by farmers, the USDA also leads a large effort to track HPAI incidence in wild ducks across the country. Wild ducks are caught along migratory routes throughout North America, and fecal samples are taken from the birds for characterization of avian influenza strains. Incidents of HPAI have occurred in 16 different states in both wild and domesticated poultry, and H5 subtypes are the most common isolates found in the United States. The most recent outbreak of HPAI in domesticated poultry in the United States was in 2004. The virus was quickly identified in one flock of chickens in the southern United States and was destroyed before it was able to spread to another flock or to humans (USDA, 2015). Because of the consistent efforts by USDA and poultry farmers across the country, HPAI is not an immediate concern for meat processors producing poultry products in the US.

Bacterial hazards associated with duck

The prevalence of *Salmonella* spp. and *Campylobacter* spp. in domesticated ducks and commercial chickens are very similar throughout the world (Ridsdale *et al.*, 1998; Alter *et al.*, 2006; Adzitey *et al.*, 2012). Occurrence of *Campylobacter* spp. in duck is estimated to be 50% worldwide followed by an estimated occurrence of *Salmonella* spp. in duck of approximately
25%. Variances in confirmation of both pathogens range from flocks being completely free to nearly 100% confirmed. Both pathogens are able to colonize the digestive tract of poultry and spread to other birds via feces in growing houses. The pathogens spread to meat surfaces via cross contamination of intestinal components, ingesta and fecal matter during slaughter and processing of birds. *Salmonella* spp. and *Campylobacter* spp. are able to cause illness in humans when the meat is consumed without proper preparation (Nguyen *et al.*, 2010). Despite needing very strict environmental conditions, *Campylobacter* spp. is able to survive for multiple weeks once attached to the surfaces of raw poultry meat (Ritz *et al.*, 2007; Landrum *et al.*, 2016). *Campylobacter* spp. has also been confirmed in multiple species of wild fowl, including wild ducks, indicating that colonization of the pathogen is not limited to ducks produced using confinement practices (Kasrazadeh and Genigeorgis, 1987; Yogasundram *et al.*, 1989). *L. monocytogenes* is occasionally found in living ducks and on raw duck meat, but occurrences are much lower than occurrences of *Salmonella* spp. and *Campylobacter* spp. (Adzitey *et al.*, 2012).

**Salmonella spp.**

*Salmonella* spp. was first discovered in the 1885 by Daniel Elmer Salmon and Theobald Smith. Salmon and Smith isolated what they called “Hog-cholerabacillus” from hogs experiencing a disease known as hog cholera. The organism isolated by Salmon and Smith was later renamed *Salmonella cholera-suis* (Fàbrega and Vila, 2013). *Salmonella* spp. was first linked to foodborne illness by Gustav Gaertner in 1888. Gaertner isolated the same strain of a bacteria, that he named *Bacillus enteritidis*, from a cow experiencing excessive diarrhea and people that experienced diarrhea linked to the meat from that cow. Gaertner’s strain of *Bacillus*
*enteritidis* was later named *Salmonella enteritidis* (Hardy, 1999). The genus *Salmonella* was given its name in honor of Salmon and his group in 1900. Ironically, the hog cholera Salmon and Smith were studying was later determined to be caused by a virus and not by *Salmonella* spp. (Fàbrega and Vila, 2013).

**Characteristics**

*Salmonella* spp. are rod-shaped, Gram negative bacteria that do not form spores. They are facultative anaerobes and can grow at temperatures ranging from 2 to 54° C (Montville *et al.*, 2012). Some *Salmonella* spp. can survive below 0° C in food for extended periods of time. *Salmonella* spp. are able to persist in all environments and are most commonly found in poultry, pork, and beef, although the presence of *Salmonella* spp. in poultry is the highest concern (Montville *et al.*, 2012). The genus *Salmonella* only contains two species: *S. enterica* and *S. bongori* (Fàbrega and Vila, 2013). *S. enterica* is further divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*. *S. enterica* subsp. *enterica* is responsible for the overwhelming majority of illness and disease in humans and animals. *Salmonella enterica* spp. *enterica* can be further divided into serovars via the Kauffmann-White identification scheme. When serovars are identified by unique lipopolysaccharides (O antigen) and flagella (H antigen), over 2,000 serovars can be identified. Serovars *Typhimurium*, *Enteritidis*, *Newport*, and *Heidelberg* are among the common serovars that cause disease in the United States (Montville *et al.*, 2012; Anna Fàbrega and Vila, 2013).
Salmonellosis

*Salmonella* spp. organisms can cause two types of disease: typhoid/enteric fever or colitis (Ohl and Miller, 2001; Gordon, 2008). Typhoid fever is caused by infection due to Typhi or Paratyphi serovars. Symptoms first present after an incubation period that lasts from seven to 28 days and can range from diarrhea and fever to failure of the respiratory system, liver, or spleen (Montville *et al.*, 2012; Fàbrega and Vila, 2013). If left untreated, mortality due to typhoid fever can reach 20%, but antibiotic and vaccine treatments have successfully increased the typhoid fever survival rate. Salmonellosis is sickness caused by non-typhoidal *Salmonella* serovars (Montville *et al.*, 2012). Infection typically requires approximately $10^3$ pathogenic cells and usually results in diarrhea, fever, and abdominal cramps that begin between 12 to 72 hours in the infected host (Agbaje *et al.*, 2011). Most colitic disease instances caused by *Salmonella* spp. are self-limiting, and severe symptoms usually clear approximately five days after the first symptom. Hospital visits may be required to replace fluids and electrolytes in severely infected persons. In the most severe cases, non-typhoidal *Salmonella* spp. can invade host cells and cause chronic diseases like reactive arthritis or ankylosing spondylitis (Montville *et al.*, 2012). In the United States, an estimated one million cases of salmonellosis from food occur with around 19,000 cases resulting in hospitalization and around 400 cases resulting in death (Montville *et al.*, 2012).

Outbreaks

The CDC has reported multiple foodborne outbreaks due to *Salmonella* spp. per year over the last decade. The most recent outbreak in meat occurred in 2019 when seven people were sickened in three states from ground turkey. One person was hospitalized in this outbreak (CDC, 2019a). Five outbreaks of *Salmonella* spp. occurred in the United States in 2018 from ground
beef, raw chicken, raw turkey, and chicken salad. A combined 1,180 people were sickened, 380 were hospitalized, and 5 were killed (CDC, 2019a). Since 1998, there has been one reported outbreak of *Salmonella* spp. in the United States due to duck. *Salmonella* Anatum isolated from duck in Massachusetts sickened eight people in 2004 (CDC, 2017). A 2015 incidence of salmonellosis in Australia was attributed to consumption of duck prosciutto, although a specific serovar was not able to be isolated. Twenty-one were sickened, and seven were hospitalized as a result of consuming the duck prosciutto (Draper *et al.*, 2017).

**Listeria monocytogenes**

**History**

*L. monocytogenes* was discovered in rabbits in 1924 by E. G. D. Murray. Murray named the newly discovered organism *Bacterium monocytogenes* (Murray *et al.*, 1926; Hof, 2003). In 1927, Harvey Pirie renamed the bacteria *Listeria hepatolytica* (Farber and Peterkin, 1991). Pirie later named the genus *Listeria* after Joseph Lister to remove confusion in the literature (Pirie, 1940). The importance of *L. monocytogenes* as a foodborne pathogen was not understood until much later. One of the first reported foodborne outbreaks of *L. monocytogenes* occurred in Canada in 1981 when 41 were infected with the pathogen causing nine stillbirths and six newborn deaths (Farber and Peterkin, 1991). Coleslaw was implicated in the outbreak, and *L. monocytogenes* collected from patients was also found in cabbage on a farm that used animal manure as fertilizer. The harvested cabbage was stored in a refrigerated shed for an extended period prior to being used in the coleslaw. This outbreak and multiple others like it in the 1980’s highlighted the vulnerability of the young, old, pregnant, and immunocompromised to severe
illness and death via *L. monocytogenes* infection and also of the pathogen’s ability to grow and persist in ready-to-eat foods (Farber and Peterkin, 1991).

**Characteristics**

*L. monocytogenes* are a Gram-positive, rod-shaped bacterium that do not form spores (Farber and Peterkin, 1991; Montville *et al.*, 2012). Although multiple species of *Listeria* are able to cause illness in animals and humans, *L. monocytogenes* causes the large majority of illness in humans and is especially problematic in food systems around the world because of its ubiquitous nature. Ninety-nine percent of illnesses caused by *L. monocytogenes* are from food (Chaturongakul *et al.*, 2008). Like *Salmonella* spp., *L. monocytogenes* are facultative anaerobes that grow across a wide range of temperatures. However, *L. monocytogenes* is able to grow at or below refrigeration temperatures (approximately 4° C) and is more resistant to the common antimicrobial hurdles used in food products (Hill *et al.*, 2002; Gandhi and Chikindas, 2007; Swaminathan and Gerner-Smidt, 2007; Ferreira *et al.*, 2014). Strains of *L. monocytogenes* have been able to grow at pH values as low as 4.4 and survive in environments with *aw* as low as 0.83. In addition to being able to grow under these conditions, exposing *L. monocytogenes* to sublethal stressors allows the pathogen to build further resistance to those stressors (Chaturongakul *et al.*, 2008). *L. monocytogenes*’ ability to colonize food processing equipment and drains in the processing environment makes the pathogen extremely problematic for food processors. Due to the ability of *L. monocytogenes* to cause mortality at a higher rate than other foodborne pathogens and grow or survive under high stress conditions, the United States has a zero-tolerance policy for the bacterium in ready-to-eat foods (FSIS, 2014). It is estimated that the
value of deli meats lost each year due to *L. monocytogenes* contamination is over $1 billion (Montville *et al.*, 2012).

**Listeriosis**

Infection by *L. monocytogenes* in humans is called listeriosis. As few as ten pathogenic cells can colonize the gut of humans and cause diarrhea and fever similar to many other types of foodborne pathogens. While listeriosis is usually self-limiting in healthy adults, individuals more susceptible to the pathogen are at a higher risk of contracting an invasive *L. monocytogenes* infection from consuming a food containing the bacterium (CDC, 2017). The bacterium is able to cause further damage to humans due to its ability to migrate out of the gut and spread infection to other areas of the body (Lecuit, 2007). Symptoms of invasive *L. monocytogenes* infection in high risk individuals usually begin between one and five weeks after a food containing the bacterium is ingested, and normal symptoms include fever, aches and pains, loss of balance, and convulsions. Very severe instances of listeriosis can result in septicemia or meningitis (Montville *et al.*, 2012). While *L. monocytogenes* may initially only cause fever, diarrhea, and/or dehydration in pregnant women, it is able to infect the fetus and cause either stillbirth or abortion. Sometimes *L. monocytogenes* may infect fetuses in pregnant women who are asymptomatic for listeriosis (Montville *et al.*, 2012). Instances of listeriosis are low compared to other foodborne pathogens. It is estimated that listeriosis occurs in 1,600 people per year; however, the mortality rate of those who contract invasive listeriosis is 20%. Miscarriage, stillbirth, or newborn death are also estimated to occur in 20% of pregnant women with listeriosis (CDC, 2019).
Outbreaks

There are no known *L. monocytogenes* outbreaks associated with duck in the United States (CDC, 2017), but there have been multiple outbreaks linked to RTE deli meats, cheeses, and fruits/vegetables. In 2011, a now infamous outbreak of *L. monocytogenes* in cantaloupes was linked to a single farm in Colorado. 147 people were sickened across 28 states. Of those sickened during the outbreak, 143 were hospitalized, and 33 died (Centers for Disease Control and Prevention, 2019). Four outbreaks of *L. monocytogenes* in various cheeses were confirmed in 2012, 2014, 2015, and 2017. Across 37 states, 66 people were sickened, 62 were hospitalized, and ten died in those four outbreaks (Centers for Disease Control and Prevention, 2019). Recent outbreaks in RTE meats have occurred in 2018 and 2019. The first outbreak occurred in RTE ham and sickened four across two states, resulting in one death. The second outbreak is ongoing as of last reporting. Sliced deli meats are expected to have caused eight hospitalizations and one death; however, the cause of this outbreak has yet to be confirmed (Centers for Disease Control and Prevention, 2019). Although improved sanitation in food production facilities appears to have decreased instances of listeriosis in the United States (Endrikat *et al*., 2010), these recent outbreaks indicate that *L. monocytogenes* is still a very serious threat to human health.

*Campylobacter* spp.

History

*Campylobacter* spp. were first discovered by Theodor Escherish in 1886 when he isolated the organism from intestines of infants who died due to a disease known as “cholera infantum” (Samie *et al*., 2007; Sheppard and Maiden, 2015). Because these organisms require specific nutrient and atmospheric conditions to be cultured in a laboratory setting, the importance of
Campylobacter spp. as a human pathogen was not realized until 1972 when it was successfully isolated from human feces for the first time (Samie et al., 2007). Since then, improvements in growth media and laboratory equipment have allowed for the successful isolation of many Campylobacter spp. isolates from human, food, and animal sources (Samie et al., 2007; Sheppard and Maiden, 2015).

Characteristics

Campylobacter spp. are Gram negative rods that do not form spores. Their cells are known for a characteristic spiral shape that makes them easy to identify under a microscope. Campylobacter spp. are microaerophilic and require approximately 5% oxygen and 10% carbon dioxide in order to grow (Montville et al., 2012; Skarp et al., 2016). Some Campylobacter spp. can grow in anaerobic conditions and some can grow in aerobic conditions, but aerophilic Campylobacter spp. are extremely rare. These organisms are also not able to grow at temperatures below 30° C and prefer to grow at temperatures ranging from 37 – 42° C. Campylobacter spp. are highly motile and utilize a single large flagellum to move throughout the environment (Montville et al., 2012; Skarp et al., 2016). Campylobacter spp. also evolve rapidly and are capable of post-translationally modifying cellular proteins to quickly adapt to changes in the environment.

Two Campylobacter species most commonly cause illness in humans: Campylobacter jejuni and Campylobacter coli. Roughly 80-90% of human illness caused by Campylobacter spp. in the United States is caused by C. jejuni. The remaining 10-20% of illnesses is caused by C. coli (Skarp et al., 2016). Despite being a difficult organism to culture in a laboratory setting, Campylobacter spp. are a leading cause of foodborne illness worldwide. The intestines of poultry
as well as other larger animals are a natural reservoir for these organisms, and they have been shown to successfully survive in raw meat for weeks after initial contamination. *Campylobacter* spp. can also thrive in drinking water if the water is contaminated with animal feces or byproducts. Ducks may also carry higher amounts of *Campylobacter* spp. at an earlier age than chickens. According to Ridsdale *et al.* (1998), *Campylobacter* spp. is colonized in all ducks by 11 days post hatching compared to first isolation in chickens at three to four weeks. *Campylobacter jejuni* and *Campylobacter coli* that have been isolated from duck in several countries around the world including the United States, the United Kingdom, Ireland, Malaysia, Taiwan, Iran, and Tanzania. This demonstrates that *Campylobacter* spp. colonization of duck and the spreading of the pathogen to duck meat is an issue that must be taken into consideration around the world (Adzitey *et al.*, 2013). The many potential sources of *Campylobacter* spp., as well as their incredible ability to persistence in meat, make this pathogen a leading cause of foodborne illness outbreaks and diseases worldwide (Montville *et al.*, 2012; Skarp *et al.*, 2016).

**Campylobacteriosis**

Disease by *Campylobacter* spp. infection (campylobacteriosis) is caused when potentially as few as 500 cells are ingested. Symptoms including fever, diarrhea, cramps, and vomiting will typically begin two to five days after the food containing the bacteria is ingested. Most cases of campylobacteriosis are self-limiting and will cease after two to ten days, but some cases will escalate with more severe side effects especially in the young, old, pregnant, and immunocompromised (Montville *et al.*, 2012; Skarp *et al.*, 2016). About one of every 1,000 cases of gut infection with *Campylobacter* spp. result in further infection of other organs in the body and could result in severe long-term complications. An estimated one in every 2,000 cases
of campylobacteriosis result in Guillain-Barre Syndrome (GBS), an autoimmune disorder. Some Campylobacter spp. antigens closely resemble components of human nerve cells. When a person contracts GBS, their immune system mistakenly attacks the nervous system as it is combating the Campylobacter spp. infection. The resulting symptoms can range from mild weakness to complete paralysis, and severe paralysis symptoms may take years to become evident (National Institute of Neurological Disorders and Stroke, 2019). Reactive arthritis is another type of autoimmune disorder that has been documented as a complication of campylobacteriosis when the infection migrates to joints and causes mild to severe inflammation in affected joints (Montville et al., 2012).

Outbreaks

It is estimated that illnesses caused by Campylobacter spp. are as high or potentially higher than illnesses caused by Salmonella spp., but because campylobacteriosis is usually less severe than salmonellosis, reported outbreaks due to Campylobacter spp. are much lower than outbreaks due to Salmonella spp. (Montville et al., 2012; Skarp et al., 2016; Centers for Disease Control and Prevention, 2019b). The CDC has confirmed 209 foodborne outbreaks in the United States due to Campylobacter spp. from 2010 to 2015. A total of 2,234 illnesses resulted from those outbreaks. Poultry and foods cross-contaminated with poultry are some of the leading sources of campylobacteriosis in the United States, and half of the outbreaks associated with duck in the United States have been due to Campylobacter spp. (CDC, 2019b). A 2011 outbreak of Campylobacter spp. from consumption of undercooked duck liver was confirmed in the United Kingdom. A total of 18 people exhibited diarrheal symptoms, and eight people were confirmed to have contracted campylobacteriosis. Interviews determined that the duck liver was
deliberately undercooked, further emphasizing the need to address *Campylobacter* spp. in duck via proper cooking techniques or other valid methods (Abid *et al.*, 2013).

**Regulations**

**United States regulation for Ready-To-Eat poultry products**

Preventing pathogenic bacteria from being present in food products is one of the biggest concerns for food regulators and processors alike. The United States now has precedent to legally punish processors who do not meet microbial standards for food products, especially if those processors are found to be negligent. Executive officers of the Peanut Corporation of America were sentenced to prison after a peanut processing plant was found to be responsible for a 2008 nationwide *Salmonella* spp. outbreak (Flynn, 2016). For meat products intended for human consumption in the United States, the Federal Meat Inspection Act and the Poultry Products Inspection Act make the sale of adulterated meat products illegal (Levine *et al.*, 2001). According to the Code of Federal Regulations (9 CFR 417.2), meat processing establishments must conduct a hazard analysis to determine food safety hazards for each product, and preventive measures must be implemented to limit or control those hazards. Food safety hazards that have higher percentages of occurring or food safety hazards that are very likely to cause illness should be given the highest priority when analyzing potential hazards. Food safety hazards that could occur in a meat product include but are not limited to the presence of pathogenic microbes, toxins, hazardous chemicals, and physical hazards from foreign material like metal or plastic. The results of the hazard analysis, as well as the methods for controlling each hazard present in a food product, must be documented in a Hazard Analysis Critical Control Point (HACCP) plan.
The HACCP plan must also establish allowable limits in the product for each hazard and
demonstrate what corrective actions will be taken if allowable limits for a particular hazard are
breached (9 CFR 417.2).

For fully cooked poultry products or raw, RTE poultry products, no viable *Salmonella*
spp. organisms can be present in a finished product. To guarantee this, a lethality process that
ensures that no viable *Salmonella* spp. organisms are present in the finished product must be
demonstrated. A $7 \log_{10}$ reduction of *Salmonella* spp. organisms is suggested, but a process that
indicates a $5 \log_{10}$ reduction of the pathogen is acceptable. *L. monocytogenes* must not be present
in the final product, since it is considered an adulterant. Adulteration can also occur if a product
comes into contact with *L. monocytogenes* in the processing environment. Demonstration that *L.
monocytogenes* is not able to grow in a product in addition to documentation that *L.
monocytogenes* is not present in food processing environments is required. Under alternatives to
the USDA *Listeria* Rule, meat processors are able to decide between multiple methods for
addressing *L. monocytogenes* in a food product. Alternative 1 utilizes a post-lethality treatment
and an antimicrobial process to eliminate the pathogen. Alternative 2 utilizes a post-lethality
treatment or an antimicrobial process to eliminate the pathogen. Lastly, Alternative 3 relies on
sanitation alone to control *L. monocytogenes* in the processing environment (United States
Department of Agriculture, 2014). For RTE products containing poultry, USDA-FSIS requires
that *Campylobacter* spp. be addressed in the product and that a $5 \log_{10}$ reduction of
*Campylobacter* spp. be achieved during processing of RTE poultry products (9 CFR 417).
Additionally, a greater than $1 \log_{10}$ growth of pathogenic organisms cannot occur in the product
(9 CFR 381.150).
Statement of the Problem

Salami products made with duck are increasing in popularity in the United States. These products use traditional salami production methods; however, there is no scientific data supporting the microbial safety of salamis containing duck. Traditional salamis containing beef or pork are made with well-established production parameters including fermentation via lactic acid starter culture, curing with sodium nitrite, and drying. There is ample scientific evidence to show that reducing water activity and pH together, in combination with the presence of nitrite in the formulation achieves shelf stability and death of pathogenic microorganisms in traditional salami. Because there is no scientific data on the microbial safety of duck salami available, a challenge study using the bacterial pathogens of concern in duck salami can validate the production parameters needed to create the product and ensure that it is safe to consume.

Statement of Objectives

The purpose of this project is to validate the safety of the production process used to make a fermented and dried duck salami. Lean duck trim and pork belly will be inoculated with high concentrations of pathogens, ground, seasoned with salt, spices, sodium nitrite and starter culture, fermented, and then dried. Measuring the pathogen concentration, water activity, and pH until the end of production will determine if the processes used to create the RTE salami are able to ensure microbial safety. The data generated will be used as scientifically valid support for the production of duck salami as a part of HACCP supporting documentation for any meat processing company that follows the processing parameters demonstrated in this research. Additionally, this project aims to begin to fill a significant gap in the knowledge about the bacterial safety of salamis that contain duck.
Figures

Figure 1: *Anas platyrhynchos* or the American Pekin duck (Department of Animal and Food Sciences, University of Kentucky, 2019)
Figure 2: Domestic *Cairina moschata* or the Muscovy duck (Copyright Nathan Tea, Macaulay Library, The Cornell Lab of Ornithology, 2018)
References:


Chapter 2
Plate Overlay Assay

Introduction

Selecting microorganisms for a challenge study is one of the most important steps in ensuring that the study is applicable to the product being tested, and that multiple hurdle approaches can be justified (National Advisory Committee on Microbiological Criteria for Foods (NACMCF), 2010). An experimental design that incorporates multiple pathogens into a single challenge study can definitively address multiple hazards at once, thus removing the need to conduct multiple studies for the same product separated by pathogens of interest. Using multiple strains of a single microorganism accounts for differences in growth patterns and potential resistance to stressors between strains ensuring that the study encompasses the diversity of a particular species of bacteria (Scott et al., 2005). Prior to conducting a challenge study that proposes to use multiple organisms and multiple strains of each organism, the existence of antagonistic effects among organisms proposed for the study must be determined (NACMCF, 2010). To prevent the designed experiment from producing a false negative result, the growth or survival of one organism cannot be inhibited by the presence of another (NACMCF, 2010; Yokoyama et al., 2005).

*Campylobacter jejuni* and *Campylobacter coli* are the most likely to be negatively impacted due to the presence of other organisms compared to other foodborne pathogens, because their narrow growth parameters and specific nutrient requirements often prevent them from flourishing in a laboratory setting (Hilbert et al., 2010). If the *C. jejuni* and *C. coli* strains chosen for a challenge study are not able to cohabitate in a mixed culture with *Salmonella enterica* serovars and *Listeria monocytogenes* strains, then these pathogens must be investigated
separately from the S. enterica serovars and L. monocytogenes strains. An experiment was
designed to compare growth characteristics of C. jejuni and C. coli strains in proximity to each
Salmonella enterica serovar and L. monocytogenes strain chosen for a challenge study. A plate
overlay assay, instead of a streak-on-lawn assay, was chosen, because it allows each strain of C.
jejuni and C. coli to be simultaneously tested against a single strain of Salmonella spp. or L.
monocytogenes, rather than conducting multiple tests for each strain of Campylobacter spp..
Conducting the assay on Campylobacter spp. growth medium helps to ensure that inhibition of
Campylobacter spp. was only due to the presence of Salmonella spp. and L. monocytogenes and
not due to inadequate Campylobacter spp. growth conditions.

**Materials and Methods**

**Culture Selection and Growth**

Cultures for the overlay assay were obtained from the Centers for Disease Control and
Prevention (CDC), the United States Department of Agriculture (USDA), or purchased from
American Type Culture Collection (ATCC) (Manassas, VA). Salmonella enterica subsp.
enterica serovar Typhimurium (ATCC 14028, isolated from chicken organs), Salmonella
enterica subsp. enterica serovar Montevideo isolate SMvo13 (CDC), Salmonella enterica
subsp. enterica serovar Derby (ATCC 7378, human isolate), Listeria monocytogenes serotype
Scott A, Listeria monocytogenes serotype 1/2a isolate FSL R2-603 (deli meats outbreak),
Listeria monocytogenes serotype 4b isolate H3396 (hotdog outbreak), Campylobacter jejuni
isolate PSU99 (isolated from chicken in Pennsylvania, species confirmed by USDA),
Campylobacter coli (ATCC 33559, pig isolate), and Campylobacter jejuni (ATCC 29428, human
isolate) were chosen to examine for antagonism in a mixed culture.
Freezer stocks of *Salmonella* spp. and *L. monocytogenes* were made by growing *Salmonella* spp. and *L. monocytogenes* cultures in 10 ml of Tryptic Soy Broth (TSB, Becton Dickinson and Company; BD, Sparks, MD) 24 hours at 37°C under aerobic conditions. After 24 hours, 1.5 mL of broth cultures were combined with 0.3 mL of a glycerol, water solution (50% glycerol) and frozen at -80°C. Overnight cultures of each *Salmonella* serovar and *L. monocytogenes* strain were grown from freezer stocks by adding a loop of culture to 10 mL of TSB (BD) and incubating for 24 hours at 37°C under aerobic conditions. Each bacterium was streaked for isolation from the overnight culture on selective media. Xylose Lysine Deoxycholate Agar (XLD; BD) was used for *Salmonella* spp., and Modified Oxford Agar (MOX; BD) was used for *L. monocytogenes*. *Salmonella* spp. and *L. monocytogenes* latex confirmation tests were performed using an isolated colony for each strain of bacteria (Microgen Bioproducts; MB, Camberley, UK). A single colony of each *Salmonella* spp. serovar and *L. monocytogenes* strain was added to 10 mL of fresh TSB (BD) and incubated under the same conditions to achieve a concentration of approximately 8 log10.

*Campylobacter* spp. cultures were grown from agar slants received from ATCC or from Penn State Food Microbiology Culture Collection freezer stocks. A loop of culture for each organism was inoculated into 10 mL of Brucella broth (Hi Media Laboratories; HML, Mumbai, India) and grown for 48 hours at 37°C under anaerobic conditions (10% CO2, 90% N2). Strains were streaked for isolation on modified Charcoal Cefoperazone Deoxycholate agar (Oxoid LTD; Basingstoke, Hampshire, England) and incubated for 48 hours under the same conditions. *Campylobacter* spp. latex confirmation tests (MB) were performed for each strain using an isolated colony. An isolated colony was also inoculated into 10 mL of fresh Brucella broth (HML) for 48 hours under the same conditions to achieve an approximate concentration of 8
log\textsubscript{10}. Additionally, freezer stocks of *Campylobacter* spp. were made using fresh broth cultures following the procedures stated above.

**Plate Overlay Assay**

The plate overlay assay was conducted with methodology adapted from Cutter (1999) using Brucella agar plates (Hardy Diagnostics; HD, Santa Maria, CA) with an overlay of 8 mL of soft Brucella agar (0.5% agar wt./vol) (HML). Prior to performing the assay, each of the strains of *Salmonella* spp. and *L. monocytogenes* were plated on to Brucella agar (HD) and incubated under anaerobic conditions (10% CO\textsubscript{2}, 90% N\textsubscript{2}) to confirm that the *Salmonella* spp. and *L. monocytogenes* were able to grow on the same media and in the same environment as *Campylobacter* spp.. Each *Salmonella* serovar and *L. monocytogenes* strain selected were able to growth under the stated conditions.

Each overlay plate tested one *Salmonella* serovar or *L. monocytogenes* strain against three *Campylobacter* organisms, and tests were conducted in duplicate. Each *Salmonella* serovar and *L. monocytogenes* strain was grown in TSB (BD) to approximately an 8 log\textsubscript{10} concentration using the methods described above. Inoculated TSB (BD) volumes of 0.8 mL, 0.4 mL, and 0.1 mL were individually added to 8 mL of soft agar that was tempered to 45\textdegree C, and the soft agar was poured over a Brucella agar plate (HD). Once the soft agar solidified, an isolated colony of each *Campylobacter* spp. was stabbed into the plate. The plates were incubated for 48 hours at 37\textdegree C under anaerobic conditions, and growth patterns around the stab locations were observed. The presence of *Salmonella* spp. and *L. monocytogenes* were presumptively confirmed by scraping each plate with a loop in an area that appeared to free of *Campylobacter* spp. growth
and streaking on to selective media. Plates were done in duplicate for each inoculum concentration, and two replications were conducted (n = 4).

Results

Overlay plates were observed for zones of clearing where *Campylobacter* spp. stabs were made into the plate. No zones of inhibition were observed between any of the organisms tested (Table 1). Plates containing *Salmonella* spp. were slightly more opaque than plates containing *L. monocytogenes*. Additionally, there was some variance in the size of *Campylobacter* spp. outgrowth around the stab area. Some *Campylobacter* spp. stabs only grew on the agar directly inoculated by the stab, and some areas were able to grow beyond the area where the agar was stabbed (Figure 2). *Campylobacter jejuni* isolate PSU99 (*Cj PSU99*) outgrew *Campylobacter jejuni* ATCC 29428 (*Cj 29428*) and *Campylobacter coli* ATCC 33559 (*Cc 33559*) in the presence of both *Salmonella* spp. and *L. monocytogenes* (Figure 1).

Discussion

Zones of inhibition similar to those observed in antibiotic resistance experiments conducted by Comeau and others (2007) (Comeau, 2007) were used as a guide for observing potential inhibition of *Campylobacter* spp. in the overlay assays. Because there were no areas around *Campylobacter* spp. stabs where *Salmonella* spp. and *L. monocytogenes* failed to grow and no stab areas where *Campylobacter* spp. failed to grow, no direct inhibition occurs between these strains of *Salmonella* spp., *L. monocytogenes*, *C. jejuni*, and *C. coli*. There were some observed differences in opacity of the soft agars between plates inoculated with *Salmonella* spp. (Figure 1A) and plates inoculated with *L. monocytogenes* and (Figure 1B), but there were not
differences in the growth of *Campylobacter* spp. in the stab locations indicating that differences in growth rates of *Salmonella* spp. and *L. monocytogenes* did not negatively impact the growth of *Campylobacter* spp.. Additionally, *C. jejuni* PSU99 performed better in the presence of *Salmonella* spp. and *L. monocytogenes* compared to *C. jejuni* 29428 and *C. coli* 33559. *C. jejuni* PSU99 is a strain isolated directly from raw farmers’ market chicken carcasses and is potentially a stronger strain than *C. jejuni* 29428 and *C. coli* 33559. The differences in growth between *C. jejuni* PSU99 compared to *C. jejuni* 29428 and *C. coli* 33559 confirm that *C. jejuni* PSU99 is the strongest strain of *Campylobacter* spp. used in this experiment. These results can be used as supporting evidence that no inhibition would occur between these organisms if they were used in a validation experiment.
Table 1: Growth (+ or -) of *Campylobacter* spp. stabbed into Brucella agar pour plates individually inoculated with 0.8 mL, 0.4 mL, or 0.1 mL of *Salmonella* spp. or *L. monocytogenes* strains (n = 2).

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>C. jejuni PSU 99</th>
<th>C. jejuni 29428</th>
<th>C. coli 33559</th>
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</thead>
<tbody>
<tr>
<td><strong>0.8 mL</strong></td>
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<tr>
<td><em>Salmonella</em> Typhimurium</td>
<td>+</td>
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<tr>
<td><em>Salmonella</em> Montevideo</td>
<td>+</td>
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<tr>
<td><em>Salmonella</em> Derby</td>
<td>+</td>
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<td>+</td>
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<tr>
<td><em>L. monocytogenes</em> Scott A</td>
<td>+</td>
<td>+</td>
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<tr>
<td>L. monocytogenes R2-603</td>
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<tr>
<td>L. monocytogenes H3396</td>
<td>+</td>
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<td><strong>0.4 mL</strong></td>
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<tr>
<td><em>Salmonella</em> Typhimurium</td>
<td>+</td>
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<td><em>Salmonella</em> Montevideo</td>
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<td><em>Salmonella</em> Derby</td>
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<td><em>L. monocytogenes</em> Scott A</td>
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<td><em>Salmonella</em> Montevideo</td>
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<td><em>Salmonella</em> Derby</td>
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<td><em>L. monocytogenes</em> Scott A</td>
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<td>L. monocytogenes R2-603</td>
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<td>L. monocytogenes H3396</td>
<td>+</td>
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</table>
Figures

**Figure 1:** Brucella agar pour plates inoculated with *Salmonella* spp. (A) and *L. monocytogenes* (B) stabbed simultaneously with isolated colonies of *C. jejuni* PSU99 (1), *C. coli* 33559 (2), and *C. jejuni* 29428 (3).
References


Chapter 3
Viability of Pathogens in Ground Pork and on Lean Pork and Pork Fat

Introduction

Contamination of raw meat and poultry by both pathogenic and spoilage microorganisms is a constant issue for meat safety and quality and is of great concern for meat processors and regulators alike (Giaouris et al., 2014). Attachment is considered to be the beginning of contamination for meat and other surfaces in the processing environment; and bacteria are able to colonize meat surfaces with which they come into contact by physically adhering to the surface of meat products (Selgas et al., 1993; Foong and Dickson, 2004). Once successful attachment has occurred, bacteria are able to survive for extended periods of time and even grow if attachment has occurred in an environment containing the necessary nutrients for growth (Foong and Dickson, 2004). Attachment of foodborne pathogens like *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. to meat products and meat processing equipment is especially concerning, because the presence of these pathogens in meat products presents a serious health risk to the end-consumer of the product (Sinde and Carballo, 2000; Foong and Dickson, 2004; Nguyen et al., 2010).

The process by which bacteria attach to surfaces is not completely understood; however, it is widely agreed upon that attachment occurs in the two distinct steps of reversible attachment and irreversible attachment (Firstenberg-Eden, 1981; Selgas et al., 1993; Nguyen et al., 2010). Reversible attachment can occur as soon as bacterial cells come into contact with a surface and is potentially caused by multiple factors. Some of the most commonly factors contributing to reversible attachment include the following: the negative charge of bacterial cell exterior
resulting in a weak van der Waals interaction between the cell and the surface; a hydrophobic or hydrophilic interaction between the cell and environmental surface; extracellular flagella or fimbriae contacting and loosely sticking to the surface; or by the cell being physically held within a crevasse of the environmental surface (Firstenberg-Eden, 1981; Dickson and Koohmaraie, 1989; Selgas et al., 1993; Nguyen et al., 2010; Giaouris et al., 2014). As the name implies, reversible attachment between bacterial cells and surfaces can be broken.

Irreversible attachment begins when cells are given time to make stronger, more permanent interactions with their surfaces. Cells can create irreversible attachments by forming ionic or covalent bonds between the cell exterior and surface components, by excreting polysaccharides to form a biofilm, or by increasing the strength of the bond between flagella or fimbriae and the surface (Firstenberg-Eden, 1981; Chung et al., 1989; Selgas et al., 1993; Foong and Dickson, 2004; Nguyen et al., 2010). The importance of flagella and fimbriae for attachment is not agreed upon, although both flagella and fimbriae are vital for attachment if cells possess them. Bacterial cells that do not possess extracellular appendages do not attach at lower levels and are able to attach to the same surfaces using other attachment methods (Firstenberg-Eden, 1981; Dickson and Koohmaraie, 1989). However, flagellated and fimbriated cells have been shown to attach in a shorter amount of time than non-flagellated or non-fimbriated cells (Firstenberg-Eden, 1981; Dickson and Koohmaraie, 1989). Once irreversible attachments are completed, physically removing the cells from the surface of an object ranges from extremely difficult to impossible, and lethality options must be used to remove or inactivate the bacteria from the environment (Chung et al., 1989).

The environmental and situational factors that impact bacterial attachment are as varied as the processes that cells use to cause attachment. Temperature, time of contact, bacterial
concentration, and the unique characteristics of the bacteria can all impact attachment. Room temperature (20-23°C) has been shown to be the optimal temperature for bacterial attachment (Firstenberg-Eden, 1981; Selgas et al., 1993; Nguyen et al., 2010), and decreasing temperature has been shown to decrease attachment, especially for *L. monocytogenes* (Nguyen et al., 2010). Although decreasing temperature can decrease attachment, variation in temperature does not translate to large variations in attached cells (Firstenberg-Eden, 1981). Increasing the time of contact between a population of bacteria and a surface increases the number of cells attached to the surface. The relationship between concentrations of attached cells and length of time of contact is usually linear, but successful attachment over extended periods of time is impacted by type of bacteria and type of surface. Interestingly, Firstenberg-Eden et. al. (1978) showed that concentrations of attached *E. coli* K12 on cut beef muscle submerged in inoculum increased from 0 to 30 minutes then decreased from 30 to 50 minutes. This demonstrates that although increasing contact time usually increases attachment, that is not always the case. Additionally, bacterial concentration is the best indicator for attachment levels, and increasing the number of bacteria available for attachment increases the number of cells that successfully attach (Selgas et al., 1993). In the context of meat processing, preventing highly concentrated sources of bacteria, e.g., the digestive tract, from coming into contact with meat is the best way to prevent large numbers of bacteria from attaching to meat surfaces (Firstenberg-Eden, 1981). Lastly, species variation of bacteria plays a significant role in attachment levels. This means that some types of bacteria are able to attach more successfully in a given environment than others due to the composition of the attachment surface, the temperature and atmospheric conditions of the environment, or any number of other factors (Firstenberg-Eden, 1981; Selgas et al., 1993; Sinde and Carballo, 2000). Understanding how all types of bacteria attach to surfaces is paramount to
preventing the attachment and improving the quality and safety of foods (Firstenberg-Eden, 1981; Giaouris et al., 2014).

Under normal conditions, bacterial attachment to meat is not desirable. However, in order to provide data supporting the conclusion that a process for ensuring the bacterial safety of a food product is sufficient, a very high starting population is needed to demonstrate concentration reductions of pathogens greater than or equal to 99.999% of the total pathogen population. Although it can be difficult to experimentally demonstrate that attachment of individual cells on a meat surface has occurred, survival of high concentrations of bacteria in meat indicates that the cells have either utilized attachment mechanisms to survive or have been held within the meat matrix without biologically attaching. For validation research, survival by attachment or by being physically held in place in meat are necessary to ensure sufficient starting populations of pathogens. Since attachment is highly variable and since there are no guarantees that specific strains of various species will successfully attach simultaneously in the same media, it should be confirmed prior to beginning a challenge study that the pathogens intended to be used for an experiment can successfully attach to meat surfaces when mixed together. Therefore, two experiments were conducted to determine if Salmonella spp., L. monocytogenes, and Campylobacter spp. could cohabit in meat and to determine if an inoculation cocktail containing Campylobacter spp. could successfully attached to meat surfaces. In the first experiment, each species of pathogen was individually inoculated into raw ground pork and compared to raw ground pork inoculated with all three pathogen species. The second experiment utilized an inoculation cocktail containing all three pathogen species that was inoculated on to lean pork and pork fat. Additionally, both experiments served to test if Campylobacter spp. could maintain the high concentrations needed to conduct a challenge study after centrifuging and
being exposed to atmospheric levels of oxygen for extended amounts of time while attaching to the pork.

Materials and Methods

Culture Selection and Growth

The following cultures were received from the United States Department of Agriculture (USDA) or Centers for Disease Control and Prevention (CDC) or purchased from American Type Culture Collection (ATCC) (Manassas, VA): *Salmonella enterica* subspp. *enterica* serovar Typhimurium (ATCC 14028, isolated from chicken organs), *Salmonella enterica* subspp. *enterica* serovar Montevideo isolate SMvo13 (CDC), *Salmonella enterica* subspp. *enterica* serovar Derby (ATCC 7378, human isolate), *Listeria monocytogenes* serotype Scott A, *Listeria monocytogenes* serotype 1/2a isolate FSL R2-603 (deli meats outbreak), *Listeria monocytogenes* serotype 4b isolate H3396 (hotdog outbreak), *Campylobacter jejuni* isolate PSU99 (isolated from chicken in Pennsylvania, species confirmed by United States Department of Agriculture), *Campylobacter jejuni* (ATCC 29428, human isolate), and *Campylobacter coli* (ATCC 33559, pig isolate).

Overnight cultures of each *Salmonella* serovar and *L. monocytogenes* strain were grown from freezer stocks by adding a loop of culture to 10 mL of Tryptic Soy Broth (TSB, Becton Dickinson and Company; BD, Sparks, MD) and incubated for 24 hours at 37° C under aerobic conditions. Each bacterium was streaked for isolation from the overnight culture on selective media. Xylose Lysine Deoxycholate Agar (XLD; BD) was used for *Salmonella* spp., and Modified Oxford Agar (MOX; BD) was used for *L. monocytogenes. Salmonella* spp. and *Listeria* latex confirmation tests were performed using an isolated colony for each strain of bacteria.
(Microgen Bioproducts; MB, Camberley, UK). A single colony of each *Salmonella* serovar and *L. monocytogenes* strain was added to 10 mL of fresh TSB (BD) and incubated under the same conditions to achieve a concentration of approximately $8 \log_{10}$.

*Campylobacter* spp. cultures were grown from agar slants received from ATCC or USDA. A loop of culture for each strain was inoculated into 10 mL of Brucella broth (Hi Media Laboratories; HML, Mumbai, India) and grown for 48 hours at 42°C under anaerobic conditions (10% CO$_2$, 90% N$_2$). Strains were streaked for isolation on modified Charcoal Cefoperazone Deoxycholate agar (CCDA; Oxoid LTD; Basingstoke, Hampshire, England) and incubated for 48 hours under the same conditions. *Campylobacter* spp. latex confirmation tests (MB) were performed for each strain using an isolated colony. An isolated colony was also inoculated into 10 mL of fresh Brucella broth (HML) for 48 hours under the same conditions to achieve an approximate concentration of $8 \log_{10}$.

**Attachment and Survival in Ground Pork**

Ground pork (~80% lean) was purchased from a local grocery store and divided into four treatment groups over five different sampling times. Three of the four experimental groups were control groups and were individually inoculated with *Salmonella* spp., *L. monocytogenes*, or *Campylobacter* spp. The final treatment group was inoculated with all three pathogens together. Each pathogen was grown in 10 mL of TSB or Brucella broth, and 3.33 mL of each pathogen were combined to make an inoculation cocktail of 10 mL for each species. 3.33 mL of all nine strains of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. were combined to make approximately 30 mL of inoculum for mixed samples. Each ground pork sample containing either *Salmonella* spp., *L. monocytogenes*, or *Campylobacter* spp. contained 90 grams of ground
pork inoculated with 10 mL of liquid culture. The mixed culture group contained 270 grams of ground pork with 30 mL of liquid culture containing all nine organisms. Samples were hand mixed inside sterile plastic bags until the inoculum was absorbed and evenly distributed and then stored in a 4°C refrigerator for the duration of the experiment. Samples were taken at 0, 12, 24, 36, and 48 hours. At each time point, 25 g from each sample were stomached for one minute at 230 rpm with 225 mL of buffered peptone water, serially diluted, and plated on to the appropriate selective media for enumeration of pathogens.

Attachment on Lean Pork and Pork Fat

Fresh overnight cultures of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. were centrifuged for 5 minutes at 10,000 g and re-suspended in 1X PBS to remove metabolites present in the growth medium. Two milliliters of each pathogen strain were combined to make an inoculation cocktail. Fresh lean pork (Institutional Meat Purchase Specifications 406a) and pork fat (Institutional Meat Purchase Specifications 410C) were obtained from the Penn State Meats Laboratory. The lean and fat were cut into pieces approximately 6x3x0.5 centimeters and UV treated (30 watt germicidal bulb, OSRAM Sylvania Inc., Wilmington, MA; 72 centimeters from tissue; 15 minutes). 0.1 milliliters of the pathogen inoculation cocktail were pipetted on to the surface of the lean and fat pieces, spread across the surface using a sterile loop, and allowed to attach for 30 minutes at 4°C. Duplicate samples for lean and fat were collected at 0, 6, and 12 hours. At each sampling time, lean and fat samples were stomached for one minute at 230 rpm, serially diluted, and plated on to selective media for pathogen enumeration.
Results

Attachment and Survival in Ground Pork

*Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. successfully survived 48 hours in ground pork, although differences in pathogen concentrations were observed between samples containing pure inoculum of one pathogenic species and mixed inoculum samples containing all pathogenic species used in the experiment (Table 1). Inoculum concentrations were determined to confirm that pathogenic cultures were able to grow to expected levels. *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. overnight cultures grew to 8.96, 8.91, and 8.67 log_{10} CFU/g, respectively. It was expected that the pathogen concentrations would decrease when inoculated into ground pork samples. Time 0 hr concentration of *Salmonella* spp. and *L. monocytogenes* in their non-mixed culture samples were both greater than 8 log_{10} CFU/g (8.05 and 8.26 log_{10} CFU/g, respectively). Time 0 hr concentration of *Salmonella* spp. and *L. monocytogenes* from mixed culture samples was lower than pure culture samples (p < 0.05), with *Salmonella* spp. achieving a 7.57 log_{10} CFU/g concentration and *L. monocytogenes* achieving a 7.82 log_{10} CFU/g concentration. *Salmonella* spp. concentrations in pure inoculum samples were not statistically different after 48 hrs (p > 0.05), with a final concentration of 7.85 log_{10} CFU/g. In contrast, concentrations of *Salmonella* spp. in mixed inoculum samples did decrease (p < 0.05). The final concentration of *Salmonella* spp. in mixed inoculum samples was 7.09 log_{10} CFU/g. *L. monocytogenes* concentrations in pure inoculum samples decreased from 0 hr to 12hr (8.26 log_{10} CFU/g to 7.89 log_{10}CFU/g) (p < 0.05) and then maintained statistically similar population levels until 48 hrs (p > 0.05). In mixed inoculum samples, *L. monocytogenes* concentrations decreased from 0hrs to 24 hrs (p < 0.05), increased from 24 to 36 hrs (p < 0.05), and decreased again from 36 to 48 hours (p < 0.05) to a final concentration of 7.48 log_{10}CFU/g.
Concentrations of *L. monocytogenes* were lower in mixed inoculum samples from 0 hrs to 24 hours (p < 0.05) and then were not statistically different from 36 hrs to 48 hrs (p > 0.05). *Campylobacter* spp. achieved time 0 hr concentrations of 7.75 log_{10} CFU/g for pure culture samples and 7.82 log_{10} CFU/g for mixed culture samples. Concentrations of *Campylobacter* spp. in pure culture samples decreased from 0 hr to 12 hr (p < 0.05) followed by no significant changes from 12 hr to 48 hr (p > 0.05), and concentrations of *Campylobacter* spp. in mixed culture samples did not change from 0hr to 48hr (p > 0.05). *Campylobacter* spp. in pure culture samples achieved a final concentration of 7.29 log_{10} CFU/g, and *Campylobacter* spp. in mixed culture samples achieved a final concentration of 7.64 log_{10} CFU/g. *Campylobacter* spp. concentrations were only statistically different between pure inoculum samples and mixed inoculum samples at 12 hrs (p < 0.05) where pure inoculum samples achieved a 7.33 log_{10} CFU/g concentration and mixed inoculum samples achieved a 7.85 log_{10} CFU/g concentration. No other statistical differences between pure culture samples and mixed culture samples containing *Campylobacter* spp. were observed. Collectively, these data indicate that *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. can coexist in a meat system.

**Attachment on Lean Pork and Pork Fat**

*Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. successfully attached to both lean and fat surfaces of pork exposed to aerobic atmosphere at 4°C (Table 2). Concentrations of *Salmonella* spp. and *L. monocytogenes* on lean pork did not decrease after 12 hours (p > 0.05), with *Salmonella* spp. achieving a final concentration of 6.22 log_{10} CFU/g and *L. monocytogenes* achieving a final concentration of 6.54 log_{10} CFU/g. *Campylobacter* spp. concentration decreased on lean pork, but still maintained a final concentration of 6.43 log_{10}
CFU/g. All three pathogen species decreased in concentration on pork fat (p < 0.05) but were still able to maintain very high levels after 12 hours. *Salmonella* spp. showed a 0.56 log$_{10}$ CFU/g reduction (p < 0.05), *L. monocytogenes* showed a 1.11 log$_{10}$ (CFU/g) (p < 0.05), and *Campylobacter* spp. showed a 0.53 log$_{10}$ CFU/g reduction (p < 0.05). Despite these reductions, all three pathogen species maintained concentrations greater than 5 log$_{10}$ CFU/g on the surface of pork fat.

**Discussion**

Preventing a false negative result from occurring in a challenge study is vital, and ensuring that the pathogens chosen for a challenge study can coexist in an inoculation cocktail and in inoculated meat is the crucial first step of conducting a challenge study (Yokoyama *et al.*, 2005). In this instance, a false negative result would most likely occur if *Campylobacter* spp. were negatively impacted by either the presence of other organisms in an inoculation cocktail or by the laboratory processes needed to prepare the inoculation cocktail. After determining that there are no direct inhibitory relationships in a pour plate assay between the taxa of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. chosen for this study (Chapter 2), additional experiments were needed to confirm that all three organisms could coexist in a meat system and to confirm that all three organisms could simultaneously attach to the surfaces of raw meat at levels sufficient to show 99.999% reductions in population. Confirmation that these strains of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. are able to successfully attach at similar concentrations is necessary since attachment can be highly variable among different species of bacteria and there is no guarantee that these specific strains will attach to meat at the same concentrations (Firstenberg-Eden, 1981; Selgas *et al.*, 1993; Sinde and Carballo, 2000).
These experiments were vital in confirming that no antagonistic relationship exists between the pathogens chosen for the study since little to no research exists on the use of *Campylobacter* spp. in a meat product challenge study. The attachment in ground pork and attachment on lean pork and pork fat experiments also served as a test for determining if these strains of *Campylobacter* spp. could maintain the high concentrations needed for a challenge study inoculation during the laboratory preparation procedures that included centrifuging and resuspending in Phosphate Buffered Saline.

The ground pork experiment (Table 1) confirmed that the nine strains of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. could successfully survive in the same meat system for an extended amount of time. Determining the populations of each pathogen inoculated into ground pork individually confirmed that there were no unknown factors negatively impacting these strains of bacteria in meat in addition to demonstrating the widely known fact that bacterial pathogens survive very well in raw meat. Comparing concentrations of each pathogen from pure inoculum samples to pathogen concentrations from samples containing all three species created the ability to determine if there were significant differences in pathogen concentrations specifically caused by the mixed inoculum. After 48 hours of refrigerated storage (4°C), only *Salmonella* spp. exhibited a statistically significant difference between pure inoculum samples and mixed inoculum samples. Although there were slight numerical differences in *L. monocytogenes* and *Campylobacter* spp. concentrations between pure inoculum and mixed inoculum samples after 48 hours, the final concentrations were not statistically different. It is equally important to note that although there were some decreases in pathogen concentrations, especially by *Salmonella* spp. in mixed inoculum samples, from 0 hrs to 48 hrs, all 48 hr samples achieved pathogen concentrations greater than 7 log\(_{10}\) CFU/g. Although there may be very small
factors causing small variations in pathogen concentrations throughout the duration of the experiment, this result confirms that a true antagonistic relationship between *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. does not manifest in raw ground meat. The experiment also confirms that the atmospheric levels of oxygen *Campylobacter* spp. are exposed to during inoculation into raw ground meat do not negatively impact the ability of the cells to successfully survive in raw meat.

The attachment onto the surfaces of lean pork and pork fat experiment (Table 2) confirmed that these strains of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. can successfully attach to meat surfaces and survive at very high concentrations for at least 12 hours. No statistical changes on lean meat were observed in *Salmonella* spp. and *L. monocytogenes*, and decreases less than 0.5 log_{10} were observed in *Campylobacter* spp. All three organisms decreased on pork fat from 0 to 12 hours. This result was intriguing, because Chung *et. al.* (1989) demonstrated that there is no difference in attachment between lean and fat tissue. One potential explanation for the decreases in concentration from this experiment is that because the samples were left uncovered, the evaporation of liquid inoculum and resulting drying on the surface of fat samples caused the death of some cells. Given that fat samples in Chung *et. al.* (1989) were sealed using Parafilm to prevent dehydration, this explanation seems reasonable. It is most important to note that all 12-hour samples contained greater than or equal to 5.5 log_{10} CFU/g concentration. Taking consideration that these samples were inoculated by pipetting inoculum onto one surface of each sample and not submerging the samples into an inoculation bath, these data show that the strains of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. utilized for this experiment can successfully attach to both lean meat and fat tissue in a mixed inoculum. Additionally, this experiment demonstrates that laboratory preparation
procedures for meat inoculation do not negatively impact the ability of *Campylobacter* spp. to attach to lean meat and fat surfaces.

These experiments conclusively demonstrate that the strains of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. used were able to successfully attach to meat at levels that would be sufficient to demonstrate a 99.999% reduction in population for process validation. Furthermore, an inhibitory relationship did not exist between the strains chosen for this study. Any death of these organisms during a challenge study would be due to negative environmental changes the bacteria would be subjected to and would not be due to the presence of the other pathogens in the meat. A false positive result in a challenge study if these strains *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. were used would be very unlikely.
### Tables

**Table 1:** Concentration of *Salmonella* spp., *Listeria monocytogenes*, and *Campylobacter* spp. (log\(_{10}\) CFU/g) in ground pork samples inoculated with individual pathogen species compared to samples inoculated with all pathogen species (\(\alpha = 0.05\))

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Inoculum</th>
<th>0 hr</th>
<th>12 hr</th>
<th>24 hr</th>
<th>36 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inoculum</td>
<td>8.96</td>
<td>8.05(^{A,a})</td>
<td>7.98(^{A,c})</td>
<td>7.99(^{A,e})</td>
<td>7.85(^{A,g})</td>
<td>7.85(^{A,i})</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>*</td>
<td>7.57(^{Z,b})</td>
<td>7.61(^{Z,d})</td>
<td>7.38(^{Y,f})</td>
<td>7.34(^{Y,h})</td>
<td>7.09(^{X,j})</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td></td>
<td>8.91</td>
<td>8.26(^{A,a})</td>
<td>7.89(^{B,c})</td>
<td>7.72(^{B,e})</td>
<td>7.66(^{B,g})</td>
<td>7.58(^{B,h})</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>*</td>
<td>7.82(^{Z,b})</td>
<td>7.61(^{Y,d})</td>
<td>7.34(^{X,f})</td>
<td>7.73(^{W,g})</td>
<td>7.48(^{X,h})</td>
</tr>
<tr>
<td><em>Campylobacter</em> spp.</td>
<td></td>
<td>8.67</td>
<td>7.75(^{A,a})</td>
<td>7.33(^{B,b})</td>
<td>7.44(^{B,d})</td>
<td>7.40(^{B,e})</td>
<td>7.29(^{B,f})</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>*</td>
<td>7.82(^{Z,a})</td>
<td>7.85(^{Z,c})</td>
<td>7.66(^{Z,d})</td>
<td>7.68(^{Z,e})</td>
<td>7.64(^{Z,f})</td>
</tr>
</tbody>
</table>

Capital letters represent comparisons among time points of the same treatment group. Lowercase letters represent comparisons made at the same time point between the pure and mixed treatment groups. No comparisons between treatment groups of different species were made.

* Mixed inoculum concentration was not calculated
Table 2: Comparison of *Salmonella* spp., *Listeria monocytogenes*, and *Campylobacter* spp. concentration (log_{10} CFU/g) in a mixed inoculum on lean meat and fat (α = 0.05)

<table>
<thead>
<tr>
<th>Time</th>
<th><em>Salmonella</em> spp.</th>
<th><em>Listeria monocytogenes</em></th>
<th><em>Campylobacter</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean</td>
<td>Fat</td>
<td>Lean</td>
</tr>
<tr>
<td>0</td>
<td>6.27a</td>
<td>6.56a</td>
<td>6.59a</td>
</tr>
<tr>
<td>6</td>
<td>6.29a</td>
<td>6.25b</td>
<td>6.57a</td>
</tr>
<tr>
<td>12</td>
<td>6.22a</td>
<td>6.00c</td>
<td>6.54a</td>
</tr>
</tbody>
</table>
References


Chapter 4
Fate of *Salmonella* spp., *Listeria monocytogenes*, and *Campylobacter* spp., During Fermentation and Drying of Duck Salami

Introduction

Salami production has been used as a method of meat preservation for thousands of years (Zeuthen, 2007), and different civilizations around the world developed unique styles and recipes for producing salami that have been preserved through generations. As the charcuterie industry in the United States has grown, the desire by European immigrant families to produce old-world charcuterie and salumi products has also grown (Maddock, 2014; Lücke, 2015). Traditional salamis like Genoa and cacciatore are produced by mixing ground pork with salt, cure, spices, and a lactic acid producing starter culture. The salamis are fermented to produce traditional flavors as well as to allow for acid coagulation of proteins by slowly decreasing the pH of the meat. Following fermentation, salamis are typically dried to remove moisture and decrease the water activity ($a_\text{w}$) of the product. Traditionally produced salamis often do not undergo a thermal processing step and are sold as raw, ready-to-eat (RTE) products (Maddock, 2014; Rust, 2014; Hierro *et al*., 2015).

Producing fermented and dried salamis with duck meat as the primary ingredient is a departure from traditional salami production methods. However, the popularity of processed poultry products is increasing in the United States in part due to the perception that poultry is healthier than beef or pork. Charcuterie companies in the United States have been able to successfully market processed poultry products that include salami (Arnaud *et al*., 2014). The use of duck in a salami presents some unique quality challenges. The main challenge is that
traditional salami relies heavily on the presence of up to 50% fat in the finished product for flavor and other sensory characteristics. Since poultry fat is at a higher risk for lipid oxidation when compared to pork or beef fat, this may present some undesirable quality traits. Poultry fat contains a high percentage of unsaturated fats that are more susceptible to oxidation than the saturated fats that make up the majority of beef and pork adipose tissue (Arnaud et al., 2014). Manufacturers that do not wish to add antioxidants to a salami can prevent the oxidation of poultry fat in a salami by removing as much fat as possible from the raw duck meat prior to production and then use another source of fat like pork that does not oxidize as readily.

Raw, RTE duck salami also faces microbial safety challenges. To prevent the survival and growth of pathogens, duck salami relies on multiple antimicrobial parameters inherent to the product and process called “hurdles” (Leistner and Gorris, 1995). Reducing pH by fermentation, reducing water activity ($a_w$) by drying, increasing osmotic stress by adding salt, and adding spices that have antimicrobial properties are traditionally used in combination to ensure the safety of salami products. The combined effect of each hurdle creates an unfavorable environment for pathogenic cells that may be present and can, over time, cause cellular exhaustion and death (Leistner and Gorris, 1995; Leistner, 2000; Khan et al., 2017). The Hurdle Effect is a widely used method to improve the microbial safety of food products; and using multiple hurdles in combination creates two practical implications for meat processors. First, the collective use of multiple hurdles allows for the strength and severity of each individual hurdle to be lessened without compromising the safety of the product (Beales, 2004; Leroy et al., 2006). For example, because salami is dried, the pH of the final product can be held at more palatable levels compared to a meat product that is not dried as severely. Second, the use of all the previously mentioned hurdles allows meat processors to produce safe products that do not
undergo a thermal treatment. Thermal treatments are effective methods for reducing the presence of pathogens in a meat product (Beales, 2004), but traditional salami are not thermally processed to maintain flavor and texture qualities that consumers expect from traditional salami products. Recently, the use of organic acid treatments on raw meat prior to salami production has been acceptable as an addition hurdle. Beefxide (Birko Corporation; Henderson, CO), which is approved as a processing aid in meat products by USDA, is commonly used for treatment of raw meat prior to salami production. When used properly, the Hurdle Effect is able to ensure that traditional salami is both safe to eat and that they meet the highest standards for quality.

*Salmonella* spp. and *Campylobacter* spp. are both commonly found in live poultry and raw poultry meat (Yogasundram et al., 1989; Benedict et al., 1990; McCrea et al., 2006; Ritz et al., 2007; Nguyen et al., 2010; Adzitey et al., 2012; Landrum et al., 2016; Skarp et al., 2016; Li et al., 2017; Wei and Kang, 2018; Shen et al., 2019); and multiple foodborne illness outbreaks have been attributed to both *Salmonella* spp. and *Campylobacter* spp. in poultry (Centers for Disease Control and Prevention (CDC), 2019a; CDC, 2019b). The CDC have reported 760 outbreaks of various types due to *Salmonella* spp. and poultry from 2000-2017. The outbreaks caused over 10,000 reported illnesses, over 1,700 reported hospitalizations, and 27 deaths (CDC, 2018). CDC has reported fewer confirmed outbreaks of *Campylobacter* spp. than *Salmonella* spp. The 478 outbreaks of *Campylobacter* spp. due to food have caused roughly 8,000 reported illnesses, 367 hospitalizations, and one death (CDC, 2018). It was determined that meat products were implicated in 100 of the outbreaks and roughly 1,000 of the illnesses were confirmed to be caused by meat products (CDC, 2018).

*Listeria monocytogenes* is a pathogen of concern for RTE meat products because of its ability to grow at refrigeration temperatures and colonize meat processing environments.
(Blackman and Frank, 1996; Gombas et al., 2003; Foong and Dickson, 2004; Ingham et al.,
2004; Zhu et al., 2005; Angelidis and Koutsoumanis, 2006; Gianfranceschi et al., 2006; Gandhi
and Chikindas, 2007; Chaturongakul et al., 2008; Endrikat et al., 2010; Ferreira et al., 2014; Goh
et al., 2014). From 2000-2017, there were five outbreaks of L. monocytogenes associated with
meat resulting in 101 confirmed illnesses, 47 hospitalizations, and 16 deaths. Of these five
outbreaks, three were confirmed to be caused by poultry deli meats (CDC, 2019c; CDC, 2018).
Additionally, there was one confirmed outbreak related to duck charcuterie products. Seven
people were hospitalized in Australia due to salmonellosis when a duck prosciutto was
improperly prepared at a restaurant (Draper et al., 2017).

To combat the presence and survival of these pathogens in meat products, the United
States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) requires that
pathogens of concern be addressed by meat processors using scientifically valid preventative
methods via a Hazard Analysis Critical Control Points (HACCP) plan (9 CFR 417.4). Challenge
studies performed in a research laboratory are a commonly utilized method by meat processors
for assistance in documenting the safety of a specific product. These studies utilize pathogens of
concern for a specific product in order to demonstrate the safety of that product. During a
challenge study, the processing parameters decided upon by the meat processor are utilized by
researchers to determine if those parameters are able to achieve adequate reductions (e.g. $\geq 5$
log$_{10}$ CFU/g) for the pathogens inoculated. Salmonella spp., L. monocytogenes, and
Campylobacter spp. are the pathogens most likely to be addressed when considering the safety of
a RTE duck salami (Benedict et al., 1990; Hill et al., 2002; Gombas et al., 2003; Foong and
Dickson, 2004; Ingham et al., 2004; McCrea et al., 2006; Nguyen et al., 2010; Adzitey et al.,
2012; Goh et al., 2014; Landrum et al., 2016; Skarp et al., 2016). Although a 7 log$_{10}$ CFU/g
reduction of *Salmonella* spp. is recommended to demonstrate that no viable *Salmonella* spp. cells are present in the final product, a $5 \log_{10} \text{CFU/g}$ reduction of the pathogen is used as a measure of validity for a “safe” process (USDA, 2017). *L. monocytogenes* is considered by USDA-FSIS to be an adulterant in RTE products for human consumption, and its presence in RTE meat products is illegal. To demonstrate that *L. monocytogenes* is not present in a RTE product, meat processors must demonstrate that *L. monocytogenes* cells are not able to grow at any point throughout a manufacturing process (USDA). Lastly, the USDA-FSIS requires that *Campylobacter* spp. be addressed in meat products containing poultry for HACCP documentation and that a $5 \log_{10} \text{CFU/g}$ reduction of *Campylobacter* spp. cells be achieved in RTE poultry products (9 CFR 417). To demonstrate those reductions in a duck salami, an experiment was designed to validate the bacterial safety of a processed used to manufacture a fermented and dried duck salami utilizing *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. as pathogens of concern.

**Materials and Methods**

**Culture Selection and Growth**

The following cultures were received or purchased from American Type Culture Collection (ATCC; Manassas, VA), CDC, or USDA: *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028, isolated from chicken organs), *Salmonella enterica* subsp. *enterica* serovar Montevideo isolate SMvo13, *Salmonella enterica* subsp. *enterica* serovar Derby (ATCC 7378, human isolate), *Listeria monocytogenes* serotype Scott A, *Listeria monocytogenes* serotype 1/2a isolate FSL R2-603 (deli meats outbreak), *Listeria monocytogenes* serotype 4b isolate H3396 (hotdog outbreak), *Campylobacter jejuni* isolate PSU99 (isolated from
chicken in Pennsylvania, species confirmed by USDA), *Campylobacter jejuni* (ATCC 29428, human isolate), and *Campylobacter coli* (ATCC 33559, pig isolate). These cultures were chosen for their role in human illness or association with meat and food products.

Overnight cultures of each *Salmonella* serovar and *L. monocytogenes* strain were grown from laboratory stocks by adding a loop of culture to 10 mL of Tryptic Soy Broth (TSB, Becton Dickinson and Company; BD, Sparks, MD) and incubating for 24 hours at 37°C under aerobic conditions. To ensure working stocks were not contaminated, each bacterium was streaked for isolation from the overnight culture on selective media. Xylose Lysine Deoxycholate Agar (XLD; BD) was used for *Salmonella* spp., and Modified Oxford Agar (MOX; BD) was used for *L. monocytogenes*. *Salmonella* spp. and *Listeria* latex confirmation tests were performed using an isolated colony for each strain of bacteria (Microgen Bioproducts; MB, Camberley, UK). A single colony of each *Salmonella* serovar and *L. monocytogenes* strain was added to 10 mL of fresh TSB (BD) and incubated under the same conditions to achieve a concentration of approximately 8 log_{10}.

*Campylobacter* spp. cultures were grown from agar slants received from ATCC or USDA. A loop of culture for each strain was inoculated into 10 mL of Brucella broth (Hi Media Laboratories; HML, Mumbai, India) and grown for 48 hours at 37°C under anaerobic conditions (10% CO_{2}, 90% N_{2}). Strains were streaked for isolation on Charcoal Cefoperazone Deoxycholate agar (CCDA; Oxoid LTD; Basingstoke, Hampshire, England) and incubated for 48 hours under the same conditions. *Campylobacter* spp. latex confirmation tests (MB) were performed for each strain using an isolated colony. An isolated colony was also inoculated into 10 mL of fresh Brucella broth (HML) for 48 hours under the same conditions to achieve an approximate concentration of 8 log_{10}.
An inoculation bath was created for the inoculation of raw duck and pork belly. Fresh 10mL overnight cultures of each of the nine strains of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. were incubated in either TSB (BD) or Brucella broth (HML). The overnight cultures were then added to a fresh 340 mL of either TSB (BD) or Brucella broth (HML) and incubated again for the same length of time. After incubation, the 350 mL broth cultures were centrifuged for 5 minutes at 21° C at approximately 11,000 g’s (Avanti J-26 CPI; Beckman Coulter, Inc.; Pasadena, CA). After centrifugation, supernatant was disposed, and the cultures were resuspended in 1x Phosphate Buffered Saline (PBS; Alfa Aesar; Tewksbury, MA). The resuspended cultures were combined in a sterilized metal bin to make an inoculation bath approximating 3.2 L.

**Duck Salami Production**

Duck trim, pork belly, seasonings, and casings were provided in confidence and separately for each of the three independent replications by a salami company in the Northeast. For a single replication, thirty pounds of duck trim and pork belly (70% duck, 30% pork belly) were thawed upon arrival and cut into pieces approximately 5cm³. On the first day of production, the duck trim and pork belly were submerged in the inoculation bath for thirty minutes, with stirring every five minutes, so that the pathogens could attach to the meat surfaces. After thirty minutes of submersion in the inoculation bath, the meat was scooped onto a draining cart and allowed to dry for 15 minutes. After drying, the meat was transferred to a clean, plastic container and manually tumbled using a spoon with 1 L of 2.5% Beefxide solution (Birko Corp.) that contained a proprietary blend lactic acid, citric acid, and potassium hydroxide for 15 minutes. When tumbling was completed, the meat was moved to a different clean, plastic container and
placed into a walk-in cooler (4°C) overnight. Twenty-four hours after the tumbling operation, the salami were prepared. The meat was ground once through a 6 mm grinding plate using a size 22 grinder (Avantco Equipment MG22; Clark Associates, Inc.; Lancaster, PA); and then mixed with salt (2.5% w/w), 0.24% cure #2 (92.75% salt, 6.25% sodium nitrite, 1% sodium nitrate), proprietary spice blend, and freeze-dried starter culture (Bactoferm ® F-LC; Chr. Hansen A/S; Hørsholm, Denmark) that was reconstituted in approximately 20 mL of distilled water. The product contained 150 ppm of ingoing sodium nitrite and 24 ppm of ingoing sodium nitrate. Following mixing, the salami batter was hand stuffed (10 LB. Sausage Stuffer; The Sausage Maker, Inc.; Buffalo, NY) into 55 mm collagen casings (Globe Packaging Inc.; Carlstadt, NJ) and hand tied using butcher twine. Each casing contained four salamis that were each approximately 260 grams in weight. A single salami in a separate casing was used as reference to measure weight loss and diameter changes during the fermentation and drying process. A total of 144 salamis were produced for all three replications. After stuffing, the salamis were hung in the drying cabinet (AS50; Impianti Condizionamento Salumifici; Camposanto, Modena, Italy) (Figure 1) and fermented for 48 hours at 23°C and 95% rH. Following fermentation, the salamis were dried at 14°C and 75% rH to a target weight loss of 44%. Drying averaged five weeks among three replications. When salamis completed drying, they were individually vacuum packaged (Ultravac UV 250; Koch Packaging; Kansas City, MO) in 3mil food grade pouches (OTR 50-70 cc/m²/24hr; UltraSource LLC.; Kansas City, MO) and stored at ~ 23°C for an additional four weeks. The pH and a_w targets for the final product were less than 5.3 and less than 0.90, respectively.
**Sampling Procedures**

For each replication, samples were taken on days 0, 1, 2, 3, 5, 10, and then every seven days until day 66. On each sampling day, three random salamis were taken for enumeration of pathogens, and one salami sample was taken for determination of pH and $a_w$. On days 0 and 1, samples were taken from duck trim and pork belly surfaces, prior to salami production. For enumeration of pathogens, salamis were cut in half, and the cores of each half were removed using a sterile plastic spoon. Twenty-five grams of meat were stomached (Seward Stomacher 400 Circulator; Fermionx Ltd; Worthing, West Sussex, UK) with 100 grams of Buffered Peptone Water (BPW; BD) for one minute at 260 rpm, and then the stomachate was serially diluted using 9 mL BPW (BD) blanks. Each sample was plated onto XLD (BD), MOX (BD), CCDA (Oxoid), and TSA (BD) plates in duplicate at the relevant dilutions. Colonies matching the morphology of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. were confirmed using latex agglutination (MB) on each sampling day. pH was measured from either the surface of duck or pork belly pieces on day 0 and day 1 (A57184 pH Electrode; Beckman Coulter Inc.; Brea, CA) or from the center of a salami for the remaining samples (Testo 206 pH2; Testo North America; West Chester, PA). Two pH measurements were taken at each sampling day, one measurement from each half of the fourth sample salami. $a_w$ samples were measured from a slice taken from the core of the salami (Aqualab 4TE; Meter Group, Inc.; Pullman, WA). Only one $a_w$ measurement was taken each sampling day.

**Statistical Analysis**

Concentrations of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. were analyzed independently using a General Linear Model with unique comparisons ($\alpha = 0.05$) (SAS...
In order to preserve statistical power, statistical comparisons were made between the average of pathogen concentration from one sampling day and the concentration of the same pathogen on the following sampling day. Statistical analysis was not utilized for \( a_w \) and pH data.

**Results**

Fermentation, drying, and vacuum packaged storage were able to achieve a 5 \( \log_{10} \) CFU/g reduction in *Salmonella* spp. (\( n = 9, p<0.0001 \)), *L. monocytogenes* (\( n = 9, p<0.0001 \)), and *Campylobacter* spp. (\( n = 9, p<0.0001 \)) (Table 1, Figure 2). *Salmonella* spp. achieved a 5.47 \( \log_{10} \) CFU/g reduction on day 38 of processing followed by a final reduction of 7.03 \( \log_{10} \) CFU/g. *L. monocytogenes* achieved a 5.20 \( \log_{10} \) CFU/g reduction on day 59 of processing with a final reduction of 5.90 \( \log_{10} \) CFU/g. Lastly, *Campylobacter* spp. achieved a 6.85 \( \log_{10} \) CFU/g reduction on day 45 of processing with a final reduction of 7.19 \( \log_{10} \) CFU/g. Pathogen concentrations did not increase by more than 1 \( \log_{10} \) CFU/g at any point during the experiment indicating that pathogens did not grow during production of duck salami. The concentration of bacteria on TSA (BD), which was presumed to be starter culture, remained greater than 5 \( \log_{10} \) CFU/g on day 66. Enrichments were conducted when pathogen concentrations reached levels below the 0.39 \( \log_{10} \) CFU/g detection limit of the plating assay (\( n=9 \)) (Table 2). By day 59, all samples produced negative *Salmonella* spp. enrichment results. *L. monocytogenes* produced positive enrichment results on all sampling days. *Campylobacter* spp. produced negative enrichment results on day 66.

In addition to achieving the previously stated reductions in pathogen concentrations, the processing parameters of fermenting and drying were able to achieve the desired reductions in
pH and a\textsubscript{w} (Table 3). Finished duck salami (Figure 3) achieved a final pH of 5.11 and a final a\textsubscript{w} of 0.81. pH first decreased below the target of 5.3 on day 3, after fermentation was completed, with a pH of 5.00. pH below 5.3 was maintained from day 3 to day 66 when processing was completed. a\textsubscript{w} first decreased below the target of 0.90 on day 31 with a a\textsubscript{w} of 0.88 and then maintained values less than 0.90 until the end of processing on day 66.

**Discussion**

The adaptation of old-world salami manufacturing methods to meet modern day pathogen safety regulations has been challenging for meat processors. The use of pure starter cultures has removed the potential for introducing potentially pathogenic organisms to the raw meat from a backslop fermentation process. Some modern starter cultures also contain bacteria that produce bacteriocins or pediocins that aid in preventing the survival and growth of *Listeria*. Modern fermentation and drying systems provide meat processors greater control and consistency for temperature and humidity settings used during processing, thus allowing for greater assurance that meat products are not able to harbor pathogens. Despite the advances in meat processing technologies and methods, the bacterial safety of salami products that are raw, RTE is still of concern. Nightingale *et al* (2016) concluded that the traditional processing steps of fermenting and drying would not be sufficient to ensure the bacterial safety of Italian salami and that a thermal processing step would be needed to prevent the presence of pathogens in the final product. They were only able to demonstrate maximum reductions of 4.5 log\textsubscript{10} CFU/g and <1 log\textsubscript{10} CFU/g of *Salmonella* spp. and *L. monocytogenes*, respectively, in traditionally processed salamis.
Instead of utilizing a thermal processing step during traditional salami production, some manufacturers have begun to use organic acid treatments of raw meat prior to grinding and stuffing. McKinney (2019) and Rivera-Reyes (2017) demonstrated that raw, RTE salami containing pork and raw, RTE landjäger (pork and beef), respectively, could be produced safely without the addition of thermal processing. Both experiments utilized an organic acid treatment of the meat trim after pathogen inoculation and prior to grinding, and both experiments were able to achieve adequate reductions of the pathogens relevant to each product. (Greater than 5 \( \log_{10} \) reductions of \textit{Salmonella} spp. and \textit{L. monocytogenes} were achieved in pork salami, and greater than 5 \( \log_{10} \) reductions of \textit{E. coli} O157:H7, \textit{Salmonella} spp., and \textit{L. monocytogenes} were achieved in landjäger.) These results demonstrated that traditional meat processing techniques were adequate to ensure bacterial safety when the use of organic acid treatments of the meat were added to the process.

The use of duck in a traditionally processed salami presents multiple additional challenges inherent to duck. Poultry is difficult to use in salami due to its higher concentration of unsaturated fat. Oxidation of poultry fats occurs much more readily than in pork or beef fats, and the use of poultry fats in a salami can potentially cause undesirable oxidized flavors and aromas in the final product. The use of poultry in traditionally processed salami also presents issues concerning bacterial safety of the product. All poultry, including duck, are a natural host of \textit{Campylobacter} spp. with \textit{C. jejuni} and \textit{C. coli} being the most common species found in poultry, respectively. Because \textit{Campylobacter} spp. is readily found in raw poultry and because it is a leading cause of foodborne illness in the United States, the pathogen must be addressed in the HACCP documentation for a RTE poultry product. Thermal treatment utilizing approved parameters for time and temperature would be sufficient to ensure the safety of a RTE poultry or
duck product; however, traditional salami manufacturing methods do not typically include a thermal treatment. Thus, a validation study for a raw, RTE duck salami utilizing Campylobacter spp. could be sufficient for validating the safety of the product, provided that appropriate reductions of Campylobacter spp. as well as Salmonella spp. and L. monocytogenes are achieved.

The results of this experiment further support the utility of organic acid treatments as additional hurdles for meat processes that do not utilize thermal treatments and confirm that the validation of traditionally produced duck salami could be achieved. Although reductions that occurred after Beefxide treatment of $0.26 \log_{10} \text{CFU/g}$, $0.29 \log_{10} \text{CFU/g}$, and $0.20 \log_{10} \text{CFU/g}$, in Salmonella spp., L. monocytogenes, and Campylobacter spp., respectively, were not significant (Table 1), the lasting effect of the acid treatment on the pathogens in combination with the reduced pH and $a_w$ hurdles was able to cause significant final reductions in pathogens. The necessary reductions for each pathogen ($> 5 \log_{10} \text{CFU/g}$) were successfully achieved in the final product. There was an increase in Campylobacter spp. from day 31 to 38 that was associated with vacuum packaging; however, the increase was less than $1 \log_{10} \text{CFU/g}$, and increases of less than $1 \log_{10} \text{CFU/g}$ are not considered to be true microbial growth.

Generic growth media, like TSA (BD), are typically used in validation experiments to determine the number of injured cells that are not able to grow on selective media. In this experiment, the addition of starter culture prevented the use of that methodology, since the presumed starter culture concentrations remained greater than $5 \log_{10} \text{CFU/g}$ on day 66 on TSA (BD) plates. Instead, enrichments (Table 2) were used to confirm the presence or absence of pathogens at the end of processing. Additionally, no distinctions were made between bacteria
that were presumed to be starter culture and bacteria that could have been natural microflora of the meat. It was also presumed that meat microflora did not impact the results of the experiment.

The decrease in both pH and a_w is influenced by multiple factors inherent to the product and the processing environment. Those values could vary at the same time across multiple replications or batches. Because of that variation, fermentation time, drying time, or water loss cannot be used as critical parameters instead of pH and aw. In duck salami, the critical parameters for final pH less than 5.3 and final a_w less than 0.90 must be followed for the reduction of Salmonella spp., L. monocytogenes, and Campylobacter spp. to be achieved. pH less than 5.3 was first achieved at the end of fermentation (day 3) and was maintained until the end of processing on day 66 (Table 3). The final pH of 5.12 meets the critical parameter of less than 5.3. Additionally, the starter culture used in this experiment produced bacteriocins and pediocins. Although no controls were conducted using a starter culture that did not produce bacteriocins or pediocins, the product should not be produced without a starter culture that is able to produce those anti-listerial compounds. There were some fluctuations in pH throughout processing, but pH never increased above 5.3 after fermentation was completed. a_w first dropped below the target of 0.90 on day 31 with a_a_w of 0.88 (Table 3). It did not increase at any point during processing and reached a final a_w of 0.81 on day 66.

These data confirm the microbiological safety of duck salami and can be used as scientifically valid evidence in a HACCP plan when meat processors are producing a raw, RTE duck salami. In addition to utilizing good manufacturing practices, processors must maintain the following critical parameters. Duck meat and pork must be treated with a 2.5% Beefxide solution prior to salami production. The duck salami must be formulated with 2.5% salt and 0.24% cure #2 (92.75% salt, 6.25% sodium nitrite, 1% sodium nitrate), fermented to a pH less than 5.2, and
dried to a \( a_w \) less than 0.87. The product must contain 150 ppm of ingoing sodium nitrite and 24 ppm of ingoing sodium nitrate. Lastly, the finished duck salami must be stored under vacuum at room temperature prior to consumption. Testing raw duck and pork for *Salmonella* spp. and *Campylobacter* spp. can be utilized to eliminate the need for vacuum packaging.
### Tables

**Table 1:** Pathogen concentration ($\log_{10}$ CFU/g ± SE) of *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. (n=9) of duck salami during sampling.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculation</td>
<td>0</td>
<td>7.41 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>*</td>
<td>7.49 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>*</td>
<td>7.57 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>*</td>
</tr>
<tr>
<td>Production</td>
<td>1</td>
<td>7.15 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26</td>
<td>7.20 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.29</td>
<td>7.37 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20</td>
</tr>
<tr>
<td>Fermentation</td>
<td>2</td>
<td>6.60 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.80</td>
<td>6.91 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.58</td>
<td>6.95 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.28 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.13</td>
<td>6.66 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83</td>
<td>6.70 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.88</td>
</tr>
<tr>
<td>Drying</td>
<td>5</td>
<td>6.05 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.35</td>
<td>6.53 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.96</td>
<td>6.67 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.90 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.51</td>
<td>5.99 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.50</td>
<td>6.50 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.07</td>
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<tr>
<td></td>
<td>17</td>
<td>4.69 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.72</td>
<td>5.89 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.60</td>
<td>5.61 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.89 ± 0.09&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.52</td>
<td>5.47 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.01</td>
<td>4.68 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.90</td>
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<td></td>
<td>31</td>
<td>2.49 ± 0.18&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.92</td>
<td>4.84 ± 0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.64</td>
<td>2.96 ± 0.15&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.61</td>
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<td>Packaging</td>
<td>38</td>
<td>1.94 ± 0.24&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5.47</td>
<td>4.14 ± 0.52&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.35</td>
<td>3.56 ± 0.33&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>45</td>
<td>0.65 ± 0.11&lt;sup&gt;h&lt;/sup&gt;</td>
<td>6.76</td>
<td>3.19 ± 0.70&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.30</td>
<td>0.73 ± 0.13&lt;sup&gt;g&lt;/sup&gt;</td>
<td>6.85</td>
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<td>0.60 ± 0.07&lt;sup&gt;h&lt;/sup&gt;</td>
<td>6.81</td>
<td>2.61 ± 0.55&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.88</td>
<td>0.61 ± 0.07&lt;sup&gt;g&lt;/sup&gt;</td>
<td>6.96</td>
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<td>59</td>
<td>0.38 ± &lt;0.01&lt;sup&gt;h&lt;/sup&gt;</td>
<td>7.03</td>
<td>2.28 ± 0.51&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>0.40 ± 0.02&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>66</td>
<td>0.38 ± &lt;0.01&lt;sup&gt;h&lt;/sup&gt;</td>
<td>7.03</td>
<td>1.58 ± 0.27&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5.90</td>
<td>0.38 ± &lt;0.01&lt;sup&gt;g&lt;/sup&gt;</td>
<td>7.19</td>
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Pathogen concentrations with different superscripts<sup>a-h</sup> are statistically different than the pathogen concentration from the preceding sampling day ($\alpha = 0.05$). Comparisons only made within column for individual pathogens.
Table 2: Enrichments for *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp. during duck salami processing. Data are reported positive (+) or negative (-) for the presence of each pathogen. (n=9)

<table>
<thead>
<tr>
<th>Sample Day</th>
<th>Salmonella spp.</th>
<th>L. monocytogenes</th>
<th>Campylobacter spp.</th>
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<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td>66</td>
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Table 3: Water loss (n=1 for each replication), salami diameter (n=3), pH (n=6), and aw (n=3) of duck salami during processing.

<table>
<thead>
<tr>
<th>Processing Step</th>
<th>Day</th>
<th>Water Loss (%) Replication 1</th>
<th>Water Loss (%) Replication 2</th>
<th>Water Loss (%) Replication 3</th>
<th>Diameter (mm)</th>
<th>pH</th>
<th>aw</th>
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<td>Inoculation</td>
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<td>*</td>
<td>*</td>
<td>5.67</td>
<td>0.99</td>
<td></td>
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<tr>
<td>Production</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>55</td>
<td>5.53</td>
<td>0.98</td>
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<tr>
<td>Fermentation</td>
<td>2</td>
<td>0.25</td>
<td>1.60</td>
<td>2.27</td>
<td>55</td>
<td>5.35</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.37</td>
<td>3.32</td>
<td>4.06</td>
<td>55</td>
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<td>Drying</td>
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<td>12.41</td>
<td>13.22</td>
<td>12.83</td>
<td>53</td>
<td>4.92</td>
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<td>31.72</td>
<td>28.43</td>
<td>47.33</td>
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<td>41.97</td>
<td>38.14</td>
<td>32.80</td>
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<td>47.31</td>
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<td>43</td>
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<td>0.88</td>
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<tr>
<td>Packaging (R2)</td>
<td>38</td>
<td>47.31</td>
<td>45.32</td>
<td>39.79</td>
<td>42.67</td>
<td>4.95</td>
<td>0.86</td>
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<td></td>
<td>45</td>
<td>47.31</td>
<td>45.32</td>
<td>42.06</td>
<td>42.33</td>
<td>4.97</td>
<td>0.83</td>
</tr>
<tr>
<td>Packaging (R3)</td>
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<td>47.31</td>
<td>45.32</td>
<td>44.55</td>
<td>42</td>
<td>5.10</td>
<td>0.82</td>
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<tr>
<td></td>
<td>59</td>
<td>47.31</td>
<td>45.32</td>
<td>44.55</td>
<td>42</td>
<td>5.05</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>47.31</td>
<td>45.32</td>
<td>44.55</td>
<td>42</td>
<td>5.12</td>
<td>0.81</td>
</tr>
</tbody>
</table>
Figure 1: Stuffed salamis hanging in drying cabinet prior to fermentation
Figure 2: Concentration of *Salmonella* spp., and *Listeria monocytogenes*, and *Campylobacter* spp. during the production of duck salami (n=9)
Figure 3: Finished duck salami
References


Chapter 5
Conclusions and Future Directions

This project proved the bacterial safety of raw, RTE duck salami against the relevant pathogenic hazards of Salmonella spp., L. monocytogenes, and Campylobacter spp.. The salami must be treated with 2.5% Beexide, fermented at a pH less than 5.2, and dried to a $a_w$ less than 0.87. The formulation must also contain at least 2.5% salt and 0.24% cure #2 (92.75% salt, 6.25% sodium nitrite, 1% sodium nitrate), and finished duck salami must be stored under vacuum. The product must contain 150 ppm of ingoing sodium nitrite and 24 ppm of ingoing sodium nitrate. Three strains of each pathogen were collectively inoculated on to raw duck and pork belly, and reductions in pathogen cell concentrations of greater than $7 \log_{10}$ were achieved for Salmonella spp. and Campylobacter spp.. A reduction of greater than $5 \log_{10}$ was also achieved for L. monocytogenes.

Although the spice blend utilized for this project was shared in confidence, meat processors can use the processing parameters established in this project as scientifically valid support for the safety of raw, RTE duck salami as a part of an established HACCP system. Citing these results would place meat processors in compliance with USDA-FSIS regulations regarding the production of this product. Meat processors could also further increase the safety of a raw, RTE duck salami in their respective facilities by establishing critical limits for pH and $a_w$ lower than those established by this research. Meat processors can utilize negative testing results for Salmonella spp. and Campylobacter spp. on raw duck and pork as evidence for the safety of a process that does not utilize vacuum packaging as a final step.

Future directions of research could include determining if decreasing the concentration of organic acid used to treat raw meat would still maintain adequate reductions in the relevant
pathogens. Also, this research fills a significant gap in the area of the bacterial safety of duck products. Future research could further explore the safety of meat products that are less commonly found like products made from domestic bison, elk, or other lesser known species.