5-2013

Development of a Predictive Modeling System for Validation of the Cumulative Mirobial Inactivation of the Salmonellae in Pepperoni Utilizing a Non-Pathogenic Surrogate Microorganism (Enterococcus faecalis)

Scott Donald Stillwell
University of Arkansas, Fayetteville

DEVELOPMENT OF A PREDICTIVE MODELING SYSTEM FOR VALIDATION OF THE CUMULATIVE MICROBIAL INACTIVATION OF THE SALMONELLAE IN PEPPERONI UTILIZING A NON-PATHOGENIC SURROGATE MICROORGANISM (*ENTEROCOCCUS FAECALIS*)

DEVELOPMENT OF A PREDICTIVE MODELING SYSTEM FOR VALIDATION OF THE CUMULATIVE MICROBIAL INACTIVATION OF THE SALMONELLAE IN PEPPERONI UTILIZING A NON-PATHOGENIC SURROGATE MICROORGANISM (*ENTEROCOCCUS FAECALIS*)

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

By

Scott Donald Stillwell
Herriot-Watt University
Master of Business Administration, 2004

May 2013
University of Arkansas

ABSTRACT

Salmonellosis is the most frequently occurring bacterial foodborne illness in the United States and the human case rate has not improved for the past two decades. The federal agency responsible for oversight of meat and poultry processors has announced the intent to extend existing pathogen reduction performance standards for *Salmonella* spp. to cover all classes of products including fermented sausages that are currently produced under HACCP plans that are validated for control of *Escherichia coli* O157:H7. The proposed regulatory modifications will require processors to revalidate HACCP plan controls to achieve either a 6.5 or a 7.0 $\log_{10}$ inactivation of the salmonellae. Validated and accepted predictive microbial inactivation models that may be used to estimate the inactivation effects achieved for different product formulations under different processing conditions hold the potential to substantially reduce the impact that the proposed regulatory changes might have on the industry. This review examines the history of food preservation; the history of fermented sausages; the pepperoni production process; the food safety hazards most often associated with fermented sausage products; recent outbreaks of illness associated with fermented meat products; and the process controls that may be employed to prevent foodborne illness from consumption of fermented sausage products. The papers intended for publication will be beneficial to processors of pepperoni, providing a modeled estimate of the $\log_{10}$ reductions achieved under a variety of processing conditions.

This dissertation is approved for recommendation

to the Graduate Council.

Dissertation Director:

_____

Dr. John Marcy

Dissertation Committee:

_____

Dr. James Denton

_____

Dr. H.L. Goodwin

_____

Dr. Richard Linton

_____

Dr. Michael Slavik

DISSERTATION DUPLICATION RELEASE

I hereby authorize the University of Arkansas Libraries to duplicate this dissertation when needed for research and/or scholarship:

Agreed _____
Scott Donald Stillwell

Refused _____
Scott Donald Stillwell

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my professor, Dr. John Marcy, for his guidance, encouragement and patience during my graduate research. I would also like to thank Dr. James Denton, Dr. H.L. Goodwin, Dr. Richard Linton and Dr. Michael Slavik for serving on my committee at program completion; Dr. Ellis Brunton (deceased) and Dr. Justin Morris for serving on my initial committee. Each of these distinguished gentlemen has provided valuable guidance, encouragement and inspiration while offering helpful suggestions throughout this process. Special thanks are reserved for Dr. Brunton, my former boss at Tyson Foods who, many years ago, saw in me an untapped potential and placed me on the path that culminates with this program of study. Absent his lofty expectations of me, I would never have set forth on this journey. I will be forever grateful for his leadership, mentoring and the opportunities for personal and professional development that he placed before me.

I am extremely grateful for the assistance I was provided by the many team members at Tyson Foods who assisted with laboratory and data analyses and provided encouragement and resources to help conduct the daily business of our department, while attending to the numerous crises in my absence: Dr. Richard Roop for his leadership and providing me the time and resources necessary to conduct my class work and research; Dr. Neal Apple, Jacquelyn Adams, John Clayborn and the research microbiologists at the Tyson Foods' Food Safety and Research Services Laboratory for their technical guidance and assistance with media preparation and microbial analyses; Dr. Daniel Zelenka and Eric Oxford for their assistance with experimental designs, data analyses and interpretations; Dr. Brian Hafley for sharing his extensive knowledge of fermented sausage manufacturing, Tyson Foods' product specifications and ensuring the experimental designs were sufficiently broad to cover the range of product formulations necessary to meet our validation needs; my staff at Tyson Foods and the many corporate and in-plant Food Safety and Quality Assurance professionals who attended to the day to day business of our department, managing our staff and responding to crises; and lastly, to all of my colleagues at Tyson Foods who have worked so hard to make our company successful so that resources were available to me and for ensuring that a strong Food Safety culture exists throughout the company so that the fruits of this research will be utilized in meaningful ways to ensure the safety of our fermented meat products and the

protection of our customers and consumers. Without their help, I would not have been able to complete this program of study.

I would also like to thank the team at Tyson Foods and the Tyson Family for the opportunity to be gainfully employed for almost 25 years at a company that values me as an individual, values the contributions I've been able to make and values continuing education for all team members by providing funding for the educational assistance program that made my research possible.

Most of all, I would like to thank my family for their unfailing love and support. Thank you for the encouragement, never-ending love, support and patience from my wonderful wife Donna, who stood by me enduring the long hours while I was away and put up with my surliness when I was home; and thank you to my son Alex for understanding all the lost weekends that were invested in research rather than father-son activities but for whom – as he prepares to start his secondary education -- I wanted to set a good example of the value of education, continuous self-improvement, hard work and tenacity. I would also like to thank all of my extended family and friends for their support during my graduate studies.

Lastly, I want to thank the Lord for blessing me with the talents and skills necessary to complete these studies while providing for my family's needs; for the gift of my wonderful wife and son who have made my life a joy; and for the gift of Salvation through our Lord and Savior Jesus Christ without whom all of this would be meaningless.

TABLE OF CONTENTS

I. INTRODUCTION 1
II. LITERATURE REVIEW 2
A. History of Food Preservation 2
B. History of Fermented Sausage 5
C. Pepperoni Manufacturing Process 7
D. Food Safety Hazards Associated with Pepperoni 12
1. Nitrite 12
2. Foreign Materials 16
3. *Trichinella* spp. 20
4. *Listeria monocytogenes* 23
5. *Clostridium* spp. 29
6. *Staphylococcus aureus* 34
7. Pathogenic *Escherichia coli* 39
8. *Salmonella* spp. 43
E. Fermented Sausage Recently Implicated in Outbreaks of Illness 49
1. Botulism 49
2. Staphylococcal Food Poisoning 50
3. Enterohemorrhagic *E. coli* 51
4. Salmonellosis 53
F. Specific Food Safety Controls for Major Hazards in Pepperoni 54
1. Sodium Nitrite 54
2. Lactic Acid Producing Starter Cultures 55
3. Degree-Hours and Control of Staphylococcal Enterotoxins 60
4. Five Options of the Blue Ribbon Task Force for Control of *E. coli* O157:H7 in Fermented Sausages 62
G. Impacts of this Research and Statement of Problem 64
H. Nonpathogenic Surrogate and Rationale for Selection 72
I. Predictive Food Microbiology 75
J. Predictive Microbial Models 76
III. REFERENCES 87
IV. CHAPTER 2 103
Evaluation of the Inactivation of a Cocktail of *Salmonella* spp. and *Escherichia coli* O157:H7 in a High Fat, Dry, Fermented Sausage Product (Pepperoni Pizza Topping with 20% Mechanically Separated Chicken)
V. REFERENCES 117
VI. CHAPTER 3 123
Developing Alternative Time / Temperature Thermal-Processing Combinations for Validation of the Inactivation of *Salmonella* spp. in a Variety of Formulations of Pepperoni
VII. REFERENCES 140
VIII. CHAPTER 4 154
Paper for Publication -- Validating a Predictive Model for Alternative Time / Temperature Thermal-Processing Combinations for Inactivation of *Salmonella* spp. in Two Formulations of Pepperoni
IX. REFERENCES 182
X. SUMMARY OF RESEARCH 186
XI. REFERENCES 190

I. INTRODUCTION

Consumers have a basic expectation that the foods they purchase will be safe to consume when handled and prepared properly; food manufacturers have a basic ethical and moral responsibility to ensure that manufacturing processes have been validated to deliver safe products to meet the consumers' expectations; and failure on the part of the manufacturer results in harm to consumers, the business and the market. The currently accepted approach to safe food process design is a scientific approach to food hazard analysis and control described by the concept of "Hazard Analysis and Critical Control Points" (HACCP).

The HACCP concept requires that the food manufacturer possess substantial process control knowledge and validate that the process is capable of producing finished products that are safe, wholesome and comply with all regulatory requirements. This review describes the process for production of pepperoni, the hazards commonly associated with the product, and the controls necessary to ensure finished product safety. Further, it discusses the role of the domestic regulatory agency responsible for meat and poultry products and details proposed changes that will require substantial effort on the part of pepperoni (and other fermented sausage) manufacturers. These changes will require processors to revalidate the controls contained within HACCP plans and potentially require process changes that will damage the finished product quality and result in substantial costs for compliance. The research associated with this dissertation aims to facilitate processors' validation work and minimize any potential costs.

II. LITERATURE REVIEW

A. HISTORY OF FOOD PRESERVATION

Throughout history and more frequently in less developed areas of the world -- food has been a scarce resource. Food lost to spoilage may lead to deprivation and nutritional inadequacy, resulting in negative health and life events (Koning *et al.* 2008) – truly a "food safety" problem. Over the previous millennia, man has sought methods of preserving food – with the unintended consequence that many of the methods subsequently developed proved to also be critical to the preservation of the safety of the food products (Rahman 2007).

Preservation of foods is intended to combat the problems associated with spoilage by extending the usable shelf life of the products. Properly done, foods previously available only during the growing season may be preserved so that they are available year round; foods that are produced in limited geographical regions of the world may be preserved for distribution to other global market places where they command higher prices, thus adding value to the products and improving the economy of the producing region; or foods that are particularly bountiful in a geographic region may be sent long distances to provide for the needs of individuals in less productive regions of the world (Rahman 2007). Food Preservation has been defined as:

> "… any of a number of methods by which food is kept from spoilage after harvest or slaughter. Such practices date to prehistoric times. Among the oldest methods of preservation are drying, refrigeration, and fermentation. Modern methods include canning, pasteurization, freezing, irradiation, and the addition of chemicals" (Encyclopaedia Britannica Online 2013).

As stated in the definition, food preservation dates to prehistoric times. As man attempted to overcome the "feast or famine" cycle of food availability, methods were discovered that extended the acceptable shelf life and helped ensure a continuous supply of nourishment. By definition, the acceptable shelf life of a food product meant that the food had to be adequately safe at the time of consumption. Thus, many of the techniques designed to ensure a basic survival requirement had the useful side benefit of reducing the risks – principally microbial risks – associated with the food (Rahman 2007).

The earliest examples of food preservation involved cooking or drying fish or meat with heat. The mechanisms of preservation were not understood for many millennia. However, through trial, error and discovery, a number of methods for extending the life of foods were devised. For example, the oldest

form of fermented meats is thought to have been produced in Samaria approximately 5000 years ago. Nomadic peoples would catch or slaughter fish, game or stock animals and discovered that salting the raw meat and stuffing it into casings would sometimes result in a meat product that – in the absence of refrigeration -- was adequately stable to carry during their journeys to areas where meat supplies were scarce (Toldrà 2002).

These nomads certainly didn't comprehend the microbiological basis of fermentation and the resulting reduction in pH; or the microbial reduction of the natural nitrates in the sea salt to form nitrite and the means by which the meat was "cured". Over the years and in different geographic regions, the availability of resources would dictate the most common methods of food preservation. In areas where ice was available, refrigeration or chilling became a common method. In areas where wood was plentiful, cooking, smoking and drying of foods became common methods for preservation. In areas where both ice and wood were abundant, foods were often cooked or smoked in combination with refrigerated storage. These combination methods often proved to be far superior to a single method of preservation (Toldrà 2002).

In the modern era, these methods became known to food producers in all geographic regions and techniques were developed to overcome the natural lack of resources so that multiple methods could be employed in combination to maximize the quality of finished products. Additional methods were also developed. For example, in 1810 after Napoleon offered an award for anyone developing a means of carrying food for his troops, the French confectioner Nicholas Francois Appert published an article entitled, "The Art of Preserving All Kinds of Animal and Vegetable Substances for Several Years". This described the process of placing foods into glass containers – which were then corked with the seal reinforced with wires and wax – and then boiling the containers for extended periods. The process, called appertization, evolved as glass jars were not practical for military transport and processors started using cylindrical tin or wrought iron "canisters". Thus, the process became what we know today as canning (Encyclopaedia Britannica Online 2013).

Today, preservation techniques allow us to provide a diverse and high quality food supply – produced in the most efficient regions of the world – distributed on a world-wide basis and delivered to the consumers in a form that is safe to consume. Foods from around the globe are now available anywhere

in the world. Knowledge of food characteristics, the impact of the preservation method on the eating quality and the available infrastructure in the areas to which the food will be distributed all dictate to the modern food process designer which means of preservation should be employed. This may be as simple as the slow chilling of fruits or vegetables to preserve maximum quality for transport to the local farmers' market – or as complex as cooking, irradiating and freeze drying foods for transport to the space station (Rahman 2007).

While apparent that the majority of food preservation methods were initially designed simply to extend the useful life of food products, beginning in the latter half of the eighteenth century with increased understanding of microbiology, it became apparent that the techniques were often quite critical to ensuring the safety of the food products. As the methods were employed primarily to reduce, eliminate or retard the replication of the spoilage microflora, it has subsequently been found that the methods have significant impacts upon the human pathogenic microflora that may be present on raw agricultural products (Rahman 2007).

Even with our expansive understanding of the controls required for shelf life extension and product safety, outbreaks and illnesses are still frequently associated with the consumption of food. According to staff at the U.S. Centers for Disease Control and Prevention (CDC), the most recent estimate of the burden of illness in the domestic population due to unsafe foods indicates that approximately 1,350 fatalities, 56,000 hospitalizations and 9.4 million illnesses occur annually (Scallan *et al.* 2011) – with a 1999 estimate of the associated economic losses at $27 billion (Mead *et al*. 1999). When the cause of foodborne illness is identifiable, it is most frequently a microbial pathogen or its toxic metabolic byproduct (Scallan *et al.* 2011). If these estimates are even within the ballpark of reality, it is apparent that food safety failures occur regularly.

How may one attribute the cause of these failures in the context of previous statements regarding the expansive knowledge we currently possess about the necessary food safety interventions and controls? Generally, one of two answers is possible:

1) The microbial pathogen or its toxin was present in the food product at infectious or toxic levels at the time of consumption and the various interventions utilized during manufacturing and distributing the product were not adequate to either:

A. Reduce the hazard to a safe level; or

B. Prevent the proliferation of the hazard to an unsafe level during the shelf life of the product; alternatively

2) the microbe attained new growth, survival or toxin formation characteristics such that either:

A. A microbe not previously considered a human pathogen attains a characteristic that infers pathogenic qualities – such as *Escherichia coli* (*E. coli*) inserting into its genome (possibly via a plasmid-induced transfer of genetics from *Shigella dysenteriae* (Neill 1997) the genes that code for a metabolic product that is toxic to man; or

B. The microbe adapts such that the previously effective interventions and controls become ineffective due to these mutations – such as *E. coli* becoming more acid tolerant and the pH reduction attained during fermentation of sausages becoming no longer adequately sufficient to inactivate the microbe present in the raw materials. This phenomenon is often responsible for what is commonly referred to as an "emerging infectious disease" – and results in the need for research to identify additional or more effective control methodologies (Tauxe 1997).

From this discussion, it is apparent that modern food manufacturers must maintain a comprehensive understanding of the currently available science associated with the food produced and marketed; the processes used to manufacture and distribute those products; as well as maintaining vigilant review of emerging science and information suggesting that hazards and controls are evolving. Lack of such knowledge and vigilance may lead to the demise of the food manufacturer (IFT 2004).

B. HISTORY OF FERMENTED SAUSAGE

The term "sausage" is used to describe numerous products – typically made from salted and cured ground meat with a variety of seasonings stuffed in a casing. The traditional methods for making sausages have an ancient history and have evolved across time and geography depending upon the availability of raw materials and preservation methods (Ordonez *et al.* 1999). While the early record of preservation methods is scant, there may be no doubt that in the earliest days of sausage making, product safety was hit or miss and certainly not well understood. As the sciences of food processing,

food microbiology and food preservation have evolved, our understanding of control measures required to ensure product safety has evolved (Cornell University 2007).

The etymology of the word “sausage” extends back approximately 5000 years possibly to the area known as Samaria (a mountainous region on the west bank of the River Jordan). Starting with the most recent derivation of the Middle English “sausage”; preceded by the Anglo-French “sauseche” and the Late Latin “salsicia”; the term ultimately derives from the Latin “salsus” -- meaning salted (Toldrà 2004).

The practice of making sausage resulted from a desire to preserve a precious and limited supply of meat, poultry or fish proteins and to ensure the efficient use of all portions of a slaughtered animal. This early form of food preservation involved the salting and spicing of ground meat often utilizing: excess meat that might spoil before consumption; scraps from butchery; excess fat; organ meats and blood; and stuffing of the meat into some form of casing; typically tissue from the gastro-intestinal or urinary tract of the slaughtered animal in which the meat would cure and ferment (Tauber 1976).

The safety of the finished products was dictated by a wide range of conditions that were undoubtedly unknown to the early practitioners. The holding of the stuffed sausages at ambient temperatures with high levels of salt resulted in proliferation of lactic acid producing bacteria such as species of the *Micrococcus*, *Streptococcus*, *Pediococcus* and *Lactobacillus*. This microbial proliferation had a three-fold effect: 1) fermentation of the available sugars to lactic acid resulted in a significant reduction in pH of the product to levels below which human pathogens were capable of proliferation; 2) microbial reduction to nitrite of the naturally occurring nitrate in the sea salt used for salting resulted in what is traditionally called “curing” providing for control of the spore-forming pathogens such as members of the genus *Clostridium*; and 3) the reduced pH and elevated salt content led to a reduction in water activity in the products to levels sufficiently low to retard pathogen growth and, in many cases, sufficiently low to retard spoilage organisms such as yeasts and molds – particularly when combined with a drying process (Deibel *et al*. 1961).

Geographic variations evolved that were dependent upon the vagaries of the climate and availability of raw materials. In this manner, a wide range of products with distinct characteristics have evolved over the ensuing years. For example, dry curing is thought to have evolved in the Mediterranean region as a result of the arid conditions; while smoking – either cold or with heat – is thought to have originated in the

northern European areas having access to cold weather refrigeration and a plentiful supply of woods for smoking and generation of heat. This evolution has resulted in a vast variety of sausages with different raw material meat stocks, spice flavorings and finished product texture, flavor and characteristic size – in many cases being specifically associated with a particular geographic area such as the case with Genoa salami. The earliest versions of sausages would have -- by necessity -- been dry or semi-dry sausages as refrigeration was unavailable (Schut 1978).

C. PEPPERONI MANUFACTURING PROCESS

A brief review of the manufacturing process for pepperoni products will allow illustration of several key processing steps that will be critical to subsequent sections of this paper related to food safety hazards and controls. While there are a wide variety of sausage formulations; different methods of reducing the pH; equipment used to manufacture sausages; casing sizes and types; and drying room designs, the following description of a typical pepperoni manufacturing process illustrates all of the major components necessary to produce any of the myriad types of dry fermented sausage types.

A question that must be addressed is, "what is pepperoni?" According to the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS), the standard of identity for pepperoni reads:

> **"PEPPERONI:**
> A dry sausage prepared from pork or pork and beef. Combinations containing more than 55 percent beef are called beef and pork pepperoni. Pepperoni made with beef must be called beef pepperoni. Pepperoni must be treated for destruction of possible live trichinae and must have an MPR of 1.6:1 or less. Antioxidants are permitted in pepperoni. The casing, before stuffing, or the finished product, may be dipped in a potassium sorbate solution to retard mold growth. Extenders and binders are not permitted in pepperoni. Hearts, tongues, and other byproducts are not acceptable ingredients."

In the event the pepperoni manufacturer desires to replace a portion of the pork or beef raw materials with a raw material derived from poultry, the standard is modified to read:

> **"PEPPERONI WITH POULTRY:**
> Poultry may be added to pepperoni if properly labeled. If the meat block contains 20 percent or less poultry, the product is labeled — "Pepperoni with Turkey (kind) Added." When poultry is over 20 percent of the meat and poultry block, the product is labeled — "Pork and Turkey (kind) Pepperoni," and an MPR of 1.6:1 is applied. If the amount of poultry exceeds that of the meat, the product label reads — "Turkey and Pork Pepperoni." This would carry a poultry legend" (USDA 2005).

Before delving into a description of the manufacturing process, a term used in the preceding definitions requires explanation. MPR is the abbreviation for "Moisture, Protein Ratio" or the percentage

moisture divided by the percentage of protein in the finished product. The greater the moisture content, the higher the ratio. Thus, to some extent, the MPR is an indicator of the degree of product drying, but is not necessarily indicative of product safety or shelf life stability as it is merely a relational proportion (USDA 2011). The MPR represents the average of a range of values obtained in a market basket survey conducted when FSIS was considering the creation of a standard of identity for a given product. In the case of pepperoni, as previously indicated, the ratio is equal to 1.6:1. However, for hard salami, it is 1.9:1; and for Genoa salami, it is 2.3:1 (USDA 1995).

From these examples, one may see that pepperoni is relatively drier than hard salami which is relatively drier than Genoa salami. As a standard of identity, it is intended to assure consumers that like-labeled products have substantially similar finished product attributes to prevent economic fraud on the part of unscrupulous manufacturers that might "short-change" a consumer by substituting less valuable ingredients or – in this instance – not removing as much water (relatively, a very inexpensive ingredient) as has been the historic standard (USDA 2006a).

A variety of meat trimmings are received and stored in a refrigerated cooler at temperatures just below freezing. For most product types, it is important for product quality to prevent the naturally present microbial flora from proliferating and spoiling the meat trimmings. Some manufacturing processes will utilize frozen meat trim as the economics of buying at certain times of the year may offset the costs of frozen storage (FAO 2007 and Toldrà 2004).

On the date of formulation, the meat trimmings are conveyed through a large aperture metal detector to identify and screen any large metal objects that might have been inadvertently included in the raw materials. If not removed, these large foreign objects have the potential to cause significant damage to the meat grinding equipment used in subsequent processing steps. Smaller objects that are not removed have the potential to be divided during the grinding operations and might form a foreign object food safety hazard (Brazell 2009).

Following metal detection, the meat trimmings are coarsely ground through large commercial grinders typically using a one-half inch grind plate. This grinding operation must be conducted under chilled temperatures using very sharp blades to prevent fat smearing which, if allowed, will have a variety of deleterious effects on product quality and consistency (Terrell *et al*. 1977).

After the meat trimmings have been coarsely ground, depending upon the specific product formulation, different meat species or meat with different proportions of fat will be conveyed to a blending hopper to be weighed for proper proportioning and mixing. Again, the mixing must be done under chilled conditions and for a short duration to avoid fat smearing. Depending upon the temperature of the meat blend, a chilling agent such as carbon dioxide ice may be added or, depending upon the blender design, gaseous carbon dioxide may be injected into the blender as the meat is mixing. It is critical to the finished product quality that the meat be maintained at temperatures very close to freezing (Hafley 2009).

After the meat blend is well mixed, several samples are typically taken to the quality control laboratory to be analyzed for fat and moisture content. The analytical data are entered into a software program designed to calculate the final meat block based upon the product formulation, the cost of the different raw materials and to adjust the lean to fat ratio to achieve consistency between batches and the least cost formulation (Brazell 2009).

Once the software has calculated the necessary formulation adjustments, additional coarse ground meat of the appropriate type or fat composition is added to the mixer and blended thoroughly. Typically, a set of verification samples are taken to the analytical lab for quality assurance and, in many cases, to meet regulatory requirements for label claims or standards of identity (Brazell 2009).

When the meat blend is confirmed to meet the manufacturing specification and relevant regulatory requirements, the non-meat ingredients are added to the meat for blending. For a modern, domestic pepperoni, these ingredients will include: oleoresin of paprika (or more traditionally, sweet paprika); tinted curing salt consisting of salt, 6.25% sodium nitrite, propylene glycol as an anti-caking agent, and red number three to distinguish the mixture from table salt so it is not inadvertently used in a formulation in place of table salt; high purity salt; and pepperoni seasoning (Terrell *et al.* 1977). The non-meat ingredients will be thoroughly blended into the meat blend and then the commercial starter culture (Niinivaara 1991, Smith and Palumbo 1983) will be added and the mixture will be blended thoroughly for the last time while maintaining appropriately low mixture temperatures (Hafley 2009).

After the meat blend and ingredients are thoroughly mixed, they are transferred to the final fine grinder where the mixture is emulsified. The meat is conveyed from the final grinder through a metal detector to screen any fine metal particles that might have been introduced during the grinding, mixing

and conveying process. The emulsified meat is transferred to the sausage stuffer and the emulsion is stuffed into a fibrous casing of the specified diameter. The stuffed casings are closed with a metal clip which is tied with cotton twine and the sticks are hung on metal sausage trees for processing. Each tree is identified with tags for lot tracking, quality and food safety verification monitoring and documentation (Brazell 2009).

The loaded sausage trees are placed into a smokehouse or oven (many pepperoni recipes do not utilize smoke) for fermentation. The fermentation process utilized will be dictated by the starter culture chosen but is typically at a higher temperature than used for many other fermented sausages as the intent is to initiate lactic acid production quickly and rapidly reduce the pH which is further aided by the inclusion of dextrose in the product formulation. The dextrose is a readily fermented substrate and a slight excess in the formula is intended to ensure complete and rapid acidification of the sausage (Niinivaara 1991, Smith and Palumbo 1983).

The seasonings in pepperoni are relatively strong compared to other fermented sausages and the subtle flavor profiles that many artisanal sausage producers prefer to develop by fermenting “low and slow” would be overwhelmed. There is no advantage to using slow fermenting starter cultures or low fermentation temperatures when manufacturing pepperoni (Marcy 2009). It is desirable to ferment the sausage as quickly as possible to maximize smokehouse throughput and to transition the sausage pH through the danger zone as quickly as possible (Hafley 2009) (additional detail will follow in the technical literature review of controls for *Staphylococcus aureus*).

Fermentation is verified by sampling the sausage to ensure the pH has dropped to below 5.3. Generally, fast-starter culture fermented pepperoni will achieve a final pH in the range of 4.3 to 4.5 immediately following the fermentation process and the pH will eventually rise to 4.8 to 5.0 due to the naturally occurring buffers in the meat components (Hafley 2009). After fermentation is verified, the pepperoni sticks are heat processed. This has not always been the case and traditional pepperoni processes do not include a terminal heat process as the finished product attributes were historically considered sufficiently hostile to control vegetative pathogens. As will be discussed in later sections, while pepperoni has not been implicated in human illnesses, in recent years, other fermented sausages have been associated with illnesses caused by a variety of vegetative pathogens and most manufacturers

now include a cook step as an additional measure of product safety. The parameters of time, temperature and humidity utilized for the cook step will depend upon the sausage diameter and specific validation for the product formulation and processing conditions (Hinkens *et al*. 1996).

After cooking, the sausage trees are transferred from the oven to the drying room and across a scale after which the total weight of the tree is recorded. This value will be used to calculate moisture loss during drying. The sausage sticks are cooled in the drying room and air drying begins under closely controlled temperature and relative humidity conditions (Brazell 2009). Drying of the sticks must be done relatively slowly to maintain product quality as a number of defects will arise if the relative humidity is maintained too high or too low. Excessively slow drying under high relative humidity conditions may allow moisture to remain on the surface of the sticks and promote mold growth while extending the time required to adequately dry the sausages. Excessively fast drying under low relative humidity conditions will result in a condition called case-hardening in which the outer surface of the sausage dries too rapidly forming a crust. This crust slows the migration of moisture from the inner portions of the sausage to the surface to be evaporated (Palumbo *et al*. 1976). Case hardening extends the drying time required and has been associated with poor product performance on pizzas during cooking due to “cupping.” Cupping is considered an unacceptable attribute as the bowl-shaped pepperoni slices will catch the melted fat and the upper edges of the pepperoni will frequently burn or char (Hafley 2009).

As the sausages approach the appropriate level of drying -- for pepperoni, the regulatory standard of identity requires the Moisture Protein Ratio (MPR) to be equal to or less than 1.6:1 – the trees are conveyed across scales, weighed and the total water weight loss is calculated to verify the MPR. If inadequate weight loss has been achieved, the trees are returned to the drying room for an additional period of time. The drying process is time, space and labor intensive making it expensive to produce dried sausages. These expenses are reflected in the selling prices (Brazell 2009).

When the required MPR has been attained, the pepperoni sticks are deep chilled and transferred to the holding cooler for further processing, slicing or packaging. The pepperoni sticks will be peeled by removing the casing and marketed in a variety of forms: sliced in bulk for food service pizza and sandwich restaurants; sliced and packaged in gas flushed plastic pouches for retail sales; cut to various lengths and

packaged for retail deli slicing; or docked by removing the rounded ends and sold to commercial frozen pizza manufacturers to slice directly onto premade pizza processing lines (Brazell 2009).

D. FOOD SAFETY HAZARDS ASSOCIATED WITH PEPPERONI

As with other foods derived from raw agricultural products, the major food safety concerns associated with pepperoni and the manufacturing process consist of those posed by microbial hazards. However, due to the complexity of the product, it poses some very interesting conceptual challenges. While fermented sausages have historically been considered microbiologically safe, there have been a large number of documented outbreaks of illness associated with microbial hazards in fermented sausages. Following are detailed descriptions of the most significant hazards commonly associated with pepperoni and fermented sausages; the current process controls utilized within the manufacturing process; and the historical perspective on how those controls came to be required in the context of HACCP. These are presented in order from least significant to the most significant acute risk, leaving the subject of my proposed research for the problem statement.

Nitrite: Nitrites have been in the foods and drinks we consume since the dawn of time as naturally occurring constituents. Leafy greens represent one of the single largest dietary sources of nitrite (as naturally occurring nitrates are reduced to nitrites during digestion) in the modern diet (McCarty *et al*. 1982) with the single largest source coming from saliva we ingest as a normal part of the function of swallowing (Tannenbaum 1972). However, there has been a long history of consumer concern with the practice of adding nitrite to foods as certain cyclic-amines that may form during processing or cooking have been demonstrated to be carcinogenic (McCarty *et al*. 1982).

The history of nitrites in fermented sausages is lost to the vagaries of the unwritten history of time and can only be postulated. As described in the introduction, it is thought that the earliest fermented sausages were the result of serendipity. Over the ensuing millennia, cured foods became an accepted and enjoyed part of man's diet. As the knowledge of food curing improved, the role of nitrates and nitrites become well understood (Toldrà 2007).

Saltpeter (potassium nitrate, KNO_3), in combination with salt (sodium chloride, NaCl) was the first curing agent deliberately added to meats. Certain microorganisms naturally present in the meat – namely species of the *Staphylococcus*, *Lactobacillus* and *Pediococcus* – metabolically synthesize nitrate

reductase (Sindelar *et al*. 2010) which acts to reduce the dissociated nitrate anion (NO_3^-) to the nitrite anion (NO_2^-) which reacts with free water to form nitric oxide (NO). Nitric oxide acts upon the oxymyoglobin pigment responsible for fresh meat color, replacing the oxygen with nitrogen forming nitrosomyoglobin which (Toldrà 2002) when heated forms a nitrosohemochrome pigment that is responsible for the distinctive cured meat color (Castellani and Niven 1954; Sofos 1981; Sebranek and Bacus 2007a).

As the scientific understanding of meat curing developed around 1930 with the discovery by Polenske of nitrite in cured meat and used curing pickle and his correct conclusion that it was formed by the microbial reduction of nitrate (Binkerd and Kolari 1975; Toldra` 2007), the suboptimal curing process relying on microbial reduction of nitrate to nitrite – which depended on the chance presence of meat microflora capable of synthesizing nitrate reductase yielding highly variable finished product results (Jones 1933) – was replaced with the direct addition of sodium nitrite ($NaNO_2$) to the meat formulation. This afforded much more precise and controlled levels of in-going and residual nitrite in the finished products which ensured the quality of the cured meat and also allowed for an overall reduction in the residual levels and human exposure. In the U.S., where direct addition of nitrite is much more prevalent than in Europe where more traditional processes dominate, the residual levels of nitrite in market meats is significantly lower providing a reduction in risks associated with cyclic-amines (Keeton *et al.* 2009). Further, strict control of the in-going levels allowed for assurances of a residual pool of nitrite remaining in the finished products to afford control of *Clostridium botulinum* throughout the shelf-life even if the products were subject to temperature abuse (Tompkin *et al.* 1978a).

In recent years in the U.S. market, demands for natural and organic foods have grown sharply. As a result, manufacturers are exploring processes to provide the traditional color and flavor of cured sausages while allowing for a “natural” claim on the product label. Commercially cured products utilizing the direct addition of nitrite are not allowed by federal regulators to make a “natural” label claim (USDA 2005; Sebranek and Bacus 2007b). However, several commercial sources of nitrite are now available either as a natural, produce-based nitrate used in combination with a commercial starter culture containing a nitrate-reducing microorganism in conjunction with a lactic acid producing bacteria (Sebranek and Bacus 2007b; Hammes 2011); or as a commercially fermented produce-based reduced

nitrate ingredient containing verified levels of nitrite per unit volume for direct addition to the meat blend (Sebranek and Bacus 2007a,b; Sebranek *et al*. 2012).

Interestingly, the first option represents a practical reversion to the traditional, highly variable and suboptimal means of nitrate reduction and conversion to nitrite while the latter option is nothing more than a legal marketing gimmick representing a means for the direct addition of nitrite, only not as a dry chemical but rather as a byproduct of the fermentation of a nitrate-containing produce juice (Sindelar *et al*. 2010). In the first instance, the process may lead to inconsistent color and flavor (Hammes 2011) formation and suboptimal microbial control. In the second instance, using a directly added, verified level of nitrite from a "natural" source yields precisely the same product – from a technical standpoint – as modern commercial direct nitrite-added processes but the alternative processes are marketed as "better for you" due to the "natural" source of the resulting nitrite. This seems at best misleading and at worst unethical.

Color is another of the traditional and expected characteristics of cured meats which is imparted due to the action of nitrite. In this role, the nitrite also acts as a very strong antioxidant stabilizing the flavor of cured meats by preventing the oxidation of lipids (Sindelar *et al*. 2010). The traditional "cured-meat" flavor is another important characteristic that develops through the action of nitrite and is readily apparent in a side-by-side comparison of the flavor of ham versus roast pork loin. The chemistry of cured meat flavor is one of the most studied and yet still incompletely understood questions remaining in the world of meat science (McCarty *et al.* 1982).

The last and most important characteristic that nitrite imparts to cured meats are the bacteriostatic and bacteriocidal roles most important for control of the spore-forming species of the *Clostridia* (Sindelar *et al.* 2010). Nitrite has a long and well-studied history of control of the human pathogens *Clostridium botulinum* and *perfringens* particularly at low pH such as is found in fermented meat products (Roberts 1975). The first is the cause of the well-known intoxication botulism, while the second is the cause of a less-well known infection causing gastroenteritis (ICMSF 1996).

Control of these microorganisms by sodium nitrite is postulated to be pH dependent and based upon the formation of nitrous acid (Castellani and Niven 1954; Sindelar *et al.* 2010) as nitrate at up to 2% of the formulation has been shown to be ineffective at preventing the germination and subsequent outgrowth of

spores (Duncan and Foster 1968). The effectiveness of nitrite is dependent upon a number of factors including: the concentration of salt; the heat treatment applied; the number of clostridial spores present in the meat matrix; the in-going and residual nitrite levels; as previously mentioned, the final product pH; competing microflora; available iron (Tompkin *et al.* 1978b); and the presence of "cure accelerators" such as ascorbate and erythorbate (Roberts and Gibson 1986). Tompkin demonstrated that the addition of isoascorbate to a perishable canned, cooked pork product significantly increased the botulinal inhibition of nitrite (Tompkin *et al*. 1978c).

The USDA's Department of Animal Industry first approved the use of nitrate as a meat curing agent on May 1, 1908 and after extensive research concerning its mode of action and chemistry, the USDA approved the direct addition of nitrite to meats in October 1925 (Sofos 1981). Further technical discussions of these microorganisms and the mode of control from nitrite are contained in a subsequent section titled *Clostridia* spp.

Consumer concern about added nitrite peaked approximately 40 years ago but still remains one of the greatest concerns related to chemicals in foods (IARC 2010) and its use was nearly banned in the 1970s (Cassens 1997). There is an abundance of literature supporting that heterocyclic-amines formed during the cooking process are potent carcinogens. These heterocyclic-amines form during high heat cooking when amino acids react with creatine and elevated nitrite levels in meats has been demonstrated to accelerate the creation of the amines as well as result in overall higher levels in the finished cooked product particularly when cook temperatures exceed 130^{o}C (Hammes 2011). At high temperature, these compounds will react with nitrite to form a group of compounds known as nitrosamines which are considered to be more potent carcinogens than heterocyclic-amines (Cassens *et al*. 1979).

Risk assessment officials in the U.S. have concluded that the use of nitrite to control microbial pathogens in cured products is a critical food safety intervention and should not be banned (Milkowski *et al.* 2010). However, additional controls have been implemented to reduce the risks associated with heterocyclic amines and N-nitroso compounds such as nitrosamines (McCarty *et al*. 1982). Strict limits have been established for the amount of nitrites or nitrates that may be added to a cured meat formulation and, in certain higher risk products – e.g. pork bacon – which are traditionally cooked at high

temperature to a well-done end point, U.S. regulations require that ingredients known to retard the formation of the compounds must be incorporated into the product formulation (USDA 1995).

The USDA has codified regulatory restrictions limiting the use of nitrate and nitrite to minimize the potential health hazards associated with nitrosamines while maintaining the critical safety intervention for control of the clostridia. For example, in products that are ground, chopped or otherwise comminuted, the maximum amount of sodium or potassium nitrate going into the raw meat formulation (weight to weight) is 1,718 parts per million (ppm), while the limits for sodium or potassium nitrite is 156 ppm. For products that are massaged, injected (pumped) or immersion-cured, the maximum in-going concentrations of nitrate and nitrite are 700 and 200 ppm, respectively. Lastly, the regulations limit in-going concentrations of nitrate and nitrite for dry-cured products to 2,187 and 625 ppm, respectively. Formulations that utilize both nitrate and nitrite must be analyzed to ensure that the combination yields no more than 200 ppm nitrite in the finished product (USDA 2006b) and, as a matter of policy, USDA requires that all cured products labeled as “Keep Refrigerated” must be formulated with a minimum of 120 ppm nitrite unless the processor can demonstrate that some other preservation characteristic of the product will ensure its safety (USDA 1995).

A review of the safety of nitrite would be incomplete without a mention of the positive role dietary nitrates and nitrites play in human health where nitrite is a metabolite. Nitric oxide is synthesized endogenously in significant quantities and plays an important role in a number of systems to maintain good health: immune response; control of blood pressure; wound repair; and neurological functions. The normal production of nitrous oxide from ingested nitrates and nitrites may be responsible for helping prevent cardiovascular diseases such as stroke or atherosclerosis (Hunault *et al*. 2009; Hammes 2011).

Foreign Materials: Foreign materials have been in the foods we consume since the dawn of time as naturally occurring defects. In very early days, the most common foreign materials likely present were represented by those naturally occurring defects associated with the harvest or preparation of the food – including stones, sand or contaminating plant materials. While relatively minor concerns compared to microbiological hazards in foods, these materials may represent a food safety hazard when the inherent characteristics – size, hardness, sharpness, orientation -- of the foreign object present the potential to cause illness or injury when the contaminated products are consumed and the foreign object is ingested.

The injuries caused by foreign objects are generally not life-threatening but may cause significant psychological trauma and may include choking, lacerations in the mouth, esophagus, gastro-intestinal tract or airway including the potential for lung collapse, broken teeth, and in rare cases, perforations of the gastrointestinal tract requiring surgical repair or antimicrobial therapy in response to a secondary infection (Goldman 2002; Olsen 1998). The review conducted by Olsen concluded that foreign objects are very rarely the direct cause of microbial infection (Olsen 1998).

In recognition of the inherent nature of these defects associated with the harvesting, storage and processing of agricultural products, the U.S. Food and Drug Administration has codified "Defect Action Levels" for common, unavoidable contaminants such as insect parts, stones and extraneous wood or stems. These limits establish acceptable levels of contamination below which the products are deemed to be acceptable for commerce and not adulterated:

> "The FDA set these action levels because it is economically impractical to grow, harvest, or process raw products that are totally free of non-hazardous, naturally occurring, unavoidable defects. Products harmful to consumers are subject to regulatory action whether or not they exceed the action levels" (FDA 2013a).

In 1995, representatives of the USDA-FSIS convened a "Public Health Hazard Analysis Board" (PHHAB) to consider when and whether bones that may be inherent in finished products might constitute a hazard and justify the recall of products that are in commerce (Goldman 2002). This specific review was initiated due to a large number of complaints received by the USDA consumer hotline for bones in ground turkey products. The initial question of whether bones present in products represented to be boneless automatically rendered the products legally adulterated or whether bones in such products would be considered inherent defects (Gregory 2012).

Representatives of the FDA had previously taken a position regarding bones in fish and fishery products concluding that the defects were inherent and the consumer should expect some number of bones in fish fillets. As such, it was expected that the consumer should take care when eating such products to avoid accidental ingestion or injury due to the presence of the defects (FDA 2005). Interestingly, in the case of FSIS and ground turkey, a similar position was taken but only up to the point at which a subjective determination concluded that the number of bones was "too numerous", representing a lack of process control based upon current technology and industry standards. As such,

the specific products that led to the formation of the PHHAB were subsequently recalled by the producing firm (Gregory 2012).

During the 1995 collaboration, the question of other foreign objects in foods was raised by representatives of the FSIS and the FDA representatives reiterated their agency's position on naturally occurring defects. However, it was agreed that in addition to the quantitative standard of "too numerous", a qualitative standard based on the size and physical characteristics of any individual object should be established. Following an exhaustive review of the literature and the conclusions drawn by the FDA's Health Hazard Evaluation Board from examination of 190 complaints of foreign objects in foods occurring during the period 1972 through 1997, Olsen concluded that, in general, hard and sharp objects between the sizes of 7.0 – 25.0 mm in food represent a food safety hazard provided the food was not intended for a particularly at risk consumer group (e.g. infant foods) (Olsen 1998).

These hazard conclusions were subsequently incorporated into guidance published by staff at the FDA detailing actions to be taken by representatives inspecting foods when foreign objects are discovered; explicitly drawing a distinction between foreign objects that are added substances versus those that are unavoidable as previously described (FDA 2005). This distinction is codified in the Code of Federal Regulations, Title 21:

> **"**§ 109.7 Unavoidability.
> (a) Tolerances and action levels in this part are established at levels based on the unavoidability of the poisonous or deleterious substance concerned and do not establish a permissible level of contamination where it is avoidable.
> (b) Compliance with tolerances, regulatory limits, and action levels does not excuse failure to observe either the requirement in section 402(a)(4) of the act that food may not be prepared, packed, or held under insanitary conditions or the other requirements in this chapter that food manufacturers must observe current good manufacturing practices. Evidence obtained through factory inspection or otherwise indicating such a violation renders the food unlawful, even though the amounts of poisonous or deleterious substances are lower than the currently established tolerances, regulatory limits, or action levels. The manufacturer of food must at all times utilize quality control procedures which will reduce contamination to the lowest level currently feasible. [42 FR 52819, Sept. 30, 1977, as amended at 55 FR 20785, May 21, 1990]" (FDA 2013a).

In modern times, particularly in meat and poultry products, naturally occurring defects such as stones have largely been supplanted by man-made objects that inadvertently become a contaminant of the finished products. These may include buckshot, glass or hard plastic, veterinary or marinate injection needles, jewelry or other personal effects, materials used during processing of the products such as writing pens, staples or wood from pallets, or more frequently, metal from harvesting, storage or

processing equipment that is poorly maintained or broken by some other foreign object. Within the context of Good Commercial Practices and the aforementioned Quality Control requirements, special consideration is made for individual bones in meat and poultry products as an inherent rather than foreign object which may cause injury – provided the number of bones present in any given lot of finished products does not exceed what would reasonably be considered to be "too numerous" (Goldman 2002; Pierson 1992).

Foreign objects in foods generally don't cause injury with an estimated 1 – 5% of ingestion exposures resulting in minor to serious injury and 80 – 90% of all ingested objects passing through the gastrointestinal tract harmlessly. However, by far they represent the most frequent source of consumer exposure to a potential hazard in foods and result in the largest number of consumer complaints received by the FDA and many food companies (Barber 2012; Olsen 1998). The number of complaints received for consumers experiencing foreign materials in finished products is generally greater than all other complaints combined (Barber 2012). Until recent years, the number of recalls of food products was dominated by those caused by the presence of foreign materials. This problem has recently been surpassed by the number of recalls for undeclared allergens (Goldman 2002).

There are a number of interventions for foreign materials that are commonly employed by representatives of the food manufacturing industry. As described in the sausage manufacturing process in the introduction, in combination with GMPs and supplier quality programs, metal detection is the single most prevalent foreign material intervention. However, screens, magnets, visual inspection and with increasing frequency due to the number of defects that occur and a desire for improved product quality, automated inspection systems and automated X-ray vision detection systems have become much more widely employed (Pierson 1992).

While the objective of such systems is to ensure effective screening for those objects that represent a food safety hazard (hard and sharp, 7.0 – 25.0 mm), members of the food processing industry expect to effectively eliminate from foods any materials that would be deemed a quality defect by the consumer. As the cost of screening technology decreases, more technology is being applied to food processing and the number of complaints for foreign materials has declined significantly over the past decade (Barber 2012).

***Trichinella* spp.:** Trichinosis is a rare human illness most frequently caused by infection with the nematode *Trichinella spiralis,* a parasitic roundworm with a direct life cycle meaning it completes its entire life cycle in one host (Gamble 2012). Human or animal infection typically occurs when raw or undercooked meat from infected animals is consumed (CDC 2012a). There are eight recognized species of *Trichinella*: *T. spiralis*; *T. pseudospiralis*; *T. britovi; T. murrelli; T. nativa*; *T. nelsoni; T. papau;* and *T. zimbabwensis*; all of which are known to infect warm-blooded omnivores, carnivores and raptorial birds. All but *T. spiralis* are commonly associated with game animals while *T. spiralis* is most commonly associated with domestic swine (USDA 2008).

The risk factors for infection of domestic swine include feeding wastes such as table or restaurant scraps that contain raw meat scraps from infected animals; and exposure of domestic swine to live or dead rodents or other infected wildlife. This risk is vastly increased for swine that are not confined (e.g. free-roaming) and due to cannibalism of swine within infected herds – including territorial and dominance-related biting behaviors. Similar and uncontrolled risks exist for wild animals; the meat from which has become the primary source of human infection in the U.S. in modern times (Gamble 2012). The number of non-pork associated cases surpassed pork-associated cases for the first time during the period 1997 to 2001 (CDC 2003). The meat from carnivorous or omnivorous wild or domesticated animals represents a potential hazard to humans.

Historically *Trichinella spiralis* has been observed as an enzootic agent in the meat of a wide variety of animals but the most frequent source of human infection was due to undercooked or uncooked pork products (CDC 2009a). At the turn of the 20th century in the U.S., it was estimated that approximately 2.5% of the domestic swine herd was infected with *T. spiralis* and consumption of pork products, particularly sausage, was the primary risk factor for human infection. A report from the National Institute for Health in 1930 described the post-mortem evaluation of human cadavers and reported that 16.2% of the human population was infected (Gamble 2000).

Trichinellosis in humans causes symptoms that are widely-varying from non-remarkable asymptomatic infection to self-resolving minor infection with nausea, vomiting and diarrhea, fever, malaise and abdominal discomfort that may evolve into more severe symptoms of headaches, chills, fevers, coughs with achy joints and muscle pains – and if the ingested meat was heavily infected with parasites,

the infected patient may experience difficulty breathing, coordinating voluntary muscle movements and heart problems that may lead to death (CDC 2003). The typical case is mild and self-limiting within a few months. Abdominal symptoms generally first are noted within 24 to 48 hours after infection with onset of more severe symptoms at 2 – 8 weeks after infection (CDC 2012b).

The direct life cycle of *Trichinella spiralis* provides an indication of how human exposure may occur, but also provides important information concerning control of this potential food safety hazard. When meat from an infected animal is ingested, acid in the stomach dissolves the cysts. The roundworms emerge in the gastrointestinal tract where the worms mate and produce offspring. The juvenile roundworms invade tissues of the intestinal mucosa, crossing into the bloodstream where they become widely disseminated throughout the host's body. They burrow into striated muscle tissues including the large muscles frequently harvested for food and become encysted. The adult parasites are eventually expelled in the stool of the host and active infection ends (Gamble 2012).

If the infected tissues are harvested and consumed without an adequate treatment to inactivate the roundworms, the lifecycle repeats with infection of tissues of the newly acquired host. Infection will generally occur in host striated muscle tissues and are concentrated in those that are the most active including the diaphragm (which may cause breathing difficulties). However, infection may extend to the heart (which may lead to death), lungs and brain (which may lead to encephalitis) (CDC 2012b).

In 1947, the number of U.S. domestic cases of trichinosis reported to government authorities peaked at approximately 500 and sausage products were thought to account for approximately 93% of the cases (Leighty 1977). Given that many sausage products have pork meat as a major component and many traditional sausage types don't receive a thermal process during manufacturing, traditional sausages represent a potential exposure to infection as many traditional curing and fermentation processes have not been demonstrated to be sufficiently lethal to the roundworms (Porto-Fett *et al.* 2010). As trichinae controls were slowly developed and implemented in the U.S., the disease burden has dropped in correlation with the decreasing proportion of infected commercial swine (CDC 2009a).

For control, it is critical to break the lifecycle between hosts such that meat animals are not infected by consumption of tissues from previously infected animals. In the wild, such controls are not available and currently animal infections in the U.S. are almost universally associated with wild animals including

bear, fox, wild swine, rats and walrus. Thus, in the U.S., foods harvested from non-domesticated meat animals are the most frequently implicated vector for human trichinosis causing practically all of the average forty cases per year (CDC 2009a).

Trichinella spiralis has practically been eradicated from the commercial swine supply by stringent application of feeding and confinement rearing controls. With the passage in 1980 of the Federal Swine Health Protection Act, the U.S. banned feeding uncooked garbage to swine to preclude the introduction into the diet of potentially infected meat (CDC 2003). Concurrently, USDA carcass inspection programs were improved for detection of heavily infected carcasses which were deemed unfit for consumption and condemned; and processing methods for pork meat were developed and validated to be effective for inactivation of the roundworms in potentially infected inspection-passed meat. For the inspection-passed products deemed to be at risk of consumption without adequate cooking by the consumer, under the supervision of the USDA, the raw meat must either be:

1) Subjected to a trichina-inactivating cook temperature ranging from 21 hours at 120^{o}F to instantaneously at 144^{o}F;
2) Frozen for a prescribed time and temperature ranging from 5^{o}F for 30 days to -35^{o}F for 0.5 hours depending upon the size and method of packing of the pork products; or
3) Salt-cured under one of seven prescribed methods of salting, holding, heating and / or drying.

Absent one of these treatments, at-risk pork from inspected and passed carcasses is not eligible to bear the mark of USDA inspection (USDA 2006c).

The raw garbage-feeding prohibition effectively eliminated a significant vector of infection for commercial swine. However, until recent years, rats were often associated with animal agriculture due to the presence of available food – either dead or down animals or the animals' feed. Both rats and swine are omnivorous and the rats have exposure to wild animals outside the confinement. As such, rats were a frequent vector of *Trichinella spiralis* from wild to domesticated animals, particularly as a vector to swine which will eat the rats. As rats are capable of consuming two to four times their weight in animal feed each day, modern commercial agricultural management practices have effectively eradicated the rat from commercial rearing houses. Elimination of the rat from the commercial rearing environment led to

significant efficiency gains by reducing the feed loss and concomitantly reduced the exposure of commercial swine to *T. spiralis* infection (Gamble 2000).

In 2008, in collaboration with the swine industry and their primary trade association, the National Pork Producers Council, representatives of the USDA's Animal and Plant Health Inspection Service (APHIS) worked to create the U.S. Trichinae Certification Program to certify swine producers' on-farm programs for control of trichinae and allow the resulting pork products to be identified as produced at a certified site. Certification of swine as "Trichinae-free" includes several elements beginning with a three-stage on-farm audit of: good production practices; security of the confinement housing to prevent exposure to rodents or other wild animals; and feeding practices to ensure meat wastes are not included. Additionally, the swine carcasses must be subjected to post-mortem inspection by representatives of the USDA's Food Safety and Inspection Service (FSIS). Lastly, laboratory testing of tissues from FSIS inspected and passed carcasses must be conducted at a frequency to ensure 99% confidence of detection of an infected carcass from the farm population assuming 0.013 percent prevalence. For a slaughter operation processing 1,000,000 certified head per year, this equates to 34,802 tests annually. For an operation processing 5,000 certified head per year, this equates to 4,996 carcass tests per year (USDA 2008).

Modern commercial swine agriculture has effectively eliminated *Trichinella spiralis* from the swine herd. The USDA routinely monitors slaughtered animals for the presence of infection with *Trichinella spiralis* and the swine infection rate has fallen from an estimated 2.5% at the turn of the century to 0.007% (Pyburn *et al.* 2005). Thus, the raw material inputs to the modern sausage manufacturer can safely be assumed to be free of this roundworm. However, prudence dictates that the manufacturer source raw materials from suppliers of meat from animals "certified trichina free"; purchase meat that is certified to have been frozen to inactivate trichinae; or utilize one of the curing and thermal drying processes previously validated to be effective for inactivation of the roundworm (Gamble 2000).

***Listeria monocytogenes*:** Listeriosis is an infection caused by the bacterium *Listeria monocytogenes* (L.m.) which is a Gram-positive, non-spore forming, facultative anaerobic rod that is motile by peritrichous flagella at room temperatures (but not at warm-blooded body temperatures) and possesses somewhat unique growth and adaptation capabilities (ICMSF 1996). Although L.m. is not responsible for causing a large number of foodborne illnesses, it can be especially virulent in "at-risk"

individuals and is recognized as the etiological agent responsible for causing the greatest number of deaths from foodborne illness (FDA 2012). L.m. is a very hardy microorganism that is salt tolerant; four times more resistant to thermal inactivation than serotypes of *Salmonella* (Wilson 1988); almost as resistant to freezing and drying as spore-forming microorganisms; capable of surviving and proliferating at refrigeration temperatures (ICMSF 1996); and is considered to be an intracellular parasite as it possesses the ability to invade and proliferate inside cells of the host immune system (Ryser and Marth 1999) and invade adjacent cells via a process termed paracytophagy which protects it from exposure to the host immune system (Jordan *et al.* 2008).

In the genus *Listeria* are six other species that are very closely related but generally not considered human pathogens: *Listeria seeligeri*; *L. grayi*; *L. welshimeri*; *L. murrayi*; *L. innocua,* and *L. ivanovii* which is considered to be a pathogen among certain ruminant species – particularly sheep (Ryser and Marth 1999). There are 13 serotypes of L.m.: 1/2a; 1/2b; 1/2c; 3a; 3b; 3c; 4a; 4ab; 4c; 4d; 4e; and 7 with approximately 90% of all foodborne illnesses caused by just three serotypes; 4b most frequently with much of the remainder of illnesses caused by 1/2a and 1/2b (FDA 2012). These microorganisms are wide-spread in nature and are considered to be ubiquitous contaminants of all raw agricultural products (ICMSF 1996).

L.m. is an opportunistic intracellular pathogen of humans and animals; often found in the gastrointestinal tract of healthy mammals and birds (ICMSF 1996) with estimates ranging from 1 – 10% of healthy, asymptomatic humans excreting L.m. in their stool (Ryser and Marth 1999). The microbe was first characterized by E.G.D. Murray in 1926 as a cause of sudden death among young rabbits that exhibited infection within white blood cells called monocytes. He named the newly discovered microbe *Bacterium monocytogenes* (Murray *et al.* 1926) which was subsequently changed to *Listeria monocytogenes* in 1940 (Pirie 1940). L.m. became a widely studied bacterium as the causative agent for disease in ruminant animals where it causes spontaneous abortions and circling disease (Wilson 1988) – a form of ataxia that causes progressive paralysis on one side of the body resulting in the standing animal "falling" toward the weak side and stumbling in a circle to remain upright (Merck 2010).

For reasons that are not fully elucidated, when introduced to a herd through a carrier or from an environmental source, the microorganism will cause wide-spread infection with up to 100% of the animals

in the herd shedding L.m. in their stool. The infection generally results in a very low proportion of animals exhibiting clinical symptoms (low morbidity). This feature explains why L.m. is ubiquitous in the environment as large numbers of warm-blooded carriers shed large numbers of viable microorganisms in their stool thus contaminating soil and plant materials. In the susceptible animal that contracts systemic infection, untreated infection leads to extremely high rates of mortality with up to 70% of clinically ill animals dying or losing their fetus to spontaneous abortion (high mortality) (Merck 2010).

The name *Listeria monocytogenes* was given in honor of Joseph Lister, the nineteenth century surgeon credited with development of the antiseptic theory; while the specie name was given due to the early observations of infection of monocytes. These cells are mononuclear, phagocytic leukocytes that form a component of the innate immune system and are capable of moving rapidly to a site of infection where they differentiate into macrophages and dendritic cells. They are responsible for phagocytosis of foreign materials or invading pathogens, antigen presentation to other aspects of the immune response, and cytokine production. Generally, phagocytosis of an invading pathogen results in the death of the pathogen through oxygen-dependent or -independent mechanisms involving, in the first instance, the production in phagosomes of reactive oxygen species or, in the second instance, degranulation and release of proteolytic enzymes (Farber and Peterkin 1991).

As previously mentioned, L.m. has somewhat unique growth and adaptation capabilities that give it particularly troublesome virulence characteristics. In contrast to the nonpathogenic species of *Listeria*, L.m. possesses genes that encode for proteins responsible for adhesion to and invasion of cells of the gastrointestinal columnar epithelium from which cell-to-cell infection may occur in a fairly unique and remarkable process called paracytophagy. This process, observed only with L.m. and *Shigella flexneri*, protects the invading microbes from exposure to the host immune system as the pathogen is safely ensconced within the host cell cytoplasm (Robbins *et al.* 1999).

After L.m. infects a host epithelial cell and replicates at least one time, the sister cell expresses a protein called ActA which is responsible for pirating the host cell actin-formation process. The host-derived actin is used by the microbe to form a tail that propels the microbe through the cytoplasm in "rocket-like" propulsion. When the pathogen reaches the cell membrane, it adheres and forms a protrusion. Given the close proximity of the columnar epithelial cells, the protrusion extends into an

adjacent cell and after a period of “fitful movement”, the original host cell membrane pinches off, depositing the pathogen inside a vacuole contained within the newly infected host cell. This vacuole eventually decays and the sister-cell L.m. successfully infects a new host cell (Robbins *et al.* 1999).

In this same manner, from the infected epithelial cells, the L.m. may infect circulating host phagocytes or dendritic cells remaining in the cytoplasm, protected from the lethal environment within the phagosomes. Circulating in the bloodstream within cells of the immune system, the microbes are effectively hidden from the remainder of the immune system. L.m. may replicate within the white blood cells and the microbes are transported throughout the host (Ramaswamy *et al.* 2007).

Survival within white blood cells allows for vectoring of the microbes via the bloodstream and tissues of the nervous system. For example, leukocytes may cross the blood brain barrier to tissues of the central nervous system where cell-to-cell infection results in encephalitis or meningitis. Similarly, in an expectant mother, the microbes may cross the placenta and infect the unborn child. Early perinatal infection of the unborn child results in death; while late term infection or infection during delivery may lead to neonatal death or other complications (Drevets 1999).

As previously described, L.m. was extensively studied in the 1900s as a cause of diseases of livestock. Although there was mounting evidence, it was not widely recognized as a cause of human foodborne illness until a large outbreak in 1985 in California that was associated with soft, Mexican-style cheese. The cheese was epidemiologically linked to 142 cases of illness and 48 deaths and the outbreak strain was subsequently isolated from the cheese and the processing facility environment. Once the cheese was removed from the market, the outbreak ceased. There were a number of previous outbreaks that had led many to believe L.m. was associated with foodborne illness including: in 1979 among 20 adult patients in hospitals in Boston, Massachusetts that were linked by case-control studies to consumption of raw vegetables (no specific food vehicle was identified); in 1981 among seven adult and thirty-four perinatal cases in Halifax, Nova Scotia epidemiologically associated with coleslaw made from cabbage that had been fertilized with sheep-manure from a herd affected by listeriosis; and in 1983 in Massachusetts a pasteurized milk associated outbreak of illnesses affected forty-two adults, seven perinatal victims and resulted in fourteen deaths. The milk was traced back to a listeriosis-affected herd.

All indications were that the milk was properly pasteurized leading to concerns (later dismissed) that L.m. may be particularly heat resistant (ICMSF 1996).

It is estimated that L.m. causes approximately 2,500 illnesses and 500 deaths each year in the U.S. The vast majority of these are now thought to be foodborne illnesses caused by the consumption of contaminated ready-to-eat foods that are eaten without adequate heating or processing to inactivate the pathogens (FDA 2012). As in ruminant animal herds, it is postulated that a large number of people are exposed to L.m. each year as most raw fruits and vegetables will be naturally contaminated with low numbers of viable cells. The vast majority of people that are exposed do not contract clinical illness (low morbidity) (Ramaswamy 2007). The reasons for low morbidity are complex and varied, ranging from contamination of food with low-virulence serotypes or at levels that are below the infectious dose; to a wide observed range of individual dose responses resulting from differences in the health status of the individual (FDA 2012).

L.m. can exist within the human host as either a pathogen or as a saprophyte. It can cause two forms of illness: invasive infection which is referred to as listeriosis; or a non-systemic intestinal saprophytic infection. Invasive listeriosis may result in death due to encephalitis, meningitis, pericarditis, septicemia or pneumonia; or in the case of an expectant mother, the death of the neonate due to spontaneous abortion, stillbirth or infection of the baby during birthing leading to systemic illness and death. Alternatively, the infant may be infected shortly before or immediately during birth and develop an infection resulting in a chronic illness known as granulamatosis infantiseptica which generally leads to physical retardation and failure to thrive.

If the infection is not systemic and the microbe exists as an intestinal saprophyte, it may result in self-limiting febrile gastroenteritis with nausea, vomiting and diarrhea during which large numbers of viable L.m. will be excreted in the stool (Schlech 1983). Systemic illness leads to death in as many as 80% of peri- or neonatal infection; 70% of the cases of meningitis; and 50% of the cases of septicemia (FDA 2012).

L.m. represents a particular risk to members of the population that are represented by the acronym YOPI: Young; Old; Pregnant; or Immunodeficient. For a number of reasons related to the specific immune status of each group and the virulence of the specific strain ingested, the immune response may

be incapable of adequately protecting the individual and as few as 1,000 viable cells may cause illness (FDA 2012). As such, the immune status of the individual exposed to L.m. in foods is considered to be one of the key reasons for why infection occurs and whether the infection progresses to systemic illness.

The very young (Y) may be exposed before a cellular immune response has developed; elderly individuals (O) often possess immune systems that are failing; pregnant women (P) have down-regulated immune systems due to the presence of "foreign" tissues; and a growing proportion of the U.S. population is considered immunodeficient (I) due to cancer, transplantation or antiretroviral (e.g. H.I.V.) therapy. If exposed to a virulent strain of L.m. at sufficiently high numbers, the cellular response is the only effective line of defense and if it is deficient for any reason, the probability of systemic illness and death is raised significantly (Doganay 2003, Robbins *et al.* 1999, and Schlech 2000).

L.m. is commonly associated with raw milk, poultry, meat, seafood, crustaceans, vegetables and fruit due to close association to soil which may contain as many as 10^4 to 10^7 Colony Forming Units (CFUs) per gram (Ryser and Marth 1999). Once the microorganism is introduced into food processing facilities, it will persist for years with eradication becoming practically impossible (Tompkin 2002). Given its association with moist, soiled areas it will persist in biofilms consisting of large numbers of the bacteria protected from the environment and adhering to surfaces due to the production of extracellular polymeric substances composed primarily of polysaccharides and proteins that form a protective matrix within which the microbes may flourish. Biofilm-associated L.m. are phenotypically distinct from planktonic forms due to genomic shifts resulting from a complex stress response system (Jordan *et al.* 2008). It has been estimated that almost 40% of the entire genome of L.m. is responsive to differential regulation making this pathogen extremely adaptive to a wide-range of environmental conditions (Harmsen *et al.* 2010).

As L.m. is capable of growth in a wide range of environmental conditions, control of this potential human pathogen is especially difficult. L.m. can grow at temperatures between negative 0.4 and positive 45^{o}C; at pH between 4.4 and 9.4; in salt concentrations as high as 10%; and at water activities as low as 0.92 (ICMSF 1996). Interestingly, the microorganism is motile via peritrichous flagella but only in the temperature range between 10 and 25^{o}C which are fairly typical for processing areas in food facilities affording L.m. the ability to translocate from the original point of introduction and establish a deeply embedded harborage. As L.m. is highly adaptive to stress, it may rapidly become resistant to common

sanitizers and, when in a biofilm, is highly resistant to manual removal during cleaning operations (Tompkin 2002).

Given its ubiquitous nature and association with raw agricultural commodities, it is practically impossible to prevent the introduction of L.m. into a food processing facility; given its adaptability, it is almost impossible to eradicate from a facility once it has become established. Thus, the food processor must have a comprehensive and holistic approach to controlling L.m. in finished ready to eat refrigerated or shelf-stable food products (Tompkin 2002).

Control of L.m. in the food supply is a complex problem and each manufacturer of ready to eat foods must have a comprehensive control methodology to reduce the risk to the maximum extent possible. In general, this methodology should consist of three critical elements:

1) An intervention such as cooking, high pressure pasteurization, irradiation or other preserving process to reduce the number of naturally occurring L.m. on raw agricultural products;
2) Facility and equipment sanitation to control the resident L.m. population sufficiently to prevent development of gross harborages and employee practices controlled to prevent recontamination of processed food products with high numbers of viable L.m.; as well as
3) Some means of ensuring that any L.m. surviving in the finished products are incapable of replicating to high numbers during the storage and distribution of the food products before consumption (Tompkin 2002).

These controls are critical to ensure that human exposures and illnesses are minimized.

***Clostridium* spp.:** Foodborne botulism is an illness caused by the ingestion of preformed neurotoxins (a simple protein composed only of amino acids (Lamanna 1959) produced during the sporulation of the bacterium *Clostridium botulinum* (*C. botulinum*); or, less frequently, by the ingestion of spores of the bacterium that germinate, infect and colonize the immature infant gut producing toxins that cause clinical symptoms (some strains of *C. butyricum* and *C. barattii* may also produce the toxins (ICMSF 1996). Botulism is a very serious but relatively rare foodborne illness causing an average 145 cases per year in the U.S. (approximately 22 cases per year are classic foodborne botulism with the remainder infant or wound botulism) that may lead to paralysis and death due to asphyxiation (CDC 2012c, FDA 2012).

Foodborne illness caused by *C. perfringens* is much more common with estimates as high as one million cases in the U.S. each year – which for bacterial causes of foodborne illness represents a number second only to the number of illnesses caused by species of the salmonellae. The illness is much less severe and almost universally self-limiting with symptoms exhibiting within 16 hours of ingestion and most frequently consisting of watery diarrhea lasting less than 24 hours. Since 1999, there have only been 24 deaths attributed to *C. perfringens* which was caused by severe dehydration. In every case, the deceased was elderly and suffering from underlying debilitating medical conditions (CDC 2012c).

Given the ubiquitous nature of these pathogens, it is imperative that food manufacturers assume that vegetative or spore-forms of the microbes will be present on or in the foods and controls are required to protect consumers. While the presence of *C. botulinum* in meat and poultry is infrequent, the illness it may cause warrants control measures. Greenberg *et al.* reported that only one of 2,358 samples of raw meat purchased throughout the U.S. and Canada in 1966 was positive for *C. botulinum*; while Taclindo *et al.* reported that only one in 75 samples of luncheon meats was positive in 1967; in 1971 Abrahamsson and Riemann reported that six of 372 samples of semi-preserved meat and poultry were positive for *C. botulinum*; and in 1980, Hauschild and Hilsheimer reported that one of 416 samples of commercial bacon were positive. The incident rates of *C. botulinum* in meat and poultry stand in marked contrast to those in fish, where it is found in up to 50% of the intestinal contents and in crabs where it may be found in 90% of samples (Rhodehamel *et al.* 1992).

However, the rates of *C. botulinum* contamination of meat and poultry are markedly different than the rates of contamination with *C. perfringens*. In 2003, Taormina *et al.* reported that almost 22% of cured or uncured meat and poultry products were positive for *C. perfringens*; with the range from 1.6% of cured whole muscle to 48.7% of ground or emulsified meat samples positive. These samples exhibited an approximately equal distribution of vegetative cells versus spores (Taormina *et al.* 2003). Further in 2003, Kalinoski *et al.* reported that of 197 ground meat samples analyzed, all but 2 had levels of *C. perfringens* below the limits of detection; while the 2 samples that were positive had spore counts of 3.3 and 66 per gram (Kalinowski *et al.* 2003).

In contrast with classic botulism in which the preformed toxins are ingested, *C. perfringens* must replicate to very high numbers in the food and be ingested as viable, vegetative cells. Illnesses are most

frequently associated with temperature abuse of previously cooked foods most often in food service or institutional settings in which foods are cooked in advance of serving and improperly held at elevated, but not growth-limiting temperatures allowing the microbes to proliferate. Capable of growth between 12 - 50 ^{o}C, an important characteristic of this organism is its ability to replicate rapidly on meat products. At 43 - 47^{o}C, this organism may double in less than 10 minutes. Thus, improper hot holding of foods may allow the number of microbes to reach infectious levels in less than one hour (ICMSF 1996).

As most of the vegetative cells of *C. perfringens* will be inactivated by the hostile environment of the stomach and upper intestinal tract, a large number of cells must be ingested ($\geq 10^6$) for illness to occur. As the environment of the mature gut is not ideal for *C. perfringens*, a sufficiently large number must survive passage to the lower intestines to colonize, replicate and produce toxin during sporulation (FDA 2012). The foodservice or institutional setting may be a requirement for most cases of human illness as it has been demonstrated by Andersson *et al.* that, for expression of the toxin to occur, the gene encoding for the toxin must be located on the chromosomes rather than on a plasmid. Strains isolated from the environment are almost universally non-toxigenic and the toxin negative strains require repeated heat shock and sporulation -- apparently to induce gene insertion into the chromosomes -- to convert to toxin positive. It has been postulated that this only routinely occurs with “kitchen strains” that have repeatedly passed through heat / reheat cycles (Andersson *et al.* 1995).

As noted by Bryan, the means of preventing foodborne illnesses from *C. perfringens* is to:

1) Hold cooked meat products at temperatures that are either too cold or too hot to allow the microbes to proliferate;
2) To hold the foods prior to consumption for a period of time insufficient for the *C. perfringens* to proliferate to numbers sufficient to cause illness; and
3) To reheat any previously chilled foods to a temperature lethal to *C. perfringens*.

Bryan established guidelines for preventing *C. perfringens* illnesses from cooked meat and poultry at foodservice establishments and concluded that with relative ease one of the most frequent causes of foodborne disease “would become a rareity” (sic) (Bryan 1972).

Both *C. botulinum* and *C. perfringens* are relatively large Gram-positive, anaerobic, motile by peritrichous flagella, endospore-forming rods that are ubiquitous in nature and often associated with soil

in any environment where organic materials are held under anaerobic conditions (Hall and Peterson 1923). Given the association with soil, both microbes are frequently present in the gastrointestinal tracts of warm-blooded animals, fish and birds and as such it must be assumed that raw agricultural commodities are contaminated with either the vegetative or spore-form of these bacteria (Rhodehamel *et al.* 1992). While any food that will support the growth of these microbes may cause illness, the most frequent cause of foodborne *C. perfringens* is a cooked, meat-containing product improperly hot-held at foodservice (CDC 2012d); while the primary cause of foodborne botulism in the U.S. is low-acid, home-canned foods (CDC 2012c) and native Alaskan fermented foods that are frequently covered in rendered seal oil and fermented at room temperature then eaten without cooking. Commercially canned food products have been associated with outbreaks in the U.S. in the 18th and 19th century before the low-acid canned food methods were developed and perfected (Sobel *et al*. 2004).

Botulism was first described in 18th century Europe and was associated with illnesses following consumption of sausages. *C. botulinum* was first isolated from an inadequately cured ham in 1895 by Emile van Ermengem following an outbreak in the village of Ellezelles in Belgium which resulted in three deaths among the 23 ill musicians affected. The microorganism was first named *Bacillus botulinus* with the genus assignment due to the shape of the cell and the species name after the Latin word for sausage, "*botulus"*. An illness called "Sausage Poisoning" was common in Europe during the eighteenth and nineteenth centuries and was suspected to have been caused by this group of microorganisms (Rhodehamel *et al.* 1992). In 1897, the microbe was first isolated from a source other than a food or wound and based upon the common association with sausages, Kempner and Pollack concluded that the microbe must be an inhabitant or transient of the swine gut (Hall and Peterson 1923). After it was observed that the microbes were strict anaerobes, the genus designation was changed from *Bacillus* which was reserved for aerobic spore-forming rods (ICMSF 1996).

Under growth-adverse conditions, *C. botulinum* forms endospores that are highly resistant to inactivation. There are seven strains designated by differences in characteristics such as toxins formed, tolerance of salt and low water activity, proteolytic activity, upper and lower growth temperature boundaries and spore heat resistance. These seven types of *C. botulinum* are given the designation A, B, C, D, E, F, and G. Strains C, D and G are not known to cause human illness. The proteolytic strains

A, B and F produce spores that are extremely heat tolerant and represent a primary concern in the food canning industry. The non-proteolytic B, E and F strains produce spores that are not very heat resistant, but the microorganism is capable of growth at refrigeration temperatures and is a concern for pasteurized or unheated foods such as sea foods and some vegetables if stored under anaerobic conditions. Most human cases of botulism are caused by *C. botulinum* type A (ICMSF 1996).

C. botulinum is recognized as a diverse grouping of microbes with a wide range of serologic and metabolic characteristics. The one thing in common across all strains is the ability to produce a potent neurotoxin. Based upon similarities within strains, Smith and Sukiyama grouped the species into four categories (I through IV) with those in Group I exhibiting proteolysis and those in Group II being non-proteolytic (ICMSF 1996). The toxin produced by all strains is a simple protein that is absorbed through the mucous membranes; binds to motor nerve endings at the neuromuscular junction; interferes with neurotransmission by preventing the release of acetylcholine; and results in a flaccid paralysis (Rhodehamel *et al.* 1992).

The botulinal toxin is considered to be the most potent toxin known to man with an estimated lethal dose for a man of approximately 0.1 – 1.0 μg while 0.0001 μg is typically lethal to a mouse. The Mouse Lethal Dose (MLD) is the standard reference for the toxin. Lamanna estimates that the toxin is at least one order of magnitude more lethal than the most potent non-protein toxin and hundreds to tens of thousands of times more toxic than the diphtheria toxin or animal venoms (Lamanna 1959).

Symptoms of botulism typically start with the eyes and face approximately 18 – 36 hours after ingestion of the toxin-contaminated food with muscles of the trunk and extremities becoming progressively affected (FDA 2012). The symptoms generally start with blurred vision, double vision (diplopia), drooping eyelid (ptosis), difficulty breathing, difficulty swallowing (dysphagia), generalized weakness, dry mouth, nausea and vomiting, inability to speak (dysphonia), fixed and dilated pupils, and abdominal pain lasting one to ten days depending upon the patient and the dose of ingested toxin. As difficulty with breathing progresses, intense respiratory assistance may be required to prevent death by asphyxiation. Mortality has decreased from approximately 65% to less than 10% of the cases due to modern mechanical ventilation support systems (FDA 2012) and administration of a trivalent antitoxin for types A, B and E (ICMSF 1996). Recovery from botulism may take weeks or months as the toxin binds

irreversibly to the nerve cells and recovery is dependent upon regeneration of new nerve pathways (Rhodehamel *et al.* 1992).

Foodborne botulism in the U.S. is most frequently associated with low-acid, home-canned foods such as asparagus, corn or green beans which are inadequately thermally processed allowing spores to survive. As the heating process drives off oxygen, the environment is made anaerobic during processing. If the heat resistant spores of *C. botulinum* strains A, B and F remain active and there is no other impediment to their growth such as refrigeration or high-acid content, the spores may germinate, replicate and ultimately produce toxins (CDC 2012c, FDA 2012). Relatively few cases of human botulism have been attributed to commercial food products (Rhodehamel *et al.* 1992).

Control of *C. botulinum* in sausages has generally been achieved through the use of curing agents. As will be described in detail in a later section, salts reduce the water activity of meat systems in which anaerobic conditions may be present and nitrite prevents the spores from germinating and growing. Since the use of modern meat cures has become wide-spread and reliance on microbial reduction of nitrates is much less frequent, residual levels of nitrite in sausages prevent *C. botulinum* germination and provide a residual nitrite store in the event that products are temperature abused (Tompkin *et al.* 1978a).

Fermentation of cured sausages adds additional margins of product safety as *C. botulinum* will not grow and produce toxins below pH 4.6 (ICMSF 1996). However, as the pH of fermented sausages may be well above 4.6, it is necessary to consider the combined effects of salt, nitrite and pH. Christiansen *et al*. demonstrated in summer sausages that commercial levels of nitrite in combination with a starter culture and 2% dextrose prevented toxin formation (Christiansen *et al*. 1975) supporting the conclusion of Drs. Hall and Peterson in 1923, who stated that control of *C. botulinum* toxin formation is “something other than acid alone” (Hall and Peterson 1923). Commercially produced cured meat products have “shown an exemplary botulism safety record” (Sofos 1981).

***Staphylococcus aureus*:** *Staphylococcus aureus* (*Staph. aureus*) is considered to be the type strain of the genus *Staphylococcus.* It is a commensal of warm-blooded animals including man where it is an opportunistic pathogen associated with a wide range of human illnesses including: minor infections of skin cuts, scratches and lesions; boils and abscesses; toxic-shock syndrome; septicemia; and toxemia (ICMSF 1996). The most common *Staph. aureus* toxemia is called staphylococcal food poisoning (SFP)

which is thought to be one of and possibly the, most common foodborne illnesses of the modern era (Dinges *et al.* 2000). SFP is estimated to affect approximately 240,000 people in the U.S. each year; though the actual number of culture-confirmed cases averages slightly more than 300 per year. For reasons discussed later, the actual number of cases is thought to be significantly underreported (Scallan *et al.* 2011). It is important to note that the number of confirmed cases has dropped substantially since the early 1970s during which period approximately 7,000 confirmed cases per year were reported (Genigeorgis 1989; Mead *et al.* 1999).

SFP is an intoxication that occurs following consumption of *Staph. aureus* contaminated foods that have been improperly stored or held under conditions that allow the pathogen to proliferate to extremely high numbers. During logarithmic growth and transition to the stationary growth phase, the microbes excrete proteins (exotoxins) that are enterotoxins to man (Batia and Zahoor 2007). Symptoms of illness manifest within one to seven hours following consumption of toxin-contaminated foods (FDA 2012).

While the mode of action has yet to be fully elucidated, the symptoms result due to the effect of the preformed toxins in the lumen of the intestines where it is thought inflammation stimulates the vagal nerve stimulating the emetic center resulting in explosive vomiting. Infection and subsequent toxin production in situ has been documented, particularly in hospital settings among patients having been treated extensively with antimicrobial therapy, where *Staph. aureus* may cause a toxicoinfection when the opportunistic pathogen colonizes the gut following elimination of the competitive normal flora (Argudin *et al.* 2010).

The most common symptoms of SFP are nausea, abdominal cramping, and explosive vomiting (with or without diarrhea). The illness is generally self-limiting within 24 -- 48 hours and is not considered to be life-threatening. However, for at-risk individuals (those with underlying medical conditions, the very young, the elderly), excessive emesis may result in hypovolemia, electrolyte imbalances and collapse requiring medical intervention in the form of I.V. fluid replacement therapy. As the illness is generally mild, self-limiting and of short duration, it is thought that most affected people don't seek medical treatment or report the illness to the health authorities. As such, it is believed that the gastroenteritis form (SFD) of *Staph. aureus* intoxication is grossly under-reported (CDC 2012e).

As an infectious agent, *Staph. aureus* is a major cause of hospital-acquired (nosocomial) and community-acquired infection including surgical or injury wounds, breaks in the integument, and with increasing frequency, a cause of pneumonia. Many strains of *Staph. aureus* have developed extensive antimicrobial resistance and medical treatment has become complicated by methicillin- and vancomycin-resistant strains (CDC 2012e). As vancomycin is an I.V. delivered antimicrobial, abuse outside of hospital settings has been minimized and the compound has had a prolonged (> 50 years) period of efficacious treatment against penicillin-resistant strains of *Staph. aureus.* However, as methicillin-resistant strains developed, vancomycin became the "drug of last resort" and its wide-spread use led to wide-spread resistance (Levine 2006). While this is causing concerns for the medical community, it is an important distinction from foodborne cases caused by the preformed toxins. The Centers for Disease Control and Prevention have ceased routine reporting of SFD due to the limited number of confirmed cases.

The term *Staphylococcus* means "grape-like clusters" which is the colony morphology of this genus when viewed under a microscope. It was first described by Alexander Ogston in a series of papers between 1878 -- 1882. Ogston was a Scottish surgeon who observed the microorganisms in pus taken from human abscesses and gave the genus name. In 1884, Rosenbach described growth of the microbes on pure culture plates that gave rise to two morphologically distinct colony-pigmentation forms: one strain resulted in golden-orange colored colonies and the species name *aureus* was given for the Latin term meaning "gold"; the other strain resulted in white colored colonies and the name *albus* was given for the Latin term meaning "white". *Staph. albus* was later renamed *Staph. epidermidis* (Bhatia and Zahoor 2007). The staphylococci are Gram-positive, non-motile, facultative anaerobic cocci that morphologically resemble species in the genus *Micrococcus.* They are phenotypically distinguishable due to metabolic, chemical and biochemical distinctions such as growth under anaerobic conditions and production of catalase (ICMSF 1996).

There are 32 species and 8 sub-species of *Staphylococcus* currently recognized. *Staph. aureus* is capable of growth in a wide range of conditions that, in the absence of competitive microflora, allows it to proliferate in a wide range of foods (Harris *et al.* 2002). These microorganisms are wide-spread among humans and common warm-blooded meat animals as commensals and opportunistic pathogens of the skin and mucosal surfaces – particularly of the nasal passages (FDA 2012).

Many authors estimate that up to 50% of the human population carries *Staph. aureus* as a commensal microorganism (Bhatia and Zahoor 2007). As reported by Casman in 1965, of 212 *Staph. aureus* strains isolated from the nasal passages of healthy subjects, 49 (23%) were toxigenic with 80% of those strains producing type-A toxin. As *Staph. aureus* is widely distributed among animals and humans, one should expect them to be present on food products that have been previously handled (Hill 1972).

During the exponential growth phase and during the transition to the stationary phase, *Staph. aureus* produces a wide range of compounds that contribute to virulence. As an agent of foodborne illness, the most significant substances formed are the low molecular weight *Staph*. enterotoxins (SE-) which are simple proteins that have been grouped into fourteen antigenic types and given letter-designations SEA through SEO (there is no SEF as that designation was assigned to a protein that was later determined not to be an enterotoxin) (Bhatia and Zahoor 2007).

SEA and SEE are considered classic *Staph. aureus* enterotoxins which are prophage-encoded by temperate bacteriophages. The bacteriophage carried genes are inserted into the chromosome of *Staph. aureus* where they function as part of the genome encoding for the production of enterotoxin-A, staphylokinase and a protein that functions as an inhibitor to complement (Schelin *et al.* 2011). It appears that among other effects, the toxins interfere with the Major Histocompatibility Complex inhibiting the release of histamine. Since the early studies of SEA, other accessory genetic mechanisms have been elucidated for toxin gene insertion, including plasmids, pathogenicity islands, genomic islands, or genes associated with the staphylococcal cassette chromosome which has been implicated in methicillin resistance. Given the wide-spread antimicrobial resistance patterns among *Staph. aureus*, its genetics are among the most broadly studied (Argudin *et al.* 2010).

Most food-poisoning outbreaks involve the toxin SEA (and to a much lesser extent SED) which is a metabolic by-product produced during the logarithmic growth phase -- as distinguished by SEB and SEC which are secondary metabolites formed during the stationary growth phase. As the genotypes that produce these toxins are tolerant of a wide range of pH, salt concentration, water activity and reduction potential, many food types will support growth of toxigenic *Staph. aureus.* Improper handling of ready to eat foods during preparation followed by poor temperature control during storage may allow the toxins to form. Illness is caused by relatively small amounts of the toxins. Depending upon individual weight and

sensitivity, it is estimated that 20 – 100 ng of ingested enterotoxin will induce symptoms (Schelin *et al*. 2011).

As *Staph aureus* are commensal microorganisms, they are frequently encountered in raw meat and poultry products which must be processed sufficiently -- generally by heat during cooking -- to inactivate the microbes to prevent proliferation during finished product storage. A wide range of meat products such as salt-cured hams are processed with no terminal heat treatment sufficient to inactivate these naturally present microbes. As such, these products were frequently found to be associated with outbreaks of SFP. A complete understanding of the illness etiology did not start to emerge until 1930 when Dack and his colleagues demonstrated that a sterile filtrate from a crème-filled cake that had been associated with illness was capable of causing illness (ICMSF 1996). Since that time, a wide range of control measures have been developed and validated and commercial food products have generally been eliminated as a source of illness with the burden now shifted to restaurant and home prepared foods (FDA 2012).

If ready to eat food products are contaminated with *Staph. aureus* during handling or processing – particularly those foods with characteristics such as high salt content that suppress the growth of potentially competitive spoilage microorganisms – and are subsequently held under conditions that allow the *Staph. aureus* to proliferate, extremely heat stable toxins may form in the foods. This is particularly problematic for foods such as puddings or pastries that are held for extended periods without refrigeration, or for hot-served products held for extended periods with inadequate temperature control (ICMSF 1996). As growth of *Staph. aureus* is optimal between 35 and 40^{o}C, certain food items represent a significant risk. These include soups, sauces and gravies that are held at warm temperatures for extended periods or cooked products that are placed into refrigeration for extended chilling when inadequate space, air flow or depth (mass) of product prevents rapid heat loss and the food stays warm for a prolonged period (FDA 2012).

In 1972, Genigeorgis documented five conditions that must exist for human illness to occur. He also demonstrated that preventing any one of the five would ensure that illness could not occur:

1) There must be a source of toxigenic *Staph. aureus*;
2) The toxigenic *Staph. aureus* must be transferred from the source to the food;
3) The food must support the proliferation of *Staph. aureus*;

4) The food must remain within the temperature range for *Staph. aureus* growth long enough for cell numbers to reach approximately 10^6 per gram for sufficient toxin to form; and
5) A sufficient amount of the contaminated food must be ingested to deliver an illness-inducing dose of toxin (Genigeorgis 1972).

In the context of this work, the temperature and time at which sausages are held during fermentation represent an ideal set of conditions for *Staph. aureus* replication and toxin formation and all of the previous five requirements may be met. These conditions have frequently been demonstrated to result in toxin formation in meat products for which the salt level in the product was lower than the growth-limiting minimum or the pH drop was insufficiently low to prevent proliferation or took too long to drop sufficiently low during which time growth occurred and stable toxins were formed (Genigeorgis 1972, Marcy *et. al* 1985).

Numerous researchers including Marcy and Rieman have demonstrated that salt levels in most product formulations are insufficient to prevent growth and toxin formation; concluding that the use of a fast-fermenting starter culture with a formulation including added sources of carbohydrates to stimulate rapid lactic acid bacteria growth, acid production and concomitant drop in pH is necessary to ensure toxins are not formed during fermentation (Genigeorgis 1972). As a result of these findings, a committee formed by the American Meat Institute (AMI) documented a concept for control that was named “Degree Hours” – the specifics of which will be described in detail in a subsequent section.

The Degree Hours protocol establishes combinations of maximum time and temperature at which the fermenting meat products should be held before the pH has been reduced to below 5.3. It is a very conservative approach to the control of staphylococcal enterotoxins in fermented meat products and has been validated effective by a number of researchers. This work has illustrated the critical role that starter cultures now play in assuring the safety of fermented meat products (Metaxopoulos *et al*. 1981).

Pathogenic *Escherichia coli*: While *Escherichia coli* (*E. coli*) is a very diverse genus with a great deal of specie variation, the most common strains are not human pathogens (Gyles 2007). These enteric microorganisms are commensal constituents of the microflora of the adult gut of all warm-blooded animals where they play a mutually important role in digestion and nutrient creation. In humans, generic (biotype I) *E. coli* are critical to proper nutrition as they synthesize essential nutrients such as biotin and

vitamin K that are relatively uncommon in the human diet (Bentley and Meganathan 1982). While survival without a gut microflora is possible, it is likely that humans would be deficient of several vitamins in an extended absence of these microbes (Steinhoff, 2005).

E. coli are Gram-negative, rod-shaped, facultative anaerobes that were first isolated in 1919 from the feces of infants by the German-Austrian pediatrician and professor Theodor Escherich who named the microorganism *Bacterium coli.* In 1920, the microorganism was removed from the genus *Bacillus* and placed into the newly named genus *Escherichia* to honor the professor amid growing evidence that the microbe was capable of causing infant mortality (ICMSF 1996). By the mid-1900s, it was apparent that certain serotypes of *E. coli* were involved in enteropathogenesis. A 1975 human case of bloody diarrhea in California was attributed to *E. coli* and following an outbreak of hemorrhagic illnesses in 1982 associated with fast food hamburgers, a clear cause and effect association was established (Doyle *et al.* 2006). Research since that time has focused on virulence factors and control measures in foods (ICMSF 1996).

Differential classifications for *E. coli* are generally based upon distinctions between the somatic (O) and flagellar (H) antigens. Occasionally, these distinctions will include the capsular (K) antigens (Gyles 2007). Following isolation and characterization, the different antigens were sequentially numbered resulting in a nomenclature in the format of *E. coli* O#:H# (e.g. *E. coli* O157:H7). These classifications are divided into several pathogenicity groupings based upon virulence and the mechanism by which the microbe causes illness (CDC 2012f). These divisions included four classifications most frequently associated with foodborne (including water) illnesses: enteropathogenic *E. coli* (EPEC); enteroinvasive *E. coli* (EIEC); enterotoxigenic *E. coli* (ETEC); and the most widely recognized enterohemorrhagic *E. coli* (EHEC) (ICMSF 1996). Additionally, there are two categories that have historically been associated with chronic malabsorptive malnutrition in underdeveloped regions (Mathusa *et al*. 2010) but are more frequently being isolated from foods implicated in illnesses, presumptively as they are acquiring toxin genes associated more commonly with the traditionally foodborne strains: enteroaggregative *E. coli* (EAEC); and diffusely adherent *E. coli* (DAEC) (FDA 2012).

Enteropathogenic *E. coli* (EPEC) are characterized by the presence of the locus of enterocyte effacement (LEE) gene which encodes for a number of virulence factors including the protein intimin

which, in conjunction with the intimin receptor, is responsible for the intimate attachment of the EPEC to the intestinal epithelium where the enzyme system for digestion and adsorption is disrupted, leading to malnutrition (Tarr *et al*. 1997). The EPEC are frequently associated with infant diarrhea and, in developing regions of the world, are important causes of infant mortality which may reach 50% of infected children and contributes significantly to the conditions associated with failure to thrive. The infectious dose for infants is thought to be very small (adults are generally not susceptible) and results in a low-grade fever with profuse, watery diarrhea lasting up to 120 days leading to dehydration and electrolyte imbalances. Illness has been associated with every food type and likely results due to fecal contamination of water used for drinking and food preparation. Controls for EPEC include proper hand-hygiene, sanitary sewer systems and adequate sources of potable water (FDA 2012).

Via an invasion gene encoded in a virulence plasmid (Neill 1997), enteroinvasive *E. coli* (EIEC) invade epithelial cells of the colon where they replicate and produce an enterotoxin that is very similar to that produced by species of *Shigella*. Both EIEC and *Shigella* spp. cause a mild form of dysentery with symptom onset within 72 hours of consumption of contaminated food. The illness is mild and self-limiting with diarrhea, vomiting, fever, chills and malaise. Humans are the only known reservoir for (EIEC), thus the fecal-oral route of exposure from contaminated water or food is the vector (FDA 2012). Controls are the same as for the EPEC.

Enterotoxigenic *E. coli* (ETEC) are motile by peritrichous flagella which is important as they are frequently water-borne. They produce several virulence factors including heat-labile and heat-stable toxins. These are a common cause of human gastroenteritis with a very large infectious dose required to cause illness resulting in onset of symptoms between 8 and 44 hours after consumption of the contaminated food or water (CDC 2012g). Symptoms include profuse, watery diarrhea caused by the toxins produced during growth of the ETEC in the small intestine. The illness is generally self-limiting but in rare instances can progress to dehydration requiring I.V. fluid-replacement therapy. Outbreaks are rare in the U.S. but ETEC is a common cause of travelers' diarrhea as a result of consumption of feces-contaminated water, foods prepared with contaminated water, or food prepared by an infected food handler (FDA 2012).

Enterohemorrhagic *E. coli* (EHEC) are the most widely recognized and notorious of the pathogenic strains of *E. coli*. The EHEC are characterized by the presence of a gene -- likely plasmid-acquired from *Shigella* (the Shiga-toxin gene (Stx) resulting in the nomenclature STEC for Shiga-Toxin producing *E. coli*) -- that encodes for the production of the Shiga-toxin (Neill 1997). The EHEC cause an enterohemorrhagic illness with typical, often grossly bloody diarrhea. While there are as many as 400 STECs, many have never been implicated in foodborne disease. Those STECs that cause severe illness are grouped as the EHEC and include the prototypical *E. coli* O157:H7 which was first declared an adulterant in ground beef products following a fast food chain related outbreak of illnesses in 1993 traced to undercooked hamburgers (Tarr *et al.* 1997). Recently, the “Big-6” non-O157 EHEC serotypes have been declared adulterants in non-intact beef products. These include: O26; O45; O103; O111; O121; and O145 which, together with O157:H7 account for the vast majority of all cases of hemorrhagic colitis in the U.S. (FDA 2012).

The EHEC possess two characteristics that distinguish them from other pathogenic and non-pathogenic *E. coli*: they possess a Shiga-toxin gene – either Stx1 and / or Stx2; and they possess the pathogenicity island LEE that encodes for the production of the protein intimin (Tarr *et al*. 1997) as previously described for the EPEC. Due to these virulence factors and the apparent acid-tolerance of the EHEC, it is estimated that the infectious dose may be as low as 10 viable cells (Gyles 2007). Many infected people are asymptomatic and may represent a carrier state shedding the microbes in their feces for extended periods.

It is estimated that the STECs cause approximately 285,000 illnesses in the United States annually with 3,600 patients admitted to hospital resulting in 30 deaths (Scallan *et al*. 2011). The route of infection is fecal-oral from ingestion of contaminated food or water or contact with infected animals (CDC 2012h). While all warm blooded animals may carry the STECs, the most frequently identified reservoir is cattle. Thus, the most often implicated food has been raw or undercooked beef products (Lake *et al.* 2007). However, a wide variety of foods including produce, unpasteurized fruit juices, milk-based products (yoghurt and cheeses), mayonnaise and fermented sausages have been implicated in outbreaks of illness (FDA 2012).

Symptoms of illness typically manifest within 3 to 9 days of consumption of contaminated foods (CDC 2012h) and, depending upon host-specific and infecting-strain specific characteristics, the illness may consist of self-limiting gastroenteritis; it may progress to severe bloody diarrhea known as hemorrhagic colitis (HC) which is a condition characterized by severe inflammation of the bowel resulting in bloody diarrhea with intense abdominal pain and afebrile vomiting. Following hemorrhagic colitis, the illness may progress to a life-threatening condition called hemolytic uremic syndrome (HUS) in up to 30 percent of the cases (predominantly among very young patients). In HUS, the shiga-toxins cause kidney damage resulting in renal insufficiency or failure, seizures, coma and death in 10 to 30 percent of the cases. Treatment may consist of palliative care for less severe cases or extend to full life-support with dialysis, serum replacement and I.V. fluid maintenance (Lake *et al*. 2007).

Evidence suggests that STECs that code for production of the toxin Stx2 and the protein intimin are more frequently associated with HUS as kidney cells have a relatively high concentration of Stx2 receptors. Many survivors of HUS will experience chronic sequelae such as renal insufficiency or neurological defects (FDA 2012).

Like all *E. coli*, the pathogenic STECs exhibit a wide-range of growth characteristics enabling them to survive and thrive in a diverse range of food products. While optimum growth is at 37^{o}C with doubling occurring every 0.4 hours (Lake *et al.* 2007), several toxigenic strains have shown growth between 10 and 50^{o}C (Palumbo *et al.* 1995) and as low as 8^{o}C (Rajkowski and Marmer 1995). The STECs are not particularly tolerant of low water activity with growth limited at a_w = 0.95. However, they may survive for long periods at reduced water activity if other interventions (such as heat) are not applied (ICMSF 1996).

Acid tolerance has emerged as an important virulence factor for the STECs (Gyles 2007, Mathusa *et al.* 2010) and survival for two months in fermented sausages at pH 4.2 has been documented with only a two $\log_{10}$ reduction in viable cell count (Lake *et al.* 2007). Thus, these microorganisms represent a particular concern for manufacturers of fermented meat products and represent a significant element in hazard identification and control during processing.

***Salmonella* spp.:** The genus *Salmonella* consists of 2,579 different serotypes (ca. 2007), many of which are not considered human pathogens. While taxonomy and nomenclature is not the focus of this paper, it is important to note that the genus consists of only two species: *Salmonella enterica* and

Salmonella bongori. The vast majority of the salmonellae with which we are concerned are serotypes of *Salmonella enterica* which is further subdivided into six subspecies:

1) (I) *Salmonella enterica* subsp. *enterica;*
2) (II) *Salmonella enterica* subsp. *salamae*;
3) (IIIa) *Salmonella enterica* subsp. *arizonae*;
4) (IIIb) *Salmonella enterica* subsp. *diarizonae*;
5) (IV) *Salmonella enterica* subsp. *houtenae*; and
6) (VI) *Salmonella enterica* subsp. *indica* (Grimont and Weill 2007).

One frequently encounters inconsistencies in the literature that may lead to confusion; and consensus on naming conventions has not been reached. For the purposes of this paper, we will use the convention of naming the microorganism of interest by use of the abbreviation for the genus and the specific serotype (e.g. *Salmonella enterica* subsp. *enterica*, serotype Kentucky will simply be referred to as *S*. Kentucky).

The salmonellae are Gram-negative, non-spore-forming, facultatively anaerobic, rod-shaped bacteria that are motile by peritrichous flagella (FDA 2012). Representatives of subspecies (I) are commonly found in the intestinal tract of humans and other warm-blooded animals with approximately 60 percent of all serotypes of *Salmonella* belonging to this group (CDC 2011a); representatives of groups (II) and (III) are often associated with cold-blooded animals; and representatives of groups (IV) and (VI) are environmental isolates that are rarely human pathogens (ICMSF 1996). *Salmonella* serotypes are distinguished based on somatic (surface) and flagellar antigens as characterized by the Kauffman-White typing scheme (FDA 2012).

S. Typhi was the first of the salmonellae to be isolated by Georg Theodor August Gaffky in 1884 following Karl Joseph Eberth's 1880 description of a bacillus that was suspected as the cause of hog cholera. In 1885, the research assistant of Dr. Daniel Elmer Salmon (Dr. Theobald Smith) isolated *S*. Choleraesuis from clinically ill swine (ICMSF 1996). In 1900, Dr. J. Lignières proposed naming the genus *Salmonella* in honor of Dr. Salmon, who had a long and distinguished career including supervising the establishment of the veterinary division at the U.S. Department of Agriculture and overseeing implementation of the federal meat inspection program (Masters 2006). In Paris in July 1930 at the First Congress of the International Society for Microbiology, the Nomenclature Committee adopted the name

for the genus in recognition of both Dr. Salmon's and Dr. Lignières' contributions to the field (*Salmonella* Subcommittee 1930).

Salmonellosis is one of the leading human foodborne illnesses with current estimates of approximately 3,500 illnesses per 100,000 people per year in the domestic population – second only to illnesses caused by viruses (Scallan *et al*. 2011). The case rate of human salmonellosis has been relatively constant since 1998 and progress has not been made toward the national goals of reducing foodborne illnesses (CDC 2011b). While any of the more than 2,500 species of *Salmonella* are considered capable of causing illness, approximately twenty species are responsible for the vast majority of human illnesses – causing approximately 1.2 million cases (approximately 42,000 culture-confirmed illnesses reported each year to the CDC), 23,000 hospitalizations and 400 deaths each year (CDC 2012i). Some have estimated that as many as 95 percent of all human cases are foodborne (Hohmann 2001 and Scallan *et al*. 2011).

In the years 1996 – 1999,"seventy-four percent of cases were caused by 8 *Salmonella* serotypes: Typhimurium, Typhi, Enteritidis, Heidelberg, Dublin, Paratyphi A, Choleraesuis, and Schwarzengrund" (Vugia *et al*. 2004). In 2009, a decade after the Vugia study, those 20 serotypes were again responsible for almost 73 percent of all human illnesses reported to the CDC with a culture-confirmed isolate. However, the serotypes responsible had shifted with *S*. Dublin, *S*. Parathyphi A and *S*. Choleraesuis having dropped off the list of the top twenty (CDC 2009b). Currently, the top 100 reported human salmonellosis serotypes account for almost 98 percent of all illnesses (CDC 2011b).

Serotypes of the salmonellae can cause 2 distinct forms of illness in humans: typhoid fever – caused by *S*. Typhi and *S*. Paratyphi; and nontyphoidal salmonellosis – caused by many other serotypes. Domestically, typhoid fever has largely been eradicated through the efforts of public health authorities. Key to control has been the disinfection of drinking water supplies, pasteurization of milk, monitoring and control of the sanitation of shellfish beds, and the introduction of sanitary sewer systems to minimize human exposure to fecal material (Tauxe 1997). In modern times, typhoid fever is associated with a carrier state as humans are the only known reservoir (Galanis *et al.* 2006). The human-carrier status was made famous in the U.S. by the story of "Typhoid Mary" Mallon, an asymptomatic cook identified as the

first carrier of the typhoid bacilli in America and ultimately blamed for at least 51 cases and 3 deaths caused by foods that she contaminated (Brooks 1996).

Infection with either *S*. Typhi or *S*. Paratyphi causes typhoid fever and if left untreated may result in death in as many as 10% of the cases. Clinical symptoms typically manifest within 7 – 28 days after ingestion of contaminated food and may include elevated temperature, malaise, headache, abdominal pain, diarrhea or constipation, anorexia and weight loss, and occasionally a rose-colored rash will develop on the trunk, abdomen and / or back. The symptoms typically resolve within 14 – 28 days after illness onset (FDA 2012).

These serotypes are highly invasive, particularly in the ileum where they replicate, cause inflammation and penetrate the intestinal mucosa becoming blood borne – a condition called septicemia. In the blood stream, the microbes are phagocytized by components of the cellular immune system and replicate to high numbers inside the phagocytes. The phagocytes carry the microorganisms to a wide range of tissue types throughout the human body where the microbes erupt from the phagocytes and cause secondary infection such as localized tissue abscess, reactive arthritis, appendicitis, endocarditis, meningitis, or cholecystitis (inflammation of the gall bladder). The gall bladder is frequently the site of long-term, chronic infection that leads to the carrier state than can persist for years (ICMSF, 1996). Current estimates indicate a burden in the U.S. of approximately 2,300 culture-confirmed cases of typhoid fever per year with most or all caused by chronic carriers. The last confirmed foodborne outbreak in the U.S. was due to contaminated tropical fruits in 1999 (FDA 2012).

Non-typhoidal salmonellosis is an illness referred to as gastroenteritis and is caused by any of the serotypes of *Salmonella* other than *S*. Typhi or *S*. Paratyphi. It is estimated the illness leads to death in less than 1% of cases except for when particularly virulent strains (e.g. *S*. Enteritidis) infect highly at-risk populations (FDA 2012). Nonetheless, even at only 0.08 deaths per 100,000 people in the U.S. population per year, it is estimated that non-typhoidal salmonellosis is responsible for 25 to 50 percent of all foodborne illness related deaths. The number of deaths is highly skewed toward “at risk” individuals such as the elderly in a nursing home or hospital environment where long-term antimicrobial therapy may have reduced the competitive gut microflora or selected for particularly antimicrobial-resistant strains of *Salmonella* (Kennedy *et al*. 2004).

Onset of symptoms typically occurs within 12 – 36 hours of ingestion of contaminated food but may range from 5 hours to as many as 5 days and includes diarrhea, vomiting, abdominal pain and cramps, low-grade fever, and chills. Symptoms are generally self-limiting lasting 2 – 7 days after onset but may lead to more severe illnesses such as dehydration and electrolyte imbalance requiring I.V. fluid-replacement therapy, reactive arthritis, and on rare occasions, septicemia and secondary tissue infection of organs or joints (ICMSF 1996, FDA 2012). Depending upon the virulence of the infecting strain, the specific characteristics of the food carrier and the host immune capacity, the infectious dose may be as low as one viable cell or as many as 10^7 per gram of food (Kothary and Babu 2001).

In contrast to typhoid, the number of cases of non-typhoidal salmonellosis has increased steadily since the mid-1900s. In 1960, there were approximately five confirmed cases per 100,000 in the U.S. population. By 1995, that number had increased to approximately 20 confirmed cases per 100,000 (Tauxe 1997) and, as previously mentioned, the case rate has remained relatively unchanged since that time.

For both typhoidal and non-typhoidal salmonellosis, the route of infection is fecal-oral due to ingestion of contaminated food, water or person-to-person contact. The controls to prevent infection include proper food handling including cooking, prevention of cross-contamination between raw and ready to eat foods, proper hand-hygiene -- particularly by care-givers in clinical, nursing or child-care care environments, an adequate sanitary sewer system to prevent waste from contaminating produce, and an adequate supply of potable water (FDA 2012).

Serotypes of *Salmonella* are not particularly virulent when compared to some of the more notorious enteric bacteria such as *Listeria monocytogenes* (L.m.) or the strains of Shiga-Toxin producing *Escherichia coli* (STECs). However, they are widely dispersed across a broad range of animal reservoirs and are considered to be ubiquitous in the raw material supply for meat and poultry processors. A recent review demonstrated that of the top ten human isolates associated with illness, six were represented by serotypes that were most frequently isolated from commercial swine and poultry in the U.S. (Kaspar *et al*. 2009). It is widely accepted that most human illnesses derive from consumption of foods of animal origin, produce contaminated by the fecal material of animals, or from cross contamination during the preparation of foods of animal origin (Voetsch *et al*. 2004). As significant human pathogens are present

in the raw materials commonly used to make fermented sausages, careful consideration must be made by the manufacturer when evaluating the production process to ensure the process is sufficiently robust to inactivate and control these enteric pathogens.

Unlike several of the other pathogens reviewed in this paper such as L.m. and the clostridia, serotypes of *Salmonella* do not exhibit any unusual growth or survival characteristics that are significant from the perspective of food safety. Refrigeration temperatures significantly slow replication of the salmonellae with most strains completely inhibited below 7^{o}C (ICMSF 1996) and significant, but highly variable levels of inactivation occur during freezing and frozen storage of meat and poultry products. The reductions range from total survival to almost total inactivation depending upon the type of meat and storage time and conditions. In those studies that assess cellular and / or metabolic damage, all frozen cells showed signs of structural damages with membrane permeability and porosity affected most substantially (Dominguez and Schaffner 2009). For the modern sausage manufacturer, these are important considerations as refrigerated and frozen storage helps ensure a high quality raw material supply and may result in injury to vegetative pathogens fostering their inactivation during fermented sausage processing.

With limited exceptions, the salmonellae are not particularly tolerant to elevated temperatures, pH or reduced water activity. The maximum temperature for replication is 49.5^{o}C under ideal conditions and inactivation will occur above that temperature with the rate of inactivation increasing as the temperature elevates. Depending upon the acidulant, the minimum pH for growth is 3.8 and the minimum growth limit for a_w is 0.94 (ICMSF 1996). However, strain characteristics, the composition of the food matrix, combinations of these growth limiting factors and the presence of competing microflora can significantly alter the growth-limiting temperature as well as the thermal resistance of the salmonellae (Doyle and Mazotta 2000). While reducing the pH of the food matrix will reduce the thermal resistance of the salmonellae, reducing a_w will significantly increase the thermal resistance (ICMSF 1996). These phenomena are important considerations when evaluating a sausage manufacturing process for finished product safety.

E. FERMENTED SAUSAGE RECENTLY IMPLICATED IN OUTBREAKS OF ILLNESS

Botulism: As previously discussed in the review of the clostridia, the name given to the organism that causes the foodborne illness botulism, *Clostridium botulinum* (*C. botulinum*), derives from the Latin word for sausage, "*botulus*." An illness called "Sausage Poisoning" was common in Europe during the eighteenth and nineteenth centuries and was suspected to have been caused by this group of microorganisms (Rhodehamel *et al*. 1992). In 1897, the microbe was first isolated from a source other than a food or wound and, based upon the common association with sausages, Kempner and Pollack concluded that the microbe must be an inhabitant or transient of the swine gut (Hall and Peterson 1923). This association with sausage products remains much stronger in Europe where traditionally fermented meat (and fish) products have a long history of causing illness (Meyer 1956); however, domestically the association to fermented sausage and meat largely came to an end during the early part of the twentieth century.

A report by Meyer in 1936 summarizes the North American situation. Between the years 1899 to 1930, 625 individuals contracted botulism and 411 succumbed to the intoxication and died. Meyer estimated that more than 75% of these cases were caused by plant-based foods and under-sterilization during home canning – principally string beans and corn, both low acid foods -- was the primary cause followed by improper non-commercial meat curing. Meyer noted that, "the commercial packing industry has done its share to remove the botulinus menace" (Meyer 1936). In a 1956 update, Meyer reports a renewed interest in botulism which had waned following the publicity that home-canning received during the depression era. This interest was attributed to a desire by public health authorities to understand the role of food sanitation. Largely as a result of the commercialization of the meat curing and fermentation industry in the period since the Meyer reports, the threat of foodborne botulism has waned significantly with an average of fewer than 25 domestic cases per year (CDC 2012c) and practically all of those cases were caused by non-commercially prepared food products (Rhodehamel *et al.* 1992).

Control of *C. botulinum* in sausages has generally been achieved through the use of curing agents. Curing salts reduce the water activity of meat systems in which anaerobic conditions may be present and nitrite prevents the spores from germinating and growing. Since the use of modern meat cures has become wide-spread and reliance on microbial reduction of nitrates is less frequent, residual levels of

nitrite in sausages prevent *C. botulinum* germination and provide a residual nitrite store in the event that products are temperature abused (Tompkin *et al.* 1978a). Fermentation of cured sausages adds additional margins of product safety as *C. botulinum* will not grow and produce toxins below pH 4.6 (ICMSF 1996).

Staphylococcal Food Poisoning: During the middle of the last century in the U.S., foodborne illnesses caused by the staphylococcal enterotoxins drew a great deal of attention. A review by Gilbert in 1974 reports that in the U.S.A. and Hungary, far more outbreaks of foodborne illness were caused by *Staphylococcus aureus* (*Staph. aureus*) than by either *Salmonella* spp. or *Clostridium welchii.* In England and Wales, he reported that the vast majority of these illnesses were caused by cold meats, including cured products. However, he states that the primary route of food contamination was post thermal-treatment contamination by poor food handling practices followed by a lack of temperature (or other) control that allowed the microbes to proliferate and produce toxins in the ready to eat food (Gilbert 1974).

The most common form of the illness is called staphylococcal food poisoning (SFP) which is thought to be one of the most common foodborne illnesses of the modern era (Dinges *et al.* 2000). SFP is estimated to affect approximately 240,000 people in the U.S. each year; though the actual number of culture-confirmed cases averages slightly more than 300 per year. It is important to note that the domestic number of confirmed cases has dropped substantially since the early 1970s during which period approximately 7,000 confirmed cases per year were reported (Genigeorgis 1989; Mead *et al.* 1999).

The curing and fermentation of meat are processes particularly prone to the hazard of toxin formation. The toxins form in foods that have been held under conditions that allow the pathogen to proliferate to extremely high numbers. During logarithmic growth and transition to the stationary growth phase, the microbes excrete proteins (exotoxins) that are enterotoxins to man (Batia and Zahoor 2007). The conditions commonly utilized for meat curing and fermentation such as elevated but not lethal temperature and elevated salt levels are particularly favorable to *Staph. aureus* growth and toxin formation.

A wide range of meat products such as salt-cured hams are processed with no terminal heat treatment sufficient to inactivate these naturally present microbes. A complete understanding of the illness etiology did not start to emerge until 1930 when Dack and his colleagues demonstrated that a

sterile filtrate from a crème-filled cake that had been associated with illness was capable of causing illness (ICMSF 1996). In the mid-1980s as many as 33% of all domestic foodborne illnesses were attributed to *Staph. aureus* with 25% of those illnesses caused by salt cured ham. Four outbreaks of staphylococcal food poisoning were document in the 1970s and 1980 associated with fermented sausages (dry salami or Genoa salami). This led to a reexamination of the sausage manufacturing process and improvements in fermentation controls (Metaxopoulos *et al.* 1981). Since that time, a wide range of control measures have been developed and validated and commercial food products have generally been eliminated as a source of illness with the burden now shifted to restaurant and home prepared foods (FDA 2012).

Numerous researchers have demonstrated that salt levels in most sausage formulations are insufficient to prevent proliferation of *Staph. aureus* and toxin formation (Marcy *et. al* 1985, Riemann *et al.* 1972); concluding that the use of a fast-fermenting starter culture with a formulation including added sources of carbohydrates to stimulate rapid lactic acid bacteria growth, acid production and concomitant drop in pH is necessary to ensure toxins are not formed during fermentation (Genigeorgis 1972). As a result of these findings, a committee formed by the American Meat Institute (AMI) published a concept for control that was called “Degree-Hours”. This protocol establishes combinations of maximum time and temperature at which the fermenting meat products should be held before the pH has been reduced to below 5.3. It is a very conservative approach to the control of staphylococcal enterotoxins in fermented meat products and has been validated effective by a number of researchers. This work has illustrated the critical role that starter cultures now play in assuring the safety of fermented meat products (Metaxopoulos *et al*. 1981).

Enterohemorrhagic *E. coli*: Pathogenic *Escherichia coli* (*E.coli*) emerged as a foodborne pathogen in the latter part of the twentieth century. The most notorious of these pathogens is *E. coli* O157:H7 described in detail in an earlier section. This human pathogen is capable of causing life-threatening illness and has been associated with fermented sausages through a number of widely publicized outbreaks. In 1994, an outbreak of 20 cases was linked to the consumption of fermented beef salami. In 1995 in Australia, a different but closely related pathotype (*E. coli* O111:NM) in mettwurst caused 150 cases which progressed in 23 ill people to hemolytic uremic syndrome (HUS) and ultimately led to one

death. In 1998, in Ontario Canada an outbreak of O157:H7 was associated with consumption of Genoa salami causing 40 illnesses and two cases of HUS (Moore 2004).

The 1994 outbreak in Oregon and Washington State led to substantial changes in the domestic regulatory requirements for production of fermented meat products (Tilden *et al*. 1996). The United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) staff worked in collaboration with industry representatives from the Blue Ribbon Task Force to develop five options designed to ensure the safety of fermented dry and semi-dry meat products. The most rigorous of the options required the processor to demonstrate a 5.0 $\log_{10}$ reduction of *E. coli* O157:H7 through the process (Nickelson *et al*. 1996, Naim *et al*. 2003). Each of these options will be described in detail in the next section of this review.

Since implementation of those changes, staff from FSIS have collected and analyzed more than 10,000 finished samples of ready to eat meat products (including cooked beef patties) for the presence of *E. coli* O157:H7 and have not reported a positive sample. Given these results, in May 2011, FSIS staff announced that they were suspending the testing of this class of products for the presence of this pathogen (FSIS 2011). Interestingly, the outbreak in Toronto occurred after the changes recommended by the Blue Ribbon Task Force were implemented in the U.S. Similar changes had not yet been required by representatives of the Canadian Food Regulatory Agency (the Canadian Food Inspection Association) (Williams *et al*. 2000).

Acid tolerance emerged as an important virulence factor for the pathogenic *E. coli* (Gyles 2007, Mathusa *et al.* 2010) and survival for two months in fermented sausages at pH 4.2 has been documented with only a two $\log_{10}$ reduction in viable cell count (Lake *et al*. 2007). Thus, these microorganisms represent a particular concern for manufacturers of fermented meat products and represent a significant element in hazard identification and control during processing.

Evidence of the need for careful validation came in early 2011 as a five-state outbreak of O157:H7 was attributed to Lebanon bologna (CDC 2011c). Investigation of the cause for pathogen-positive finished products revealed that the manufacturer used as validation a scientific publication based upon processing conditions that were substantially different than those used in manufacturing the suspect products. Particular differences included: the diameter of the sausages manufactured ranged from 52 to 119 mm

while the scientific paper was based upon a sausage of 27 mm diameter and officials postulated that the difference in heat penetration would result in significantly reduced pathogen inactivation; further, the scientific study was conducted in sealed glass tubes while the production process employed permeable casings to facilitate smoking the products; lastly the manufacturer employed a large smokehouse that did not have sophisticated controls for heat and humidity while the literature source research was conducted in a very well controlled hot water bath. For these reasons, FSIS staff published a compliance guideline for manufacture of Lebanon bologna and concluded that, "it is important when an establishment designs its HACCP system during the initial validation period that it identify supporting documentation that is representative of the actual process so that results can be repeatable" (FSIS 2013a).

Salmonellosis: A variety of serotypes of *Salmonella* have been implicated, both domestically and internationally, in illnesses and outbreaks associated with consumption of RTE fermented dry and semi-dry sausages. In 1995, an outbreak of *Salmonella typhimurium* in South Central Pennsylvania was associated with Lebanon bologna and the pathogen was isolated from opened packages of the product taken from the refrigerators of several ill patients. This outbreak and the associated product illustrate a number of critical process controls that have demonstrated to be necessary to ensure an adequate level of product safety. The manufacturer utilized a traditional, natural fermentation based upon the existing microflora naturally present on the raw materials. The salted meat was ground and pan-aged at a temperature of less than 45^{o}F (7^{o}C) for 10 to 14 days during which a natural fermentation was expected to lower the pH to below 6.0. The aged meat was further ground and stuffed into casings before loading into wooden smokehouses fueled by smoldering hardwood and smoked for 52 to 72 hours to a final product temperature of 110^{o}F (43^{o}C). The manufacturer failed to verify raw meat pH and did not monitor the pH during aging. There were no temperature checks of the smoked products and finished product status was verified by visual and tactile characteristics and by taste. Lastly, the process did not employ a thermal process for lethality (cook step). Once the implicated products were recalled, the outbreak came to an end (Sauer *et al.* 1997).

The previously described outbreak of illnesses clearly illustrates the need for robust process controls designed into the manufacturing process and that is the intent of the HACCP-based approach to food safety. In all instances since the implementation of *E. coli* O157:H7 controls following the 1994 outbreak,

it was apparent that the manufacturing process was not sufficiently robust to inactivate the vegetative pathogen load presented on the raw materials (>10^4 CFU/g) (Sauer *et al.* 1997) or resulted in finished product characteristics – pH, water activity or lack of maturation time -- that allowed *Salmonella* spp. to survive or proliferate to numbers sufficient to cause illness upon consumption (Bremer *et al*. 2004, Emberland *et al*. 2006, Luzzi *et al*. 2007, and Pontello *et al*. 1998). Traditional processes with no thermal-lethality step are more prone to result in pathogen positive finished products and a number of authors have concluded that a thermal-lethality (cook) step is necessary for adequate inactivation of vegetative pathogens in these products (Hinkens *et al*. 1996, Nightingale *et al*. 2006, Smith *et al*. 1975).

F. SPECIFIC FOOD SAFETY CONTROLS FOR MAJOR HAZARDS IN PEPPERONI

Sodium Nitrite: Foodborne botulism is an illness generally caused by the ingestion of preformed neurotoxins which are simple proteins composed only of amino acids (Lamanna 1959) produced during the sporulation of the bacterium *Clostridium botulinum* (*C. botulinum*). Under growth-adverse conditions, *C. botulinum* forms endospores that are highly resistant to inactivation. Control of *C. botulinum* in fermented sausages has generally been achieved through the use of curing agents to prevent the germination and thus, the proliferation of the pathogen.

As defined by Toldrà, the term curing typically means “the use of a curing salt (sodium chloride and nitrate/nitrite) which generates or produces characteristic color or flavor in the product” (Toldrà 2002). However, curing imparts more than color or organoleptic effects as the residual nitrite is a strong inhibitor to the growth of *C. botulinum*. Nitrite may be generated in the meat product through the microbial reduction of nitrates or it may be directly added to the meat. However, as recently as 1979 the precise mechanism or mode of action to explain how nitrite prevents toxin formation was unknown (Sofos *et al*. 1979). Tompkin postulated that nitrite in meat systems serves as a reservoir for nitric oxide which is able to react with iron and the inhibitory effect on *C. botulinum* may be associated with reaction of nitric oxide with ferredoxin within the germinated cell. This reaction could interfere with cellular metabolism and energy production and prevent cell proliferation. Tompkin concluded that this general mechanism agreed with previous research that demonstrated that in the presence of nitrite, spores of *C. botulinum* germinated but failed to elongate and ultimately lysed (Tompkin 1978d).

The specific effects exerted by nitrite to prevent cell growth and toxin formation are to this day not well understood. The effectiveness of nitrite as a bacteriostatic and bacteriocidal agent is dependent on several interrelated factors including; the residual nitrite level; the number of spores present; the pH and specific acids responsible for lowering the pH; the iron content of the meat matrix; and the salt concentration. As has been repeatedly demonstrated, the inhibitory effects of nitrite are more effective at lower pH levels (Allaker *et al.* 2001, Roberts 1975, Roberts and Gibson 1986). It has become apparent that nitrite acts in two phases. The first of which is to inhibit endospores from germinating into vegetative cells; while the second of which is to prevent cell division of vegetative cells that may arise from spores, thus preventing proliferation (Duncan and Foster 1968, Pierson and Smoot 1982).

Fermentation of cured sausages adds additional margins of product safety as *C. botulinum* will not grow and produce toxins below pH 4.6 (ICMSF 1996). However, as the pH of fermented sausages may be well above 4.6, it is necessary to consider the combined effects of salt, nitrite and pH. Christiansen *et al.* demonstrated in summer sausages that commercial levels of nitrite in combination with a starter culture and 2% dextrose prevented toxin formation (Christiansen *et al*. 1975) supporting the conclusion of Drs. Hall and Peterson in 1923, who stated that control of *C. botulinum* toxin formation is "something other than acid alone" (Hall and Peterson 1923). Commercially produced cured meat products have "shown an exemplary botulism safety record" (Sofos 1981).

Lactic Acid Producing Starter Cultures: As previously mentioned, it has been repeatedly demonstrated that nitrite is more effective at lower pH levels and *Clostridium botulinum* will not grow and produce toxins at pH lower than 4.6. The pH of fermented sausages plays other critical roles in the assurance of finished product microbiological safety and product quality. It's beyond the scope of this paper to delve into the biochemistry of protein gelation; or the isoelectric point of proteins and the impact on water holding capacity in a fermented sausage meat matrix that is intended to be dried. However, all of these characteristics imparted by the drop in pH that results from fermentation have substantial impact beyond the organoleptic or sensory characteristics that result. These processes that occur during fermentation, thermal processing and subsequent drying of sausages such as pepperoni play key roles in the multi-hurdle approach to finished product safety (Leistner 1992).

In the first patent application for the “Art of Curing Meat”, Frederick Kurk stated that the results of curing meat have been, “variable, irregular and uncontrollable” and he had developed a means of making the cure of meat “standard or substantially uniform” by use of a “virile bacterium” not previously described or isolated but identifiable by the following characteristics: it is a non-putrefactive; non-pathogenic; nitrate-reducing; micrococcus. He had found that consistent cure could be achieved by inoculating each fresh pickle or cure with the selected bacteria producing a predominant growth of the inoculated culture to prevent excessive proliferation of undesirable bacteria normally present in the raw materials. This approach resulted in improvements in color, flavor and uniformity of cured products (Kurk 1921).

From this first patented application of a undefined microbial culture to improve the traditional uncontrolled fermentations that often resulted from back-slopping or pan-curing arose a great deal of research and applied science in a drive to understand the role played by various microorganisms. The first documented discussion of the use of specific lactic acid producing starter cultures was a patent application submitted by Lloyd Jensen and Levi Paddock in April, 1939. Employing mono- or mixed-cultures of lactobacilli, the two claimed that they could reproduce sausages of a type previously thought to be geographically dependent upon some unknown characteristic of weather, geography or other “trade secret” such as Lebanon bologna which was considered unique to Pennsylvania. The patent application suggested that rather than some trade secret or happenchance of geography, the likely reason certain regional sausage types had arisen was due to the “dumb luck” of chance inoculation with microorganisms that imparted unique finished product characteristics. They further explained the benefits of using a known culture to outcompete some of the heterofermentative contaminating microflora responsible for gas production and casing ruptures (Jensen and Paddock 1939).

Since those early days of isolating and understanding the role of specific microorganisms, the art and science of microbiology and meat fermentation have become intertwined. The search for desirable starter cultures began with the isolation of microorganisms present in high quality, naturally fermented sausages. For example Laban *et al*. studied a large number of naturally occurring *Lactobacillus* strains isolated from French sausages. They concluded that use of such strains as the basis for commercial starter cultures would be more efficient and provide for higher quality finished products than if isolates from dairy products were used (Nordal and Slinde 1980). The first commercial starter culture introduced

to the market was a lyophilized culture of *Pediococcus cerevisiae* in 1957. However, by the late 1960s, concentrated, frozen cultures of *Lactobacillus plantarum* and *Pediococcus cerevisiae* were available and delivering significant improvements in finished product consistency and reduced fermentation times (Wardlaw *et al*. 1973) which is a critical consideration when we consider the concept of degree hours and control of the formation of staphylococcal enterotoxins.

The direct introduction of microbial starter cultures has a number of different purposes. In the context of this review, we will focus on the aspects of improved food safety, but there are at least three other benefits that derive from the use of starter cultures:

1) To reduce the impact by out-competing and overgrowing undesirable microflora that may be present on the raw materials or processing equipment which, if allowed to proliferate, might result in unacceptable product quality or, as previously mentioned, gas production and casing rupture – improvements of sensory attributes or economics;
2) To provide for additional varieties of sausage products or ensure availability of formerly regional products; and
3) Potentially improved consumer health through the effects of probiotics or protective cultures on the intestinal microflora (Lucke 2000).

In 1977, Terrell *et al.* defined eight requirements for necessary characteristics of a commercial starter culture:

1) “Salt tolerant and rapid growth in a 6% brine;
2) Ability to grow well in the presence of 80 – 100 ppm sodium nitrite;
3) Optimum growth at 90^{o}F; range 80 to 110^{o}F;
4) Homofermentative (produce only lactic acid);
5) Must not be proteolytic or lipolytic;
6) Must not produce off-flavors as by-products of fermentation;
7) Must not be harmful to health and should be inactivated around 135 to 140^{o}F;
8) May or may not survive freeze drying and subsequent reconstitution” (Terrell *et al.* 1977).

These attributes remain important today with very slight modifications in the range of optimum growth as several fast fermenting strains have been identified that are used at fermentation temperatures as high

as 115^{o}F. The commercial starter culture SAGA 200 (*Pediococcus acidilactici*, Kerry Ingredients and Flavors, Beloit, WI) intended to be utilized in the conduct of the proposed research has a specified fermentation target temperature of 112^{o}F for optimum performance. The expected benefits of using a commercial starter culture were described as:

1) "Color formation was speeded up;
2) The pH of the system was lowered more rapidly;
3) The desired consistency was achieved more rapidly;
4) Total processing time could be shortened considerably, a great economic advantage; and
5) The process became fail-safe in view of the antagonistic nature of the starter culture which inhibited many spoilage or pathogenic organisms" (Niinivaara 1991).

While by one estimate, fewer than 50% of the commercial fermented meat product manufacturers were utilizing a commercial starter culture in 1983 (Smith and Palumbo 1983), by as early as 1975 research was demonstrating the benefits of use of a commercial starter culture for product safety. Smith *et al*. demonstrated that the pH drop achieved with the starter culture fermentation was much more rapid than in the naturally fermented pepperoni – dropping from pH 6.1 and 0.24% acid to pH 4.5 and 0.72% acid in 24 hours; whereas the naturally fermented pepperoni dropped from pH 6.2 and 0.23% acid to pH 5.1 and 0.47% acid in 48 hours. This rapid pH drop conferred an advantage to the inactivation of artificially inoculated strains of *Salmonella* with approximately 1.0 $\log_{10}$ greater inactivation demonstrated over the naturally fermented product after 42 days of drying. These researchers further demonstrated that the traditional pepperoni process of fermentation and drying was not reliably capable of inactivating the 10^{3} – 10^{4} CFU/gram of *Salmonella* spp. inoculated into the raw meat batter. However, they demonstrated that following fermentation a thermal processing (cook) step at a temperature considered to be sufficient as a control for *Trichinella spiralis* in pork products (60^{o}C) ensured the total inactivation of any viable *Salmonella* spp. that survived the fermentation process (Smith *et al*. 1975).

The 1975 work of Smith *et al*. was an important step in what subsequently became the commercial industry standard for control of vegetative pathogens in fermented meat products. Subsequent research supported the conclusion that fermentation and drying alone are insufficient to provide an adequate margin of finished product safety. Domestically, Levine *et al*. reported that between the years of 1990 to

1999 approximately 1.5% of fermented sausage products tested as part of the USDA verification program were positive for the presence of Salmonella spp. (Levine *et al*. 2001). Calicioglu *et al*. reported that fermentation and drying of soudjouk sausage only achieved about a 2.0 $\log_{10}$ inactivation of *E. coli* O157:H7 (Calicioglu *et al*. 2001). In the research that forms the basis of HACCP plan validation for the commercial manufacture of most of the pepperoni in the U.S., Hinkens *et al*. reported that the traditional pepperoni process only delivered a 1.2 $\log_{10}$ inactivation of *E. coli* O157:H7 and concluded, similarly to the work done by Smith *et al.* in 1975, that a post-fermentation thermal process for control of *Trichinella spiralis* in pork (specifically, 128^{o}F (53.3^{o}C) for 60 minutes or 145^{o}F (62.8^{o}C) instantaneously) was sufficient to ensure a 5.0 $\log_{10}$ inactivation of *E. coli* O157:H7 in pepperoni (Hinkens *et al*. 1996). Recently, the impact of commercial starter culture was demonstrated by Porto-Fett *et al*. who concluded that "it is strongly suggested that manufacturers use a starter culture to improve the quality, consistency, and safety of their products" (Porto-Fett *et al*. 2008).

The effect of pH on pathogens has been researched extensively as has the role of the different organic acids. It is beyond the scope of this paper to exhaustively review the mechanisms involved in cellular injury and energy depletion, but the general consensus indicates that at a minimum, ion regulation to maintain homeostasis places a large energy drain on the pathogens and sensitizes them to other stresses such as reduced water activity and the elevated temperature of thermal processing (Ross *et al.* 2008, Shadbolt *et al*. 2001).

Before moving on to the concept of degree hours and control of the staphylococcal toxins, it is important to note a final key attribute calling for the use of commercial starter cultures for fermentation of cured meats. As previously discussed in the section on sodium nitrite, concerns exist for the potential formation of biogenic amines in nitrite-cured meat systems. A number of researchers have documented that undesirable contaminating microflora possessing the genes expressing for production of amino acid decarboxylases may lead to elevated levels of the biogenic amines in finished products. Eitenmiller *et al*. demonstrated that use of a commercial starter culture consisting of a *Pediococcus cerevisiae* that does not produce amino acid decarboxylase yields finished products with lower levels of biogenic amines (Eitenmiller *et al*. 1978). Utilization of a defined starter culture prevents contaminating microflora from

proliferating during fermentation and producing undesirable and potentially carcinogenic amines (Ordonez *et al*. 1999, Smith and Palumbo 1983).

Degree-Hours and Control of Staphylococcal Enterotoxins: As previously described, due to their inherent growth characteristics, production of enterotoxins by the species of *Staphylococcus* – particularly *Staphylococcus aureus* (*Staph. aureus*) – represents a very real risk during manufacture of cured and fermented meat products. Also as previously discussed, up to the end of the last century, this risk often manifested itself in cured products as staphylococcal food poisoning was considered one of the most frequent forms of foodborne illness. Very stringent controls are necessary to prevent proliferation of *Staph. aureus* in foods in which competitive microflora have been suppressed by elevated salt content or reduced water activity as *Staph. aureus* is considered one of the most osmotolerant microorganisms known. These conditions are present in cured and fermented meats and necessitated the identification of a means of controlling the proliferation of the enterotoxin producing species of *Staphylococcus*.

As the HACCP regulations were being finalized in the late 1990s, an industry trade association convened a group of manufacturers and academics to identify controls that could be included in a HACCP system to prevent microbiological hazards associated with the production of fermented dry and semi-dry sausage products. Those controls were published in October 1997 under the title "Good Manufacturing Practices for Fermented Dry and Semi-Dry Sausage Products." This document contained the time-temperature control for fermentation that was captured in the concept of degree-hours. The "degree-hours" is defined as the cumulative number of hours the product is maintained in the range between the minimum and maximum growth temperature for *Staph. aureus* multiplied by the number of degrees (in Fahrenheit) above the minimum (AMI 1997). This is most frequently described as the time above the minimum -- 60^{o}F or 15.6^{o}C -- as until very recently, fermentations were not routinely conducted at a temperature above the maximum growth temperature for *Staph. aureus* (~115^{o}F or 46.1^{o}C). A fermentation process is considered acceptable if:

1) The pH is reduced to below 5.3 within fewer than 1,200 degree-hours when the fermentation temperature is less than 90^{o}F (32.2^{o}C);
2) The pH is reduced to below 5.3 within fewer than 1,000 degree-hours if the fermentation temperature is between 90 and 100^{o}F (32.2 – 37.8^{o}C); or

3) The pH is reduced to below 5.3 within fewer than 900 degree-hours if the fermentation temperature is above 100^{o}F (37.8^{o}C) (FSIS 2011).

Failure of the process to achieve these performance standards raises concerns about the possible presence of staphylococcal enterotoxins and extensive testing of the surface of the fermented products is recommended to assess the number of coagulase-positive staphylococci present. If the numbers are elevated, it is recommended that extensive product testing be conducted for the presence of the thermonuclease proteins. If the presence of thermonuclease is detected, it is recommended that the products be destroyed (AMI 1997). Fortunately, modern commercial starter cultures that are supplied a sufficient source of carbohydrates in the product formulation ferment rapidly and seldom fail to rapidly reduce the pH to below 5.3. Most commercial HACCP plans require the reduction to proceed to below pH 5.0 to prevent the products' inherent buffering capacity to allow the pH to rise above 5.3 during maturation and drying.

Extensive work has demonstrated the efficacy of the rapid pH drop in controlling proliferation of *Staph. aureus* and production of thermonucleases during fermentation. In the late 1960s and early 1970s, inhibition of toxin production was postulated to be due to either the production of some type of inhibitory compound by the lactic acid-producing starter culture or competition for essential nutrients such as biotin and niacin (Smith and Palumbo 1976). Niskanen and Nurmi demonstrated that the initial level of contamination of the sausage with *Staph. aureus* had to be at levels greater than the starter culture inoculation level in order for the starter culture to fail to prevent proliferation. Further, they documented that contamination with more than 10^5 CFU/gram was necessary during the production stage and more than 10^6 CFU/gram in the final product before detectable thermonuclease was present in a 200 gram sample (Niskanen and Nurmi 1976).

The effect of the acid produced during fermentation was documented by Smith and Palumbo in 1978 when they were able to restore acid-injured cells to a viable status and concluded that both starter culture and glucose were necessary to cause injury and prevent proliferation of *Staph. aureus*. They concluded that the sublethal acid-injury would prevent the growth of the *Staph. aureus* even if the sausage product were placed for an extended period at a temperature conducive to their growth; demonstrating that the injured cells eventually were inactivated (Smith and Palumbo 1976).

Thus, the focus of fermented sausage manufacturers for prevention of staphylococcal enterotoxins in finished products has been two-fold. The first is to ensure a source of high quality raw materials and maintenance of a sanitary processing environment to ensure the pre-fermentation load of *Staph. aureus* is kept as low as possible. The second is to ensure a rapid pH drop through the application of a commercial starter culture and addition of a simple carbohydrate source, while ensuring the total degree-hours limits are met.

Five Options of the Blue Ribbon Task Force for Control of *E. coli* O157:H7 in Fermented Sausages: Following the 1994 outbreak of illnesses caused by *E. coli* O157:H7 in beef salami, the industry convened a group of experts that collaborated with representatives from the Department of Agriculture's Food Safety and Inspection Service to develop options for control of the pathogen during the production of fermented meat products. While most domestic manufacturers eventually elected to implement a thermal process step to achieve a 5.0 $\log_{10}$ inactivation of *E. coli* O157:H7 (or equivalent probability of it being present in the finished product), it is important to note that the other four options remain available to the manufacturer and are likely being used by smaller processors or "artisanal" sausage makers that are concerned with the development of specific flavor profiles that may be unacceptably impacted by the application of a thermal process.

The five options developed by the task force are:

1) "Utilize a heat process as listed in 9 CFR 318.17 (145^{o}F for 4 minutes);
2) Include a validated 5D inactivation treatment;
3) Conduct a hold and test program for finished product;
4) Propose other approaches to achieve at least a 5D inactivation; or
5) Initiate a Hazard Analysis Critical Control Point (HACCP) system that includes raw batter testing and at least a 2D inactivation (Nickelson *et al*. 1996)."

Option 1 is probably the simplest to achieve as it merely refers to the existing regulatory requirements for production of certain meat products (cooked beef, roast beef or cooked corned beef) that are eligible to bear the label declaration "fully cooked" or "ready to eat" (or other indications that the product is safe to consume without any further pathogen lethality intervention). At the time these options were developed, the regulations for these products were highly prescriptive and the stated 145^{o}F for 4 minutes was

required as a minimum for these products. Effectively, this option treated the sausage product like other fully cooked meat products and did not take account of any additional inactivation of vegetative pathogens achieved by sausage fermentation, maturation or drying.

Option 2 has become a widely-adopted selection based upon the work of a number of researchers. As previously described, a number of authors have published work demonstrating processing conditions that are capable of achieving a cumulative 5.0 $\log_{10}$ inactivation of *E. coli* O157:H7 in a variety of fermented sausage products under a variety of conditions. The most widely adopted process is described by Hinkens *et al*. for the production of pepperoni (Hinkens *et al.* 1996). The Blue Ribbon Task Force arranged for funding to conduct research into a wide variety of process parameters to identify acceptable processing conditions to achieve a "5D reduction" and published those process parameters in their final report (Nickelson *et al.* 1996).

Option 3 has been widely discredited and was discounted in the Task Force's final report. It would be prohibitively expensive to implement as the sample size would necessarily be extremely large to provide sufficient confidence in the sample results – as testing to support "zero" presence of a pathogen is statistically meaningless in the context of a commercial production process. Further, the option is not consistent with the process control principles of the HACCP approach to ensuring food safety; with product testing for safety assurances already having been proven inadequate. The task force identified several research needs that might make such an approach feasible. It is not known whether researchers have attempted to fulfill those needs.

Option 4 has become the second most widely adopted approach to control of the pathogen as it affords the processor with flexibility to "mix and match" process elements in a manner that accumulates a 5.0 $\log_{10}$ inactivation. This task has become much more practicable as research and publications have proliferated in the intervening years between the Task Force report and the present creating more mix and match options.

Option 5 was recommended by the representatives from the task force to provide flexibility for the manufacture of certain products for which no combination of process controls had previously been identified as achieving a "5D reduction". It was the most complicated of the options as several statistical assumptions had to be made and supported. For example, for the testing of the raw meat batter, a

method had to be devised that was demonstrably capable of detecting contamination at a level sufficient to ensure the pathogen was present at less than 1.0 CFU/gram. Certainly, it was expected that the blending and grinding typical to production of sausages would ensure better homogeneity in the distribution of the pathogen in the meat batter than in the raw materials; but, it was unknown to what degree a lack of homogeneity needed to be incorporated into the sampling assumptions – particularly given that the final report stated that, "the definition of a "lot" for the purpose of sampling must be statistically sound". The report further states that a procedure must be developed for dealing with lots of raw meat batter that test positive; and that "at a minimum, all positive lots must be subjected to conditions that will provide a total 5D process" (Nickelson *et al.* 1996). Given the logistical problems associated with creating options for specific meat formulations that might potentially test positive, this option was not widely adopted.

Due to the determination made by representatives of the USDA that *E. coli* O157:H7 is an adulterant in raw beef products intended for grinding or other "non-intact" further processing, substantial investments in interventions have been made by the beef slaughter and processing industry over the last two decades. The incidence of *E. coli* O157:H7 contamination of beef raw materials has fallen significantly during that period and the quantitative level of contamination in positive lots of raw meat has dropped significantly as well. These upstream interventions have undoubtedly made the fermented sausage interventions substantially more robust, leading to reduced risk to the consuming public.

G. IMPACTS OF THIS RESEARCH AND STATEMENT OF PROBLEM

The HACCP concept was first applied to food production in the United States during the 1960s in a combined effort between the Pillsbury Corporation and the U.S. National Aeronautics and Space Administration (NASA). The HACCP concepts derived from an engineering approach called Failure Mode Effect Analysis (FMEA) that had previously been applied to quality assurance of war munitions. This engineering approach required the analysis of the reasons that war munitions were defective with feedback to the manufacturing process to drive quality improvements. It did not rely on the traditional quality control concept of lot acceptance based on finished product testing which had proven inadequate (Pearson and Dutson 1999).

Scientists at the Pillsbury Corporation were challenged by procurement officials at NASA to provide absolute assurances that the food products to be sent on manned space missions would prove to be safe as foodborne illness in a space module would likely have proven catastrophic. The Pillsbury scientists recognized, particularly for the assurance of microbial product safety, that the traditional lot quality acceptance approach based on Military Sampling Plans and finished product testing would prove insufficiently robust. In looking for an alternative to lot acceptance sampling plans, they turned to the concepts of FMEA; modified them to align more closely to food manufacturing and the associated hazards; and defined what has come to be known as the Hazards Analysis, Critical Control Points approach to the design of production processes to ensure safe finished food products (Pierson and Corlett 1992).

The approach proved to be sufficiently robust and, to varying and often limited extent, was subsequently adopted by forward-looking, progressive food manufacturing companies outside the Pillsbury Corporation and the space exploration program. In subsequent years, a large number of national and international organizations and regulatory agencies recommended that all food manufacturers apply the HACCP approach to food process design and manufacturing process control. As many manufacturers deemed the approach to be too complex or too costly, wide-spread voluntary adoption of the HACCP approach was never achieved (Mortimore and Wallace 1994).

On February 03, 1995, following a number of large and well publicized outbreaks of foodborne illnesses, the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) published a proposed rule that would require the implementation of HACCP programs in all meat and poultry processing facilities. FSIS staff partnered with representatives of international organizations and the food processing industry through a variety of trade associations to develop what eventually was finalized and published on July 25, 1996 as the "Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems; Final Rule" (FSIS 1996). This controversial rule required, among other things, that management at FSIS-inspected meat and poultry facilities develop and implement programs and procedures intended to ensure that the food products were safe and wholesome when released into interstate commerce; effectively placing total responsibility for the safety of products on the food manufacturer.

The HACCP rule was based on seven principles that, if properly administered during the food process design phase, would ensure product safety. In the simplest terms, those seven principles required that:

1) Management review the food manufacturing process and identify anything that could go wrong that might introduce a hazard and make the finished products unsafe for consumption (Hazard Analysis);
2) Identify steps or procedures in the process that could be controlled (Critical Control Points -- CCPs) to prevent the hazard;
3) Define the level of control at each CCP (e.g. a Critical Limit) necessary to prevent the hazard;
4) Establish the monitoring requirements at each CCP sufficient to ensure the process is adequately controlled to prevent the hazard;
5) Predefine corrective actions that are to be implemented in the event of a deviation from a critical limit at a CCP;
6) Establish record-keeping procedures sufficient to demonstrate that products were manufactured with a process that was “under control” whereby the critical control points were monitored and all critical limits were met; and
7) Define procedures to verify, including the subset of procedures to validate, that the HACCP program is properly implemented and working to ensure product safety (FSIS 1996, Scott 2005).

The HACCP final rule included performance standards for specie-specific pathogens of human concern; as well as for indicator organisms intended to demonstrate slaughter process control. These requirements were intended to drive systematic improvements in the microbial safety of raw meat and poultry products for two reasons:

1) To reduce the consumer exposure to these pathogens during preparation and handling of the raw agricultural commodities while cooking to reduce the potential for cross-contamination of other food products or food processing surfaces in the consumer kitchen; and
2) To reduce the pathogen load contained within raw meat and poultry products intended for further processing, including fully cooking, at FSIS-inspected food processing establishments

with the expectation that this would reduce the probability of processing deviations resulting in pathogen-positive finished ready to eat products (FSIS 1996).

Before the HACCP rule was implemented, FSIS staff had established a “zero tolerance” for human pathogens in meat and poultry products that were classified as ready to eat. This zero tolerance policy resulted in a series of regulatory requirements that dictated, for a limited number of product types, specific time at temperature requirements for the products to meet during processing to reduce pathogenic microorganisms and qualify the products to be labeled as fully cooked (FSIS 1999a, FSIS 2001). FSIS staff conducted limited microbiological monitoring for verification of compliance with the zero tolerance policy and, with some regularity, ready to eat finished products tested positive for the pathogen of concern. FSIS staff intended that the HACCP rule would reduce the frequency of pathogen positive finished ready to eat products and stated that they intended to revise the regulations as required to continuously drive improvements in food safety (USDA 1996).

For ready to eat meat and poultry products that were not covered by the prescriptive regulations, the food processing industry had used defined processes that had been proven adequate either due to historic lack of association with illnesses or by scientific validation studies, most often consisting of microbial challenge studies. For instance, the processes used for manufacturing dry fermented sausages had been considered for millennia to result in microbiologically safe products (Toldrà 2007). Unfortunately, in the early 1990s, several outbreaks of illness caused by the newly emerging pathogen *E. coli* O157:H7 associated with consumption of dry fermented sausage products (Tilden *et al*. 1996) led to the conclusion that the traditional processes were no longer adequate to ensure product safety from this particular hazard associated with the beef ingredients (Hinkens, *et al*. 1996, Naim *et al.* 2003).

As a result, in 1994 members of the processing industry and a beef trade association (The Blue Ribbon Task Force) collaborated with FSIS staff to establish five processing options that were intended to assure dry fermented sausage safety by achieving sufficient control during the manufacturing process to achieve a 5.0 $\log_{10}$ reduction in viable *E. coli* O157:H7 or an equivalent probability of pathogen positive finished product (Nickelson *et al*. 1996). Following the implementation of the HACCP rule beginning in 1998, these five processing options were considered to be validated to ensure dry fermented sausage safety and were included as part of the processor’s HACCP plan. Given that dry fermented sausages

were not covered by the pathogen reduction performance standards, the species of the salmonellae were considered to be adequately controlled by the *E. coli* O157:H7 controls (FSIS 2012).

On January 06, 1999, FSIS staff published rules that converted the prescriptive time at temperature regulations for certain fully cooked meat and poultry products into newly defined performance standards requiring specific levels of pathogen inactivation to be achieved during processing. These standards did not prescribe the means by which the microbial inactivation was to be achieved but did require that the process be defined in either a process schedule or within the processing facility's HACCP plan. The old regulatory requirements were converted into two compliance guidelines that could be considered as validated to achieve the required levels of microbial control during the manufacture of ready to eat meat and poultry products (FSIS 1999b).

Both the pathogen reduction performance standards and the newly established compliance guidelines applied to a limited number of well-defined ready to eat fully cooked products. Thus, for a manufacturer of cooked roast beef, the regulatory requirements were well defined. However, for products not included within the old prescriptive regulations, such as dry fermented sausages, the requirements were less well-defined (FSIS 1999b, FSIS 2012).

On February 27, 2001 FSIS staff published a proposed rule entitled "Performance Standards for the Production of Processed Meat and Poultry Products" that would have extended the 1999 performance standards associated with the limited number of well-defined fully cooked products to cover all products that are considered ready to eat including those that did not include a thermal-lethality (cook) step in the manufacturing process. This proposal would have established similar pathogen reduction requirements across all ready to eat products and specifically stated that the more rigorous reduction standards for poultry products would be applied to all products containing poultry meat, regardless of the level of inclusion in the product formulation (FSIS 2001).

If finalized, this proposed rule would result in significant challenges to the dry fermented sausage industry as numerous products – both traditional and recently formulated – contain poultry as an inexpensive component of the meat block. These products have been validated for control of *E. coli* O157:H7 with the conclusion that the salmonellae are adequately controlled as evidenced by the lack of outbreak association following full implementation of those control measures. However, there are very

limited scientific data that might be drawn upon as validation that the processes achieve the more rigorous *Salmonella* spp. reductions required for poultry products.

In recent years in the United States, the food safety regulations and control systems employed by industry have become more science-based and less prescribed by regulation. As a result, the need for validated methodologies for ensuring the safety of new product formulations or processing systems has become more pressing. The publication of the HACCP rule established the requirement that food processors conduct a thorough review of the product and processing methods to identify hazards that may be inherent to the product or arise during the manufacturing process, storage and distribution, or the preparation and consumption stage. All hazards that are identified as reasonably likely to occur must have defined process controls and food manufacturers have increasingly been held accountable for demonstrating that the control measures in place are sufficient to "prevent, eliminate or reduce to an acceptable level the hazards reasonably likely to occur" in the production of the particular food product (FSIS 1996, Scott 2005).

In the years following implementation of the HACCP rule, both industry and the federal food safety regulatory agencies – principally the United States Department of Agriculture's Food Safety and Inspection Service (FSIS) and the House and Human Service's Food and Drug Administration (FDA) – have identified numerous knowledge or data gaps to be addressed to ensure processing controls are adequate to assure product safety. As the science of food safety is dynamic, emergent food safety hazards -- primarily biological hazards in the U.S.A. although chemical hazards have recently been highlighted in certain products within, and exported from the People's Republic of China (Ingelfinger 2008) -- may arise resulting in the need for additional scientific validation of process controls.

Thus, there is a need for additional research to demonstrate that the traditional sausage processes achieve the proposed 6.5 $\log_{10}$ or 7.0 $\log_{10}$ reduction of the salmonellae under the specific conditions applied during manufacture. As there are a very wide variety of processes, equipment and ingredients utilized in this category of products, conducting microbial challenge studies under all possible combinations is simply not feasible. Therefore, a properly constructed and accepted modeling tool would prove to be very beneficial to the processing industry.

A variety of serotypes of *Salmonella* have been implicated, both domestically and internationally, in illnesses and outbreaks associated with consumption of "Ready-to-Eat" (RTE) fermented dry and semi-dry sausages. The most recent domestic outbreak caused by *Salmonella* Montevideo was apparently the result of a contaminated ingredient (black and red pepper) added to the fermented sausage as a coating after the lethality treatment. Interestingly, a second *Salmonella* serotype (Senftenberg, known for its heat tolerance) was recovered from a subset of ill patients and the salami; indicating there may have been pathogens that survived the lethality processes (CDC 2010). During the recent decades there have been a small number of documented domestic outbreaks of salmonellosis associated with fermented meat products; such as the 1995 outbreak of *Salmonella* Typhimurium attributed to Lebanon bologna that afflicted 26 people, in which it was apparent that the manufacturing process was not sufficiently robust to inactivate the vegetative pathogen load presented on the raw materials ($>10^4$ CFU/g) (Sauer *et al.* 1997).

On a more frequent basis, *Salmonella* spp. outbreaks associated with fermented sausage products have been reported from Europe. These outbreaks were also caused by inadequate manufacturing processes that resulted in finished product characteristics – pH, water activity or lack of maturation time -- that allowed *Salmonella spp.* to survive in adequate numbers to cause illness upon consumption (Bremer *et al*. 2004, Emberland *et al*. 2006, Luzzi *et al*. 2007, and Pontello *et al*. 1998). Many European manufacturers continue to use "old world" traditional manufacturing processes that don't employ a thermal treatment for pathogen inactivation; relying solely on reductions achieved during fermentation, drying and maturation. These processes are likely to be more prone to result in pathogen-positive finished products as a number of authors have concluded that a thermal processing step is necessary for adequate decimal reductions (Hinkens *et al*. 1996, Nightingale *et al*. 2006, Smith *et al*. 1975). Domestic testing of finished RTE fermented sausages by federal government authorities for the presence of *Salmonella* spp. during the period 1990 through 1999 indicated that approximately 1.43% were positive (Levine *et al.* 2001), but that number fell to 0.0% in the following year (FSIS 2000). While direct comparisons for fermented sausages are not possible from the FSIS data as the reporting system changed following the year 2000, the number of *Salmonella* spp. positive reports from testing of all RTE meat and poultry products has remained extremely low (FSIS 2013b).

As a result of these and other concerns, on March 02, 2005, FSIS staff issued Notice 16-05 indicating their intent to modify the existing processing regulations pertaining to ready to eat sausage products specific to the biological validation of the microbial inactivation of human pathogens present in the raw materials. The FSIS staff was made aware that many sausage manufacturers were adjusting formulations to incorporate lower priced poultry raw materials in attempts to either lower the finished product cost or other characteristics such as fat or sodium content. Therefore, FSIS announced its intention to modify current policy and include performance standards for the microbial inactivation of the species of the salmonellae in ready to eat fermented sausage products containing poultry ingredients (FSIS 2005).

The proposed changes have yet to be enacted. However, in January 2013, USDA staff published an announcement in the Semi-Annual Regulatory Agenda of their intent to move forward with an Interim Final Rule codifying these requirements and extending the Pathogen Reduction Performance Standards to all RTE meat and poultry products (FSIS 2013c). These proposed regulatory changes, if enacted, are expected to have significant cost impacts that will no doubt pass to the consuming public. The regulated industry will be required to revalidate HACCP plans for a wide variety of products that are currently validated to achieve a 5.0 $\log_{10}$ inactivation of *E. coli* O157:H7 to demonstrate an adequate reduction in the salmonellae.

This intended policy change revealed a deficiency in the existing scientific literature in that these traditional formulations and manufacturing methods have previously been thoroughly validated for adequate control of both O157:H7 and trichina; yet there is little to be found regarding the effects of the processing methods on survival of *Salmonella* spp. Therefore, the proposed rule could require that a processor revert to a safe harbor provision found in the 1999 document for control of the salmonellae in poultry products – namely, cooking the products to a minimum internal temperature of 160^{o}F as the Appendix-A time and temperature tables only cover products formulated with up to 12% fat and most fermented sausage products contain much greater proportions of fat in the formulation.

Given that traditional dry and fermented sausages contain considerable fat content, and the fat is often not emulsified and present in the finished products as discernible particulate pieces, cooking these type products to this temperature would result in an unacceptable quality for the finished products as the

fat would “grease out” – separating from the meat matrix; becoming trapped immediately between the casing and the defatted meat. As this would not result in an acceptable finished product and would substantially interfere with the product drying cycle given the fat would form a moisture barrier, a need has become apparent for validation studies to determine the microbial inactivation of the *Salmonella* spp. in traditional, fermented dry and semi-dry ready to eat sausage products manufactured with traditional methods.

The research projects proposed herein will primarily focus on pepperoni with the intent to:

1) Establish the microbial reduction of the salmonellae in a variety of product formulations under a variety of processing conditions; and
2) Develop a statistical modeling system based upon a non-pathogenic microbial surrogate for the salmonellae such that a processor may:
    A. Formulate to any specification;
    B. Include a sufficient number of the surrogate microorganisms; and
    C. Process the products under the individual conditions unique to the processing facility and company.

In this manner, other sausage manufacturers will be provided a means by which they may validate an adequate reduction of the salmonellae under the specific and unique conditions present in their processing facility while utilizing a non-pathogenic microorganism for culture and recovery analyses to demonstrate the required reduction as proposed by FSIS.

H. NONPATHOGENIC SURROGATE AND RATIONALE FOR SELECTION

In the context of understanding how processes affect the inactivation kinetics of pathogens in foods, the term surrogate has been previously defined as, “an organism, particle, or substance used to study the fate of a pathogen in a specific environment” (Sinclair *et al*. 2012). As other authors have previously stated, it is not appropriate to work with pathogen-inoculated products in a commercial processing facility and the identification of appropriate surrogates that might be used to model the behavior of pathogens in food processing systems would be beneficial for furthering the understanding of process controls and combinations of controls that effectively provide a desired level of pathogen reduction (Gurtler *et al*. 2010, Murphy *et al.* 2001). As stated by Cabrera-Diaz *et al*., “surrogates can allow the researcher to quantify

the effect of interventions on nonpathogenic organisms in commercial food processing environments where pathogens cannot be utilized because of safety concerns (Cabrera-Diaz *et al*. 2009).

The first conceptual use of a surrogate or indicator microorganism was described in 1885 when Theodor Escherich studied the spread of enteric pathogens in water by developing methods for the recovery and enumeration of the microorganism that would subsequently bear his name – *Escherichia coli*. It was suggested that these microbes could be used to determine the efficacy of water treatment systems for removing enteric pathogens from drinking water. In 1892, Franz Schardinger described how the motile, rod-shaped bacteria isolated from the feces of babies could be used as an indication of fecal contamination of water with fecal coliforms being used as an indicator of the presence of vegetative enteric pathogens (Sinclair *et al*. 2012). The U.S. Food and Drug Administration states in the Glossary of Kinetics of Microbial Inactivation for Alternative Food Processing Technologies that, "surrogates enable biological verification of a given antimicrobial process or treatment without introducing pathogens into a food processing area (FDA 2013b).

The staff at FSIS has previously indicated that non-pathogenic strains of microorganisms may be used in product formulations as surrogates for pathogens under conditions that do not result in the creation of a food safety hazard, insanitary condition or otherwise cause the food to become adulterated (FSIS 2010). As defined by previous authors, an appropriate surrogate microorganism should be non-pathogenic; genetically stable; easily grown to high-density and easily enumerated in the lab; easily distinguishable from the expected background microflora; and most importantly, it should possess similar inactivation kinetics in the model system as the pathogenic microorganism it is intended to model (Kim and Linton 2008). There is a long history of the successful use of nonpathogenic surrogate microorganisms in the food processing industry with perhaps the best known and most widely used example being the use of *Clostridium sporogenes* endospores as a thermal processing surrogate for the less thermotolerant *Clostridium botulinum* in the canning of low-acid foods (Borowski *et al*. 2009).

Researchers have used a wide variety of non-pathogenic microorganisms as surrogates in foods for model development including a commercial lactic acid bacteria (LAB) starter culture which was shown to be effective. In one study, the authors demonstrated a $\geq$ 5.0 $\log_{10}$ inactivation of a cocktail of serotypes of *Salmonella* during fermentation of summer sausage (Borowski *et al*. 2009). Others have used generic

Bio-type I *E. coli* in a fermented summer sausage where a 5.0 $\log_{10}$ inactivation of *Salmonella* spp. was achieved with lesser reductions in the generic *E. coli* indicating suitability for use as a surrogate (Niebuhr *et al*. 2008); and in ground beef where cooking was demonstrated to achieve inactivation levels not statistically different between *E. coli* O157:H7 and five strains of generic Biotype I *E. coli,* while the inactivation achieved during fermentation was lesser for the potential surrogates indicating suitability for use (Keeling *et al.* 2009). Lastly, *Enterococcus faecium* was used in moist air heating of almonds where it was demonstrated to be an acceptable surrogate (Jeong *et al*. 2011).

Of the non-spore forming bacteria, species of *Enterococcus* are among the most thermotolerant and we would expect inactivation levels to be substantially less than the inactivation achieved for the vegetative pathogens (Mangus *et al*. 1988) which achieves one of the criteria for surrogate selection --- the need to ensure we have surviving surrogates at the end-point of the pathogen survivor curve so that correlations and predictions may be made for model development. We intend to use a strain of *Enterococcus faecalis* (*E. faecalis*) from the Tyson Foods' culture collection for a variety of reasons including the following:

1) We have previous experience using this strain in thermal processing studies, having correlated the thermal inactivation to the inactivation of *Salmonella* Senftenberg in ground chicken meat;
2) We have experience with culture, recovery and enumeration of the strain and it is easy to work with;
3) It has been used as a human probiotic as it is considered a part of the normal adult gut flora (Nueno-Palop and Narbad 2011) and as such, should be acceptable to representatives of the USDA for inclusion as a safe surrogate in meat products; and
4) Most importantly, it is considered to be a common contaminate of foods, both raw and ready to eat as it is a common constituent of food animal gastro-intestinal tract contents and has been associated with fermented meats and cheeses for millennia (Gomes *et al*. 2010).

As an important contributor to the flavor profile of a variety of traditional fermented meats and cheeses, it is expected that the microorganism will possess inactivation kinetics in fermented meat systems that are comparable to the *Salmonella* spp. targeted in this research.

I. PREDICTIVE FOOD MICROBIOLOGY

As previously described, the HACCP approach to food safety is predicated upon the identification of potential hazards associated with foods and the processes to which those foods are exposed and the application of process controls to eliminate, prevent or reduce a particular hazard to an acceptable level with the intent to ensure foods are safe to consume (FSIS 1996). For the food manufacturer to properly validate that the HACCP plan achieves the appropriate food safety objectives, it is necessary to measure or evaluate the effect that process interventions have on the hazard to be controlled. For example, if the intent is to prevent a microbial hazard from increasing due to temperature abuse (e.g. *Clostridium botulinum* during the cooling of non-cured, fully cooked meat products), the manufacturer must have knowledge of the time and temperature to which the products will be exposed and the impact of the product formulation and processing conditions on the ability of the pathogenic hazard to proliferate. Likewise, if the intent is to reduce a known microbial hazard (e.g. *Salmonella* spp. on raw meat or poultry products) by application of a process intervention, the processor must have knowledge of the processing conditions, product attributes and microbial response to the intervention under those conditions.

The traditional approach used to demonstrate the safety of food products has been the microbial challenge study to estimate the effect of various process control interventions on either the microbial proliferation or inactivation under controlled laboratory conditions. There are a number of recognized shortcomings to microbial challenge studies including cost; lack of representation of the actual food processing conditions or formulation; and the level of process control achievable in the lab versus under actual processing conditions (Baranyi and Roberts 1995). Predictive Food Microbiology is a science that has arisen in part to address these shortcomings. It is defined as the “quantitative microbial ecology of foods” (McMeekin and Ross 2002). A predictive food microbiological model has been defined as a, “mathematical expression that describes the growth, survival, inactivation or biochemical process of a foodborne microorganism” (McDonald and Sun 1999).

When conducted under conditions representing food products and processes, predictive microbial modeling can inform the food manufacturer about the efficacy of interventions and whether the process is capable of achieving the desired food safety objectives. As such, predictive models may provide guidance to the HACCP and food safety management team and may allow for substantial reductions in

the demand for challenge studies (Elliott 1996). According to Bernaerts *et al*., mathematical modeling is used to quantify the microbial ecology in foods to predict food safety and shelf-life and to guide the development of food safety assurance systems such as HACCP noting that the key to the development of a successful model is user friendliness and computational manageability (Bernaerts *et al.* 2004).

This concept apparently was first practiced in the 1920s in the canning industry using a first-order kinetics model developed by Esty and Meyer for predicting the 12-D inactivation of spores of *Clostridium botulinum* (McMeekin *et al*. 2002). Microbial inactivation kinetics have since been shown to not be log-linear as originally assumed; the inactivation curves are not based on first-order kinetics derived from knowledge of chemical reactions (McMeekin *et al.* 2010). Rather, under many conditions, the plot of survivors is best represented by a curvilinear inactivation response where shoulders and tails are observed (Linton *et al*. 1996, Humpheson *et al*. 1998). The 12-D canning process is still widely accepted and practiced in large part due to the sizable safety factor built into the original model (McMeekin *et al*. 2002).

The concept of a more broad approach to the concept of predictive microbiology such as using microbiological knowledge to predict future outcomes based on process changes was first articulated by W.J. Scott in 1937 while studying the impact of the cold chain on spoilage of beef, noting that with knowledge “it would be feasible to predict the possible extent of the changes” in microbial populations during the initial cooling of sides of beef (Scott 1937). Predictive microbiology has most recently been defined as, “the integration of traditional microbiology knowledge with those found in the disciplines of mathematics, statistics and information systems and technology to describe microbial behavior in order to prevent food spoilage as well as food-borne illnesses” (Fukruddin *et al*. 2011). The process of gathering information and using the information to develop a predictive model to assess the potential impact of process or environmental changes has evolved extensively with the aid of computational power to become predictive software programs such as the Pathogen Modeling Program or ComBase (Baranyi 2003).

J. PREDICTIVE MICROBIAL MODELS

As stated by Munoz-Cuevas *et al*., predictive food microbiology, “aims at describing mathematically, the effect of environmental conditions on the bacterial response to the food environment”. Since the first

mathematical models of microbial growth and inactivation were developed decades ago, increases in computational power have allowed for more detailed and more comprehensive models, often incorporating data obtained from other researchers. From simple primary log-linear models based upon first-order kinetics; to more complex models based on sigmoidal functions such as the Gompertz; to coupled differential equations attempting to estimate the impact of fluctuations in the microbial environment; to secondary models capable of accounting for a larger number of variables and thus, becoming more accurate; to multi-factor models or multivariate response surface models; the models have become better, more accurate and more robust predictors of the behavior of microorganisms under a variety of environmental conditions (Munoz-Cuevas *et al*. 2012). However, models have limitations and a complete understanding of the appropriate use of models is necessary before undertaking the considerable effort to accumulate data through experimentation so that proper experimental design may be considered (Geeraerd *et al*. 2000).

Typically, predictive microbial models are grouped as primary, secondary or tertiary. The model classification system most widely adopted was first proposed by Whiting and Buchanan in 1983. An example of a primary model is the standard microbial growth curve showing the changes in bacterial concentration over time (Black and Davidson 2008) and consisting of:

1) A lag phase during which time the microbial cells are acclimating to their new environment and producing the necessary enzymes and metabolic precursors to initiate energy production and then replication;
2) Followed by an exponential growth phase during which time the daughter cells produced from the original microbial cells and possessing all the necessary enzymes and metabolic precursors are capable of rapid, exponential increases in cell concentration;
3) A stationary phase during which time the cell numbers neither increase nor decrease as cell replication is balanced by cell death – a phase typically achieved when nutrients have become relatively scarce or metabolic waste products have built up in the environment to levels that are growth-limiting; and
4) Finally, a death phase during which time there is a net loss of cell numbers due to nutrient depletion or environmental toxicity (Buchanan *et al*. 1997).

A secondary model considers environmental variables and their effect on the phases of growth; while a tertiary model creates an interface between the secondary model and the user through a computer program to output primary model predictions – e.g. cell density over time (Black and Davidson 2008).

As the proposed research is focused on the inactivation of vegetative pathogens in a complex food matrix, this review will turn to the models commonly utilized to predict cell inactivation. The kinetics of inactivation in a complex food system are likely very complex with differences in rates of inactivation due to the micro-environment any individual or colony of cells might experience – differences such as those the cells might encounter in the water phase versus the fat phase where diffusion of solutes will be limited. There are a wide number of models available based upon two primary hypotheses concerning the shape of the inactivation curve: the mechanistic hypothesis asserts that the inactivation curve will best be represented by a straight line; while the vitalistic hypothesis asserts that the survivor curve may take many different curvilinear shapes depending upon differences in cell density, genetic variants in the population, the environment and changes in the environment over time.

The mechanistic hypothesis is founded upon theories of chemical kinetics and cell inactivation is assumed to be the result of a single event – the “one hit kill” which results in a cell population in which all cells are either active or inactive. The vitalistic hypothesis is based upon the theory that cell inactivation under extreme conditions may be the result of a single event; while under other conditions may be the result of cumulative damage or metabolic stress resulting in a mixed cell population of inactive, injured and fully active cells with a wide range of metabolic and replication capabilities. This mixed culture results in varying cell density under a range of environmental conditions and yields curvilinear survivor curves (Lambert 2003, Peleg and Cole 1998).

Predictive microbial models are generally classified as either empirical or mechanistic. Empirical models are defined as those based upon observations of experimental data. Mechanistic models are those that are based upon a presumption that a physical (e.g. cell wall disruption) or molecular (e.g. protein denaturation or DNA damage) mechanism affects the microbial response. Mechanistic models are further divided between probabilistic and deterministic; with probabilistic models designed to predict the cell population at a given time; and deterministic models designed to utilize the known history of the cell population to predict the future behavior of the population (Heldman and Newsome 2003).

A number of models have been developed to describe the inactivation kinetics of microbial populations. These include relatively simple log-linear models such as the Bigelow model first published in the 1920s which is still in use as it is simple (van Boekel 2008). The model equation includes the D-value for the decimal-reduction in cell density over time, most commonly defined to be the time required at a given temperature to achieve a 1.0 $\log_{10}$ reduction in population density (van Asselt and Zwietering 2006, Mazzotta 2000) which is calculated as the negative inverse of the slope of the least sum of squares best-fit line to the experimental data (Linton *et al*. 1995) and takes the form:

$$S(t) = \exp\left(-\frac{t}{D}\right) \qquad (1)$$

or

$$\log S(t) = -\frac{t}{D} \qquad (2)$$

in which

$$S(t) = \frac{N}{N_0} \qquad (3)$$

where N is the cell density at a given time and N_0 is the cell density at time zero. The mechanistic explanation for why microbial populations might be expected to exhibit log-linear inactivation is that each cell has an identical probability of inactivation at a given time (Buzrul *et al.* 2005). As is apparent from equation 2, a plot of log S(t) will yield a straight line which is seldom seen in plots of actual experimental results where curvilinear survivor curves are the norm (van Boekel 2008).

Microbial populations often exhibit inactivation rates that are not log-linear and do not produce a straight line when plotted. The more commonly observed inactivation curves are:

1) Shoulders representing a period of delay under the experimental conditions before inactivation is measured and is often attributed to cell clumping and reduced penetration of the lethal effect (e.g. heat) yielding a convex survivor curve;
2) Tails representing extended time with reduced rate of inactivation most frequently attributed to a subpopulation of resistant cells yielding a concave survivor curve; and
3) Both shoulders and tails with the microbial population exhibiting both a lag in the rate of inactivation and an extended period of survivors with a slower rate of inactivation yielding a sigmoidal survivor curve (Xiong *et al*. 1999a, Xiong *et al.* 1999b).

As an attempt to address this incongruity between the model and experimental observations of non-linearity, Peleg and Cole described a model based upon the Weibull distribution which has a long history of use in the quality sciences as the basis of reliability engineering for describing and predicting failure rates and calculation of the mean time between failures. In reliability engineering, the typical curve is represented as the "bathtub curve" which represents three distinct phases of failure:

1) High failure rate during the "infant mortality" or early failure period;
2) Followed by a longer duration constant (or random) lower failure rate period which is linear;
3) Followed by the "wear-out" phase which is typically a normally distributed function (Krishnamoorthi 1992, and Kececioglu 1994).

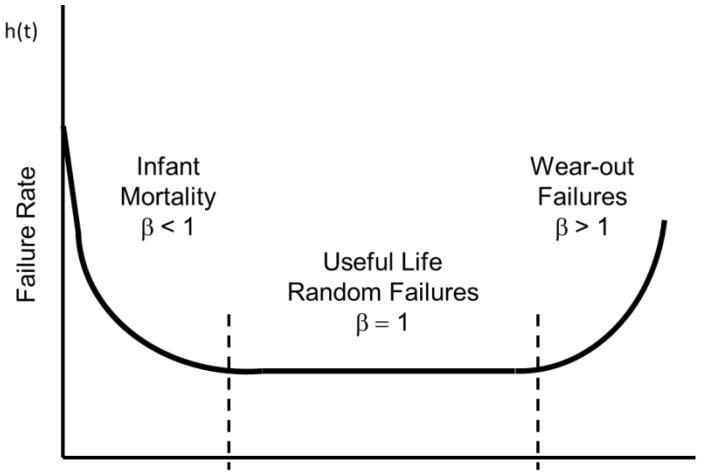


Figure 1 – The hazard function over time e.g. the classical "bathtub" curve (adapted from Klutke *et al*. 2003).

Given that the model includes inflection points between each of the phases, Peleg and Cole saw the potential for accounting for the transitions at shoulders and tails in microbial populations that have been observed in plots of survivors during thermal inactivation experiments (Peleg and Cole 1998).

The Weibull model has frequently been used in studies of thermal and nonthermal microbial inactivation and assumes that the rate of inactivation is not constant with an underlying premise that every individual microbial cell has a unique resistance to the effects of the lethal environmental (Marks 2008). The model can be used to describe survivor curves that are linear (when $\beta = 1.0$), convex or concave (Coroller *et al*. 2006) and takes the form:

$$S(t) = \exp\left[-\left(\frac{t}{\alpha}\right)^{\beta}\right] \qquad (4)$$

and

$$log\ S(t) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \qquad (5)$$

In comparing equation 4 to equation 2, the primary difference is the use of α as the scale parameter with units time and the non-dimensional shape factor β, which as previously stated when equal to 1.0 yields a straight line survivor curve and reduces mathematically to the Bigelow equation (van Boekel 2008). By altering the shape factor β, the researcher may obtain the least sums of squares best fit line to the experimental data including the shouldering and tails to produce the typical sigmoidal survivor curve that is frequently, but not always, experimentally observed. Van Boekel applied the Weibull model to 55 different data sets and observed a concave downward survivor curve in 39 of the data sets; a concave upward curve in 14 data sets; and only one data set which exhibited a linear inactivation curve (van Boekel 2002).

Other primary inactivation models that have been published include: the Modified Gompertz which takes the form (Bhaduri *et al*. 1991):

$$\log N(t) = A - Ce^{-B(t-M)} \qquad (6)$$

or

$$\log\frac{N(t)}{No} = Ce^{-e^{BM}} - Ce^{-e^{-B(t-M)}} \qquad (7)$$

The Kamau model which has been used to predict thermal inactivation of *Listeria monocytogenes* and *Staphylococcus aureus* and takes the general form (Kamau *et al*. 1990) for nonlinear survival curves:

$$\log\frac{N(t)}{N_0} = \frac{2}{1+e^{\beta t}} \qquad (8)$$

The Membre Equation used to model the inactivation of *Salmonella* Typhimurium in reduced calorie mayonnaise and takes the form (Membre *et al*. 1997):

$$\log N(t) = (1 + \log N_0) - e^{kt} \qquad (9)$$

The Buchanan model which has been widely used to estimate the interaction of water activity and pH on the thermal inactivation of *Listeria monocytogenes* (Buchanan *et al*. 1993) and takes the form:

$$\log N(t) = \begin{cases} \log N_0, & t \le t_{lag} \\ \log N_0 - \frac{t - t_{lag}}{D}, & t > t_{lag} \end{cases} \quad (10)$$

And lastly is the Modified Weibull (Albert and Mafart 2005) taking the form:

$$\gamma(t/\delta) = (N_0 - N_{res}) 10^{\left(-\left(\frac{1}{\delta}\right)^p\right)} + N_{res} \quad (11)$$

Polynomial models are the most widely utilized secondary modeling approaches and usually take the form of multivariate quadratic equations. These are used to further develop and describe the kinetics described by the primary models from which they are derived and attempt to estimate the effects of changes in the microbial ecology (Ross and McMeekin 1994). However, one of the earliest secondary models is represented by the z-value, the increase in temperature required to reduce the D-value by one order of magnitude (von Boekel 2002). The typical approach to the estimation of a z-value is to replicate the determination of the D-value at multiple temperatures; assess the effect of the temperature change on the slope of the log-linear plot of survivors; and calculate the temperature change required to reduce the D-value by a factor of 10 (Blackburn *et al*. 1997).

The polynomial secondary models are much more complex that the primary models. As previously described, this is expected to make the predictions more robust. However, full model development generally requires substantially more data and care must be taken not to extrapolate outside the boundaries of the measured environmental variables. As a representative example, Pond *et al*. (2001) developed a series of polynomial models utilizing quadratics and response surface models to predict the inactivation of *E. coli* O157:H7 in fermented sausage. The general model takes the form:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \in \quad (12)$$

where y = *E. coli* O157:H7 log reduction; $x_1 = a_w$; x_2 = pH; x_3 = time of processing at specific stages; β_0 = estimate of the y-intercept; $\beta_1 x_1$ = estimate for the linear effect of the independent variable aw; $\beta_2 x_2$ = estimate for the linear effect of the independent variable pH; $\beta_3 x_3$ = estimate for the linear effect of the independent variable time; $\beta_{22} x_2^2$ = estimate for the quadratic curvature effect of independent variable pH; $\beta_{33} x_3^2$ = estimate for the quadratic curvature effect for independent variable time; and $\in$ = the error term. This model was well correlated with the validation data but slightly over-predicted the total $\log_{10}$ reductions achieved (Pond *et al.* 2001).

The second model of Pond *et al*. separated the process into two distinct phases, fermentation and drying that allowed for the inclusion of a variable to estimate the effect of the time during fermentation, taking the form:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \in \qquad (13)$$

where y = *E. coli* O157:H7 log reduction during the fermentation stage; x_1 = fermentation time multiplied by temperature; x_2 = pH during fermentation; β_0 = estimate of the y-intercept; β_1 = estimate for the linear effect of the time at temperature during fermentation; β_2 = estimate for the linear effect of pH; β_{12} = estimate for the interactive effect of time at temperature and pH; and $\in$ = the error term. This model explained 83% of the inactivation achieved during validation testing with the remainder explained as random error. The validation data and equation prediction agreed well with $R^2 = 0.965$ for the best fit regression through the scatter plot (Pond *et al*. 2001).

For the dual phase model, a second model was hypothesized for the drying phase of sausage manufacturing with the model taking the form:

$$y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \in \qquad (14)$$

where y = *E. coli* O157:H7 log reduction during the drying stage; x_1 = aw of the fermented salami; x_2 = time during the drying stage; β_0 = estimate of the y-intercept; β_1 = estimate for the linear effect of a_w during the drying stage; β_2 = estimate of the linear effect of time during the drying stage; β_{12} = estimate for the interactive effect between a_w and time for the drying stage; and $\in$ = the error term. This model also predicted a slightly greater inactivation than achieved during validation ($R^2 = 0.930$) and there was good agreement between the predicted values and the scatter plot with $R^2 = 0.976$ (Pond *et al*. 2001).

A third model was proposed by Pond *et al*. to estimate the effect of the time the samples were held at pH $\leq$ 5.3. The model takes the form:

$$y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \in \qquad (15)$$

Where y = *E. coli* O157:H7 log reduction; x_1 = aw of the fermented salami; x_2 = time at pH $\leq$ 5.3; β_0 = estimate of the y-intercept ; β_1 = estimate for the linear effect of a_w; β_2 = estimate of the linear effect of time at pH $\leq$ 5.3; β_{12} = estimate for the interactive effect between a_w and time at pH $\leq$ 5.3; and $\in$ = the error term. The final equation for modeling the inactivation of O157:H7 in uncooked fermented salami takes the form:

$$y = 10.6 - 10.5x_1 - 0.148x_2 + 0.208x_1x_2 + \in \quad (16)$$

Validation of this model indicates it slightly underestimates the inactivation achieved through fermentation and initial drying; however, it slightly overestimates the inactivation achieved through the remainder of the drying phase. The regression through the scatter plot shows reasonably good agreement with $R^2 = 0.941$ (Pond *et al*. 2001).

The models of Pond *et al.* clearly demonstrate the utility of the use of predictive microbial models. The work further illustrates the substantial effort and expense required to develop such a model set and readers should be reminded that the experimental conditions should not be extrapolated to other processes or formulations – indicating that such an approach may require a custom model for each possible combination of processing conditions and formulations (Fukruddin *et al*. 2011).

Tertiary predictive microbial models are intended to provide access to predictive models to the broad audience of food safety professionals, academics and regulatory policy-makers that are not proficient in microbial modeling methods. These models are intended to be utilized by individuals with adequate microbiological knowledge to properly interpret the predictive outputs of the computer-based modeling program and understand the limitations of the specific models being utilized (Isabelle and Andre 2006).

The most widely known and utilized domestic predictive microbial modeling program was developed by researchers at the U.S. Department of Agriculture's Agricultural Research Service at the Eastern Regional Research Center and is called the Pathogen Modeling Program (PMP). It is available online or as a computer downloaded version. It has thermal inactivation models for *E. coli* in ground beef with additives; *Listeria monocytogenes* in ground beef and in simulated beef gravy; and *Salmonella* serotypes in ground beef (as well as many other types of models for a variety of microbial specie). The program is user-friendly and interactive with drop down menus and slide bars allowing a wide range of product and process variables to be modeled. All models are built upon the published literature and the database currently contains over 250 peer-reviewed data sources. The PMP is a useful research tool; however, the developers are quick to warn users about the limitations of microbial models. In response to a question concerning whether the models can be used for HACCP plan validation, the online version states:

> "The PMP models are only valid for the conditions used to produce the model. The reference(s) found in the "Source and/or Related Publications" window presents an explanation for the methodologies used to produce the model. Therefore, if the conditions (e.g., food formulation) used to produce the PMP model do not match your food system, then you must validate the

> model for your specific application. Validation normally involves laboratory studies where your product is inoculated with a specific bacteria and then you record the levels of growth or inactivation. These data can then be compared to the PMP model predictions to see if they are within the predicted 95% confidence intervals. If they do not match, then the PMP model is not valid for your application. In this case, assuming sufficient experimental data have been collected, your data may be used to develop a new model that it would be valid for your food product" (USDA 2013).

Other widely recognized tertiary models include a spreadsheet based model called GInaFIT, which stands for the "Geeraerd and Van Impe Inactivation Model Fitting Tool." This modeling program is a free add-in for Microsoft® Excel and incorporates nine different modeling tools for predicting the inactivation of vegetative bacteria that exhibit inactivation kinetics that will result in all the previously described survivor curves. It incorporates advanced statistics to assist researchers with understanding the precision expected from the predictions and, for anyone comfortable with the Excel spreadsheet software, is very user-friendly (Geeraerd *et al*. 2005).

ComBase is a collaborative database utilizing the combined efforts of several governmental program areas including the USDA-ARS researchers responsible for the PMP; the UK's Food Standards Agency; and the Institute of Food Research in Norwich, UK. The core of the ComBase program consists of the models contained with both the PMP and a defunct program formerly called the Food MicroModel (FMM). ComBase is also a complex set of Excel spreadsheets creating a relational database with user-friendly interface and descriptive statistics. It is anticipated that the ComBase database will offer a repository for experimental data and illuminate data gaps to stimulate ongoing research to further develop tertiary modeling and expand its utility (Baranyi and Tamplin 2004).

Other lesser known and utilized tertiary modeling programs include the *Pseudomonas* Predictor at the University of Tasmania under the supervision of Neumeyer (Neumeyer *et al.* 1997); Sym'Previus is a modeling program developed by the French Departments of Research and Agriculture to predict microbial behavior (Leporq *et al.* 2005); and the Seafood Spoilage and Safety Predictor (SSSP) developed by the Danish Institute for Fisheries Research to predict microbial growth under constant and fluctuating temperature conditions (Dalgaard *et al.* 2002).

The primary challenge faced by users of predictive microbial modeling programs is their basis on data derived from growth or inactivation experiments conducted under very specific laboratory conditions. The applicability of the model predictions to a commercial food processor leaves a great deal of uncertainty

concerning the value and use of the output of these tertiary models. However, as a starting point for understanding the impact of process or formulation changes, the models have a great deal of utility. Further, when in-process data can be collected and compared directly against one of the model outputs, the user has a distinct advantage of being able to assess the predictions against a (likely) limited set of observational data. These models will continue to evolve and develop and we expect greater utility and wider acceptance of the outputs. The simplest model is the best model; and every model is, at best, an estimate of what happens in the real world under actual food processing conditions.

III. REFERENCES:

Albert, I., and P. Mafart. 2005. A Modified Weibull Model for Bacterial Inactivation. Int. J. Food Microbiol. 100:197-211.

Allaker, R.P., L.S. Silva Mendez, J.M. Hardie, and N. Benjamin. 2001. Antimicrobial Effect of Acidified Nitrite on Periodontal Bacteria. Oral Microbiol. Immunol. 16:253-256.

AMI. 1997. Good Manufacturing Practices for Fermented Dry and Semi-Dry Sausage Products. www.meathaccp.wisc.edu/assets/Heat_Treated_Shelf_Stable/AMIF_degreehours.pdf (accessed 03/24/2013).

Andersson, A., U. Ronner, and P. Granum. 1995. What Problems does the Food Industry Have with the Spore-Forming Pathogens *Bacillus cereus* and *Clostridium perfringens*? Int. J. Food Microbiol. 28:145-155.

Argudin, M., M. Mendoza, and M. Rodicio. 2010. Food Poisoning and *Staphylococcus aureus* Enterotoxins. Toxins. 2:1751-1773.

Baranyi, J., and T.A. Roberts. 1995. Mathematics of Predictive Food Microbiology. Int. J. Food Microbiol. 26:199-218.

Baranyi, J. 2003. Predictive Modeling to Control Microbial Hazards in the Food Processing Industry. http://smas.chemeng.ntua.gr/files/webdoc_9_25_8_2003.pdf (accessed 07/15/2012).

Baranyi, J., and M.L. Tamplin. 2004. ComBase: A Common Database on Microbial Responses to Food Environments. J. Food Protect. 67(9):1967-1971.

Barber, Willie. 2012. Tyson Foods, Inc. Personal Communication.

Bentley, R. and R. Meganathan, 1982. Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiol. Rev. 46(3):241-283.

Bernaerts, K. E. Dens, K. Vereecken, A.H. Geeraerd, A.R. Standaert, F. Devlieghere, J. Debevere, and J.F. Van Impe. Concepts and Tools for Predictive Modeling of Microbial Dynamics. J. Food Protect. 67(9):2041-2052.

Bhaduri, S.P.W.S., S.A. Palumbo, C.O. Turner-Jones, J.L. Smith, B.S. Marmer, R.L. Buchanan, L.L. Zaika, and A.C. Williams. 1991. Thermal Destruction of *Listeria monocytogenes* in Liver Sausage Slurry. Food Microbiol. 8:75-78.

Bhatia, A., and S. Zahoor. 2007. *Staphylococcus aureus* Enterotoxins: A Review. J. Clin. Diagnost. Res. 1(2):188-197.

Binkerd, E., and O. Kolari. 1975. The History and Use of Nitrate and Nitrite in the Curing of Meat. Food Cosmet. Toxicol. 13:655-661.

Black, D.G., and P.M. Davidson. 2008. Use of Modeling to Enhance the Microbiological Safety of the Food System. Comp. Rev. Food Sci. Food Safe. 7:159-167.

Blackburn, C.W., L.M. Curtis, L. Humpheson, C. Billon, and P.J. McClure. 1997. Development of Thermal Inactivation Models for *Salmonella enteritidis* and *Escherichia coli* O157:H7 with Temperature, pH, and NaCl as Controlling Factors. Int. J. Food Microbiol. 38:31-44.

Borowski, A.G., S.C. Ingham, and B.A. Ingham. 2009. Validation of Ground-and-Formed Beef Jerky Processes Using Commercial Lactic Acid Bacteria Starter Cultures as Pathogen Surrogates. J. Food Protect. 72(6):1234-1247.

Brazell, Ernest. 2009. Tyson Foods, Inc. Personal Communication.

Bremer, V., K. Lietmeyer, E. Jensen, U. Metzel, H. Meczulat, E. Weise, D. Werber, H. Tschaepe, L. Kreienbrock, S. Glaser, and A. Ammon. 2004. Outbreak of *Salmonella* Goldcoast Infections Linked to Consumption of Fermented Sausage, German 2001. Epidemiol. Infect. 132(5):881-887.

Brooks, J. 1996. The Sad and Tragic Life of Typhoid Mary. J. Can. Med. Assoc. 154(6):915-916.

Bryan, F. 1972. *Clostridium perfringens* in Relation to Meat Products. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 25. p.323-341.

Buchanan, R.L., M.H. Golden, and R.C. Whiting. 1993. Differentiation of the Effects of pH and Lactic or Acetic Acid Concentrations on the Kinetics of *Listeria monocytogenes* Inactivation. J. Food Protect. 56:474-478.

Buchanan, R.L., R.C. Whiting, and W.C. Damert. 1997. When is Simple Good Enough: A Comparison of the Gompertz, Baranyi, and Three-Phase Linear Models for Fitting Bacterial Growth Curves. Food Microbiol. 14:313-326.

Buzrul, s., H. Alpas, and F. Bozoglu. 2005. Use of Weibull Frequency Distribution Model to Describe the Inactivation of *Alicyclobacillus acidoterrestris* by High Pressure at Different Temperatures. Food Res. Int. 38:151-157.

Cabrera-Diaz, E., T.M. Moseley, L.M. Lucia, J.S. Dickson, A. Castillo, and G.R. Acuff. 2009. Fluorescent Protein-Marked *Escherichia coli* Biotype I Strains as Surrogates for Enteric Pathogens in Validation of Beef Carcass Interventions. J. Food Protect. 72(2):295-303.

Calicioglu, M., N.G. Faith, D.R. Buege, and J.B. Luchansky. 2001. Validation of a Manufacturing Process for Fermented, Semi-Dry Turkish Soudjouk to Control *Escherichia coli* O157:H7. J. Food Protect. 64:1156-1161.

Cassens, R., M. Greaser, T. Ito, M. Lee. 1979. Reactions of Nitrite in Meat. Food Technol. 33(7):48-57.

Cassens, R. 1997. Composition and Safety of Cured Meats in the USA. Food Chem. 59:561-566.

Castellani, A. and C. Niven. 1954. Factors Affecting the Bacteriostatic Action of Sodium Nitrite. Journal Paper No. 105, American Meat Institute Foundation. Personal collection.

CDC. 2003. Morbidity and Mortality Weekly Report. Trichinellosis Surveillance – United States, 1997-2001. Department of Health and Human Services. Centers for Disease Control and Prevention. 52(SS06):1-8.

CDC. 2009a. Morbidity and Mortality Weekly Report. Trichinellosis Surveillance – United States, 2002-2007. Department of Health and Human Services. Centers for Disease Control and Prevention. 58:SS-9.

CDC. 2009b. Laboratory-confirmed *Salmonella* isolates from human sources reported to CDC, with the 20 most frequently reported serotypes listed individually. www.cdc.gov/ncezid/dfwed/PDFs/SalmonellaAnnualSummaryTables2009.pdf.

CDC. 2010. Investigation Update: Multistate Outbreak of Human *Salmonella* Montevideo Infections. www.cdc.gov/salmonella/montevideo/index.html (accessed 07/15/2012).

CDC. 2011a. National Enteric Disease Surveillance: *Salmonella* Surveillance Overview. Atlanta, Georgia: US Department of Health and Human Services, CDC. www.cdc.gov/nationalsurveillance/PDFs/NationalSalmSurveillOverview_508.pdf.

CDC. 2011b. Trends in Foodborne Illness, 1996-2010. Atlanta, Georgia: US Department of Health and Human Services, CDC. www.cdc.gov/foodborneburden/PDFs/FACTSHEET_B_TRENDS.PDF.

CDC. 2011c. Investigation Announcement: Multistate Outbreak of *E. coli* O157:H7 Infections Associated with Lebanon Bologna. http://www.cdc.gov/ecoli/2011/O157_0311/index.html (accessed 01/12/2013).

CDC. 2012a. Trichinellosis FAQs. http://www.cdc.gov/parasites/trichinellosis/gen_info/faqs.html (accessed December 15, 2012).

CDC. 2012b. Trichinellosis Epidemiology and Risk Factors. http://www.cdc.gov/parasites/trichinellosis/epi.html (accessed December 15, 2012).

CDC. 2012c. Botulism, General Information. Centers for Disease Control and Prevention. http://www.cdc.gov/nczved/divisions/bfdmd/diseases/botulism/ (accessed January 2, 2012).

CDC. 2012d. *Clostridium perfringens*. Centers for Disease Control and Prevention. http://www.cdc.gov/foodborneburden/clostridium-perfringens.html (accessed January 2, 2012).

CDC. 2012e. Staphylococcal Food Poisoning. www.cdc.gov/ncidod/dbmd/diseaseinfo/staphylococcus_food_g.htm (accessed December 23, 2012).

CDC. 2012f. National Shiga toxin-producing *Escherichia coli* (STEC) Surveillance Overview. Atlanta, Georgia: US Department of Health and Human Services, CDC, 2012. www.cdc.gov/ncezid/dfwed/PDFs/national-stec-surveillance-overiew-508c.pdf (accessed 01/12/2013).

CDC. 2012g. Enterotoxigenic *Escherichia coli* (ETEC), Frequently Asked Questions. www.cdc.gov/ncidod/dbmd/diseaseinfo/etec_g.htm (accessed 12/21/2012).

CDC. 2012h. General Information *Escherichia coli* (*E. coli*). www.cdc.gov/ecoli/general/index.html (accessed 01/12/2013).

CDC. 2012i. *Salmonella*. Technical Information. www.cdc.gov/salmonella/general/technical.html (accessed 07/15/2012).

Christiansen, L., R. Tompkin, and A. Shaparis. 1975. Effect of Sodium Nitrite and Nitrate on *Clostridium botulinum* Growth and Toxin Production in a Summer Style Sausage. J. Food Sci. 40:488-490.

Cornell University. 2007. Fermented Sausages, Fact Sheets for the Small Scale Food Entrepreneur. http://necfe.foodscience.cornell.edu/publications/pdf/FS_FermentedSausages.pdf (accessed 01/13/2013).

Coroller, L., I. leguerinel, E. Mettler, N. Savy, and P. Mafart. 2006. General Model, Based on Two Mixed Weibull Distributions of Bacterial Resistance, for Describing Various Shapes of Inactivation Curves. App. Env. Microbiol. 72(10):6493-6502.

Dalgaard, P., P. Buch, S. Silberg. 2002. Seafood Spoilage Predictor - Development and Distribution of a Product Specific Application Software. Int. J. Food Microbiol. 73:227-233.

Deibel, R., C. Niven, Jr., and G. Wilson. 1961. Microbiology of Meat Curing. III. Some Microbiological and Related Technological Aspects in the Manufacture of Fermented Sausages. Appl. Microbiol. 9(2):156-161.

Dinges, M., P. Orwin, and P. Schlievert. 2000. Exotoxins of *Staphylococcus aureus*. Clin. Microbiol. Rev. 13(1):16-34.

Doganay, M. 2003. Listeriosis: Clinical Presentation. FEMS Immunol. Med. Microbiol. 35:173-175.

Dominguez, S., and D. Schaffner. 2009. Survival of *Salmonella* in Processed Chicken Products during Frozen Storage. J. Food Protect. 72(10):2088-2092.

Doyle, M. and A. Mazotta. 2000. Review of Studies of the Thermal Resistance of Salmonellae. J. Food Protect. 63(6):779-795.

Doyle, M., J. Archer, C. Kaspar, and R, Weiss. 2006. Human Illness Caused by *E. coli* O157:H7 from Food and Non-Food Sources. Food Research Institute, UW-Madison. Funded in part by the American Meat Institute Foundation. http://fri.wisc.edu/docs/pdf/FRIBrief_EcoliO157H7humanillness.pdf (accessed 01/12/ 2013).

Drevets, D. 1999. Dissemination of *Listeria monocytogenes* by Infected Phaocytes. Infect. Immun. 67(7):3512-3517.

Duncan, C.L. and E.M. Foster. 1968. Effect of Sodium Nitrite, Sodium Chloride, and Sodium Nitrate on Germination and Outgrowth of Anaerobic Spores. App. Microbiol. 16(2):406-411.

Eitenmiller, R.R., P.E. Koehler, J.O. Reagan. 1978. Tyramine in Fermented Sausages: Factors Affecting Formation of Tyramine and Tyrosine Decarboxylase. J. Food Sci. 43(3):689-693.

Elliott, P.H. 1996. Predictive Microbiology and HACCP. J. Food Protect. 1996 (Suppl.):48-53.

Emberland, K.E., K. Nygard, B.T. Heier, P. Aavitsland, J. Lassen, T.L. Stavnes, and B. Gondrosen. 2006. Outbreak of *Salmonella* Kedougou in Norway Associated with Salami, April – June 2006. Euro Surveill. 11(27):2995.

Encyclopaedia Britannica Online. 2013. Food Preservation. www.britannica.com/EBchecked/topic/212684/food-preservation (accessed 01/13/2013).

FAO. 2007. Small-Scale Sausage Production, Fermented Sausage Production. FAO Corporate Document Repository. Agriculture and Consumer Protection. www.fao.org/docrep/003/x6556e/X6556E05.htm (accessed 10/01/2007).

Farber, J., and P. Peterkin. 1991. *Listeria monocytogenes*, a Food-Borne Pathogen. Microbiol. Rev. 55(3):476-511.

FDA. 2005. Inspections, Compliance, Enforcement, and Criminal Investigations. Compliance Policy Guide Section 555.425 Foods, Adulteration Involving Hard or Sharp Foreign Objects. http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074554.htm (accessed December 15, 2012).

FDA. 2012. Bad Bug Book -- Foodborne Pathogenic Microorganisms and Natural Toxins Handbook – Second Edition.

www.fda.gov/downloads/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/UCM297627.pdf.

FDA. 2013a. Code of Federal Regulations. Title 21 - Food and Drugs. Chapter I - Food and Drug Administration, Department of Health and Human Services, Subchapter B - Food for Human Consumption. Part 109 - Unavoidable Contaminants in Food for Human Consumption and Food-Packaging Material. Subpart A - General Provisions. www.gpo.gov/fdsys/pkg/CFR-2002-title21-vol2/xml/CFR-2002-title21-vol2-sec109-7.xml.

FDA. 2013b. Kinetics of Microbial Inactivation for Alternative Food Processing Technologies. www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm100158.htm (accessed 03/30/2013).

FSIS. 1996. 9 CFR Part 304 *et al.*, Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems; Final Rule. U.S. Department of Agriculture, Food Safety and Inspection Service. Fed. Reg. 61(144):38806-38855.

FSIS. 1999a. 9 CFR Parts 301, 317, 318, 320, and 381.Docket No. 95-033F. Production Standards for the Production of Certain Meat and Poultry Products. Fed. Reg. 64(3):732-749.

FSIS. 1999b. Appendix A, Compliance Guidelines for Meeting Lethality Performance Standards for Certain Meat and Poultry Products. www.fsis.usda.gov/Frame/FrameRedirect.asp?main=http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/95-033F/95-033F_Appendix_A.htm (accessed 02/16/2013).

FSIS. 2000. Percent Positive *Salmonella* Tests for RTE meat and poultry products, CY 1990-2000. www.fsis.usda.gov/Science/Table2_Micro_Testing_RTE_1990-2004/index.asp (accessed 02/26/2013).

FSIS. 2001. 9 CFR Parts 301, 303, 317, 318, 319, 320, 325, 331, 381, 417, and 430. Docket No. 97-013P. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg. 66(39):12590-12636.

FSIS. 2005. Time and Temperature Tables for Cooking Ready to Eat Poultry Products. Original Paper Copy -- Online version has had the fermented poultry references removed.

FSIS. 2010. Use of Non-pathogenic *Escherichia coli* (*E. coli*) Cultures as Surrogate Indicator Organisms in Validation Studies. http://askfsis.custhelp.com/app/answers/detail/a_id/1392/kw/surrogate%20indicator%20organisms/session/L3RpbWUvMTM2MzU0Mzk5Mi9zaWQvZFM4TDhwbGw%3D (accessed 08/03/2011).

FSIS. 2011. Principles of Preservation of Shelf-Stable Dried Meat Products. www.fsis.usda.gov/PDF/FSRE_SS_7Principles.pdf (accessed 03/24/2013).

FSIS. 2012. FSIS *Salmonella* Compliance Guidelines for Small and Very Small Meat and Poultry Establishments that Produce Ready-to-Eat (RTE) Products. www.fsis.usda.gov/PDF/Salmonella_Comp_Guide_091912.pdf (accessed 01/13/2013).

FSIS. 2013a. FSIS Compliance Guideline: Lebanon bologna. www.fsis.usda.gov/PDF/Compliance_Guideline_Lebanon_Bologna.pdf (accessed 03/23/2013).

FSIS. 2013b. The FSIS Microbiological Testing Program for Ready-to-Eat (RTE) Meat and Poultry Products, 1990–2011. www.fsis.usda.gov/Science/Micro_Testing_RTE_Continuation/index.asp#previous (accessed 02/26/2013).

FSIS. 2013c. Proposed Rule. 155. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg. 78(5):1532.

Fukruddin, Md., R.M. Mazumder, and K.S. Bin Mannan. 2011. Predictive Microbiology: Modeling Microbial Responses in Food. Ceylon J. Sci. 40(2):121-131.

Galanis, E., D. Lo Fo Wong, M. Patrick, N. Binsztein, A. Cieslak, T. Chalermchaklit, A. Aidara-Kane, A. Ellis, F. Angulo, and H. Wegener. 2006. Web-based Surveillance and Global *Salmonella* Distribution, 2000-2002. Emerg. Infect. Dis. 12(3):381-388.

Gamble, H. 2000. Facts. Trichinae. National Pork Producers Council. www.pork.org/filelibrary/Factsheets/PorkSafety/facttrichinae04377.pdf (accessed December 15, 2012).

Gamble, H. 2012. Pork Facts – Food Quality and Safety. USDA, Agricultural Research Service. http://www.aphis.usda.gov/vs/trichinae/docs/fact_sheet.htm (accessed December 15, 2012).

Geeraerd, A.H., C.H. Herremans, and J.F. Van Impe. 2000. Structural Model Requirements to Describe Microbial Inactivation during a Mild Heat Treatment. Int. J. Food Microbiol. 59:185-209.

Geeraerd, A.H., V.P. Valdramidis, and J.F. Van Impe. 2005. GInaFiT, a Freeware Tool to Assess Non-log-Linear Microbial Survivor Curves. Int. J. Food Microbiol. 102:95-105.

Genigeorgis, C. 1972. Factors Influencing Growth and Toxin Production by *S. aureus*. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 25. p.306-322.

Genigeorgis, C. 1989. Present State of Knowledge on Staphylococcal Intoxication. Int. J. Food Microbiol. 9:327-360.

Gilbert, R.J. 1974. Staphylococcal Food Poisoning and Botulism. Postgrad. Med. J. 50:603-611.

Goldman, D. The Physical Hazards of Foreign Materials. Presentation for the Public Meeting on Foreign Material Contamination, September 24, 2002. www.fsis.usda.gov/OPPDE/rdad/FRPubs/02-033N/ThePhysicalHazardsofForeignMaterials.pdf.

Gomes, B.C., B.D.G. de Melo Franco, and E.C.P De Martinis. 2010. Dualistic Aspects of *Enterococcus* spp. In Foods. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Technology. A. Mendez-Vilas (ed.). www.formatex.info/microbiology2/1119-1125.pdf (accessed 01/13/2013).

Gregory, Michael. 2012. Tyson Foods. Personal communication.

Grimont, P. and F. Weill. 2007. Antigenic Formulae of the *Salmonella* Serovars, 9th Edition. World Health Organization. WHO Collaborating Center for Reference and Research on Salmonella. Institute Pasteur, Paris, France. www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089.

Gurtler, J.B., R.B. Rivera, H.Q. Zhang, and D.J. Geveke. 2010. Selection of Surrogate Bacteria in Place of *E. coli* O157:H7 and *Salmonella Typhimurium* for Pulsed Electric Field Treatment of Orange Juice. Int. J. Food Microbiol. 139:1-8.

Gyles, C. 2007. Shiga toxin-producing *Escherichia coli*: An overview. J. Anim. Sci. 85(E. Suppl.):E45-E62.

Hafley, Brian. 2009. Tyson Foods, Inc. Personal Communication.

Hall, I., and E. Peterson. 1923. The Effect of Certain Bacteria upon the Toxin Production of *Bacillus botulinus* in Vitro. J. Bacteriol. 8(4):319-341.

Hammes, W. 2011. Metabolism of Nitrate in Fermented Meats: The Characteristic Feature of a Specific Group of Fermented Foods. Food Microbiol. 29:151-156.

Harmsen, M., M. Lappann, S. Knochel, S. Molen. 2010. Role of Extracellular DNA during Biofilm Formation by *Listeria monocytogenes*. App. Environ. Micro. 76(7):2271-2279.

Harris, L., S. Foster, and R. Richards. 2002. An Introduction to *Staphylococcus aureus*, and techniques for Identifying and Quantifying *S. aureus* Adhesins in Relation to Adhesion to Biomaterials: Review. Europ. Cells Mater. 4:39-60.

Heldman, D.R., and R.L. Newsome. 2003. Kinetic Models for Microbial Survival during Processing. Food Tech. 57:40-47.

Hill, W. 1972. The Significance of Staphylococci in Meats. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 25. p.300-305.

Hinkens, J., N. Faith, T. Lorang, P. Bailey, D. Buege, C. Kaspar, and J, Luchansky. 1996. Validation of Pepperoni Processes for Control of *Escherichia coli* O157:H7. J. Food Protect. 59(12):1260-1266.

Hohmann, E. 2001. Nontyphoidal Salmonellosis. Clin. Infect. Dis. 32:263-269.

Humpheson, L., M.R. Adams, W.A. Anderson, and M.B. Cole. 1998. Biphasic Thermal Inactivation Kinetics in *Salmonella enteritidis* PT4. App. Env. Microbiol. 64(2):459-464.

Hunault, C., A. van Velzena, A. Sips, R. Schothorst, and J. Meulenbelt. 2009. Bioavailability of Sodium Nitrite from an Aqueous Solution in Healthy Adults. Toxicol. Lett. 190:48-53.

IARC. 2010. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Ingested Nitrate and Nitrite and Cyanobacterial Peptide Toxins. World Health Organization. International Agency for Research on Cancer. Vol. 94: http://monographs.iarc.fr/ENG/Monographs/vol94/mono94.pdf (accessed December 08, 2012).

ICMSF, 1996. Microorganisms in Foods 5. Characteristics of Microbial Pathogens. Blackie Academic & Professional, London (ISBN 0412 47350 X).

IFT. 2004. Bacteria Associated with Foodborne Diseases. Institute of Food Technologists. 525 West Van Buren St., Suite 1000, Chicago, IL 60607. www.ift.org/Knowledge-Center/Read-IFT-Publications/Science-Reports/Scientific-Status-Summaries/~/media/Knowledge%20Center/Science%20Reports/Scientific%20Status%20Summaries/bacteriafoodborne_0704.pdf (accessed 01/13/2013).

Ingelfinger, J.R. 2008. Melamine and the Global Implications of Food Contamination. N. Engl. J. Med. 359(26):2745-2748.

Isabelle, L., and L. Andre`. 2006. Quantitative Prediction of Microbial Behavior during Food Processing Using an Integrated Modeling Approach: A Review. Int. J. Refrig. 29:968-984.

Jay, J.M. 1996. Modern Microbiology, Third Edition. Van Nostrand Reinhold Co., New York, NY. p.331-345.

Jensen, L.B., and L.S. Paddock. 1939. Sausage Treatment. Patented December 24, 1940. Serial Number 266,537. U.S. Patent Office. Patent Number 2,225,783.

Jeong, S., B.P. Marks, and E.T. Ryser. 2011. Quantifying the Performance of *Pediococcus* sp. (NRRL B-2354: *Enterococcus faecium*) as a Nonpathogenic Surrogate for *Salmonella Enteritidis* PT30 during Moist-Air Convection Heating of Almonds. J. Food Protect. 74(4):603-609.

Jones, O. 1933. Nitrite in Cured Meats. The Analyst. 58(684):140.

Jordan, S., S. Perni, S. Glenn, I. Fernandes, M. Barbosa, M. Sol, R. Tenreiro, L. Chambel, B. Barata, I. Zilhao, T. Aldsworth, A. Adriao, M. Faleiro, G, Shuma, and P. Andrew. 2008. *Listeria monocytogenes* Biofilm-Associated Protein (BapL) May Contribute to Surface Attachment of *L. monocytogenes* but is absent from Many Field Isolates. App. Enviro. Microbiol. 74(17):5451-5456.

Kalinowski, R., R. Tompkin, P. Bodnaruk, and W. Pruett. 2003. Impact of Cooking, Cooling, and Subsequent Refrigeration on the Growth or Survival of *Clostridium perfringens* in Cooked Meat and Poultry Products. J. Food Protect. 66(7):1227-1232.

Kamau, D.N., S. Doores, and K.M. Pruitt. Enhanced Thermal Destruction of *Listeria monocytogenes* and *Staphylococcus aureus* by the Lactoperoxidase System. App. Envir. Microbiol. 59(9):2711-2716.

Kaspar, C., M. Doyle, J. Archer, and R. Klos. 2009. White Paper on Human Illness Caused by *Salmonella* from all Food and Non-Food Vectors. Food Research Institute, UW-Madison. www.pork.org/FileLibrary/ResearchDocuments/08-173-Kaspar-UofWis-FULL%20report.pdf.

Kececioglu, D. 1994. Reliability and Life Testing Handbook, Volume 2. PTR Prentice Hall, Inc. Simon and Schuster Company, Englewood Cliffs, NJ. pp.149-176.

Keeling, C., S.E. Niebuhr, G.R. Acuff, and J.S. Dickson. 2009. Evaluation of *Escherichia coli* Biotype I as a Surrogate for *Escherichia coli* O157:H7 for Cooking, Fermentation, Freezing, and Refrigerated Storage in Meat Processes. J. Food Protect. 72(4):728-732.

Keeton, J., W. Osburn, M. Hardin, N. Bryan, and M. Longnecker. 2009. A National Survey of the Nitrite/Nitrate Concentrations in Cured Meat Products and Non-meat Foods Available at Retail. National Pork Board Project #08-124. http://www.pork.org/FileLibrary/ResearchDocuments/08-124-KEETON-TxA-M.pdf (accessed December 08, 2012).

Kennedy, M., R. Villar, D. Vugia, T. Rabatsky-Her, M. Farley, M. Pass, K. Smith, P. Smith, P. Cieslak, B. Imhoff, and P. Griffin. 2004. Hospitalizations and Deaths Due to *Salmonella* Infections, FoodNet, 1996-1999. Clin. Infect. Dis. 38(Suppl 3):S142-S148.

Kim, J-M, and R.H. Linton. 2008. Identification of a Non-pathogenic Surrogate organism for Chlorine Dioxide (ClO2) Gas Treatment. Food Microbiol. 25:597-606.
Klutke, G.A., P.T. Kiessler, and M.A. Wortman. 2003. A Critical Look at the Bathtub Curve. IEEE Transact. Reliabil. 52(1):125-129.

Koning, N., M. Van Ittersum, G. Becx, M. Van Boekel, W. Brandenburg, J. Van Den Broek, J. Goudriaan, G. Van Hofwegen, R. Jongeneel, J. Schiere, and M. Smies. 2008. Long-term Global Availability of Food: Continued Abundance or New Scarcity? NJAS. 55(3):229-292.

Kothary, M., and U. Babu. 2001. Infective Dose of Foodborne Pathogens in Volunteers: A Review. J. Food Safety. 21:49-73.

Krishnamoorthi, K.S. 1992. Reliability Methods for Engineers. ASQC Quality Press. Milwaukee, WI. pp.82-87.

Kurk, F.W. 1921. Art of Curing Meat. Specification of Letters Patent. Patented May 31, 1921. Serial Number 441,503. U.S. Patent Office. Patent Number 1,380,068.

Lake, R., A. Hudson, and P. Cressy. 2007. Risk Profile: Shiga-Like Toxin Producing *Escherichia coli* in Uncooked Comminuted Fermented Meat Products. Institute of Environmental Science and Research Limited. Christchurch Science Center. Christchurch, NZ. www.foodsafety.govt.nz/elibrary/industry/Risk_Profile_Shiga_Toxin_Producing_Escherichia-Science_Research.pdf (accessed 01/12/2013).

Lamanna, C. 1959. The Most Poisonous Poison. Science. 130:763-772.

Lambert, R.J.W. 2003. A Model for the Thermal Inactivation of Micro-Organisms. J. App. Microbiol. 95:500-507.

Leighty, J. 1977. Update – Trichinosis. Food Safety and Quality Service, USDA. American Meat Science Association. Reciprocal Meat Science Proceedings. Vol. 30. p.177-180.

Leistner, L.E.E. 1992. Linkage of Hurdle-Technology with HACCP. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 45. pp.1-3.

Leporq, B., J.M. Membre, C. Dervin, P. Buche, J.P. Guyonnet. 2005. The ‘‘Sym’Previus’’ Software, a Tool to Support Decisions to the Foodstuff Safety. Int. J. Food Microbiol.100:231-237.

Levine, D. 2006. Vancomycin: A History. Clin. Infect. Dis. 42(S1):S5-S12.

Levine, P.B., B. Rose, S. Green, G. Ransom, and W. Hill. 2001. Pathogen Testing of Ready-to-Eat Meat and Poultry Products Collected at Federally Inspected Establishments in the United States, 1990-1999. J. Food Protect. 64:1188-1193.

Linton, R.H., W.H. Carter, M.D. Pierson, and C.R. Hackney. 1995. Use of a Modified Gompertz Equation to Model Nonlinear Survival Curves for *Listeria monocytogenes* Scott A. J. Food Protect. 58(9):946-954.

Linton, R.H., W.H. Carter, M.D. Pierson, C.R. Hackney, and J.D. Eifert. 1996. Use of a Modified Gompertz Equation to Predict the Effects of Temperature, pH, and NaCl on the Inactivation of *Listeria monocytogenes* Scott A Heated in Infant Formula. J. Food Protect. 59(10):16-23.

Lucke, F.K. 2000. Utilization of Microbes to Process and Preserve Meat. Meat Sci. 56:105-115.

Luzzi, I., P. Galetta, M. Massari, C. Rizzo, A.M. Dionisi, E. Feletici, A. Cawthorne, A. Tozzi, M. Argentieri, and S. Bilei. 2007. An Easter Outbreak of *Salmonella* Typhimurium DT104A Associated with Traditional Pork Salami in Italy. Euro Surveill. 12(3-6):149-152.

Mangus, C.A., A.R. McCurdy, and W.M. Ingledew. 1988. Further Studies on the Thermal Resistance of *Streptococcus faecium* and *Streptococcus faecalis* in Pasteurized Ham. J. Can. Inst. Food Sci. Technol. 21:209-212.

Marcy, J.A., A.A. Kraft, D.G. Olson, H.W. Walker, and D.K. Hotchkiss. 1985. Fate of *Staphylococcus aureus* in Reduced Sodium Fermented Sausage. J. Food Sci. 50:316-320.

Marcy, John. 2009. University of Arkansas. Personal Communication.

Marks, B.P. 2008. Status of Microbial Modeling in Food Process Models. Comp. Rev. Food Sci. Food Safe. 7:137-143.

Masters, B. 2006. Remarks prepared for Dr. Barbara J. Masters, Administrator, FSIS, at the American Veterinary Medical Association's Annual Convention, Event 2425, July 18, 2006, in Honolulu, Hawaii. www.fsis.usda.gov/News_&_Events/Speech_071806_Masters_01/index.asp.

Mathusa, E., Y. Chen, E. Enache, and L. Hontz. 2010. Non-O157 Shiga Toxin-Producing *Escherichia coli* in Foods. J. Food Protect. 73(9):1721-1736.

Mazzotta, A. 2000. D- and z- Values of *Salmonella* in Ground Chicken Breast Meat. J. Food Safe. 20:217-223.

McCarty, M., L. Bartoshuk, F. Busta, D. Clayson, D. Fine, S. Goldfarb, J. Gray, P. Greenwald, R. Hall, M. Kelsey, B. La Du, F. Oeime, D. Rowley, and J. Ryzin. 1982. Alternatives to the Current Use of Nitrite in Foods. Part 2 of a 2-Part Study by the Committee on Nitrite and Alternative Curing Agents in Food. Assembly of Life Sciences. National Academy Press. Washington, DC. p.1.3-1.5.

McDonald, K., and D-W Sun. 1999. Predictive Food Microbiology for the Meat Industry: A Review. Int. J. Food Microbiol. 52:1-27.

McMeekin, T.A., and T. Ross. 2002. Predictive Microbiology: Providing a Knowledge-Based Framework for Change Management. Int. J. Food Microbiol. 78:133-153.

McMeekin, T.A., J. Olley, D.A. Ratkowsky, and T. Ross. 2002. Predictive Microbiology: Towards the Interface and Beyond. Int. J. Food Microbiol. 73:395-407.

McMeekin, T.A., C. Hill, M. Wagner, A. Dahl, and T. Ross. 2010. Ecophysiology of Food-Borne Pathogens: Essential Knowledge to Improve Food Safety. Int. J. Food Microbiol. 139:S64-S78.

Mead, P., L. Slutsker, V. Dietz, L. McCaig, J. Bresee, C. Shapiro, P. Griffin, and R. Tauxe. 1999. Food-Related Illness and Death in the United States. Emerg. Infect. Dis. 5(5):607-625.

Membre, J.M., J. Thurette, and M. Catteau. 1997. Modeling the Growth, Survival and Death of *Listeria monocytogenes*. J. App. Microbiol. 82(3):345-350.

Merck. 2010. Listeriosis – Circling Disease. The Merck Veterinary Manual, Tenth Edition. Merck and Company. Whitehouse Station, NJ.

Metaxopoulos, J., C. Genigeorgis, M. Fanelli, C. Franti, and E. Cosma. 1981. Production of Italian Dry Salami: Effect of Starter Culture and Chemical Acidulation on Staphylococcal Growth in Salami under Commercial Manufacturing Conditions. App. Environ. Microbiol. 42(5):863-871.

Meyer, K.F. 1936. The Frequency of Botulism. California and Western Medicine. 44(5):385-388.

Meyer, K.F. 1956. The Status of Botulism as a World Health Problem. Bull. Wld. Hlth. Org. 15:281-298.

Milkowski, A., H. Garg, J. Coughlin, and N. Bryan. 2010. Nutritional Epidemiology in the Context of Nitric Oxide Biology: A Risk-Benefit Evaluation for Dietary Nitrite and Nitrate. Nitric Oxide 22:110-119.

Moore, J.E. 2004. Gastrointestinal Outbreaks Associated with Fermented Meats. Meat Sci. 67:565-568.

Mortimore, S., and C. Wallace. 1994. HACCP, A Practical Approach. Chapman and Hall. London, England. pp.xiii-50.

Munoz-Cuevas, M., A. Metris, and J. Baranyi. 2012. Predictive Modeling of *Salmonella*: From Cell Measurements to E-Models. Food Res. Int. 45:852-862.

Murphy, R.Y., E.R. Johnson, B.P. Marks, M.G. Johnson, and J.A. Marcy. 2001. Thermal Inactivation of *Salmonella senftenberg* and *Listeria innocua* in Ground Chicken Breast Patties Processed in an Air Convection Oven. Poultry Sci. 80:515-521.

Murray, E., R. Webb, and M. Swann. 1926. A Disease of Rabbits Characterized by a Large Mononuclear Luekocytosis, Caused by a Hitherto Undescribed Bacillus *Bacterium monocytogenes.* J. Pathol. Bacteriol. 29:407-439.

Naim, F., S. Messier, L. Saucier, and G. Piette. 2003. A Model Study of *Escherichia coli* O157:H7 Survival in Fermented Dry Sausages -- Influence of Inoculum Preparation, Inoculation Procedure, and Selected Process Parameters. J. Food Protect. 66(12): 2267-2275.

Neill, M. 1997. Overview of Verotoxigenic *Escherichia coli*. J. Food Protect. 60(11):1444-1446.

Neumeyer, K., T. Ross, and T.A. McMeekin. 1997. Development of *Pseudomonas* Predictor. Aus. J. Dairy Tech. 52:120-122.

Nickelson, R., J. Luchansky, C. Kaspar, and E. Johnson. Dry Fermented Sausage and *E. coli* O157:H7. Blue Ribbon Task Force. National Cattlemen's Beef Association. Research Report no.11-316.

Niebuhr, S.E., A. Laury, G.R. Acuff, and J.S. Dickson. 2008. Evaluation of Nonpathogenic Surrogate Bacteria as Process Validation Indicators for *Salmonella enterica* for Selected Antimicrobial Treatments, Cold Storage, and Fermentation of Meat. J. Food Protect. 71(4):714-718.

Nightingale, K.K., H. Thippareddi, R.K. Phebus, J.L. Marsden, and A.L. Nutsch. 2006. Validation of a Traditional Italian-Style Salami Manufacturing Process for Control of *Salmonella* and *Listeria monocytogenes.* J. Food Protect. 69(4):794-800.

Niinivaara, F.P. 1991. Starter Cultures in the Processing of Meat by Fermentation and Dehydration. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 44. pp.59-63.

Niskanen, A., and E. Nurmi. 1976. Effecti of Starter Culture on Staphylococcal Enterotoxin Thermonuiclease Production in Dry Sausage. App. Env. Microbiol. 31(1):11-20.

Nordal, J., and E. Slinde. 1980. Characteristics of Some Lactic Acid Bacteria Used as Starter Cultures in Dry Sausage production. App. Env. Microbiol. 40(3):472-475.

Nueno-Palop, C., and A. Narbad. 2011. Probiotic Assessment of *Enterococcus faecalis* CP58 Isolated from Human Gut. Int. J. Food Micribiol. 145:390-394.

Olsen, A. 1998. Regulatory Action Criteria for Filth and Other Extraneous Materials. I. Review of Hard or Sharp Foreign Objects as Physical Hazards in Food. Reg. Toxicol. Pharmacol. 28:181-1989.

Ordonez, J.A., E.M. Hierro, J.M. Bruna, and L. de la Hoz. 1999. Changes in the Components of Dry-Fermented Sausages during Ripening. Crit. Rev. Food Sci. Nutr. 39(4):329-367.

Palumbo, S., J. Smith, and L. Zaika. 1976. Sausage Drying: Factors Affecting the Percent Yield of Pepperoni. J. Food Sci. 41:1270-1272.

Palumbo, S., J. Call, F. Schultz, and A. Williams. 1995. Minimum and Maximum Temperatures for Growth on Verotoxin Production by Hemorrhagic Strains of *Escherichia coli*. J. Food Protect. 58(4):352-356.

Pearson, A.M., and T.R. Dutson. 1999. HACCP in Meat, Poultry and Fish Processing, Advances in Meat Research Series, Volume 10. A Chapman and Hall Food Science Book. Aspen Publishers, Inc., Gaithersburg, MD. pp.1-7.

Peleg, M., and M.B. Cole. 1998. Reinterpretation of Microbial Survival Curves. Crit. Rev. Food Sci. 38(5):353-380.

Pierson, M.D., and L.A. Smoot. 1982. Nitrite, Nitrite Alternatives, and the Control of *Clostridium botulinum* in Cured Meats. CRC Crit. Rev. Food Sci. Nutr. 17:141-187.

Pierson, M.D., and D.A. Corlett, Jr. 1992. HACCP, Principles and Application. Chapman and Hall, New York, NY. pp.1-27.

Pirie, J. 1940. *Listeria*: Change of Name for a Genus of Bacteria. Nature. 145:264.

Pond, T.J., D.S. Wood, I.M. Mumin, S. Barbut, and M.S. Griffith. 2001. Modeling the Survival of *Escherichia coli* O157:H7 in Uncooked, Semidry, Fermented Sausage. J. Food Protect. 64(6):759-766.

Pontello, M., L. Sodano, N. Nastasi, C. Mammina, and the working group: M. Astuti, M. Domenichini, G. Belluzzi, E. Soccini, M.G. Silvestri, M. Gatti, E. Gerosa, and A. Montagna. 1998. A Community-Based Outbreak *Salmonella enterica* Serotype Typhimurium Associated with Salami Consumption in Northern Italy. Epidemiol. Infect. 120:209-214.

Porto-Fett, A.C.S., C.A. Hwang, J.E. Call, V.K. Juneja, S.C. Ingham, B.H. Ingham, and J.B. Luchansky. 2008. Viability of Multi-Strain Mixtures of *Listeria monocytogenes*, *Salmonella typhimurium*, or *Escherichia coli* O157:H7 Inoculated into the Batter or Onto the Surface of a Soudjouk-Style Fermented Semi-Dry Sausage. Food Microbiol. 25:793-801.

Porto-Fett, A., J. Call, B. Shoyer, D. Hill, C. Pshebniski, G. Cocoma, and J. Luchansky. 2010. Evaluation of Fermentation, Drying, and/or High Pressure Processing on Viability of *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp., and *Trichinella spiralis* in Raw Pork and Genoa Salami. Int. J. Food Microbiol. 140:61-75.

Pyburn, D., H. Gamble, E. Wagstrom, L. Anderson, and L. Miller. 2005. Trichinae Certification in the United States Pork Industry. Vet. Parasit. 132:179-183.

Rahman, M. 2007. Handbook of Food Preservation, Second Edition. CRC Press, Taylor and Francis Group, 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, Florida.

Rajkowski, K., and B. Marmer. 1995. Growth of *Escherichia coli* O157:H7 at Fluctuating Incubation Temperatures. J. Food Protect. 58(12):1307-1313.

Ramaswamy, V., V. Cresence, J. Rejitha, M. Lekshmi, K. Dharsana, S. Prasad, and H. Vijila. 2007. *Listeria* – A Review of Epidemiology and Pathogenesis. J. Microbiol. Immunol. Infect. 40:4-13.

Rhodehamel, E., N. Reddy, and M. Pierson. 1992. Botulism: The Causative Agent and its Control in Foods. Food Control. 3(3):125-143.

Riemann, H.L., W.H. Lee, and C. Genigeorgis. 1972. Control of *Clostridium botulinum* and *Staphylococcus aureus* in Semi-Preserved Meat Products. J. Milk Food Technol. 35:514-523.

Robbins, J., A. Barth, H. Marquis, E. de Hostos, W. Nelson, and J. Theriot. 1999. *Listeria monocytogenes* Exploits Normal Host Cell Processes to Spread from Cell to Cell. J. Cell Biol. 146:1333-1349.

Roberts, T.A. 1975. The Microbial Role of Nitrite and Nitrate. J. Sci. Food Agric. 26:1755-1760.

Roberts, T.A., and A.M. Gibson. 1986. Chemical Methods for Controlling *Clostridium botulinum* in Processed Meats. Food Technol. 40:163-171, 176.

Ross, T., and T.A. McMeekin. 1994. Predictive Microbiology: A Review. Int. J. Food Microbiol. 23:241-264.

Ross, T., D. Zhang, and O.J. McQuestin. 2008. Temperature Governs the Inactivation Rate of Vegetative Bacteria under Growth-Preventing Conditions. Int. J. Food Microbiol. 128:129-135.

Ryser, E., and E. Marth. 1999. *Listeria*, Listeriosis, and Food Safety, Second Edition. Marcel Dekker, Inc. New York, NY.

Salmonella Subcommittee. 1930. The Genus *Salmonella* Lignières, 1900. International Society for Microbiology, Nomenclature Subcommittee. MS received for publication 8. v. 1934. – ED. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2170865/pdf/jhyg00240-0053.pdf.

Sauer, C.J., J. Majkowski, S. Green, and R. Eckel. 1997. Foodborne Illness Outbreak Associated with a Semi-Dry Fermented Sausage Product. J. Food Protect. 60(12):1612-1617.

Scallan, E., R. Hoekstra, F. Angulo, R. Tauxe, M. Widdowson, S. Roy, J. Jones, and P. Griffin. 2011. Foodborne Illness Acquired in the United States – Major Pathogens. Emerg. Infect. Dis. 17(1):7-15.

Schelin, J., N. Wallin-Carlquist, M. Cohn, R. Lindqvist, G. Barker, and P. Radstrom. 2011. The Formation of *Staphylococcus aureus* Enterotoxin in Food Environments and Advances in Risk Assessment. Virulence. 2(6):580-592.

Schlech, W., P. Lavigne, R. Bortolussi, A. Allen, E. Haldane, A. Wort, A. Hightower, S. Johnson, S. King, E. Nichols, and C. Broome. 1983. Epidemic Listeriosis – Evidence for Transmission by Food. N. Engl. J. Med. 308:203-206.

Schlech, W. 2000. Foodborne Listeriosis. Clin. Infect. Disease. 31:770-775.

Schut, J. 1978. The European Sausage Industry. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 31. p.5-12.

Scott, V.N. 2005. How does Industry Validate Elements of HACCP Plans? Food Control 16:497-503.

Scott, W.J. 1937. The Growth of Microorganisms on Ox Muscle: II. The Influence of Temperature. J. Council Sci. Indust. Res., Austral. 10:338-350.

Sebranek, J., and J. Bacus. 2007a. Natural and Organic Cured Meat Products: Regulatory, Manufacturing, Marketing, Quality and Safety Issues. American Meat Science Association White Paper Series. http://www.meatscience.org/page.aspx?id=403 (accessed December 08, 2012).

Sebranek, J., and J. Bacus. 2007b. Cured Meat Products without Direct Addition of Nitrate or Nitrite: What are the Issues? Meat Sci. 77:136-147.

Sebranek, J., A. Jackson-Davis, K. Myers, and N. Lavieri. 2012. Beyond Celery and Starter Culture: Advances in natural/Organic Curing Processes in the United States. Meat Sci. 92(3):267-273.

Shadbolt, C., T.Ross, and T.A. McMeekin. 2001. Differentiation of the Effects of Lethal pH and Water Activity: Food Safety Implications. Let. Appl. Microbiol. 32:99-102.

Sinclair, R.G., J.B. Rose, S.A. Hasham, C.P. Gerba, and C.N. Haas. 2012. Criteria for Selection of Surrogates Used to Study the Fate and Control of Pathogens in the Environment. Appl. Env. Microbiol. 78(6):1969-1977.

Sindelar, J., J. Sebranek, J. Bacus. 2010. Meat Processing Technology Series. Uncured, Natural, and Organic Processed Meat Products. American Meat Science Association. Champaign, IL.

Smith, J.L., C.N. Huhtanen, J.C. Kissinger, and S.A. Palumbo. 1975. Survival of *Salmonella* during Pepperoni Manufacture. App. Microbiol. 30(5):759-763.

Smith, J.L., and S.A. Palumbo. 1976. Injury to *Staphylococcus* aureus During Sausage Fermentation. App. Env. Microbiol. 36(6):857-860.

Smith, J.L. and S.A. Palumbo. 1983. Use of Starter Cultures in Meats. J. Food. Protect. 46:997-1006.

Sobel, J., N. Tucker, A. Sulka, J. McLaughlin, and S. Maslanka. 2004. Foodborne Botulism in the United States, 1990-2000. Emerg. Infect. Dis. 10(9):1606-1611.

Sofos, J.N., F.F. Busta, and C.E. Allen. 1979. Sodium Nitrite and Sorbic Acid Effects on *Clostridium botulinum* Spore Germination and Total Microbial Growth in Chicken Frankfurter Emulsions During Temperature Abuse. App. Environ, Microbiol. 37(6):1103-1109.

Sofos, J. 1981. Nitrite, Sorbate and pH Interaction in Cured Meat Products. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 34. p.104-120.

Steinhoff, Ulrich, 2005. Who controls the crowd? New findings and old questions about the intestinal microflora. Immunology Letters. 99:12-16.

Tannenbaum, S. 1972. Nitrite and Nitrosamine Content of Foods: Unsolved Problems and Current Research. American Meat Science Association. Reciprocal Meat Science Proceedings. Vol. 25. p.96-110.

Taormina, P., G. Bartholomew, and W. Dorsa. 2003. Incidence of *Clostridium perfringens* in Commercially Produced Cured Raw Meat Product Mixtures and Behavior in Cooked Products during Chilling and Refrigerated Storage. J. Food Protect. 66(1):72-81.

Tarr, P., T. Besser, D. Hancock, W. Keene, and M. Goldoft. 1997. Verotoxigenic *Escherichia coli* Infection: U.S. Overview. J. Food Protect. 60(11):1466-1471.

Tauber, F. 1976. The History of Sausage. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 29. p.55-60.

Tauxe, R. 1997. Emerging Foodborne Diseases: An Evolving Public Health Challenge. Emerg. Infect. Dis. 3(4):425-434.

Terrell, R.N., G.C. Smith, and Z.L. Carpenter. 1977. Practical Manufacturing Technology for Dry and Semi-Dry Sausage. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 30. pp.39-44.

Tilden, J., W. Young, A.M. McNamara, C. Custer, B. Boesel, M.A. Lambert-Fair, J. Majkowski, D. Vugia, S.B. Werner, J. Hollingsworth, and J.G. Morris. 1996. A New Route of Transmission for *Escherichia coli*: Infection from Dry Fermented Salami. Am. J. Public Health. 80(8):1142-1145.

Toldrà, F. 2002. Dry Cured Meat Products. Food and Nutrition Press. Trumbull, CT. p.30-146.

Toldrà, F. 2004. Food Processing: Principles and Applications. Meat: Fermented Meats. J. Smith and Y. Hui (eds.). Blackwell Publishing. 9600 Garsington Road, Oxford, United Kingdom. p.399-415.

Toldrà, F. 2007. Handbook of Fermented Meat and Poultry. Y.H. Yui, I. Astiasaran, W.K. Nip, J.G. Sebranek, E.T.F. Silveira, L.H. Stahnke, and R. Talon (eds.).Blackwell Publishing, Ames, IA.

Tompkin, R.B., L. Christiansen, and A. Shaparis. 1978a. Causes of Variation in Botulinal Inhibition in Perishable Canned Cured Meat. Appl. Environ. Microbiol. 35:886-889.

Tompkin, R.B., L. Christiansen, and A. Shaparis. 1978b. The Effect of Iron on Botulinal Inhibition in Perishable Canned Cured Meat. J. Food Sci. Technol. 13(6):521-527.

Tompkin, R.B., L. Christiansen, and A. Shaparis. 1978c. Enhancing Nitrite Inhibition of *Clostridium botulinum* with Isoascorbate in Perishable Canned Cured Meat. Appl. Environ. Microbiol. 35:59-61.

Tompkin, R.B. 1978d. The Role and Mechanism of the Inhibition of *C. botulinum* by Nitrite – Is a Replacement Available? American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 31. pp.135-147.

Tompkin, R. 2002. Control of *Listeria monocytogenes* in the Food-Processing Environment. J. Food Protect. 65(4):709-725.

USDA. 1995. Processing Inspectors' Calculations Handbook. United States Department of Agriculture, Food Safety and Inspection Service. www.fsis.usda.gov/OPPDE/rdad/FSISDirectives/7620-3.pdf (accessed December 08, 2012).

USDA. 2005. Food Standards and Labeling Policy Book. United States Department of Agriculture, Food Safety and Inspection Service. www.fsis.usda.gov/OPPDE/larc/Policies/Labeling_Policy_Book_082005.pdf (accessed December 08, 2012).

USDA. 2006a. FSIS Directive 7000.1, Verification of Non-Food Safety Consumer Protection Regulatory Requirements. United States Department of Agriculture, Food Safety and Inspection Service. www.fsis.usda.gov/OPPDE/rdad/FSISDirectives/7000.1.pdf

USDA. 2006b. Code of Federal Regulations. Title 9, Animals and Animal Products. Subpart C – Food Ingredients and Sources of Radiation. Section 424.21. Office of the Federal Register, National Archives and Records Administration. U.S. Government Printing Office. Washington, DC. p.636-660.

USDA. 2006c. Code of Federal Regulations. Title 9, Animals and Animal Products. Part 318 – Entry Into Official Establishments; Reinspection and Preparation of Products. Subpart A. Section 318.10 Prescribed Treatment of Pork and Products Containing Pork to Destroy Trichinae. p.246-256.

USDA. 2008. Trichinae Certification Program. Animal and Plant Health Inspection Service. Final Rule. Fed. Reg. 73(198):60464-60488.

USDA. 2011. Principles of Preservation of Shelf-Stable Dried Meat Products. United States Department of Agriculture, Food Safety and Inspection Service. www.fsis.usda.gov/PDF/FSRE_SS_7Principles.pdf.

USDA. 2013. Pathogen Modeling Program (PMP) Online. USDA-ARS Eastern Regional Research Center (ERRC) in Wyndmoor, Pennsylvania. http://pmp.arserrc.gov/PMPHome.aspx (accessed 03/31/2013).

Van Asselt, E.D., and M.H. Zwietering. 2006. A Systematic Approach to Determine Global Thermal Inactivation Parameters for Various Food Pathogens. Int. J. Food Microbiol. 107(1):73-82.

Van Boekel, M.A.J.S. 2002. On the Use of the Weibull Model to Describe Thermal Inactivation of Microbial Vegetative Cells. Int. J. Food Microbiol. 74(1):139-159.

Van Boekel, M.A.J.S. 2008. Kinetic Modeling of Food Quality: A Critical Review. Comp. Rev. Food Sci. Food Safe. 7:144-158.

Voetsch, A., T. VanGilder, F. Angulo, M. Farley, S. Shallow, R. Marcus, P. Cieslak, V. Deneen, and R. Tauxe. 2004. FoodNet Estimate of the Burden of Illness Caused by Nontyphoidal *Salmonella* Infections in the United States. Clin. Infect. Dis. 38(Suppl 3):S127-S134.

Vugia, D.J., Michael Samuel, Monica Farley, Ruthanne Marcus, Beletshachew Shiferaw, Sue Shallow, Kirk Smith and Fredrick Angulo, 2004. Invasive *Salmonella* Infections in the United States, FoodNet, 1996–1999: Incidence, Serotype Distribution, and Outcome. Clin. Infect. Dis. 38:(Suppl 3)S149-156.

Wardlaw, F.B., G.C. Skelley, M.G. Johnson, and J.C. Acton. 1973. Changes in Meat Components During Fermentation, Heat Processing and Drying of Summer Sausage. J. Food Sci. 38:1228-1231.

Williams, R.C., S. Isaacs, M.L. Decou, E.A. Richardson, M.C. Buffett, R.W. Slinger, M.H. Brodsky, B.W. Ciebin, A. Ellis, J. Hockin, and the E. coli O157:H7 Working Group. 2000. Illness Outbreak Associated with *Escherichia coli* O157:H7 in Genoa Salami. Can. Med. Assoc. J. 162(10):1409-1413.

Wilson, G. 1988. *Listeria monocytogenes* – 1988. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 41. p.11-13.

Xiong, R., G. Xie, A.E. Edmondson, R.H. Linton, and M.A. Sheard. 1999a. Comparison of the Baranyi Model with the Modified Gompertz Equation for Modeling Thermal Inactivation of *Listeria monocytogenes* Scott A. Food Microbiol. 16:269-279.

Xiong, R., G. Xie, A.E. Edmonston, and M.A. Sheard. 1999b. A Mathematical Model for Bacterial Inactivation. Int. J. Food Microbiol. 46:45-55.

IV. Chapter 2

Inactivation of Enteric Pathogens in Fermented Pepperoni Formulated with Chicken

Evaluation of the Inactivation of a Cocktail of *Salmonella* spp. and *Escherichia coli* O157:H7 in a High Fat, Dry, Fermented Sausage Product (Pepperoni Pizza Topping with 20% Mechanically Separated Chicken)

Scott Stillwell*

Tyson Foods, Inc., Springdale, Arkansas 72762, USA

Key words: *Salmonella* spp., *E. coli* O157:H7, pepperoni, microbial inactivation, fermentation, sausage

*Author for correspondence. Tel: (479) 290-7186; Fax: (479) 757-6888
E-mail: scott.stillwell@tyson.com

ABSTRACT

Fermented meats have been associated with outbreaks of illnesses due to surviving strains of *Salmonella* and enteropathogenic *Escherichia coli* O157:H7. Following an outbreak of *E. coli* O157:H7 associated with salami in 1994, the Blue Ribbon Task Force developed options for manufacturers of fermented meat products to ensure adequate control of *E. coli* O157:H7. Over the course of the last decade, manufacturers have been modifying fermented sausage formulations to include poultry products as a means of reducing the product cost. During this same period, the USDA's Food Safety Inspection Service staff announced their intent to extend the meat and poultry pathogen reduction performance standards to all Ready to Eat (RTE) meat and poultry products. Given the scarcity of data associated with fermented products formulated with poultry and current process capability to meet a 7.0 Log_{10} inactivation of the salmonellae, it is necessary to assess the cumulative inactivation achieved during processing. A high fat pepperoni product was formulated with 20% poultry; fermented utilizing a starter culture to pH below 4.95; heat treated; diced and dried. Through the cook step, the process achieved a cumulative inactivation of a cocktail of strains of *Salmonella* and *E. coli* O157:H7 of at least 7.5 and 6.5 Log_{10} respectively.

Despite significant improvements in the past two decades, the burden of foodborne illness in the United States continues to be significant with a recent estimate of greater than 9 million annual cases of domestic illness acquired from the consumption of foods contaminated with one of the major foodborne pathogens. Salmonellosis is one of the leading human foodborne illnesses with current estimates of approximately 3,500 illnesses per 100,000 people per year in the domestic population – second only to illnesses caused by viruses (Scallan *et al*. 2011). The case rate of human salmonellosis has been relatively constant since 1998 and, unlike other notable enteric pathogens such as *Escherichia coli* (*E. coli*) O157:H7, progress has not been made toward the national goals of reducing foodborne illnesses caused by the salmonellae (CDC 2011a). While any of the more than 2,500 species of *Salmonella* are considered capable of causing illness, approximately twenty species are responsible for the vast majority of human illnesses – causing approximately 1 million cases (approximately 42,000 culture-confirmed illnesses are reported each year to the CDC), 19,000 hospitalizations and 400 deaths each year (CDC 2012). Some have estimated that as many as 95 percent of all human cases of salmonellosis are foodborne (Hohmann 2001 and Scallan *et al*. 2011).

Enterohemorrhagic *E. coli* (EHEC) are the most widely recognized and notorious of the pathogenic strains of *E. coli*. The EHEC are characterized by the presence of genes that (among other virulence factors) encode for the production of a Shiga-like toxin (Neill 1997) leading to the currently accepted nomenclature Shiga-toxin producing *E. coli* (STEC). Those STECs that cause severe illness are grouped as the EHEC and include the prototypical *E. coli* O157:H7 which was first declared an adulterant in ground beef products following a fast food chain related outbreak of illnesses in 1993 traced to undercooked hamburgers (Tarr *et al.* 1997). Recently, the “Big-6” non-O157 EHEC serotypes have been declared adulterants in non-intact beef products. These include: O26; O45; O103; O111; O121; and O145 which, together with O157:H7 account for the vast majority of all cases of hemorrhagic colitis in the U.S. (FDA 2012).

Both pathogenic strains of *E. coli* and a variety of serotypes of *Salmonella* have been implicated in illnesses and outbreaks associated with “Ready to Eat” (RTE) fermented dry and semi-dry sausages. Most recently in the United States, a multi-state outbreak of *E. coli* O157:H7 was responsible for 14 illnesses epidemiologically associated with a regional brand of Lebanon bologna that was subsequently

recalled (CDC 2011b). Beginning in July, 2009 a nationwide outbreak caused by *Salmonella* Montevideo with matching Pulsed-Field Gel Electrophoresis (PFGE) patterns (same genotype) was confirmed to have been caused by a salami product apparently contaminated by pepper used as a coating on the finished sausage. During this outbreak, the same genotype of *Salmonella* Montevideo was isolated from the salami, ill consumers of the salami, and subsequently, from samples of the black and red pepper used to manufacture the salami. The outbreak resulted in 272 illnesses in 44 states and the District of Columbia and ended when the salami products were recalled. Interestingly, during the outbreak investigation, a genotypically-indistinguishable strain of *Salmonella* Senftenberg was isolated from 11 ill patients and the implicated salami products indicating that the pepper was contaminated with at least two different serotypes of *Salmonella* (CDC 2010).

A primary protein-based ingredient of many dry and semi-dry fermented sausages – particularly in the U.S. domestic market – is beef. Over the past two decades, *E. coli* O157:H7 has emerged as the principle microbial hazard associated with foods comprised of beef or – as is often the case with sausage products -- mixtures of meats containing beef. This is particularly true for fermented meat products as it has been demonstrated repeatedly that *E. coli* O157:H7 exhibits substantial acid and salt tolerance and can survive and in many cases proliferate under the conditions achieved by many traditional meat fermentation processes (Erickson and Doyle 2007, Glass *et al*. 1992, Riordan *et al*. 1998). As such, domestic regulatory policy and scientific research has focused on the control of the hazard associated with the presence of this pathogen. The regulatory approach has been predicated upon the concept that adequate control of *E. coli* O157:H7 in meat products would ensure an adequate level of control for other types of vegetative human microbial pathogens likely to be associated with raw red meat protein components (FSIS 2001a).

Following a 1994 outbreak of illnesses caused by *E. coli* O157:H7 in fermented beef salami sausages (Tilden *et al*. 1996), the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) staff worked in collaboration with industry representatives from the "Blue Ribbon Task Force" to develop five options designed to ensure the safety of fermented dry and semi-dry meat products. The most rigorous of the options required the processor to demonstrate a 5.0 $\log_{10}$ reduction of *E. coli* O157:H7 through the process (Nickelson *et al*. 1996, Naim *et al*. 2003).

Beginning in January 1999, following the implementation of the HACCP regulations (FSIS 1996), FSIS converted prescriptive time and temperature processing requirements for certain RTE meat and poultry products into performance standards requiring the process to achieve either a 6.5 $\log_{10}$ (meat products) or a 7.0 $\log_{10}$ (poultry products) inactivation of serotypes of *Salmonella* in the finished product. These standards applied to RTE roast beef, corned beef, cooked beef and all fully cooked poultry products (FSIS 1999a). Specific time and temperature combinations that were validated to achieve those reductions were published in a FSIS Directive as "Appendix-A" (FSIS 1999b).

In February 2001, FSIS staff proposed to extend these performance standards to all RTE meat and poultry products (FSIS 2001b). Further, the time and temperature guidance in Appendix-A was expanded based upon the work of Juneja *et al*. who had modeled the effects of varying fat levels in poultry product formulations on the thermal inactivation of *Salmonella* spp. These models, which included data for fat levels between 1 – 12% of the formulation, indicated that inactivation kinetics were altered by fat in the product and postulated that higher fat levels provided a thermal-protective effect. The staff at FSIS modified Appendix-A to include time and temperatures tables based on fat levels and poultry specie that provided a variety of combinations validated to achieve the required 7.0 $\log_{10}$ lethality of *Salmonella* spp. Since the publication of the Juneja paper in 2001, it has become a standard expectation that higher fat formulations will result in slower and/or lower cumulative vegetative pathogen inactivation.

In March 2005, the FSIS staff published Notice 16-05 notifying the regulated industry of a draft proposal to update Appendix-A to include (among other things): a requirement to address in HACCP plans the specific hazards associated with serotypes of *Salmonella* in products currently validated to control *E. coli* O157:H7 such as fermented sausages containing poultry; and specific requirements to process such fermented products containing poultry derived from chicken to a minimum of 136^{o}F (57.8^{o}C) for 76.7 minutes; or for such fermented products containing poultry derived from turkey to a minimum of 136^{o}F (57.8^{o}C) for 68.4 minutes (FSIS 2005). These proposed changes have yet to be enacted. However, in January 2013, the FSIS staff published in the Semi-Annual Regulatory Agenda an announcement of their intent to publish in September 2013 an Interim Final Rule codifying these requirements (FSIS 2013).

In the years immediately after the 1994 salami outbreak, most research on the safety of fermented sausages focused on adequate control of *E. coli* O157:H7 (Barbut and Griffiths 2001, Faith *et al*. 1997, Hinkens *et al*. 1996, and Riordan *et al*. 2000). However, as the regulatory proposals have evolved and other pathogens such as *Listeria monocytogenes* have "emerged" in RTE meat and poultry products, researchers have more frequently included data on the inactivation or control of a more diverse variety of microbial hazards associated with dry and semi-dry fermented sausages (Barbuti and Parolari 2002, Deumier and Collignan 2003, Porto-Fett *et al*. 2008, and Smith *et al*. 1975). Unfortunately, scant data exist in published literature concerning fermented meat products formulated to contain poultry and whether manufacturing processes are validated to be capable of reliably meeting the proposed regulatory performance standard of a 7.0 $\log_{10}$ inactivation of the salmonellae.

In recent years, domestic production of fermented sausage products formulated to contain raw materials of poultry origin has increased substantially. This is due, in part, to the proliferation of low cost; frozen; ready to heat and serve pizzas (and other products such as hand-held dough-enrobed "pizza pockets") which contain pepperoni as a principle meat ingredient. The manufacturers of these pizza products compete on the basis of quality and taste – with a very heavy advertising emphasis on the low price of the products. As such, a great deal of interest in formulating lower cost pepperoni products has led to the inclusion of alternative protein ingredients such as mechanically separated chicken or turkey in place of some portion of the beef and/or pork components (the total proportion of each frequently depending on a least cost formulation and current commodity prices).

The March 2005 draft modifications to the *Compliance Guidelines for Meeting Lethality Performance Standards for Cooked, Ready to Eat Meat and Poultry Products* ("Appendix A") represent a significant departure from some of the most commonly utilized processing parameters and, if codified in the 2013 Interim Final Rule, are expected to result in significant reductions in product quality and yield with a concomitant increase in product cost. The validated processing conditions most widely utilized in commercial processes for control of *E. coli* O157:H7 in stick pepperoni are 128^{o}F (53.3^{o}C) for 60 minutes or 145^{o}F (62.8^{o}C) for an instantaneous lethality (5.0 $\log_{10}$ inactivation) (Hinkens *et al*.). The standards proposed by the staff at FSIS are expected to result in significant "fatting out" (the melting of globular fat with subsequent migration from the meat matrix with accumulation between the sausage and its casing);

color and texture changes; and reductions in throughput with particular difficulties during drying due to the product fatting out (the fat creates a moisture vapor barrier and retards drying). Therefore, it has been deemed advantageous to provide alternative time at temperature combinations that are validated to achieve the proposed minimum microbial inactivation (e.g. a 6.5 $\log_{10}$ reduction of serotypes of *Salmonella* in products formulated solely with pork and beef or a 7.0 $\log_{10}$ reduction in products formulated with poultry in any ratio).

The present study was designed to assess the cumulative microbial inactivation of a cocktail of serotypes of *Salmonella* and a cocktail of strains of *E. coli* O157:H7 resulting from the application of a unique and proprietary commercial manufacturing process utilized to make a diced pepperoni pizza topping containing poultry meat in the formulation. The data derived from the study were analyzed to assess whether the multi-step manufacturing process – consisting of fermentation, heat processing, drying, and freezing – is sufficiently robust to deliver at least a cumulative 7.0 $\log_{10}$ inactivation of the serotypes of *Salmonella* and a 5.0 $\log_{10}$ inactivation of the strains of *E. coli* O157:H7. These data will form the basis for validation of the commercial process (a time and temperature combination differing from the FSIS proposal) to achieve the required microbial inactivation in an extremely high fat formulation to ensure the worst-case estimate of cumulative inactivation. Further, the data will help guide additional research efforts intended to develop a mathematical model for predicting inactivation of serotypes of *Salmonella* in fermented meat products utilizing a non-pathogenic surrogate microorganism appropriate for inclusion in a commercial product formulation such that in-plant process validations may be conducted.

MATERIALS AND METHODS

Approximately 100 pounds of raw, commercially processed non-standard pepperoni pizza topping "batter" was obtained from a large manufacturing operation owned by Tyson Foods, Inc. operating in Dallas, Texas. The batter was prepared by the processing plant staff utilizing the commercial manufacturing equipment and divided from a much larger batch immediately prior to the introduction of microbial starter culture (all other processing steps had been completed). The formulation (in order of predominance) consisted of pork (approximately 75% of the meat block), mechanically separated chicken (approximately 20% of the meat block), beef (approximately 5% of the meat block), water, soy protein

isolate, salt, spices, dextrose, oleoresin of paprika, sodium ascorbate, dehydrated garlic, sodium nitrite, BHA, BHT, and citric acid with a finished product specification of 34 – 44% fat (Tyson Product Code 11054-0043). The meat batter was divided into two portions, placed into plastic bags and transported in refrigerated coolers to the Tyson Foods corporate laboratory in Springdale, Arkansas where it was held overnight in a commercial walk-in cooler at 34^{o}F (1.1^{o}C).

On the following day, twelve individual 1,500 gram portions of pepperoni batter were weighed into plastic sample bags and placed into a retail-style reach-in display cooler at approximately 40^{o}F (4.4^{o}C) until inoculated; whereupon they were formed into loaves approximately 6 inches wide, by 12 inches long, and 3.5 inches thick (to approximate the thickness of the commercial product); covered with foil; and returned to the cooler pending transport to the research cookhouse at the University of Arkansas pilot facility in Fayetteville, Arkansas.

Inocula Preparation: Three concentrated microbial cultures for inoculation were prepared. The first culture consisted of a commercially obtained lactic acid starter culture (SAGA 200 containing the homo-fermentative lactic acid-producing bacteria *Pediococcus acidilactici* from Kerry Ingredients and Flavors, Beloit, WI 53511) maintained prior to use frozen in a commercial, retail ice cream freezer (less than -15^{o}F (-8.3^{o}C)). The second culture consisted of an overnight (18 -- 24 hours after the concentrated microorganisms were washed, re-suspended in sterile saline and refrigerated) culture of a five-serotype cocktail of *Salmonella* comprised of: *Salmonella* Senftenberg ATCC 43845 obtained from the Tyson Foods collection; and four serologically distinct “wild” strains of *Salmonella* of unknown serotype isolated from routine whole-bird carcass rinse samples of commercial broilers from Tyson Foods’ commercial poultry slaughter operations. The third culture consisted of an overnight culture of a three-serotype cocktail of *E. coli* O157:H7 comprised of three serologically distinct “wild” strains isolated from routine samples of beef trim from Tyson Foods’ commercial fed cattle slaughter, fabrication and grinding operations.

Immediately before inoculating the meat portions, the frozen puck of starter culture was suspended in a volume of room temperature tap water per the supplier’s directions. The pathogen cultures had been concentrated by Tyson Foods’ research microbiologists to provide an initial inoculation level of approximately $10^{7.5}$ colony forming units (CFUs) per gram of pepperoni batter. In no particular order, one

of the twelve samples of raw pepperoni batter was removed from the holding cooler and the meat was transferred to a large baking sheet covered with clean aluminum foil and divided into two approximately equal portions which were flattened by gloved hand into disks.

Sample Preparation: The inocula were transferred by pipette with half the volume applied to each of the two meat portions; starting with the cocktail of *Salmonella* strains and ending with the commercial starter culture; slowly dripping the inocula across the surface of the meat batter. Once the inocula were transferred to the meat, each portion was thoroughly mixed to ensure homogenous distribution of the microorganisms. This was accomplished by repetitively kneading and flattening the portion by hand; then using the knife-edge of the hands, forming the sample into a rope which was rolled or coiled upon itself and kneaded and flattened again for a total of 10 repetitions. After each portion had been thoroughly mixed, the two portions were combined and mixed in the same manner for a total of ten repetitions. After mixing, the sample was formed by hand into a loaf in a 10 inch wide, by 14 inch long, by 4 inch deep heavy foil baking pan; covered with foil; and transferred to the holding cooler.

Once each sample was inoculated, each pan was randomly assigned to one of six sample types with two replicates (A and B): 0) inoculated raw; 1) inoculated fermented; 2) inoculated fermented and cooked; 3) inoculated fermented, cooked, diced and dried; 4) inoculated fermented, cooked, diced and re-inoculated; 5) inoculated fermented, cooked, diced, re-inoculated and dried; with each pan permanently marked appropriately. Samples 1-A and B through 5-A and B remained in the cooler pending transfer to the pilot plant cookhouse; while samples 0-A and B were used to weigh 20 individual 25 gram samples into stomacher bags for microbiological enumeration to establish the initial (time zero) pathogen count per gram of pepperoni batter in each of the two replicates.

Sample Processing: The remaining samples were transported to the U of A pilot processing plant, uncovered, and placed in randomly chosen locations on a rack (all samples at the same level for consistent heat exposure) inside the Alkar smokehouse (Model #1000, Lodi, WI). The smokehouse was programmed to operate at 112^{o}F (44.4^{o}C) (wet bulb) for 12 hours after the center of mass thermocouple indicated the samples had reached 112^{o}F (44.4^{o}C) for fermentation which was allowed to proceed overnight.

The next morning, after an elapsed 12 hours held at 112°F (44.4°C) internal temperature, the pH was obtained from each sample to ensure a successful fermentation. Once the pH of each sample was verified to be less than 4.95 by surface probe (range 4.40 – 4.69, data not shown), the fermentation samples 1-A and B were placed into a blast freezer and rapidly chilled to below 28°F (-2.2°C) then transferred to a 34°F (1.1°C) holding cooler. The remaining samples were returned to the Alkar smokehouse and the cook cycle was programmed: 145°F (62.8°C) dry bulb; and 140°F (60.0°C) wet bulb temperatures were programmed with the center of mass thermocouple to be maintained at 137°F (58.3°C) for 75 minutes after the target temperature was reached. After the cook cycle was completed, the samples were immediately transferred to a blast freezer and rapidly cooled to below 28°F (-2.2°C) before transporting them back to the Tyson Foods lab for further processing and microbiological analyses.

Sample sets 1-A and B and 2-A and B were used to weigh 20 individual 25 gram samples into stomacher bags for microbiological enumeration to establish the pathogen count per gram of pepperoni in each replicate after 1) fermentation and after 2) cooking. Samples 3-A and B through 5-A and B were individually aseptically hand diced into (approximately) 3/8 inch cubes. After dicing, each of sample 4-A and B and 5-A and B were placed into individual plastic sample bags and re-inoculated with a volume of the overnight cultures containing cocktails of *Salmonella* and *E. coli* O157:H7 serotypes to achieve (approximately) 10^4 CFUs per gram. From each of the re-inoculated samples 4-A and B, 20 individual 25 gram samples were removed and weighed into stomacher bags for microbial enumeration.

For drying the diced pepperoni, a food dehydrator (Model FD-75 PR Nesco Professional Dehydrator) was purchased new. The circular drying trays were divided in two and marked with a permanent marker to indicate replicate A or B. Each of the samples 3-A and B and 5-A and B were separately placed onto the designated drying tray; each tray was weighed; and the weight of each tray was recorded. The drying trays were loaded onto the dehydrator in random order from bottom to top and the dehydrator was set to operate at 110°F (43.3°C). After 75 minutes, the trays were weighed and the Moisture:Protein Ratio was calculated and determined to be less than 1.6:1 as required for pepperoni. From each replicate of samples 3-A and B and 5-A and B, 20 individual 25 gram samples were weighed into stomacher bags for microbiological enumerations.

Microbiological Analyses: Using standard protocols for sample preparation, serial dilution and spread-plate enumeration, Tyson Foods corporate research microbiologists prepared each individual sample for recovery and enumeration. Trypticase Soy Agar plates (in duplicate for each sample and dilution) were serially inoculated; dried for approximately 2 hours in a 35^{o}C incubator; overlaid with selective media (Xylose-Lysine-Tergitol 4 for recovery of the *Salmonella* strains or Sorbitol MacConkey Agar for recovery of the *E. coli* O157:H7 strains); and incubated for 24 hours at 35^{o}C. Following incubation, each set of plates was examined for typical colonies. Any plates showing no typical colony growth or indications of microbial stress were returned to the incubator for an additional 24 hours. Following standard micro-lab protocols, the research microbiologists reported quantitative results for *Salmonella* spp. and *E. coli* O157:H7 recovered from each replicate of each sample type.

Statistical Analyses: Utilizing the Statistical Analysis System (SAS) version 9.1 software for Windows (SAS Institute, Cary NC), the data were log-transformed with all "none detected" samples reported as 1.0 CFU/g. The mean values for microbial reduction in viable count were analyzed for each replicate and processing treatment to determine whether replicates exhibited significantly different levels of microbial inactivation; to assess the treatment effect on microbial inactivation; and to determine the cumulative microbial inactivation achieved. The SAS General Linear Model was utilized for the Means Test, Analysis of Variance and the Duncan's Multiple Range Test.

RESULTS

Rather unexpectedly, the level of inactivation achieved through the fermentation and thermal (cooking) processing was sufficient to provide a 7.5 $\log_{10}$ inactivation of the *Salmonella* spp. and a 6.5 $\log_{10}$ inactivation of the *E. coli* O157:H7. The initial (time zero) raw inoculated meat batter had a mean starting load of 7.5 and 6.5 $\log_{10}$ respectively and after the cook step, there were no recoverable survivors (Figure 1). The reductions measured between each treatment were highly significant ($p < 0.0001$). The SAS summary statistics are presented in Table 1. None of the reps differed, allowing us to combine the data from the two replications at each treatment.

DISCUSSION

Unfortunately, the direct plate method of enumeration provides discrimination only down to 10^{-1} dilution (as the 25 gram sample is stomached in 225 ml of buffered peptone; a 10:1 dilution). Thus, by

having no survivors in the after cooking samples (at the 10^{-1} dilution level), it is not possible for us to estimate the total microbial inactivation (decimal reduction) achieved as we have no data to estimate the end point for the inactivation curve. Thus, all statistical analyses are confounded and there are no data for construction of a best fit model. Given the review of literature on inactivation achieved in fermented sausages and the initial inoculation levels, it was expected that countable numbers of survivors would have been present in the cooked samples.

This leaves a very limited number of conclusions that may be drawn from the statistical analyses of the data set; the most commercially important of which is that this unique, proprietary process achieves a reduction through inactivation of both vegetative pathogens sufficient to comply with the existing regulations for fermented products: at least a 5.0 $\log_{10}$ reduction of *E. coli* O157:H7; and also achieves the level of reduction proposed to be required in the Interim Final Rule for products containing poultry meat: at least a 7.0 $\log_{10}$ reduction of *Salmonella* spp. Even with the limitations of the direct plate count method previously discussed, as illustrated in Figure 1, this conclusion is supported by the cumulative inactivation data from the fermentation and thermal processing treatments alone. It is not necessary to take into account any additional reductions demonstrated by these data to occur during drying; and we may completely disregard any inactivation hypothesized to occur during freezing and storage (data were not obtained to assess those treatment effects).

The inactivation achieved through fermentation and cooking – particularly given the inclusion of the heat resistant strain *Salmonella* Senftenberg in the inoculums -- is more than adequate to meet regulatory requirements. Additional inactivation that occurs during drying, when accumulated with the reduction achieved during fermentation and thermal processing, serve to illustrate that this unique, proprietary process for the manufacture of pepperoni pizza topping is extremely robust.

These data represent an initial assessment of the microbial inactivation achieved during processing of a high fat pepperoni pizza topping containing poultry meat. The process applied is unique, proprietary and patented. Given the stated intent of representatives of the USDA's FSIS to require microbial reduction performance standards to fermented products containing poultry meat that are more stringent than those that are currently in effect, it is necessary to assess whether the existing process parameters are sufficiently lethal to the pathogens of concern. As is evident by the data represented in Figure 1, the

process achieves microbial inactivation sufficient to meet the current and proposed standards merely by fermenting and cooking the product (discounting the reductions achieved during drying, freezing and storage). As an alternative process to the time and temperature combinations published in FSIS Notice 16-05, the time and temperature processing conditions utilized to produce this product are validated to achieve the necessary levels of microbial inactivation. Given that the product formulation utilized for this trial is extremely high fat, based upon the conclusions that the FSIS staff have used to develop the process tables contained in Appendix-A, the results of this study should extend to product formulations with lower fat levels which are expected to achieve similar, if not greater, levels of microbial inactivation.

The reduction achieved during fermentation and cooking was surprising to the investigators as the literature reviewed would not have suggested such a large reduction would have been achieved under the conditions described. However, as this process is unique, there are no data currently in the literature that are representative of the product manufactured in this process. The data from the literature for microbial inactivation when manufacturing pepperoni are generally derived from traditional stick pepperoni products with a cross section of no greater than 25 – 30 mm. The process examined in this work consists of loaves of raw pepperoni batter with a minimal cross section approximately double a traditional stick. The time to achieve the core temperature in the loaf is much greater than with a stick – thus, the accumulated time at temperature will be much greater. Given that thermal penetration is going to occur most rapidly across the shortest distance, the miniature loaves utilized in this study were deemed representative of the commercial process as the loaf thickness was maintained to the processing specification of 3.5 inches. Thus, we were assured that the target core cook temperature would be achieved no faster than the commercial process and the results obtained would be representative and conservative.

These data clearly demonstrate previously published concerns about the acid tolerance of *E. coli* O157:H7 as potential explanation for outbreaks caused by fermented products in years prior to the changes recommended by the Blue Ribbon Task Force. As is apparent in Figure 1 and Table 1, the microbial inactivation achieved during the fermentation treatment was significantly greater ($p < 0.001$) for the strains of *Salmonella* than for the strains of *E. coli* O157:H7 (reductions of 3.4 versus 1.53 $\log_{10}$,

respectively). No strain difference was observed in the data obtained from drying and no assessment of the cook treatment could be made given there were no after cook survivors to assess the end point.

With the exception of the *Salmonella* Senftenberg which was included due to its notable thermal tolerance, the strains of *Salmonella* and *E. coli* O157:H7 utilized in the present study were isolated from commercial samples and are expected to possess characteristics representative of the strains present in raw materials utilized for the manufacture of fermented meat products. As such, these data provide additional support for previous researchers (Ellajosyula et al. 1998, Hinkens *et al*. 1996, Incze 1998, Smith *et al*. 1975) that have concluded a thermal processing step is a critical component necessary to achieve sufficient pathogen inactivation during manufacture of RTE fermented meat products.

These data demonstrate that the manufacturing process utilized by Tyson Foods to make this pepperoni pizza topping is adequate to ensure finished RTE product microbial safety and achieve all relevant regulatory requirements for pathogen reduction in fermented meat products containing poultry in the formulation. The data will help guide future research intended to assist with validation trials of commercial manufacturing processes.

V. REFERENCES

Barbut, S., and M.W. Griffiths. 2001. Developing Validation Models for *E. coli* O157 Inactivation in Dry Fermented Sausages. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 54. p. 44-48.

Barbuti, S., and G. Parolari. 2002. Validation of Manufacturing Process to Control Pathogenic Bacteria in Typical Dry Fermented Products. Meat Sci. 62:323-329.

CDC. 2010. Investigation Update: Multistate Outbreak of Human *Salmonella* Montevideo Infections. http://www.cdc.gov/salmonella/montevideo/index.html (accessed 07/15/2012).

CDC. 2011a. Trends in Foodborne Illness, 1996-2010. Atlanta, Georgia: US Department of Health and Human Services, CDC. www.cdc.gov/foodborneburden/PDFs/FACTSHEET_B_TRENDS.PDF

CDC. 2011b. Investigation Announcement: Multistate Outbreak of E. coli O157:H7 Infections Associated with Lebanon Bologna. http://www.cdc.gov/ecoli/2011/O157_0311/index.html (accessed 01/12/2013).

CDC. 2012. *Salmonella*. Technical Information. www.cdc.gov/salmonella/general/technical.html (accessed 07/15/2012).

Deumier, F., and A. Collignan. 2003. The Effects of Sodium Lactate and Starter Cultures on pH, Lactic Acid Bacteria, *Listeria monocytogenes* and *Salmonella* spp. Levels in Pure Chicken Dry Fermented Sausage. Meat Sci. 65: 1165-1174.

Ellajosyula, K.R., S. Doores, E.W. Mills, R.A. Wilson, R.C. Anantheswaran, and S.J. Knabel. 1998. Destruction of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in Lebanon Bologna by Interaction of Fermentation pH, Heating Temperature, and Time. J. Food Protect. 61(2): 152-157.

Erickson, E.C., and M.P. Doyle. 2007. Food as a Vehicle for Transmission of Shiga Toxin-Producing *Escherichia coli*. J. Food Protect. 70(10): 2426-2449.

Faith, N., N. Parniere, T. Larson, T. Lorang, and J. Luchansky. 1997. Viability of *Escherichia coli* O157:H7 in Pepperoni during the Manufacture of Sticks and the Subsequent Storage of Slices at 21, 4 and -20^{o}C Under Air, Vacuum and CO_2. Int. J. Food Microbiol. 37:47-54.

FDA, 2012. Bad Bug Book -- Foodborne Pathogenic Microorganisms and Natural Toxins Handbook – Second Edition. www.fda.gov/downloads/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/UCM297627.pdf

FSIS. 1996. 9 CFR Part 304, *et al*. Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) System; Final Rule. Fed. Reg. 61(144): 38805-38855.

FSIS. 1999a. 9 CFR Parts 301, 317, 318, 320, and 381.Docket No. 95-033F. Production Standards for the Production of Certain Meat and Poultry Products. Fed. Reg. 64(3): 732-749.

FSIS. 1999a. Appendix A, Compliance Guidelines for Meeting Lethality Performance Standards for Certain Meat and Poultry Products. www.fsis.usda.gov/Frame/FrameRedirect.asp?main=http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/95-033F/95-033F_Appendix_A.htm (accessed 02/16/2013).

FSIS. 2001a. Risk Assessment of the Public Health Impact of *Escherichia coli* O157:H7 in Ground Beef. http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/00-023N/00-023NReport.pdf

FSIS. 2001b. 9 CFR Parts 301, 303, 317, 318, 319, 320, 325, 331, 381, 417, and 430. Docket No. 97-013P. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg 66(39): 12590-12636.

FSIS. 2005. Time and Temperature Tables for Cooking Ready to Eat Poultry Products. Original Paper Copy -- Online version has had the fermented poultry references removed.

FSIS. 2013. Proposed Rule. 155. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg. 78(5): 1532.

Glass, K., J. Loeffelholz, J.P. Ford, and M.P. Doyle. 1992. Fate of *Escherichia coli* O157:H7 as Affected by pH or Sodium Chloride and in Fermented, Dry Sausage. App. Environ. Microbiol. 58(8): 2513-2516.

Hinkens, J.C., N. Faith, T. Lorang, P. Bailey, D. Buege, C. Kaspar, and J. Luchansky. Validation of Pepperoni Processes for Control of *Escherichia coli* O157:H7. J. Food Protect. 59(12):1260-1266.

Hohmann, E. 2001. Nontyphoidal Salmonellosis. Clin. Infect. Dis. 32:263-269.

Incze, K. 1998. Dry Fermented Sausages. Meat Sci. 49(Suppl. 1): S169-S177.

Juneja, V.K., B.S. Eblen, and H.M. Marks. 2001. Modeling Non-Linear Survival Curves to Calculate Thermal Inactivation of *Salmonella* in Poultry of Different Fat Levels. Int. J. Food Microbiol. 70: 37-51.

Naim, F., S. Messier, L. Saucier, and G. Piette. 2003. A Model Study of *Escherichia coli* O157:H7 Survival in Fermented Dry Sausages -- Influence of Inoculum Preparation, Inoculation Procedure, and Selected Process Parameters. J. Food Protect. 66(12): 2267-2275.

Neill, M. 1997. Overview of Verotoxigenic *Escherichia coli*. J. Food Protect. 60(11):1444-1446.

Nickelson, R., J. Luchansky, C. Kaspar, and E. Johnson. Dry Fermented Sausage and *E. coli* O157:H7. Blue Ribbon Task Force. National Cattlemen's Beef Association. Research Report no. 11-316.

Porto-Fett, A.C.S., C.-A. Hwang, J.E. Call, V.K. Jenuja, S.C. Ingham, B.H. Ingham, and J.B. Luchansky. 2008. Viability of Multi-Strain Mixtures of *Listeria monocytogenes*, *Salmonella typhimurium*, or *Escherichia coli* O157:H7 Inoculated into the Batter or Onto the Surface of a Soudjouk-Style Fermented Dry Sausage. Food Microbiol. 25: 793-801.

Riordan, D., G. Duffy, J. Sheridan, B.S. Eblen, R.C. Whiting, I.S. Blair, and D.A. McDowell. 1998. Survival of *Escherichia coli* O157:H7 during the Manufacture of Pepperoni. J. Food Protect. 61(2): 146-151.

Riordan, D., G. Duffy, J. Sheridan, R.C. Whiting, I.S. Blair, and D.A. McDowell. 2000. Effects of Acid Adaptation, Product pH, and Heating on Survival of *Escherichia coli* O157:H7 in Pepperoni. App. Environ. Microbiol. 66(4): 1726-1729.

Scallan, E., R. Hoekstra, F. Angulo, R. Tauxe, M. Widdowson, S. Roy, J. Jones, and P. Griffin. 2011. Foodborne Illness Acquired in the United States – Major Pathogens. Emerg. Infect. Dis. 17(1):7-15.

Smith, J.L., C.N. Huhtanen, J.C. Kissinger, and S.A. Palumbo. 1975. Survival of *Salmonella* during Pepperoni Manufacture. App. Microbiol. 30(5): 759-763.

Tarr, P., T. Besser, D. Hancock, W. Keene, and M. Goldoft. 1997. Verotoxigenic *Escherichia coli* Infection: U.S. Overview. J. Food Protect. 60(11):1466-1471.

Tilden, J., W. Young, A.M. McNamara, C. Custer, B. Boesel, M.A. Lambert-Fair, J. Majkowski, D. Vugia, S.B. Werner, J. Hollingsworth, and J.G. Morris. 1996. A New Route of Transmission for *Escherichia coli*: Infection from Dry Fermented Salami. Am. J. Public Health. 80(8):1142-1145.

Figure legends

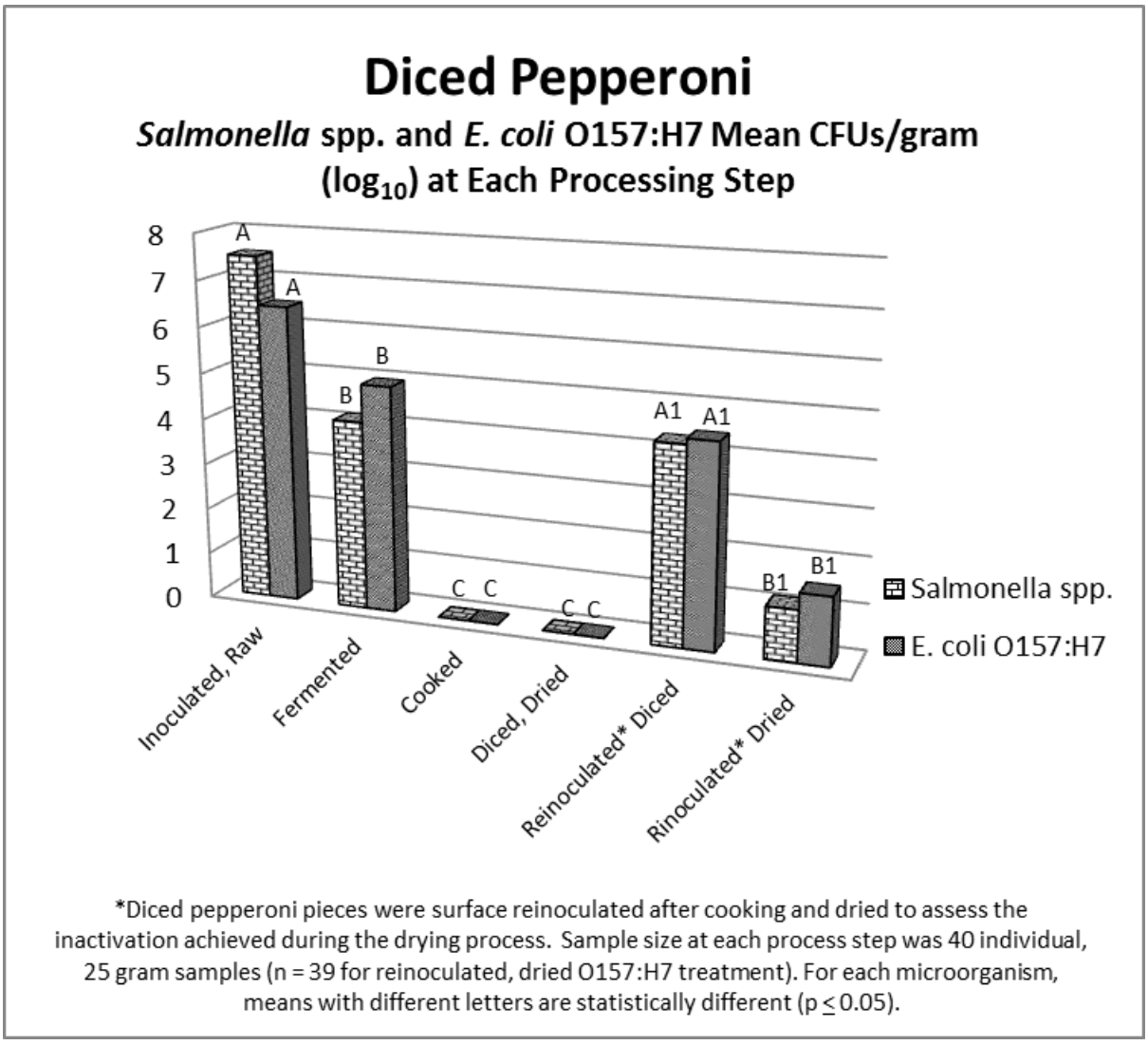
Figure 1. Inactivation of experimentally inoculated *Salmonella* strains and *E. coli* O157:H7 during processing of diced pepperoni pizza topping.

Table legends

Table 1. Summary statistics from SAS output.

Figures

Figure 1.



*Diced pepperoni pieces were surface reinoculated after cooking and dried to assess the inactivation achieved during the drying process. Sample size at each process step was 40 individual, 25 gram samples (n = 39 for reinoculated, dried O157:H7 treatment). For each microorganism, means with different letters are statistically different ($p \leq 0.05$).

Tables

Table 1.

	Salmonella spp.				*E. coli* O157:H7			
	Sample Size	Mean log_{10} CFUs/g	Standard Deviation	Inactivation Achieved log_{10} CFUs/g	Sample Size	Mean log_{10} CFUs/g	Standard Deviation	Inactivation Achieved log_{10} CFUs/g
Inoculated Raw	n = 40	7.5097	0.311	N.A.	n = 40	6.4643	0.319	N.A.
Fermented	n = 40	4.1133	0.266	3.3964	n = 40	4.9342	0.293	1.5301
Cooked	n = 40	0	0	~4.1133	n = 40	0	0	~4.9342
Diced, Dried	n = 40	0	0	Unknown	n = 40	0	0	Unknown
Reinoculated* Diced	n = 40	4.2792	0.307	N.A.	n = 40	4.4013	0.228	N.A.
Rinoculated* Dried	n = 40	1.1249	0.641	3.1543	n = 39	1.4766	0.859	2.9247

VI. Chapter 3

Alternative Time / Temp Combinations for Inactivation of *Salmonella* spp. in Pepperoni

Developing Alternative Time / Temperature Thermal-Processing Combinations for Validation of the Inactivation of *Salmonella* spp. in a Variety of Formulations of Pepperoni

Scott Stillwell*, Eric Oxford, and Daniel Zelenka

Tyson Foods, Inc., Springdale, Arkansas 72762, USA

Key words: *Salmonella* spp., microbial inactivation, pepperoni, fermentation, validation

*Author for correspondence. Tel: (479) 290-7186; Fax: (479) 757-6888
E-mail: scott.stillwell@tyson.com

ABSTRACT

Fermented meats have been associated with outbreaks of illnesses due to surviving strains of *Salmonella*. Following an outbreak of *E. coli* O157:H7 associated with salami in 1994, a Blue Ribbon Task Force developed options for manufacturers of fermented meat products that have proven sufficient to control *E. coli* O157:H7 and these controls were deemed sufficient for all vegetative pathogens present in raw materials. Over the course of the last decade, manufacturers have been modifying fermented sausage formulations to include poultry products as a means of reducing the product cost. During the last decade, the Food Safety Inspection Service staff announced their intent to extend the pathogen reduction performance standards to all Ready-to-Eat (RTE) meat and poultry products. Given the scarcity of data associated with fermented products formulated with poultry and process capability to meet a 7.0 $\log_{10}$ inactivation of the salmonellae, it is necessary to develop tools to facilitate validation of HACCP plans. Utilizing two meat block formulations (pork\beef and pork\chicken\beef) and three percentage fat formulations (8\25\42), the present study compared the inactivation of *Salmonella* spp. with fermentation followed by a variety of time and temperature combinations (0 – 120 minutes; 116^{o}F (46.7^{o}C) – 148^{o}F (64.4^{o}C)) to facilitate validation in the event proposed regulatory changes become effective.

The term “sausage” is used to describe numerous products – made from salted and cured ground meat with a variety of seasonings stuffed in a casing. The traditional methods for making sausages have an ancient history and have evolved across time and geography depending upon the availability of raw materials and preservation methods (Ordonez *et al.* 1999). While the ancient record of preservation methods is scant, there may be no doubt that in the earliest days of fermented sausage making, product safety was hit or miss and certainly not well understood. As the sciences of food processing, food microbiology and food preservation have evolved, our understanding of control measures required to ensure product safety has also evolved (Cornell University 2007, Toldrà *et al*. 2007).

Pepperoni is a modern iteration of fermented, dried spicy meat sausage typically formulated with paprika and garlic which may be (but is often not) lightly smoked. With estimates of domestic consumption reaching to well over 250 million pounds per year, it is the most widely consumed type of dried fermented sausage in the U.S. (Buccheri 2012) with its primary use as toppings for pizza products or as filling for bread-enrobed products such as calzones. In the U.S., labeling laws have been established for a wide variety of products and there exists a legal standard of identity for pepperoni which establishes key product characteristics such as the allowed meat components and degree of finished product dryness. These labeling requirements preclude substandard products from being labeled with the name pepperoni (FSIS 2003).

Manufacturers of pepperoni have a duty to ensure that microbiological safety controls are designed into their production processes and their Hazard Analysis, Critical Control Point (HACCP) plans are robust or risk brand damage and liabilities in the event of illnesses caused by failures to adequately control pathogens present in raw materials. As the scientific understanding of the curing and fermentation process has developed, many controls have been introduced over the years -- e.g. curing with sodium nitrite for control of toxigenic spore-forming pathogens (Sofos 1981) and added carbohydrates and use of commercial lactic acid producing starter cultures to rapidly acidify the products during fermentation for control of toxigenic *Staphylococcus aureus* (Metaxopoulos *et al*. 1981). Currently, the primary vegetative enteric pathogens of concern are serotypes of *Salmonella* and strains of enterohemorrhagic *Escherichia coli* (*E. coli*) (Hwang *et al*. 2009).

Salmonellosis is one of the leading human foodborne illnesses in the U.S. with estimates of approximately 3,500 illnesses per 100,000 people per year; second only to foodborne illnesses caused by viruses (Scallan *et al*. 2011). The case rate of human salmonellosis has been relatively constant since 1998 and unlike for other notable enteric pathogens such as *E. coli* O157:H7, progress has not been made toward the national goals of reducing foodborne illnesses caused by the salmonellae (CDC 2011a).

A variety of serotypes of *Salmonella* have been implicated, both domestically and internationally, in illnesses and outbreaks associated with consumption of "Ready to Eat" (RTE) fermented dry and semi-dry sausages. The most recent domestic outbreak caused by *Salmonella* Montevideo was apparently the result of a contaminated ingredient (pepper) added to the fermented sausage post-lethality treatment. Interestingly, a second *Salmonella* serotype (Senftenberg, known for its heat tolerance) was recovered from a subset of ill patients and the salami, indicating there may have been pathogens that survived the lethality processes (CDC 2010). During recent decades there have been a small number of documented domestic outbreaks of salmonellosis associated with fermented meat products such as the 1995 outbreak of *Salmonella* Typhimurium attributed to Lebanon bologna that afflicted 26 people, in which it was apparent that the manufacturing process was not sufficiently robust to inactivate the vegetative pathogen load presented on the raw materials ($>10^4$ CFU/g) (Sauer *et al.* 1997).

On a more frequent basis, *Salmonella* spp. outbreaks associated with fermented sausage products have been reported from Europe. These outbreaks were also caused by inadequate manufacturing processes that resulted in finished product characteristics – pH, water activity or lack of maturation time -- that allowed *Salmonella spp.* to survive in adequate numbers to cause illness upon consumption (Bremer *et al*. 2004, Emberland *et al*. 2006, Luzzi *et al*. 2007, and Pontello *et al*. 1998). Many European manufacturers continue to use "old world" traditional manufacturing processes that don't employ a thermal treatment for pathogen inactivation, relying solely on reductions achieved during fermentation, drying and maturation. These processes are likely to be more prone to result in pathogen positive finished products as a number of authors have concluded that a thermal processing step is necessary for adequate decimal reductions (Hinkens *et al*. 1996, Nightingale *et al*. 2006, Smith *et al*. 1975). Domestic testing of finished RTE fermented sausages by federal government authorities for the presence of *Salmonella* spp. 1990 – 1999 indicated that approximately 1.43% were positive (Levine *et al.* 2001), but

that number fell to 0.0% in the following year (FSIS 2000). While direct comparisons for fermented sausages are not possible from the FSIS data as the reporting system changed following 2000, the number of *Salmonella* spp. positive reports from testing of all RTE meat and poultry products has remained extremely low (FSIS 2013a).

Following a 1994 outbreak of illnesses caused by *E. coli* O157:H7 in a fermented beef salami (Tilden *et al*. 1996), the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) staff worked in collaboration with industry representatives from the "Blue Ribbon Task Force" to develop five options designed to ensure the safety of fermented dry and semi-dry meat products. The most rigorous of the options required the processor to demonstrate a 5.0 $\log_{10}$ reduction of *E. coli* O157:H7 through the process (Nickelson *et al*. 1996, Naim *et al*. 2003). Since that time, staff from FSIS have collected and analyzed more than 10,000 finished RTE samples (including cooked beef patties) for the presence of *E. coli* O157:H7 and have not reported a positive sample. Given these results, in May 2011, FSIS staff announced that they were suspending the testing of this class of products for the presence of this pathogen (FSIS 2011).

Beginning in January 1999, following the implementation of the HACCP regulations (FSIS 1996), the staff at FSIS converted prescriptive time and temperature processing requirements for certain RTE meat and poultry products into performance standards requiring the process to achieve either a 6.5 $\log_{10}$ (meat products) or a 7.0 $\log_{10}$ (poultry products) inactivation of serotypes of *Salmonella* in the finished product. These standards applied to RTE roast beef, corned beef, cooked beef and all fully cooked poultry products (FSIS 1999a). Specific time and temperature combinations that were validated to achieve those reductions were published in a FSIS Directive as "Appendix-A" (FSIS 1999b). In February 2001, FSIS staff proposed to extend these performance standards to all RTE meat and poultry products (FSIS 2001). Further, the time and temperature guidance in Appendix-A was expanded based upon the work of Juneja *et al*. who had modeled the effects of varying fat levels in poultry product formulations on the thermal inactivation of *Salmonella* spp. These models, which included data for fat levels between 1 – 12% of the formulation, indicated that inactivation kinetics were altered by fat in the product and postulated that higher fat levels provided a thermal-protective effect. The staff at FSIS modified Appendix-A to include time and temperatures tables based on fat levels and poultry specie that provided a variety of

combinations validated to achieve the required 7.0 $\log_{10}$ lethality of *Salmonella* spp. Since the publication of the Juneja paper in 2001, it has become a standard expectation that higher fat formulations will result in slower and/or lower cumulative vegetative pathogen inactivation.

In March 2005, the FSIS staff published Notice 16-05 notifying the regulated industry of a draft proposal to update Appendix-A to include (among other things): a requirement to address in HACCP plans the specific hazards associated with serotypes of *Salmonella* in products currently validated to control *E. coli* O157:H7 such as fermented sausages containing poultry; and specific requirements to process such fermented products containing poultry derived from chicken to a minimum of 136^{o}F (57.8^{o}C) for 76.7 minutes; or for such fermented products containing poultry derived from turkey to a minimum of 136^{o}F (57.8^{o}C) for 68.4 minutes (FSIS 2005). The proposed changes have yet to be enacted. However, in January 2013, USDA staff published an announcement in the Semi-Annual Regulatory Agenda of their intent to move forward with an Interim Final Rule codifying these requirements and extending the Pathogen Reduction Performance Standards to all RTE meat and poultry products (FSIS 2013b). These proposed regulatory changes, if enacted, are expected to have significant cost impacts that will no doubt pass to the consuming public. The regulated industry will be required to revalidate HACCP plans for a wide variety of products that are currently validated to achieve a 5.0 $\log_{10}$ inactivation of *E. coli* O157:H7 to demonstrate an adequate reduction in the salmonellae.

In the years immediately after the salami outbreak in 1994, most research on the safety of fermented sausages focused on adequate control of *E. coli* O157:H7 (Barbut and Griffiths 2001, Faith *et al*. 1997, Hinkens *et al*. 1996, and Riordan *et al*. 2000). However, as regulatory proposals have evolved, researchers have more frequently included data on the inactivation or control of a variety of microbial hazards associated with dry and semi-dry fermented sausages (Barbuti and Parolari 2002, Deumier and Collignan 2003, Porto-Fett *et al*. 2008, and Smith *et al*. 1975). Unfortunately, scant data exist in published literature concerning fermented meat products formulated to contain poultry meat; any impact the inclusion may have on pathogen inactivation kinetics; and whether manufacturing processes are validated to be capable of reliably meeting the proposed regulatory performance standard of a 7.0 $\log_{10}$ inactivation of the salmonellae.

In recent years, domestic production of fermented sausage products formulated to contain raw materials of poultry origin has increased substantially. This is due, in part, to the proliferation of low cost, frozen, heat and serve pizzas (and other products such as hand-held dough-enrobed "pizza pockets") which contain pepperoni as a principle meat ingredient. The manufacturers of these pizza products compete on the basis of quality and taste – with a very heavy advertising emphasis on the low price of the products. As such, a great deal of interest in formulating lower cost pepperoni products has led to the inclusion of alternative protein ingredients such as mechanically separated chicken or turkey in place of some portion of the beef and/or pork components (the total proportion of each frequently depending on a least cost formulation and current commodity prices). Given current market conditions, it is anticipated that this trend will persist.

The March 2005 draft modifications to the *Compliance Guidelines for Meeting Lethality Performance Standards for Cooked, Ready to Eat Meat and Poultry Products* ("Appendix A") represent a significant departure from some of the most commonly utilized processing parameters and, despite evidence indicating modifications are necessary, if codified in the 2013 Interim Final Rule, those changes are expected to result in significant reductions in product quality and yield with additional increase in product cost. The validated thermal processing conditions most widely utilized in commercial processes for control of *E. coli* O157:H7 in stick pepperoni are 128^{o}F (53.3^{o}C) for 60 minutes or 145^{o}F (62.8^{o}C) for an instantaneous lethality (5.0 $\log_{10}$ inactivation of E. coli O157:H7) (Hinkens *et al*.). The standards proposed in 2005 by the staff at FSIS (and subsequently removed from the official on-line version of Notice 16-05 and Appendix-A) to achieve a 7.0 $\log_{10}$ reduction of the salmonellae are expected to result in significant "fatting out" (the melting of globular fat with subsequent migration from the meat matrix with accumulation between the sausage and its casing); color and texture changes; and reductions in throughput with particular difficulties during drying due to the fatting out (the fat creates a moisture vapor barrier and retards drying). The vast majority of fermented sausage products have a finished formulation well in excess of 12% fat, thus the currently published Appendix-A time temperature tables will be inapplicable. Therefore, it has been deemed advantageous to identify alternative time at temperature combinations that are validated to achieve the proposed minimum microbial inactivation (e.g. a 6.5 $\log_{10}$

reduction of serotypes of *Salmonella* in products formulated solely with pork and beef; or a 7.0 $\log_{10}$ reduction in products formulated with poultry in any ratio).

The present study was designed to assess the impact of formulation differences on the *Salmonella* spp. inactivation dynamics in a range of pepperoni products. Product meat specie differences (pork/beef vs. pork/chicken/beef) and fat levels in the formulation (low – 15%; mid – 33%; and high – 50%) as well as thermal processing times and temperatures were varied (0 – 120 minutes; 116°F (46.7°C) – 148°F (64.4°C)) to conservatively predict the time at temperature combination at which the appropriate level of inactivation is achieved. This is expected to be beneficial to processors of similar products as a tool for assessing their current process parameters against the proposed microbial reduction performance standards. The data derived will inform additional research intended to develop a mathematical model for predicting inactivation of serotypes of *Salmonella* in fermented meat products utilizing a non-pathogenic surrogate microorganism appropriate for inclusion in a commercial product formulation for in-plant commercial process validations.

MATERIALS AND METHODS

Approximately 50 pounds of six different formulations of raw, commercially processed standard pepperoni "batter" was prepared at a USDA-inspected manufacturing operation owned by Tyson Foods, Inc. operating in Springdale, Arkansas. The batter was prepared with the assistance of the processing plant staff utilizing commercial manufacturing equipment. All ingredients with the exception of the starter culture were blended to achieve the following (approximate) blend formulations: a pork (70%) and beef (30%) blend with low (15%), medium (33%) or high (50%) fat; and a pork (50%), chicken (45%) and beef blend (5%) with low (17.5%), medium (33.5%) or high (49%) fat. As illustrated in Table 1, the slight differences in formulated fat levels are due to the commercial availability of common fat/lean ratio raw materials and the requirements to formulate to a common 50 pound batch size. Each unique formulation of meat batter was divided into twelve portions of approximately 4.25 pounds (~ 1.9 kg) representing samples for five time/temperature processing combinations in replicate with two samples held in reserve. The batter was placed into plastic bags, labeled, placed into plastic storage tubs, covered with granular dry ice to rapidly freeze the meat batter, then transported to Tyson Foods' corporate laboratory in

Springdale, Arkansas where the samples were held in a commercial walk-in freezer at 0°F (-17.8°C) pending processing.

For inactivation curve development, five cook-temperatures each with four cook-times were selected to cover a full range of expected fermented sausage processing conditions (see Table 2):

1) 116°F (46.7°C) thermal processing temperature for 48, 72, 96 and 120 minutes;
2) 124°F (51.1°C) thermal processing temperature for 36, 60, 84, and108 minutes;
3) 132°F (55.6°C) thermal processing temperature for 24, 48, 72, and 96 minutes;
4) 140°F (60.0°C) thermal processing temperature for 12, 36, 60, and 84 minutes; and
5) 148°F (64.4°C) thermal processing temperature for 0, 24, 48, and72 minutes.

All samples were processed under identical conditions for fermentation and then thermally processed at a set temperature with samples obtained after four different time periods per formulation type. Each formulation type treatment was processed in duplicate replications in random order over the course of several weeks (see Table 2).

Sample Preparation: On the date of processing, one sample bag of each of the six formulation types was randomly selected and removed from frozen storage to thaw for approximately two hours in a commercial, reach-in display holding refrigerator at approximately 40°F (4.4°C). During this period, the processing vessel was set up and sample materials were prepared. Thermal processing was conducted in a water bath contained in a 150 quart marine-grade chest cooler (Rubbermaid Commercial Products Model 2B8001) fitted with a thermal cycler and digital thermometer (PolyScience Professional Chef Series Model SVC-AC1B) to maintain the water bath at the target temperature. In addition to the thermal cycler digital temperature display, the water bath temperature was monitored with two calibrated mercury thermometers (Thermco Water Bath Thermometer Model ACC711SSC +20 -- +100°C) and four digital recording thermometers (Ellab TrackSense Pro, Ellab A/S, Hilleroed, Denmark). The initial water bath temperature was set for 112°F (44.4°C) for the fermentation process and the water temperature was maintained (once it reached steady state) at +/- 0.2°F based upon the thermal cycler display (through all ten trials at any location in the water bath, with or without samples loaded, no discernible temperature difference could be detected with the certified mercury thermometer with 0.5°C scale increments).

Inocula Preparation: Three concentrated microbial cultures for inoculation were prepared. The first culture consisted of a commercially obtained lactic acid starter culture (SAGA 200 containing the homo-fermentative lactic acid-producing bacteria *Pediococcus acidilactici* from Kerry Ingredients and Flavors, Beloit, WI 53511) maintained prior to use frozen in a commercial, retail ice cream freezer (less than -15^{o}F (-8.3^{o}C)). The second culture consisted of an overnight (18 -- 24 hours after the concentrated microorganisms were washed, re-suspended in sterile saline and refrigerated) culture of a five-serotype cocktail of *Salmonella* comprised of: *Salmonella* Senftenberg ATCC 43845 obtained from the Tyson Foods collection; and four serologically distinct "wild" strains of *Salmonella* of unknown serotype isolated from routine whole-bird carcass rinse samples of commercial broilers from Tyson Foods' commercial poultry slaughter operations. The third culture consisted of an overnight culture of *Enterococcus faecalis* ATCC29212 intended for future modeling purposes and obtained from the Tyson Foods collection.

Sample Inoculation: Immediately before inoculating the meat portions, the frozen puck of starter culture was suspended in a volume of room temperature tap water per the supplier's directions. The pathogen and surrogate cultures had been concentrated by Tyson Foods' research microbiologists to provide an initial inoculation level of approximately $10^{7.5}$ colony forming units (CFUs) per gram of pepperoni batter. In randomized order, one of the six samples of raw pepperoni batter was removed from the holding cooler; 650 grams of batter was removed from the sample and weighed (+/- 0.1 gram); and the meat was transferred to a large baking sheet covered with clean aluminum foil. The sample was divided into two approximately equal portions which were flattened by gloved hand into disks.

The inocula were transferred by pipette with half the volume applied to each of the two meat portions; starting with the cocktail of *Salmonella* strains and ending with the commercial starter culture; slowly dripping the inocula across the surface of the meat batter. Once the inocula were transferred to the meat, each portion was thoroughly mixed to ensure homogenous distribution of the microorganisms. This was accomplished by repetitively kneading and flattening the portion by hand; then using the knife-edge of the hands, forming the sample into a rope which was rolled or coiled upon itself and kneaded and flattened again for a total of 10 repetitions. After each portion had been thoroughly mixed, the two portions were combined and mixed in the same manner for a total of ten repetitions.

Vacuum bags (Cryovac Sealed Air Corporation, Duncan SC; 8"x16" standard curved end; Model B2470T) were labeled with sample identification and thoroughly mixed to randomize the order of selection. From the inoculated, mixed meat sample, individual 25 gram samples were weighed into the labeled bags as represented in Table 2: five (5) samples for microbial enumeration for determination of the initial inoculation level (t_0); two (2) samples for verification of pH after fermentation; five (5) samples for enumeration after fermentation; and three (3) samples for each of four (4) time at temperature obtained after the water bath was adjusted to the target cook temperature. The samples in the bags were flattened by hand into approximately 2 mm thick disks; and the bags were vacuum sealed (MulitVac Inc., Kansas City, MO; Model C-350). After all bags were sealed and grouped according to type, they were placed in a plastic sample container and returned to the holding refrigerator.

Sample Processing: In random order, all six meat formulations were prepared in a similar manner. After all of the samples were inoculated, weighed and vacuum sealed, they remained in the holding refrigerator for a minimum of 30 minutes so that all samples were temperature equilibrated. Following equilibration, all time zero, initial-inoculation level samples were segregated and placed into the holding refrigerator for subsequent microbial enumeration. The remaining samples were then grouped together according to treatment type and duration, clipped together with weighted binder clips (United Stationers Supply Co., Des Plaines, IL; Model #10220); and strung together using nylon twine for ease of removal from the water bath. Once all sample sets were similarly prepared and the water bath temperature was verified to be 112^{o}F (44.4^{o}C) for the start of fermentation, in a pre-randomized order and location in the water bath, the samples were placed into the water. Once all samples were placed into the water bath and the water temperature was restored to 112^{o}F (44.4^{o}C) (maximum deviation observed for all ten trials was 0.3^{o}F), a countdown timer was started for 12 hours of fermentation.

After twelve hours, the string of samples for pH measurement was pulled from the water bath and each of the six formulation types were verified to ensure the pH was below 4.95 (pH model number DPH230SD, General Tools and Instruments Co, LLC, New York, NY). Once successful fermentation was verified, the string of samples for microbial enumeration after fermentation was pulled and immediately chilled in an ice-slush water bath.

The hot water bath temperature was then raised to the target cook temperature in the following manner: the thermal cycler set point was adjusted to the target cook temperature; a volume of water equal to approximately one-third of the capacity was siphoned out to a floor drain; a volume of boiling water was slowly added to the water bath near the circulation pump to ensure rapid mixing while observing the digital temperature reading on the thermal cycler. Boiling water was added until the water bath temperature was approaching the target cook temperature (without exceeding it). As soon as the thermal cycler indicated the target temperature had been achieved and the temperature was verified by certified mercury thermometer, a countdown timer was started to indicate when the first set of cooked samples were to be removed from the hot water bath (in each trial, this process took less than ten minutes). After the designated time at temperature for the trial conditions had elapsed, the string of cooked samples was removed and immediately chilled in an ice-slush water bath. Once all samples were cooked and chilled, they were placed into the holding refrigerator for microbiological analyses.

Microbiological Analyses: Using standard protocols for sample preparation, serial dilution and spread-plate enumeration, Tyson Foods' corporate research microbiologists prepared each individual sample for recovery and enumeration. Trypticase Soy Agar plates (in duplicate for each sample and dilution) were serially inoculated, dried for approximately 2 hours in a 35^{o}C incubator, overlaid with selective media (Xylose-Lysine-Tergitol 4 for recovery of the *Salmonella* strains (DIFCO, Beckton Dickenson, and Company, Franklin Lakes, NJ)), and incubated for 24 hours at 35^{o}C. Following incubation, each set of plates was examined for typical colonies. Any plates showing no typical colony growth or indications of microbial stress were returned to the incubator for an additional 24 hours. Following standard micro-lab protocols, the research microbiologists reported quantitative results for *Salmonella* spp. recovered from each replicate of each sample type.

Statistical Analyses: Utilizing the Statistical Analysis System (SAS) version 9.3 software for Windows (SAS Institute, Cary NC), the data were log-transformed (all "none detected" samples were reported as one) for analyses. Due to slight differences in the initial inoculation levels, enumerated counts at fermentation and during cooking were deviated from the average of the $\log_{10}$ transformed values for the beginning level for each time at temperature, fat-level and composition combination. All further statistical evaluations were directed toward the differences from the mean value for each

treatment to minimize the differences in results that are due only to differences in the initial (time zero) inoculation levels.

The General Linear Model (PROC GLM) was used to evaluate the deviated differences as each step (fermentation and cooking) were evaluated independently to determine the impact of the main effects of composition, fat content, replicate, temperature and the two way interactions of comp*fat and fat*temp. The value for pH was included as a covariate in the model. Means were output for significant terms and interactions at each step. No repeated measures were represented as the testing of the samples was destructive; thus, no two measures were taken on the same sample.

To fit the reduction over time, multiple nonlinear models were evaluated through the PROC NLIN procedure of SAS, but the best fit model was determined using the Gauss-Newton algorithm to be a log model with the following equation: $\{\log_{10} \text{difference} = A \times (\log(\text{time})) + \beta\}$; where alpha (scale factor for adjusting the shape of the curve to derive least sums of squares) and beta (y-intercept) were determined for each cook temperature through minimization of the sums of squares over multiple iterations.

The mean values for microbial reduction in viable count were analyzed for each replicate and processing treatment to determine whether replicates exhibited significantly different levels of microbial inactivation; to assess the treatment effect on microbial inactivation; and to determine the cumulative microbial inactivation achieved. The SAS General Linear Model was utilized for the Means Test, Analysis of Variance and the Duncan's Multiple Range Test.

RESULTS

As expected, there were no differences in mean reduction achieved between any of the replicates of a treatment. Thus, data from both replicates were combined for all further evaluations and graphical representations. Further, there was no significant treatment effect due to the protein portion of the formulation indicating that the reductions in *Salmonella* spp. achieved across all time and temperature combinations was unaffected by the inclusion of poultry meat in the formulation. Thus, the model need not consider meat block differences. However, as expected, there was a highly significant effect ($p > 0.001$) on the inactivation achieved during the fermentation treatment due to the fat composition; as well as the expected time and temperature effects.

The mean $\log_{10}$ numbers for each targeted thermal processing time and temperature combination are graphically represented in Figure 1 – 5 as survivor curves. In Figures 6 – 10, the output of the best fit model of the survivor data graphically represents the predicted cumulative reduction achieved over time for evaluating the potential of a particular formulation, time and temperature combination to achieve the proposed regulatory pathogen reduction performance standard for the salmonellae. The spreadsheet containing these models is available from the author by request.

DISCUSSION

As previously noted, the prescriptive time and temperature standards that were published in the 2005 FSIS Notice (16-05) for fermented sausages containing poultry are no longer associated with the FSIS online version of either Notice 16-05 or Appendix-A. Given the general policy move away from prescriptive definitions and toward industry-defined and validated processing controls to achieve a codified regulatory pathogen reduction performance standard, one must conclude that the staff at FSIS doesn't intend to publish those previously defined time and temperature combinations as "safe harbors". As such, it becomes even more imperative for processors to identify and validate controls across a broad range of fermented meat product formulations that will provide conservative estimates of pathogen inactivation.

Consistent with the findings of other authors (Smith *et al*. 1975), differing meat species in the matrix -- in this case the inclusion of poultry meat in the formulation -- did not alter the rate or extent of inactivation. Using mixed meat species in a fermented system with ten total trials across a multitude of fat levels and time at temperature combinations, we failed to support the previous work of Juneja and others that have observed significantly lower rates of inactivation in higher fat formulations. As is evident from the survivor curves in Figures 1 – 5, at any combination of time at temperature where a statistically significant difference in the level of inactivation was measured, the higher fat formulation always resulted in greater inactivation. Similar results as ours have been observed by a number of other investigators (Kotrola and Conner 1997) and additional consideration of this variable appears to be warranted.

The phenomena of greater inactivation observed with higher fat formulations may be the result of bacteriocins; in this case pediocins which are known to be produced by strains of *Pediococcus acidilactici* (Albano *et al*. 2007). These peptides will be closely associated with the proliferation of the starter culture

in the water phase of the meat matrix rather than the fat phase due to available water (Abee *et al*. 1995, Biswas *et al*. 1991). In the relatively low temperature fermentation model, the postulated thermal-protective effect of higher fat formulations may not be as meaningful; and the relative proportion of the water phase versus the proportion of the fat phase will be lower in the high fat formulations potentially leading to higher concentrations of the pediocins in the water phase. While pediocins are thought to be more effective against Gram-positive bacteria, there are a number that have demonstrated antimicrobial activity against strains of *Salmonella* (Nitisinprasert *et al.* 2000).

At a post-fermentation thermal processing temperature of 116^{o}F, we failed to achieve a level of inactivation that would meet the proposed pathogen reduction standards. However, even at a thermal processing temperature as low as 128^{o}F, we achieved levels of inactivation that would meet the standards for either all red meat or red meat formulations containing poultry at all fat levels. As expected, the level of inactivation achieved increased both with increasing thermal processing temperature and time. With the exception of 116^{o}F, all thermal processing temperatures studied resulted in an adequate level of inactivation if the product was held at temperature for a sufficient period of time. These experimental conditions are specific to the inactivation achieved and caution should be exercised when attempting to extrapolate these data to formulations outside these ranges of fat, or exhibiting extreme levels of cure, salt or other inherently synergistic formulation attributes that may contribute to the cumulative pathogen lethality.

For a conservative estimate of the level of inactivation one might expect to achieve under conditions of varying fat formulations and combinations of time at temperature, we developed a mathematical model to predict the level of inactivation of the salmonellae through fermentation followed by thermal processing at the temperatures studied. The experimental data were assessed against a wide range of existing inactivation models. Due to the conditions represented by fermentation and the differences in inactivation achieved in the different fat formulations at elevated processing temperatures, none of the existing models adequately described the shape of the inactivation curves obtained. Therefore, we developed and selected a two-phase model based on a simple linear regression to predict inactivation achieved through fermentation followed by a log-linear model with a scalar factor determined through multiple iterations to deliver the least sums of squares of deviations from the observed values.

The model takes the form {$\log_{10}$ difference = α x (log(time)) + β}; where alpha is a scale factor for adjusting the shape of the curve to derive the least sums of squares and beta is the y-intercept. This approach resulted in three different models for inactivation of *Salmonella* spp. due to the differences in inactivation achieved for the three different fat levels through fermentation (the linear regressed portion of the two-phase model) and differences in the shape of the inactivation curves due to the different fat levels. The mathematical models are contained within a spreadsheet that is available upon request.

The outputs of the models are graphically represented in Figures 6 – 10 for each of the post-fermentation thermal processing conditions. Each illustrates the different level of inactivation achieved through fermentation represented as the starting point of the inactivation curve on the $\log_{10}$ reduction scale (the bottom curve is always for the low fat formulation, while the top curve is always for the high fat formulation). To estimate the level of inactivation a particular process is predicted to achieve, one would simply select the temperature used for thermal processing and the time at which the product was held after reaching that temperature and estimate on the inactivation curve the cumulative inactivation achieved.

On two levels, the models were constructed and intended to be conservative. The first is illustrated by taking the actual experimental data and comparing to the predicted inactivation under the same conditions. For illustration purposes, one may refer to the data in Table 3 which provide the estimated time at temperature required to achieve either a 6.5 or 7.0 $\log_{10}$ inactivation of the salmonellae. From Figure 3 of the inactivation data, it is apparent that at 24 minutes, levels of reduction sufficient to meet the proposed pathogen reduction performance standard were achieved for all fat formulations and specie combinations. In comparison, using Figure 8 as reference, neither the low-fat nor the mid-fat formulations would be predicted to achieve the required 7.0 $\log_{10}$ inactivation if formulated with poultry. Thus, a processor relying solely on these models to estimate reductions to be achieved versus having obtained through direct observation actual experimental data derived from the specific formulation and processing conditions would be expected to conservatively underestimate the actual inactivation achieved, requiring additional time at temperature to be applied to ensure safety.

On the second level, these models predict the accumulated lethality through fermentation and thermal processing. There is a substantial body of data demonstrating that additional inactivation occurs in

pepperoni during the drying process and during storage and distribution (Hwang *et al*. 2009, Ihnot *et al*. 1998, and Porto-Fett *et al*. 2010). The results achieved varied from approximately 0.3 – 2.4 $\log_{10}$ inactivation during drying to 4.6 – 6.6 $\log_{10}$ inactivation during storage under vacuum for 56 days. Given the highly variable and process specific reductions achieved, we chose a conservative approach and elected to model the time required to achieve full inactivation during the portion of the process for which processors have the greatest levels of process control.

These models will prove useful to manufacturers wishing to estimate the level of inactivation of the salmonellae any particular combination of formulation and processing conditions might achieve. One should use caution and never rely solely on the estimates of a model. In-plant data will always be preferred and these results are intended to be further utilized in the development of a model based upon a non-pathogenic surrogate microorganism that might be employed to predict the inactivation achieved in specific formulations under actual commercial processing conditions.

VII. REFERENCES

Abee, T., L. Krockel, and C. Hill. 1995. Bacteriocins: Modes of Action and Potentials in Food Preservation and Control of Food Poisoning. Int. J. Food Microbiol. 28: 169-185.

Albano, H., S.D. Todorov, C.A. van Reenen, T. Hogg, L.M.T. Dicks, and P. Teixeira. 2007. Characterization of Two Bacteriocins Produced by *Pediococcus acidilcatici* Isolated from "Alheira", a Fermented Sausage Traditionally Produced in Portugal. Int. J. Food Microbiol. 116: 239-247.

Barbut, S., and M.W. Griffiths. 2001. Developing Validation Models for *E. coli* O157 Inactivation in Dry Fermented Sausages. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 54. p. 44-48.

Barbut, S., and G. Parolari. 2002. Validation of Manufacturing Process to Control Pathogenic Bacteria in Typical Dry Fermented Products. Meat Sci. 62:323-329.

Biswas, S.R., P. Ray, M.C. Johnson, and B. Ray. 1991. Influence of Growth Conditions on the Production of Bacteriocin, Pediocin AcH, by *Pediococcus acidilactici* H. App. Environ, Microbiol. 57(4): 1265-1267.

Bremer, V., K. Lietmeyer, E. Jensen, U. Metzel, H. Meczulat, E. Weise, D. Werber, H. Tschaepe, L. Kreienbrock, S. Glaser, and A. Ammon. 2004. Outbreak of *Salmonella* Goldcoast Infections Linked to Consumption of Fermented Sausage, German 2001. Epidemiol. Infect. 132(5): 881-887.

Buccheri, Mike. 2012. Tyson Foods, Inc. Personal Communication.

CDC. 2010. Investigation Update: Multistate Outbreak of Human *Salmonella* Montevideo Infections. www.cdc.gov/salmonella/montevideo/index.html (accessed 07/15/2012).

CDC. 2011a. Trends in Foodborne Illness, 1996-2010. Atlanta, Georgia: US Department of Health and Human Services, CDC. www.cdc.gov/foodborneburden/PDFs/FACTSHEET_B_TRENDS.PDF

CDC. 2011b. Investigation Announcement: Multistate Outbreak of E. coli O157:H7 Infections Associated with Lebanon Bologna. www.cdc.gov/ecoli/2011/O157_0311/index.html (accessed 01/12/2013).

CDC. 2012. *Salmonella*. Technical Information. www.cdc.gov/salmonella/general/technical.html (accessed 07/15/2012).

Cornell University. 2007. Fermented Sausages, Fact Sheets for the Small Scale Food Entrepreneur. necfe.foodscience.cornell.edu/publications/pdf/FS_FermentedSausages.pdf (accessed 01/13/2013).

Deumier, F., and A. Collignan. 2003. The Effects of Sodium Lactate and Starter Cultures on pH, Lactic Acid Bacteria, *Listeria monocytogenes* and *Salmonella* spp. Levels in Pure Chicken Dry Fermented Sausage. Meat Sci. 65: 1165-1174.

Ellajosyula, K.R., S. Doores, E.W. Mills, R.A. Wilson, R.C. Anantheswaran, and S.J. Knabel. 1998. Destruction of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in Lebanon Bologna by Interaction of Fermentation pH, Heating Temperature, and Time. J. Food Protect. 61(2): 152-157.

Emberland, K.E., K. Nygard, B.T. Heier, P. Aavitsland, J. Lassen, T.L. Stavnes, and B. Gondrosen. 2006. Outbreak of *Salmonella* Kedougou in Norway Associated with Salami, April – June 2006. Euro Surveill. 11(27): 2995.

Faith, N., N. Parniere, T. Larson, T. Lorang, and J. Luchansky. 1997. Viability of *Escherichia coli* O157:H7 in Pepperoni during the Manufacture of Sticks and the Subsequent Storage of Slices at 21, 4 and -20^{o}C Under Air, Vacuum and CO_2. Int. J. Food Microbiol. 37:47-54.

FSIS. 1996. 9 CFR Part 304, *et al*. Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) System; Final Rule. Fed. Reg. 61(144): 38805-38855.

FSIS. 1999a. 9 CFR Parts 301, 317, 318, 320, and 381.Docket No. 95-033F. Production Standards for the Production of Certain Meat and Poultry Products. Fed. Reg. 64(3): 732-749.

FSIS. 1999b. Appendix A, Compliance Guidelines for Meeting Lethality Performance Standards for Certain Meat and Poultry Products. www.fsis.usda.gov/Frame/FrameRedirect.asp?main=http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/95-033F/95-033F_Appendix_A.htm (accessed 02/16/2013).

FSIS. 2000. Percent Positive *Salmonella* Tests for RTE meat and poultry products, CY 1990-2000. www.fsis.usda.gov/Science/Table2_Micro_Testing_RTE_1990-2004/index.asp (accessed 02/26/2013).

FSIS. 2001. 9 CFR Parts 301, 303, 317, 318, 319, 320, 325, 331, 381, 417, and 430. Docket No. 97-013P. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg 66(39): 12590-12636.

FSIS. 2003. Part 319 – Definitions and Standards of Identity or Composition. Code of Federal Regulations, Title 9, Volume 2. 9CFR319.

FSIS. 2005. Time and Temperature Tables for Cooking Ready to Eat Poultry Products. Original Paper Copy -- Online version has had the fermented poultry references removed.

FSIS. 2011. Agency to Suspend *E. coli* Testing Program for Certain RTE Products. Constituent Update, May 13, 2011. www.fsis.usda.gov/News_&_Events/Const_Update_051300/index.asp (accessed 02/23/2013).

FSIS. 2013a. The FSIS Microbiological Testing Program for Ready-to-Eat (RTE) Meat and Poultry Products, 1990–2011. www.fsis.usda.gov/Science/Micro_Testing_RTE_Continuation/index.asp#previous (accessed 02/26/2013).

FSIS. 2013b. Proposed Rule. 155. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg. 78(5): 1532.

Hinkens, J.C., N. Faith, T. Lorang, P. Bailey, D. Buege, C. Kaspar, and J. Luchansky. Validation of Pepperoni Processes for Control of *Escherichia coli* O157:H7. J. Food Protect. 59(12):1260-1266.

Hohmann, E. 2001. Nontyphoidal Salmonellosis. Clin. Infect. Dis. 32:263-269.

Hwang, C.A., A.C.S. Porto-Fett, V.K. Kuneja, S.I. Ingham, B.H. Ingham, and J.B. Luchansky. 2009. Modeling the Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium during Fermentation, Drying, and Storage of Soudjouk-Style Fermented Sausage. Int. J. Food Microbiol. 129: 244-252.

Incze, K. 1998. Dry Fermented Sausages. Meat Sci. 49(Suppl. 1): S169-S177.

Ihnot, A.M., A.M. Roering, R.K. Wierzba, N.G. Faith, and J.B. Luchansky. 1998. Behavior of *Salmonella typhimurium* DT104 during the Manufacture and Storage of Pepperoni. Int. J. Food Microbiol. 40:117-121.

Juneja, V.K., B.S. Eblen, and H.M. Marks. 2001. Modeling Non-Linear Survival Curves to Calculate Thermal Inactivation of *Salmonella* in Poultry of Different Fat Levels. Int. J. Food Microbiol. 70: 37-51.

Kotrola, J.S., and D.E. Conner. 1997. Heat Inactivation of *Escherichia coli* O157:H7 in Turkey Meat as Affected by Sodium Chloride, Sodium Lactate, Polyphosphate, and Fat Content. J. Food Protect. 60(8):898-902.

Levine, P., B. Rose, S. Green, G. Ransom, and W. Hill. Pathogen Testing of Ready-to-Eat Meat and poultry Products Collected at Federally Inspected Establishments in the United States, 1990 to 1999. J. Food Protect. 64(8): 1188-1193.

Luzzi, I., P. Galetta, M. Massari, C. Rizzo, A.M. Dionisi, E. Feletici, A. Cawthorne, A. Tozzi, M. Argentieri, and S. Bilei. 2007. An Easter Outbreak of *Salmonella* Typhimurium DT104A Associated with Traditional Pork Salami in Italy. Euro Surveill. 12(3-6): 149-152.

Metaxopoulos, J., C. Genigeorgis, M. Fanelli, C. Franti, and E. Cosma. 1981. Production of Italian Dry Salami: Effect of Starter Culture and Chemical Acidulation on Staphylococcal Growth in Salami under Commercial Manufacturing Conditions. App. Environ. Microbiol. 42(5):863-871.

Naim, F., S. Messier, L. Saucier, and G. Piette. 2003. A Model Study of *Escherichia coli* O157:H7 Survival in Fermented Dry Sausages -- Influence of Inoculum Preparation, Inoculation Procedure, and Selected Process Parameters. J. Food Protect. 66(12): 2267-2275.

Nickelson, R., J. Luchansky, C. Kaspar, and E. Johnson. Dry Fermented Sausage and *E. coli* O157:H7. Blue Ribbon Task Force. National Cattlemen's Beef Association. Research Report no. 11-316.

Nightingale, K.K., H. Thippareddi, R.K. Phebus, J.L. Marsden, and A.L. Nutsch. 2006. Validation of a Traditional Italian-Style Salami Manufacturing Process for Control of *Salmonella* and *Listeria monocytogenes*. J. Food Protect. 69(4): 794-800.

Nitisinprasert, S., V. Nilphai, P. Bunyun, P. Sukyai, K. Doi, and K. Sonomoto. 2000. Screening and Identification of Effective Thermotolerant Lactic Acid Bacteria Producing Antimicrobial Activity against *Escherichia coli* and *Salmonella* sp. Resistant to Antibiotics. Kasetsart J. (Nat. Sci.) 34: 387-400.

Ordonez, J., E. Hierro, and J. Bruna. 1999. Changes in the Components of Dry-Fermented Sausages During Ripening. Crit. Rev. Food Sci. Nutr. 39(4):329-367.

Pontello, M., L. Sodano, N. Nastasi, C. Mammina, and the working group: M. Astuti, M. Domenichini, G. Belluzzi, E. Soccini, M.G. Silvestri, M. Gatti, E. Gerosa, and A. Montagna. 1998. A Community-Based Outbreak *Salmonella enterica* Serotype Typhimurium Associated with Salami Consumption in Northern Italy. Epidemiol. Infect. 120: 209-214.

Porto-Fett, A.C.S., C.-A. Hwang, J.E. Call, V.K. Jenuja, S.C. Ingham, B.H. Ingham, and J.B. Luchansky. 2008. Viability of Multi-Strain Mixtures of *Listeria monocytogenes*, *Salmonella typhimurium*, or *Escherichia coli* O157:H7 Inoculated into the Batter or Onto the Surface of a Soudjouk-Style Fermented Dry Sausage. Food Microbiol. 25: 793-801.

Porto-Fett, A.C.S., J.E. Call, B.E. Shoyer, D.E. Hill, C. Pshebniski, G.J. Cocoma, and J.B. Luchansky. 2010. Evaluation of Fermentation, Drying, and/or High Pressure Processing on Viability of *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp., and *Trichinella spiralis* in Raw Pork and Genoa Salami. Int. J. Food Microbiol. 140:61-75.

Riordan, D., G. Duffy, J. Sheridan, R.C. Whiting, I.S. Blair, and D.A. McDowell. 2000. Effects of Acid Adaptation, Product pH, and Heating on Survival of *Escherichia coli* O157:H7 in Pepperoni. App. Environ. Microbiol. 66(4): 1726-1729.

Sauer, C.J., J. Majkowski, S. Green, and R. Eckel. 1997. Foodborne Illness Outbreak Associated with a Semi-Dry Fermented Sausage Product. J. Food Protect. 60(12): 1612-1617.

Scallan, E., R. Hoekstra, F. Angulo, R. Tauxe, M. Widdowson, S. Roy, J. Jones, and P. Griffin. 2011. Foodborne Illness Acquired in the United States – Major Pathogens. Emerg. Infect. Dis. 17(1):7-15.

Smith, J.L., C.N. Huhtanen, J.C. Kissinger, and S.A. Palumbo. 1975. Survival of *Salmonella* during Pepperoni Manufacture. App. Microbiol. 30(5): 759-763.

Sofos, J. 1981. Nitrite, Sorbate and pH Interaction in Cured Meat Products. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 34. p. 104-120.

Tarr, P., T. Besser, D. Hancock, W. Keene, and M. Goldoft. 1997. Verotoxigenic *Escherichia coli* Infection: U.S. Overview. J. Food Protect. 60(11):1466-1471.

Tilden, J., W. Young, A.M. McNamara, C. Custer, B. Boesel, M.A. Lambert-Fair, J. Majkowski, D. Vugia, S.B. Werner, J. Hollingsworth, and J.G. Morris. 1996. A New Route of Transmission for *Escherichia coli*: Infection from Dry Fermented Salami. Am. J. Public Health. 80(8):1142-1145.

Toldrà, F., Y. Hui, I. Astiasaran, W. Nip, J. Sebranek, E. Silveira, L. Stahnke, and R. Talon. 2007. Handbook of Fermented Meat and Poultry. Blackwell Publishing. Ames, IA.

Figure legends

Figure 1. Inactivation of experimentally inoculated *Salmonella* strains during post-fermentation thermal processing of pepperoni at 116^{o}F.

Figures 2. Inactivation of experimentally inoculated *Salmonella* strains during post-fermentation thermal processing of pepperoni at 124^{o}F.

Figure 3. Inactivation of experimentally inoculated *Salmonella* strains during post-fermentation thermal processing of pepperoni at 132^{o}F.

Figure 4. Inactivation of experimentally inoculated *Salmonella* strains during post-fermentation thermal processing of pepperoni at 140^{o}F.

Figure 5. Inactivation of experimentally inoculated *Salmonella* strains during post-fermentation thermal processing of pepperoni at 148^{o}F.

Figure 6. Predicted cumulative post-fermentation reduction of *Salmonella* spp. in product held at 116^{o}F for the time indicated (top curve = high fat formulations; middle curve = mid-range fat formulations; and bottom curve = low fat formulations).

Figure 7. Predicted cumulative post-fermentation reduction of *Salmonella* spp. in product held at 124^{o}F for the time indicated (top curve = high fat formulations; middle curve = mid-range fat formulations; and bottom curve = low fat formulations).

Figure 8. Predicted cumulative post-fermentation reduction of *Salmonella* spp. in product held at 132^{o}F for the time indicated (top curve = high fat formulations; middle curve = mid-range fat formulations; and bottom curve = low fat formulations).

Figure 9. Predicted cumulative post-fermentation reduction of *Salmonella* spp. in product held at 140^{o}F for the time indicated (top curve = high fat formulations; middle curve = mid-range fat formulations; and bottom curve = low fat formulations).

Figure 10. Predicted cumulative post-fermentation reduction of *Salmonella* spp. in product held at 148^{o}F for the time indicated (top curve = high fat formulations; middle curve = mid-range fat formulations; and bottom curve = low fat formulations).

Table legends

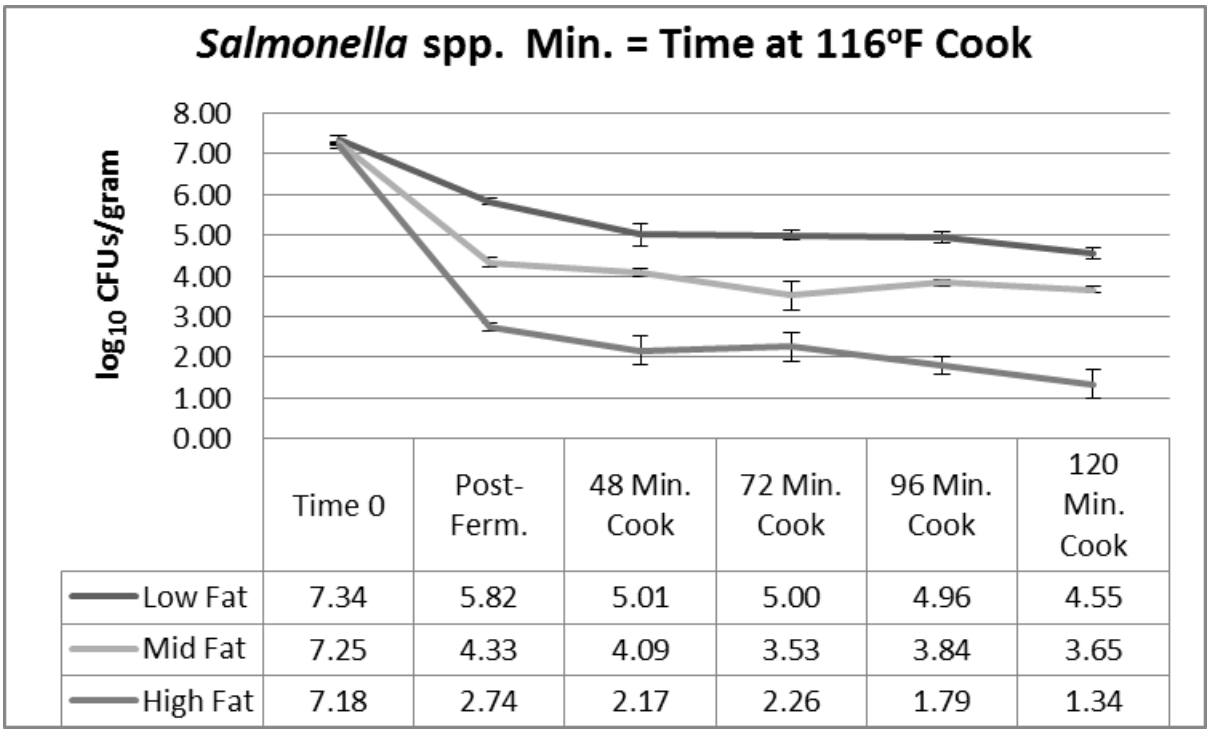
Table 1. Meat block formulations to achieve desired finished product fat proportion (all other ingredients were held constant across all formulations).

Table 2. Sample matrix indicating the number of samples obtained for microbiological analyses at each time and temperature.

Table 3. Predicted time (minutes) to achieve the proposed minimum inactivation of *Salmonella* spp. at the indicated post-fermentation cook temperature.

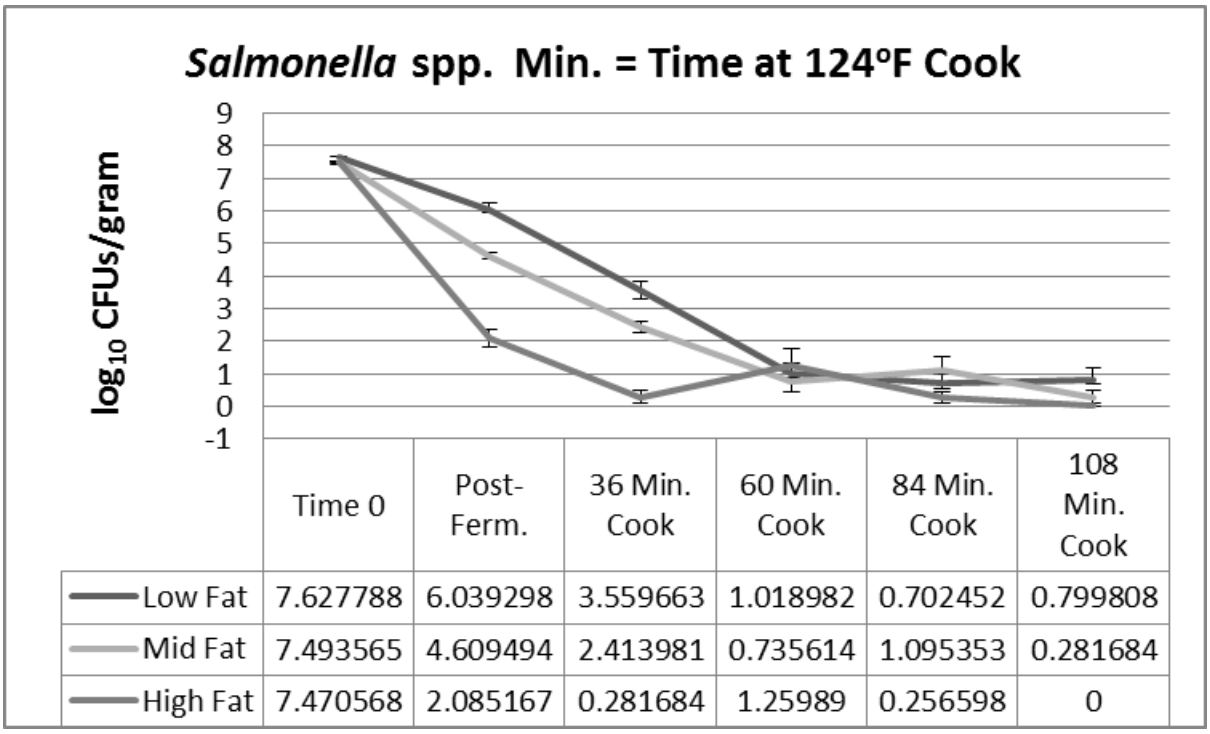
Figures

Figure 1.



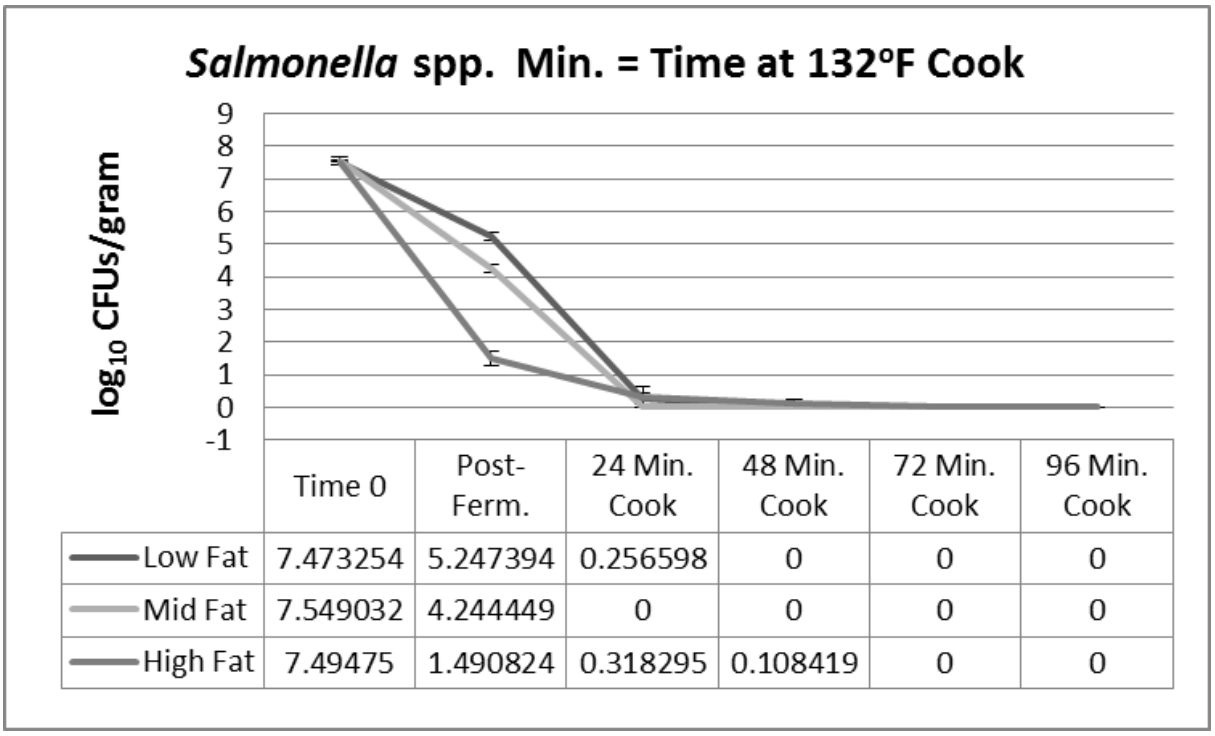
	Time 0	Post-Ferm.	48 Min. Cook	72 Min. Cook	96 Min. Cook	120 Min. Cook
Low Fat	7.34	5.82	5.01	5.00	4.96	4.55
Mid Fat	7.25	4.33	4.09	3.53	3.84	3.65
High Fat	7.18	2.74	2.17	2.26	1.79	1.34

Figure 2.



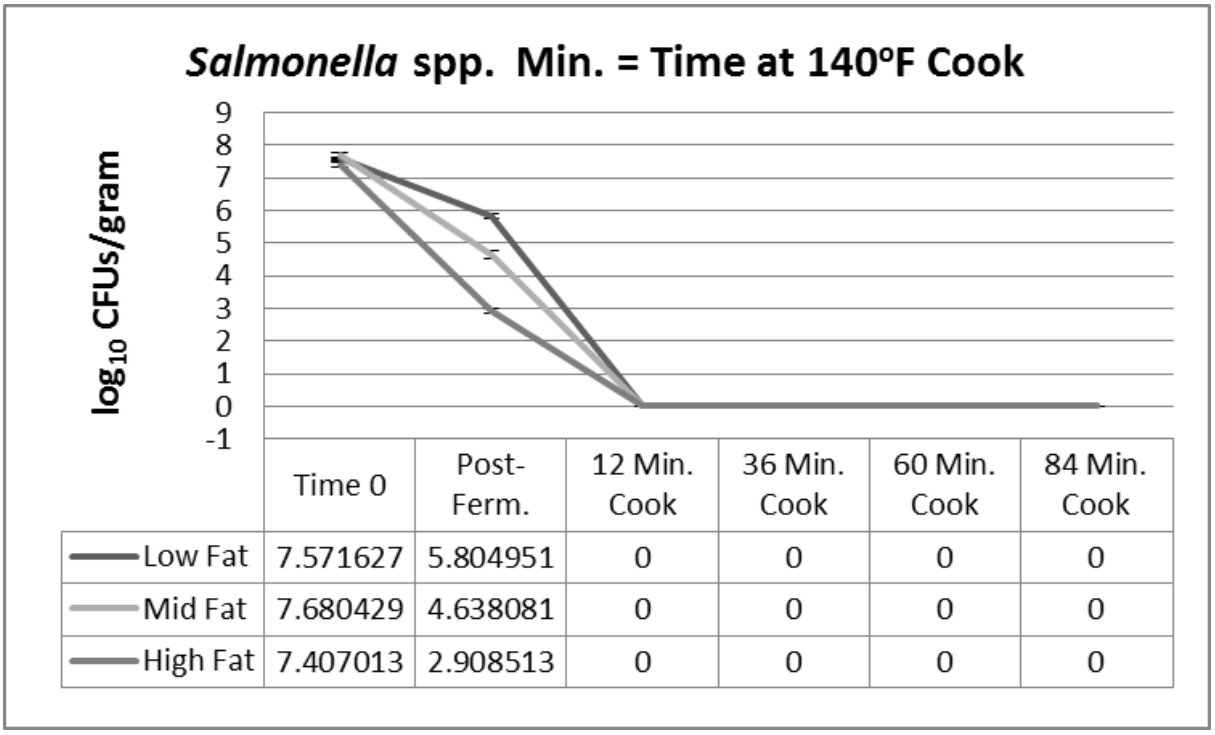
	Time 0	Post-Ferm.	36 Min. Cook	60 Min. Cook	84 Min. Cook	108 Min. Cook
Low Fat	7.627788	6.039298	3.559663	1.018982	0.702452	0.799808
Mid Fat	7.493565	4.609494	2.413981	0.735614	1.095353	0.281684
High Fat	7.470568	2.085167	0.281684	1.25989	0.256598	0

Figure 3.



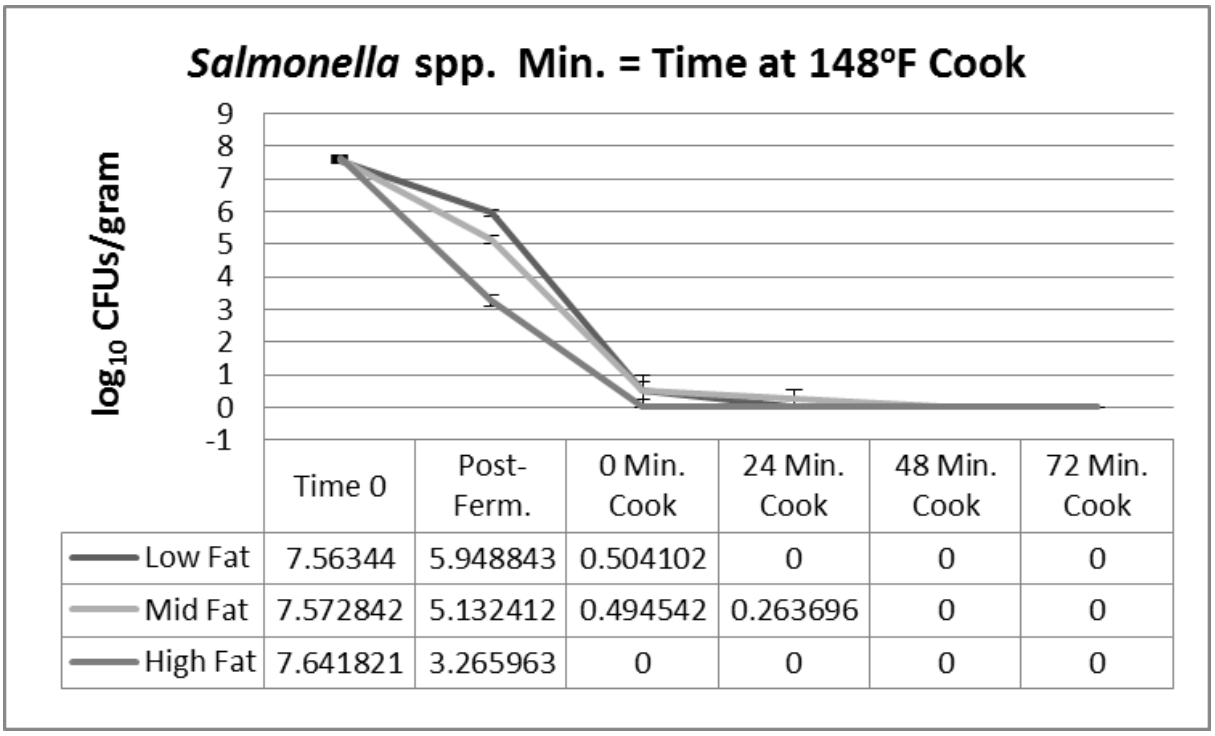
	Time 0	Post-Ferm.	24 Min. Cook	48 Min. Cook	72 Min. Cook	96 Min. Cook
Low Fat	7.473254	5.247394	0.256598	0	0	0
Mid Fat	7.549032	4.244449	0	0	0	0
High Fat	7.49475	1.490824	0.318295	0.108419	0	0

Figure4.



	Time 0	Post-Ferm.	12 Min. Cook	36 Min. Cook	60 Min. Cook	84 Min. Cook
Low Fat	7.571627	5.804951	0	0	0	0
Mid Fat	7.680429	4.638081	0	0	0	0
High Fat	7.407013	2.908513	0	0	0	0

Figure 5.



	Time 0	Post-Ferm.	0 Min. Cook	24 Min. Cook	48 Min. Cook	72 Min. Cook
Low Fat	7.56344	5.948843	0.504102	0	0	0
Mid Fat	7.572842	5.132412	0.494542	0.263696	0	0
High Fat	7.641821	3.265963	0	0	0	0

Figure 6.

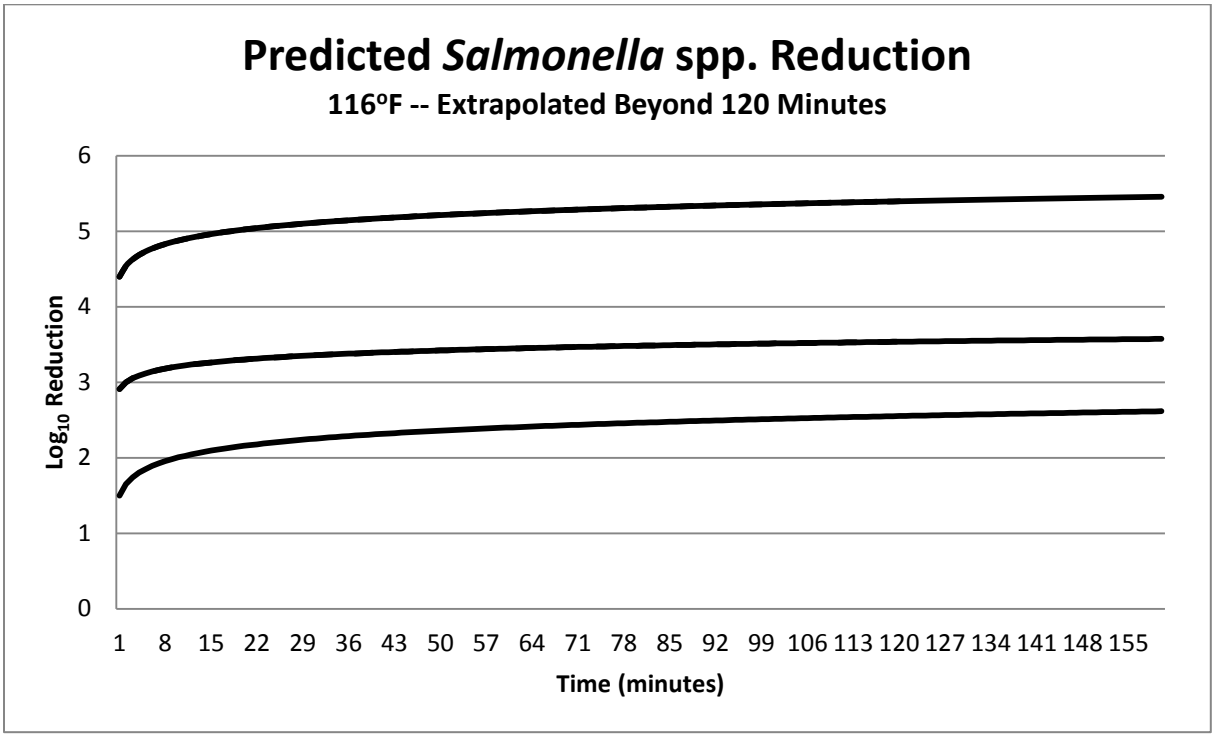


Figure 7.

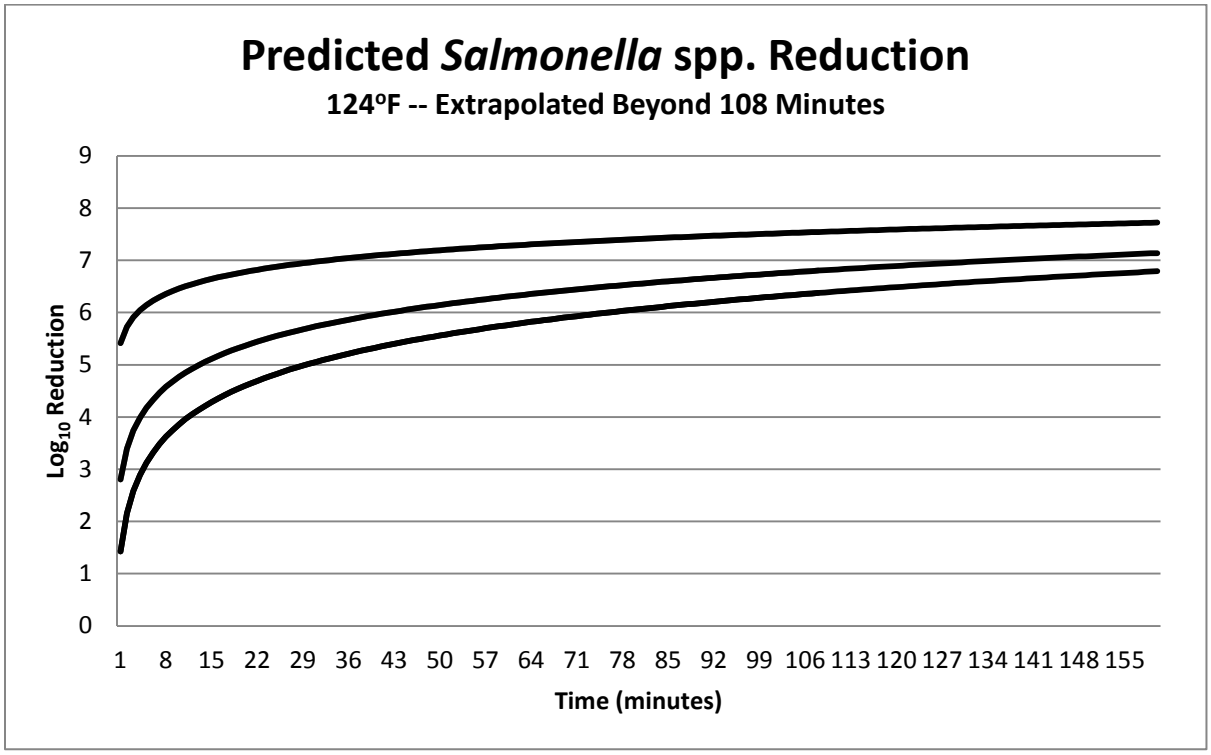


Figure 8.

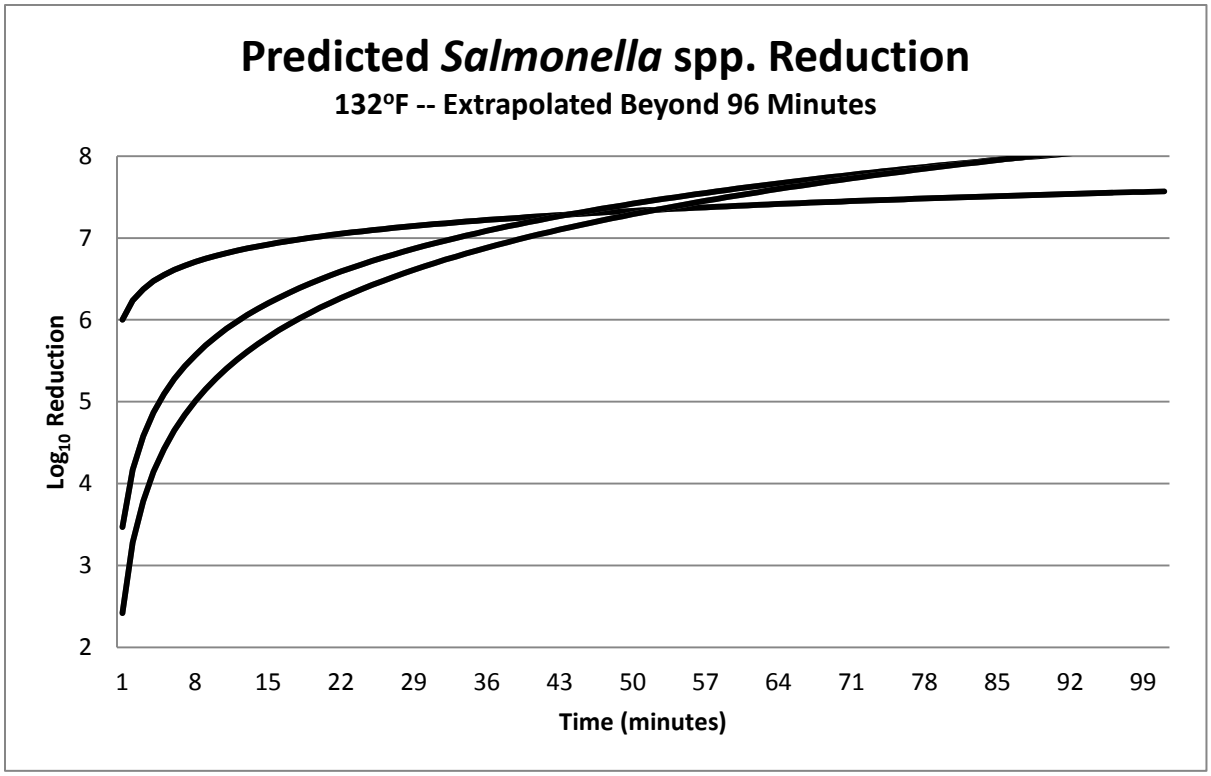


Figure 9.

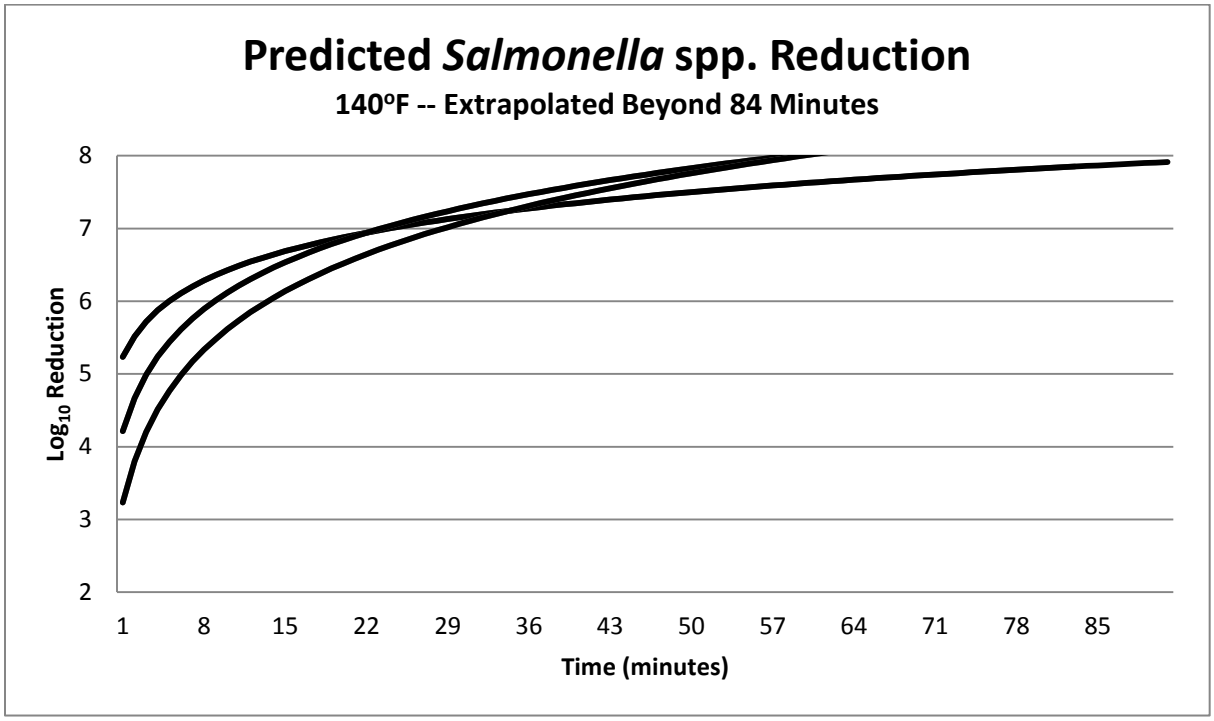
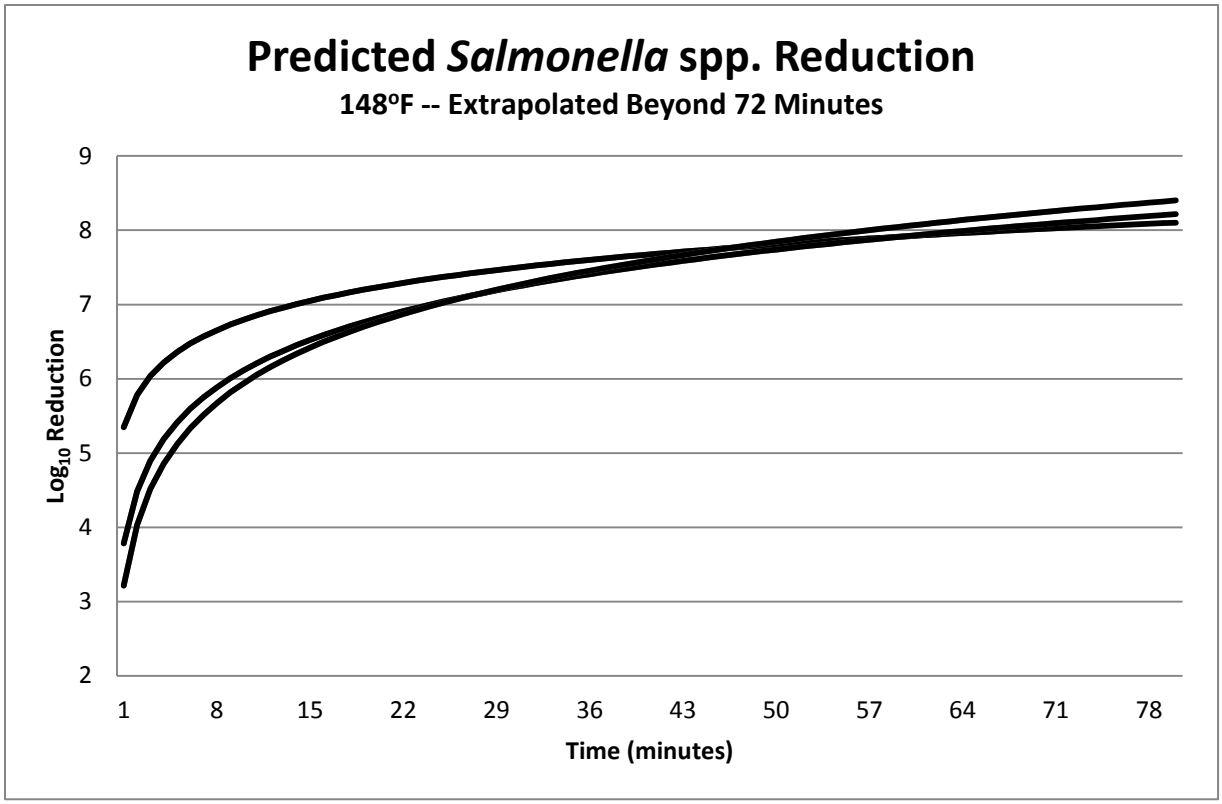


Figure 10.



Tables

Table 1.

		Pork 42s		Pork 72s		Pork 90s		MSC		Beef 50s		Beef 90s	
	Target % Finished Fat	Pounds	Percent	Pounds	Percent	Pounds	Percent	Pounds	Percent	Pounds	Percent	Pounds	Percent
PB-Low	15.1%	--	--	--	--	35	70.0%	--	--	--	--	15	30.0%
PB-Mid	33.3%	--	--	--	--	35	70.0%	--	--	15	30.0%	--	--
PB-Hi	50.0%	--	--	28.25	56.5%	6.75	13.5%	--	--	15	30.0%	--	--
PCB-Low	17.6%	--	--	--	--	25	50.0%	22.5	45.0%	--	--	2.5	5.0%
PCB-Mid	33.6%	--	--	22.5	45.0%	2.5	5.0%	22.5	45.0%	2.5	5.0%	--	--
PCB-Hi	49.1%	16.5	33.0%	8.5	17.0%	--	--	22.5	45.0%	2.5	5.0%	--	--

PB = Pork and Beef; PCB = Pork, Chicken and Beef; Low, Mid, High = Finished fat target level.

Table 2.

Pepperoni Validation -- High / Mid / Low Fat; Traditional / Chicken Formulation										
	Temperature (Degrees F) -- (Numeral) next to Trial Number is order the trials were run. Numerals in the matrix indicate the number of microbiological samples analyzed at each time / temperature combination.									
Time (Minutes)	Trials 1 (8) & 2 (10)		Trials 3 (1) & 4 (5)		Trials 5 (6) & 6 (2)		Trial 7 (4) & 8 (7)		Trials 9 (9) & 10 (3)	
	5	5	5	5	5	5	5	5	5	5
	5	5	5	5	5	5	5	5	5	5
$Time_0$	5	5	5	5	5	5	5	5	5	5
Ferment	112^o		112^o		112^o		112^o		112^o	
	5	5	5	5	5	5	5	5	5	5
Fermented	5	5	5	5	5	5	5	5	5	5
$Time_{720}$	5	5	5	5	5	5	5	5	5	5
Cook	116^o		124^o		132^o		140^o		148^o	
									3	3
Cooked									3	3
Time Instant									3	3
							3	3		
Cooked							3	3		
Time 12							3	3		
					3	3			3	3
Cooked					3	3			3	3
Time 24					3	3			3	3
			3	3			3	3		
Cooked			3	3			3	3		
Time 36			3	3			3	3		
	3	3			3	3			3	3
Cooked	3	3			3	3			3	3
Time 48	3	3			3	3			3	3
			3	3			3	3		
Cooked			3	3			3	3		
Time 60			3	3			3	3		
	3	3			3	3			3	3
Cooked	3	3			3	3			3	3
Time 72	3	3			3	3			3	3
			3	3			3	3		
Cooked			3	3			3	3		
Time 84			3	3			3	3		
	3	3			3	3				
Cooked	3	3			3	3				
Time 96	3	3			3	3				
			3	3						
Cooked			3	3						
Time 108			3	3						
	3	3								
Cooked	3	3								
Time 120	3	3								

Legend	
PB_{HI}	PCB_{HI}
PB_{MID}	PCB_{MID}
PB_{LOW}	PCB_{LOW}

Key
PB = Pork & Beef Formula: 70% / 30%
PCB = Pork, Chicken & Beef Formula: ~50% / ~45% / ~5%
Subscript "HI", "MID" & "LOW" = Fat: ~50% / ~33% / ~15%

Table 3.

	116°F		124°F		132°F		140°F		148°F	
	6.5 $\log_{10}$	7 $\log_{10}$	6.5 $\log_{10}$	7 $\log_{10}$	6.5 $\log_{10}$	7 $\log_{10}$	6.5 $\log_{10}$	7 $\log_{10}$	6.5 $\log_{10}$	7 $\log_{10}$
High Fat	--	--	11	33	5	19	13	25	7	14
Mid Fat	--	--	76	137*	21	34	16	25	15	25
Low Fat	--	--	122*	--	27	40	21	30	17	25

-- There were no time/temp combinations at this cook temperature that provided the minimum necessary inactivation.

* Represents a time value outside the range of experimental observations (extrapolated data).

VIII.Chapter 4

Model Validation Alternative Time / Temp Combinations for Inactivation of *Salmonella* spp. in Pepperoni

Validating a Predictive Model for Alternative Time / Temperature Thermal-Processing Combinations for Inactivation of *Salmonella* spp. in Two Formulations of Pepperoni

Scott Stillwell*, Eric Oxford, and Daniel Zelenka

Tyson Foods, Inc., Springdale, Arkansas 72762, USA

Key words: *Salmonella* spp., microbial inactivation, pepperoni, fermentation, validation, predictive model

*Author for correspondence. Tel: (479) 290-7186; Fax: (479) 757-6888
E-mail: scott.stillwell@tyson.com

ABSTRACT

Validation of processing controls to achieve the desired or required level of finished product food safety is a critical component of HACCP Principles. Proposed FSIS policy modifications related to the pathogen reduction requirements in Ready-to-Eat meat and poultry products have the potential to impose significant cost on manufacturers of fermented sausage products. The processing conditions for these products have historically been validated to meet one of the options described by the Blue Ribbon Task Force for control of *Escherichia coli* O157:H7 that were developed following a 1994 outbreak of illnesses associated with a fermented beef salami. Limited data exist to assess whether the processing conditions are sufficient to deliver a 6.5 or 7.0 $\log_{10}$ inactivation of the salmonellae as proposed. This study was designed to further develop models for assessing the pathogen-inactivation achieved by current processing conditions for comparison to the proposed pathogen reduction performance standards and to assess whether the proposed changes have merit. Previously developed predictive inactivation models were expanded and challenged with inoculated pepperoni formulations to evaluate the cumulative inactivation achieved through fermentation and thermal processing. These results indicate that the processes validated to achieve a 5.0 $\log_{10}$ inactivation of *Escherichia coli* O157:H7 are sufficient to achieve at least the proposed levels of inactivation of the salmonellae leading us to conclude that policy changes for fermented meat and poultry products are unnecessary.

Pepperoni is a modern iteration of a cured and fermented, dried spicy meat sausage typically formulated with paprika and garlic. With estimates of domestic consumption reaching well over 250 million pounds per year, it is the most widely consumed type of dried fermented sausage in the U.S. (Buccheri 2012) and is primarily use as toppings for pizza products or as a filling for bread-enrobed products such as calzones. Like all manufacturers of Ready-to-Eat (RTE) products, those that make pepperoni have a duty to ensure that microbiological safety controls are designed into their production processes. HACCP plans must be robust or the processor risks brand damage and liabilities in the event of illnesses due to consumption of the products. As the scientific understanding of the curing and fermentation process has developed, many process controls have been introduced -- e.g. curing with sodium nitrite for control of toxigenic spore-forming pathogens (Sofos 1981) and added carbohydrates and use of commercial lactic acid producing starter cultures to rapidly acidify the products during fermentation for control of toxigenic *Staphylococcus aureus* (Metaxopoulos *et al*. 1981). Currently, the primary vegetative enteric pathogens of concern for manufacturers of fermented RTE meat and poultry products are the serotypes of *Salmonella* and strains of enterohemorrhagic *Escherichia coli* (*E. coli*) (Hwang *et al*. 2009).

Salmonellosis is one of the leading human foodborne illnesses in the U.S. with estimates of approximately 3,500 illnesses per 100,000 people per year; second only to foodborne illnesses caused by viruses (Scallan *et al*. 2011). The case rate of human salmonellosis has been relatively constant since 1998 and unlike other notable enteric pathogens such as *E. coli* O157:H7, progress has not been made toward the national goals of reducing foodborne illnesses caused by the salmonellae (CDC 2011a). A variety of serotypes of *Salmonella* have been implicated, both domestically and internationally, in illnesses and outbreaks associated with consumption of RTE fermented dry and semi-dry sausages. In recent instances, it was apparent that the manufacturing process was not sufficiently robust to inactivate the vegetative pathogen load present on the raw materials ($>10^4$ CFU/g) (Sauer *et al.* 1997) or resulted in finished product characteristics – pH, water activity or lack of maturation time -- that allowed *Salmonella* spp. to survive or proliferate to numbers sufficient to cause illness upon consumption (Bremer *et al*. 2004, Emberland *et al*. 2006, Luzzi *et al*. 2007, and Pontello *et al*. 1998). Traditional processes with no thermal-lethality step are more prone to result in pathogen positive finished products and a number of authors

have concluded that a thermal-lethality (cook) step is necessary for adequate inactivation of vegetative pathogens in these products (Hinkens *et al*. 1996, Nightingale *et al*. 2006, Smith *et al*. 1975).

As previously described in previous chapters of this dissertation, numerous federal regulatory policy changes have evolved in the U.S. following a 1994 outbreak of illnesses caused by *E. coli* O157:H7 in a fermented beef salami (Tilden *et al*. 1996). For example, the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) staff worked in collaboration with industry representatives from the "Blue Ribbon Task Force" to develop five options designed to ensure the safety of fermented dry and semi-dry meat products (Nickelson *et al*. 1996, Naim *et al*. 2003). Since those process changes, staff from FSIS have collected and analyzed more than 10,000 finished RTE samples (including cooked beef patties) for the presence of *E. coli* O157:H7 and have not reported a positive sample. As a result, FSIS staff announced in May 2011 that they were suspending the testing of fermented meat products for *E. coli* O157:H7 (FSIS 2011), thus leaving the serotypes of *Salmonella* as the primary pathogen of concern in this class of products.

In 1999, the staff at FSIS converted prescriptive time and temperature processing requirements for certain RTE meat and poultry products into performance standards requiring the process to achieve either a 6.5 $\log_{10}$ (meat products) or a 7.0 $\log_{10}$ (poultry products) inactivation of serotypes of *Salmonella* in the finished product (FSIS 1999a). Specific time and temperature combinations that were validated to achieve those reductions were published in a FSIS Directive as "Appendix-A" (FSIS 1999b). In January 2013, USDA staff published an announcement in the Semi-Annual Regulatory Agenda of their intent to move forward with an Interim Final Rule codifying new requirements and extending the Pathogen Reduction Performance Standards to all RTE meat and poultry products (FSIS 2013b). These proposed regulatory changes, if enacted, are expected to have significant impacts. The regulated industry will be required to revalidate HACCP plans for a wide variety of products that are currently validated to achieve a 5.0 $\log_{10}$ inactivation of *E. coli* O157:H7 to demonstrate an adequate reduction in the salmonellae and as previously documented, these changes are expected to impose significant cost and quality impacts.

The present study was designed to assess the ability of a modified and expanded version of previously developed inactivation models (previous chapters of this dissertation) to predict the *Salmonella* spp. inactivation dynamics in two different formulations of pepperoni products and assess the potential for

the use of a surrogate, nonpathogenic microorganism, *Enterococcus faecalis*, for in-plant trials where heavily pathogen-inoculated formulations would be inappropriate.

The staff at FSIS has previously indicated that non-pathogenic strains of microorganisms may be used in product formulations as surrogates for pathogens under conditions that do not result in the creation of a food safety hazard, insanitary condition or otherwise cause the food to become adulterated (FSIS 2010). As defined by previous authors, an appropriate surrogate microorganism should be non-pathogenic; genetically stable; easily grown to high-density and easily enumerated in the lab; easily distinguishable from the expected background microflora; and most importantly, it should possess similar inactivation kinetics in the model system as the pathogenic microorganism it is intended to model (Kim and Linton 2008).

We have previous experience with modeling the heat inactivation of *Enterococcus faecalis* (*E. faecalis*) as a surrogate for *Salmonella* Senftenberg having shown it to be substantially more thermo-tolerant and having correlated the inactivation over a wide temperature range (unpublished data). Other authors have used a wide variety of non-pathogenic microorganisms as surrogates for model development. These include commercial lactic acid bacteria (LAB) starter cultures which were shown to be effective. They demonstrated a similar level of inactivation ($\geq$ 5.0 $\log^{10}$) of a cocktail of serotypes of *Salmonella* during fermentation of summer sausage (Borowski *et al*. 2009) as we had demonstrated during fermentation of pepperoni (previoous chapters of this dissertation). Others have used generic Bio-type I *E. coli* (Niebuhr *et al*. 2008) and *Enterococcus faecium* (Jeong *et al*. 2011). We selected the *E. faecalis* given our knowledge of its thermo-tolerance and did not elect to use one of these from the literature for the following reasons: the LAB starter culture to be utilized in the trials (SAGA 200) and our stock non-pathogenic Bio-type I *E. coli* cultures were difficult to distinguish on the selective media for enumeration; and *Enterococcus faecium* is known to produce a bacteriocin (Leroy *et al.* 2003) which may have affected the inactivation kinetics in the model system.

The previously produced models predicting the inactivation of *Salmonella* spp. in pepperoni were challenged with two different pepperoni formulations at a cook time and temperature not used for model development. Product meat specie differences (pork/beef vs. pork/chicken/beef) with a mid-range fat level in the formulation (~ 33%) were studied to assess the level of inactivation achieved versus the

prediction of the models. Inclusion of a potential surrogate (*E. faecalis*) was included in the formulation with the intent to develop correlations to the inactivation kinetics of the species of *Salmonella* for further model development.

This work is expected to be beneficial particularly to small processors of similar products as a tool for assessing current process and product parameters against the proposed microbial reduction performance standards. The data derived will inform additional research intended to develop a mathematical model for predicting inactivation of serotypes of *Salmonella* in fermented meat products.

MATERIALS AND METHODS

The previously developed models were extensively expanded by interpolation of the existing data to estimate the inactivation achieved under conditions of time and temperature at given proportions of formulated fat. Approximately 20 pounds of raw commercially processed standard pepperoni (pork and beef) and 20 pounds of non-standard pizza topping (pork, chicken and beef) meat "batter" were obtained from a large manufacturing operation owned by Tyson Foods, Inc. operating in Dallas, Texas. The batter was prepared by the processing plant staff utilizing the commercial manufacturing equipment and divided from a much larger batch immediately after all raw processing steps had been completed. All ingredients were blended to achieve the following (approximate) blend formulations: pork (70%) and beef (30%) blend with medium (33%) fat; and pork (50%), chicken (45%) and beef blend (5%) with medium (33%) fat. The batter was placed into plastic bags, labeled, placed into a foam shipping cooler with multiple cold packs, and transported by overnight courier to Tyson Foods' corporate laboratory in Springdale, Arkansas where the samples were held in a commercial walk-in cooler at 34^{o}F (1.2^{o}C) pending processing.

Sample Preparation: Two concentrated microbial cultures for inoculation were prepared. The first culture consisted of an overnight (18 -- 24 hours after the concentrated microorganisms were washed, re-suspended in sterile saline and refrigerated) culture of a five-serotype cocktail of *Salmonella* comprised of: *Salmonella* Senftenberg ATCC 43845 obtained from the Tyson Foods collection; and four serologically distinct "wild" strains of *Salmonella* of unknown serotype isolated from routine whole-bird carcass rinse samples of commercial broilers from Tyson Foods' commercial poultry slaughter operations. The second culture consisted of an overnight culture of *Enterococcus faecalis* ATCC29212 intended for modeling purposes and obtained from the Tyson Foods collection. The raw meat batter had previously been

inoculated at the processing plant with a commercial lactic acid starter culture (SAGA 200 containing the homo-fermentative lactic acid-producing bacteria *Pediococcus acidilactici* from Kerry Ingredients and Flavors, Beloit, WI 53511). The pathogen and surrogate cultures had been concentrated by Tyson Foods' research microbiologists to provide an initial inoculation level of approximately $10^{7.5}$ colony forming units (CFUs) per gram of pepperoni batter.

Sample Inoculation: On the date of processing, each of the two formulation types were removed from refrigerated storage and 1,125 gram units were weighed and inoculated as previously described (Stillwell *et al.* 2013). The four trial samples (2 trials with 2 replicates) were prepared in a random order predetermined by roll of the die.

Five time$_0$ samples of 25 grams each were weighed from each trial and replicate of the inoculated batter into vacuum bags (Cryovac Sealed Air Corporation, Duncan SC; 8"x16" standard curved end; Model B2470T) for inoculated enumeration. The remaining 1,000 grams of inoculated pepperoni batter was hand-stuffed into pre-soaked (20 minutes in ~ 100^{o}F (38^{o}C) water) 56 mm fibrous casings (Viskase Companies, Inc., Chicago, IL; DS/SL Fibrous). Four inoculated pepperoni sticks were prepared consisting of two replicates of each of the formulations (standard and non-standard); and three additional non-inoculated sticks were prepared: one of each formulation for pH verification following fermentation; and one as a processing temperature monitor after insertion of the smokehouse digital product thermometer probe. After all sticks were stuffed and tied, they were placed in a plastic sample container and returned to the holding refrigerator for a minimum of 60 minutes to equilibrate pending processing.

Sample Processing: Thermal processing was conducted in a commercial, home-style smoker with digital temperature control (Masterbuilt Electric Smokehouse Model 20070910) fitted with a digital thermometer to monitor the product for maintaining the target temperature and water pan for establishing high relative humidity (not controlled or monitored). The initial smoker digital temperature-controller was set for 120^{o}F (48.9^{o}C) to start the fermentation process. Once the product temperature reached the target fermentation temperature, the digital temperature-controller was reset for 112^{o}F (44.4^{o}C) and the product temperature was maintained (once it reached steady state) at +/- 1.5^{o}F based upon observation of the indwelling thermometer probe display.

After twelve hours, a representative stick (non-pathogen inoculated) of each of the two formulas were verified to ensure the pH was below 4.95 (pH model number DPH230SD, General Tools and Instruments Co, LLC, New York, NY). Once successful fermentation was verified, the smoker digital temperature controller was reset at 136^{o}F (57.8^{o}C).

As soon as the indwelling thermocouple indicated the product temperature had reached 124^{o}F (to allow for equilibration due to heat penetration), the smoker temperature controller was set to 128^{o}F (53.3^{o}C). After the product temperature at the center of mass reached 128^{o}F (53.3^{o}C), the digital timer on the smokehouse was started to indicate when the cooked samples were to be removed from the smoker. After the designated time at temperature for the trial conditions had elapsed (60 minutes), the sticks of cooked samples were removed; immediately vacuum packaged and chilled in an ice-slush water bath. After the samples were chilled, 25 gram samples were aseptically cut from the pepperoni sticks; placed into vacuum bags labeled with sample-designation information; and placed into the holding refrigerator for microbiological analyses.

Microbiological Analyses: Using standard protocols for sample preparation, serial dilution and spread-plate enumeration, Tyson Foods' corporate research microbiologists prepared each individual sample for recovery and enumeration. Trypticase Soy Agar plates (in duplicate for each sample and dilution) were serially inoculated, dried for approximately 2 hours in a 35^{o}C incubator, overlaid with Xylose-Lysine-Tergitol 4, a selective medium for recovery of the *Salmonella* strains (DIFCO, Beckton Dickenson, and Company, Franklin Lakes, NJ) and incubated for 24 hours at 35^{o}C. Trypticase Soy Agar plates (in duplicate for each sample and dilution) were serially inoculated; dried for approximately 2 hours in a 35^{o}C incubator, overlaid with KF Streptococcus Agar for recovery of the *E. faecalis*, (Neogen Corporation, Lansing, MI), and incubated for 24 hours at 35^{o}C. Following incubation, each set of plates was examined for typical colonies. Any plates showing no typical colony growth or indications of microbial stress were returned to the incubator for an additional 24 hours. Following standard microbiological laboratory protocols, the research microbiologists reported quantitative results for *Salmonella* spp. and *E. faecalis* recovered from each replicate of each sample type.

Statistical Analyses: Utilizing the Statistical Analysis System (SAS) version 9.3 software for Windows (SAS Institute, Cary NC), the data were log-transformed (all "none detected" samples were

reported as one) for analyses. Mean values were output for significant terms at each step. No repeated measures were represented as the testing of the samples was destructive; thus, no two measures were taken on the same sample.

The mean values for microbial reduction in viable count were analyzed for each replicate and processing treatment to determine whether replicates exhibited significantly different levels of microbial inactivation; to assess the treatment effect on microbial inactivation; and to determine the cumulative microbial inactivation achieved. The SAS General Linear Model was utilized for the Means Test, Analysis of Variance and the Duncan's Multiple Range Test.

RESULTS

As expected given that all post-cook samples analyzed were reported as none-detected for *Salmonella* spp., there were no differences in mean reduction achieved between replicates of a treatment. Thus, the *Salmonella* spp. data from both replicates were combined for all further evaluations and graphical representations. Consistent with the first iteration of the model, there was no significant treatment effect due to the protein portion of the formulation indicating that the reduction in *Salmonella* spp. achieved was unaffected by the inclusion of poultry meat in the formulation. Thus, the model for inactivation of *Salmonella* spp. need not consider meat block differences.

There was a significant effect ($p > 0.05$) on the inactivation of *E. faecalis* compared to the *Salmonella* spp. with the mean reduction of *E. feacalis* only 1.48 $\log_{10}$ CFU/gram while the reduction of the *Salmonella* spp. was at least 7.14 $\log_{10}$ CFU/gram (as all samples were reported "none detected"). Further, there was a difference in the reduction achieved depending upon the formulation with a significantly smaller effect on *E. faecalis* in the standard (pork and beef) formulation (1.26 $\log_{10}$) than in the non-standard formulation (1.69 $\log_{10}$).

DISCUSSION

In the event that the staff at FSIS moves forward with extending the pathogen reduction performance standards to all RTE meat and poultry products, there will be a large class of fermented products for which validation data don't exist. It will become imperative for processors to validate across a broad range of fermented meat product formulations that controls are adequate to ensure a 6.5 or 7.0 $\log_{10}$ inactivation of *Salmonella* spp. Challenge studies are the most frequently cited examples to demonstrate

the efficacy of a particular process but must consider the effect of a wide variety of formulation and processing differences. To validate the wide variety of fermented products by challenge study would represent an enormous undertaking (Baranyi and Roberts 1995). Development of predictive models that are validated is a means to minimize the economic impact and disruption to the processing industry that such a policy change might bring and help ensure HACCP plans are robust and effective in protecting public health (McMeekin *et al*. 2002).

We previously developed a two-phase model taking the form {$\log_{10}$ difference = α x (log(time)) + β}. This approach resulted in different models for inactivation of the *Salmonella* spp. To challenge the models, we selected two formulations of mid-level fat pepperoni; one traditional formulation containing pork and beef and one nontraditional formulation containing pork, chicken and beef. We selected a thermal processing (cook) temperature and time that was not included in the original model development data set to challenge the predictive capacity of the models.

As illustrated in Figure 1 for a mid-level fat formulation, it is estimated at 124^{o}F (51.1^{o}C) to take approximately 137 minutes to achieve a 7.0 $\log_{10}$ inactivation; while at 132^{o}F (55.6^{o}C), it is estimated to take approximately 34 minutes for the same level of inactivation of the *Salmonella* spp. for a product containing poultry in the formulation. Figure 2 illustrates an interpolated estimate of the inactivation achieved at 128^{o}F (53.3^{o}C) as the mean reduction of each of the two bounding temperatures at each time. From that estimate, it is apparent that it will take 64 minutes at a post-fermentation thermal processing temperature of 128^{o}F (53.3^{o}C) to achieve the required 7.0 $\log_{10}$ inactivation for a formulation containing poultry.

The results from the trials conducted at 128^{o}F (53.3^{o}C) for 60 minutes post-fermentation thermal processing indicate an inactivation of the *Salmonella* spp. of at least 7.14 $\log_{10}$ as there were no detected survivors. This illustrates that the interpolated estimate is conservative but realistic. The interpolation approach was applied to all of the previously generated model estimates to develop a surface response model as illustrated in Figure 3. This allows for the continuous estimation of the level of inactivation achieved under conditions of fat formulation that range from 17% to 49%; and post-fermentation thermal processing temperature that ranges from 116^{o}F (46.7^{o}C) to 148^{o}F (64.4^{o}C) for a duration of from 1 to160 minutes.

From this model, we extracted the minimum time estimate at a given fat formulation and post-fermentation thermal processing temperature required to achieve either a 6.5 or 7.0 $\log_{10}$ inactivation of *Salmonella* spp. in pepperoni. Those data are represented in Table 1. As is apparent, the table illustrates that longer time or higher temperature processing conditions and higher fat formulations require less time to achieve the targeted level of inactivation.

This approach is highly conservative as these models predict the accumulated lethality through fermentation and thermal processing. A substantial body of data demonstrates that additional inactivation occurs in pepperoni during the drying process and during storage and distribution (Hwang *et al*. 2009, Ihnot *et al*. 1998, and Porto-Fett *et al*. 2010). The results achieved varied from approximately 0.3 – 2.4 $\log_{10}$ inactivation during drying to 4.6 – 6.6 $\log_{10}$ inactivation during storage under vacuum for 56 days. Given the highly variable and process specific reductions achieved, we previously chose a conservative approach and elected to model the time required to achieve full inactivation during the portion of the process for which processors have the greatest levels of process control (previous chapters of this dissertation). However, processors may have a substantial amount of control over critical factors such as drying conditions; or temperature, time and conditions of storage before products are distributed and for quality, yield or throughput, may wish to afford themselves of a less aggressive thermal process and account for the additional inactivation achieved as part of the cumulative requirement.

As such, we have provided two additional tables illustrating the minimum required time at a post-fermentation thermal processing temperature under a given fat formulation to achieve a 4.0, 4.5, 5.0 or 5.5 log10 inactivation of the salmonellae in pepperoni. The models are available by request from the author in the event that a processor has unique needs for assessing any other levels of log inactivation. Recognizing that the underlying models were developed to be conservative, these estimates are intended to ensure a considerable margin for safety.

Given that we have experienced inconsistent results in the inactivation data for the *E. faecalis*, it may be that a different choice for a surrogate is necessary. In the original model data set, there were significant differences in the level of inactivation achieved due to the meat formulation. Under some conditions, a greater inactivation was achieved in the pork and beef formulation; while in others, a greater inactivation was achieved in the nontraditional pork, chicken and beef formulation. These difference

appeared to have a relationship with the post-fermentation thermal processing temperature as the lower temperatures always favored E.f. inactivation in the traditional beef and pork formulation; while the higher temperatures (140 and148^{o}F / 60 and 64.4^{o}C) favored inactivation of the E.f. in the nontraditional pork, chicken and beef formulation.

In the current trial we picked up a significant difference due to the meat formulation. This holds the potential to confound the correlation between *E. faecalis* inactivation and *Salmonella* spp. inactivation or necessitate different models for each of the different formulation conditions. Without an adequate understanding of why these results were achieved, further development of a surrogate model using *E. faecalis* in a fermentation system with differing meat components may not be feasible given that it appears the inactivation kinetics are affected by some unknown formulation attribute. Certainly, further examination is warranted.

This model and data table should prove useful to manufacturers for estimating the level of inactivation of the salmonellae any particular combination of formulation and processing conditions might achieve within the minimum and maximum bounds of the model data set. One should use caution and never rely solely on the estimates of a model. In-plant data will always be preferred and these experimental conditions are specific to the inactivation achieved. Caution should be exercised when attempting to extrapolate these data to formulations utilizing a different lactic acid bacterial starter culture, outside these ranges of fat, or exhibiting extreme levels of cure, salt or other inherently synergistic formulation attributes that may contribute to the cumulative pathogen lethality.

Figure legends

Figure 1. Predicted cumulative post-fermentation reduction of *Salmonella* spp. in product held at 124^{o}F and 132^{o}F for the time indicated and the time at which a cumulative 7.0 $\log_{10}$ inactivation is first achieved.

Figure 2. Predicted cumulative post-fermentation reduction of *Salmonella* spp. in product held at 128^{o}F for the time indicated from the interpolation of the 124^{o}F and 132^{o}F cook model data and the time at which a cumulative 7.0 $\log_{10}$ inactivation if first achieved.

Figure 3. Surface response model of the estimated inactivation achieved at a given time with varying fat formulations and post-fermentation thermal processing temperatures.

Table legends

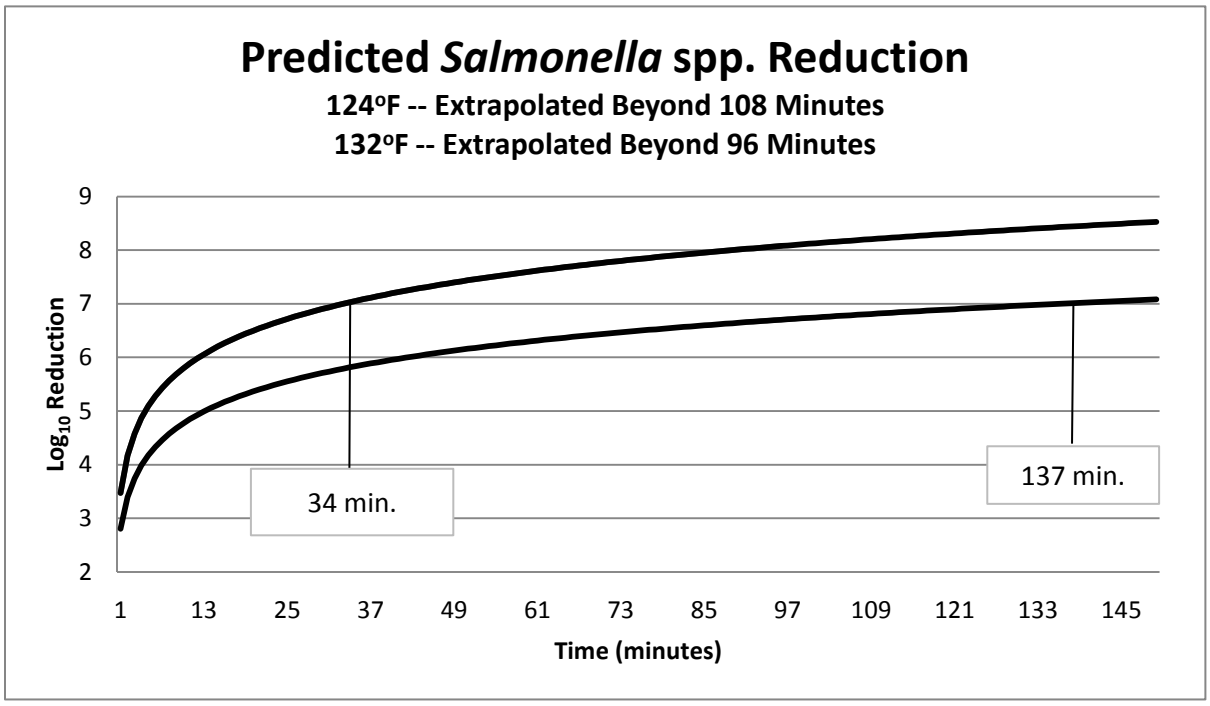
Table 1. Predicted time (minutes) to achieve the proposed minimum inactivation of *Salmonella* spp. at the indicated post-fermentation cook temperature in a formulation containing the indicated proportion of fat.

Table 2. Predicted time (minutes) to achieve a 5.5 or 6.0 $\log_{10}$ inactivation of *Salmonella* spp. at the indicated post-fermentation cook temperature in a formulation containing the indicated proportion of fat.

Table 3. Predicted time (minutes) to achieve a 4.5 or 5.0 $\log_{10}$ inactivation of *Salmonella* spp. at the indicated post-fermentation cook temperature in a formulation containing the indicated proportion of fat

Figures

Figure 1.



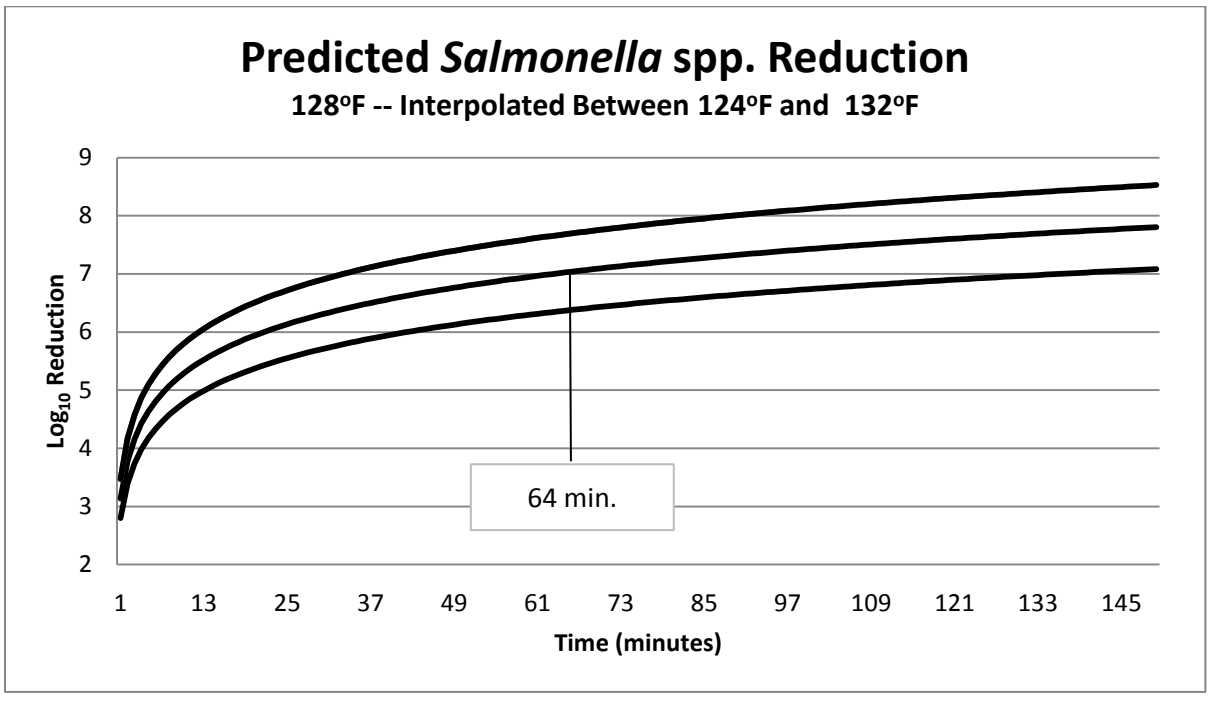
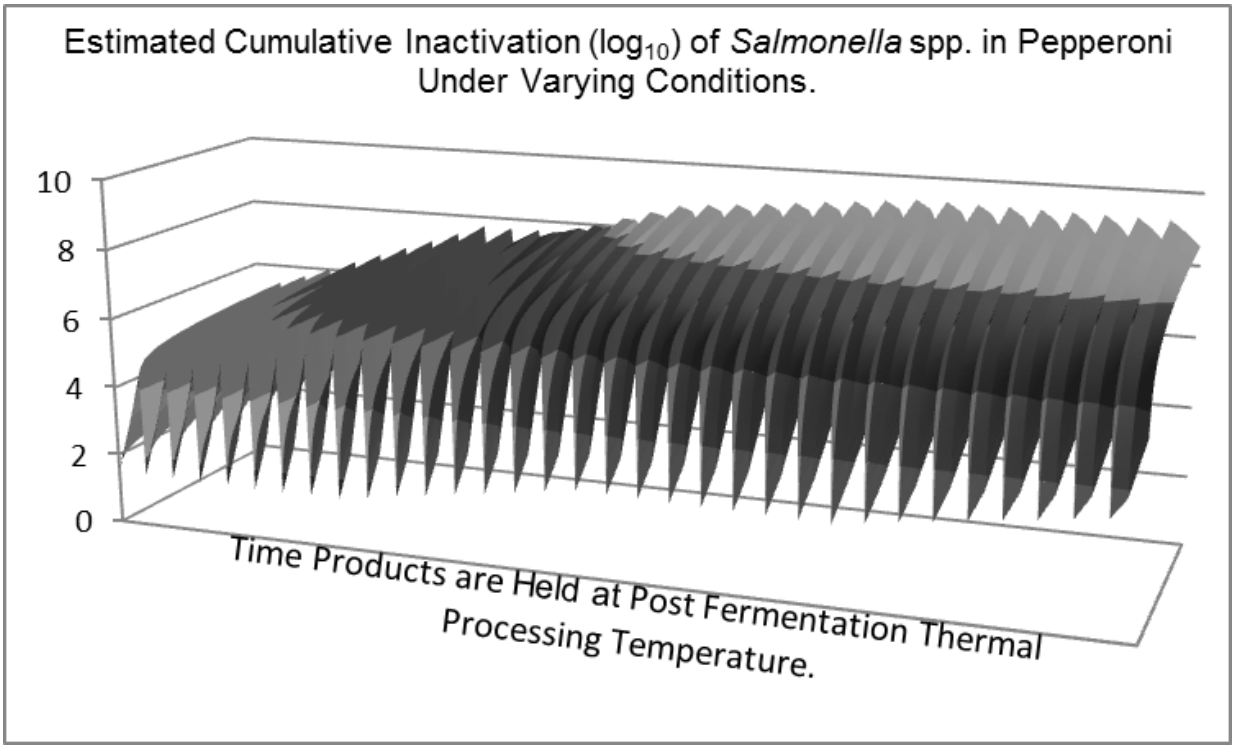


Figure 3.


Estimated Cumulative Inactivation (log_{10}) of *Salmonella* spp. in Pepperoni Under Varying Conditions.
10
8
6
4
2
0
Time Products are Held at Post Fermentation Thermal Processing Temperature.

Tables

Table 1.

Estimated time required (min.) to achieve a 6.5 or 7.0 $\log_{10}$ cumulative inactivation of *Salmonella* spp. in Pepperoni.

Temperature °F / °C		Percentage Fat in Formulation																
		17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47	49
121 / 49.4	6.5 $\log_{10}$	--	--	--	--	--	--	--	--	--	--	--	--	--	--	125	87	58
	7.0 $\log_{10}$	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
122 / 50.0	6.5 $\log_{10}$	--	--	--	--	--	--	--	--	--	--	--	--	110	85	64	46	31
	7.0 $\log_{10}$	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	108
123 / 50.6	6.5 $\log_{10}$	--	--	--	--	--	154	144	134	125	108	92	77	62	49	37	27	18
	7.0 $\log_{10}$	--	--	--	--	--	--	--	--	--	--	--	--	144	120	98	77	57
124 / 51.1	6.5 $\log_{10}$	122	116	112	105	99	93	87	82	76	67	57	48	39	31	23	17	13
	7.0 $\log_{10}$	--	--	--	--	--	159	152	144	137	123	110	97	83	70	57	45	33
125 / 51.7	6.5 $\log_{10}$	98	94	89	85	80	76	72	67	63	56	49	41	34	28	21	15	10
	7.0 $\log_{10}$	155	150	145	139	134	128	123	117	111	102	93	83	73	61	52	42	32
126 / 52.2	6.5 $\log_{10}$	80	76	73	70	66	63	59	56	53	47	41	36	30	25	19	14	10
	7.0 $\log_{10}$	125	121	117	113	109	105	101	96	92	85	79	71	64	56	47	39	30
127 / 52.8	6.5 $\log_{10}$	65	63	60	57	55	52	50	47	44	40	36	31	26	22	17	13	9
	7.0 $\log_{10}$	101	98	95	92	88	87	83	79	76	72	66	61	56	50	43	36	27
128 / 53.3	6.5 $\log_{10}$	54	52	50	48	46	44	42	40	37	34	31	27	23	19	15	12	8
	7.0 $\log_{10}$	83	81	78	76	74	71	69	66	64	61	57	53	49	44	39	33	26
129 / 53.9	6.5 $\log_{10}$	45	43	42	40	39	37	35	34	32	29	27	24	21	17	14	10	7
	7.0 $\log_{10}$	68	67	65	63	61	59	58	56	54	51	49	46	43	39	35	30	25
130 / 54.4	6.5 $\log_{10}$	38	36	35	34	33	31	30	29	27	25	23	21	18	15	12	9	6
	7.0 $\log_{10}$	57	55	54	53	51	50	49	47	46	44	42	40	38	35	31	28	23
131 / 55	6.5 $\log_{10}$	32	31	30	29	28	27	26	25	24	22	20	18	16	14	11	8	6
	7.0 $\log_{10}$	48	47	46	45	43	42	41	40	39	38	36	35	33	31	28	25	21
132 / 55.6	6.5 $\log_{10}$	27	26	25	25	24	23	22	21	21	19	18	16	14	12	10	7	5
	7.0 $\log_{10}$	40	39	39	38	37	36	35	34	34	33	32	31	29	27	25	23	19

133 / 56.1	6.5 log_{10}	26	25	25	24	23	22	22	21	20	19	17	16	14	12	10	8	6
	7.0 log_{10}	39	38	37	36	36	35	34	33	32	31	31	30	28	27	25	23	21
134 / 56.7	6.5 log_{10}	25	25	24	23	22	22	21	20	19	18	17	16	14	13	11	9	7
	7.0 log_{10}	37	36	36	35	34	33	33	32	31	30	30	29	28	27	25	24	22
135 / 57.2	6.5 log_{10}	24	24	23	22	22	21	20	19	19	18	17	16	14	13	12	10	8
	7.0 log_{10}	36	35	34	34	33	32	31	31	30	29	29	28	27	26	25	24	23
136 / 57.8	6.5 log_{10}	24	23	22	22	21	20	20	19	18	17	16	15	14	13	12	11	9
	7.0 log_{10}	33	33	32	31	31	30	29	28	28	27	27	27	26	26	25	24	23
137 / 58.3	6.5 log_{10}	23	22	22	20	20	20	19	18	17	17	16	15	14	14	13	11	10
	7.0 log_{10}	33	33	32	31	31	30	29	28	28	27	27	27	26	26	25	24	23
138 / 58.9	6.5 log_{10}	22	22	21	20	20	19	18	18	17	16	16	15	15	14	13	12	11
	7.0 log_{10}	32	32	31	30	30	29	28	27	27	26	26	26	26	26	25	25	24
139 / 59.4	6.5 log_{10}	22	21	21	20	19	19	18	17	16	16	16	15	15	14	13	13	12
	7.0 log_{10}	31	30	30	29	29	28	27	26	26	26	26	25	25	25	25	25	25
140 / 60.0	6.5 log_{10}	21	21	20	10	19	18	17	17	16	16	15	15	15	14	14	13	13
	7.0 log_{10}	30	30	29	28	28	27	26	25	25	25	25	25	25	25	25	25	25
141 / 60.6	6.5 log_{10}	21	20	20	19	18	18	17	17	16	16	15	15	14	13	13	12	12
	7.0 log_{10}	30	29	28	27	27	26	25	25	25	25	24	24	24	24	24	24	24
142 / 61.1	6.5 log_{10}	20	20	19	19	18	17	17	16	16	15	15	14	14	13	12	12	11
	7.0 log_{10}	29	28	28	27	27	26	26	25	25	24	24	24	24	23	23	23	22
143 / 61.7	6.5 log_{10}	20	19	19	18	18	17	17	16	16	15	15	14	13	13	12	11	10
	7.0 log_{10}	28	28	27	27	27	26	26	25	25	24	24	24	23	23	22	21	21
144 / 62.2	6.5 log_{10}	19	19	18	18	17	17	16	16	16	15	14	14	13	12	11	10	9
	7.0 log_{10}	28	27	27	27	26	26	25	25	25	24	24	23	22	22	21	20	19
145 / 62.8	6.5 log_{10}	18	18	18	17	17	17	16	16	15	15	14	13	12	11	11	10	9
	7.0 log_{10}	27	27	26	26	26	26	25	25	25	24	23	23	22	21	20	19	18
146 / 63.3	6.5 log_{10}	18	17	17	17	17	16	16	16	15	15	14	13	12	11	10	9	8
	7.0 log_{10}	26	26	26	26	26	25	25	25	25	24	23	22	21	20	19	18	17

147 / 63.9	6.5 $\log_{10}$	17	17	17	16	16	16	16	16	15	14	13	12	11	10	9	8	7
	7.0 $\log_{10}$	26	26	25	25	25	25	25	25	25	24	23	22	21	19	18	17	16
148 / 64.4	6.5 $\log_{10}$	17	16	16	16	16	16	16	15	15	14	13	12	11	10	9	8	7
	7.0 $\log_{10}$	25	25	25	25	25	25	25	25	25	23	22	21	20	19	17	16	14

Table 2.

Estimated time required (min.) to achieve a 5.5 or 6.0 $\log_{10}$ cumulative inactivation of *Salmonella* spp. in Pepperoni.

Temperature °F / °C		Percentage Fat in Formulation																
		17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47	49
116 / 46.7	5.5 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	–	–	--	--
	6.0 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	–	–	--	--
117 / 47.2	5.5 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	–	–	144	59
	6.0 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	–	–	--	--
118 / 47.8	5.5 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	–	100	49	24
	6.0 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	–	–	--	147
119 / 48.3	5.5 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	118	70	40	22	11
	6.0 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	–	–	106	58
120 / 48.9	5.5 $\log_{10}$	–	–	–	–	–	–	--	–	--	157	112	77	51	33	20	12	8
	6.0 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	–	–	–	116	76	47	28
121 / 49.4	5.5 $\log_{10}$	–	–	–	–	157	140	125	110	96	73	55	40	28	19	12	7	4
	6.0 $\log_{10}$	–	–	–	–	–	–	--	–	--	–	141	107	79	56	38	25	15
122 / 50.0	5.5 $\log_{10}$	120	110	101	92	84	75	68	60	53	42	32	24	17	12	8	5	3
	6.0 $\log_{10}$	–	–	–	–	–	148	135	123	111	91	73	57	43	32	22	15	9
123 / 50.6	5.5 $\log_{10}$	72	67	61	56	52	47	42	38	34	27	21	16	13	8	6	4	2
	6.0 $\log_{10}$	121	114	106	99	92	86	79	71	65	54	44	35	27	20	15	9	6
124 / 51.1	5.5 $\log_{10}$	48	44	41	39	35	32	29	27	24	20	16	12	9	6	4	3	2
	6.0 $\log_{10}$	76	72	67	63	59	55	51	47	43	36	30	24	18	14	10	7	4
125 / 51.7	5.5 $\log_{10}$	39	37	34	32	29	27	25	23	20	16	13	11	8	6	4	2	2
	6.0 $\log_{10}$	62	59	55	52	48	45	42	39	36	31	26	21	16	12	9	6	4
126 / 52.2	5.5 $\log_{10}$	32	30	28	27	25	23	21	19	18	15	12	9	7	5	3	2	1
	6.0 $\log_{10}$	51	48	45	43	40	38	35	33	30	26	22	18	14	11	8	5	3
127 / 52.8	5.5 $\log_{10}$	27	26	24	22	21	19	18	16	15	13	10	8	6	5	3	2	1
	6.0 $\log_{10}$	42	40	38	36	34	32	30	28	26	22	19	16	13	10	7	5	3

128 / 53.3	5.5 $\log_{10}$	23	22	20	19	18	17	15	14	13	11	9	7	6	4	3	2	1
	6.0 $\log_{10}$	35	33	32	30	29	27	25	24	22	19	17	14	11	9	6	4	3
129 / 53.9	5.5 $\log_{10}$	19	18	17	16	15	14	13	12	11	10	8	6	5	4	2	2	1
	6.0 $\log_{10}$	29	28	27	26	24	23	22	20	19	17	15	12	10	8	6	4	2
130 / 54.4	5.5 $\log_{10}$	17	16	15	14	13	12	12	11	10	9	7	6	4	3	2	1	1
	6.0 $\log_{10}$	25	24	23	22	21	20	19	18	17	15	13	11	9	7	5	3	2
131 / 55	5.5 $\log_{10}$	14	14	13	12	12	11	10	10	9	8	6	5	4	3	2	1	1
	6.0 $\log_{10}$	21	20	20	19	18	17	16	15	15	13	11	10	8	6	4	3	2
132 / 55.6	5.5 $\log_{10}$	12	12	11	11	10	10	9	9	8	7	6	5	4	3	2	1	1
	6.0 $\log_{10}$	18	18	17	16	16	15	14	14	13	11	10	9	7	5	4	3	1
133 / 56.1	5.5 $\log_{10}$	12	12	11	10	10	9	9	8	8	7	6	5	4	3	2	1	1
	6.0 $\log_{10}$	18	17	16	16	15	14	14	13	12	11	10	9	7	6	4	3	2
134 / 56.7	5.5 $\log_{10}$	12	11	11	10	10	9	9	8	8	7	6	5	4	3	2	2	1
	6.0 $\log_{10}$	17	17	16	15	15	14	13	13	12	11	10	9	7	6	5	4	3
135 / 57.2	5.5 $\log_{10}$	12	11	11	10	10	9	9	8	7	7	6	5	4	3	3	2	1
	6.0 $\log_{10}$	17	16	16	15	14	14	13	12	12	11	10	9	8	7	5	4	3
136 / 57.8	5.5 $\log_{10}$	11	11	10	10	9	9	8	8	7	7	6	5	4	4	3	2	2
	6.0 $\log_{10}$	16	16	15	15	14	13	13	12	11	11	10	9	8	7	6	5	4
137 / 58.3	5.5 $\log_{10}$	11	11	10	10	9	9	8	8	7	7	6	5	5	4	3	3	2
	6.0 $\log_{10}$	16	15	15	14	14	13	12	12	11	10	10	9	8	7	6	5	4
138 / 58.9	5.5 $\log_{10}$	11	11	10	10	9	9	8	8	7	7	6	5	5	4	4	3	3
	6.0 $\log_{10}$	16	15	15	14	13	13	12	12	11	10	10	9	8	8	7	6	5
139 / 59.4	5.5 $\log_{10}$	11	10	10	9	9	8	8	7	7	6	6	6	5	5	4	3	3
	6.0 $\log_{10}$	15	15	14	14	13	12	12	11	11	10	10	9	8	8	7	7	6
140 / 60.0	5.5 $\log_{10}$	11	10	10	9	9	8	8	7	7	6	6	6	5	5	4	4	3
	6.0 $\log_{10}$	15	14	14	13	13	12	12	11	10	10	10	9	9	8	8	7	6
141 / 60.6	5.5 $\log_{10}$	10	10	9	9	8	8	8	7	7	6	6	5	5	5	4	4	3
	6.0 $\log_{10}$	14	14	13	13	12	12	11	11	10	10	9	9	8	8	7	7	6

142 / 61.1	5.5 $\log_{10}$	10	9	9	9	8	8	7	7	7	6	6	5	5	4	4	3	3
	6.0 $\log_{10}$	14	14	13	13	12	12	11	11	10	10	9	9	8	7	7	6	6
143 / 61.7	5.5 $\log_{10}$	9	9	9	8	8	8	7	7	7	6	6	5	5	4	4	3	3
	6.0 $\log_{10}$	14	13	13	12	12	11	11	10	10	9	9	8	8	7	6	6	5
144 / 62.2	5.5 $\log_{10}$	9	9	8	8	8	7	7	7	6	6	6	5	4	4	3	3	2
	6.0 $\log_{10}$	13	13	12	12	12	11	11	10	10	9	9	8	7	7	6	5	5
145 / 62.8	5.5 $\log_{10}$	9	8	8	8	7	7	7	7	6	6	5	5	4	4	3	3	2
	6.0 $\log_{10}$	13	12	12	12	11	11	11	10	10	9	8	8	7	6	6	5	4
146 / 63.3	5.5 $\log_{10}$	8	8	8	7	7	7	7	6	6	6	5	5	4	3	3	3	2
	6.0 $\log_{10}$	12	12	11	11	11	11	10	10	10	9	8	8	7	6	5	5	4
147 / 63.9	5.5 $\log_{10}$	8	8	7	7	7	7	7	6	6	5	5	4	4	3	3	2	2
	6.0 $\log_{10}$	12	11	11	11	11	10	10	10	10	9	8	7	7	6	5	4	4
148 / 64.4	5.5 $\log_{10}$	7	7	7	7	7	7	6	6	6	5	5	4	4	3	3	2	2
	6.0 $\log_{10}$	11	11	11	10	10	10	10	10	9	9	8	7	6	5	5	4	3

Table 3.

Estimated time required (min.) to achieve a 4.5 or 5.0 log_{10} cumulative inactivation of *Salmonella* spp. in Pepperoni.

Temperature °F / °C		Percentage Fat in Formulation																
		17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47	49
116 / 46.7	4.5 log_{10}	--	–	--	–	--	–	–	–	–	–	–	–	146	40	13	5	2
	5.0 log_{10}	--	–	--	–	--	–	–	–	–	–	–	–	–	--	–	--	--
117 / 47.2	4.5 log_{10}	--	–	--	–	--	–	–	–	–	–	–	80	33	13	6	3	1
	5.0 log_{10}	--	–	--	–	--	–	–	–	–	–	–	–	–	107	43	18	8
118 / 47.8	4.5 log_{10}	--	–	--	–	--	–	–	–	–	95	50	26	13	6	3	2	1
	5.0 log_{10}	--	–	--	–	--	–	–	–	–	–	–	136	70	35	17	8	4
119 / 48.3	4.5 log_{10}	--	–	--	–	145	118	95	75	58	36	22	13	7	4	2	1	1
	5.0 log_{10}	--	–	--	–	--	–	–	–	–	129	81	49	29	16	9	5	3
120 / 48.9	4.5 log_{10}	117	101	87	75	63	53	44	36	29	19	13	8	5	3	2	1	1
	5.0 log_{10}	--	–	--	–	152	131	111	94	78	55	37	25	16	10	6	3	2
121 / 49.4	4.5 log_{10}	61	54	47	41	35	30	25	21	18	13	9	6	4	2	2	1	1
	5.0 log_{10}	119	107	95	84	74	65	56	48	41	30	22	15	10	6	4	2	1
122 / 50.0	4.5 log_{10}	37	33	30	26	23	20	17	15	12	9	7	5	3	2	1	1	1
	5.0 log_{10}	67	61	55	49	44	39	34	30	26	19	14	10	7	5	3	2	1
123 / 50.6	4.5 log_{10}	25	23	21	18	16	14	13	11	10	7	5	4	3	2	1	1	1
	5.0 log_{10}	44	39	36	32	29	26	23	20	18	14	11	8	5	4	2	2	1
124 / 51.1	4.5 log_{10}	19	17	16	14	13	11	10	9	8	6	4	3	2	2	1	1	1
	5.0 log_{10}	30	28	25	23	21	19	17	15	14	11	8	6	4	3	2	1	1
125 / 51.7	4.5 log_{10}	16	15	13	12	11	10	9	8	7	5	4	3	2	1	1	1	1
	5.0 log_{10}	25	23	21	19	18	16	15	13	12	9	7	5	4	3	2	1	1
126 / 52.2	4.5 log_{10}	13	12	11	10	9	8	8	7	6	5	4	3	2	1	1	1	1
	5.0 log_{10}	21	19	18	16	15	14	13	11	10	8	6	5	4	2	2	1	1
127 / 52.8	4.5 log_{10}	11	11	10	9	8	7	7	6	5	4	3	2	2	1	1	1	1
	5.0 log_{10}	18	16	15	14	13	12	11	10	9	7	6	4	3	2	2	1	1

128 / 53.3	4.5 $\log_{10}$	10	9	8	8	7	7	6	5	5	4	3	2	2	1	1	1	1
	5.0 $\log_{10}$	15	14	13	12	11	10	9	9	8	6	5	4	3	2	1	1	1
129 / 53.9	4.5 $\log_{10}$	9	8	7	7	6	6	5	5	4	3	3	2	1	1	1	1	1
	5.0 $\log_{10}$	13	12	11	11	10	9	8	8	7	6	5	4	3	2	1	1	1
130 / 54.4	4.5 $\log_{10}$	7	7	7	6	6	5	5	4	4	3	2	2	1	1	1	1	1
	5.0 $\log_{10}$	11	10	10	9	9	8	7	7	6	5	4	3	2	2	1	1	1
131 / 55	4.5 $\log_{10}$	7	6	6	5	5	5	4	4	4	3	2	2	1	1	1	1	1
	5.0 $\log_{10}$	10	9	9	8	8	7	7	6	6	5	4	3	2	2	1	1	1
132 / 55.6	4.5 $\log_{10}$	6	5	5	5	4	4	4	4	3	3	2	2	1	1	1	1	1
	5.0 $\log_{10}$	8	8	8	7	7	6	6	5	5	4	3	3	2	1	1	1	1
133 / 56.1	4.5 $\log_{10}$	6	5	5	5	4	4	4	4	3	3	2	2	1	1	1	1	1
	5.0 $\log_{10}$	8	8	7	7	7	6	6	5	5	4	3	3	2	2	1	1	1
134 / 56.7	4.5 $\log_{10}$	6	5	5	5	4	4	4	4	3	3	2	2	1	1	1	1	1
	5.0 $\log_{10}$	8	8	7	7	7	6	6	5	5	4	4	3	2	2	1	1	1
135 / 57.2	4.5 $\log_{10}$	5	5	5	5	4	4	4	3	3	3	2	2	2	1	1	1	1
	5.0 $\log_{10}$	8	8	7	7	6	6	6	5	5	4	4	3	3	2	1	1	1
136 / 57.8	4.5 $\log_{10}$	6	5	5	5	4	4	4	3	3	3	2	2	2	1	1	1	1
	5.0 $\log_{10}$	8	8	7	7	6	6	6	5	5	4	4	3	3	2	2	1	1
137 / 58.3	4.5 $\log_{10}$	6	5	5	5	4	4	4	3	3	3	2	2	2	1	1	1	1
	5.0 $\log_{10}$	8	7	7	7	6	6	5	5	5	4	4	3	3	2	2	2	1
138 / 58.9	4.5 $\log_{10}$	6	5	5	5	4	4	4	3	3	3	2	2	2	2	1	1	1
	5.0 $\log_{10}$	8	7	7	7	6	6	5	5	5	4	4	3	3	3	2	2	1
139 / 59.4	4.5 $\log_{10}$	5	5	5	5	4	4	4	3	3	3	3	2	2	2	1	1	1
	5.0 $\log_{10}$	8	7	7	6	6	6	5	5	5	4	4	3	3	3	2	2	2
140 / 60.0	4.5 $\log_{10}$	5	5	5	5	4	4	4	3	3	3	3	2	2	2	2	1	1
	5.0 $\log_{10}$	8	7	7	6	6	6	5	5	5	4	4	4	3	3	3	2	2
141 / 60.6	4.5 $\log_{10}$	5	5	5	4	4	4	4	3	3	3	2	2	2	2	2	1	1
	5.0 $\log_{10}$	7	7	7	6	6	5	5	5	4	4	4	3	3	3	3	2	2

142 / 61.1	4.5 $\log_{10}$	5	5	4	4	4	4	3	3	3	3	2	2	2	2	1	1	1
	5.0 $\log_{10}$	7	7	6	6	6	5	5	5	4	4	4	3	3	3	2	2	2
143 / 61.7	4.5 $\log_{10}$	5	4	4	4	4	4	3	3	3	3	2	2	2	2	1	1	1
	5.0 $\log_{10}$	6	5	5	5	4	4	4	4	3	3	3	2	2	2	1	1	1
144 / 62.2	4.5 $\log_{10}$	4	4	4	4	4	3	3	3	3	3	2	2	2	1	1	1	1
	5.0 $\log_{10}$	6	6	6	5	5	5	5	4	4	4	3	3	3	2	2	2	1
145 / 62.8	4.5 $\log_{10}$	4	4	4	4	3	3	3	3	3	2	2	2	2	1	1	1	1
	5.0 $\log_{10}$	6	6	5	5	5	5	5	4	4	4	3	3	3	2	2	2	1
146 / 63.3	4.5 $\log_{10}$	4	4	4	3	3	3	3	3	3	2	2	2	2	1	1	1	1
	5.0 $\log_{10}$	6	5	5	5	5	5	4	4	4	4	3	3	3	2	2	1	1
147 / 63.9	4.5 $\log_{10}$	4	4	3	3	3	3	3	3	3	2	2	2	1	1	1	1	1
	5.0 $\log_{10}$	5	5	5	5	5	4	4	4	4	3	3	3	2	2	2	1	1
148 / 64.4	4.5 $\log_{10}$	3	3	3	3	3	3	3	3	3	3	2	2	2	1	1	1	1
	5.0 $\log_{10}$	5	5	5	5	4	4	4	4	4	3	3	3	2	2	2	1	1

IX. REFERENCES

Abee, T., L. Krockel, and C. Hill. 1995. Bacteriocins: Modes of Action and Potentials in Food Preservation and Control of Food Poisoning. Int. J. Food Microbiol. 28: 169-185.

Albano, H., S.D. Todorov, C.A. van Reenen, T. Hogg, L.M.T. Dicks, and P. Teixeira. 2007. Characterization of Two Bacteriocins Produced by *Pediococcus acidilcatici* Isolated from "Alheira", a Fermented Sausage Traditionally Produced in Portugal. Int. J. Food Microbiol. 116: 239-247.

Baranyi, J., and T.A. Roberts. 1995. Mathematics of Predictive Food Microbiology. Int. J. Food Microbiol. 26:199-218.

Barbut, S., and M.W. Griffiths. 2001. Developing Validation Models for *E. coli* O157 Inactivation in Dry Fermented Sausages. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 54. p. 44-48.

Barbut, S., and G. Parolari. 2002. Validation of Manufacturing Process to Control Pathogenic Bacteria in Typical Dry Fermented Products. Meat Sci. 62:323-329.

Biswas, S.R., P. Ray, M.C. Johnson, and B. Ray. 1991. Influence of Growth Conditions on the Production of Bacteriocin, Pediocin AcH, by *Pediococcus acidilactici* H. App. Environ, Microbiol. 57(4): 1265-1267.

Borowski, A.G., S.C. Ingham, and B.A. Ingham. 2009. Validation of Ground-and-Formed Beef Jerky Processes Using Commercial Lactic Acid Bacteria Starter Cultures as Pathogen Surrogates. J. Food Protect. 72(6): 1234-1247.

Bremer, V., K. Lietmeyer, E. Jensen, U. Metzel, H. Meczulat, E. Weise, D. Werber, H. Tschaepe, L. Kreienbrock, S. Glaser, and A. Ammon. 2004. Outbreak of *Salmonella* Goldcoast Infections Linked to Consumption of Fermented Sausage, German 2001. Epidemiol. Infect. 132(5): 881-887.

Buccheri, Mike. 2012. Tyson Foods, Inc. Personal Communication.

CDC. 2010. Investigation Update: Multistate Outbreak of Human *Salmonella* Montevideo Infections. www.cdc.gov/salmonella/montevideo/index.html (accessed 07/15/2012).

CDC. 2011a. Trends in Foodborne Illness, 1996-2010. Atlanta, Georgia: US Department of Health and Human Services, CDC. www.cdc.gov/foodborneburden/PDFs/FACTSHEET_B_TRENDS.PDF

CDC. 2011b. Investigation Announcement: Multistate Outbreak of E. coli O157:H7 Infections Associated with Lebanon Bologna. www.cdc.gov/ecoli/2011/O157_0311/index.html (accessed 01/12/2013).

CDC. 2012. *Salmonella*. Technical Information. www.cdc.gov/salmonella/general/technical.html (accessed 07/15/2012).

Deumier, F., and A. Collignan. 2003. The Effects of Sodium Lactate and Starter Cultures on pH, Lactic Acid Bacteria, *Listeria monocytogenes* and *Salmonella* spp. Levels in Pure Chicken Dry Fermented Sausage. Meat Sci. 65: 1165-1174.

Ellajosyula, K.R., S. Doores, E.W. Mills, R.A. Wilson, R.C. Anantheswaran, and S.J. Knabel. 1998. Destruction of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in Lebanon Bologna by Interaction of Fermentation pH, Heating Temperature, and Time. J. Food Protect. 61(2): 152-157.

Emberland, K.E., K. Nygard, B.T. Heier, P. Aavitsland, J. Lassen, T.L. Stavnes, and B. Gondrosen. 2006. Outbreak of *Salmonella* Kedougou in Norway Associated with Salami, April – June 2006. Euro Surveill. 11(27): 2995.

Faith, N., N. Parniere, T. Larson, T. Lorang, and J. Luchansky. 1997. Viability of *Escherichia coli* O157:H7 in Pepperoni during the Manufacture of Sticks and the Subsequent Storage of Slices at 21, 4 and -20^{o}C Under Air, Vacuum and CO_2. Int. J. Food Microbiol. 37:47-54.

FSIS. 1996. 9 CFR Part 304, *et al*. Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) System; Final Rule. Fed. Reg. 61(144): 38805-38855.

FSIS. 1999a. 9 CFR Parts 301, 317, 318, 320, and 381.Docket No. 95-033F. Production Standards for the Production of Certain Meat and Poultry Products. Fed. Reg. 64(3): 732-749.

FSIS. 1999b. Appendix A, Compliance Guidelines for Meeting Lethality Performance Standards for Certain Meat and Poultry Products. www.fsis.usda.gov/Frame/FrameRedirect.asp?main=http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/95-033F/95-033F_Appendix_A.htm (accessed 02/16/2013).

FSIS. 2000. Percent Positive *Salmonella* Tests for RTE meat and poultry products, CY 1990-2000. www.fsis.usda.gov/Science/Table2_Micro_Testing_RTE_1990-2004/index.asp (accessed 02/26/2013).

FSIS. 2001. 9 CFR Parts 301, 303, 317, 318, 319, 320, 325, 331, 381, 417, and 430. Docket No. 97-013P. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg 66(39): 12590-12636.

FSIS. 2003. Part 319 – Definitions and Standards of Identity or Composition. Code of Federal Regulations, Title 9, Volume 2. 9CFR319.

FSIS. 2005. Time and Temperature Tables for Cooking Ready to Eat Poultry Products. Original Paper Copy -- Online version has had the fermented poultry references removed.

FSIS. 2010. Use of Non-pathogenic *Escherichia coli* (*E. coli*) Cultures as Surrogate Indicator Organisms in Validation Studies. http://askfsis.custhelp.com/app/answers/detail/a_id/1392/kw/surrogate%20indicator%20organisms/session/L3RpbWUvMTM2MzU0Mzk5Mi9zaWQvZFM4TDhwbGw%3D (accessed 08/03/2011).

FSIS. 2011. Agency to Suspend *E. coli* Testing Program for Certain RTE Products. Constituent Update, May 13, 2011. www.fsis.usda.gov/News_&_Events/Const_Update_051300/index.asp (accessed 02/23/2013).

FSIS. 2013a. The FSIS Microbiological Testing Program for Ready-to-Eat (RTE) Meat and Poultry Products, 1990–2011. www.fsis.usda.gov/Science/Micro_Testing_RTE_Continuation/index.asp#previous (accessed 02/26/2013).

FSIS. 2013b. Proposed Rule. 155. Performance Standards for the Production of Processed Meat and Poultry Products. Fed. Reg. 78(5): 1532.

Hinkens, J.C., N. Faith, T. Lorang, P. Bailey, D. Buege, C. Kaspar, and J. Luchansky. Validation of Pepperoni Processes for Control of *Escherichia coli* O157:H7. J. Food Protect. 59(12):1260-1266.

Hohmann, E. 2001. Nontyphoidal Salmonellosis. Clin. Infect. Dis. 32:263-269.

Hwang, C.A., A.C.S. Porto-Fett, V.K. Kuneja, S.I. Ingham, B.H. Ingham, and J.B. Luchansky. 2009. Modeling the Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella*

Typhimurium during Fermentation, Drying, and Storage of Soudjouk-Style Fermented Sausage. Int. J. Food Microbiol. 129: 244-252.

Incze, K. 1998. Dry Fermented Sausages. Meat Sci. 49(Suppl. 1): S169-S177.

Ihnot, A.M., A.M. Roering, R.K. Wierzba, N.G. Faith, and J.B. Luchansky. 1998. Behavior of *Salmonella typhimurium* DT104 during the Manufacture and Storage of Pepperoni. Int. J. Food Microbiol. 40:117-121.

Jeong, S., B.P. Marks, and E.T. Ryser. 2011. Quantifying the Performance of *Pediococcus* sp. (NRRL B-2354: *Enterococcus faecium*) as a Nonpathogenic Surrogate for *Salmonella* Enteritidis PT30 during Moist-Air Convection Heating of Almonds. J. Food Protect. 74(4):603-609.

Juneja, V.K., B.S. Eblen, and H.M. Marks. 2001. Modeling Non-Linear Survival Curves to Calculate Thermal Inactivation of *Salmonella* in Poultry of Different Fat Levels. Int. J. Food Microbiol. 70: 37-51.

Kim, J-M, and R.H. Linton. 2008. Identification of a Non-pathogenic Surrogate organism for Chlorine Dioxide (ClO2) Gas Treatment. Food Microbiol. 25: 597-606.

Leroy, F., M.R. Foulquie Moreno, and L. De Vuyst. 2003. *Enterococcus faecium* RZS C5, An Interesting Bacteriocin Producer to be used as a Coculture in Food Fermentation. Int. J. Food Microbiol. 88: 235-240.

Levine, P., B. Rose, S. Green, G. Ransom, and W. Hill. Pathogen Testing of Ready-to-Eat Meat and poultry Products Collected at Federally Inspected Establishments in the United States, 1990 to 1999. J. Food Protect. 64(8): 1188-1193.

Luzzi, I., P. Galetta, M. Massari, C. Rizzo, A.M. Dionisi, E. Feletici, A. Cawthorne, A. Tozzi, M. Argentieri, and S. Bilei. 2007. An Easter Outbreak of *Salmonella* Typhimurium DT104A Associated with Traditional Pork Salami in Italy. Euro Surveill. 12(3-6): 149-152.

Metaxopoulos, J., C. Genigeorgis, M. Fanelli, C. Franti, and E. Cosma. 1981. Production of Italian Dry Salami: Effect of Starter Culture and Chemical Acidulation on Staphylococcal Growth in Salami under Commercial Manufacturing Conditions. App. Environ. Microbiol. 42(5):863-871.

McMeekin, T.A., J. Olley, D.A. Ratkowski, and T. Ross. 2001. Predictive Microbiology: Toward the Interface and Beyond. Int. J. Food Microbiol. 73:395-407.

Naim, F., S. Messier, L. Saucier, and G. Piette. 2003. A Model Study of *Escherichia coli* O157:H7 Survival in Fermented Dry Sausages -- Influence of Inoculum Preparation, Inoculation Procedure, and Selected Process Parameters. J. Food Protect. 66(12): 2267-2275.

Nickelson, R., J. Luchansky, C. Kaspar, and E. Johnson. Dry Fermented Sausage and *E. coli* O157:H7. Blue Ribbon Task Force. National Cattlemen's Beef Association. Research Report no. 11-316.

Niebuhr, S.E., A. Laury, G.R. Acuff, and J.S. Dickson. 2008. Evaluation of Nonpathogenic Surrogate Bacteria as Process Validation Indicators for *Salmonella enterica* for Selected Antimicrobial Treatments, Cold Storage, and Fermentation of Meat. J. Food Protect. 71(4):714-718.

Nightingale, K.K., H. Thippareddi, R.K. Phebus, J.L. Marsden, and A.L. Nutsch. 2006. Validation of a Traditional Italian-Style Salami Manufacturing Process for Control of *Salmonella* and *Listeria monocytogenes*. J. Food Protect. 69(4): 794-800.

Nitisinprasert, S., V. Nilphai, P. Bunyun, P. Sukyai, K. Doi, and K. Sonomoto. 2000. Screening and Identification of Effective Thermotolerant Lactic Acid Bacteria Producing Antimicrobial Activity against *Escherichia coli* and *Salmonella* sp. Resistant to Antibiotics. Kasetsart J. (Nat. Sci.) 34: 387-400.

Pontello, M., L. Sodano, N. Nastasi, C. Mammina, and the working group: M. Astuti, M. Domenichini, G. Belluzzi, E. Soccini, M.G. Silvestri, M. Gatti, E. Gerosa, and A. Montagna. 1998. A Community-Based Outbreak *Salmonella enterica* Serotype Typhimurium Associated with Salami Consumption in Northern Italy. Epidemiol. Infect. 120: 209-214.

Porto-Fett, A.C.S., C.-A. Hwang, J.E. Call, V.K. Jenuja, S.C. Ingham, B.H. Ingham, and J.B. Luchansky. 2008. Viability of Multi-Strain Mixtures of *Listeria monocytogenes*, *Salmonella typhimurium*, or *Escherichia coli* O157:H7 Inoculated into the Batter or Onto the Surface of a Soudjouk-Style Fermented Dry Sausage. Food Microbiol. 25: 793-801.

Riordan, D., G. Duffy, J. Sheridan, R.C. Whiting, I.S. Blair, and D.A. McDowell. 2000. Effects of Acid Adaptation, Product pH, and Heating on Survival of *Escherichia coli* O157:H7 in Pepperoni. App. Environ. Microbiol. 66(4): 1726-1729.

Sauer, C.J., J. Majkowski, S. Green, and R. Eckel. 1997. Foodborne Illness Outbreak Associated with a Semi-Dry Fermented Sausage Product. J. Food Protect. 60(12): 1612-1617.

Scallan, E., R. Hoekstra, F. Angulo, R. Tauxe, M. Widdowson, S. Roy, J. Jones, and P. Griffin. 2011. Foodborne Illness Acquired in the United States – Major Pathogens. Emerg. Infect. Dis. 17(1):7-15.

Smith, J.L., C.N. Huhtanen, J.C. Kissinger, and S.A. Palumbo. 1975. Survival of *Salmonella* during Pepperoni Manufacture. App. Microbiol. 30(5): 759-763.

Sofos, J. 1981. Nitrite, Sorbate and pH Interaction in Cured Meat Products. American Meat Science Association. Reciprocal Meat Conference Proceedings. Vol. 34. p. 104-120.

Tarr, P., T. Besser, D. Hancock, W. Keene, and M. Goldoft. 1997. Verotoxigenic *Escherichia coli* Infection: U.S. Overview. J. Food Protect. 60(11):1466-1471.

Tilden, J., W. Young, A.M. McNamara, C. Custer, B. Boesel, M.A. Lambert-Fair, J. Majkowski, D. Vugia, S.B. Werner, J. Hollingsworth, and J.G. Morris. 1996. A New Route of Transmission for *Escherichia coli*: Infection from Dry Fermented Salami. Am. J. Public Health. 80(8):1142-1145.

X. SUMMARY OF RESEARCH

The research project intended to result in a predictive model for the inactivation of *Salmonella* spp. in pepperoni utilizing a surrogate (*Enterococcus faecalis*) appropriate for inoculation into the raw pepperoni for in-plant validation of process controls and HACCP plans. High-level inoculation challenge studies are generally the only acceptable baseline means of demonstrating that a particular set of processing conditions delivers a given level of microbial inactivation. Unfortunately, high-level inoculation using pathogenic microorganisms has substantial limitations. Researchers are barred from conducting validation work under specific commercial processing conditions in a processing facility (Murphy *et al.* 2001) as such work may only be conducted in microbiological laboratory facilities under pathogen-appropriate biosecurity protocols. As seen in the current work, even with high-level inoculation, one always runs the risk that under the most stringent processing conditions, viable surviving pathogens may be below the limits of detection or the cumulative inactivation is greater than the initial inoculation level. This combination results in samples reported as “none detected” and creates significant obstacles to data analyses and model development.

It would be advantageous to the commercial food processor to have available alternatives to in-plant challenge studies with high-levels of pathogens (Keeling *et al*. 2009). In-lab studies correlating pathogen inactivation to a surrogate with similar properties holds the potential for development of a predictive model that would facilitate in-plant validation. This could take the form of non-pathogen surrogate inoculation of raw materials followed by processing the raw materials under actual commercial conditions to assess the level of inactivation achieved in the surrogate microorganism then correlating that back to predict the level of pathogen inactivation that would have been achieved under those conditions. Such was the goal of this project.

Selection of the surrogate microorganism is critical to the success of such an endeavor. As previously described, the surrogate must be nonpathogenic, easily cultured and possess characteristics that provide for a robust correlation to the target pathogen (Kim and Linton 2008). When preparing to search for new surrogate models in food systems that have not previously been studied, the selection of the particular microorganism may be informed by previous work with the potential surrogate; literature demonstrating successful work with the surrogate under similar food processing conditions; and

information contained in texts concerning the biochemical or phylogenetic similarities (homologies) between the potential surrogate and the target pathogen – essentially making the selection an educated guess.

We elected to use a strain of *Enterococcus faecalis* (*E. faecalis*) from the Tyson Foods' culture collection for a variety of reasons including the following: we have previous experience using this strain in thermal processing studies, having correlated the thermal inactivation to the inactivation of *Salmonella* Senftenberg in ground chicken meat; we have experience with culture, recovery and enumeration of the strain and it is easy to work with; it has been used as a human probiotic as it is considered a part of the normal adult gut flora (Nueno-Palop and Narbad 2011) and as such, should be acceptable to representatives of the USDA for inclusion as a safe surrogate in meat products; and most importantly, it is considered to be a common contaminate of foods, both raw and ready to eat as it is a common constituent of food animal gastro-intestinal tract contents and has been associated with fermented meats and cheeses for millennia (Gomes *et al*. 2010). As an important contributor to the flavor profile of a variety of traditional fermented meats and cheeses, it was expected that the microorganism would possess inactivation kinetics in fermented meat systems that were comparable to the *Salmonella* spp. targeted in this research.

Unfortunately, we have found that it did not possess similar inactivation kinetics as the specie formulation (pork and beef versus pork, chicken and beef) introduced a statistically significant effect on the level of inactivation *Enterococcus faecalis* achieved while there was no similar effect on the level of inactivation of the *Salmonella* spp. Causing further concern is the apparent pattern that the effect exhibited. Namely, we obtained significantly greater inactivation at the three lower post-fermentation thermal processing temperatures in the traditional pork and beef formulations; while we obtained a significantly greater inactivation at the two higher temperatures in the nontraditional pork, chicken and beef formulations. Thus, we don't know if the effect is attributable to processing temperature or meat specie differences. There was no apparent effect due to the chronology (order) in which the trials were processed. Greater inactivation was achieved in the pork and beef formulation in trial 1 (124^{o}F); trial 2 (132^{o}F); trial 5 (124^{o}F); trial 6 (132^{o}F); trial 8 (116^{o}F); and trial 10 (116^{o}F); while greater reductions were

achieved in the pork, chicken and beef formulation in trial 3 (148^{o}F); trial 4 (140^{o}F); trial 7 (140^{o}F) and trial 9 (148^{o}F).

In work published by other authors, it is common to see decimal reduction values that differ depending upon the substrate used for the trials (Murphy *et al*. 2004). In meat product research, a possible explanation for the phenomenon has been given as differences in the level of fat in the types of meat studied (Juneja *et al.* 2001) and we measured a highly significant effect due to the level of fat in the formulation. However, this would not explain the differences in the current work as we deliberately designed the protocol with three fat levels within a meat specie formulation with similar differences in fat between the meat specie formulae specifically expecting to detect a significant effect and expecting that effect to be consistent -- which it was.

James Jay has documented that other formulation differences may provide some type of protective effect. Carbohydrates, proteins, fats, salt and other curing ingredients, etc. may all influence the inactivation kinetics in foods (Jay 1996). This provides some opportunity to speculate as the two meat specie formulas varied in a number of potentially important characteristics: the traditional formulas contained 3.5% salt (as sodium chloride) while the nontraditional formulas contained 3.28%; and the traditional formulas were seasoned with ground mustard and paprika with dextrose separately added while the nontraditional formulae were seasoned with a proprietary commercial pepperoni seasoning blend consisting of a wide variety of seasonings and ingredients. However, given that the formulae were similarly different across all temperature treatments, an effect due to an ingredient would be anticipated to affect the formulae similarly across the entire range of temperatures observed – e.g. if the slight difference in salt made the *E. faecalis* more thermotolerant, one would have expected to see the difference at all cook temperatures utilized.

As other authors have previously noted, complex food matrices are difficult to model (Murphy *et al.* 1999). Walker *et al.* theorized that competing microflora, immobilization of the target cells within differing food matrices, and differences in patterns of diffusion of nutrients, gases or metabolites might create differences in growth. As such, similar differences between food matrices could explain inactivation differences. Again, we would have expected differences of these types to affect all the similar formulae in a similar manner. Given our inability to explain the reason for the unpredictability of inactivation and the

lack of similar inactivation characteristics in the target pathogen, we've concluded that the *Enterococcus faecalis* strain chosen is likely not an appropriate surrogate for the *Salmonella* spp. in pepperoni formulations utilizing different meat specie components and processed at a variety of post-fermentation thermal processing temperatures.

The work that was completed has important contributions for processors of pepperoni products, providing a means for assessing the potential inactivation expected to be achieved under a variety of formulation and processing conditions. Further, the work illustrates that common current processing conditions validated to control *E. coli* O157:H7 are sufficient for control of the salmonellae and additional extension of the regulatory requirements of the pathogen reduction performance standards to this class of products is unnecessary.

Further work is anticipated as we assess different modeling tools to determine if the differences in level of inactivation under the treatment conditions can be accounted for in the model. It may be possible to interpolate the results in *E. faecalis* inactivation as we previously have done with the *Salmonella* spp. and provide "meaningful" estimates for correlation and prediction even if the data set contains dissimilar data. Development of a model of the type anticipated with this work remains an important goal, particularly in the event the performance standards are extended to this class of products.

X. REFERENCES:

Gomes, B.C., B.D.G. de Melo Franco, and E.C.P De Martinis. 2010. Dualistic Aspects of *Enterococcus* spp. In Foods. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Technology. A. Mendez-Vilas (ed.). www.formatex.info/microbiology2/1119-1125.pdf (accessed 01/13/2013).

Jay, J.M. 1996. Modern Microbiology, Third Edition. Van Nostrand Reinhold Co., New York, NY. p.331-345.

Juneja, V.K., B.S. Eblen, and G.M. Ransom. 2001. Thermal Inactivation of *Salmonella* spp. in Chicken Broth, Beef, Pork, Turkey, and Chicken: Determination of D- and z-values. J. Food Sci. 66(1):146-152.

Keeling, C., S.E. Niebuhr, G.R. Acuff, and J.S. Dickson. 2009. Evaluation of *Escherichia coli* Biotype I as a Surrogate for *Escherichia coli* O157:H7 for Cooking, Fermentation, Freezing, and Refrigerated Storage in Meat Processes. J. Food Protect. 72(4):728-732.

Kim, J-M, and R.H. Linton. 2008. Identification of a Non-pathogenic Surrogate organism for Chlorine Dioxide (ClO2) Gas Treatment. Food Microbiol. 25:597-606.

Klutke, G.A., P.T. Kiessler, and M.A. Wortman. 2003. A Critical Look at the Bathtub Curve. IEEE Transact. Reliabil. 52(1):125-129.

Murphy, R.Y., B.F. Marks, E.R. Johnson, and M.G. Johnson. 1999. Inactivation of *Salmonella* and *Listeria* in Ground Chicken Breast Meat during Thermal Processing. J. Food Protect. 62(9):980-985.

Murphy, R.Y., E.R. Johnson, B.P. Marks, M.G. Johnson, and J.A. Marcy. 2001. Thermal Inactivation of *Salmonella senftenberg* and *Listeria innocua* in Ground Chicken Breast Patties Processed in an Air Convection Oven. Poultry Sci. 80:515-521.

Murphy, R.Y., M.A. Davidson, and J.A. Marcy. 2004. Process Lethality Prediction for *Escherichia coli* O157:H7 in Raw Franks during Cooking and Fully Cooked Franks during Post-Cook Pasteurization. J. Food Sci. 69(4):112-116.

Nueno-Palop, C., and A. Narbad. 2011. Probiotic Assessment of *Enterococcus faecalis* CP58 Isolated from Human Gut. Int. J. Food Micribiol. 145:390-394.

Walker, S.L., T.F. Brockelhurst, and J.W.T. Wimpenny. 1997. The Effects of Growth Dynamics Upon pH Gradient Formation Within and Around Subsurface Colonies of *Salmonella typhimurium*. J. Appl. Microbiol. 82:610-614.