

## ABSTRACT

PEÑA CEVALLOS, LUIS ENRIQUE. The Effects of Cooling Parameters, pH and NaCl on the Germination and Outgrowth of *Clostridium perfringens* in Cooked Turkey Breast. (Under the direction of Arritt, F.M. PhD).

As of 2010, according to the Centers for Disease Control and Prevention (CDC) using the Surveillance for Foodborne Disease Outbreaks in the United States, *Clostridium perfringens* in poultry is the second most important pathogen-commodity pair responsible for 281 illnesses outbreak-related illnesses. Additionally, the surveillance showed that in 2007, poultry was the second most common commodity implicated in foodborne disease associated with 40 outbreaks and 691 illnesses and the largest outbreak related to a single item was a chicken dish contaminated with *C. perfringens* causing 132 illnesses (8)

A primary concern for ready-to-eat (RTE) turkey producers is fulfilling USDA/FSIS guidance during the stabilization process (cooling time-temperature combination) known as Appendix B. This guidance establishes a limit for the growth of *C. perfringens* to less than one log (factor of 10) during the cooling process of meat products (12). Currently, there are numerous publications identifying *C. perfringens* ability to grow under various conditions of pH, temperature and NaCl concentration in beef and pork; however, there is a limited amount data addressing how this microorganism is affected in turkey meat under similar conditions.

The main objective of this research is two evaluate the viability of *C. perfringens* to germinate and grow in cooked whole turkey muscle under certain conditions and is divided in two components. The first component is to validate if the manufacturing process of turkey deli meat by a company in North Carolina is capable of fulfilling the Appendix B guidance for the stabilization process (cooling down step). The company has a cooling cycle of 48.89°C (120°F) to 26.67°C (80°F) in 120 min.; 26.67°C (80°F) to 12.80°C (55°F) in 240

min followed by a second cooling after frying of 21.11°C (70°F) to 12.80°C (55°F) in 120 min. The second part is the study of the effect of 1, 1.5 and 2% NaCl at a pH range between 5.76 and 6.40 formulated with 0.5% wt/wt of SAPP and 0.5% wt/wt Brifisol® respectively, on the germination and outgrowth of *C. perfringens* using two different inoculum sizes (2.48 log<sub>10</sub> per gram and 6.48 log<sub>10</sub> per gram). A three-strain cocktail of *C. perfringens* (NTCC8238, NTCC8239 and ATCC10388) was used at 4, 5 and 6 hours of cooling time. The cooling temperatures are according to the Appendix B USDA/FSIS guidance for the stabilization of meat products; such products must be cooled down from 120°F (48.89°C) to 55°F (12.77°C) in cooked whole muscle turkey breast as the safety temperatures to assure not more than one log growth of *C. perfringens* (66).

In the first study, results indicate that the combined cooling curves and the use of phosphate in combination with NaCl showed less than a total of one log<sub>10</sub> growth of *C. perfringens* in accordance with USDA policy. This also indicates that the product cooled under these parameters would be safe.

In the second study, more than one log growth was obtained in all the treatments at 5 and 6 h cooling times for both inocula (2.48log<sub>10</sub> and 6.48 log<sub>10</sub> CFU/g). At a cooling time of four hours, none of the treatments grew more than one log<sub>10</sub> for an inoculum of 2.48 log<sub>10</sub> and no statistically significant difference was found between any treatments. With an inoculum of 6.48 log<sub>10</sub> at a cooling time of 4h, T1(1% NaCl an 0.5% Sodium acid pyrophosphate SAPP) and T4 (1% NaCl an 0.5% of an agglomerated blend of sodium phosphates named as Brifisol®) grew more than one log<sub>10</sub> (1.05 log<sub>10</sub> and 1.13 log<sub>10</sub> respectively); however, there was not a statistically significant difference between these two treatments. Treatment 3 with 1.5%NaCl at a pH of 5.85 (SAPP) showed the best response

with the lowest growth ( $0.35 \log_{10}$ ) at 4 h with the  $2.48 \log_{10}$  inoculum. For an inoculum of  $6.48 \log_{10}$ , T2 with 1% NaCl and 0.5% Sodium acid pyrophosphate SAPP (had the lowest growth ( $0.4 \log_{10}$ )). Low growth was also obtained on treatment T0 (without NaCl or Phosphate) at 5 h ( $1.2 \log_{10}$ ).

The time needed to reduce turkey whole muscle temperature to those established by the USDA in order to obtain less than one  $\log_{10}$  growth of *C. perfringens* is dependent on the pH and NaCl interaction and should be reduced to less than four hours muscle pH is between 5.76 and 6.40 formulated with 1, 1.5 and 2% NaCl.

© Copyright 2012 by Luis Enrique Peña Cevallos

All Rights Reserved

The Effects of Cooling Parameters, pH and NaCl on the Germination and Outgrowth of  
*Clostridium perfringens* in Cooked Turkey Breast

by  
Luis Enrique Peña Cevallos

A thesis submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

Food Science

Raleigh, North Carolina

2013

APPROVED BY:

---

Fletcher Arritt, PhD  
Committee Chair

---

Dana Hanson, PhD

---

Trevor Phister, PhD

# **DEDICATION**

To my Lord and Savior Jesus Christ.

To my parents, Leonel and Nohemy.

# **BIOGRAPHY**

Luis Enrique Peña Cevallos was born on August 12, 1971 in San Salvador, El Salvador. He graduated with a bachelor degree in Mechanical Engineering and a Minor in Industrial Engineering at Saltillo Institute of Technology, Saltillo Mexico in 1994. He obtained his master degree in Manufacturing Systems at Monterrey Institute of Technology on December, 1998. He worked as Quality Manager for Cetron El Salvador, in San Salvador El Salvador. He worked as Quality Manager at Avicola Salvadoreña, a poultry company with slaughter facilities and RTE plants across El Salvador between 2005 and 2008. From 2008 to 2009 he worked as a Quality Auditor at ICONTEC, providing QMS ISO 9000:2000 certifications to the local industry. In 2009 moved to North Carolina to study a master of science in food science at Dr. Fletcher Arritt's laboratory doing research in food pathogens and food safety.

# ACKNOWLEDGMENTS

I would like to thank BK Guilini for providing the sodium phosphates. Dr. V.J. Juneja and the Microbial Food Safety Research Unit, Eastern Regional Research Center USDA-Agricultural Research Service also graciously provided the *Clostridium perfringens* strains.

Dr. Fletcher M. Arritt III, for giving me the opportunity to study again. For his patience and support even when I thought I could not make it.

My Parents, Nohemy for her unconditional love and support on everything I've done; and Leonel, because I'll never forget his guidance and his words "el chompipe más preparado es el que se comen primero".

Dr. Dana Hanson for his support and help teaching me about meat. The meat lab crew, Travis, Cat, Johny, Iryna, and Daniela, some from the past and others from the present. Thank you guys for all your support.

Dr. Lee-Ann Jaykus and her laboratory crew, especially Blanca Escudero for her support doing this research.

Dr. Trevor Phister for his support and help as a committee member throughout my research. Thank you so much Dr. Phister.

Caitlin Alberts for being a huge help in the laboratory, for all the hard work she put on this research helping me to do every experiment and for making my life in the lab a lot easier.

My special gratitude to Robin Siletzky for all the knowledge she shared with me, her patience when I asked the questions over and over again and for helping me to understand how the microbes work. Thank you so much Robin, you are awesome.

My friends Jose Pablo and David, for their support and help during my time at NCSU.

Brian, Brad, Paul, William, Andrew, Thomas and Jen, Thank you friends for your support and help.

Finally, the most important, the one who deserves all Glory and Honor, my God, my Savior, my shelter and friend Jesus Christ, for being there for me all the time. This is because of Him, He made this possible, not because I'm capable to do it.

I'll worship you God all my life!!

# TABLE OF CONTENTS

LIST OF TABLES .....	viii
LIST OF FIGURES .....	ix
Chapter 1 .....	1
Literature Review.....	1
Introduction.....	1
<i>Clostridium perfringens</i> .....	3
Virulence and Pathogenicity .....	4
Reservoirs .....	6
Outbreaks .....	6
Contributing factors leading food poisoning .....	7
Factors that affect the growth and survival of <i>Clostridium perfringens</i> .....	8
Spores and their importance.....	11
D-values .....	13
Combase©.....	15
The effects of hurdles.....	15
Phosphates.....	15
The use of phosphate. ....	16
The use of phosphates in poultry and as antimicrobial.....	18
NaCl .....	20
Rationale and Significance .....	21
Hypothesis and Objective .....	22
Previous Studies.....	23
Chapter 2.....	28
Validation of cooling curves.....	28
Abstract .....	28
Introduction.....	30
Materials and Methods.....	33
The Product.....	33
Preparation of the turkey samples.....	33

Inoculum preparation .....	34
Inoculation .....	36
Plating Procedure .....	38
Results and conclusions .....	40
Chapter 3.....	45
Germination and outgrowth of <i>Clostridium perfringens</i> in cooked whole turkey muscle .....	45
Abstract.....	45
Introduction.....	48
The turkey industry.....	49
pH.....	50
Objective.....	51
Materials and Methods.....	53
Preparation of the turkey samples.....	53
Inoculum preparation .....	54
Inoculation .....	56
Plating Procedure .....	59
Results and Discussion .....	62
Statistical Analysis.....	62
Control Analysis .....	62
Statistical Analysis of Treatments .....	63
TABLES AND FIGURES .....	69
Tables.....	69
Figures.....	73
REFERENCES .....	79
APPENDICES .....	86
Appendix A.....	87
Statistical Analysis.....	87

## LIST OF TABLES

<b>Table 1-1.</b> Different factors that influence the growth of <i>Clostridium perfringens</i> vegetative cells. Adapted from “Microorganisms in foods 5. Characteristics of microbial pathogens” (50).....	69
<b>Table 1-2.</b> Top five pathogens causing domestically acquired foodborne illnesses. (32, 58, 75) .....	69
<b>Table 2-1.</b> Moisture, NaCl, pH and phosphate content of sampled RTE whole muscle turkey breast. ....	70
<b>Table 2-2.</b> Bacteria population log and log change between replicates and controls. ( red indicates growth).....	70
<b>Table 2-3.</b> Growth average, standard deviation and Standard error per run. ....	70
<b>Table 3-1.</b> pH values of Sodium Acid Pyrophosphate (SAPP) and Brifisol® used to prepare the brine injected in the turkey breast in a wt/wt ratio.....	71
<b>Table 3-2.</b> Water, NaCl and Phosphate used to prepare the brine injected in the turkey breast in a wt/wt ratio. ....	71
<b>Table 3-3.</b> Mean, standard error (SE) and standard deviation (SD) of the growth of <i>Clostridium perfringens</i> in cooked turkey breast for an inoculum of 3.48 log <sub>10</sub> .....	71
<b>Table 3-4.</b> Mean, standard error (SE) and standard deviation (SD) of the growth of <i>Clostridium perfringens</i> in cooked turkey breast for an inoculum of 7.48log <sub>10</sub> . ....	72

## LIST OF FIGURES

- Figure 2-1.** Graphs of the cooling curves required by the USDA in Appendix B and the experimental curve..... 73
- Figure 2-2.** *Clostridium perfringens* growth during the first stage of the cooling process using Combase© Perfringens Predictor simulation tool. pH of 6.15 and 1.3% NaCl for a not cured product (A) step 1, from 48.89°C (120°F) to 26.67°C (80°F) in 120 min. (B) step 2, from 21.11°C (70°F) to 12.8°C (55°F) in 120 min.(20) ..... 74
- Figure 3-1.** *Clostridium perfringens* log<sub>10</sub> growth from in 4 h cooling time from 120°F (48.9°C) to 50°F (12.8°C) for two different inocula. Columns that have different letters are significantly different (p<0.05)..... 75
- Figure 3-2.** *Clostridium perfringens* log<sub>10</sub> growth from in 5 h cooling time from 120°F (48.9°C) to 50°F (12.8°C) for two different inocula. Columns that have different letters are significantly different (p<0.05)..... 76
- Figure 3-3.** *Clostridium perfringens* log<sub>10</sub> growth from in 6 h cooling time from 120°F (48.9°C) to 50°F (12.8°C) for two different inocula. Columns that have different letters are significantly different (p<0.05)..... 77

# Chapter 1

## Literature Review

---

### Introduction

During the production of ready to eat meats, one of the concerns of the meat industry, including the turkey industry, is the growth of *C. perfringens* to levels that can cause illness. This bacterium is ubiquitous in nature and its spores can be naturally found in meat products. Inappropriate cooling of cooked meat products could result in the germination and outgrowth of the spores and consequent replication of bacteria to unacceptable levels (67). "A risk assessment for *C. perfringens* in ready to eat (RTE) and partially cooked meat and poultry products", published by the United States Department of Agriculture (USDA) in 2005, reported that improper cold storage of these meat products is responsible for 90% of the predicted illnesses (67).

*C. perfringens* has been targeted by the industry for years, specifically outlined under the guidance by the USDA. On January 6, 1999 the Food Safety Inspection Service (FSIS) published, their final rule "The Performance Standards for the Production of Certain Meat and Poultry Products" in the Federal Register. This rule addressed the standards for *C. perfringens* in cooked beef, roast beef, and cooked corned beef products; fully and partially cooked meat patties; and certain fully and partially cooked poultry products. This

performance standard limits the growth of *C. perfringens* to one log<sub>10</sub> in the product, while being produced specifically by limiting the time and temperature during the stabilization process (cooling) for certain fully and partially cooked meat products (63). The Food Safety Inspection Services (FSIS) is the public health regulatory agency portion of the Department of Agriculture responsible for the safety and wholesomeness of meat, poultry and egg products in the United States and it is their responsibility to develop rules or regulations for this type of products.

Perfringens Predictor ® is an application of the web-based predictive models tool ComBase in which a simulation of the growth of *C. perfringens* in bulk meat takes into account the maximum heating temperature (70-95°C), pH of the meat (5.2-8.0), concentration of NaCl (0-4%) and if the product is cured or not. This model was developed by Y. Le Mark (36) by using different media prepared with beef, pork and turkey; however this modeling has not been accepted by the USDA as a validation for all turkey products. More reliable data is needed to describe if *C. perfringens* germinates and grows in specific meat products, under specific conditions, i.e. whole turkey muscle meat.

Many studies have conducted using various parameters such as time, temperature and ingredients to evaluate the level of *C. perfringens* growth under different conditions during the heating and cooling of many meat products; however very few have been performed with poultry products, specifically turkey.

As we know more about various products and how the pathogens could grow in certain food matrix we understand their behavior better and can apply effective hurdles, like ingredients or treatments in order to control their growth or eliminate them. To assure the

performance of certain products according to rules and regulations for These types of studies could open up a better understanding of the organism to apply accurate control processes and design adequate facilities to assure food safety.

Our research consisted of two main studies: first, the validation of cooling time after the cooking and browning cycle during the production of turkey deli meat to guarantee no more than one  $\log_{10}$  growth as indicated in the USDA appendix B guidance; second, the evaluation of the effects of pH in turkey meat by the addition of two different types of phosphate (each of them with different pH) and how this variable interacts with three different levels of NaCl in order to limit the ability of *C. perfringens* to germinate and grow under these conditions during the stabilization process of the product (cooling time after cooking). As a result the development of appropriate time-temperature combinations during the stabilization, to be used as a reference by the turkey industry when designing accurate and effective cooling curves during the production of turkey deli meat to remain in compliance with the USDA Appendix B.

### ***Clostridium perfringens.***

*C. perfringens* is a rod shaped, spore forming, gram positive encapsulated bacterium; considered anaerobic but can tolerate moderate oxygen exposure. A high number of bacteria ( $>10^6$  vegetative cells) are needed to cause illness (2), and because of the mildness of the disease, cases could be underestimated as many affected individuals do not seek hospital care, reducing reporting and subsequent detection used for statistics.

This pathogen requires a relatively short time frame to complete a replication (<10 min for some strains) and has the ability to sporulate making it highly tolerant to environmental stresses such as, heat, desiccation and even radiation. These characteristics increase the frequency in may cause foodborne disease (50)

This pathogen has also the ability to produce toxin that could persist in foods that have not been cooked well or not completely reheated (67). As mentioned this bacterium also tolerates a moderate exposure to oxygen and requires a slight reduction in Eh potential (oxidation-reduction) in order to grow (37).

It was not until the 1940's when *Clostridium perfringens* was recognized as an important cause of foodborne disease was it discovered it causes two distinctive foodborne diseases in humans, one of them known as a food borne toxico-infection and the other one identified as Necrotic enteritis, known as Darmbrand or Pig-Bel(37). Infection with *C. perfringens* can cause tissue necrosis or gas gangrene none as clostridial mionecrosis.

### **Virulence and Pathogenicity**

There are five different types of *C. perfringens*, identified by their toxin (A-E) that cause a toxico-infection. Type A, C and D cause disease in humans, with type A and C the most common groups to cause disease (50) The gastroenteritis type perfringens disease produces relatively mild gastrointestinal tract illness; including profuse diarrhea, fever, vomiting and abdominal spasms, for approximately 24 hours after an incubation time of 8 to 12 h. The disease usually resolves spontaneously after 12 or 24 h however, mortalities can occur in people who are immunocompromised, elderly or taking certain medications.

Typically, in order succumb to the disease, an individual needs to ingest large quantities of vegetative cells as they need to survive harsh acidic conditions in the stomach, pass into the small intestine, colonize it, multiply, then sporulate and produce toxin. The infectious dose is considered  $>10^6$  organisms/g of food (spores or vegetative cells).

*C. perfringens* enterotoxin (CPE) quickly attaches to intestinal epithelial cells inducing tissue damage subsequently causing fluid loss. The CPE typically affects cells at the villus tip, which are old intestinal cells that can be easily substituted by young cells in patients that are healthy (37). Also, the diarrhea that is caused helps remove toxin and *C. perfringens* cells that are not attached to the intestinal lining. In the case of patients with constipation the intestine could be exposed to the toxin longer resulting in necrotizing enteritis (37). Necrotic enteritis (pig-bel) is associated with Type C,  $\beta$ -toxin and is fortunately not very common in the US as it is usually fatal. Symptoms not only include necrosis of the small intestine but bloody diarrhea and distention, accompanied with abdominal pain and vomiting (2).

Type A  $\alpha$ -toxin is more related to gas gangrene (myonecrosis), peripheral pyrexia, septicemia, necrotizing colitis, and food poisoning and often denoted as CPE. CPE is a single 35 kDa (kiloDalton) polypeptide consistent of 19 amino acids primarily aspartic acid, serine, leucine and glutamic acid (35, 50). There is no evidence of immunity in patients previously exposed to the toxin; although an individual may develop serum antibody after infection (4).

## **Reservoirs**

*C. perfringens*, being a ubiquitous organism, it can be found in the soil at levels between  $10^3$  to  $10^4$  CFU/g (13). It is also found in ~50% of raw or frozen meat and in the intestinal tract of animals. Human feces usually contain  $10^4$  to  $10^6$  organisms/g. Less than 5% of global isolates carry the enterotoxin gene (CPE gene located on the chromosome nearly all human food poisoning isolates or a plasmid]). However, currently there is deficient knowledge on the main reservoir of *C. perfringens* type A food poisoning (37)

## **Outbreaks**

According to the Morbidity and Mortality Weekly Report published in 2009 which summarizes epidemiologic data on food borne disease outbreaks (FBDOs) reported during 2006, the Public health officials identified a food vehicle in 528 (42%) FBDOs, of which 243 (46%) outbreaks with 6,395 (50%) cases were classified as having ingredients belonging to only one food commodity. Among the 243 outbreaks attributed to a single commodity, the most outbreaks were attributed to fish with 47 outbreaks, poultry with 35 outbreaks, and beef with 25 outbreaks, and the most cases were attributed to poultry with 1,355 cases. Pathogen-commodity pairs responsible for the most outbreak-related cases were *C. perfringens* in poultry with 902 cases (9).

In the 2011 CDC report “Food borne illness acquired in the United States” it was estimated, that 31 pathogens caused 37.2 million illnesses in the United States. *C. perfringens* caused almost an estimated 1.0 million illnesses annually (965,958 illnesses)

being the third most important pathogen after Norovirus (5,461,731 illnesses) and *Salmonella* nontyphoidal (1,027,561 illnesses) (**Table 2.1**) (54).

Generally, in order to identify *C. perfringens* outbreaks, stools of two or more ill persons and/or implicated foods have to contain  $10^5$  organisms/g (13). However, it is possible to find high levels of *C. perfringens* in healthy individuals. Therefore, to define an outbreak, CPE belonging to the genotype being investigated (i.e., A, C or D) must also be found in a certain number of ill people. A multiplex toxin genotyping PCR for CPE positive could be used, as well as a CPE genotyping PCR assay to establish that those type A, C or D disease isolates transport the chromosomal CPE gene, distinctive of food poisoning isolates. Additionally, it is common practice to use Western blots to check that the isolates express CPE when the sporulation occurs.(37).

According to the Center for Disease Control and Prevention (CDC), the actual number of cases of *C. perfringens* is 10 times the average of the reported cases related to outbreaks, due to the mildness of the disease. The total number of cases is assumed to be 38 times the number of cases reported, if the numbers are calculated, based on the studies of *Salmonella* spp diseases (39)

### **Contributing factors leading food poisoning**

There are several factors that contribute to the high levels of *C. perfringens* growth needed to cause food borne illness; including temperature abuse after cooking or cooling or improperly hot holding food. Incorrect handling temperatures during storage has been found

in 100% of outbreaks, improper cooking in around 30% of outbreaks and the use of contaminated equipment has been found as a factor in around 15% of the outbreaks. In order to prevent this from occurring, refrigeration temperatures should be kept below 4°C and the temperatures during serving should be kept above 70°C (2, 37, 48).

### **Factors that affect the growth and survival of *Clostridium perfringens***

#### ***Temperature***

The spores of *C. perfringens* have a relatively high heat resistance. It has been found that spores survive one hour at boiling temperatures in a protective environment, such as meat medium (36). It is known, that spores may become more resistant to heat depending upon the temperature at which they have sporulated; this condition has been confirmed in some studies where spores from food poisoning isolates are found to be, much more heat resistant than spores of *C. perfringens* isolated from sources other than foods that have been heated (50). This higher heat resistance of spores found in foods related to illness may contribute to their virulence (53). It is also important to mention, that the spores of the strains associated with food borne diseases are those which have shown higher heat resistance. This ability gives them a better chance of survival in foods that have not been cooked properly. On the other hand, the enterotoxin has a relatively mild heat resistance and can be inactivated when it is held at 29°-65°C for 1.5 to 72.8 min in gravy and 2.4 to 149.4 min in buffer (6).

The optimal growth temperature for vegetative cells is between 43 to 47°C but may grow at temperatures as high as 50°C. On the other hand, vegetative cells do not replicate well in cold temperatures. The growth rate of this bacteria decreases significantly at temperatures below 15°C, showing no growth at 6°C (**Table 1.1**) (13, 37). Juneja et. al. showed that heat shocking the spores increases the heat resistance of this organism allowing the vegetative cells to survive longer (up to 1.5 fold) (24). Interestingly, the vegetative cells isolated from food poisoning outbreaks are also more heat resistant than those not related with foodborne illness (53).

### ***Chemicals***

Chemicals applied as sanitizers to eliminate bacteria during cleaning procedures can have a detrimental effect on bacteria; however the spores of *C. perfringens* can tolerate chlorine dioxide exhibiting even more tolerance than other bacteria like *B. cereus* or *B. stearothermophilus* (20-80mg ClO<sub>2</sub>/L at pH 4.50, 6.50, 8.50) *C. perfringens* is however sensitive to ingredients commonly used in meat products such as sodium chloride and nitrite. These ingredients have a synergistic effect when they work together inhibiting a low levels of injured spores/cells, in combination with a mild heat treatment and sensitivity to curing agent which is the reason why there is little information about outbreaks associated with cured meats However, it has been found that un-germinated spores can still be active or viable in products containing ingredients used for preservation of food that normally would prevent cell growth. Under the right conditions, those spores could eventually germinate and replicate, if the growth-inhibiting factors are removed from the food matrix or have been

eliminated during the cooking process of the food (37). For example, there are cases when *C. perfringens* has been found in cured and smoked ham containing 17% (wt/v) NaCl, 1500ppm NaNO<sub>2</sub> and 980ppm NaNO<sub>3</sub> (10).

### ***E<sub>h</sub>***

In order to grow, *C. perfringens* does not need an exceptionally reduced environment ( $E_h$ ). The precise value of  $E_h$  required to allow growth of this bacterium depends on the influence of environmental conditions like pH. *C. perfringens* can produce compounds like ferredoxin as reducing molecules in order to change the  $E_h$  of its environment and adjust the conditions to promote its growth (13). The redox potential that limits the growth of *C. perfringens* is in the range of -125 to 287 mv at a pH between 6.0 and 7.6. These  $E_h$  values depend on cultural conditions; in this case, five different types of media were used (59).

### ***pH***

*C. perfringens* can grow at an optimal pH range between 6.0 and 7.0. This bacterium does not grow well or even stops growing at a pH values less than 5.0 and greater than 8.3 (37). pH also has an effect in the production of heat resistant spores. At a pH of 7.0 or 8.0 the number of heat resistance spores starts to increase after between 2 and 4 h; the maximum value reached was 10<sup>7</sup> spores/ml after 6 to 7 h. For a pH between 3.0 and 6.0, heat resistant spores were produced after 17 to 20 h at a maximum level of 1x10<sup>6</sup> to 5x10<sup>6</sup> spores/ml; production of spores started after a lag time of 13-15h (18). Controlled pH also has an effect in the production of CPE in *C. perfringens*. In the case of *C. perfringens* type D at a

controlled pH between 6.0 and 7.4, the growth of bacteria was the same as when the pH was not controlled; however, the production of toxins were increased two times for alpha toxin, six times for epsilon, two times for kappa and 8 times for theta with controlled pH at the optimum levels of 7.0 to 7.2 for alpha, 6.7 to 7.4 for epsilon and kappa, and 7.0 to 7.4 for theta toxin than without controlled pH The effect of pH in the growth of vegetative cells is shown on **Table 1.1**.

### **Spores and their importance**

The characteristic that gives *Clostridium perfringens* an advantage over many organisms is the ability to form spores. A spore is the stage intended to protect and preserve the viability of the cell when environmental conditions are not optimal for the growth and reproduction of the vegetative cell. Approximately 12 different stages have been identified in the sporulation of *C. perfringens*; the most important ones are cell enlargement, reduction of the cytoplasmic basophilia, forespores terminal location, enlargement of the forespore, encystment of the forespore, progressive sporangium contraction around the forespore and spore maturation. The mature spore is comprised of a central core with an external cortical layer and a laminated spore wall created by the sporangium (57).

The spore has the ability to become a vegetative cell after a chemical or heat activation. This action interrupts the spore's dormant stage and initiates the process to become a vegetative cell. Monohidric and some polyhydric alcohols could help in spore's activation at 37°C after 15 min of exposure (11); also, 100 mmol l<sup>-1</sup> KCl could help in the activation of the spores with no heat, however only 2% of the spores germinate (72).

Seventy-five degrees Celsius for 20 min are necessary to heat activate the spores of *C. perfringens* (33). It has been found that more than 80% of spores germinate in the first 15 min after heat shock at 80°C for 10 or 30 min; the same percentage germinates in the first 30 min after activation for 5 min at 80°C or at 70°C for 10 min; and in the first 40 min after activation for 10 min at 60°C (49).

It has been suggested that the formation of the enterotoxin responsible for causing disease is associated with the ability to form spores (15). In some species of clostridia, toxin production can only occur at the end of the exponential growth (55).

An important factor in the sporulation of the bacterium is the incubation time. Research with strain NCTC8238 shows that there was not a change in the quantity of spores harvested after an incubation time of 24 at 42°C using 3 different levels of bacteria in the inoculum; however there was an increase in the amount of spores at 44 h of incubation at the same temperature. An inoculum of  $10^2$ ,  $10^4$  and  $10^6$  of viable cells at a pH of 6.90, 6.92 and 6.99 respectively produced final counts of  $8.20 \times 10^6$ ,  $7.30 \times 10^6$  and  $5.7 \times 10^6$  spores/ml after 24h of incubation time at 42°C. On the other hand, the number of spores increased to  $1.27 \times 10^7$ ,  $1.31 \times 10^7$  and  $1.02 \times 10^7$  spores/ml respectively after 44h of incubation at 42°C (17).

### ***Spore culture***

The most commonly used and widely accepted method to culture vegetative cells of *C. perfringens* to induce spore production uses a volume (800 ml) of sporulation media with a headspace of H<sub>2</sub>:N<sub>2</sub> (10:90, v/v) (36) and heat activated spores. The procedure indicates that 0.1ml of the stock cell spore culture must be transferred into 9 ml of Fluid Thioglycolate

Medium (FTM) in screw cap test tubes; then heat shocked at 75°C for 20 min to activate the germination of the spores. The Duncan-Strong media used is composed of proteose peptone, yeast extract, sodium thioglycollate, disodium hydrogen phosphate, heptahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ), starch and water (14).

The use of starch as carbohydrate source was changed to raffinose due to its ability to increase the amount of spores between 2% and 93%; a level of variation commonly seen when different strains of *C. perfringens* are used (14). It has also been found that the use of 50-200µg of caffeine ( $\text{C}_8\text{H}_{10}\text{N}_4\text{O}_2$ ) is effective in increasing spore yields from 4.18-4.89 to 6.00-7.13  $\log_{10}$  spores/ml (33)

### **D-values**

The decimal-reduction times (D-values) for vegetative cells of *C. perfringens* in pork luncheon meats vary from 0.90 min (65°C) to 16.30 min (55°C). The D-values for *C. perfringens* spores range between 2.20 min (100°C) to 34.20 min (90°C) (7). The z-values were calculated to be 7.80 and 8.40°C for *C. perfringens* vegetative cells and spores, respectively. This data suggests that a relatively mild cook at 70°C for 1.3 min would cause a 6 log reduction of vegetative cells (7). In turkey, decimal reduction times (D-values) for *C. perfringens* range from 17.5 min at 55°C to 1.3 min at 62.5°C. In the presence of 0.15 sodium pyrophosphate (SPP) without NaCl, D-values ranged from 16.2 min at 55°C to 1.1 min at 62.5°C. When a concentration of 0.3% SPP was used, a reduction of >8  $\log_{10}$  CFU/g was observed at 65°C for 1 min, indicating a decline in heat resistance (25)

The USDA-FSIS developed standards for the inactivation of *Salmonella spp.* with a reduction of 6.50-D in meat products (beef or pork) and a 7-D for poultry products known as Appendix A (12). Juneja et al., reported decimal reduction times (D -values) of 1.15-1.60 min for 10 strains of *C. perfringens* vegetative cells in beef gravy (34). These times are below the D -values for *Salmonella*, therefore, those lethalties should also be effective in destroying *C. perfringens* vegetative cells. However, if this organism is present in its spore stage, those spores will survive the inactivation step. The cooking process could actually activate the spores, which could germinate, outgrow and multiply during stabilization. This situation could be exacerbated if the chilling process improperly executed by not reaching the appropriate temperatures at the right time intervals. This is the primary reason why the USDA/FSIS established additional guidelines to ensure that not more than one log<sub>10</sub> growth of *C. perfringens* will occur during the stabilization stage in meat products. This guideline is referred to as Appendix B (68). On the other hand, the enterotoxin, has a relatively mild heat resistance, and can be inactivated when it is held at 29-65°C for 1.5 to 72.80 min in gravy and 2.40 to 149.40 min in buffer (6). Based on the study of Le Marc et al. (36), a predictive model of the growth of *C. perfringens* was developed and simulates growth of this pathogen over time at various conditions of temperature, NaCl, pH, with or without cure. However this model is recognized only as a reference tool as the medium used to compile the data was beef medium, ground cooked beef with 5%fat, pork or turkey in reinforced clostridia medium and not in actual whole muscle products (36).

## **Combase©**

ComBase© is an on-line international resource for quantitative and predictive microbiology in food. The database was created by using information on how different microorganisms responded to related conditions and applied them in predictive models and it is a collaboration between the USDA Agricultural Research Service (USDA-ARS), the Institute of Food Research (IFR) out of the United Kingdom, and the University of Tasmania Food Safety Centre (FSC) in Australia (64)

## **The effects of hurdles**

Research has shown that changes in pH, NaCl content and temperature could affect the growth of *C. perfringens* in beef (36); however, the influence of these hurdles in the behavior of this pathogen has to be validated in turkey. The use of previous models, like those found in ComBase Perfringens Predictor© give a great overview of the effect of these hurdles in beef, pork or turkey media, however the USDA has some concerns about the use of this type of internet/computer based predictors, as a reliable way to validate other types of meat products and processes.

## **Phosphates**

It is not really known when phosphates were initially used in foods but it is well known that ancient civilizations used salting or drying as preservation processes in meat products. The first historical mention of the use of phosphates with NaCl and nitrates is in Roman sausage manufacture, known as Botulus (41).

Phosphates have been used in modern meat products processing due to their capacity of increasing the water holding capacity (WHC) within the muscle. When they are used together, sodium and chloride ions are absorbed at a higher degree, exceeding the sum of the absorption if both are used separately (41). Phosphates also protect against lipid oxidation in cooked meat and improve stability. Due to their flexibility to have an impact in the properties of muscle proteins, phosphates are widely used in the production of restructured products and pigment protection in meat (41).

Phosphates also protect against lipid oxidation in cooked meat and improve stability. Due to their impact on the properties of muscle proteins, phosphates are widely used in the production of restructured products and for pigment protection in meat. Between the decades of 1950's and 1960's several studies were done that established the scientific basis that promoted the rapid adoption of phosphates use in various products outside the meat industry (41).

The use of phosphates also has a significant impact on ATP (adenosine triphosphate). ATP has an important role in the disassociation of myosin and actin promoting the onset of rigor mortis. If pyrophosphates are added to the muscle, the ATP breakdown rate and the glycogen depletion rate will be increased; as a result the rigor in the muscle will be decreased (41).

### **The use of phosphate.**

It is widely known that phosphates have sequestering properties, being capable of forming complexes with poly and monovalent metal cations. This property is also known as

chelation and it is the product of a competitive reaction for the metal ion between a precipitating and a sequestrant anion in a solution. In other words, chelation is the action of a phosphate removing a cation permanently or temporarily; this can be assessed using a solution or a combination of inorganic and organic compounds. When cations are removed, they become unavailable to meet the nutritional needs of microorganisms. (47). It has been shown that this property is not related to the increase in the WHC of meat. According to Inklaar (19), around 60% of calcium and 20% of magnesium, naturally presented in meat are so firmly attached to proteins that they are unavailable for a reaction with any added phosphate.

The contraction of the skeletal muscle is controlled and based on the interaction of calcium in the troponin-tropomyosin complex and the high energy phosphate bond of magnesium-ATP. The ability of phosphates to chelate metal ions affects the availability of calcium and magnesium to be used in muscle contraction, increasing tenderness by relaxing the muscle (41)

Phosphates are also important acidifying and buffering agents, used extensively in the food industry. The dibasic sodium NaCl of pyrophosphoric acid (SAPP) are preferably used as acidifying agents over acid NaCl of polyphosphoric acids (47). This acidifying property is particularly important in the inhibition of microbial growth, due to the effect of pH on cells (47).

In previous research, it has been observed that minor variations in pH of pork meat could have a significant impact on the germination and outgrowth of spores of *C. perfringens*. The pH could be impacted by the type and concentration of phosphate and the

muscle type (56). More research needs to be performed in turkey meat to better understand how the pH of the meat, due to the addition of phosphates affects the germination of spores and outgrowth of *C. perfringens*.

K. A. Nelson et al., studied the synergetic effect of phosphates, like sodium acid pyrophosphate (SAPP) with other compounds like sodium nitrite and sorbic acid or potassium sorbate. They determined there was an inhibitory effect on the production of toxin of *C. botulinum*. Other important factors that were taken into account, while evaluating the use of preservatives, were the effect of pH and the addition of nitrite. When nitrite was not added, there was no inhibitory effect on toxin production by SAPP. At the same pH a combination of potassium sorbate and nitrite was more effective inhibiting the production of the toxin than the sorbic acid and nitrate treatment. The use of SAPP with potassium sorbate and nitrite was the most effective combination. It was suggested that the combination of SAPP, sorbic acid and nitrite may give the best results in controlling botulinum toxin, due to the reduction in pH (41, 47). The same effect is expected in the case of *C. perfringens*. The reduction in pH and the synergetic effect of SAPP, sodium nitrate and sorbic acid or potassium sorbate could have the same inhibitory characteristic for the production of toxin due to similarities in the formation of endospore, cell wall, biochemical properties, common to *Clostridium* spp. (3)

### **The use of phosphates in poultry and as antimicrobial**

It has been shown that the use of phosphates in chicken meat at concentrations, between 1 and 3% improve yield, flavor, tenderness and total moisture (41). In the case of

turkey, all the same benefits have been observed with the exception of tenderness (41). Additionally, some studies have shown that phosphates slow down lipid oxidation in freshly cooked and reheated meat. Juiciness was also increased and together with meaty-brothy flavor and aroma was positively correlated by a panel ( $p < 0.05$ ) (21). Additionally, juiciness and tenderness had a positive correlation. It has also been shown that the addition of sodium chloride positively impacts the flavor (22).

Phosphates have been shown to exhibit antimicrobial properties when used in fresh poultry products due to the lack of release of the membrane phosphatases, that normally occurs in red meat processing (cutting or grinding) due to the massive disruption of cells (41). Another contributing factor is that the water holding capacity decreases the water activity values ( $a_w$ ) and the protein hydration effect, which may have an effect on the swelling of the molecules, inhibiting the movement of bacteria into the muscle (41).

The use of phosphates has a positive result when is used to inhibit the germination and outgrowth of *C.perfringens* in certain meat products. Pork meat was treated with three different blends of phosphates including tetrasodium pyrophosphate (TSPP) and sodium acid pyrophosphate (SAPP) (0.15% of each for a total of 0.3% of phosphate and 1% NaCl). The combination of two different types of SAPP was better than the other blends to inhibit this pathogen (56). Akhtar et al.,2008, showed similar results with the use of other polyphosphates, like STPP, however the concentration of the chemical was 1%, which is higher than regulatory limits in food (0.5 %) (1,66).

Skepticism exists with the hypothesis that the antimicrobial properties of phosphates due to the hydrolysis of phosphates by meat phosphatases in fresh meat, where the meat and

microbial enzymes are not inactivated by cooking (43); however there are some studies showing that using 0.5% of Sodium Acid Pyro Phosphate (SAPP), Tetrasodium Pyro Phosphate (TSPP), Sodium Tripolyphosphate (STPP) or Sodium polyphosphate glasy (SPG) have some positive inhibitory effect on spoilage bacteria and *Staphylococcus aureus* in raw refrigerated bratwurst (43).

Kena, a commercial blend from Innophos Inc. which include 75% STPP and 25% TSPP has shown antimicrobial properties on turkey steaks when they were submerged in a 6% solution for 6 h and stored at 5°C extending its shelf life (61).

## **NaCl**

The use of NaCl in meat products has been extensively studied, however publications on the effect of NaCl in turkey products is scarce. It appears to some extent that *C. perfringens* prefers the presence of some NaCl. No growth is observed in beef products containing 3% NaCl. In the same type of product *C. perfringens* grew  $\geq 3 \log_{10}$  CFU/g, in 15 h; 5  $\log_{10}$  CFU/g in 18 h and 21h when <2% NaCl was used. In cooked ham normally formulated with >2% NaCl, the population of this pathogen increased <1  $\log_{10}$  CFU/g during 15h cooling time. It has also been shown in cooked ham and beef that NaCl at a concentration ranging from 6 – 8% inhibits *C. perfringens* growth (75).

The preparation of food also has an impact in the germination and outgrowth of *C. perfringens*. Apparently the ingredients used in pork scrapple formulated with 1.11% NaCl and potato starch (0.25%) at a 12% pump rate (no potassium tetra pyrophosphate), have a positive effect in the inhibition of this bacterium. Less than one  $\log_{10}$  growth was observed

during 12 or 14 h of cooling time from 54.4°C to 7.2°C. However, after 21h the spores germinated and grew from  $\sim 3.00 \log_{10}$  to  $\sim 7.80 \log_{10}$  (30).

It also has been found that 3% NaCl inhibit the growth of *C. perfringens* at a pH of 5.5 for 24 h in beef. At a pH of 7.0, the growth of this bacterium was delayed for 8 days when kept at 15°C of storage temperature using 3% NaCl. Vegetative cells were not observed after 21 days of storage at 15°C with 3% NaCl and pH 7.0 (27).

## **Rationale and Significance**

Although significant and abundant research has been completed to determine cooling curves for *C. perfringens* in different meat products, there is still a need to obtain data relating the growth of this microorganism in turkey meat with the influence of commonly used ingredients in the industry, to determine if there are limitations in growth. This research could have a significant impact for the turkey industry, as it could assist firms in complying with current regulations. Having more accurate information about *C. perfringens* growth in turkey meat will aid in the design of better processes, controls and better food production methodologies in order to assure a safe supply of products to the consumer. Validating cooking and cooling curves of turkey products will also give the regulatory agencies and consumers more confidence about the safety of production practices and of the product.

As mentioned before, NaCl and phosphates are widely used in the meat industry because of the diverse physicochemical and sensorial properties that are imparted in the products. It has also shown that different combinations of phosphates and NaCl are being

used in the meat industry, and the use of them could have antimicrobial properties in meat products. (1, 40, 41, 42, 43, 47, 56, 62, 75). The effect of pH and NaCl in the inhibition of *C. perfringens* has been widely evaluated in beef and pork but less research has been done in turkey.

Due to the obvious research gaps relating to the behavior of this pathogen in turkey products, this research was designed to evaluate primarily the ability of *C. perfringens* to germinate and grow under the effects of NaCl and the interaction with different levels of pH obtained by adding two different types of phosphates in order to simulate current formulations that exist in turkey products in addition to the determining the combined effects of pH and NaCl in the inhibition of *C. perfringens* in turkey.

## **Hypothesis and Objective**

Current USDA stabilization performance standards (1999) state that, no more than one log<sub>10</sub> growth of *C. perfringens* is allowed during the processing of beef and poultry meat products. If the time-temperature combinations used by the turkey industry are developed including hurdles like pH (as an effect of the addition of phosphate) and NaCl content at the appropriate levels, it could be possible to achieve no more than one log<sub>10</sub> growth in *C. perfringens* populations in RTE turkey meat much easier. In this case, it would be possible to extend the cooling process time, required to limit the growth of this bacterium and have a greater safety margin.

The main objective of this research is to study the effect of NaCl and pH in the germination and outgrowth of *C. perfringens* during four different cooling curves in cooked

turkey whole muscle (breast). This effect was studied following the stabilization temperatures required for the Appendix B of the USDA/FSIS. The NaCl percentages used were 1%, 1.5% and 2% NaCl, based on the percentages used in two different turkey facilities in North Carolina. The pH range was obtained using two different phosphates formulated for the industry in compliance with the concentrations established by the USDA/FSIS (66); 0.5% wt/wt of SAPP (Sodium Acid Pyrophosphate) for a pH of 5.76 (lower level); and 0.5% wt/wt Brifisol® (agglomerated blend of sodium phosphates) for a pH of 6.4. Seven different treatments resulted from the combination of three different percentages of NaCl (Salt) and the two different phosphates at the same percentage (0.5%), including a control treatment without NaCl of phosphate.

Additionally this research will:

- Determine the possible synergic effects between different pH and NaCl concentration at different cooling times in turkey meat in order to limit a one  $\log_{10}$  increase in the population of *C. perfringens* at a specific temperature adhering to USDA's Appendix B.
- Establish baseline data to assist in the further development of software that will allow the government and the industry to simulate formulations and cooling conditions of turkey meat in order to validate different production practices.

## **Previous Studies**

Several studies evaluating cooling practices pointed out that cooked ready-to-eat turkey deli roasts require a maximum cooling time of 8.9 h from 48.9°C (120°F) and 12.8°C

(55°F) to prevent the potential outgrowth of *C. perfringens* and subsequent foodborne illness (58). Cooking of meat products is usually adequate in destroying vegetative forms of the organism; however, the spores of *C. perfringens* typically survive and utilize the heat during the cooking process to activate germination of the spore. The germinated spore may then outgrow and multiply into toxin-producing vegetative cells, if the product is not cooled rapidly enough. There is a lack of appropriate and rapid cooling especially in the case of dense, large-muscle meat products with reduced heat transfer capabilities (63). It has been found, that after the cooling process, the spores of this bacterium can grow from  $\sim 3.0 \log_{10}$  to  $\sim 7.8 \log_{10}$  CFU/g, proving its capacity to grow rapidly under the right conditions in certain types of food (31). More specifically, in the case of the strain NTCC8238, data has shown that it possesses a generation time of 7.1 min at 41°C. (74)

Other research has studied the germination and outgrowth of *C. perfringens* in whole turkey breasts of approximately 4kg, without using any ingredient or antimicrobials. Regression analysis of the information obtained from the 8h and 10 h curves has estimated, with 95% confidence that the whole breast could be cooled down from 48.9° to 12.8°C in no more than 9.6 h (58)

Juneja et al. indicated that beef when pasteurized must be cooled down to 7.2°C in no more than 15h, in order to achieve less than one  $\log_{10}$  growth of this bacterium. *C. perfringens* grew from  $1.5 \log_{10}$  to around  $6.0 \log_{10}$  CFU/g after 18 h of cooling time until reaching 7.2°C. Again, no phosphate or NaCl was used in this research (28).

The growth inhibition of *C. perfringens* was also studied in raw cured bologna made of 70% Chicken with sodium nitrate. Samples were inoculated with spores of three different

strains, then cooked and cooled down for 20h then stored at 4.4°C, under vacuum. The population of the bacteria remained constant without significant changes, during chilling from 54.5° to 7.2°C and the population was reduced slightly during storage under vacuum at 4.4°C (60). The impact of anaerobic conditions in the growth of this pathogen has been evaluated on the strains NTCC8238 and NTCC8239 in uncured ground turkey (29). With an inoculum of ~3.0 log<sub>10</sub>, both strains grew to about 7.0 log<sub>10</sub>, after 9 h anaerobically and 24 h aerobically, at 28°C. They concluded that if the products were temperature abused for 5h, no significant growth would be observed; however if the abuse occurred for a longer period of time, there was a risk of elevated levels of the bacteria in the product. (29).

Cured and uncured turkey meat has also been studied. Raw meat blends were inoculated with 100 spores per gram and cooked at 73.9°C. After cooling, according to the USDA/FSIS guidelines, 2.25 and 2.44 log<sub>10</sub> growth was obtained for uncured turkey that was cooled down during a period of 6h in two different experiments. In a third experiment just a 0.83 log<sub>10</sub> increase was observed from an inoculum of 10 spores per gram. Based on these results, it was concluded that with an initial low inoculum level, lower growth was obtained. On the other hand, no growth was obtained in the cured samples irrespective of initial load (35).

The use of antimicrobials has also been investigated in turkey meat due to their ability to inhibit the germination and outgrowth of bacteria. In a study that evaluated the effects of lactates on the germination and outgrowth of *C. perfringens* and compared to the effects of acetates, lactates regardless of their type, inhibited the growth of *C. perfringens* when used in different concentrations in injected turkey (1%, 2%, 3% and 4.8%) (71). However, calcium

lactate showed better inhibition with respect to germination and outgrowth of *C. perfringens* at  $\geq 1\%$  concentration, than sodium and potassium lactates (71). The use of 1% sodium lactate, 1% sodium acetate, 1% Ional (buffered sodium citrate) or Ional Plus (buffered sodium citrate containing sodium diacetate) inhibited the germination and outgrowth of *C. perfringens* spores in marinated turkey breast considerably (23). With an inoculum of 2.8 log<sub>10</sub> final counts were 3.12, 3.10, 2.38, and 2.92 log<sub>10</sub> respectively after 15 h of chilling. The same inhibition was obtained after 18 and 21 h (23)

. The combination of 0.25% sodium diacetate and 1.25% sodium lactate in cook-in-bag turkey breast samples, preserved them in storage at 4°C for a period longer than 22 weeks, without off odors and anaerobic plate counts lower than 2.3 log<sub>10</sub>. This treatment inhibited the growth of psychrotrophic spoilage organisms like a nonproteolytic, psychrotrophic *Clostridium* isolate, designated strain OMFRI1(40).

In roast beef without any antimicrobial added under an anaerobic environment, *C. perfringens* can germinate and grow from 3.1 log<sub>10</sub> to 2.00, 3.44, 4.04, 4.86 and 5.72 log<sub>10</sub> after 9, 12, 15, 18 and 21 h of cooling time respectively (51). The use of organic acid NaCl's inhibit the germination and growth of this bacteria; however when the meat that contains organic acid NaCl's is used as an ingredient to produce other type of food, the amount of this NaCl is diluted; in this particular case, *C. perfringens* can germinate and grow if not more of this NaCl is added to the final product (51).

Another study in cooked ground beef indicated that the product must be cooled down to 7.2°C in not more than 15h in order to achieve not more than one log<sub>10</sub> growth of *C.*

*perfringens* (28) as the population grew from 1.5 log<sub>10</sub> to around 6.0 log<sub>10</sub> with 18 h of cooling time to (7.20°C).

## Chapter 2

# Validation of cooling curves

---

### Abstract

The turkey industry in the US has grown significantly in the last four decades as turkey consumption has increased 102 percent since 1970 (46). This level of turkey consumption has placed the US as the most important turkey producer in the world (46). As a growing industry, more research is needed to provide accurate information about how pathogenic organisms behave in turkey products. The Food Safety and Inspection Service of the United States Department of Agriculture (USDA/FSIS) has developed a standard for the stabilization of meat products in order to limit the growth of *C. perfringens* to no more than one log<sub>10</sub> in cooked products (63). This standard was developed based on 9 CFR 318.17(h) (10) which was intended originally to be used in cooked beef, cooked corned beef and cooked roast beef but not for poultry products, therefore it is important to validate if this organism has the same response in animal products other than beef (63).

Ready to Eat (RTE) whole muscle turkey roast with 1- 2% NaCl (NaCl) and 0.25 – 0.50% Sodium Phosphate was evaluated to determine if *C. perfringens* would grow more than one log<sub>10</sub> using a specific cooking cooling process.

The product was cooked, chilled then fried in 365-385°F (185 – 196.1°C) soybean oil, to create a browned surface colored product. The primary concern of this process is the double cooling curve that is needed, since product temperature rises to 50-80°F (10-26.7°C), during the superficial frying procedure.

A three strain cocktail of *C. perfringens* (NTCC8268, NTCC8239, ATCC10388) using a 1:1:1 ratio was injected in the center of 10.00g cooked turkey roast cores, vacuum packaged and placed in a programmable water bath to simulate a specific cooling curve used by a North Carolina company. There was no statistical significance difference in *C. perfringens* growth between the controls and the replications for the data collected ( $p > 0.05$ ). Results indicate that the combined cooling curves showed less than a total of one  $\log_{10}$  growth in accordance with USDA policy. Therefore, the formulation of the product combined with the cooking and cooling conditions used yield a safe product.

## Introduction

*Clostridium perfringens* is a gram-positive, spore-forming, anaerobic, non-motile, bacillus shaped bacterium, which is ubiquitous in nature. Depending on the strain, some spores of this pathogenic organism have enough heat resistance to survive the cooking process (37). Heat treatments can also be the stressor required for spores to become activated and germinate into vegetative cells, capable of growing during the stabilization of food products to levels that could cause disease (50).

. This microorganism is one of the most common bacterium, associated with food related diseases in the United States, being responsible for an estimated 1,000,000 cases of food borne illness, annually (54). It is the second most important bacterial pathogen associated with outbreaks, after *Salmonella* spp., according to the most recent surveillance from the Centers for Disease Control and Prevention (CDC) (54).

In 2010 the Morbidity and Mortality Weekly Report published by the CDC reported that meat and poultry products are the food vehicles, most commonly associated with *C. perfringens* food poisoning (9). Out of the 235 outbreaks in 2007, 17% were attributed to poultry which was the highest percentage, caused by a single commodity. During cooling of RTE products prepared commercially or at residences, *C. perfringens* can potentially grow to numbers that become a significant threat to public health. *C. perfringens* appears to be nationally present in 28.10% of 296 samples of ground turkey meat (25 g/sample) (70). The average number of this bacteria found in the samples was 39 CFU/g with a maximum of  $3.5 \times 10^3$  CFU/g (69).

The turkey industry has changed in the last 30 years from being a holiday-season business into a completely integrated industry with a diversified line of products, successfully competing with other sectors in the production of a reliable source of protein (46).

The US is the largest turkey producer in the world generating 2.59 billion metric tons (MT) in 2011. From those, 2.29 billion MT were consumed locally, according to the United States Department of Agriculture, Foreign Agricultural Service (USDA/FAS) (65). In the US during the first quarter of 2012 alone turkey meat production was 635,000 MT; representing 3.1% more than the first quarter in 2011(73). North Carolina was the second largest turkey producer in the US raising 30 million turkeys, just behind Minnesota (47 million), in 2010 (46). The average consumption of turkey meat per capita is 7.3 kg in the US, followed by Canada with 4.3 kg and Europe with 3.7 kg (65).

One of the primary concerns of Ready to Eat (RTE) turkey deli meat processors is compliance with the USDA stabilization (cooling) guideline, commonly referred to as Appendix B, which limits the growth of *C. perfringens* to less than one log<sub>10</sub> (68).

Cooling curves that deviate from the guidance must be validated to ensure products remain under the growth parameters established. Appendix B provides two different cooling curves that can be used to achieve a rapid enough reduction in temperature to limit *C. perfringens* growth. Within Appendix B the first cooling guideline is the most conservative cooling curve. The internal product temperature has to be cooled from 130°F (54.4°C) to 80°F (26.7°C) in no more than 1.5 hours and cannot remain between 80°F (26.7°C) and 40°F

(4.4°C) for more than 5 hours. The second Appendix B cooling guideline is based on 9 CFR 318.17(h) (10) “Requirements for the production of cooked beef, roast beef, and cooked corned beef”. This document can be applied to whole muscle products like beef or pork loin and turkey breast; however as its name indicates, this regulation is based on data obtained from beef. This guideline establishes that the center of the product must not remain between 120°F (48.9°C) and 55°F (12.8°C) for more than 6 h. Then the product should be cooled down to a temperature of 40°F (4.4°C) and not be packed for shipment until it has reached this temperature (**Figure 2-2**) (68). There is not a specific time limitation to perform this final cooling step to 40.00° F (4.44° C), due to the limited ability of *C. perfringens* to grow below 12.00°C (50).

There is however a need to generate accurate data, regarding cooling curves specifically in turkey meat as commonly formulated. This research focuses on the evaluation of turkey meat, after undergoing specific cooking and cooling processes to determine if more than one log<sub>10</sub> growth of *C. perfringens* occurs during dual cooling curves.

## Materials and Methods

### The Product

The product that was being validated was a Ready-To-Eat (RTE) turkey deli breast roast which has been cooked –cooled- fried-cooled. The additional frying process provides a browned layer outside the product to improve its appearance.

The turkey breast roasts are typically formulated with 1%- 2% NaCl (NaCl) and 0.25% – 0.5% Sodium Phosphate. The product is cooked-in-bag to an internal temperature of 165°F (73.9°C) then chilled to 40°F or below prior to the browning step. Cooked and chilled products are held at 35°- 40°F (1.7°– 4.4°C) for 8 to 72 h prior to the oil browning. The product is then removed from the cook in bag and passed through 365°- 385°F (185° – 196.1°C) soybean oil to create surface color, typical of fried product. During this step, the internal temperature of the product rises to a temperature range between 50°-80°F (10°- 26.7°C).

### Preparation of the turkey samples

Turkey deli meat roast formed with whole turkey breasts, injected with NaCl and phosphate (**Table 2-1**), was cooked and fried according to Appendix A and cooled according to Appendix B (12). Finished product was received from a company in North Carolina placed in a cooler and transported at or below 4°C to the North Carolina State University (NCSU) meat pathogen laboratory. The deli meat was cut into 10g cores using a 23.8 mm diameter core tool (Humboldt Science Education products, Schiller Park, Illinois) placed into individual sterile 7.0oz Whirl Pak bags (OTR 0.125 cc/100 in<sup>2</sup>/24 h at 22.8°C and 0% RH;

Part #B00992WA; Nasco, Fort Atkinson, Wisconsin) and stored at -20°C, until use. Prior to inoculation, cores were thawed by placing them at 4°C in an Isotemp Low Temperature Incubator (Fischer Scientific model 307C Cat No 11-679-25C, Dubuque, Iowa) overnight. The cores used as controls were cut and stored under the same conditions as the treatment cores.

### **Inoculum preparation**

Three strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and ATCC 10388 (Hobbs serotype 13), were obtained from the Microbial Food Safety Research Unit, Eastern Regional Research Center USDA-Agricultural Research Service. The spores were suspended in deionized water, kept at 4°C in an Isotemp Low Temperature Incubator (Fischer Scientific model 307C Cat No 11-679-25, Dubuque, Iowa) and used to produce additional spore crops according to Juneja et al. (33).

One milliliter of the spore solution was transferred to a screw cap tube with 10 ml of Fluid Thioglycollate medium (FTG) (Becton Dickinson Microbial Systems Difco™ Ref 225650, 500g, Franklin Lakes, New Jersey). The tubes were heated to 75°C for 20 min to heat shock the spores; then, the tubes were incubated at 37°C for 18 h. Following incubation, one ml of this culture was transferred into a new 10ml tube with FTG and incubated for 4h at 37°C. Due to the fact that thioglycollate provides the necessary environment for the growth of the bacteria, it was not necessary to incubate under anaerobic conditions during these initial steps. The bacterial culture was then transferred to Modified Duncan Strong (DS)

sporulation media at a 1% ratio (one ml of culture in 100 ml of sporulation media) and incubated for 5 days at 37°C.

Duncan Strong media was made using 15g of proteose peptone (Becton Dickinson Microbial Systems BD Bacto™ REF. 211684, 500g, Sparks, Maryland), 4g of yeast extract (Becton Dickinson Microbial Systems BD Bacto™ REF. 212750, 500g, Franklin Lakes, New Jersey), one gram of sodium thioglycollate (Becton Dickinson Microbial Systems BD Bacto™ REF. 212081, 100g, Franklin Lakes, New Jersey), 10g of sodium phosphate dibasic heptahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ) (Fischer Bioreagents ® ChemAlert ® BP331-500, 500g, Fair Lawn, New Jersey), 4g raffinose (Becton Dickinson Microbial Systems BD Bacto™ REF. 217410(0174-15) 100g, Sparks, Maryland) and one liter of deionized water. The pH was adjusted to  $7.8 \pm 0.1$  with filter-sterilized 0.66M sodium carbonate ( $\text{CO}_3\text{Na}_2$ ) (Across 42428-5000, Geel, Belgium) using a 25 mm 0.22µm Syringe filter (MCE, Sterile, FisherBrand, Fisher Scientific, Ireland). The media was also supplemented with 100µg/ml of caffeine (Sigma-Aldrich, product code 1000739445 CO750-1002 Caffeine- kofeina 1, 3, 7-trimethylxanthine, St Louis, Missouri) in order to stimulate the production of spores.

Spores were harvested by centrifugation at 6900 x g for 20 min at 4°C using an Eppendorf centrifuge (Eppendorf, Model Number 5810R, 15 amp version, Germany). After centrifugation the spores were washed twice and suspended with 5.0-ml sterile deionized water. The spore crops were kept at 4°C and enumerated following the plating procedure described below.

Three strain spore cocktails were prepared in a 1:1:1 ratio through serial dilution of the spore stocks of each strain to assure the same log level in all the strains solutions. Actual

spore count was determined to be  $3.4 \times 10^3$  CFU/ml ( $3.53 \log_{10}$ ) after heat shocking and plating the day of the inoculation.

### **Inoculation**

Six experiments were performed using four individual cores (one control and three replications) inoculated with 0.1 mL of the aforementioned non-heat-shocked *C. perfringens* spore cocktail, containing  $3.5 \log_{10}$  of spores, using a BD Medical Luer-Lok 1mL disposable syringe, which was inserted down the center of the core. The plunger of the syringe was slowly depressed as the needle was carefully removed from the core to ensure equal spore distribution along the center of the core. All the inoculated cores were placed into 7oz Whirl Pak bags (OTR 0.13 cc/100 in<sup>2</sup>/24 h at 22.8°C and 0% RH; Part #B00992WA; Nasco, Fort Atkinson, Wisconsin) and vacuum packaged at 99% vacuum (7.60 mmHg) in a Kock Ultravac vacuum package machine (Model UV225, Item No 903225-070, Koch Equipment, Kansas, Missouri).

The inoculated cores were heat shocked at 75°C (167.00°F) for 20 min using a water bath (Fisher Scientific ISOtemp Model 128, 8.5 Amps, 120 Volts, Dubuque, Iowa). Center core temperature was confirmed using a data logger (Omega Engineering, Inc. Stamford, Connecticut) with a type T thermocouple inserted into one core. The thermometer was calibrated at 0°C using an ice water slurry and at 100°C using boiling water. After an appropriate heat shock was achieved, the actual spore count was determined by enumerating the control core, following the method described below under plating procedure. The replicate cores were then transferred to iced water to reduce their temperature to 60°C to reduce the time needed begin the cooling curve. The cores were then submerged completely

in a RTE-17 circulating water bath unit; model RTE-17 D+ 115/60 BST (Thermo Scientific Brand, Dubuque, Iowa) for 10 min until 120°F (48.9°C) was achieved to initiate the stabilization process. The water bath was controlled using Neslab NESCOM 3.0 Temperature control software (Thermo Fisher Scientific, Newington, New Hampshire) to simulate cooling curves. The program simulates both cooling curves, after cooking and after frying; raising the temperature of the cores after the first cooling step. The cooling process of the product was achieved in two stages. The first stage contained two steps, from 120°F (48.9°C) to 80°F (26.7°C) in 120 min. and from 80°F (26.7°C) to 55°F (12.8°C) in 240 min. The second stage simulated the cooling cycle after frying from 70°F (21.1°C) to 55°F (12.7°C) in 120 min. All the temperatures were verified with the data logger described above, placing the thermocouple at the center of an additional turkey core as a surrogate to the inoculated product. After the first cooling stage was achieved, the samples were heated in the same programmable water bath for 10 min to achieve the highest temperature the product reaches during the frying process (70.0°F/21.1°C) then the second cooling stage was initiated.

As mentioned previously, the inoculated control sample was used to determine the initial population of viable *C. perfringens* cells at the beginning of the aforementioned cooling processes. After the heat shock treatment (activation of the spores), the control core was cooled down with the other cores in ice water until they reached approximately 60°C. The replicate cores were then transferred to the programmable water bath to start the cooling process while the control core was left in the ice water to assure a temperature below 12°C was achieved to prevent multiplication. (50).

## **Plating Procedure**

Once the cooling cycles were completed in both two stages, the samples were transferred to ice water in order to cool them down below 12°C or less to stop the growth of bacteria. Each individual bag containing an inoculated 10g core was opened aseptically using a sterile scalpel (Feather disposable scalpel, stainless steel blade with plastic handle, Feather safety razor CO., LTD. Osaka, Japan) and immediately transferred into an autoclaved stainless steel blender cup (Waring Mini-sample Containers MC-2, 12-37ml capacity, Conair Corp. East Windsor, New Jersey) with 20 ml of Phosphate Butterfield's buffer (99ml Butterfield's Buffer R23601, Remel Inc. Lenexa, Kansas) and blended on a Waring laboratory Blender at maximum speed for approximately 20 sec in order to minimize chunks of meat (Waring LB, model LB10G, 240 V, Conair Corp. East Windsor, New Jersey).

Enumeration for *C. perfringens* was performed by serial dilution, transferring 1ml of the blended solution into 9ml of Phosphate Butterfield's buffer (3M™ Mini Flip-Top Vial with Butterfield's Buffer BPPFV9BFD, St. Paul, Minnesota); then 0.25 ml was spread plated onto four duplicate plates from three different dilutions ( $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  or  $10^{-5}$ ) to assure a total of 1 ml was plated for each dilution. This procedure was selected due to the low recovery rate from samples in preliminary studies. Triptose Sulfite Cycloserine Agar plates were used to enumerate the bacterial population (TSC) (EMD Chemicals TSC Agar Base, 1.11972.0500, Darmstadt, Germany, Gibbstown, New Jersey). The media was supplemented with *C. perfringens* Selective Supplement (EMD Chemicals Inc. EM1.00888.0001 Darmstadt, Germany, Gibbstown, New Jersey) and egg yolk (Egg Yolk suspension, R450290, 50% 100.00ml, Remel Inc. Lenexa, Kansas).

After the sample plates had dried with lids on for approximately 5 min, plates were overlaid with 10 ml egg yolk free TSC agar. The plates were allowed to solidify before placing them lid side up, in a non-vented, O-ring gasketed, polycarbonate anaerobic jar sealed with a clamp and thumb screw (BBL Gaspak 150 Large Anaerobic System 260628, Sparks Maryland) with three anaerobic generation sachets (BD Diagnostic Systems, GasPak EZ anaerobic Container System 260678, Sparks, Maryland). The jar was incubated for 24 h at 35°C then the plates were enumerated. The lower limit of detection was calculated as 10 CFU/g ( $1.0 \times 10^1$  CFU/g) using this plating procedure. The protocols followed were as directed by the USDA/FSIS Microbiology Laboratory Guidebook 3rd Edition/1998 (38).

## Results and conclusions

Six experiments were performed equaling a total of 18 replications and six controls. Data was analyzed using SAS software (version 9.1, SAS Institute, Cary, North Carolina) and analysis of variance was carried out at 95% significance level ( $P < 0.05$ ) with a Tukey test being used for separation of means.

The effect of NaCl in beef and cooked ham, which is normally formulated to contain  $>2\%$  NaCl, suggest that a 15 h cooling time would yield an acceptable product (with an increase of  $<one \log_{10}$  CFU/g in the *C. perfringens* count); however, for beef containing  $<2\%$  NaCl, *C. perfringens* populations may reach levels high enough to cause illness (33).

From the food safety point of view in RTE turkey deli breasts, previous work has suggested a cooling period between 129°F (53.9°C) and 55°F (12.7°C) in no more than 8.9 h. (58). However, the regulation that applies to this type of products is the USDA's Appendix B which is more conservative (68). The data obtained in this study showed that the production process of a turkey meat product formulated with 1- 2% NaCl (NaCl) and 0.25 – 0.50% Sodium Phosphate including two cooking and cooling steps is able to fulfill the Appendix B guidance of not more than one  $\log_{10}$  growth after the final cooling step. The highest growth obtained was 0.53  $\log_{10}$ . Seventy two percent (13 out of 18 replicates) showed a reduction in the population of viable *C. perfringens* cells after the second step of the total cooling time. It has been observed however that the size of the inoculum is a factor that increases variation. In the data obtained by Kalinowski et al. where the impact of the inoculum in the growth level of *C. perfringens* has been tested, three different experiments were done in uncured raw turkey emulsion. Experiment 1 and 2 were inoculated with 100 spores per gram of turkey

meat ( $2 \log_{10}$ ). During 6 h of cooling time from  $48.9^{\circ}$  to  $12.8^{\circ}$  C, 2.44 and  $2.25 \log_{10}$  growth were observed respectively. The third experiment was done with an inoculum of 10 spores per gram ( $1 \log_{10}$ ); in this case a  $0.83 \log_{10}$  growth was observed, where the effect of the size of the initial population was evident (35). In other hand, there are different aspects that could cause this data to indicate reduction instead of growth. The presence of phosphate with potential antimicrobial effects (depending on the type of phosphate), the recovery method used to process the samples (stomacher, blender), contamination of the spore stock solution, competitive strains within the cocktail, the sample matrix, poor sporulation obtained during the preparation stage of the spores stock solutions and the variability of the inoculum due to the use of a syringe with inadequate scale causing accurate volumes to be dispensed. In the case of phosphates, 0.5% of Sodium Acid Pyrophosphate showed antimicrobial properties in Bratwurst when handled at an abuse temperature of  $24^{\circ}$  C for 48 h. Sodium tripolyphosphate, trisodium phosphate and sodium phosphate glasy display the same characteristics, however the time is reduced to 24 h of temperature abuse at  $24^{\circ}$  C in the same product.(41)

Conversely, the same cooling curve as this study was run on Combase Perfringens predictor© for the two stages of the cooling of the product which was based on Y. Le Mark (36) studies. The two limits of the range of pH and NaCl used by the company were the input for this simulation. For a pH of 6.15 and 1.3% of NaCl with temperature values according to a cooling process of  $120^{\circ}$  F ( $48.9^{\circ}$  C) at 0h,  $80^{\circ}$  F ( $26.7^{\circ}$  C) at 2h and  $55^{\circ}$  F ( $12.7^{\circ}$  C) at 6h showed one  $\log_{10}$  growth when the simulator reached  $66^{\circ}$  F ( $18.9^{\circ}$  C) after 4.24 h of cooling time. At the end of the first stage used by the firm total growth was predicted at  $1.06 \log_{10}$ . The second stage after frying only when entered into the program represented a  $0.002 \log_{10}$

growth under the same conditions yielding a total growth for both stages of  $1.06\log_{10}$ . (20). For the same pH of 6.15 and a NaCl concentration of 1.81% the total growth was  $0.82\log_{10}$  for both stages combined compared to  $1.12\log_{10}$  growth was obtained at a pH of 6.18 and 1.3% NaCl and with 1.8% NaCl the predictor showed a total growth of  $0.87\log_{10}$ . All simulations were run with the uncured option and revealed more than one  $\log_{10}$  growth was shown for a 1.3% NaCl at both values of pH (6.15-6.18) as opposed to  $0.53\log_{10}$  as the highest growth achieved in this study (an average of  $-0.40\log_{10}$  growth among all the replicates). As mentioned before Combase Perfringens Predictor © was developed using beef medium and ground cooked beef with 5% fat, pork or turkey in reinforced clostridial medium and not using actual whole muscle products (36). It is also important to note that there is not option in Perfringens Predictor© to consider the type and amount of phosphate in the product. When used in combination with NaCl, sodium and chloride ions are absorbed more effectively, exceeding the sum of the absorption if both are used separately (41). This characteristic decreases the water activity values ( $a_w$ ) and the protein hydration effect, which may have an effect on the swelling of the molecules, inhibiting the movement of bacteria into the muscle (41). This reduction in water activity could also have a positive impact in the inhibition of bacterial populations.

NaCl has an inhibitory effect on the growth of *C. perfringens* as shown by Zaika et al. when it is used at levels greater than 2% in cooked ham. (75). In ground turkey, 3% NaCl completely inhibited the outgrowth of *C. perfringens* at 15°C; it also inhibited growth at 28°C for 12 h with the addition of 0.3% of Sodium pyrophosphate. This pathogen was also inhibited in ground turkey meat with 1% and 2% of NaCl and 0.3% of sodium pyrophosphate

at 15°C.(26). These results are similar to the data obtained in this research indicating levels of NaCl between 1.30 and 1.80% may result in a better inhibition of the outgrowth of the bacteria.

The use of some types of phosphate has also been proven to have an inhibitory effect in the outgrowth of *C. perfringens* in pork meat as indicated by Singh et. al. where a blend of two different sodium acid pyrophosphate's (SAPP) held a population of *C. perfringens* to less than one log<sub>10</sub> growth (56). More specifically ground pork loin of three different types (pale, soft, and exudative [PSE]; normal; and dark, firm, and dry [DFD]) were mixed with a cocktail of three strains of *C. perfringens* and taken from 54.40° to 4.00°C in a 6.50, 9, 12, 15, 18, or 21 h. cooling period. Two types of SAPP showed less than one log<sub>10</sub> growth when the samples were cooled down in all cooling cycles for PSE; in 6.5, 9.0 and 12.0h for normal; and in 6.5h for DFD. The interaction between phosphates and NaCl on the heat resistance of the spores is described by Juneja et al. where 0.3% of sodium pyrophosphate at a pH of 6.0 with levels of NaCl between 0% and 3% in turkey slurry showed a change in the D value from 23.2 min (no NaCl) to 17.7 min (3% NaCl) at 99°C (26). Although this study did not apply a thermal treatment 72% of the data (13 samples) actually showed a decrease in colony count compared with the control while 22% (4 samples) had <0.50 log<sub>10</sub> growth and only 5% (1 sample) expressed growth greater than 0.50 log<sub>10</sub>. None of the 16 replications presented growth greater than one log (max. growth was 0.53 log<sub>10</sub> with one replication).

The mean count of the 8 controls obtained during the research was 3.10 +/- 0.19 log<sub>10</sub> and the 16 replications had a mean count of 2.70 +/- 0.12 log<sub>10</sub>. There was no statistical significance difference between the controls and the replications for of the data collected

( $p=0.1221$ ) (**Table 2-2, 2-3**). In conclusion, a cooling cycle of 120°F (48.9°C) to 80°F (26.7°C) in 120 min.; 80°F (26.7°C) to 55°F (12.8°C) in 240 min followed by a second cooling after frying of 70°F (21.1°C) to 55°F (12.8°C) in 120 min. results in less than one log increase in *C. perfringens* indicating that product cooled under the these parameters would be safe.

## Chapter 3

# Germination and outgrowth of *Clostridium perfringens* in cooked whole turkey muscle

---

### Abstract

The turkey industry has an important role on the economy of the US due to its rapid growth and significant impact on public health due to its close association with *C. perfringens*, a causative agent of foodborne disease (46). This pathogen is responsible of close to 1.0 million illnesses annually which represents 10% of the total number of foodborne illnesses, according to the January 2011 study published by the Center for Disease Control and Prevention (CDC) **Table 1.2** (54). Mortality and Morbidity Weekly (MMWR) in 2007 reported that two of the four largest outbreaks assigned to a single food commodity were caused by *C. perfringens* and the third pathogen-commodity pair with the most illnesses related to an outbreak was *C. perfringens* in poultry (9). The Food Safety and Inspection Service of the United States Department of Agriculture (USDA/FSIS) developed a guide for

the stabilization of meat products known as Appendix B. This guideline helps prevent more than one  $\log_{10}$  growth of *C. perfringens* in cooked products. Appendix B was developed based on 9 CFR 318.17(h) (10) which was originally applied to cooked beef, cooked corned beef and cooked roast beef, not poultry products (68).

The turkey industry uses ingredients like NaCl and phosphates in order to improve tenderness and flavor in their products. The use of these ingredients affects the pH of the meat ultimately impacting the germination and outgrowth of *C. perfringens*. The effect of pH and NaCl in the germination and outgrowth of *C. perfringens* during cooling according to the Appendix B was evaluated in this study. Whole muscle turkey with 1%, 1.5% and 2% NaCl (NaCl) and 0.5% of Sodium Acid Pyrophosphate (SAPP) and 0.5% of Brifisol® (agglomerated blend of sodium phosphates) was tested in order to validate if *C. perfringens* would germinate and grow more than one  $\log_{10}$  under these conditions. These two types of phosphates at 0.5% wt/wt produced an intramuscular pH of 6.17- 6.76 that represent typical values of turkey products within the industry (**Table 3-1**).

Three *C. perfringens* strains (NTCC8268, NTCC8239, and ATCC10388) were combined in a cocktail of a 1:1:1 ratio with a concentration of approximately 8.48  $\log_{10}$  spores per gram, heat shocked at 75°C for 20 min then injected into the center of 10g of cooked turkey breast cores (two controls and three replicates). Samples were then chilled following the less conservative cooling curve established in Appendix B in 4, 5, 6 and 9 h.

There was significant interaction between phosphate and NaCl in effecting *C. perfringens* growth in all treatments. The effect of NaCl at 1.5% showed more inhibition in

growth of the bacterium at 4 and 5 h of cooling time with SAPP as part of an interaction among NaCl, phosphate and cooling times.

More than one  $\log_{10}$  of *C. perfringens* was obtained in all the treatments at 5, 6 and 9 h of cooling time which suggest the need for validation of different meat products under various conditions to assure safe products.

## Introduction

*C. perfringens* is a gram-positive, spore-forming bacterium that is anaerobic, non-motile, bacillus shaped and ubiquitous. Depending on the strain some spores of this pathogenic organism may survive the cooking process. Additionally, this heat treatment could be the stress required for the spore to become activated and germinate into a vegetative cell capable of growing during the cooling step of the food product to levels that could cause disease (37, 50). This organism is one of the most common bacterium associated with food related disease in the United States being responsible for an estimated of 1,000,000 cases of food borne illness annually (54). It is the second most important bacterial pathogen associated with outbreak illnesses after only *Salmonella spp.* according to the most recent from the Centers for Disease Control and Prevention (CDC) (54).

In the Morbidity and Mortality Weekly Report of 2010 the CDC reported that meat and poultry products are the food vehicles most commonly associated with *C. perfringens* food poisoning (9). Out of the 235 outbreaks in 2007, 17% were attributed to poultry which was the highest percentage caused by a single commodity. During cooling of RTE products prepared industrially or at home, *C. perfringens* can potentially grow to numbers that become a significant threat to public health. *C. perfringens* was found to be present nationally in 28.1% of 296 samples of ground turkey meat (25 g/sample) (70). with the average amount being 39 CFU/g with a maximum number of  $3.50 \times 10^3$  CFU/g (69).

## **The turkey industry**

The turkey industry has changed in the last 30 years from being a holiday-season business into a completely integrated meat production industry with a diversified line of products competing strongly with other sectors in the production of a reliable source of protein (46). The US is the largest turkey producer in the world with 2.59 billion of metric tons (MT) in 2011 and 2.29 billion MT consumed locally according to the United States Department of Agriculture, Foreign Agricultural Service (USDA/FAS) (65). North Carolina was the second largest turkey producer in the US with 30 million of turkeys just after Minnesota with 47 million in 2010 (46). The average consumption of turkey meat per capita is 7.30 kg in the USA followed by Canada with 4.30 kg and Europe with 3.70 kg (65). In the first quarter of 2012 turkey meat production in the US was 1.40 billion pounds; this amount represents 3.10% more than the first quarter in 2011(73).

One of the major concerns of Ready to Eat (RTE) turkey deli meat processors is compliance with the USDA stabilization (cooling) guideline commonly referred to as Appendix B, which limits the growth of *C. perfringens* to less than one  $\log_{10}$  during the cooling down step in the production of meat products (68).

Cooling curves that deviate from the guidance must be validated; therefore manufacturers should know if their products remain under parameters established. Appendix B provides two different cooling curves that can be used to assure a rapid reduction in temperature to a level where *C. perfringens* would cease to grow. Within Appendix B the first cooling temperature-time combination is the most conservative cooling curve. In order to be in compliance with this guidance, firms need to assure that the internal temperature of the

product is cooled down from 130°F (54.4°C) to 80°F (26.7°C) in not more than 1.5 hours; then, the product has to be cooled down from 80°F (26.7°C) to 40°F (4.4°C) in not more than 5 hours. On the other hand, the second Appendix B cooling guideline is based on previous data according to the 9CFR 318.17(h) (10) “Requirements for the production of cooked beef, roast beef, and cooked corned beef”. Appendix B applies to whole muscle products like beef or pork loin and turkey breast. This guideline establishes that the center of the product must not remain between 120°F (48.9°C) and 55°F (12.8 °C) for more than 6 h; then, the product should be cooled down to a temperature of 40°F (4.4°C) and not be packed for shipment if it has not reached this temperature (**Figure 2-2**) (68). There is not an specific time to perform this last cooling step to 40°F (4.4° C) due to the limited ability of *C. perfringens* growth at temperatures below 53.6°F (12°C) (50).

There is also a need to generate accurate information specific to turkey meat due to the fact that the guidelines derived from the 9 CFR 318.17(h) (10) was originally intended to be used for cooked beef, cooked corned beef and cooked roast beef not to poultry products (68).

## **pH**

pH has an impact in the growth of *C. perfringens* as it does for most microorganisms. The range of pH on which this bacterium can grow is relatively wide with a minimum pH of 5.5-5.8 and a maximum of 8.0-9.0 with an optimum pH value of 7.2 depending upon the strain (50). This range matches with the pH of different types of cooked meats which can change from 6.0-6.5 to 5.0-5.3 during storage due to the presence of lactic acid bacteria (5).

In the case of turkey, raw breast pH can range between 6.66 and 6.69 (in a range between 3 and 20 min post mortem)(52).

In order to simulate the range of pH in whole muscle turkey meat products, SAPP and an agglomerated blend of sodium phosphates (Brifisol®) were used. The pH of the raw turkey breast was between 5.49 and 5.63 however the buffering capacity of turkey meat has been observed in previous studies. Once the meat was cooked, pH reached values between 5.82 and 5.89 without additional ingredients. The brine used with 0.50% SAPP had a pH of 4.43; once it was added to the meat the pH of the turkey meat changed to 5.65 after 24 h and dropped down to 5.56 after 65h. The brine with Brifisol® had a pH of 11 and changed the pH of the meat to 6.14 after 24 h and then reduced the pH to 6.09 after 65 h. Once the samples were cooked the values increased to a pH of 5.98 for SAPP and 6.60 for Brifisol®. It has been found that the pH of turkey meat changes an average of 12.02% in SAPP and 10.30% in Brifisol® after cooking (**Figure 3-4**). The buffering capacity of the meat is the reason why even having phosphate with values as low as 4 and as high as 11 has less of an effect on the pH of the turkey meat as it remained in a range between 5.98 and 6.60. These final pH values are in agreement with Santé et al. who compared different pH measurement in raw and frozen turkey *Pectoralis superficialis* at different stages after slaughter (52). In our research a final pH of 5.87 was obtained for fresh muscle

### **Objective**

The intention of this research was to determine the ability of *C. perfringens* to germinate and grow more than one log<sub>10</sub> in a whole muscle RTE turkey breast product. Three different cooling times were evaluated according to the temperatures established in Appendix

B by the USDA. The study included the use of 3 levels of NaCl and 2 different phosphates in order to simulate different types of products. The cooling times evaluated were 4, 5 and 6 hours. Nine hours was also evaluated, but because more than one  $\log_{10}$  growth was obtained at 5 and 6 hours as well as at 9 hours, those data points were not included. Samples of 6 different product treatments with a combination of 1.00%, 1.50% or 2.00% NaCl and 0.50% of two different phosphates were tested in order to have two different pH values representing high and low ends of pH range of existing products in the market. The maximum level of phosphate allowed by the USDA to be used in meat products is 0.50% (66).

## Materials and Methods

### Preparation of the turkey samples

Whole turkey breasts were received from a company in North Carolina transported in a cooler with ice packs at or below 4°C to the NCSU meat processing laboratory. The turkey breasts were weighed using a digital scale (Smart Scale Interface, Model Infinity GS9200 class III/III L . n<sub>max</sub> 10,000, NTEP CC 06-119, Serial 100448, Gainco Inc. Gainesville, Georgia), identified with a consecutive numbered paper tag and placed in cook-in bags (BT4770, size 12x19 in, clear color, product number PYU42, Gauge HVY, Sealed Air Cryovac, Duncan, South Carolina). The breasts were frozen and stored at -18.°F (-27.°C) in the meat processing laboratory until used.

Individual brine solutions were prepared for six treatments using 15% wt/wt of water; 1%, 1.5% and 2% NaCl; 0.5% Sodium Acid Pyrophosphate (SAPP. pH (1%) 4.05 ± 0.35) and 0.5% of Brifisol® (agglomerated blend of sodium phosphates, pH(1%) 11.5±0.5). The turkey breasts were injected with the corresponding brine using a manual Pump (Brine Pump, 1 Stück, No 900500, Friedr Dick GmbH + Co KG, Diezisaue, Germany) **Table 3.2**; placed in two cook-in bags, vacuum packaged at approximately 60% vacuum (Vacuum package machine model MVS50, MV501A02, 220V, 60 Hz, 28000W, 13.5 A, Minipack-Torre SPA Food division, Dalmine (BG) Italy); tumbled for 20 min in a SIMPROMAC tumbler (Mod ET-5, TYP K820, 120 Volt, 6Hz, Ser 7188, Canada) and then cooked in a Smokehouse (Enviro Pak, Series Max Pak, model MP-2000, Clackamas Oregon) to an internal temperature of 170°F (76.7°C). The cooked turkey breast were immediately cooled down to

an internal temperature of 40°F (4°C) then transferred into a walk-in cooler (37°F/2.8°C). Seven treatments were analyzed identified as Treatment 0 (T0) without NaCl and phosphate used as control; Treatment 1 (T1) with 1% NaCl and 0.5% of sodium acid pyrophosphate (SAPP); Treatment 2 (T2) with 1.5% NaCl and 0.5% SAPP; Treatment 3 (T3) with 2% NaCl and 0.5% of SAPP; Treatment 4 (T4) with 1% NaCl and 0.5% of an agglomerated blend of sodium phosphates (Brifisol®); Treatment 5 (T5) with 1.5% NaCl and 0.5% of Brifisol®; and Treatment 6 (T6) with 1% NaCl and 0.5% Brifisol®.

Each turkey breast was cut into 10g cores using a 23.8 mm diameter core tool (Humboldt Science Education products, Schiller Park, Illinois) placed into labeled individual sterile 7oz Whirl Pak bags (OTR 0.125 cc/100 in<sup>2</sup>/24 h at 22.8°C and 0% RH; Part #B00992WA; Nasco, Fort Atkinson, Wisconsin) and stored at -20°C until use. Prior to inoculation cores were thawed at 4°C in an Isotemp Low Temperature Incubator (Fischer Scientific model 307C Cat No 11-679-25C, Dubuque, Iowa) overnight.

### **Inoculum preparation**

Three strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and ATCC 10388 (Hobbs serotype 13), were obtained from the Microbial Food Safety Research Unit, Eastern Regional Research Center USDA-Agricultural Research Service. The spores were kept at 4°C in deionized water (DiH<sub>2</sub>O) in an Isotemp Low Temperature Incubator (Fischer Scientific model 307C Cat No 11-679-25, Dubuque, Iowa)

and used to produce spore crops according to Juneja et al (1993) (33). This spore crop production process is described below.

One milliliter of the spore solution was transferred to a screw cap tube with 10 ml of Fluid Thioglycollate medium (FTG) (Becton Dickinson Microbial Systems Difco™ Ref 225650, 500g, Franklin Lakes, New Jersey). The tubes were heated to 75°C for 20 min to activate the spores; then, the tubes were incubated at 37°C for 18h. One ml of this culture was transferred to a new 10ml tube with FTG and incubated for 4h at 37°C. It was not necessary to provide any anaerobic conditions during these steps as the thioglycollate provides the necessary anaerobic environment for the growth of the bacteria. This bacterial culture was transferred to the sporulation media at a 1% ratio (one ml of culture in 100 ml of sporulation media) and incubated for 24 h at 37°C. Modified Duncan Strong media (DS) was used to induce sporulation of the bacteria. To assure complete sporulation the incubation time was extended to a 5 day period.

DS media was made using 15g of proteose peptone (Becton Dickinson Microbial Systems BD Bacto™ REF. 211684, 500g, Sparks, Maryland), 4g of yeast extract (Becton Dickinson Microbial Systems BD Bacto™ REF. 212750, 500g, Franklin Lakes, New Jersey), one gram of sodium thioglycolate (Becton Dickinson Microbial Systems BD Bacto™ REF. 212081, 100g, Franklin Lakes, New Jersey) , 10g of sodium phosphate dibasic heptahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ) (Fischer Bioreagents ® ChemAlert ® BP331-500, 500g, Fair Lawn, New Jersey), 4g raffinose (Becton Dickinson Microbial Systems BD Bacto™ REF. 217410(0174-15) 100g, Sparks, Maryland) and one liter of deionized water ( $\text{DiH}_2\text{O}$ ). The pH was adjusted to  $7.8 \pm 0.1$  with filter-sterilized 0.66M sodium carbonate ( $\text{CO}_3\text{Na}_2$ ) (Across 42428-5000,

Geel, Belgium) using a 25 mm Syringe filter 22µm ( MCE, Sterile, FisherBrand, Fisher Scientific, Ireland). The media was supplemented with 100µg/ml of caffeine (Sigma-Aldrich, product code 1000739445 CO750-1002 Caffeine- kofeina 1, 3, 7-trimethylxanthine, St Louis, Missouri).

The spores were harvested by centrifugation at 6900 x g for 20 min at 4°C using an Eppendorf centrifuge (Eppendorf, Model Number 5810R, 15 amp version, Germany). After centrifugation the spores were washed twice with sterile deionized water and then re-suspended in 5ml of sterile DiH<sub>2</sub>O. These spore crops were kept at 4°C and enumerated following the plating procedure described in the plating procedure section.

The three strains spore cocktail was prepared in a 1:1:1 ratio through serial dilution of the spore stocks of each strain to assure the same log level in all the strains solutions and the same amount of bacteria using the following formula:

$$V_1 C_1 = V_2 C_2$$

Where,

$V_1$  = Volume of the spore stock solution

$C_1$  = CFU/ml of the spore stock solution

$V_2$  = Amount of volume to be removed from the spore stock

$C_2$  = CFU/ml of bacteria needed.

In order to know the exact amount of diluent to add it was necessary to subtract this new volume from the desired volume  $V_2 - V_1$ .

### **Inoculation**

Two different inocula were used in order to evaluate two different levels of initial population of bacteria. Cocktail I with 8.48 log<sub>10</sub> spores (~3.00x10<sup>8</sup> CFU/ml) and cocktail II

with  $4.48 \log_{10}$  spores ( $\sim 3.00 \times 10^4$  CFU/ml) were prepared and stored at  $4^\circ\text{C}$ . At the time of the inoculation, 2ml screw cap vials (Vial, Cryogenic; Fisherbrand; Storage; Externally threaded; 2mL; Polypropylene; HDPE closure, Fisher Scientific, Dubuque, Iowa) containing the *C. perfringens* spore cocktail were placed in a water bath (Fisher Scientific ISOtemp Model 128, 8.50 Amps, 120 Volts, Dubuque, Iowa) at  $167^\circ\text{F}$  ( $75^\circ\text{C}$ ) for 20 min to heat activate the spores.

Five 10g cores (two control and three replicates) per treatment were then placed in a RTE-17 circulating water bath unit, model RTE-17 D+ 115/60 BST (Thermo Scientific Brand, Dubuque, Iowa) for approximately 20 min in order to stabilize them at the starting temperature of the cooling curve ( $120^\circ\text{F}/48.9^\circ\text{C}$ ). The cores were then inoculated with 0.1 mL of the aforementioned heat-shocked spore cocktail using a calibrated syringe (1710RN Syringe Calibrates CAL81030, SN16875, Hamilton, Reno, Nevada) inserted down the center of the core. The final inoculum for cocktail I was  $7.48 \log_{10}$  CFU/g ( $\sim 3.00 \times 10^7$  CFU/g) and cocktail II with  $3.48 \log_{10}$  CFU/g ( $\sim 3.00 \times 10^3$  CFU/g).

The inoculated cores were placed into 7oz Whirl Pak bags (OTR 0.125 cc/100 in<sup>2</sup>/24h at  $22.80^\circ\text{C}$  and 0% RH; Part #B00992WA; Nasco, Fort Atkinson, Wisconsin) and vacuum packaged at 99% vacuum (7.60 mmHg) in a vacuum package machine (Koch Ultravac, model UV225, Item No 903225-070, Koch Equipment, Kansas, Missouri). The cores were then transferred back to the RTE-17 circulating water bath unit (model RTE-17 D+ 115/60 BST, Thermo Scientific Brand, Dubuque, Iowa) to initiate the stabilization process. The water bath was controlled using Neslab NESCOM 3.0 Temperature control software (Thermo Fisher Scientific, Newington, New Hampshire) to simulate cooling curves. The less

conservative cooling curve recommended in Appendix B was used in order to evaluate the worst case scenario (68). The cooling process of the product consisted in cooling down the cores from 120°F (48.9°C) to 55.0°F (12.9°C) in 4, 5 and 6 hours.

The two control cores per treatment were used to determine the initial population of viable *C. perfringens* cells at the beginning of each of the aforementioned cooling processes. Control cores were cut and stored under the same conditions as the replicate cores.

All the control cores were injected at the same time as the replicates with 0.1 mL of the spore cocktail for each of the two inoculum tested. After the inoculation, the control cores were placed in ice water and then plated to determine the initial population of viable *C. perfringens* cells. The remainder of the cores was put in the programmable water bath to initiate the cooling process.

The controls gave an initial population of viable cells at the beginning of each experiment detailed in **Table 3.3**. The lower limit of detection was calculated as one log ( $1.0 \times 10^1$  CFU/g) using the following the plating procedure.

### ***Size of the Inoculum***

An important aspect of an experiment is the amount of bacteria to be inoculated. In the study of Steele et.al, the author makes a prediction based on regression analysis that the turkey breast could be cooled down from 48.9°C to 12.8°C in 9.6h without allowing more than one log cumulative growth of *C. perfringens*. This research recommended a cooling period  $\leq 8.9$  h (58). The use of a whole muscle product with a relatively small inoculum (one  $\log_{10}$  CFU/g) could be the factor that would have influenced this result. In the Steele et al.

study an inoculum of less than one spore per g of turkey breast was used. The same results were obtained in the study of Juneja et al. where the author suggests cooling cooked beef to 7.2°C in less than 15 h.(28). Kalinowski et al. tested the impact of the inoculum in growth level of *C. perfringens* during three different experiments in uncured raw turkey emulsion. Experiment 1 and 2 were inoculated with 100 spores per gram of turkey meat (2.0 log<sub>10</sub>). During 6 h of cooling time from 48.9 to 12.8°C, 2.44 and 2.25 log<sub>10</sub> growth was observed, respectively. The third experiment was done with an inoculum of 10 spores per gram (one log<sub>10</sub>); in this case only 0.83log<sub>10</sub> growth was observed, where the effect of the size of the initial population was evident (35).

In the canning industry the term “skip” describes where no survival is observed between survival times of spore formers when thermal death times are evaluated. The smaller the amount of bacterial spores initially heated increases the incidence of skips.(45). According to this conclusion, it is better to use a larger level of initial inoculum in order to avoid the presence of skips that could lead to incorrect conclusions.

This is the reason why in this study two different inoculums were used in order to validate the results at a lower and higher initial populations of the bacteria.

### **Plating Procedure**

Once the cooling cycles were completed, the samples were cooled down to 4°C or less using the same water bath used in the cooling process in order to stop the growth of the bacteria. Each bag containing the inoculated 10g cores were opened aseptically using a sterile scalpel (Feather disposable scalpel, stainless steel blade with plastic handle, Feather safety

razor CO., LTD. Osaka, Japan) and immediately transferred into an autoclaved stainless steel blender cup (Waring Mini-sample Containers MC-1, 12-37ml capacity, Conair Corp. East Windsor, New Jersey). Twenty ml of Phosphate Butterfield's buffer (99.00ml Butterfield's Buffer R23601, Remel Inc. Lenexa, Kansas) were added to the blender cups and the samples were blended in a Waring laboratory Blender (Wareing LB, model LB10G, 240 V, Conair Corp. East Windsor, New Jersey) in order to improve recovery of the bacteria.

Enumeration for *C. perfringens* was performed by serial dilution by transferring 1ml of the blended solution into screw cap tubes with 9 ml of Butterfield's Phosphate-Buffered Dilution Water (BPB) when necessary depending upon the level of dilution to be plated.

Spread plating was done on Tryptose Sulfite Cycloserine Agar plates (TSC) (EMD Chemicals TSC Agar Base, 1.11972.0500, Darmstadt, Germany, Gibbstown, New Jersey) supplemented with *C. perfringens* Selective Supplement (EMD Chemicals Inc. EM1.00888.0001 Darmstadt, Germany, Gibbstown, New Jersey) and egg yolk (Egg Yolk suspension, R450290, 50.00% 100.00ml, Remel Inc. Lenexa, Kansas).

After the inoculum had slightly dried, the plates were overlaid with approximately 10 ml egg yolk free TSC agar. The plates were allowed to solidify before placing them lid side up, in a non-vented, O-ring gasketed, polycarbonate anaerobic jar closed with a clamp and thumb screw lid (BBL Gaspak 150 Large Anaerobic System 260628, Sparks Maryland) with three anaerobic environment generation sachets (BD Diagnostic Systems, GasPak EZ anaerobic Container System 260678, Sparks, Maryland). The jar was incubated for 24 h at 35° to 37°C. The protocols followed were as directed by the USDA/FSIS Microbiology Laboratory Guidebook 3rd Edition/1998 (38).

Three experiments were performed (4, 5 and 6 hours) for each of the NaCl and phosphate combinations (6 treatments with different combinations of NaCl and phosphate and one control treatment without NaCl or phosphate) in two different runs. This represents 84 controls and 126 replications (210 cores) for each of the inoculum tested. The total amount of samples was 168 controls and 252 replicates (420 cores).

## **Results and Discussion**

### **Statistical Analysis**

Analyses of variance ANOVA were performed using SAS statistical software (version 9.1, SAS Institute, Cary, North Carolina) and a 95% significance level ( $P < 0.05$ ) was used to determine significance.

### **Control Analysis**

The controls for all the different treatments at different cooling times were analyzed using ANOVA. The null hypothesis “there is no significance difference between the controls among cooling times” was rejected with a p value  $< 0.001$  ( $p < 0.05$ ). In conclusion there was a statistically significant difference between controls at different cooling times; even though there was not difference in cooling time since these controls were plated immediately after inoculation with the three strain spore cocktail. The sum of squares was relatively small (Sum of Squares = 3.32) which indicates a small variation between all the observations among the cooling times; this is an indicator of the presence of hidden errors not considered in the experiment. The human error, the instrument and the method variation could be those hidden errors. This result was expected and is the main reason why it is necessary to have controls each experiment for each individual cooling time.

In other hand, there was no statistically significant difference between controls among treatments. This also was expected since there was not enough time for the treatments to affect the bacterial growth.

## Statistical Analysis of Treatments

Some *C. perfringens* strains can grow in products up to 8% NaCl (**Table 1-1**). The range used in this research (1%, 1.5%, 2%) are included within the optimal growth range for this pathogen and could be the reason why there is not an evident statistical effect in the growth of bacteria among different NaCl concentrations. However, for an inoculum of  $7.48 \log_{10}$ , when the effect of NaCl is analyzed among all the cooling times, the lowest average of bacteria population was obtained at 1.5% NaCl using SAPP ( $7.12 \log_{10}$ ). In the case of using an inoculum of  $3.48 \log_{10}$ , the lowest average of total bacterial count was observed at 2% NaCl using as well SAPP ( $3.35 \log_{10}$ ). The inhibitory effect due to the interaction between NaCl and SAPP was clearly observed at the lowest inoculum; in this case, the most inhibitory combination of NaCl and phosphate was 2% NaCl and 0.5% SAPP with an average growth of  $1.32 \log_{10}$  among cooling times. The results agreed with the data obtained by Zaika (75) where it was found that *C. perfringens* grew more or equal to  $3.0 \log_{10}$  during cooling times of 15 h,  $5.0 \log_{10}$  for 18 h and  $5.0 \log_{10}$  for 21 h in beef with  $<2\%$  NaCl. In the same study, less than one  $\log_{10}$  growth of *C. perfringens* was obtained for 15 h of cooling time for cooked ham which normally contains  $>2\%$  NaCl. (75).

For turkey whole muscle without NaCl and phosphate (T0, Control treatment) the average of growth was more than one  $\log_{10}$  ( $2.83 \log_{10}$  and  $1.63 \log_{10}$  for  $3.48 \log_{10}$  and  $7.48 \log_{10}$  inoculum size respectively). In contrast, Steele et.al reported not more than one  $\log_{10}$  of *C. perfringens* was obtained during a 6hr cooling time (no data available of the exact log growth). . While performing a regression analysis the author stated that the turkey breast

could be cooled down from 120 °F(48.9°C)to 55°F (12.8°C) in 9.6h without violating the Appendix B cooling times however they recommended a cooling period  $\leq 8.9$  h (58).

It is known that the use of a whole muscle product with a relatively small inoculum (one  $\log_{10}$  CFU/g) could be the factor that influences survival times of spores due to the possible presence of “skips”. The recommendation therefore is always to accept the longest survival time in thermal death studies. Therefore a larger number of initial spores it is desired to minimize the presence of “skips” (32, 45).

Kalinowski et al.(35) tested the impact of the inoculum in the growth level of *C. perfringens* during three different experiments in uncured raw turkey emulsion. Experiment 1 and 2 were inoculated with 100 spores per gram of turkey meat ( $2.0 \log_{10}$ ). During 6 h of cooling time from 48.9 to 12.8°C, 2.44 and 2.25  $\log_{10}$  growth was observed, respectively. The third experiment was done with an inoculum of 10 spores per gram (one  $\log_{10}$ ); in this case only 0.83 $\log_{10}$  growth was observed, where the effect of the size of the initial population was evident. This third experiment is unrealistic according to the USDA the standard of  $10^4$  organism/g of *C. perfringens* defined for a worst-case scenario (63) (USDA, 1999). There is also no consideration in Kalinowski research about any issue during recovery. With that amount of organism (10 spores per gram) it is very difficult to have good recovery due to the loss of approximately one  $\log_{10}$  obtained in our research.

In our study the use of two different levels of inoculum did not seem to have an effect in the trend of the results but in the level of growth. The use of a large inoculums ( $7.48 \log_{10}$ ) showed lower growth than when a lower quantity of spores was used ( $3.48 \log_{10}$ ).In either case, *C. perfringens* grew more than one  $\log_{10}$  for both inocula and in all the treatments for 5

and 6 hours of cooling time. There is only a certain amount of bacterial growth that can be supported in a food matrix depending upon the nutrients available. This could be a reason why lower growth was observed at a higher inoculum. Having a small amount of initial population of bacteria could leave more nutrients available for more bacteria to grow reducing the effect of bacterial competition in a saturated system.

The use of cores of whole muscle and injecting the inoculum in the center of them seemed to have an impact in the recovery of cells. The justification use whole muscle was to assure simulation of the actual product and to obtain a better anaerobic environment for the bacteria. Approximately one  $\log_{10}$  was lost during the recovery of the bacteria from the cores. With an inoculum of  $7.48 \log_{10}$  controls had an average of  $6.36 \log_{10}$ . The same situation was observed for an inoculum of  $3.48 \log_{10}$  where controls had an average of  $2.32 \log_{10}$ . This loss in recovery has not being commented on by other researchers.

At a cooling time of 4h and an inoculum of  $3.48 \log_{10}$ , none of the treatments grew more than one  $\log_{10}$ ; and growth ranged from  $0.35 \log_{10}$  (T3) to  $0.95 \log_{10}$  (T4). There was not however statistical difference between treatments with an inoculum of  $3.48 \log_{10}$ . At an inoculum of  $7.48 \log_{10}$ , 4h of cooling time allowed *C. perfringens* to grow more than one  $\log_{10}$  in treatments T1 ( $1.05 \log_{10}$ ) and T4 ( $1.13 \log_{10}$ ) but T1 was not statistically different than the other treatments. The only treatments that showed statistically less growth were T4 and T2 with a p-value =0.03(p<0.05). When making comparisons between T1 and T2 (p=0.057), and T4 and T6 (p = 0.08), there was not statistical difference between them (p>0.05); however, their p values are slightly greater than 0.05 however with more replications a statistical difference may have been observed. Treatments T0 (control without

any phosphate or NaCl), T2, T3, T5 and T6 had a less than one  $\log_{10}$  growth compared to the control at 4 hours (**Fig. 3.1**).

At 5 hours of cooling time and an inoculum of  $3.48 \log_{10}$ , treatment T0 was statistically different than T1, T4, T5, T6 ( $p < 0.05$ ) but not statistically different from T2 and T3 ( $p > 0.05$ ). Treatment T0, T2 and T3 allowed the least amount of growth ( $1.22, 1.23, 1.43 \log_{10}$ , respectively). There was no evidence of an effect on the growth of this bacterium when 0.5% SAPP was used with 1.5% and 2% of NaCl compared to the control. A small effect of the phosphate was observed since all the treatments with SAPP had lower growth compared to those treatments with Brifisol® for both levels of inoculum. Using an inoculum of  $7.48 \log_{10}$ , there was not a statistically significant difference between all the treatments ( $p > 0.05$ ). Treatment 2 has the lowest growth ( $1.18 \log_{10}$ ) compared to the highest levels of growth obtained in T4 and T5 with  $1.63 \log_{10}$  and  $1.64 \log_{10}$  respectively (**Fig. 3.2**).

At 6 hours cooling time and an inoculum of  $3.48 \log_{10}$ , all of the treatments grew more than one  $\log_{10}$  with ranges from  $2.18 \log_{10}$  (T3) to  $3.18 \log_{10}$  (T6). Treatment 3 was statistically different than treatments T0, T1, T2, T5 and T6 ( $p < 0.05$ ). On other hand, with an inoculum of  $7.48 \log_{10}$ , the treatments with the least growth were T0 ( $1.63 \log_{10}$ ) and T3 ( $1.70 \log_{10}$ ) and the treatments with most growth were T5 and T6 ( $3.17 \log_{10}$  and  $3.18 \log_{10}$  respectively) however here was not statistically significant difference between the treatments ( $p > 0.05$ ). (**Fig 3.3**)

The combination of SAPP with NaCl has greater effect in minimizing the growth of *C. perfringens* in turkey meat. Similar results were obtained by Singh et al. where the use of SAPP allowed an increase of just  $0.40 \log_{10}$  in fresh pork meat during a cooling time of 6.5h

(56). The reduction of pH due to the use of SAPP (pH 5.76, 5.84, 5.87 for 1%, 1.5% and 2% of NaCl respectively) versus Brifisol® (pH 6.21, 6.26, 6.40 for 1%, 1.5% and 2% of NaCl respectively) had an antagonistic impact on the growth of *C. perfringens*. Thippareddi et al. confirmed the this effect on meat with a lower pH; the population of *C. perfringens* in beef and pork after a cooling time of 18h (from 54.4 to 7.2 °C) increased 1.51 and 3.70 log<sub>10</sub> respectively. The change in the amount of growth indicated a possible beneficial influence of a lower pH in beef versus pork (5.62 and 6.11 respectively) as well as the impact of intrinsic differences between these two animal species (62).

In cooked whole turkey muscle it is necessary to cool down the product in less than 5 hours. In this particular case, it would be important to evaluate the use of antimicrobials in order to achieve inhibition of this pathogen during the stabilization process. Products like Kena, a commercial blend of 75% STPP and 25% TSPP has shown antimicrobial properties on turkey steaks when they are submerged in a 6% solution for 6h and stored at 5°C and could be used in further analysis however Appendix B remains the standard with a limit of 6h of cooling time (61).

This study was done using a linear cooling curve instead of an exponential cooling curve as used by Zaika, Steele and Write, and Juneja et al. The use of a linear cooling curve produced a more realistic process of cooling and a worst case scenario of what could happen in the turkey industry; this could be the reason why in the aforementioned research showed less than one log<sub>10</sub> growth of *C. perfringens* during 6h of cooling time. (32, 58, 75).

This research confirms the importance of evaluating how *C. perfringens* responds differently in various types of products. The pH and NaCl content has a significant

interaction and impact on growth at different cooling times. In future studies, additional variables could be taken into account such as differences in the type of muscle and fat content and how this could impact the pH of the meat and subsequently the germination and outgrowth of this microorganism.

It has been determined in this study and others that the amount of spores in the initial inoculum also impacts the level of growth of this pathogen. This information should be confirmed with further research on different types of meat and with different combinations of ingredients. The significant interaction among pH, NaCl and cooling times described how multiple parameters should be considered in the development of new products to assure safety. It is also important to validate if the cooling times described in the USDA/FSIS Appendix B remains adequate depending on the product and ingredients used by the industry

## TABLES AND FIGURES

### Tables

**Table 1-1.** Different factors that influence the growth of *Clostridium perfringens* vegetative cells. Adapted from “Microorganisms in foods 5. Characteristics of microbial pathogens” (50).

	Min	Optimum	MAX	Turkey meat values	Generation Time (min)
<b>Temperature (°C)</b>	12.00	43.00 – 47.00	50.00	--	7.10-10.00 (at optimum)
<b>pH</b>	5.50 – 5.80	6.00-7.20	8.00 – 9.00	~5.90	25.00
<b>NaCl (%)</b>		Up to 5.00% strain variation up to 8.00%		--	--
<b>Water Activity (a<sub>w</sub>)</b>	0.97	0.95 – 0.96	0.93	0.95	34.00-46.00

**Table 1-2.** Top five pathogens causing domestically acquired foodborne illnesses. (32, 58, 75)

Pathogen	Estimated annual number of illnesses	90% Credible Interval	%
Norovirus	5,461,731	3,227,078–8,309,480	58
<i>Salmonella</i> , nontyphoidal	1,027,561	644,786–1,679,667	11
<b><i>Clostridium perfringens</i></b>	<b>965,958</b>	<b>192,316–2,483,309</b>	<b>10</b>
<i>Campylobacter spp.</i>	845,024	337,031–1,611,083	9
<i>Staphylococcus aureus</i>	241,148	72,341–529,417	3
		Subtotal	91

**Table 2-1.** Moisture, NaCl, pH and phosphate content of sampled RTE whole muscle turkey breast.

pH	6.15-6.18
Moisture	72.94-78.08%
NaCl content	1.30 – 1.81%
Sodium Tripolyphosphate (STPP)	0.25-0.50% of total product weight (0.43% avg.)

**Table 2-2.** Bacteria population log and log change between replicates and controls. ( red indicates growth)

<i>Experiment</i>	<i>Sample</i>	<i>Average of CFU/g</i>	<i>Average of Log</i>	<i>Log change</i>
1	Control	8.90x10 <sup>2</sup>	2.95	
	Replicates	1.37x10 <sup>3</sup>	3.14	<u>0.19</u>
2	Control	1.73x10 <sup>3</sup>	3.24	
	Replicates	2.77x10 <sup>2</sup>	2.44	-0.80
3	Control	1.18x10 <sup>3</sup>	3.07	
	Replicates	9.37x10 <sup>2</sup>	2.97	-0.10
4	Control	4.48x10 <sup>3</sup>	3.65	
	Replicates	9.13x10 <sup>2</sup>	2.96	-0.69
5	Control	2.42x10 <sup>3</sup>	3.38	
	Replicates	7.07x10 <sup>2</sup>	2.85	-0.54
6	Control	2.00x10 <sup>2</sup>	2.30	
	Replicates	3.67x10 <sup>2</sup>	2.56	<u>0.27</u>

**Table 2-3.** Growth average, standard deviation and Standard error per run.

	<i>Run1</i>	<i>Run2</i>	<i>Run3</i>	<i>Run4</i>	<i>Run5</i>	<i>Run6</i>
Mean	0.12	-1.09	-0.18	-0.69	-0.68	0.12
Standard Error	0.19	0.39	0.20	0.04	0.25	0.27
Standard Deviation	0.32	0.67	0.35	0.07	0.44	0.47

**Table 3-1.** pH values of Sodium Acid Pyrophosphate (SAPP) and Brifisol® used to prepare the brine injected in the turkey breast in a wt/wt ratio.

	Name	Phosphate pH (1%)	% (wt/wt)	pH after cooking	Mean	STDEV	Standard error
SAPP	Sodium Acid Pyrophosphate	3.70-4.40	0.50	6.17-6.38	6.26	0.08	0.03
Brifisol®	Blend of Sodium Phosphates	11.00-12.00	0.50	6.54-6.76	6.60	0.08	0.03

**Table 3-2.** Water, NaCl and Phosphate used to prepare the brine injected in the turkey breast in a wt/wt ratio.

<i>Treatment</i>	<i>Water</i>	<i>NaCl</i>	<i>Phosphate 0.5%</i>
T0	0%	0%	---
T1	15%	1.00%	SAPP
T2	15%	1.50%	SAPP
T3	15%	2.00%	SAPP
T4	15%	1.00%	BRIFISOL
T5	15%	1.50%	BRIFISOL
T6	15%	2.00%	BRIFISOL

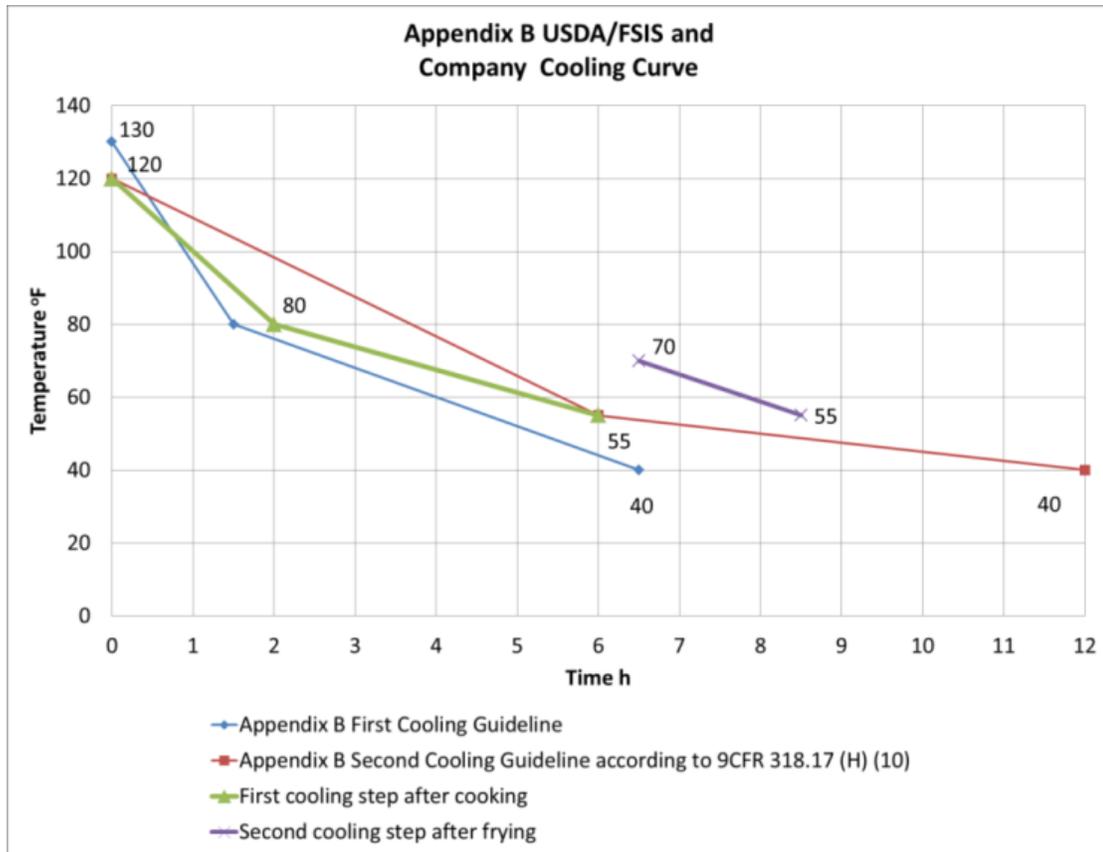
**Table 3-3.** Mean, standard error (SE) and standard deviation (SD) of the growth of *Clostridium perfringens* in cooked turkey breast for an inoculum of 3.48 log<sub>10</sub>.

<i>Treatment</i>	<i>Cooling times</i>								
	<i>4h</i>			<i>5h</i>			<i>6h</i>		
	<i>Mean</i>	<i>SE</i>	<i>SD</i>	<i>Mean</i>	<i>SE</i>	<i>SD</i>	<i>Mean</i>	<i>SE</i>	<i>SD</i>
<i>T0</i>	0.48	0.15	0.37	1.22	0.18	0.43	2.83	0.12	0.29
<i>T1</i>	0.75	0.09	0.23	2.10	0.09	0.22	3.02	0.10	0.24
<i>T2</i>	0.70	0.14	0.34	1.28	0.09	0.21	2.83	0.10	0.24
<i>T3</i>	0.35	0.12	0.28	1.43	0.29	0.71	2.18	0.22	0.53
<i>T4</i>	0.96	0.16	0.40	2.38	0.24	0.58	2.75	0.20	0.49
<i>T5</i>	0.68	0.09	0.21	1.96	0.12	0.28	3.17	0.29	0.71
<i>T6</i>	0.35	0.28	0.68	1.92	0.17	0.43	3.18	0.32	0.79

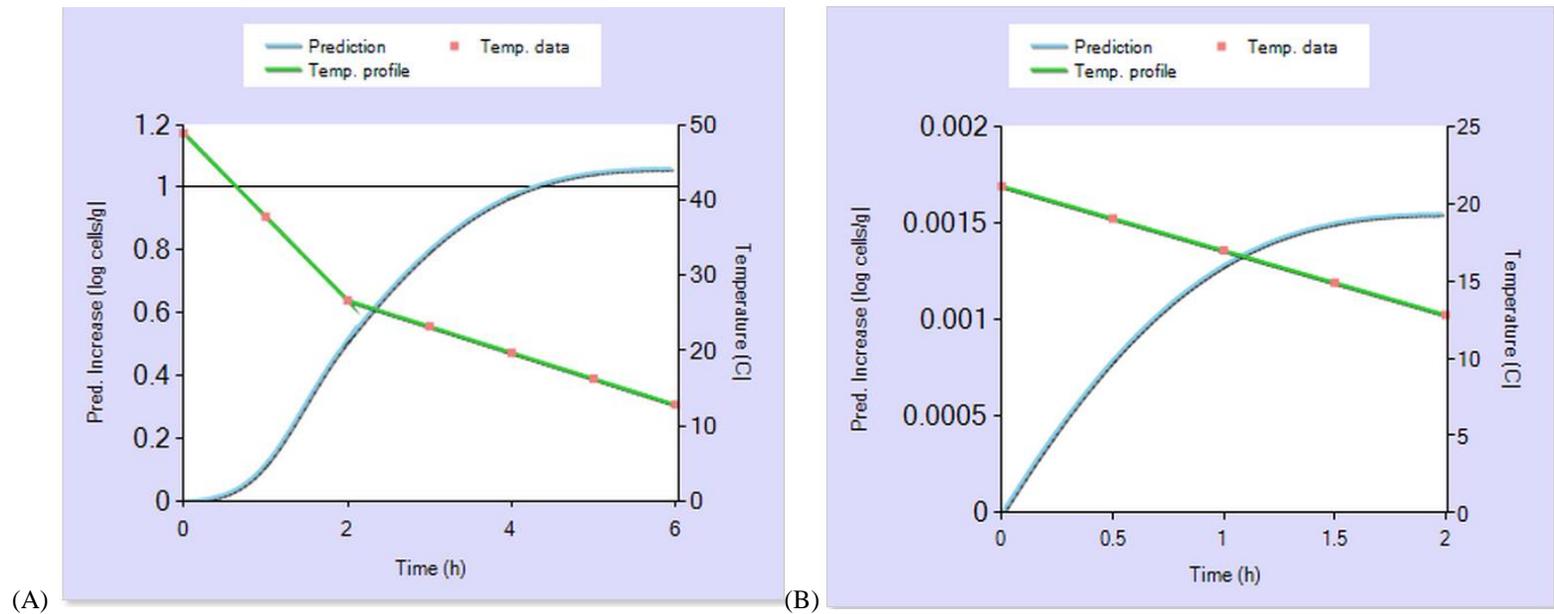
**Table 3-4.** Mean, standard error (SE) and standard deviation (SD) of the growth of *Clostridium perfringens* in cooked turkey breast for an inoculum of  $7.48\log_{10}$ .

<i>Treatment</i>	<i>Cooling times</i>								
	<i>4h</i>			<i>5h</i>			<i>6h</i>		
	<i>Mean</i>	<i>SE</i>	<i>SD</i>	<i>Mean</i>	<i>SE</i>	<i>SD</i>	<i>Mean</i>	<i>SE</i>	<i>SD</i>
<i>T0</i>	0.75	0.14	0.35	1.25	0.18	0.45	1.60	0.09	0.22
<i>T1</i>	1.05	0.09	0.22	1.32	0.09	0.21	1.78	0.08	0.19
<i>T2</i>	0.41	0.14	0.35	1.18	0.04	0.10	1.87	0.10	0.24
<i>T3</i>	0.90	0.14	0.35	1.29	0.19	0.46	1.71	0.05	0.11
<i>T4</i>	1.13	0.15	0.36	1.63	0.12	0.30	1.96	0.05	0.12
<i>T5</i>	0.95	0.03	0.08	1.64	0.14	0.35	2.11	0.09	0.23
<i>T6</i>	0.54	0.11	0.26	1.45	0.10	0.26	1.98	0.12	0.29

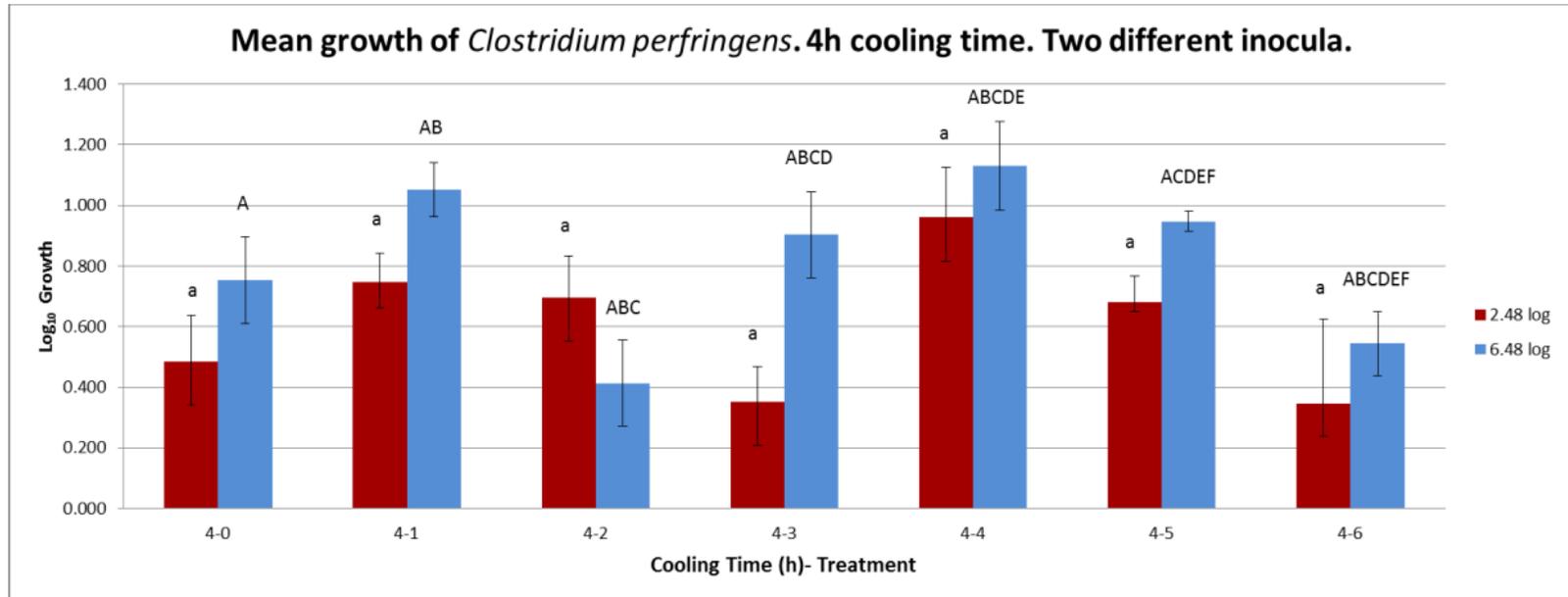
# Figures



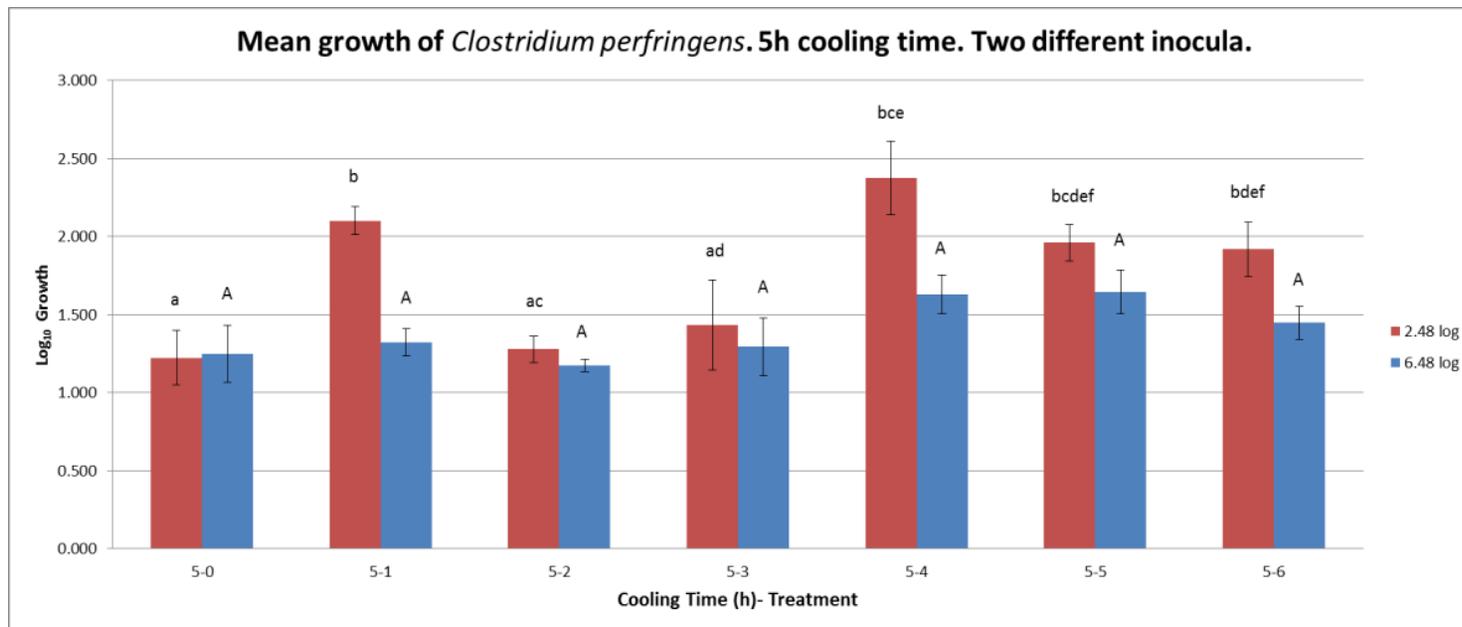
**Figure 2-1.** Graphs of the cooling curves required by the USDA in Appendix B and the experimental curve. Within Appendix B the first cooling guideline is the most rigorous cooling curve (blue diamonds). The second Appendix B cooling guideline is based on previous data and according to “Requirements for the production of cooked beef, roast beef, and cooked corned beef” 9 CFR 318.17(h) (10) represented (red squares). For the experimental curves the first cooling stage after cooking (green triangles); and the second cooling stage after frying (purple X).



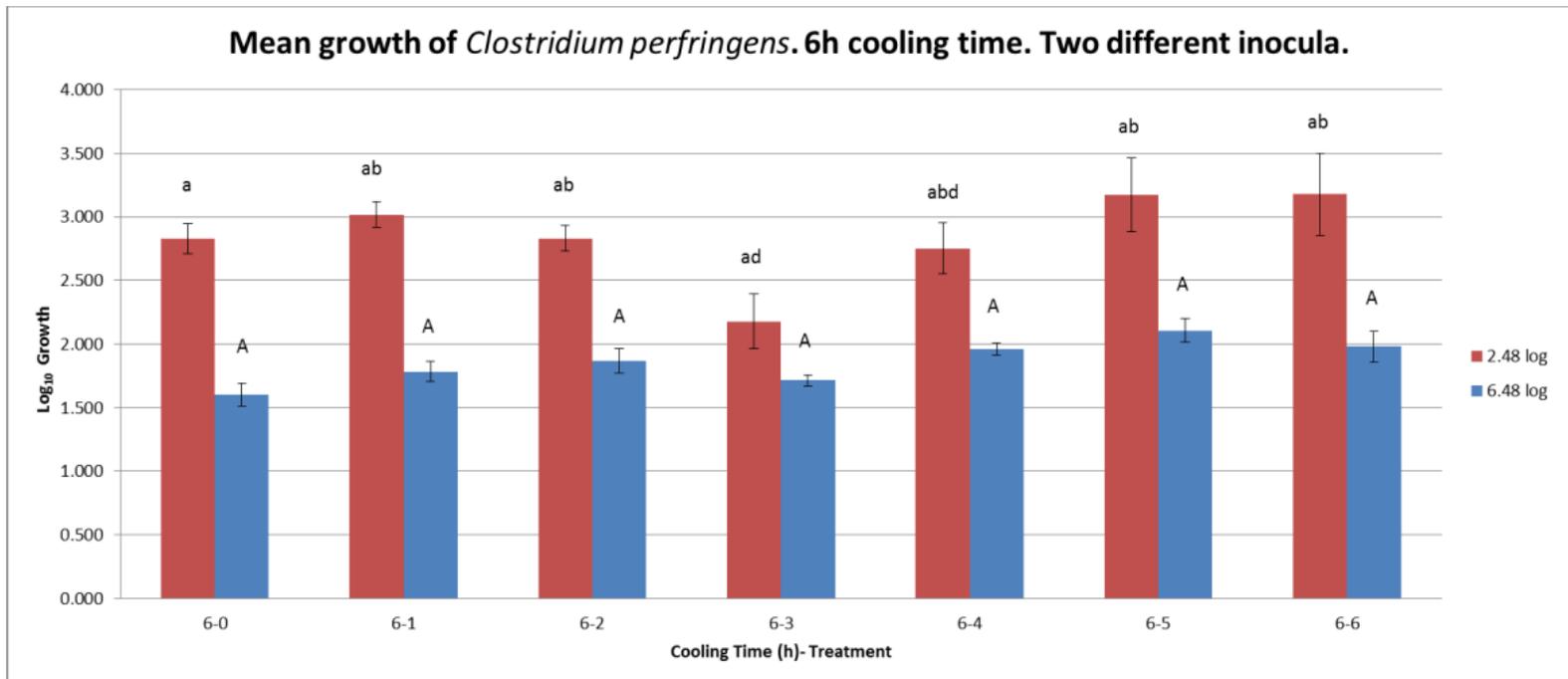
**Figure 2-2.** *Clostridium perfringens* growth during the first stage of the cooling process using Combase© Perfringens Predictor simulation tool. pH of 6.15 and 1.3% NaCl for a not cured product (A) step 1, from 48.89oC (120°F) to 26.67°C (80°F) in 120 min. (B) step 2, from 21.11°C (70°F) to 12.8°C (55°F) in 120 min.(20)



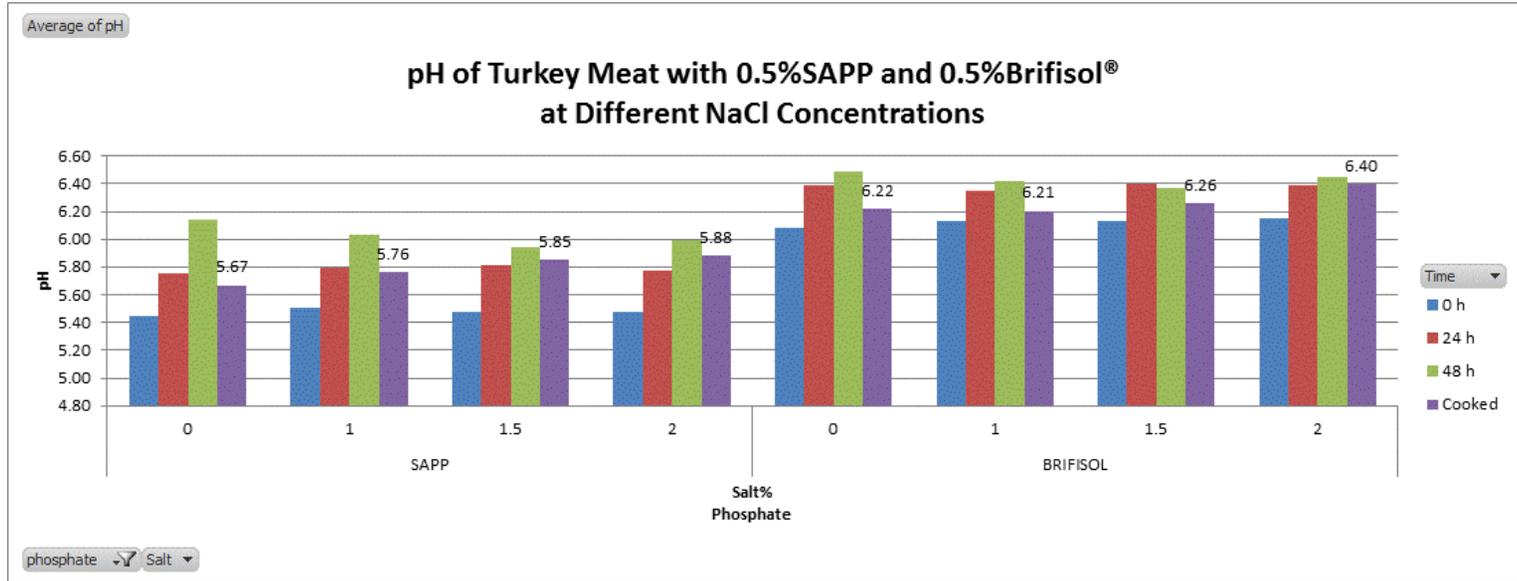
**Figure 3-1.** *Clostridium perfringens* log<sub>10</sub> growth from in 4 h cooling time from 120°F (48.9°C) to 50°F (12.8°C) for two different inocula. Columns that have different letters are significantly different (p<0.05)



**Figure 3-2.** *Clostridium perfringens*  $\log_{10}$  growth from in 5 h cooling time from 120°F (48.9°C) to 50°F (12.8°C) for two different inocula. Columns that have different letters are significantly different (p<0.05)



**Figure 3-3.** *Clostridium perfringens* log<sub>10</sub> growth from in 6 h cooling time from 120°F (48.9°C) to 50°F (12.8°C) for two different inocula. Columns that have different letters are significantly different (p<0.05)



**Figure 3-4.** Second pH study of turkey meat with 0.5% SAPP and 0.5% Brifisol® and 0%, 1%, 1.5% and 2% NaCl (% wt/wt) after 0, 24 and 48 h of storage and after cooking.

## REFERENCES

1. Akhtar, S., D. Paredes-Sabja, and M. R. Sarker. 2008. Inhibitory effects of polyphosphates on *Clostridium perfringens* growth, sporulation and spore outgrowth. *Journal of Food Microbiology* 25:802-808.
2. Al-Khaldi, S. 2010. Bad Bug Book - Foodborne Pathogenic Microorganisms and Natural Toxins Handbook, *In: Anonymous*.
3. Bhunia, A. K. 2008. *Clostridium botulinum* and *Clostridium perfringens*, p. 149-164. *In: Anonymous Foodborne Microbial Pathogens*. Springer, New York.
4. Birkhead, G., R. L. Vogt, E. M. Heun, J. T. Snyder, and B. A. McClane. 1988. Characterization of an Outbreak of *Clostridium perfringens* Food Poisoning by Quantitative Fecal Culture and Fecal Enterotoxin Measurement. *Journal of Clinical Microbiology* Vol. 26, No. 3:471-474.
5. Borch, E., M. L. Kant-Muemans, and Y. Blixt. 1996. Bacterial spoilage of meat products and cured meat. *International Journal of Food Microbiology* 33:103-120.
6. Bradshaw, J. G., G. N. Stelma, V. I. Jones, J. T. Peeler, J. C. Wimsatt, J. J. Corwin, and R. M. Twedt. 1982. Thermal inactivation of *Clostridium perfringens* Enterotoxin in Buffer and in Chicken Gravy. *Journal of Food Science* 47:914-916.
7. Byrne, B., G. Dunne, and D. J. Bolton. 2006. Thermal inactivation of *Bacillus cereus* and *Clostridium perfringens* vegetative cells and spores in pork luncheon roll. *Journal of Food Microbiology* 23:803-806.
8. Center for Disease Control and Prevention, CDC. 2011. Surveillance for Foodborne Disease Outbreaks. *Morbidity and Mortality Weekly Report (MMWR)* 60, No 35:1197-1202.
9. Center for Disease Control and Prevention, CDC. 2010. Surveillance for Foodborne Disease Outbreaks. *Morbidity and Mortality Weekly Report (MMWR)* 59, No 31:973-1008.
10. Cough, B. J., J. A. Alford. 1965. Effect of Curing Agents on the Growth and Survival of Food-Poisoning Strains of *Clostridium perfringens*. *Journal of Food Science* 3:1025-1028.

11. Craven, S. E., L. C. Blankenship. 1985. Activation and Injury of *Clostridium perfringens* Spores by Alcohols. *Applied and Environmental Microbiology* 50, No. 2:249-256.
12. Department of Agriculture, Food Safety and Inspection Services. USDA/FSIS. 2001. Performance Standards for the Production of Processed Meat and Poultry Product. *9CFR* 39:301,303,317-320,325,331,381,417 and-430.
13. Doyle, M. P. (ed). 1989. Food borne bacterial pathogens. Marcel Dekker, Inc., New York, NY.
14. Duncan, C. L., D. H. Strong. 1968. Improved Medium for Sporulation of *Clostridium perfringens*. *Applied Microbiology* 16, No1:82-89.
15. Duncan, C. L., D. H. Strong, and M. Sebald. 1972. Sporulation and Enterotoxin Production by Mutants of *Clostridium perfringens*. *Journal of Bacteriology* 110, No.1:378-391.
16. Gary, G. A., T. E. Cloete, and A. Von Holy. 1991. Quantification of microbial populations associated with the manufacture of vacuum-packaged, smoked Vienna sausages. *International Journal of Food Microbiology* 13:239-248.
17. Goldner, S. B., M. Solberg, S. Jones, and L. S. Post. 1986. Enterotoxin Synthesis by Nonsporulating Cultures of *Clostridium perfringens*. *Applied and Environmental Microbiology* 52:407-412.
18. Hickey, C. S., M. G. Johnson. 1981. Effects of pH Shifts, Bile Salts, and Glucose on Sporulation of *Clostridium perfringens* NCTC 8798. *Applied and Environmental Microbiology* 41, No. 1:124-129.
19. Inklaar, P. A. 1967. Interaction between polyphosphates and meat. *Journal of Food Science* 32:525-526.
20. Institute of Food Research, IFR. 2012. Combase. Perfringens Predictor. Available at: [http://modelling.combase.cc/Perfringens\\_Predictor.aspx](http://modelling.combase.cc/Perfringens_Predictor.aspx). Accessed July 24 2012. .
21. Johnson, P. G., J. A. Bowers. 1974. Freshly cooked and reheated turkey breast muscle. 1. Cooking losses and sensory evaluation of muscle and expressed juice. *Journal of Poultry Science* 53:343-348.
22. Johnson, P. G., J. A. Bowers. 1974. Freshly cooked and reheated turkey breast muscle. 2. Cooking losses and sensory evaluation of muscle and expressed juice. *Journal of Poultry Science* 53:349-354.

23. Juneja, V. K., H. Thippareddi. 2004. Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. *International Journal of Food Microbiology* 93:155-163.
24. Juneja, V. K., J. S. Novak. 2001. Heat resistance of *Clostridium perfringens* vegetative cells as affected by prior heat shock. *Journal of Food Safety* 21:127-139.
25. Juneja, V. K., B. S. Marmer. 1998. Thermal inactivation of *Clostridium perfringens* vegetative cells in ground beef and turkey as affected by sodium pyrophosphate. *Journal of Food Microbiology* 15:281-287.
26. Juneja, V. K., B. S. Marmer. 1996. Growth of *Clostridium perfringens* from spore inocula in sous-vide turkey products. *International Journal of Food Microbiology* 32:115-123.
27. Juneja, V. K., W. M. Majka. 1995. Outgrowth of *Clostridium perfringens* spores in COOK-IN-BAG beef products. *Journal of Food Safety* 15:21-34.
28. Juneja, V. K., O. P. Snyder, and M. Cygnarowicz-Provost. 1994. Influence of cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ground beef. *Journal of Food Protection* 57, No 12:1063-1067.
29. Juneja, V. K., J. E. Call, B. S. Marmer, and A. J. Miller. 1994. The effect of temperature abuse on *Clostridium perfringens* in cooked turkey stored under air and vacuum. *Journal of Food Microbiology* 11:187-193.
30. Juneja, V. K., A. C. S. Porto-Fett, K. Gartner, L. Tufft, and J. B. Lucahnsky. 2010. Potential for growth of *Clostridium perfringens* from spores in pork scrapple during cooling. *Foodborne pathogens and disease* 7, No 2:153-157.
31. Juneja, V. K., A. C. S. Porto-Fett, K. Gartner, L. Tufft, and J. B. and Luchansky. 2009. The Potential for Growth of *Clostridium perfringens* from Spores in Pork Scrapple During Cooling. .
32. Juneja, V. K., O. P. Snyder, and M. Cygnarowicz-Provost. 1994. Influence of Cooling Rate on Outgrowth of *Clostridium perfringens* Spores in Cooked Ground Beef. *Journal of Food Protection* 57:1063-1067.
33. Juneja, V. K., J. E. Call, and A. J. Miller. 1993. Evaluation of methylxanthines and related compounds to enhance *Clostridium perfringens* sporulation using a modified Duncan and Strong medium. *Journal of Rapid Methods and Automation in Microbiology* 203-218.

34. Juneja, V. K., J. S. Novak, B. S. Eblen, and B. A. McClane. 2001. Heat resistance of *Clostridium perfringens* vegetative cells as affected by prior heat shock. *Journal of Food Safety* 21:127-139.
35. Kalinowski, R. M., R. B. Tompkin, P. W. Bodnaruk, and J. Pruett W.P. 2003. Impact of cooking, coling and subsequent refrigeration on the growth or survival of *Clostridium perfringens* in cooked meat and poultry products. *Journal of Food Protection* 66, No 7:1227-1232.
36. Le Mark, Y., J. Plowman, C. F. Aldus, M. Munoz-Cuevas, J. Baranyi, and M. W. Peck. 2008. Modelling the growth of *Clostridium perfringens* during the cooling of bulk meat. *International Journal of Food Microbiology* 128:41-50.
37. McClane, B. A. 2007. *Clostridium perfringens*, p. 423-444. In: M. P. Doyle and L. R. Beuchat (eds), *Food Microbiology. Fundamentals and Frontiers*, vol. 3rd Edition. ASM Press, Washington, D.C.
38. McNamara, A. M., C. P. Lattuada. 1998. Chapter 13: Examination of Meat and Poultry Products for *Clostridium perfringens*, p. 13-1-13-8. In: B. P. Dey and C. P. Lattuada (eds), *Microbiology Laboratory Guidebook*, vol. 1 & 2. USDA/FSIS Office of Public Health and Science. Microbioly Division, .
39. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases* 5, No 5:607-625.
40. Meyer, J. D., J. G. Cerveny, and J. B. Lucahnsky. 2003. Inhibition of nonproteolytic, psychrotrophic Clostridia and anaerobic sporeformers by sodium diacetate and sodium lactate in cook-in-bag turkey breast. *Journal of Food Protection* 66, No 8:1474-1478.
41. Molins, R. A. 1991. Phophates in Food. CRC Press, Inc., Florida USA.
42. Molins, R. A., A. A. Kraft, and J. A. Marcy. 1987. Extension of the Shelf-Life of Fresh Ground Pork with Polyphosphates. *Journal of Food Science* 52 No 2:513-514.
43. Molins, R. A., A. A. Kraft, and D. G. Olson. 1985. Effect of Phosphates on Bacterial Growth in Refrigerated Uncooked Bratwurst. *Journal of Food Science* 50:531-532.
44. Molins, R. A., A. A. KRAFT, H. W. Walker, and D. G. OLSON. 1985. Effect of Poly- and Pyrophosphates on the Natural Bacterial Flora and Inoculated *Clostridium sporogenes* PA 3679 in Cooked Vacuum packaged Bratwurst. *Journal of Food Science* 50:876-880.

45. National Canners Association Research Laboratories. 1968. Chapter 7. Thermal Death Time, p. 198. *In*: Anonymous. Laboratory Manual for Food Canners and Processors, vol. 1. The AVI Publishing Company, INC., Westport, Connecticut.
46. National Turkey Federation, N. 2010 2012. Turkey Statistics. Available at: <http://www.eatturkey.com/consumer/stats/stats.html>. Accessed July 12 2012. .
47. Nelson, K. A., F. F. Busta, J. N. Sofas, and M. K. Wagner. 1983. Effect of polyphosphates in combination with nitrite·sorbate or sorbate on *Clostridium botulinum* growth and toxin production in chicken frankfurter emulsions. *Journal of Food Protection* 46:846-855.
48. Olsen, S. J., L. C. MacKinnon, J. S. Goulding, N. H. Bean, and L. Slutsker. 2000. Surveillance for Foodborne-Disease Outbreaks — United States, 1993–1997. *Morbidity and Mortality Weekly Report (MMWR)* 44, No SS-1:1-51.
49. Paredes-Sabja, D., J. A. Torres, P. Setlow, and M. R. Sarker. 2007. *Clostridium perfringens* spore Germination: Characterization of Germinants and Their Receptors. *Journal of Bacteriology* 190, No. 4:1190-1201.
50. Roberts, T. A. (A. C. Baird-Parker, and R. B. Tompkin (eds). 1998. Micro-organisms in Foods Characteristics of Microbial Pathogens. Blackie Academic & Professional, Great Britain.
51. Sanchez-Plata, M. X., A. Amezcuita, E. Blankenship, D. E. Burson, V. K. Juneja, and H. Thippareddi. 2005. Predictive model for *Clostridium perfringens* growth in roast beef during cooling and inhibition of spore germination and outgrowth by organic acid salts. *Journal of Food Protection* 68, No 12:2594-2605.
52. Santé, V., X. Fernandez. 2000. The measurement of pH in raw and frozen turkey *Pectoralis superficialis* muscle. *Meat Science* 55:503-506.
53. Sarker, M. R., R. P. Shivers, S. G. Sparks, V. J. Juneja, and B. A. McClane. 2000. Comparative Experiments To Examine the Effects of Heating on Vegetative Cells and Spores of *Clostridium perfringens* Isolates Carrying Plasmid Genes versus Chromosomal Enterotoxin Genes. *Applied and Environmental Microbiology* 66, No 8:3234-3240.
54. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. F. Tauxe, M. Widdowson, and S. L. Roy. 2011. Food borne illness acquired in the United States - Major pathogens. *Emerging Infectious Disease* 17, No 1:7-15.

55. Schaeffer, P. 1969. Sporulation and the Production of Antibiotics, Exoenzymes, and Exotoxins. *Bacteriological Reviews* 33, No. 1:48-71.
56. Singh, A., N. R. Korasapati, V. K. Juneja, and H. Thippareddi. 2010. Effect of Phosphate and Meat (Pork) types on the Germination and Outgrowth of *Clostridium perfringens* Spores during Abusive Chilling. *Journal of Food Protection* 73, No 5:879-887.
57. Smith, A., G., P. D. Ellner. 1956. Cytological observations on the sporulation process of *Clostridium perfringens*. *Journal of Bacteriology* 73 No 1:1-7.
58. Steele, F. M., K. H. Wright. 2001. Cooling Rate Effect on Outgrowth of *Clostridium perfringens* in Cooked, Ready-to-Eat Turkey Breast Roasts. *Journal of Poultry Science* 80:813-816.
59. Tabatabai, L. B., H. W. Walker. 1970. Oxidation-Reduction Potential and Growth of *Clostridium perfringens* and *Pseudomonas fuorescens*. *Applied Microbiology* 20, No. 3:441-446.
60. Taormina, P. J., G. W. Bartholomew, and W. J. Dorsa. 2003. Incidence of *Clostridium perfringens* in commercially produced cured raw meat product mixtures and behavior in cooked products during chilling and refrigerated storage. *Journal of Food Protection* 66, No 1:72-81.
61. Taylor, M. H., L. T. Smith, and J. D. Mitchell. 1965. The effect of packaging materials and a tripolyphosphate (Kena) on the shelf life of turkey steaks. *Journal of Poultry Science* 44, No 1:297-298.
62. Thippareddi, H., V. K. Juneja, R. K. Phebus, J. L. Marsden, and C. L. Kastner. 2003. Control of *Clostridium perfringens* Germination and Outgrowth by Buffered Sodium Citrate during Chilling of Roast Beef and Injected pork. *Journal of Food Protection* 66 No 3:376-381.
63. USDA. 1999. Performance Standards for the Production of Certain Meat and Poultry Products. 9 *CFR* 301,317-318,320, 381.
64. USDA/ARS, FSC, and IFR. 2012 2012. Combase. Perfringens Predictor. Available at: <http://www.combase.cc/index.php/en/about-combase>. Accessed July, 29 2012. .
65. USDA/FAS. 2012. Production, Supply and Distribution Online PSD. Available at: <http://www.fas.usda.gov/psdonline/psdQuery.aspx>. Accessed July 12 2012. .

66. USDA/FSIS. 2012. Safe and suitable ingredients used in the production of meat, poultry, and egg products. *FSI Directive* Revision 12:7120.1.
67. USDA/FSIS. 2005. A Risk Assessment for *Clostridium perfringens* in Ready-to-Eat and Partially Cooked Meat and Poultry Products. .
68. USDA/FSIS. 1999. Performance Standards for the Production of Certain Meat and Poultry Products. Final Rule. *CFR* 9:CFR Parts 301, 317, 318, 320, and 381.
69. USDA/FSIS. 1998. Lethality and Stabilization performance Standards for Certain Meat and Poultry Products: Technical Paper. *Technical paper* 1-23.
70. USDA/FSIS Microbiology Division. 1996. Nationwide Raw Ground Turkey Microbiological Survey. *Baseline Data Reports* 1-9.
71. Velugoti, P. R., L. K. Bohra, V. K. Juneja, and H. Thippareddi. 2007. Inhibition of germination and outgrowth of *Clostridium perfringens* spores by lactic acid salts during cooling of injected turkey. *Journal of Food Protection* 70, No 4:923-929.
72. Wang, G., P. Zhang, D. Paredes-Sabja, C. Green, P. Setlow, M. R. Sarker, and Y. Q. Li. 2011. Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity. *Journal of Applied Microbiology* 111:1212-1223.
73. Watt Publishing, C. May 12, 2012 2012. US turkey production to grow in 2013 First-quarter 2012 numbers up over 2011 production. Available at: [http://www.wattagnet.com/US\\_turkey\\_production\\_to\\_grow\\_in\\_2013.html](http://www.wattagnet.com/US_turkey_production_to_grow_in_2013.html). Accessed July 20 2012. .
74. Willardsen, R. R., F. F. Busta, C. E. Allen, and L. B. Smith. 1978. Growth and survival of *Clostridium perfringens* during constantly rising temperatures. *Journal of Food Science* 43:470-475.
75. Zaika, L. L. 2003. Influence of NaCl content and cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ham and beef. *Journal of Food Protection* 66, No 9:1599-1603.

# APPENDICES

## Appendix A

### Statistical Analysis

Analyses of variance ANOVA were performed using SAS statistical software (version 9.1, SAS Institute, Cary, North Carolina) at 95% confidence level ( $P < 0.05$ ). The model developed for this analysis is shown in two different ways; the first one is the more general way considering the effects of treatments, cooling times, salt, phosphate and the error external to the model. However this model could be described in a more detailed way to show all the possible interactions between all the variables nested on each of them. In both equations the last term “ $E$ ” represents the error from factors outside the variables or the model; these could be error proper of the method used, the equipment or the person who was done the experiments.

$$y_{ijl} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + E_{ijl}$$

Where

$i = 1, 2, 3, 4, 5, 6, 7$  (Treatments T2, T4, T7, T12, T13, T17, T19)

$j = 1, 2, 3, 4$  (Cooling times 4, 5, 6, 9 h.)

$l = 1, 2, 3$  (for  $j = 1, 2, 4$ )

or

$l = 1, 2, 3, 4, 5, 6$  (for  $j = 3$ )

“ $l$ ” or the number of replicates could take values from 1 -3 for the cooling times 4, 5, 9 and from 1-6 for 6 h because this cooling time was performed twice as a way to verify the data.

And,

$\alpha_i$  = main effects of treatment

$\tau_j$  = main effects of cooling time

$(\alpha\tau)_{ij}$  = interaction effects

The model describes investigated effects of salt and phosphate effects among treatments with no NaCl and phosphate by creating an indicator variable for treatment control T0 and nesting factorial effects of salt and phosphate within this indicator variable. This enabled to recover the ANOVA for a complete crossed 3x2x4 factorial experiment with 3 levels of NaCl, 2 levels of phosphate and 4 cooling times (including 9h) while still including information about variability from the controls in a single model.

This model could be decomposed as an alternative parameterization according to its separates factorial effects:

$$y_{ijklm} = \mu + C_l + \alpha_{i(l)} + \beta_{ij(l)} + (\alpha\beta)_{ij(l)} + \tau_k + (C\tau)_{lk} + (\alpha\tau)_{ik(l)} + (\beta\tau)_{jk(l)} + (\alpha\beta\tau)_{ijk(l)} + E_{ijklm}$$

Where

$C_l$  = Control

$\alpha_{i(l)}$  = Interaction effects of Salt nested in the control treatment  $T_{19}$

$\beta_{ij(l)}$  = Interaction effects of phosphate nested in the control treatment  $T_{19}$

$(\alpha\beta)_{ij(l)}$  = Interaction effect of salt and phosphate interaction nested in  $T_{19}$

$\tau_k$  = main effects of cooling time

$(C\tau)_{lk}$  = Control cooling time interaction

$(\alpha\tau)_{ik(l)}$  = Salt and cooling time interaction nested in  $T_{19}$

$(\beta\tau)_{jk(l)}$  = phosphate cooling interaction nested in  $T_{19}$

$(\alpha\beta\tau)_{ijk(l)}$  = Salt, phosphate and cooling time interaction nested in  $T_{19}$

$E_{ijklm}$  = All the error of the variables that affects the system without being considering in the model.