SELECTION AND APPLICATION OF NATURAL ANTIMICROBIALS TO CONTROL *CLOSTRIDIUM PERFRINGENS* IN SOUS-VIDE CHICKEN BREASTS

by

CLAYTON J. SMITH

(Under the Direction of Francisco Diez-Gonzalez)

ABSTRACT

Current consumer preferences provide an opportunity to explore novel natural ingredients against *Clostridium perfringens* in sous vide foods. A formulation of vinegar and lemon juice concentrate and of vinegar and citrus extract were found effective at inhibiting *C. perfringens* growth in pure culture, reducing endospore and vegetative cell counts, and decreasing sporulation efficiency for three strains. The ingredients also inhibited *C. perfringens* growth in a sous vide chicken breast model exposed to temperature abuse conditions. Both formulations inhibited spore germination and outgrowth during a period of 16 days of relatively mild temperature abuse and 24 h of extreme abuse. The ingredients also upheld accordance to the USDA FSIS stabilization guidelines (<1 log CFU/g. outgrowth of *C. perfringens*) during defective chilling regimens of 12 and 18 h. This research identified two natural food preservatives capable of inhibiting *C. perfringens* in a vacuum-sealed chicken product while maintaining a clean label.

INDEX WORDS: *Clostridium perfringens*, Sous vide, Antimicrobial, Natural

SELECTION AND APPLICATION OF NATURAL ANTIMICROBIALS TO CONTROL *CLOSTRIDIUM PERFRINGENS* IN SOUS-VIDE CHICKEN BREASTS

by

CLAYTON J. SMITH

B.S., Auburn University, 2018

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

SELECTION AND APPLICATION OF NATURAL ANTIMICROBIALS TO CONTROL *CLOSTRIDIUM PERFRINGENS* IN SOUS-VIDE CHICKEN BREASTS

by

CLAYTON J. SMITH

Major Professor: Francisco Diez-Gonzalez
Committee: Hendrik C. den Bakker
Harshavardhan Thippareddi

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
May 2020

ACKNOWLEDGEMENTS

I would first like to thank my major professor, Dr. Francisco Diez-Gonzalez for all of his advice and mentorship throughout the past two years. I would not have been able accomplish everything I have without his constant support. I would also like to thank the other two members of my graduate committee, Dr. Hendrik C. den Bakker and Dr. Harshavardhan Thippareddi for their expert guidance along the way. I very much appreciate the constant support from the Diez lab as a whole from our undergraduate workers and from Meghan den Bakker and Magdalena Olszewska. The friendly, collaborative environment was extremely conducive to research and a joy to be a part of. I would also like to thank WTI Inc. for funding my graduate career through the Wolf Ludwig scholarship and for providing us with the ingredients used within this project. Lastly, I would like to thank my friends and family for always supporting me and providing me with the opportunity to be able to pursue my aspirations.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF TABLES viii

LIST OF FIGURES xi

CHAPTER

1 INTRODUCTION 1

2 LITERATURE REVIEW 3

Clostridium perfringens Information 3

Clostridium perfringens in the Poultry Industry 15

Methods of Controlling *Clostridium* perfringens 19

Sous vide Foods 25

3 MATERIALS AND METHODS 29

Clostridium perfringens Strains 29

Antimicrobial Treatments 29

Antimicrobial Screening 30

Endospore and Vegetative Cell Harvest 31

Mode of Action Experiments 32

Sporulation Efficiency Experiment 33

Chicken Sample Procurement and Storage 34

Clostridium perfringens Spore Cocktail Preparation 34

Chicken Sample Preparation 34

Sample Cooking Method 35

Abusive Storage Trials Sample Chilling and Storage……35
Abusive Storage Trials Enumeration……36
Defective Cooling Trials……36
Measurement of pH and Water Activity……37
Statistical Analyses……37
4 RESULTS……38
Antimicrobial Screening……38
Pure Culture pH Readings……40
Growth Curve Determination……42
Endospore Mode of Action……43
Vegetative Cell Mode of Action……44
Sporulation Efficiency……46
Chicken Samples pH and Water Activity Readings……47
Abusive Storage Experiment……48
Defective Chilling Trials……51
5 DISCUSSION……56
Antimicrobial Screening Comparison……56
pH and Water Activity Significance……59
Mode of Action Comparison……60
Sporulation Efficiency……60
Protection Against Temperature-Abuse Storage……61
Protection Against Defective Chilling……62
Abusive Storage Comparison……64

Defective Chilling Comparison 65

6 CONCLUSION 68

Conclusion 68

Future Research 69

REFERENCES 70

APPENDICES

A Antimicrobial Screening and Pure Culture pH Measurements 85

B Growth Curve, Endospore, and Vegetative Cell Experiment Data 93

C Chicken Matrix Experiment Data 101

LIST OF TABLES

Page

Table 1: *C. perfringens* toxin-based typing scheme based on production of six major toxins and commonly associated diseases....11-12

Table 2: List of natural antimicrobials used in this project....29-30

Table 3: pH values of FTG media after mixing with vinegar and citrus extract (VCE) ingredient at different concentrations....40-41

Table 4: pH values of FTG media after mixing with vinegar and cinnamaldehyde (VC) ingredient at different concentrations....41

Table 5: pH values of FTG media after mixing with vinegar and natural flavors (VNF) ingredient at different concentrations....41

Table 6: pH values of FTG after mixing with vinegar and lemon juice concentrate (VLJC) ingredient at different concentrations....41

Table 7: pH values of FTG after mixing with liquid vinegar (LV) ingredient at different concentrations....41

Table 8: pH values of FTG media after mixing with dried vinegar (DV) ingredient at different concentrations....42

Table 9: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the sporulation efficiency of *C. perfringens* strain NCTC 8239....46

Table 10: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the sporulation efficiency of *C. perfringens* strain ATCC 10240....47

Table 11: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the sporulation efficiency of *C. perfringens* strain NCTC 8238.....47

Table 12: pH and water activity measurements of control chicken samples (chicken and commercial seasoning blend) and chicken samples with different natural antimicrobial ingredient concentrations and seasoning blend. Treatments are vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC).....48

Table A1: *C. perfringens* strain 8239 antimicrobial screening data.....85-86

Table A2: *C. perfringens* strain 10240 antimicrobial screening data.....87-88

Table A3: *C. perfringens* strain 8238 antimicrobial screening data.....89-90

Table A4: pH measurements of natural antimicrobial ingredients in FTG media.....91-92

Table B1: *C. perfringens* growth curve data.....93-94

Table B2: Mode of action endospore experiment data.....94-95

Table B3: Mode of action vegetative cell experiment data.....96-97

Table B4: Sporulation efficiency experiment data.....98-100

Table C1: Chicken water activity measurements.....101

Table C2: Chicken pH measurements.....101

Table C3: Defective chilling cooling profiles.....102

Table C4: Extreme abusive storage (42 °C) experiment data.....103

Table C5: Abusive storage experiment positive control data.....104

Table C6: Abusive storage experiment VCE 1.0% data.....105

Table C7: Abusive storage experiment VCE 3.0% experiment……..106

Table C8: Abusive storage VLJC 1.5% experiment……..107

Table C9: Abusive storage VLJC 3.0% experiment……..108

Table C10: Defective chilling 6.5 h experiment data……..109

Table C11: Defective chilling 12 h experiment data……110

Table C12: Defective chilling 18 h experiment data……111

LIST OF FIGURES

Page

Figure 1: Bacterial endospore structure 5

Figure 2: The sporulation process 7

Figure 3: The germination and cellular outgrowth process 9

Figure 4: CpAL regulation of *C. perfringens* virulence factors by population density induced autoinducers 10

Figure 5: Unit operations for primary poultry processing and commonly associated sources of contamination 17

Figure 6: A simplified outline of the sous-vide process 26

Figure 7: Inhibition of *C. perfringens* strain NCTC 8239 at 42 °C from naturally derived food ingredients in FTG media 39

Figure 8: Inhibition of *C. perfringens* strain ATCC 10240 at 42 °C from naturally derived food ingredients in FTG media 39

Figure 9: Inhibition of *C. perfringens* strain NCTC 8238 at 42 °C from naturally derived food ingredients in FTG media 40

Figure 10: Growth curve of three *C. perfringens* strains incubated at 37 °C for 24 h in FTG media 42

Figure 11: Effect of treatments vinegar and lemon juice concentrate (VLJC) and vinegar and citrus extract (VCE) on viable endospore counts of three strains of *C. perfringens* 43-44

Figure 12: Effect of treatments vinegar and lemon juice concentrate (VLJC) and vinegar and citrus extract (VCE) on vegetative cell counts of three strains of *C. perfringens*……45

Figure 13: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 4 °C for 16 days……49

Figure 14: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 12 °C for 16 days……50

Figure 15: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 16 °C for 16 days……50

Figure 16: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viability of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 42 °C for 10 and 24 h……51

Figure 17: Exponential cooling rate used for abusive chilling experiments. Samples chilled from 54.4 °C to 4 °C in 6.5 h……52

Figure 18: Exponential cooling rate used for abusive chilling experiments. Samples chilled from 54.4 °C to 4 °C in 12 h……53

Figure 19: Exponential cooling rate used for abusive chilling experiments. Samples chilled from 54.4 °C to 4 °C in 18 h……53

Figure 20: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* in sous-vide style cooked chicken breasts chilled from 54.4 °C to 4 °C in 6.5 h……54

Figure 21: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* in sous-vide style cooked chicken breasts chilled from 54.4 °C to 4 °C in 12 h……54

Figure 22: Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* in sous-vide style cooked chicken breasts chilled from 54.4 °C to 4 °C in 18 h……55

CHAPTER 1

INTRODUCTION

Since the late-2000s and the early-2010s sous-vide cooking has undergone a rapid increase in popularity for both home and industry settings. Sous-vide cooking involves vacuum sealing meats and other ingredients inside plastic bags and then cooking in a temperature-controlled medium for an extended period of time. This method offers multiple advantages to shelf life, food quality, consumer convenience, and repeatability, but there are disadvantages. The step of vacuum sealing involves evacuating all atmospheric gases from the bag, and then heat-sealing. As a result of vacuum sealing, the sous-vide process creates an environment with very low oxygen content, or anaerobic conditions. Additionally, the sous-vide process cooks the foods at relatively low external temperatures compared to traditional cooking methods, such as a convection oven. While the process is effective at killing bacterial vegetative cells, heat resistant spores may not be destroyed. Both of these factors may create an ideal environment for anaerobic spore-forming bacteria, such as *Clostridium perfringens*, to germinate and potentially grow within sous-vide food products, especially if the cold supply chain is compromised.

A *C. perfringens* outbreak in a hospital in 1995 was traced back to vacuum-packaged pork, and numerous studies have demonstrated the possibility of spore germination inside vacuum-sealed and sous-vide products for both *C. perfringens* and *C. botulinum* (Regan et al. 1995; Juneja et al. 2006, 2007; Juneja and Friedman 2007). Therefore, as sous-vide cooking becomes even more popular the likelihood of

these outbreaks will also increase. One possible intervention to reduce the likelihood of spore germination in sous-vide products is the incorporation of food-grade antimicrobials. “Clean label” natural antimicrobials are also experiencing an increase in consumer popularity and previous studies have reported some to be effective in inhibiting *C. perfringens* spore germination (Juneja and Friedman 2007; Juneja et al. 2007; Valenzuela-Martinez et al. 2010).

This study was aimed to test the efficacy of various natural antimicrobials, such as vinegar and lemon juice, on *C. perfringens* germination and growth inhibition. This goal was achieved by completing three objectives. First, to identify effective natural antimicrobial concentrations against *C. perfringens* by antimicrobial screening in pure culture. Second, to determine the extent of the bactericidal and sporicidal activity of selected antimicrobial treatments on *C. perfringens* vegetative cells and spores, respectively. And third, to determine the most effective antimicrobial treatment that inhibits *C. perfringens* in a sous-vide cooked, temperature-abused chicken breast model. The findings of this work will contribute to advancing our knowledge of the food safety of sous-vide products and provide companies with a “clean label” solution to minimize risk.

CHAPTER 2

LITERATURE REVIEW

Clostridium perfringens Information

Clostridium perfringens Characteristics

Clostridium perfringens is an anaerobic, Gram-positive, non-motile, encapsulated, spore-forming rod-shaped bacterium (Ray and Bhunia 2008). Formerly referred to as *Bacillus aerogenes* and *Clostridium welchii*, the pathogen was finally classified as *C. perfringens* in the 1970s. *C. perfringens* is a mesophile that grows optimally at temperatures between 43 and 47 °C. This bacterium can grow at temperatures as low as 6 °C and requires a water activity level of at least 0.93 is required for cell proliferation (Garcia et al. 2019). The optimum pH range for bacterial multiplication occurs between values of 6 and 7. Little to no replication has been reported at pH values of $\leq$5 and $\geq$8.3. *C. perfringens* is a relatively oxygen tolerant microorganism, because *C. perfringens* can produce reducing agents, such as ferredoxin, to lower the redox potential (E_h) value of the environment to a more suitable level (Montville et al. 2012).

Taxonomy

The species *C. perfringens* is taxonomically classified into the phylum *Firmicutes,* the class *Clostridia*, the family *Clostridiaceae*, and the genus *Clostridium*. The *Firmicutes* phylum is mostly composed of bacteria with Gram-positive cell wall structures, of which many possess the capability to produce endospores. This phylum is usually divided into three classes, *Clostridia*, *Bacilli,* and *Erysipelotrichia.*

The *Clostridia* class includes strictly anaerobic organisms, while the *Bacillus* class contains facultative anaerobes. The *Erysipelotrichia* class has low rRNA similarity with other groups within the phylum but has similar cell wall and phenotypic characteristics (Ludwig et al. 2009).

The *Clostridium* genus consists of many species important to both human and animal health. For example, the species *Clostridium leptum* and *Clostridium coccoides*, when part of a host's microbiota, have been found to provide a number of health benefits and immune system protections (Garcia et al. 2019). However, even though the majority of species belonging to this genus are harmless, there are some recognized human pathogens. These include *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, and *Clostridium tetani*. Among all of the pathogenic *Clostridium* spp., *C. perfringens* is the most abundant toxin-producing species (Ray and Bhunia 2008).

Metabolism

Clostridium spp. can either be saccharolytic or proteolytic. Toxin-producing species, such as *C. perfringens*, are usually strongly proteolytic. *C. perfringens* acquires energy through the process of anaerobic respiration. This means *C. perfringens* utilizes a compound other than oxygen as a terminal electron acceptor in the electron transport chain, such as nitrate. Though anaerobic respiration is the main way *C perfringens* produces energy, it can also undergo a variety of metabolic reactions to sustain its energy needs such as fermentation. For example, *C. perfringens* can ferment sugars, such as glucose, to produce carbon dioxide or

electron carriers to establish more ideal environmental growing conditions (Titball et al. 1999).

Sporulation and Germination

C. perfringens has the ability to undergo the process of sporulation. Sporulation is a survival mechanism of many bacteria to persist within an unfavorable environment by forming a highly resistant, metabolically inactive endospore. A bacterial endospore is highly resilient to environmental factors due to its unique structure, that is comprised of seven distinct layers (Figure 1). The ultimate function of the bacterial spore is to protect the cell's DNA, ribosomes, and other molecules necessary for vegetative cell growth until favorable external growth conditions are met (Ray and Bhunia 2008).

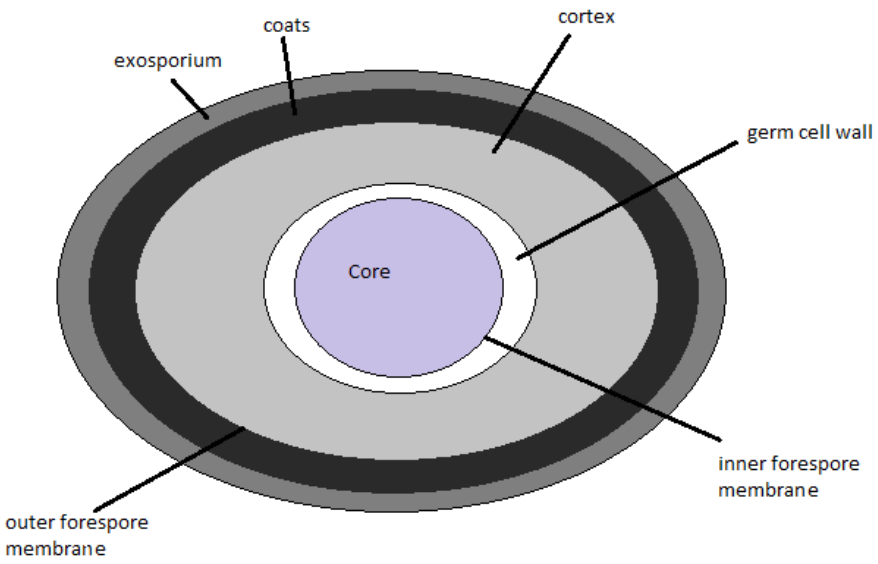


Figure 1: Bacterial endospore structure taken from (Montville et al. 2012).

The outermost layer of the spore is the exosporium. The exosporium is composed of lipids, carbohydrates, and proteins. It plays a role in excluding large

molecules, spore surface adhesion, and spore pathogenesis. Under the exosporium, various layers of proteins are known as the spore coats. The exact function of the spore coats is not fully understood, but some of the constitutive enzymes have been found to facilitate the detoxification of reactive oxygen species and influence the ability of germinants to bind to their respective receptors (Paredes-Sabja et al. 2014).

Under the spore coats, the cortex is found, which is surrounded by an outer forespore membrane (OM) and an inner forespore membrane (IM). The function of the OM is not yet fully known, but the IM plays a pivotal role in protecting the spore core from numerous molecules, including even water. The cortex is structurally similar to the peptidoglycan layer in the cell wall. It contains various amino acids residues suspected to contribute to spore germination. Preceding the core is the germ cell wall, which resembles its vegetative cell counterpart in both structure and function. Finally, the innermost layer is known as the spore core. The core contains the endospore's DNA, ribosomes, enzymes, and divalent cations. An extremely low water content, elevated levels of dipicolonic acid (DPA), and the saturation of DNA with α/β small acid soluble proteins (SASP) are all conditions present within the core. All of these factors are hypothesized to play a major role in both spore dormancy and environmental resistances (Paredes-Sabja et al. 2014; Setlow and Johnson 2019).

Commonly referred to as the model organism for cell differentiation and bacterial chromosome replication, much of the understanding of the sporulation mechanism is based on previous work involving *Bacillus subtilis* (Higgins and

Dworkin 2012; Setlow 2014; Krawczyk et al. 2017). Those studies on *B. subtilis* have provided a simplified model to explain sporulation in seven distinct morphological stages, illustrated in Figure 2. The first three stages involve the replication and condensation of the bacterial cell chromosomes and an asymmetrical cell division creating a smaller forespore and a larger mother cell. In the following three stages the spore cortex and spore coats surround the forespore and the spore is allowed to "mature". Lastly, in stage seven the mother cell lyses, and the dormant, developed endospore is released into the environment.

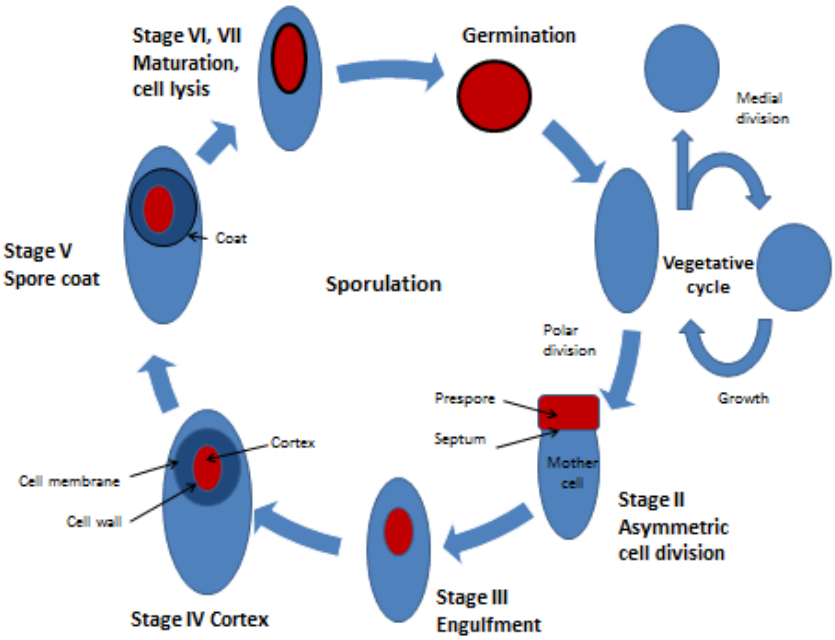


Figure 2: The sporulation process taken from (Errington 2003).

Adverse environmental stimuli, such as nutrient exhaustion, thermal and chemical stress, and radiation can induce the sporulation process. The exact mechanism of endospore formation is not fully understood, but a multitude of researchers have contributed to its elucidation (Jiang et al. 2000; Stephenson and Hoch 2002; Molle et al. 2003). The DNA-binding protein Spo0A is regularly denoted

as the master regulator for the initiation of sporulation because it influences over 500 genes responsible for the formation of an endospore. The activity of Spo0A is regulated by multiple phosphorelays and a cascade of phosphorylation events. Once Spo0A is phosphorylated to its active form, Spo0A~P, the regulator protein will bind to a DNA sequence element known as the "0A-box". This will then act as a repressor to various genes involved in vegetative cell growth and an activator of genes responsible for sporulation (Molle et al. 2003).

Even though bacterial endospores can remain present within the environment for years, exposure to certain agents can cause the spore to lose dormancy and resistances, or germinate, within minutes. Vegetative cell outgrowth can then quickly begin. To begin germination the spore must first be activated. Numerous stimuli can activate the endospores such as, heat, high pressure, acids, nutrients, among others (Setlow 2014). Once activated, specific nutrients will bind to the receptors responsible for germination. These nutrients are called germinants and are typically single amino acids, sugars, or purine nucleosides (Setlow and Johnson 2019).

There are four phases of the germination and outgrowth cycle (Figure 3). The activation and outgrowth phases are considered pre and post steps respectively to the defined germination events. This distinction is characterized by the lack of metabolic energy needed in both stage 1 and stage 2 of germination (Setlow and Johnson 2019). Ultimately, the spore will transform into metabolically active cells in an environment highly conducive to rapid bacterial growth.

The first phase of the germination cycle is the activation step. This can be initiated from a variety of agents, most commonly sub-lethal heat. The exact cellular changes are not fully known, but it is theorized that the stimulus may enact an alteration to protein structure. This change would allow the appropriate nutrient germinants to bind to their receptors and begin germination (Krawczyk et al. 2017).

The first stage of endospore germination involves the release of spore core H^+, monovalent cations, DPA, and divalent cations (Ca^{2+}). Also, water will replace the DPA within the core. In the second stage, the spore cortex is hydrolyzed, and the core and germ cell wall swell as more water is taken up. Both of these stages result in an elevation of the spore core pH and hydration levels, mutually essential for metabolic enzyme mobility and action. The initiation of enzyme activity allows for the beginning of spore metabolism and cellular outgrowth. SASPs are degraded and macromolecules are synthesized, successfully converting the germinated spore to a growing cell (Setlow 2003; Paredes-Sabja et al. 2014; Setlow and Johnson 2019).

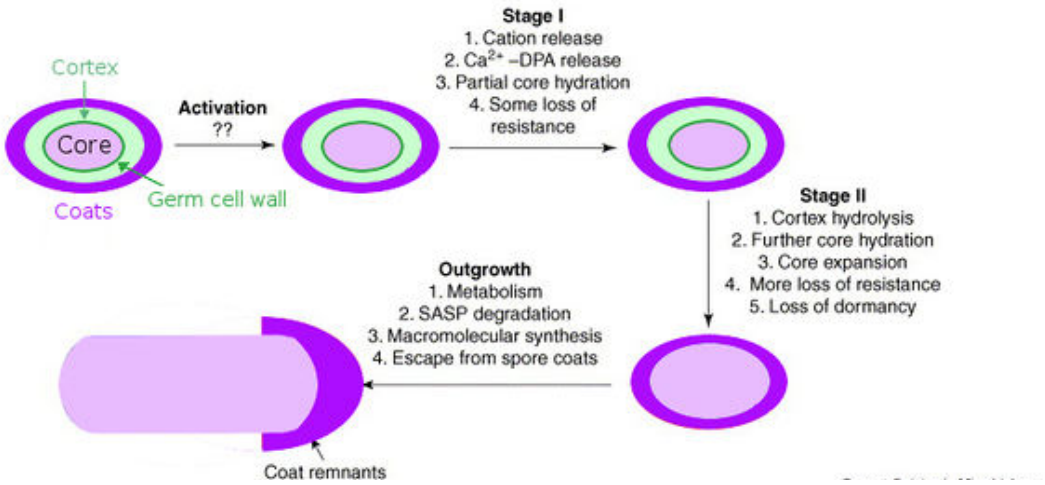


Figure 3: The germination and cellular outgrowth process taken from (Setlow 2003).

Virulence Factors

Bacterial virulence factors are molecules produced by a pathogen to invade and colonize a host, cause diseases, and evade host immune defenses. Some examples of virulence factors include, adherence factors, invasion factors, capsules, and toxins (Mascaretti 2014). *C. perfringens* is capable of producing more than 16 different toxins all of which allow it to survive in the host. The toxin production in *C. perfringens* is mediated by a functional Agr-like quorum-sensing system (CpAL). The system functions by secreting autoinducer signaling molecules when a high bacterial population is detected ($\geq 10^6$). These peptides (AgrD) are sensed by a membrane sensor (VirS), which in turn phosphorylates VirR, the response regulator. Activated VirR then directly upregulates the transcription of several toxin genes (Figure 4) (Uzal et al. 2014; Garcia et al. 2019).

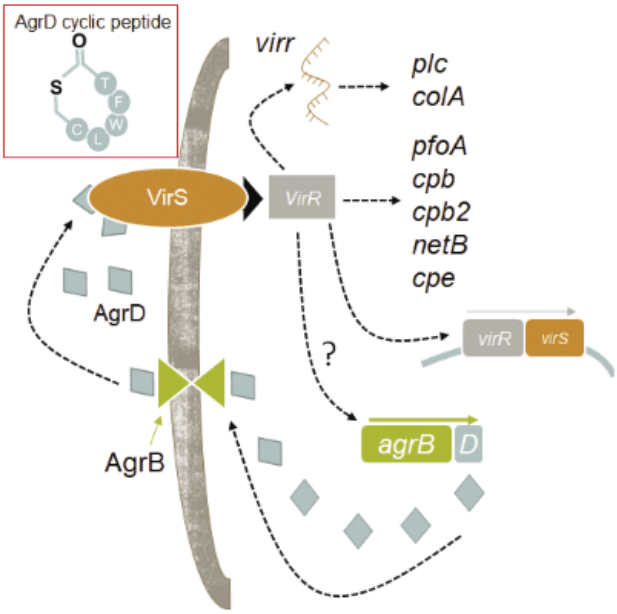


Figure 4: CpAL regulation of *C. perfringens* virulence factors by population density-induced autoinducers taken from (Garcia et al. 2019). Autoinducer AgrD is sensed

by sensor VirS within the cell membrane (gray curved structure), phosphorylating regulator VirR and activating toxin genes.

Clostridium perfringens Toxins and Toxinotyping

Since the 1960s, *C. perfringens* has been classified into five different strain types based on the production of four major toxins. An updated toxinotyping system has been proposed based upon the production of six major toxins (CPA, CPB, ETX, ITX, CPE, and NetB) and introduces two new toxinotypes (F and G) (Table 1). The toxinotypes are organized by the types of diseases they cause. Type A strains are associated with human gas gangrene. Type B, C, and D are mainly associated with different animal enteric diseases. Type F stains are responsible for human foodborne illness and antibiotic-resistant diarrhea; and type G stains are linked to necrotic enteritis in chickens (Rood et al. 2018).

Table 1: *C. perfringens* toxin-based typing scheme based on production of six major toxins and commonly associated diseases (X, production of toxin; -, no production of toxin) adapted from (Garcia et al. 2019).

***C. perfringens* Toxinotype**	**Presence of Toxin**						**Disease**
	CPA	**CPB**	**ETX**	**ITX**	**CPE**	**NetB**	
A	X	-	-	-	-	-	Human gas gangrene
B	X	X	X	-	-	-	Enteric animal disease, lamb
C	X	X	-	-	X	-	Enteric animal disease
D	X	-	X	-	X	-	Enteric animal disease, sheep, goats

E	X	-	-	X	X	-	Ovine and bovine hemorrhagic enteritis
F	X	-	-	-	X	-	Human food poisoning
G	X	-	-	-	-	X	Chicken necrotic enteritis

Clostridium perfringens **Enterotoxin**

C. perfringens Type F strains (formerly type A) have been linked with human foodborne illness. The causative agent of *C. perfringens* food poisoning is *C. perfringens* enterotoxin (CPE). Strong evidence has been discovered to correlate CPE with human gastrointestinal illness. For example, CPE has been found in the feces of human adults suffering from *C. perfringens* foodborne illness compared to healthy human adults, and when healthy adults were fed purified CPE they developed the characteristic gastrointestinal symptoms associated with *C. perfringens* (Skjelkvale and Uemura 1977; Smedley et al. 2004).

CPE is a 319-amino-acid (~35-kDa) single polypeptide encoded by the *cpe* gene. *C. perfringens* food poisoning is a toxicoinfection caused by the intracellular accumulation of CPE produced during sporulation. When a host consumes *C. perfringens* spores, the conditions for spore germination are achieved. The enterotoxin is then released into the host when the vegetative cells are lysed by stomach acid (Duncan 1973). CPE will then cause illness by damaging the host's intestinal epithelial and endothelial cells.

Toxin Effect Mechanism

Overall, there are two individual phases of the cytotoxic activity of CPE. Once the enterotoxin is released into the intestinal lumen, the toxin first binds to receptors on the host enterocyte proteins. A complex containing CPE, receptor claudins, and nonreceptor claudins, forms on the membrane of the epithelial cells. Next, the complex undergoes a conformation change into a large complex, containing CPE hexamers. This large complex is inserted into the enterocyte cell, creating a pore. The pore induces cell death by hydrolyzing phospholipids and permeabilizing the plasma membrane. This ultimately promotes an influx of Ca^{2+} and the loss of essential metabolites and ions from the host cell, finalizing cell death (McClane and McDonel 1980; Chakrabarti and McClane 2005).

***Clostridium perfringens* Foodborne Illness Incidence**

Clostridium perfringens is the third most prevalent foodborne bacterial pathogen in the United States and has been recognized as a cause of food poisoning since the 1940s. It is estimated to cause approximately one million cases of foodborne illness per year (Scallan et al. 2011). Typically, outbreaks are traced to large gathering settings, such as nursing homes, cafeterias, or prisons. These scenarios are frequently caused by improper holding temperature or inadequate cooling of cooked foods (Doyle 2002). The most common form of foodborne illness associated with *C. perfringens* is a toxicoinfection that involves acute gastrointestinal symptoms experienced 8 to 12 hours after ingesting contaminated foods. Symptoms typically are resolved within 24 hours, unless an immunocompromised individual, the elderly, or infants are infected. The most

common form of treatment is oral hydration to counteract the rapid loss of fluid and electrolytes (Uzal et al. 2010; Montville et al. 2012).

C. perfringens is a ubiquitous microorganism that can be found in soil, dust, sewage, water, food (nearly 50% of raw meats), spices, in the intestinal tracts of animals and humans, and on almost every natural environmental surface examined so far (Allaart et al. 2013; Garcia et al. 2019). *C. perfringens* is particularly associated with protein-rich foods since the bacterium can only produce 7 of the essential 20 amino acids. This bacterium is of particular concern to the meat and poultry industries based on epidemiological data. The Center for Disease Control (CDC) estimates from outbreak data gathered between 1998 and 2008 that the greatest percent of food commodities associated with *C. perfringens* outbreaks are beef and poultry (33% and 31% respectively) (Painter et al. 2013).

Multiple factors contribute to the ability of *C. perfringens* to survive and proliferate in foods. *C. perfringens* has a comparatively wider growing temperature range for a mesophilic microorganism (15-50 °C compared to 25-40 °C). So, *C. perfringens* can start to grow before its competitors in certain scenarios. Additionally, *C. perfringens* has one of the shortest generation times of all currently known microorganisms, being able to duplicate within 8-12 minutes at optimum temperatures (Ray and Bhunia 2008). *C. perfringens* tolerates oxygen exposure to a certain extent, and it has been observed to have the capability to form biofilm-like structures on host tissues and abiotic surfaces (Charlebois et al. 2014). These characteristics coupled with the capability to form highly environmentally resistant

endospores allows *C. perfringens* to persist in food manufacturing plants if contamination takes place.

Clostridium perfringens in the Poultry Industry

Poultry Industry Trends

As the world population increases so does the demand for cost effective protein sources. Since 1909, an increase in the total meat consumption in the United States has been observed. The last available data from 2007 estimates the total meat consumption in the United States to be approximately 250 g daily per-capita. Red meat is the most popular option, but poultry consumption has been steadily increasing since the 1940s. Most recently, poultry consumption was reported to be approximately 80 g daily per-capita, triple the amount reported in 1944 (Daniel et al. 2011). This same consumption trend can also be seen in the European Union where poultry consumption has approximately doubled since the 1970s (Rouger et al. 2017).

Of the entire poultry industry, chicken meat accounts for the highest percentage of consumption comprising approximately 75% of total sales (Rouger et al. 2017). The rapid growth of the broiler industry can be attributed to multiple factors. First, vertical integration, in which one company controls most aspects of operations, allows supply chain costs to be dramatically decreased. Additionally, chickens have one of the most efficient feed to protein conversion ratios of 2:1 kg of feed to kg of consumable tissue. Another advantage of poultry production is that broilers have a relatively short production time (6-8 weeks) compared to other

farmed meat sources. These aspects and more contribute to the low relative cost of chicken meat and its increasing popularity (Singh et al. 2019).

Incidence of *Clostridium perfringens* in the Poultry Industry

According to findings from numerous studies, the prevalence of *C. perfringens* in the poultry industry is high. An analysis of the intestinal tracts of broiler hens found approximately 75% to 95% of the tested flocks to be positive for *C. perfringens,* and 40% of the isolates were enterotoxigenic strains (Shane et al. 1984; Miwa et al. 1997). An examination of retail poultry meat revealed high percentages of positive *C. perfringens* samples, upwards of 84% (Miwa et al. 1998). Another study which followed 16 separate flocks from hatching to post carcass chill reported 13 of the 16 flocks (81%) to be positive for *C. perfringens* (Craven et al. 2001). Evidence also suggests that the *C. perfringens* colonization of broilers occurs as early as the hatchery stage and can be transmitted throughout the entire operation (Figure 5). For example, *C. perfringens* has been isolated from eggshell fragments, chicken fluff, and paper pads placed on the bottom of hatchery floors further showcasing that contamination can occur at even the earliest stages of processing (Craven et al. 2003; van Immerseel et al. 2004).

Furthermore, *C. perfringens* is also frequently isolated from environmental samples of poultry farms and slaughter plants. This is because *C. perfringens* is widespread in the environment, most commonly found in soil and water. Data acquired from the environmental swabbing of a poultry farm indicated high incidences of *C. perfringens* around the facility. The uppermost values were wall swabs (53%), fan swabs (46%), fly strips (43%), dirt outside facility entrance swabs

(43%), and worker boots swabs (29%) (Craven et al. 2001). Also, a study completed by Gaucher and team over the span of 6 months reported that out of 16 visits to a broiler slaughter plant an average of 5% of 217 total environmental samples were confirmatory for *cpe*-positive *C. perfringens* strains and the highest incidence was 25% (Gaucher et al. 2018).

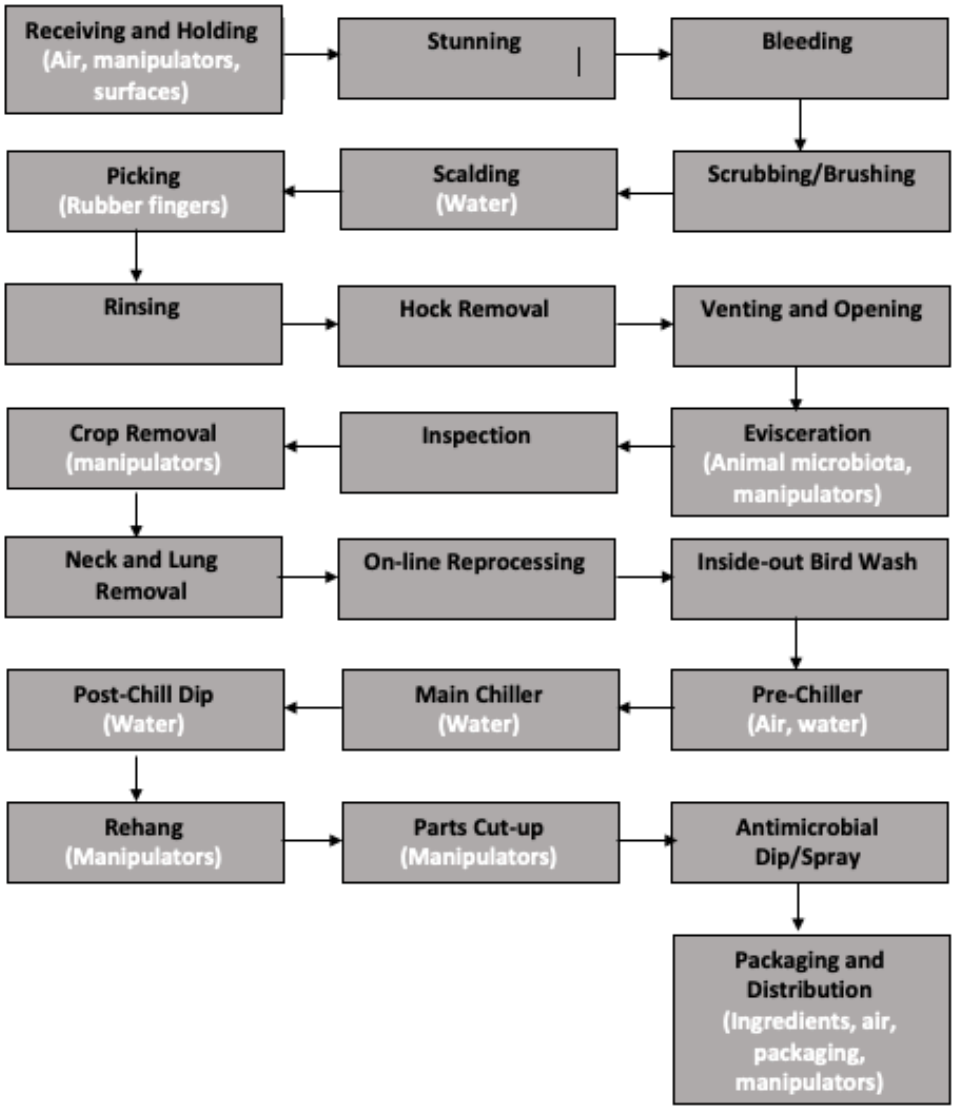


Figure 5: Unit operations for primary poultry processing. Most common sources of contamination for a particular step are in parentheses and white. Adapted from (Rouger et al. 2017; Singh et al. 2019).

Sources of Contamination

In healthy birds the muscle tissue is sterile, but the slaughterhouse environment is not. The chicken meat can become contaminated from a wide variety of sources including the broiler skin, feathers, digestive tract, processing equipment, workers, aerosols, water, etc. This contamination can occur at any step within the slaughter process. Figure 5 shows steps from slaughter to final further processing and the most commonly associated contamination routes. Additionally, products can be re-contaminated postprocessing. One survey conducted by the World Health Organization (WHO) discovered that approximately 25% of foodborne outbreaks can be traced to the recontamination of processed meat products (Tirado and Schmidt 2001). Therefore, the most effective method to control contamination is by proper sanitation and environmental control (Rouger et al. 2017).

Clostridium perfringens Outbreaks Associated with Poultry

As the poultry industry continues to expand, ensuring microbial safety will become even more important. A report from the CDC utilizing data from the United States' Foodborne Disease Outbreak Surveillance System from 1998 to 2012 implicates poultry as the most common food in foodborne illness outbreaks. Of a total 1,114 outbreaks, 279 (25%) were traced to poultry. According to those figures, the second most common pathogen reported was *C. perfringens* (26%) after *Salmonella* (43%) (Chai et al. 2017). The most frequent causative factor for the *C. perfringens* outbreaks was improper holding temperature, or temperature abuse, of cooked meat products (approx. 80%) (Doyle 2002).

Methods of Controlling *Clostridium perfringens*

Physical Antimicrobial Interventions

The effects of physical antimicrobial interventions, such as thermal processing, pressure treatment, and irradiation have all been observed on *C. perfringens.* Thermal processing is largely intended for cooking meats and for the destruction of deleterious microbes; however, cooking temperatures are frequently not sufficient to inactivate thermally resistant endospores. *C. perfringens* spores have been found to be highly heat resistant and are of particular concern. For example, one study reported *C. perfringens* spores to be able to survive thermal treatments as high as 100 °C (Adams 1973). Fortunately, spore populations on raw meats are largely regarded to be low. Therefore, since the spore population is low and vegetative cells are killed during thermal processing, it is acceptable to control for spore germination and vegetative cell outgrowth through other means (Singh et al. 2019).

One method to control spore germination and outgrowth is to ensure the United States Department of Agriculture Food Safety Inspection Services (USDA FSIS) compliance guidelines for stabilization are followed. The guidelines state that as long as a food, particularly meat, is cooled from 54.5 °C to 26.6 °C in one and a half hours and from 26.6 °C to 4.4 °C in five hours the food should be safe for consumption. This performance standard is based upon controlling the outgrowth of *C. perfringens* in a food product to less than 1 log CFU/g during the cooling process. It is safe to assume that the control of *C. perfringens* outgrowth applies to

other spore-formers as well, such as *Clostridium botulinum* and *Bacillus cereus* (Sánchez-Plata et al. 2005; Singh et al. 2019).

High hydrostatic processing (HHP) is another standard pasteurization technique. HHP has been reported to be more effective at killing vegetative cells and inactivating spores of *C. perfringens* than thermal processing while also maintaining the sensory and nutritional quality of foods (Sarker et al. 2015). Two findings in particular have shown that by combining heat and pressure treatments as much as a 5.1-fold reduction of *C. perfringens* spores can be achieved (Paredes-Sabja et al. 2007; Gao et al. 2011). Conversely, *C. perfringens* vegetative cells and spores are radiation resistant. Irradiation has been found to make spores slightly more heat susceptible, but the vegetative cells are more resistant to radiation than other pathogens because no significant decrease was noted after a treatment of 1.75 kGy (Monk et al. 1995; Labbe and Garcia 2001).

Chemical Antimicrobial Interventions

Several chemical interventions have also been observed to control *C. perfringens* in food matrices. Some examples of effective treatments are nitrite, lactates, and phosphates. Nitrite is widely used in the meat industry to control spore-forming bacteria due to its antimicrobial activity (Talukdar et al. 2017). The precise inhibitory mechanism is not fully understood; however, nitrite does increase the oxidative stress upon microorganisms when it is converted to the strong oxidizing agent peroxynitrite. While Gram-positive bacteria have been found to be more susceptible to nitrites than Gram-negative, endospores are generally more resistant (Majou and Christieans 2018). Although, it has been reported that sodium

nitrite (≥50 ppm) had a significant effect on the germination and outgrowth of *C. perfringens* in a vacuum-sealed ham product exposed to abusive cooling conditions (Redondo-Solano et al. 2013). Even though nitrites are successful at limiting pathogen growth, they are limited to levels ≤200 ppm within meats due to the potential of the formation of carcinogenic nitrosamines (Dahle 1979).

Lactic acid salts such as potassium lactate, sodium lactate, and calcium lactate have also all been shown to inhibit *C. perfringens* at ≤2.0% in temperature abused pork samples (Reddy Velugoti et al. 2007). The proposed mechanism of action of lactates is the reduction of intracellular pH. The acid molecules are lipophilic, allowing diffusion across the cell membrane, dissociation, and release of protons into the cytoplasm. In order to maintain homeostasis, the affected cells expend energy to remove the protons. The removal of protons also disrupts the cell proton gradient, impacting the transport of essential molecules such as amino acids (Shelef 1994).

Inorganic phosphates have been utilized as food additives for years due the functions of emulsification, oxidative protection, flavor stabilization, increasing water-holding capacity, and antimicrobial activity (Talukdar et al. 2017). Phosphates have been found to have a much higher degree of inhibition on Gram-positive bacteria than Gram-negative possibly due to their ability to chelate metal ions. Polyphosphates have higher affinities for metal cations than the cation-binding sites within the cell wall of Gram-positive bacteria. This could disrupt the cellular uptake of essential metals and negatively impact enzymatic reactions necessary for cell colonization and growth (Knabel 1989; Lorencová et al. 2012). Ortho-, pyro-,

and polyphosphates have all been examined to inhibit *C. perfringens* in both pure culture and food matrices (Akhtar et al. 2008; Singh et al. 2010). Unfortunately, phosphates are limited to a maximum of 0.5% in processed meat products because excess consumption could affect the phosphorous level within the body and hinder thyroidal hormone regulation and bone density, though evidence is limited (Long et al. 2011; Calvo et al. 2014).

Natural Antimicrobial Interventions

Due to recent health-conscious consumer trends, interest in novel natural food antimicrobial agents has increased. Several researchers have studied the inhibitory effects of various natural ingredients on foodborne pathogens, suggesting their potential as food preservatives (Tiwari et al. 2009; Lucera et al. 2012; Juneja et al. 2012; Davidson et al. 2013). Moreover, a number of these ingredients have been observed to be effective at controlling *C. perfringens*. Some of the most significant treatments against *C. perfringens* are essential oils (EOs), tannins, plant extracts, bacteriocins, and organic acids (Talukdar et al. 2017).

EOs, tannins, and plant extracts are all examples of phytoantimicrobials. Specifically, EOs are secondary metabolites produced from aromatic plants usually isolated through steam-distillation. EOs contain numerous molecules such as terpenes, terpenoids, phenols, and aliphatic compounds (Bakkali et al. 2008). The proposed method of action is mostly attributed to their lipophilic nature which allows for passage through the cellular membrane and subsequent membrane permeabilization. This will lead to the disruption of the bacterial membrane potential and the interruption of proton pumps, resulting in ATP depletion and

cytotoxicity (di Pasqua et al. 2006). Cinnamaldehyde, carvacrol, thymol, and oregano oils have all exhibited antimicrobial activity on *C. perfringens* between 1.0% and 2.0%, though the results are variable depending upon the food matrix (Juneja et al. 2006; Juneja and Friedman 2007).

Tannins are water-soluble polyphenolic compounds frequently found in woody plants such as chestnut and oak trees. A mechanism of antimicrobial activity has not been established, but tannins have been observed to have a strong iron binding capacity which may hinder bacterial biochemical reactions. Some studies have indicated that tannic acid had inhibitory activity against *C. perfringens* vegetative cells at ≥3.1 μM concentration, but its effects on spores or spore germination have not yet been studied (Scalbert 1991; Elizondo et al. 2010). Furthermore, various plant extracts including green tea, grapefruit, and grape seed have been reported to have inhibitory qualities against *C. perfringens*. Treatment with grape seed extract significantly reduced the heat resistance of *C. perfringens* vegetative cells, and all three compounds caused significantly reduced viable counts in vacuum-sealed meat products exposed to temperature abuse conditions (Juneja et al. 2006, 2007; Cosansu and Juneja 2018; Cosansu et al. 2019).

Bacteriocins are peptides synthesized by virtually all bacterial genera to kill or inhibit similar microbes for resources. Bacteriocins are generally harmless to the human body and the surrounding environment and very few have received Generally Recognized as Safe (GRAS) status from the U.S. Food and Drug Administration (FDA). Bacteriocins have been observed to be bacteriostatic and bactericidal against both Gram-positive and Gram-negative bacteria but are either

narrow spectrum (effective for the same species) or broad spectrum (effective across genera) (Cotter et al. 2005; Yang et al. 2014). Therefore, a Gram-positive bacteriocin is likely to have little to no effect on a Gram-negative bacterium and vice-versa. Nisin and lacticin are examples of bacteriocins that have been tested against *C. perfringens* (Talukdar et al. 2017).

Nisin is a class I broad spectrum Gram-positive bacteriocin produced by *Lactococcus* and *Streptococcous* species. Nisin produced by certain strains of *Lactococcus lactics* subsp. *lactis* has been recorded to significantly inhibit *C. perfringens* spore outgrowth and vegetative cells, but no such effect has been observed in a food matrix (Udompijitkul et al. 2012; Shin et al. 2016). Lacticin is also a class I broad spectrum Gram-positive bacteriocin produced by strains of *L. lactis*. It is very similar to nisin in terms of inhibitory effects but is more active at a physiological pH (Rea et al. 2007). Lacticin alone and in combination with organic acids has been noted to significantly inhibit the growth of *C. perfringens* in a pork sausage product (Scannell et al. 2000). The bactericidal action of both bacteriocins is believed to be attributed to the generation of pores in the cell membrane and ultimate disruption of cell-wall biosynthesis (Ruhr and Sahl 1985).

Organic acids have been commercially produced since the late 1800s and their antimicrobial efficacy is well-studied (Lucera et al. 2012). It is proposed that the bactericidal properties of organic acids are because of their ability to cross the cellular membrane and acidify the cell cytoplasm with protons when in an undissociated state. This would cause a decrease in the cell cytoplasmic pH, below the level required for vital biochemical enzyme function. The bacterial cell will then

not be able to efflux protons rapidly enough to regulate the cytoplasmic pH, resulting in cellular death (Russell and Diez-Gonzalez 1997).

Two of the most common organic acids utilized in the food industry as preservatives are acetic and citric acids. To attain a "clean label" some manufacturers have chosen to incorporate these acids into product formulations in the forms of vinegar and citrus extracts or juices respectively. Both have demonstrated inhibitory action against Gram-positive and Gram-negative bacteria, including *C. perfringens* (Kang et al. 2003; Valenzuela-Martinez et al. 2010; Gomes et al. 2018). Specifically, a blend of vinegar and lemon concentrate has shown significant efficacy in controlling the spore germination and outgrowth of *C. perfringens* in vacuum-sealed and temperature abused roast beef and turkey products (Valenzuela-Martinez et al. 2010; Smith et al. 2018).

Sous-vide Foods

The Sous-vide Process

Originally a French culinary technique, sous-vide translates as "under vacuum," and sous-vide cooking is defined as "raw materials or raw materials with intermediate foods that are cooked under controlled conditions of temperature and time inside heat-stable vacuumized pouches" (Baldwin 2012). Therefore, sous-vide cooking involves the utilization of vacuum sealing a food product, and then submerging the product into a controlled heating medium (water bath or convection steam oven) for an extended period of time. Meat and poultry are most commonly prepared with this technique, but vegetables and fruits can be as well. In fact, sous-vide cooked vegetables have been observed to maintain nearly all of their

nutritive value and have a more concentrated flavor since the plant cell walls are left mostly intact (Creed 1995).

A simplified overview of the sous-vide process can be seen below in Figure 6. This cooking method begins with the preparation of raw ingredients and can either be completed as cook-serve or cook-chill. Cook-chill is the most popular method for food manufacturers since the finished products can be transported and stored for extended periods of time, while cook-serve is more prevalent in ready-to-serve restaurants and consumer homes.

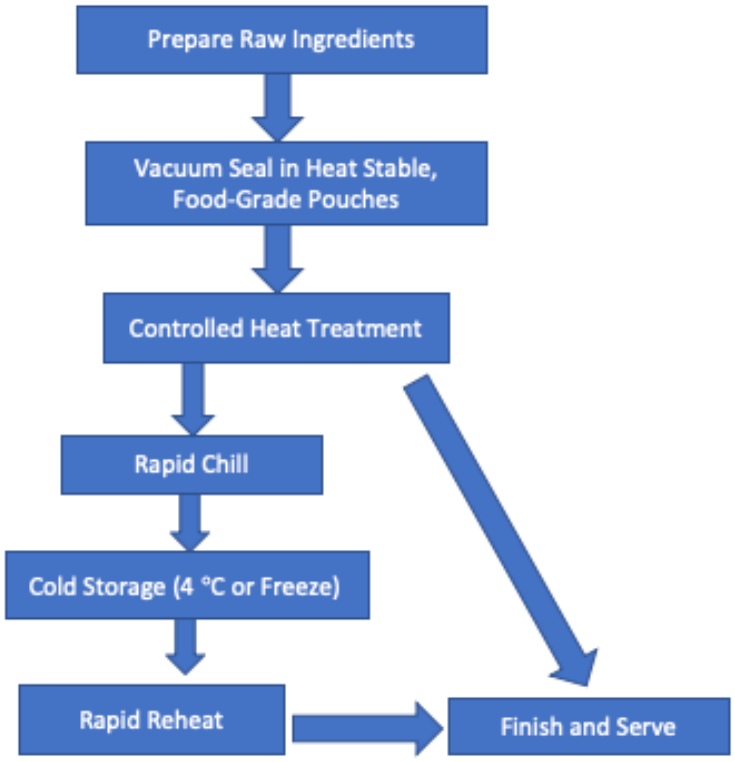


Figure 6: A simplified outline of the sous-vide process adapted from (Baldwin 2012).

Over the past three decades the utilization of sous-vide cooking has increased in both home and industry settings (Singh et al. 2019). Sous-vide has

become popular because it is convenient, has superior reproducibility, extends shelf life, and the products have been found to generally have equal, or better, sensory characteristics compared to traditionally prepared foods (Creed 1995; Baldwin 2012). Defined temperature control allows more precision in doneness and texture because the possibility of uneven cooking or overcooking is drastically reduced.

Sous-vide Food Safety Concerns

Commercial thermal processing for sous-vide pasteurization is typically 70 °C for 100 minutes or 90 °C for 10 minutes, though there is no overall standard due to product variability and differing desired levels of doneness (Singh et al. 2019). It is just recommended that a food receive an acceptable temperature and hold time combination to have a 7 $\log_{10}$ CFU/g reduction of *Salmonella* spp. and a 6 $\log_{10}$ CFU/g reduction of *Listeria monocytogenes* (Baldwin 2012). These relatively mild heat treatments are effective at eliminating vegetative pathogens but not at deactivating bacterial endospores. For example, *C. perfringens* spores have been observed to be susceptible to wet heat, but only to a certain extent. Wang and team reported only a 30% *C. perfringens* spore population reduction when heating an endospore suspension in a water bath set to 90 °C for 60 minutes (Wang et al. 2012).

The survival and germination of *C. perfringens* endospores during the sous-vide process has been observed in several studies in various food matrices (Juneja and Marmer 1996; Juneja 2006; Juneja et al. 2006; Cosansu and Juneja 2018; Cosansu et al. 2019). Additionally, a specific *C. perfringens* outbreak in 1995 in a hospital was traced back to a vacuum-sealed and heated pork product (Regan et al.

1995). While vacuum packaging does inhibit food oxidation, prevent product recontamination, and eliminate growth competition from aerobic microbes, it also establishes ideal conditions for anaerobes. Thus, the main food safety concern with sous-vide products is the survival and outgrowth of anaerobic heat resistant bacterial spores, especially in temperature abuse scenarios.

The largest contributing factor for documented *C. perfringens* outbreaks (approx. 80%) is holding cooked foods at improper temperatures or temperature abuse (Doyle 2002). This abuse can either occur because of a deviation from the USDA FSIS Stabilization cooling standards (abusive chilling) or from prolonged exposure to elevated storage conditions (abusive storage) (>6 °C). *C. perfringens* spores have been examined to have the potential to germinate and outgrow in various vacuum-sealed sous-vide food matrices in both abusive cooling and abusive storage scenarios (Sánchez-Plata et al. 2005; Juneja et al. 2006, 2011; Akhtar et al. 2008). So proper adherence to cooling guidelines and monitoring of temperature are vital steps to ensure *C. perfringens* does not outgrow enough to cause human illness.

CHAPTER 3

MATERIALS AND METHODS

Clostridium perfringens Strains

Three strains of *Clostridium perfringens* were used in this project. The strains were NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13). All three strains are known enterotoxin producing strains. All three are were contributed by Dr. Harshavardhan Thippareddi, Department of Poultry Science, University of Georgia in the form of spore suspensions. The suspensions were verified for spore presence by phase-contrast microscopy (Olympus BH2-RFCA, Japan).

Antimicrobial Treatments

A total of six commercial natural antimicrobial treatments were formulated and provided by World Technology Ingredients Inc. (WTI Inc.) from Jefferson, GA for use in this project. The names of the antimicrobial ingredients, ingredient preparations, and concentrations tested can be seen in Table 2.

Table 2. List of natural antimicrobials used in this project. Commercial names of ingredients, active ingredients, and concentrations tested are all shown.

Commercial ingredient name	Active ingredients	Concentrations tested (%w/v, or %w/w)
DV	Dried vinegar	0.5-3.0
NatureIn LV1X	Vinegar and lemon juice concentrate	0.5-3.0
NatureIn V	Liquid vinegar	0.5-3.0
Prototype VKO	Vinegar and citrus extract	0.5-3.0

Prototype VKM	Liquid vinegar with natural flavors	0.5-3.0
Prototype VKC	Vinegar and cinnamaldehyde	0.5-3.0

Antimicrobial Screening

A 96-well plate (Falcon 351172 Microtest Flat Bottom Franklin Lakes, NJ) format was used for the antimicrobial efficacy screening of all the treatment combinations against the strains of *C. perfringens* separately. The treatments were prepared to desired concentrations (0.5%-3.0%) in fluid thioglycolate medium (FTG) (Neogen 111624E). Bacterial cultures were prepared by aliquoting 100 μL of spore suspensions into 10 mL of FTG broth and heat shocking for 20 min at 75 °C in a heated water bath (Precision Scientific Dubnoff Chicago, IL). The testing wells contained 75 μL of prepared FTG media containing the individual antimicrobial ingredients at specific concentrations and 75 μL of heat shocked spore suspensions. The positive control wells contained 75 μL of FTG broth and 75 μL of heat shocked spore suspensions. The negative control wells consisted of just 150 μL of uninoculated FTG media. Negative control wells of 150 μL of prepared FTG media with antimicrobial ingredients for each concentration were also made. Strains were kept on separate plates to minimize risk of cross contamination.

To establish anaerobic conditions the prepared plates were placed into an anaerobic chamber (Gaspack System BBL) with anaerobic atmosphere packs (Pack-Anaero Mitsubishi Gas Chemical Company, Inc. New York, NY) and flushed with nitrogen gas (Airgas NI 200 Radnor, PA) for approximately two minutes prior to incubation at 42 °C for 24 h. Resazurin anaerobic indicators were used to verify that

anaerobic conditions were maintained (Thermo Scientific BR0055B Germany) during incubation. A 96-well plate reader (BioTek Cytation 3 Winooski, VT) was used to obtain optical density measurements at 600 nm (OD_{600}). Each treatment concentration had three replicates per test and the experiment was repeated three independent times. The OD_{600} values were averaged and compared to determine statistical significance between treatments and strains and determine the minimum inhibitory concentrations (MIC). The two most effective formulations were utilized for following experiments vinegar and lemon juice concentrate (VLJC) and vinegar and citrus extract (VCE).

Endospore and Vegetative Cell Harvest

The sporulation of *C. perfringens* was based on the methods developed by Duncan and Strong (Duncan and Strong 1968), but slightly modified according to recommendations by (Juneja et al. 1993). All incubations were done in an anaerobic chamber at 37 °C and all three strains were harvested separately. Working cultures were prepared by aliquoting 0.1 mL of suspended spore cultures into 10 mL tubes of FTG broth and heat shocking for 20 min at 75 °C. These cultures were incubated for approximately 18 h at 42 °C. A 1 mL quantity of the cultures were then transferred to 10 mL tubes of FTG media and incubated for 4 h at 42 °C to facilitate vegetative cell growth. An aliquot of 0.1 mL from the new working culture was added to 10 mL (1% inoculum) of prepared modified Duncan-Strong (DS) (Himedia M1237 India) media supplemented with 0.1 g/L caffeine (Sigma-Aldrich BCBZ5624 St. Louis, MO) to enhance sporulation (Juneja et al. 1993). The inoculated DS tubes were incubated anaerobically at 37 °C for 24 h and were confirmed for spore presence by phase-

contrast microscopy. Spore level was evaluated for each strain by enumerating heat shocked portions of culture on prepared FTG agar plates. Spore crops were maintained in DS media at 4 °C until use.

C. perfringens vegetative cells were harvested by first establishing a growth curve for each of the three strains. A 1 mL portion of the DS spore stocks were aliquoted into 9 mL FTG tubes and heat activated for 20 min at 75 °C. These tubes were incubated at 37 °C; aliquots were taken at time points (0, 2, 4, 6, 8, and 24 h) and OD_{600} values were obtained with a spectrophotometer (Thermo Spectronic Genesys 10uv Madison, WI). This experiment was done three independent times and the final absorbance value was averaged from the three values for each strain. Exponential cell cultures were deemed to be established approximately at between 4 and 5 h, so vegetative cultures were obtained by inoculating an FTG media tube, heat shocking, and incubating for 4 h at 37 °C prior to use in subsequent experiments.

Mode of Action Experiments

The effect of the previously chosen effective treatments were utilized for the experiments. VCE 1.0% and 1.5% was used and VLJC 1.5% and 2.0%. Vegetative cells and bacterial endospores were obtained using the methods established in the vegetative cell and spore harvest section. The same 96-well plate layout was utilized as in the antimicrobial screening experiments, but plates were separated by vegetative cells and endospores for all three strains. Well plates were filled in the same manner as described previously. Prior to incubation, treated and negative control wells were serially diluted with 0.1% peptone water and sub-cultured to

prepared FTG agar plates to acquire pre-incubation viable cells counts. The FTG agar plates were overlain with 8-10 mL of FTG agar prior to being incubated anaerobically at 37 °C for 24 h.

After incubation at 42 °C for 24 h, the well plates were visually analyzed for turbidity and non-turbid wells were again serial diluted in 0.1% peptone water, sub-cultured to prepared FTG agar plates, overlain with 8-10 mL of FTG agar, and incubated anaerobically at 37 °C for 24 h. Negative control wells were also plated on FTG agar to ensure no viable cells were present. Viable cells counts were obtained for both vegetative cells and endospores and the average values of three independent trials were calculated to determine bacteriostatic, bactericidal, sporicidal, or spore germination inhibitory activities of the treatments.

Sporulation Efficiency Experiment

The procedure was adapted from Akhtar (Akhtar et al. 2008). The effect of treatments VCE and VLJC on the sporulation efficiency on three strains of *C. perfringens* was measured by first preparing 10 mL tubes of DS media with sublethal antimicrobial concentrations (VCE 0.05%, 0.1% and VLJC 0.5%, 0.75%). DS tubes were inoculated with 0.1 mL of 4-h FTG cultures separate for each of the three tested strains. Positive controls with no treatment and negative controls with just DS media were also prepared. The DS tubes were then incubated anaerobically for 24 h at 37 °C. To determine viable cell counts in 24 h incubated DS cultures, aliquots were serially diluted in 0.1% peptone water, plated onto FTG agar plates and overlain with 8-10 mL FTG agar. Heat-resistant spore counts were estimated by heat shocking the 24 h DS cultures for 20 min at 75 °C then plating appropriate serial

dilutions onto FTG plates with overlay. All plates were incubated anaerobically for 24 h at 37 °C. The experiment was repeated three independent times and values were averaged together. Percent values of sporulation efficiency were determined by dividing heat-resistant spore counts (CFU/mL) by viable cell counts (CFU/mL).

Chicken Sample Procurement and Storage

Sample portioning, preparation, cooking, and enumeration were adapted from Valenzuela-Martinez (Valenzuela-Martinez et al. 2010). Fresh boneless, skinless chicken breast samples were obtained from a local grocery store in Griffin, GA. The breasts were stored in a refrigerator at 4 °C for no longer than one day and portioned in 5 g ± 0.2 g pieces and placed into vacuum pouches (Clarity nylon; 3-mil standard barrier). The pouches were vacuum sealed at 12 mbar with a vacuum-sealer (Multivac NG00059 Wolfertschwenden, Germany) and frozen at -20 °C until use. Samples were frozen for a maximum of one week. Fresh chicken breasts were obtained for each experiment trial.

***C. perfringens* Spore Cocktail Preparation**

A three-strain *C. perfringens* spore cocktail was made by mixing equal parts of prepared spore suspensions (NCTC 8238, NCTC 8239, and NCTC 10240). This cocktail was stored at 4 °C and used within 30 days.

Chicken Sample Preparation

Prior to sample preparation, pouches were thawed overnight in a refrigerator at 4 °C. Sample pouches were opened and inoculated with 100 μL of the prepared 3-strain *C. perfringens* cocktail and manually massaged for 30 s to ensure spore distribution. Samples were then seasoned (0.1 g ± 0.05 g) with a commercial

chicken seasoning blend (Newly Weds Foods G98187-1; salt, dehydrated onion, dehydrated garlic, spice, garlic powder, onion powder, canola oil, silicon dioxide). For appropriate samples antimicrobial treatments VCE (1.0%, 3.0%) and VLJC (1.5%, 3.0%) were applied and samples were manually massaged once more for 30 s to ensure distribution of ingredients. Positive control samples were prepared with just chicken, spore inoculum, and seasoning, while negative controls were prepared with just chicken and seasoning. Once prepared all samples were vacuum sealed again and briefly stored at 4 °C until use.

Sample Cooking Method

All samples for each trial were heat shocked in a circulating heated water bath (Precision 51221035 Winchester, VA) for 20 min at 75 °C. To ensure samples had reached the recommended internal temperature of 73.9 °C, a temperature probe (ThermoWorks ChefAlarm China) was inserted into one uninoculated chicken portion, sealed with cell foam tape (md Oklahoma City, OK), and continuously monitored. Pouches were arranged in baskets and secured with clips to ensure samples were constantly submerged and not overcrowded in the water bath.

Abusive Storage Trials Chilling and Storage

Once samples were appropriately thermally processed, they were pulled and immediately chilled in an ice bath. An uninoculated sample was continuously monitored for internal temperature with a temperature probe. When the probed sample reached 4 °C (approx. 8 min) all samples were pulled and stored in incubators set at either 4, 12, or 16 °C until scheduled sampling. An additional

abusive storage condition trial was also performed at 42 °C with the same preparation, cooking, and chilling procedures.

Abusive Storage Trials Enumeration

Chicken portions were sampled after 0, 1, 4, 7, 10, 13, and 16 days. For the 42 °C storage experiment samples were taken at time points 0, 10, and 24 h. On sampling times, the pouches were pulled from the appropriate incubators, opened, and filled with 20 mL of 0.1% peptone water. The samples were then stomached for 1 min (Stomacher 80, Seward Biomaster, London, UK) and serial dilutions were made with 0.1% peptone water and appropriately plated onto selective tryptose-sulfite-cycloserine (TSC) (Himedia M837 India) agar without egg yolk. Plates were then overlain with 8-10 mL of TSC agar and incubated anaerobically at 37 °C for 24 h. *C. perfringens* colonies (black colonies) were enumerated and counts were expressed as CFU/g of meat. The experiment was repeated three independent times and final values were obtained by averaging the three trials.

Defective Cooling Trials

Chicken samples were prepared and cooked according to methods stated above, however, once the samples were fully cooked, they were immediately moved to a circulating refrigerated water bath (RTE 10, Thermo Neslab, Portsmouth, NH) set at 54.4 °C and allowed to equilibrate to this temperature for 10 min. After equilibration, the samples were chilled from 54.4 °C to 4.0 °C within 6.5, 12, and 18 h. Exponential chilling rates were used (Figures 18-20) to simulate the cooling of meat products in the meat industry. Sampling was done by following the same procedure as the abusive storage experiment explained above, but samples were

taken immediately after the cook step and immediately after exponential chilling. The experiment was repeated three independent times and final values were obtained by averaging the three trials.

Measurement of pH and Water Activity

Uninoculated FTG media and uninoculated FTG media with treatment concentrations were prepared and and pH values were taken by immersing the electrode of a pH meter (OakIon pH 510 Series Vernon Hills, IL) into the tubes. Additionally, uninoculated 5 g chicken portions were processed and chilled according to previous procedures. A control of chicken and seasoning was compared to chicken and seasoning samples with treatments of VCE (1.0%, 3.0%) and VLJC (1.5%, 3.0%). Pouches were opened, filled with 20 mL 0.1% peptone water, stomached for 1 min, and pH value was taken by immersing electrode into the homogenate. The water activity of identically prepared samples was taken using an Aqua Lab a_w meter (AquaLab CX-2 Pullman, WA) according to manufacturer's instructions. All measurements were taken in triplicate and the experiments were repeated three independent times. The final values were the mean of the three trials and three replicate measurements.

Statistical Analyses

To analyze the results the statistical software JMP (SAS, Cary, NC) was used. Since all results were mean values a one-way ANOVA test, followed by Tukey-Kramer HSD test were used to find significant differences between strains and treatments where applicable ($p \leq 0.05$).

CHAPTER 4

RESULTS

Antimicrobial Screening

The results of the antimicrobial screening experiment for all three strains of *C. perfringens* can be seen in Figures 7-9. The maximum optical density (OD_{600}) measured for all three strains in FTG in the absence of treatments ranged between 0.7 and 0.95 after 24 h at 42 °C. For all three *C. perfringens* strains tested, The final OD_{600} measurements for dried vinegar (DV) and liquid vinegar and natural flavors (LVNF) treated FTG cultures were never below 0.5 at any of the concentrations tested (0.5-3.0%). A similar result was obtained with the vinegar and cinnamaldehyde (VC) formulation except that strain 10240 cultures reached 0.25 OD_{600} at 3.0%. Treatment with liquid vinegar (LV) did not reduce the maximum density of all three strains from 0.5 to 2.5%, but at 3.0% growth was markedly reduced. No detectable growth was observed when cultures were treated with VCE and VLJC at concentrations greater than 1.0 and 1.5% respectively. VCE treatment consistently resulted in complete inhibition (0.076 OD_{600}) of all three bacterial strains at 1.0% and similarly VLJC at 1.5%. Minimum inhibitory concentrations for VCE and VLJC for the tested 3 strains of *C. perfringens* was determined to be 1.0 and 1.5% respectively in FTG media.

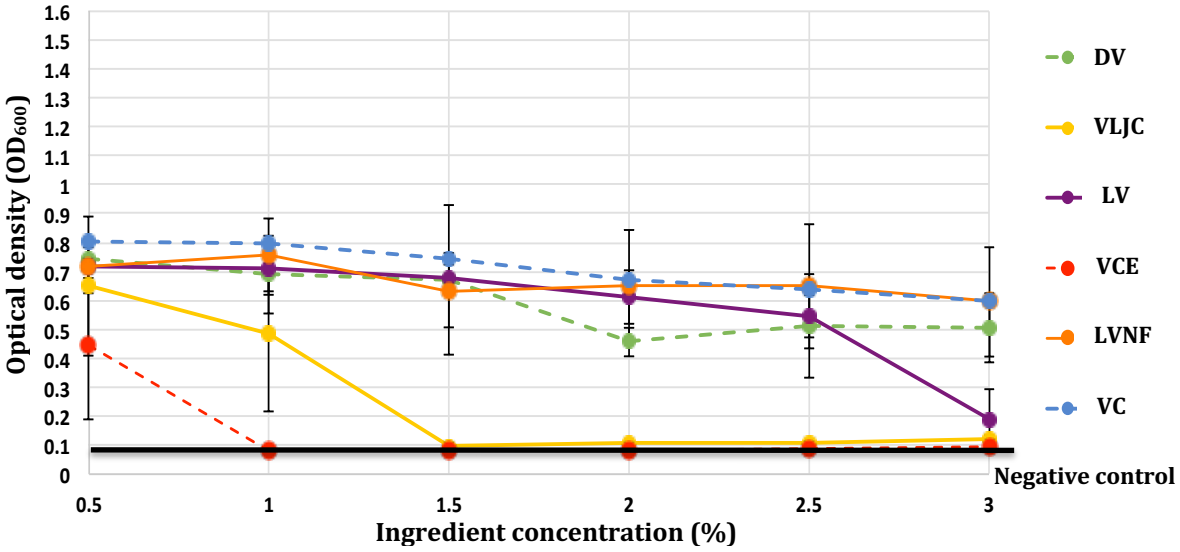


Figure 7. Inhibition of *C. perfringens* strain NCTC 8239 after incubation for 24 h at 42 °C from naturally derived food ingredients in FTG media. Negative control (FTG media) approx. 0.076. Abbreviations: DV, dry vinegar; VLJC, vinegar with lemon juice concentrate; LV, liquid vinegar; VCE, vinegar with citrus extract; LVNF, liquid vinegar with natural flavors; VC, vinegar with cinnamaldehyde.

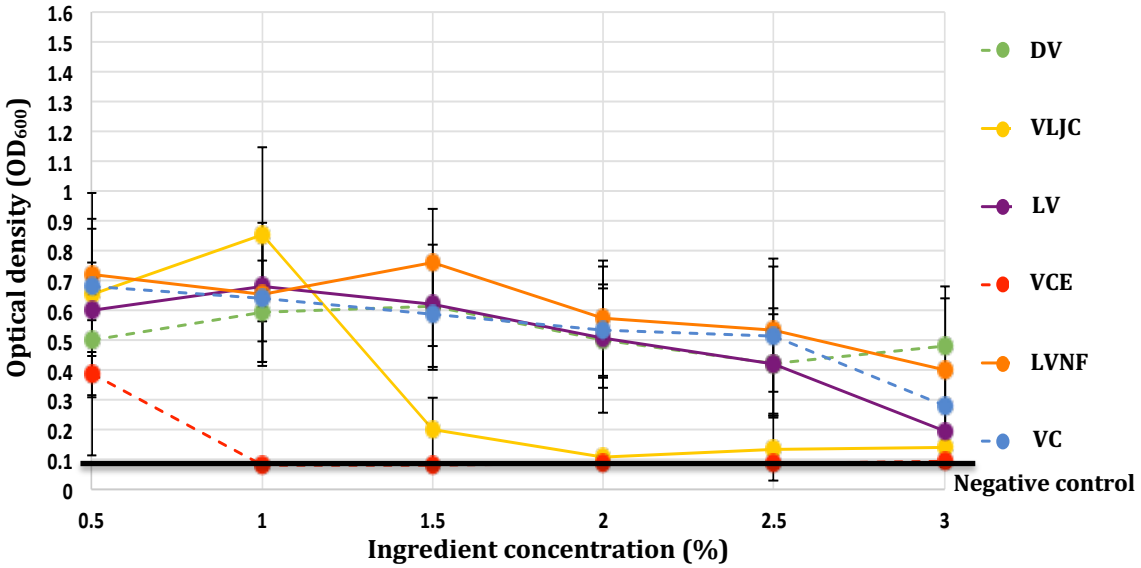


Figure 8. Inhibition of *C. perfringens* strain ATCC 10240 after incubation for 24 h at 42 °C from naturally derived food ingredients in FTG media. Negative control (FTG media) approx. 0.076. Abbreviations: DV, dry vinegar; VLJC, vinegar with lemon juice concentrate; LV, liquid vinegar; VCE, vinegar with citrus extract; LVNF, liquid vinegar with natural flavors; VC, vinegar with cinnamaldehyde.

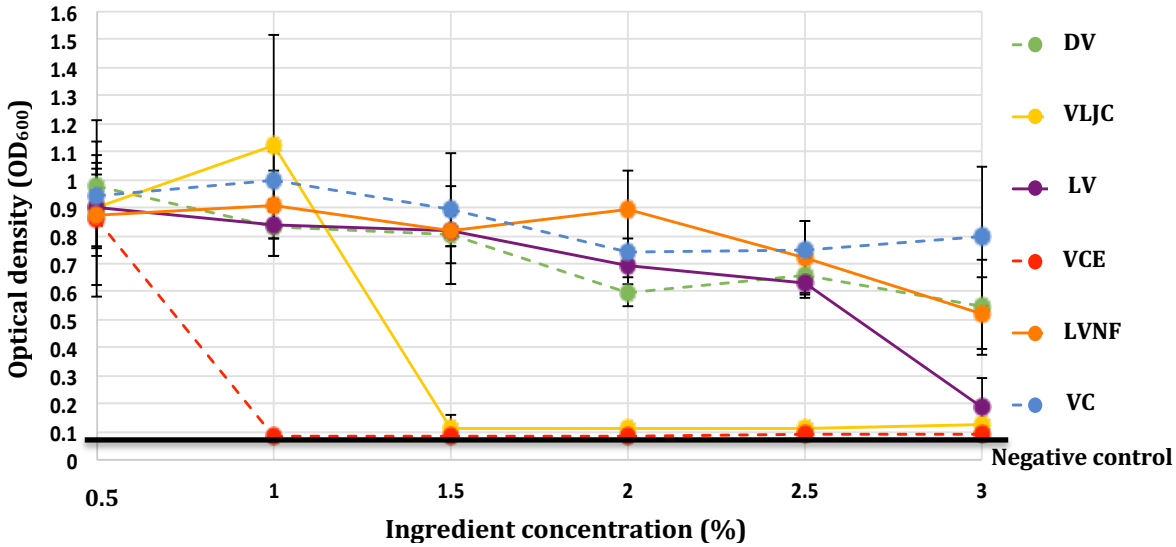


Figure 9. Inhibition of *C. perfringens* strain NCTC 8238 after incubation for 24 h at 42 °C from naturally derived food ingredients in FTG media. Negative control (FTG media) approx. 0.076. Abbreviations: DV, dry vinegar; VLJC, vinegar with lemon juice concentrate; LV, liquid vinegar; VCE, vinegar with citrus extract; LVNF, liquid vinegar with natural flavors; VC, vinegar with cinnamaldehyde.

Pure Culture pH Readings

The pH values of the natural antimicrobial ingredients prepared in FTG media and FTG media itself can be seen below in Tables 3-8. The pH value of the control FTG media was found to be 6.83. The pH values of the treatments ranged from 6.66 to 5.63 from 0.5 to 3.0%. Treatments at all concentration levels were found to have pH values that were significantly ($p \geq 0.05$) different from the pH value of the FTG media control.

Table 3. pH values of FTG media after mixing with vinegar and citrus extract (VCE) ingredient at different concentrations.

Concentration (%)	pH
0.0	6.83 ± 0.06^{a}
0.5	6.66 ± 0.03^{b}
1.0	6.17 ± 0.04^{c}
1.5	5.86 ± 0.03^{d}
2.0	5.77 ± 0.01^{e}

2.5	5.69 ± 0.02^{e}
3.0	5.65 ± 0.02^{f}

Table 4. pH values of FTG media after mixing with vinegar and cinnamaldehyde (VC) ingredient at different concentrations.

Concentration (%)	pH
0.0	6.83 ± 0.06^{a}
0.5	6.42 ± 0.01^{b}
1.0	6.09 ± 0.01^{c}
1.5	5.86 ± 0.01^{d}
2.0	5.69 ± 0.03^{e}
2.5	5.68 ± 0.01^{e}
3.0	5.63 ± 0.02^{f}

Table 5. pH values of FTG media after mixing with vinegar and natural flavors (VNF) ingredient at different concentrations.

Concentration (%)	pH
0.0	6.83 ± 0.06^{a}
0.5	6.31 ± 0.01^{b}
1.0	6.07 ± 0.02^{c}
1.5	5.85 ± 0.01^{d}
2.0	5.75 ± 0.01^{e}
2.5	5.67 ± 0.02^{f}
3.0	5.63 ± 0.01^{g}

Table 6. pH values of FTG after mixing with vinegar and lemon juice concentrate (VLJC) ingredient at different concentrations.

Concentration (%)	pH
0.0	6.83 ± 0.06^{a}
0.5	6.33 ± 0.02^{b}
1.0	6.27 ± 0.01^{c}
1.5	5.91 ± 0.01^{d}
2.0	5.78 ± 0.01^{e}
2.5	5.72 ± 0.01^{f}
3.0	5.66 ± 0.01^{g}

Table 7. pH values of FTG after mixing with liquid vinegar (LV) ingredient at different concentrations.

Concentration (%)	pH
0.0	6.83 ± 0.06^{a}
0.5	6.32 ± 0.01^{b}
1.0	6.13 ± 0.01^{c}
1.5	5.84 ± 0.01^{d}

2.0	5.76 ± 0.01^{e}
2.5	5.71 ± 0.01^{f}
3.0	5.68 ± 0.01^{f}

Table 8. pH values of FTG media after mixing with dried vinegar (DV) ingredient at different concentrations.

Concentration (%)	pH
0.0	6.83 ± 0.06^{a}
0.5	6.54 ± 0.01^{b}
1.0	6.21 ± 0.01^{c}
1.5	6.11 ± 0.01^{d}
2.0	6.04 ± 0.01^{e}
2.5	5.99 ± 0.01^{f}
3.0	5.98 ± 0.01^{f}

Growth Curve Determination

Growth curves were determined to identify the duration of the exponential growth phase for vegetative cell collection. The relationship between OD_{600} and time is shown in Figure 10. The cell density of all three strains increased rapidly after 2 hours and the exponential phase continued at 4 h when cultures reached maximum density. Based on these information, vegetative cells were harvested between 3.5 and 4 hours for subsequent experiments.

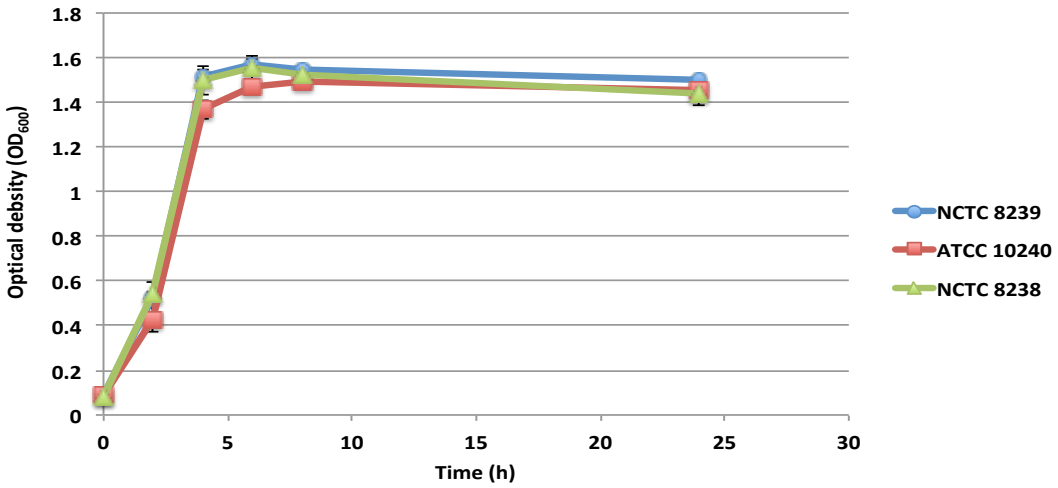
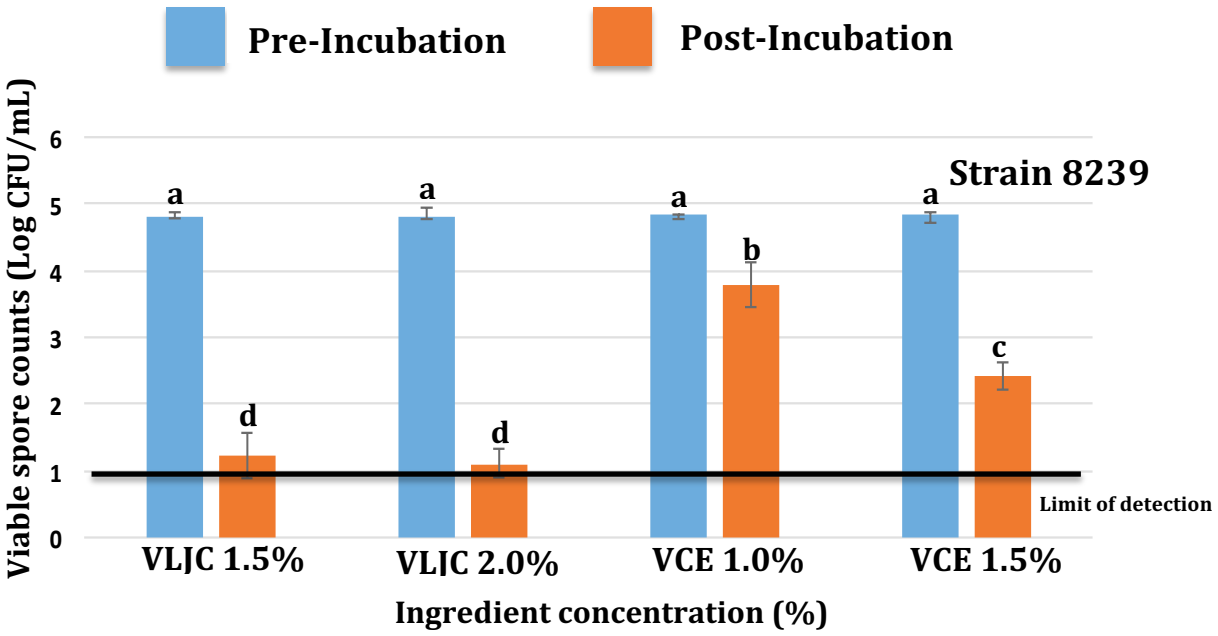


Figure 10. Growth curve of three *C. perfringens* strains incubated at 37 °C for 24 h in FTG media.

Endospore Mode of Action

The results from the endospore mode of action experiments can be found below in Figure 11. Treatments VCE (1.0 and 1.5%) and VLJC (1.5 and 2.0%) were chosen for testing based on results from the antimicrobial screening experiments. A trend of reduction of viable spore counts varied depending on the strain and antimicrobial ingredient All treatments caused a significant reduction in viable spore counts. Treatment VCE at 1.0 and 1.5% only reduced the viable spore count of strain 8239 by roughly 1 and 2 log CFU/mL respectively. The highest reductions in viability caused by VLJC 2.0% and VCE 1.5% treatments were more than 4 log CFU/mL for strain 8238.



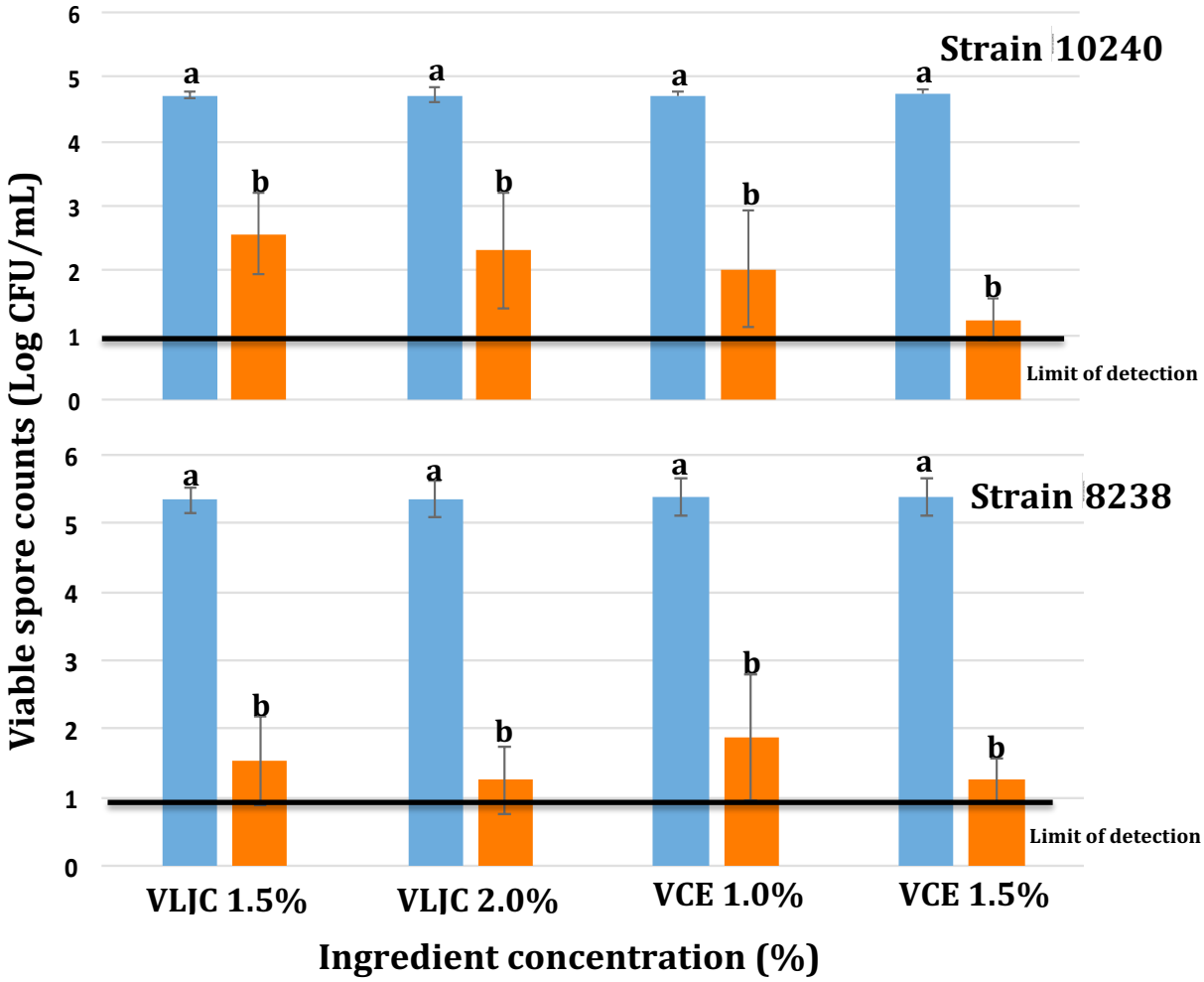


Figure 11. Effect of treatments vinegar and lemon juice concentrate (VLJC) and vinegar and citrus extract (VCE) on viable endospore counts of three strains of *C. perfringens*. Culture were incubated anaerobically at 42 °C for 24 h. Limit of detection was 1 log CFU/mL.

Vegetative Cell Mode of Action

The results from the vegetative cell mode of action experiments can be found below in Figure 12. The same treatments used in the endospore mode of action experiments above were also utilized in this experiment. A trend of reduction in cell counts was observed for all strains and treatments tested. All treatments tested significantly reduced viable cell counts consistently for all three strains of *C. perfringens* used. The initial level of vegetative cells was observed to be

approximately 6.0 to 6.5 log CFU/mL. The antimicrobial ingredients reduced cell populations in FTG media ranging from below the level of detection (1.0-log CFU/mL) to final counts of approx. 1.7 log CFU/mL.

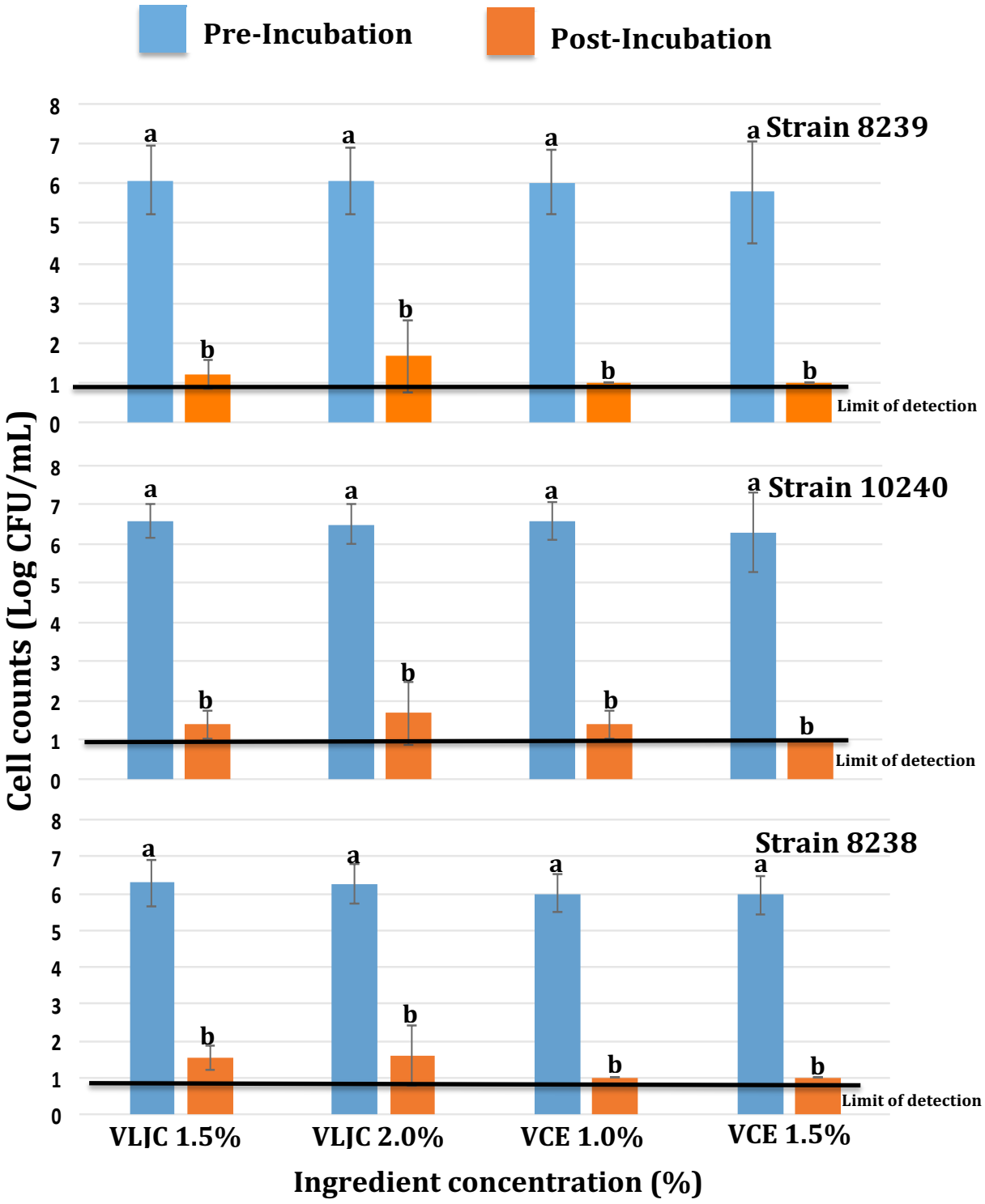


Figure 12. Effect of treatments vinegar and lemon juice concentrate (VLJC) and vinegar and citrus extract (VCE) on vegetative cell counts of three strains of *C. perfringens*. Cultures were incubated anaerobically at 42 °C for 24 h. Limit of detection was 1 log CFU/mL.

Sporulation Efficiency

The results of the sporulation efficiency experiments can be found below in Tables 9-11. Overall, all antimicrobial ingredients tested at all concentrations demonstrated significant reductions in the sporulation efficiency of all three *C. perfringens* strains when compared to the control samples. The percent sporulation values for the positive control DS cultures were 6.20% (NCTC 8239), 8.38% (ATCC 10240), and 12.27% (NCTC 8238). The DS culture with antimicrobial ingredient concentrations had sporulation efficiency percentages ranging from 0.01 to 2.20%. No significant differences in sporulation efficiency was seen between treatments for strain 10240. Treatments VCE 0.1, VLJC 0.5, and VLJC 0.75% (0.01-0.43%) did exhibit a significant effect compared to treatment VCE 0.05% (2.20%). Additionally, ingredient formulation VLJC 0.75% had the most pronounced decrease in percent sporulation efficiency for strain 8238 (0.01%) when compared to the other treatments tested (0.18-0.76%).

Table 9. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the sporulation efficiency of *C. perfringens* strain NCTC 8239.

Treatment	Viable cell count ($CFU/mL \times 10^7$)	Heat-resistant cell count ($CFU/mL \times 10^6$)	Percent sporulation (%)
Positive control	5.42 ± 3.28	2.98 ± 0.88	6.20 ± 2.08^a
0.05% VCE	3.13 ± 0.73	0.73 ± 0.38	2.20 ± 0.82^b
0.1% VCE	3.62 ± 1.02	0.048 ± 0.018	0.14 ± 0.04^c
0.5% VLJC	1.52 ± 1.60	0.077 ± 0.0844	0.43 ± 0.18^c
0.75% VLJC	5.06 ± 2.73	0.003 ± 0.002	0.01 ± 0.01^c

Table 10. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the sporulation efficiency of *C. perfringens* strain ATCC 10240.

Treatment	Viable cell count (CFU/mL × 10^7)	Heat-resistant cell count (CFU/mL × 10^6)	Percent sporulation (%)
Positive control	1.62 ± 0.551	1.40 ± 1.00	8.38 ± 3.89[a]
0.05% VCE	0.0608 ± 0.0176	0.000006 ± 0.000002	0.01 ± 0.006[b]
0.1% VCE	0.0567 ± 0.0169	0.000003 ± 0.000002	0.005 ± 0.003[b]
0.5% VLJC	2.86 ± 3.99	0.00627 ± 0.00504	0.14 ± 0.23[b]
0.75% VLJC	0.652 ± 0.163	0.00638 ± 0.00319	0.11 ± 0.07[b]

Table 11. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the sporulation efficiency of *C. perfringens* strain NCTC 8238.

Treatment	Viable cell count (CFU/mL × 10^7)	Heat-resistant cell count (CFU/mL × 10^6)	Percent sporulation (%)
Positive control	2.15 ± 1.21	2.89 ± 2.16	12.27 ± 6.39[a]
0.05% VCE	0.769 ± 0.983	0.083 ± 0.127	0.76 ± 0.41[b]
0.1% VCE	0.741 ± 1.22	0.0035 ± 0.0045	0.18 ± 0.13[bc]
0.5% VLJC	1.39 ± 0.973	0.0246 ± 0.0219	0.31 ± 0.34[bc]
0.75% VLJC	1.14 ± 0.618	0.00211 ± 0.00328	0.01 ± 0.02[c]

Chicken Samples pH and a_w Readings

The results of the pH and water activity readings of prepared chicken are reported in Table 12. The pH value of the control chicken samples (chicken + seasoning) was 6.24 while the pH values of the chicken samples with natural antimicrobial ingredients ranged from 6.02 to 6.09. All chicken samples with antimicrobial ingredients had statistically different pH values when compared to the control samples. The water activity reading of the control chicken samples was 0.990 and the samples with ingredients ranged from 0.986 to 0.989. Only samples

with higher ingredient concentrations (VCE and VLJC 3.0%) were observed to have statistically different a_w when compared to the control samples.

Table 12. pH and water activity measurements of control chicken samples (chicken and commercial seasoning blend) and chicken samples with different natural antimicrobial ingredient concentrations and seasoning blend. Treatments are vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC).

Treatment	pH	a_w
Control	6.24 ± 0.079^{a}	0.990 ± 0.002^{a}
1.0% VCE	6.09 ± 0.075^{b}	0.989 ± 0.002^{a}
3.0% VCE	6.02 ± 0.085^{b}	0.986 ± 0.002^{b}
1.5% VLJC	6.09 ± 0.069^{b}	0.988 ± 0.002^{ab}
3.0% VLJC	6.03 ± 0.062^{b}	0.986 ± 0.002^{b}

.

Abusive Storage Experiment

The results of the experiments that simulated storage under temperature abuse conditions can be seen below in Figures 14-16. The initial inocula level on chicken samples were approximately 3.5 log CFU/g. No significant change of *C. perfringens* counts was observed in all control and treated chicken samples stored at 4 °C over the 16-day sampling period. Significant *C. perfringens* outgrowth was first determined in positive control samples stored at 12 °C on day 7 (3.86 log CFU/g) and at 16 °C on day 4 (4.38 log CFU/g). The viable cell count of *C. perfringens* increased gradually in control chicken samples stored at 12 and 16 °C, and reached 5.1 and 5.9 CFU/g, respectively, by the end of the 16-day period. In contrast, chicken samples treated with any of the two concentrations tested for VCE and VLJC had *C. perfringens* levels that never increased above the initial count and at most sampling times slight reductions were observed. At 12 °C, the final count of all four treatments ranged between 2.3 and 2.7 log CFU/g, and at 16 °C the count after 16

days ranged between 2.5 and 3.2 log CFU/g. No significant differences were determined between treatments tested.

An additional set of experiments were conducted at 42 °C, the optimal temperature for *C. perfringens* growth, to evaluate the extent of protection of antimicrobial treatments at the most extreme conditions (Figure 17). Significant *C. perfringens* outgrowth after 10 and 24 h, with final counts of 6.52 and 6.58 log CFU/g, respectively, were measured in chicken samples containing no antimicrobial ingredients. All four treatments resulted in significant inhibition of *C. perfringens* in chicken samples. At 3.0% both VCE and VLJC ingredients caused a reduction in the final count of 1.9 and 2.1 log CFU/g, respectively after 24 h compared to control samples.

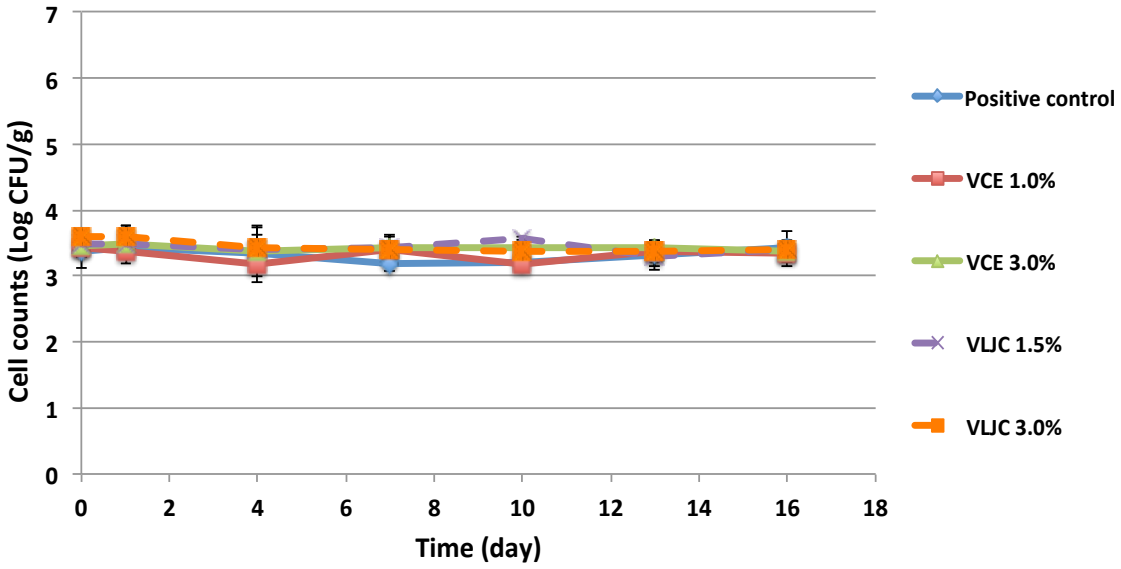


Figure 13. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 4 °C for 16 days.

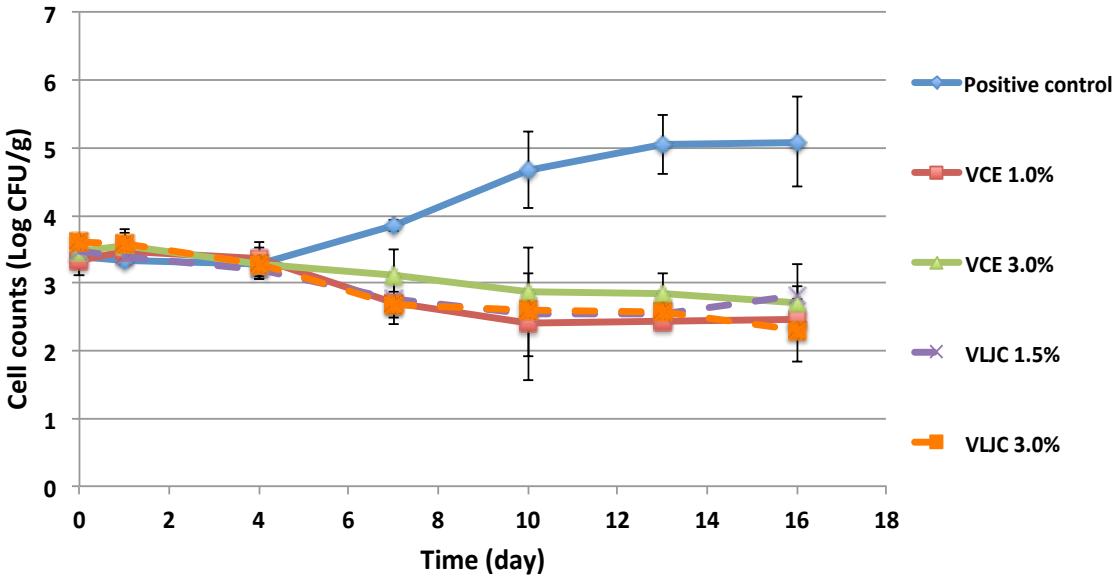


Figure 14. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 12 °C for 16 days.

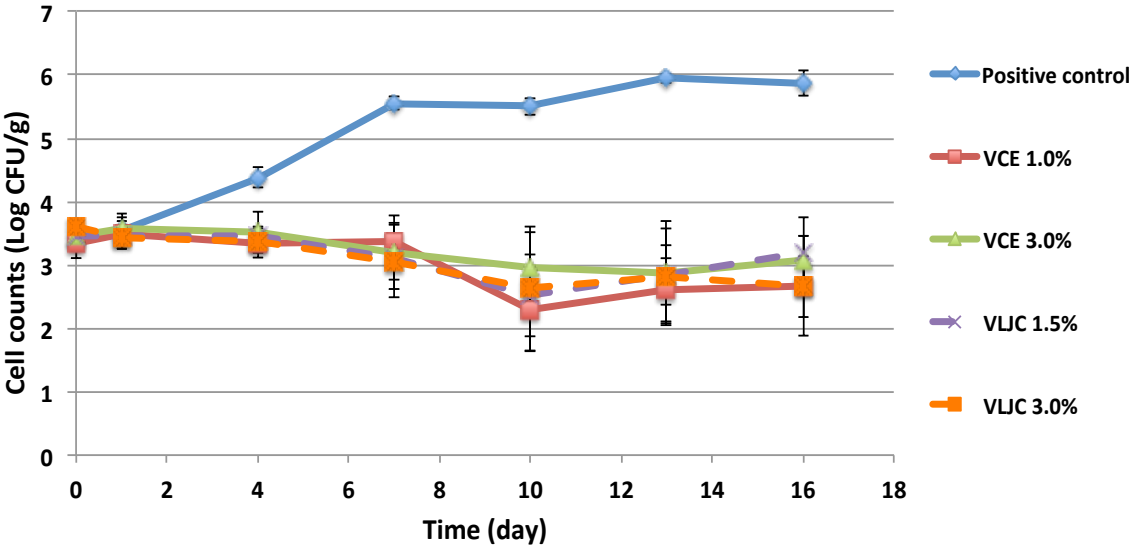


Figure 15. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 16 °C for 16 days.

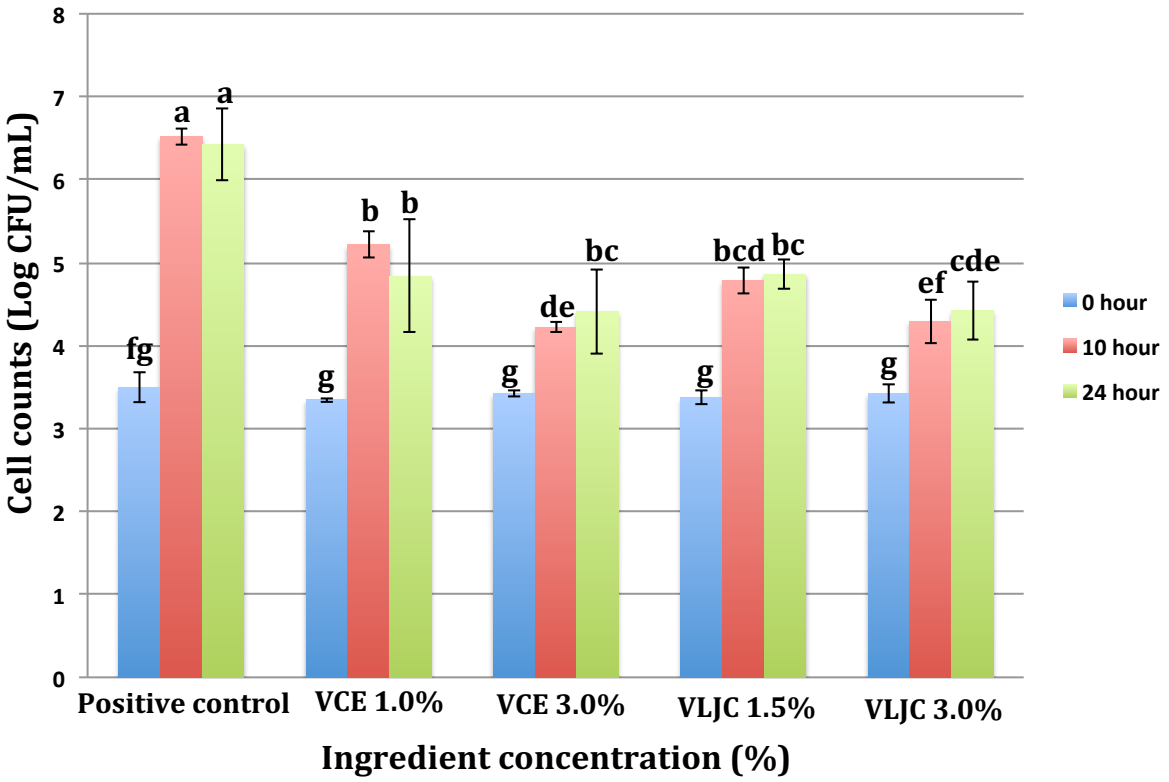


Figure 16. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viability of *C. perfringens* inoculated in sous-vide style cooked chicken breasts stored at 42 °C for 10 and 24 h.

Defective Chilling Trials

The exponential cooling profiles for the defective chilling trials can be found below in Figures 18-20. Chicken samples were chilled from 54.4 °C to 4 °C within 6.5, 12, or 18 h. The results of these cooling experiments can also be found below in Figures 21-23. The initial *C. perfringens* levels were approximately 3.5-log CFU/g. The count of positive control samples chilled within 6.5 h did not increase significantly (3.92-log CFU/g), but all chicken samples with antimicrobial ingredients had *C. perfringens* count reductions of less than 0.5 log CFU/g that were statistically different from control samples. Chilling within 12 h, resulted in

significant growth with control samples having final counts of 4.97 log CFU/g. In contrast, treated samples had significant reductions with final counts ranging from 2.80 to 3.00 log CFU/g compared to controls, but no significance was detected among treatments.

When positive control samples were chilled to 4 °C within 18 h the final *C. perfringens* count reached was 7.21 log CFU/g. Under the conditions, the 1.0% VCE and 1.5% VLJC treatments, *C. perfringens* growth, the final count increased to 4.80 and 5.64 log CFU/g, respectively. At 3.0%, growth was markedly inhibited, and the final average counts of samples treated with VCE and VLJC were 3.76 and 4.35 log CFU/g, respectively. No significant difference was determined between these two treatments.

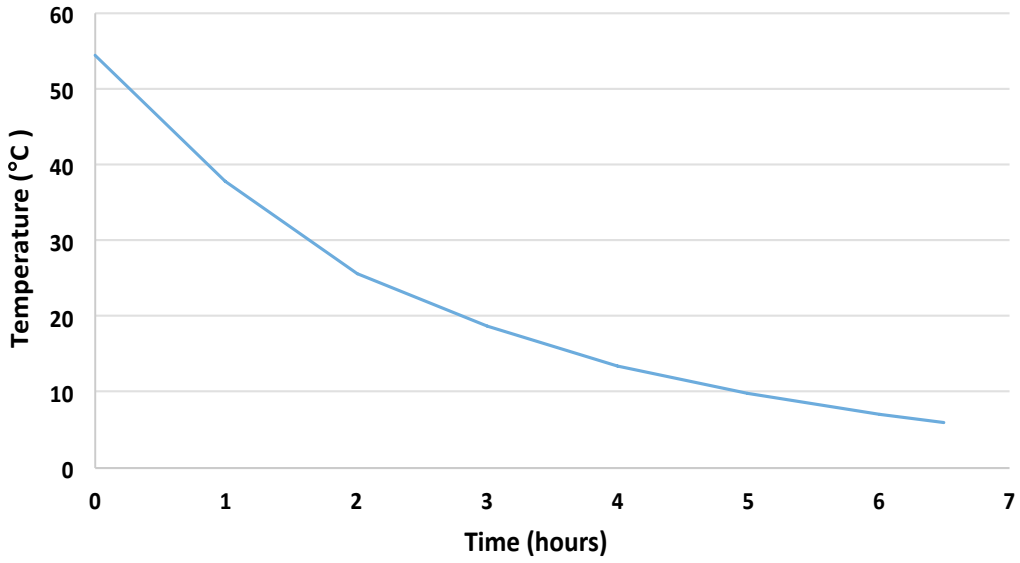


Figure 17. Exponential cooling rate used for defective chilling experiments. Samples chilled from 54.4 °C to 4 °C in 6.5 h.

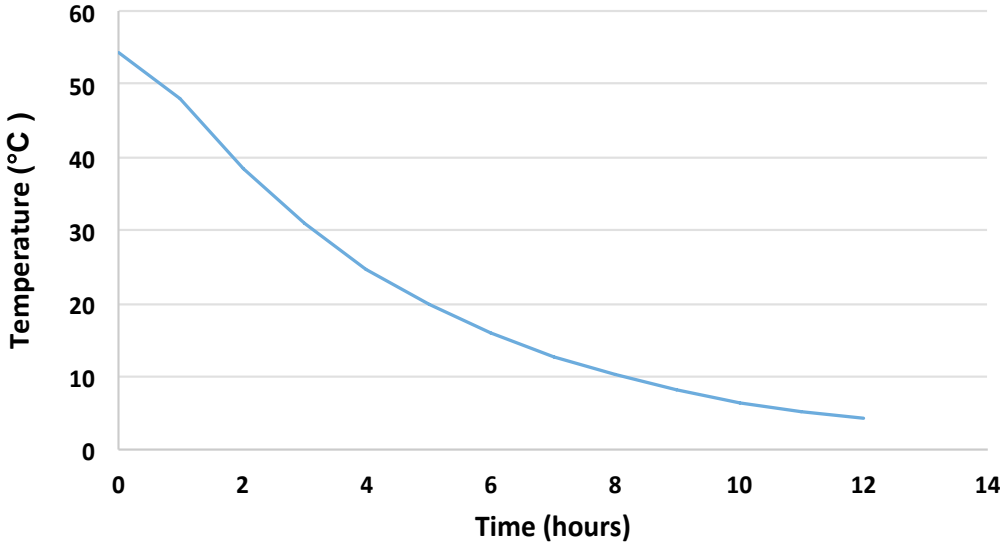


Figure 18. Exponential cooling rate used for defective chilling experiments. Samples chilled from 54.4 °C to 4 °C in 12 h.

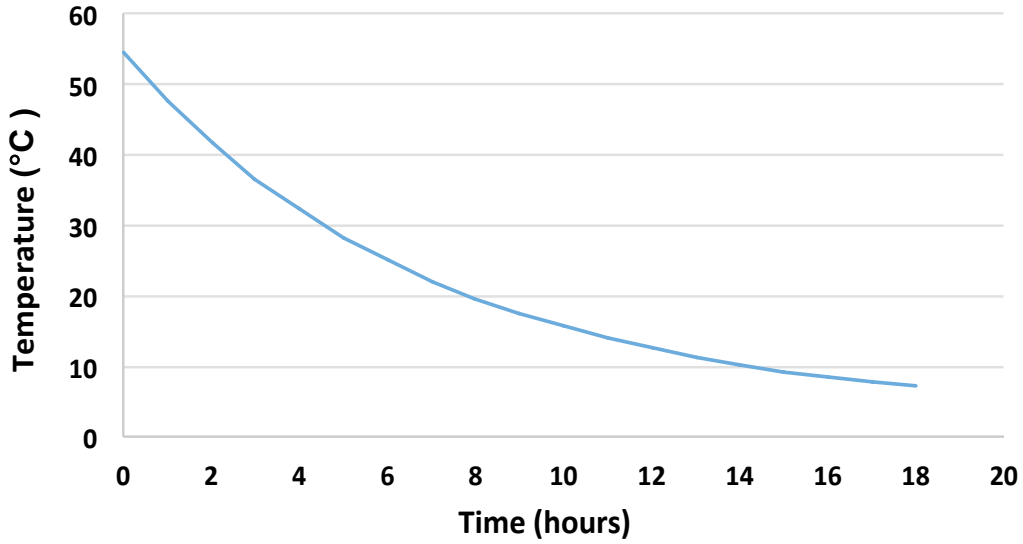


Figure 19. Exponential cooling rate used for defective chilling experiments. Samples chilled from 54.4 °C to 4 °C in 18 h.

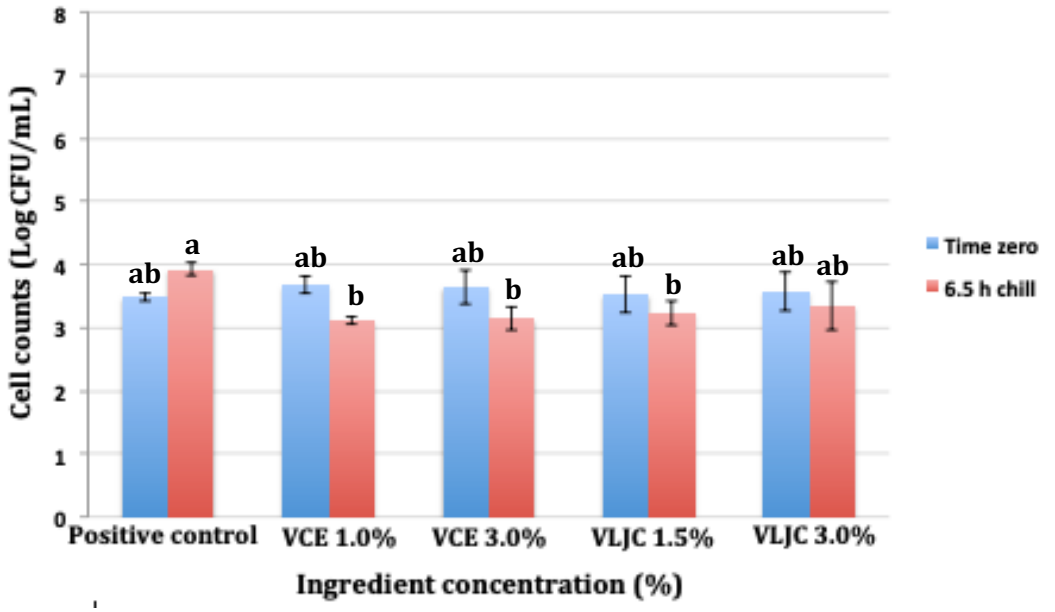


Figure 20. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* in sous-vide style cooked chicken breasts chilled from 54.4 °C to 4 °C in 6.5 h.

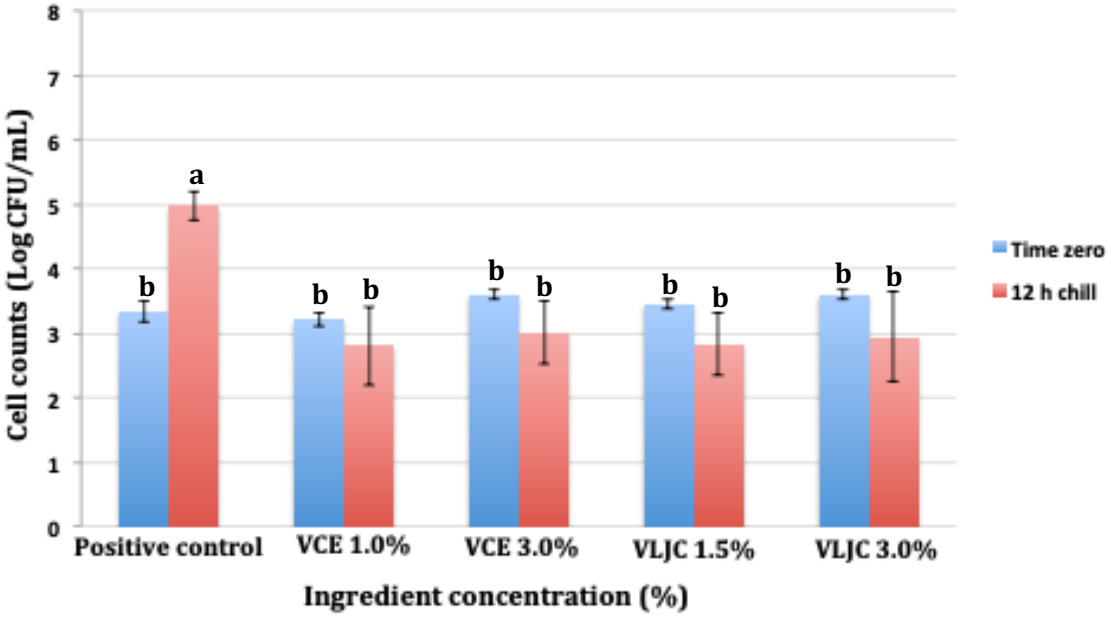


Figure 21. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* in sous-vide style cooked chicken breasts chilled from 54.4 °C to 4 °C in 12 h.

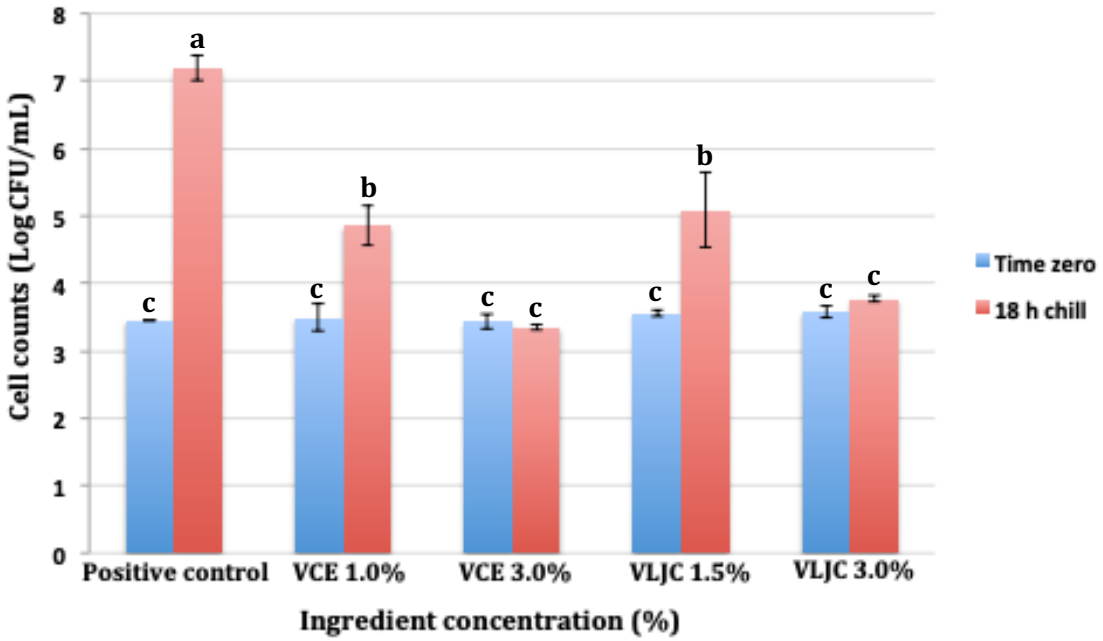


Figure 22. Effect of natural antimicrobial ingredients vinegar and citrus extract (VCE) and vinegar and lemon juice concentrate (VLJC) on the viable count of *C. perfringens* in sous-vide style cooked chicken breasts chilled from 54.4 °C to 4 °C in 18 h.

CHAPTER 5

DISCUSSION

Antimicrobial Screening Comparison

A total of six natural antimicrobial ingredient formulations were tested against *C. perfringens* in pure culture (Table 2). The ingredient preparations of dried vinegar (DV), liquid vinegar and natural flavors (LVNF), and vinegar and cinnamaldehyde (VC) did not markedly inhibit *C. perfringens* growth in FTG media. The findings that DV and VC treatments were not effective at inhibiting *C. perfringens* growth contrast with findings of previously published works.

Cinnamaldehyde has previously been reported to be highly effective at controlling *C. perfringens* in both pure culture and food matrices (Juneja et al. 2006; Juneja and Friedman 2007; Unlu et al. 2010). However, in those studies cinnamaldehyde was tested alone and not in combination with other ingredients as was done in this project. The additional ingredients within the buffered vinegar formulation may be affecting the antimicrobial efficacy of the cinnamaldehyde against the *C. perfringens* strains tested. Another study also reported dried vinegar (0.7%) to be effective at inhibiting *C. perfringens* growth in turkey roast alone and in combination with celery juice powder (50 ppm) (King et al. 2015). These observations differ from the results of the antimicrobial screening of this study that

clearly indicated the absence of inhibition for any of the three *C. perfringens* strains for DV concentrations as high as 3.0% in FTG media.

The buffered liquid vinegar treatment (LV) was used as the base for the formulations of the liquid vinegar and natural flavors (LVNF), vinegar and citrus extract (VCE), and vinegar and lemon juice concentrate (VLJC) treatments. LV was not observed to inhibit *C. perfringens* growth at concentrations smaller than 3.0% for the three strains in FTG media. These findings were similar to results from studies involving the incorporation of a concentrated, buffered vinegar ingredient into a *C. perfringens* inoculated meat product. Two different teams reported significant *C. perfringens* inhibition in roast turkey meat for a buffered vinegar ingredient at concentrations of at least 2.5% and 2.7% (King et al. 2015; Smith et al. 2018). Conversely, treatment with LVNF had no growth inhibition of *C. perfringens* strains. We can speculate that this difference may be due to the added dextrose organoleptic masker included in the LVNF formulation acting as a glucose source for the *C. perfringens* strains, promoting bacterial growth conditions.

From the six possible antimicrobial ingredients screened in this study, two antimicrobial ingredients were capable of complete inhibition of *C. perfringens* growth in FTG media at minimal concentrations. The two treatments were VLJC (1.5%) and VCE (1.0%). These treatments were combinations of vinegar (acetic acid) and a source of citric acid. The exact formulations of the ingredients are unknown due to being proprietor information, but the antimicrobial effect may be due to a synergistic effect from the acetic and citric acids.

The observed greater efficacy of the combinations of both acetic and citric acid opposed to just acetic acid could be attributed to the differences in the proposed modes of action. Acetic acid is thought to exert the majority of its antimicrobial action by the weak acid mechanism of the undissociated molecule. The undissociated molecules of the acid can diffuse through the cellular membrane much easier than the dissociated form, thus liberating more protons in the cell cytoplasm and decreasing intercellular pH. In comparison, citric acid is believed to exert the majority of its antimicrobial action by chelation. Metal chelating compounds have been reported to be highly effective at disrupting *C. perfringens* growth and CPE production, so the combination of both modes of action may be causing a synergistic effect between the acetic and citric acids against *C. perfringens* (Moskowitz et al. 1956; Kang et al. 2003).

A lower MIC for the VCE formulation may be due to the citrus extract component. It is reported that plant extracts are highly variable in their concentrations of active compounds and can include various other natural antimicrobial secondary plant metabolites such as terpenoids and terpenes. In addition, EOs and plant extracts have been reported to be hydrophobic, which enables them to effectively partition the lipids of cellular membranes and render them permeable resulting in leakage of cellular contents (Burt 2004). Organic acids also possess the ability to cross the cellular membrane and acidify the cell cytoplasm, disrupting homeostatic conditions and forcing the microbe to expend energy to pump out protons (Russell and Diez-Gonzalez 1997). Therefore, a

synergistic effect between the two could be creating a heightened antimicrobial effect on *C. perfringens*.

pH and Water Activity Significance

The pH values of all six ingredient formulations at all concentrations tested (0.5-3.0%) in FTG media were taken to determine if antimicrobial activity was due mainly to just a reduction in pH (Tables 3-8) or other means. While all treatments were found to have a statistically significant decrease in pH values when compared to the FTG media control, the practical significance seems to be limited. The pH values of the MICs for VLJC and VCE were 5.91 and 6.17 respectively in FTG media. The growth range of pH for *C. perfringens* is between 5.0 and 9.0 and the optimum growth range is between 6.0 and 7.0 (Ray and Bhunia 2008). Therefore, both treatments are maintaining the suitable pH range of growth for *C. perfringens* and VCE 1.0% is still within the optimum pH range.

This same situation is seen from the pH and a_w readings in chicken meat samples (Table 12). All antimicrobial treatments tested caused statistically significant reductions in both pH and a_w values when compared to control chicken samples, but its potential effect is most likely negligible. Chicken samples with antimicrobial ingredients had pH values between 6.02-6.09 which is still within the optimum pH range for *C. perfringens*. Additionally, treated samples had a_w values between 0.986 and 0.989 which is well above the minimum growth requirement for *C. perfringens* (0.930) (Ray and Bhunia 2008).

Mode of Action Comparison

Natural antimicrobial ingredients VCE 1.5% and VLJC 2.0% proved extremely effective at reducing both *C. perfringens* endospore and vegetative cells counts for all three strains tested. Endospore count reductions ranged from 2 to 4 log CFU/mL and vegetative cell reductions were consistently 5 log CFU/mL in FTG media. These results parallel previous work with similar vinegar and lemon juice ingredient formulations. One study reported 6 to 7 log CFU/g. reductions in *C. perfringens* counts with a 2.0% ingredient concentration and another study reported 3 to 5 log CFU/g reductions from a 3.5% ingredient concentration (Valenzuela-Martinez et al. 2010; Li et al. 2012).

Sporulation Efficiency

Treatments VCE 0.1% and VLJC 0.75% had a significant effect on the sporulation efficiency of the three strains of *C. perfringens* comparable to previous reported results understanding the inhibitory effects of polyphosphates on *C. perfringens*. Akhtar and team reported sodium tripolyphosphate at 0.6% to have approximately a 100-fold decrease in percent sporulation. In comparison, treatments VCE 0.1% and VLJC 0.75% had approximately a 44- to 1,500-fold decrease in sporulation efficiency of the three strains in FTG media. Akhtar also observed that treatments with sodium polyphosphate, tetra sodium pyrophosphate, sodium acid pyrophosphate, and sodium tripolyphosphate at concentrations ranging from 0.9 to 1.3% consistently caused approximately 5 log CFU/mL reductions in in heat-resistant cells when compared to control cultures. Comparatively, treatments VCE 0.1% and VLJC 0.75% reduced heat-resistant cell

counts from approximately 2 to 5 log CFU/mL This result clearly indicates that the natural ingredients affect the sporulation efficiency of *C. perfringens* strains comparatively to traditional polyphosphate ingredients (Akhtar et al. 2008).

Protection Against Temperature-Abuse Storage

Sous vide chicken samples stored at a recommended refrigeration temperature of 4 °C did not have noticeable growth *of C. perfringens* throughout the 16-day storage period. This result was expected because the lowest temperature reported for *C. perfringens* growth is 6 °C (Garcia et al. 2019). Natural antimicrobial ingredients VCE (1.0 and 3.0%) and VLJC (1.5 and 3.0%) were found to be highly effective at controlling *C. perfringens* growth in sous vide chicken breasts exposed to extended temperature abuse conditions (12 and 16 °C). Slight reductions in counts were even observed in treated temperature abuse chicken samples while no such effect was seen in 4 °C storage samples. This suggests the degree of antimicrobial efficacy of these ingredients is affected by temperature. Higher storage temperatures allowed for inhibition and reductions in *C. perfringens* counts possibly due to an increase in metabolic activity of spores and/or to the bactericidal effects of antimicrobial treatments on vegetative cells.

A higher storage temperature provides better conditions for the *C. perfringens* spores to germinate and outgrow than the 4 °C storage samples. Both VCE and VLJC were very effective killing *C. perfringens* strains in pure culture (Figure 12). Additionally, when comparing the positive control chicken samples to the treated samples exposed to temperature abuse on a day-by-day basis, at each time the positive control counts increased while the treated samples had less viable

counts. This suggests that as the positive control samples were germinating and outgrowing the treated samples which followed similar growth pattern, but the ingredients killed the vegetative cells as they were produced. This could mean the main inhibitory activity of these treatments in the chicken model is from the antimicrobial effect on the vegetative cells of *C. perfringens*.

The results of treated chicken samples stored at extreme temperature abuse conditions (42 °C) strongly indicated a significant inhibition in *C. perfringens* counts, but no reductions from initial inocula levels. In contrast from the milder temperature abuse experiments, in the extreme condition experiments a dose-dependent effect of the ingredients can be identified. No significant differences were reported between treatments in the mild temperature abuse trials, but in treated samples stored at 42 °C for 10 h a statistically significant difference is observed in the 3.0% concentrations of both ingredients when compared the lower concentration ingredients. The positive performance of both ingredients at mild and extreme temperature abuse conditions provides evidence these treatments could ensure the safety of a vacuum-sealed chicken product during shipping.

Protection Against Defective Chilling

The efficacy of these ingredients was also explored when deviations from the USDA FSIS stabilization guidelines occur. According to these guidelines, a cooked, vacuum-sealed meat product is considered to be safe from the outgrowth of spore-forming bacteria when the outgrowth of *C. perfringens* is controlled to <1 log CFU/g during the chilling process. Three exponential cooling profiles were created and used (Figures 17-19) to simulate the cooling process a meat product would

undergo in a production plant. A 6.5 h chilling cycle was chosen to explore the maximum limit of the stabilization guidelines (Juneja et al. 2006).

This chilling schedule resulted in no significant differences in counts between time zero and 6.5 h chilled samples. The positive control chicken samples had slight increases in counts, but the increase was <1 log CFU/g. In contrast, treated samples had slight reductions in counts similar to the trend seen in the storage trials. These results corroborate that that accordance the stabilization guidelines are adequate to control the outgrowth of *C. perfringens* in vacuum-sealed chicken products.

The extent of natural antimicrobial ingredients VCE (1.0 and 3.0%) and VLJC (1.5 and 3.0%) effect was elucidated when deviations from the stabilization guidelines were used. Two 12 and 18 h exponential cooling profiles was used for these trials. The *C. perfringens* counts of positive control chicken samples at those two schedules increased substantially by approx. 1.5 and 3.5 log CFU/g, respectively. Conversely, all treated samples chilled for 12 h had slight reductions in counts and would be considered safe according to the stabilization guidelines.

Treatments VCE 1.0% and VLJC 1.5% reduced the final *C. perfringens* counts when compared to positive control samples, but both treatments allowed count increases of approximately 1.5 log CFU/g. Therefore, these treatments were considered not effective in controlling *C. perfringens* growth in a sous vide chicken model chilled in 18 h. Treatments with 3.0% VCE and VLJC controlled *C. perfringens* growth as the initial inocula levels (3.5 log CFU/g.) were maintained after 18 h chilling. Again, a dose-dependent effect is observed in more extreme temperature

abuse conditions. These findings corroborated our previous results that ingredients VCE and VLJC could be utilized in a vacuum-sealed chicken product to effectively control *C. perfringens* when deviations from the FSIS stabilization guidelines occur.

Abusive Storage Comparison

The results from this project correspond to conclusions previously reported from other research teams. The effect of antimicrobial ingredients on the growth of *C. perfringens* in food matrices exposed to extended temperature abuse storage is lacking, but a study conducted by Juneja and team reported success of a grapefruit extract food ingredient within a marinated chicken product. In that study, grapefruit extract (200 ppm) effectively controlled *C. perfringens* growth when samples were stored at 19 and 25 °C for a maximum of 21 and 10.5 h respectively. At the end of 48 h the treated 19 °C samples reached a total increase of 3.03 log CFU/g. and at the end of 16 h the treated samples had a 3.99 log CFU/g. increase in *C. perfringens* counts (Juneja et al. 2006). Additionally, Akhtar and team reported significant inhibition of *C. perfringens* growth in a vacuum-sealed chicken matrix when using various polyphosphates and storage at 37 °C for 12 h. In that publication, sodium tripolyphosphate (0.8 and 1.0%) reduced initial inocula counts of *C. perfringens* by approximately 1 and 1.8 log CFU/g. respectively in chicken samples (Akhtar et al. 2008).

Comparatively, in this project natural treatments VCE and VLJC 3.0% both effectively inhibited *C. perfringens* growth in a vacuum-sealed chicken matrix for a maximum of 24 h at the optimum growth temperature of 42 °C. While sodium tripolyphosphate does seem to be preforming better at a lower concentration, this

ingredient is not considered clean label and can only be used in a processed meat product at a maximum of 0.5%. When compared to the grapefruit extract ingredient (200 ppm) both VCE and VLJC appear to be more effective at controlling *C. perfringens* growth in temperature abused storage conditions. In this project, treatments VCE and VLJC 3.0% only allowed approximately 1 log CFU/g. increases in final counts at the end of the 24 h storage at 42 °C.

Defective Chilling Comparison

The incorporation of antimicrobial ingredients into *C. perfringens* inoculated vacuum-sealed food products exposed to defective chilling conditions has been reported in the literature. Research teams have tested traditional and naturally derived antimicrobial food ingredients against *C. perfringens* in a variety of food matrices. Some of the traditional food preservatives tested were polyphosphates, organic acid salts, sodium nitrite, and sodium erythorbate. A combination of sodium pyrophosphate dibasic and sodium pyrophosphate tetrabasic (0.15% + 0.15%) was reported to be effective at controlling *C. perfringens* spore germination and outgrowth within stabilization guidelines at both a 12 and 18 h exponential cooling regimen as well as calcium, potassium, and sodium lactates ranging from 2.0 to 4.8% in pork meat (Reddy Velugoti et al. 2007; Singh et al. 2010).

Sodium nitrite (200 ppm) and a combination of sodium nitrite (200 ppm) and sodium erythorbate (557 ppm) were also both reported to control *C. perfringens* growth in a ham product exponentially chilled over a 15 h period (Redondo-Solano et al. 2013). Additionally, buffered sodium citrate (1.3%), buffered

sodium citrate (1.3%) plus sodium diacetate (0.8%), sodium lactate (2.5%), and sodium lactate plus sodium diacetate (2.5% total) were all also reported to safely control *C. perfringens* growth in roast beef over 12, 18, and 21 h cooling profiles (Sánchez-Plata et al. 2005).

Comparatively, natural treatments VCE 1.0% and VLJC 1.5% were found to be effective in this project during a 12 h chilling profile and 3.0% concentrations for both were effective during an 18 h chill. These treatments performed similarly to a few of the traditional preservative such as sodium lactate and sodium lactate plus sodium diacetate at near concentrations, but most traditional preservatives inhibited *C. perfringens* growth at relatively lower concentrations. However, the incorporation of these ingredients into a food product would not allow clean labelling.

The antimicrobial activity of various natural antimicrobial has also been assessed against *C. perfringens* during exponential cooling regimens in both beef and turkey. Essential oils such as thymol, cinnamaldehyde, oregano oil, and carvacrol at 0.1% were reported to safely control *C. perfringens* growth in a beef product over 12 h exponential cooling. Additionally, thymol (2.0%), cinnamaldehyde (0.5%), oregano oil (2.0%), and carvacrol (2.0%) also inhibited *C. perfringens* growth during an 18 h cooling profile (Juneja et al. 2006). A buffered concentrated vinegar product and a vinegar and lemon juice concentrate product was also reported to effectively control *C. perfringens* growth for 12 h chilling at 2.5% for both and for 18 h chilling for a 2.5 and a 3.5% respectively in turkey (Valenzuela-Martinez et al. 2010). The results from this project parallel those findings that the natural ingredients between

approximately 2.0 to 3.0% concentrations are effective at inhibiting *C. perfringens* growth during defective cooling scenarios.

CHAPTER 6

CONCLUSION

Current consumer preferences for both clean label food ingredients and convenience-based foods has provided a unique opportunity to explore the application of novel natural food preservatives in sous vide products. The anaerobic environment and relatively low thermal processing which is characteristic of the sous vide process creates a favorable scenario for the survival, germination, and outgrowth of spore-forming bacteria such as *C. botulinum* and *C. perfringens*. The germination and outgrowth of *C. perfringens* in vacuum-sealed and sous vide style cooked meats has been reported in multiple studies when the foods were exposed to temperature abuse conditions (Juneja et al. 1994, 2006a; Taormina and Dorsa 2004; Singh et al. 2010; Redondo-Solano et al. 2013). An estimated 80% of foodborne *C. perfringens* cases have been traced to temperature abuse conditions either in the form of improper holding/storage conditions or improper cooling of cooked products (Doyle 2002). Therefore, the scope of this project was to identify effective novel natural ingredient formulations against *C. perfringens*, understand the antimicrobial activity, and implement the ingredients within a vacuum-sealed sous vide chicken model exposed to both abusive storage and abusive chilling conditions.

Ingredients VCE (1.0%) and VLJC (1.5%) were identified as the most effective at inhibiting *C. perfringens* at in pure culture. Treatments with these ingredients resulted in significant reductions ($p \leq 0.05$) in both endospore and

vegetative cell counts for the three strains tested as well as significant ($p \leq 0.05$) reductions in sporulation efficiency. Once incorporated into a chicken meat matrix VCE (1.0 and 3.0%) and VLJC (1.5 and 3.0%) controlled *C. perfringens* growth ($p \leq 0.05$) during extended storage temperature conditions (12 and 16 °C) over 16 days and even at extreme conditions (42 °C) for 24 h. VCE 1.0% and VLJC 1.5% effectively controlled *C. perfringens* outgrowth within the USDA FSIS stabilization guidelines during a 12 h defective chilling profile and both VCE and VLJC 3.0% inhibited growth during an 18 h cooling profile. Overall, treatments VCE and VLJC could be implemented within the formulation of a sous vide chicken product to provide an extra layer of protection against *C. perfringens* while maintaining a clean label.

Future Research

Additional experiments could be conducted to help elucidate the extent the citrus extract and lemon juice concentrate components of the ingredient formulations are having on the inhibition of the *C. perfringens* strains. For example, creating formulations with no vinegar and just either citrus extract or lemon juice concentrate and comparing the results could provide additional insight. Experiments utilizing *C. botulinum* could also be performed to see if these ingredients could ensure protection from anaerobes beyond *C. perfringens*.

REFERENCES

Adams DM (1973) Inactivation of *Clostridium perfringens* type A spores at ultrahigh temperatures. Applied Microbiology 26:282–287.

Akhtar S, Paredes-Sabja D, Sarker MR (2008) Inhibitory effects of polyphosphates on *Clostridium perfringens* growth, sporulation and spore outgrowth. Food Microbiology 25:802–808. https://doi.org/10.1016/j.fm.2008.04.006

Allaart JG, van Asten AJAM, Gröne A (2013) Predisposing factors and prevention of *Clostridium perfringens*-associated enteritis. Comparative Immunology, Microbiology and Infectious Diseases 36:449–464.

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils - A review. Food and Chemical Toxicology 46:446–475.

Baldwin DE (2012) Sous vide cooking: A review. International Journal of Gastronomy and Food Science 1:15–30. https://doi.org/10.1016/J.IJGFS.2011.11.002

Burt S (2004) Essential oils: Their antibacterial properties and potential applications in foods - A review. International Journal of Food Microbiology 94:223–253.

Calvo MS, Moshfegh AJ, Tucker KL (2014) Assessing the health impact of phosphorus in the food supply: issues and considerations. Advances in Nutrition 5:104–113. https://doi.org/10.3945/an.113.004861

Chai SJ, Cole D, Nisler A, Mahon BE (2017) Poultry: The most common food in outbreaks with known pathogens, United States, 1998-2012. Epidemiology and Infection 145:316–325. https://doi.org/10.1017/S0950268816002375

Chakrabarti G, McClane BA (2005) The importance of calcium influx, calpain and calmodulin for the activation of CaCo-2 cell death pathways by *Clostridium perfringens* enterotoxin. Cellular Microbiology 7:129–146. https://doi.org/10.1111/j.1462-5822.2004.00442.x

Charlebois A, Jacques M, Archambault M (2014) Biofilm formation of *Clostridium perfringens* and its exposure to low-dose antimicrobials. Frontiers in Microbiology 5:. https://doi.org/10.3389/fmicb.2014.00183

Cosansu S, Juneja VK (2018) Growth of *Clostridium perfringens* in sous vide cooked ground beef with added grape seed extract. Meat Science 143:252–256. https://doi.org/10.1016/j.meatsci.2018.05.013

Cosansu S, Juneja VK, Osoria M, Mukhopadhyay S (2019) Effect of grape seed extract on heat resistance of *Clostridium perfringens* vegetative cells in sous vide processed ground beef. Food Research International 120:33–37. https://doi.org/10.1016/J.FOODRES.2019.02.014

Cotter PD, Hill C, Ross PR (2005) Bacteriocins: developing innate immunity for food. Nature Reviews Microbiology 3:777–788. https://doi.org/10.1038/nrmicro1240

Craven SE, Cox NA, Bailey JS, Cosby DE (2003) Incidence and tracking of *Clostridium perfringens* through an integrated broiler chicken operation. Avian Diseases 47:707–711. https://doi.org/10.1637/6010

Craven SE, Stern NJ, Bailey JS, Cox NA (2001) Incidence of *Clostridium perfringens* in broiler chickens and their environment during production and processing. Avian Diseases 45:887–96

Creed PG (1995) The sensory and nutritional quality of 'sous vide' foods. Food Control 6:45–52. https://doi.org/10.1016/0956-7135(95)91453-R

Dahle HK (1979) Nitrite as a food additive. NIPH annals 2:17–24.

Daniel CR, Cross AJ, Koebnick C, Sinha R (2011) Trends in meat consumption in the USA. Public Health Nutrition 14:575–583. https://doi.org/10.1017/S1368980010002077

Davidson PM, Critzer FJ, Taylor TM (2013) Naturally occurring antimicrobials for minimally processed foods. Annual Review of Food Science and Technology 4:163–190. https://doi.org/10.1146/annurev-food-030212-182535

di Pasqua R, Hoskins N, Betts G, Mauriello G (2006) Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. Journal of Agricultural and Food Chemistry 54:2745–2749. https://doi.org/10.1021/jf052722l

Doyle E (2002) Survival and growth of *Clostridium perfringens* during the cooling step of thermal processing of meat products. A review of the scientific literature. Food Research Institute. https://www.meatinstitute.org/index.php?ht=a/GetDocumentAction/i/1442

Duncan CL (1973) Time of enterotoxin formation and release during sporulation of *Clostridium perfringens* type A. Journal of Bacteriology 113:932–6.

Duncan CL, Strong DH (1968) Improved medium for sporulation of *Clostridium perfringens*. Applied Microbiology 16:82–9.

Elizondo AM, Mercado EC, Rabinovitz BC, Fernandez-Miyakawa ME (2010) Effect of tannins on the in vitro growth of *Clostridium perfringens*. Veterinary Microbiology 145:308–314. https://doi.org/10.1016/j.vetmic.2010.04.003

Errington J (2003) Regulation of endospore formation in *Bacillus subtilis*. Nature Reviews Microbiology 1:117–126. https://doi.org/10.1038/nrmicro750

Gao Y, Qiu W, Wu D, Fu Q (2011) Assessment of *Clostridium perfringens* spore response to high hydrostatic pressure and heat with nisin. Applied Biochemistry and Biotechnology 164:1083–1095. https://doi.org/10.1007/s12010-011-9196-0

Garcia S, Vidal JE, Heredia N, Juneja VK (2019) Fundamentals and Frontiers *Clostridum* Chapter. In: Doyle MP, Diez-Gonzalez F, Hill C (eds) Food Microbiology Fundamentals and Frontiers, 5th edn. ASM Press, Washington, D.C., pp 513–540.

Gaucher M-L, Thibodeau A, Fravalo P, et al (2018) Broiler chicken carcasses and their associated abattoirs as a source of enterotoxigenic *Clostridium perfringens*: Prevalence and critical steps for contamination. AIMS Microbiology 4:439–454. https://doi.org/10.3934/microbiol.2018.3.439

Gomes RJ, Borges M de F, Rosa M de F, et al (2018) Acetic acid bacteria in the food industry: systematics, characteristics and applications. Food Technology and Biotechnology 56:139–151.

Higgins D, Dworkin J (2012) Recent progress in *Bacillus subtilis* sporulation. FEMS Microbiology Reviews 36:131–148.

Jiang M, Shao W, Perego M, Hoch JA (2000) Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. Molecular Microbiology 38:535–542. https://doi.org/10.1046/j.1365-2958.2000.02148.x

Juneja VK (2006) Delayed *Clostridium perfringens* growth from a spore inocula by sodium lactate in sous-vide chicken products. Food Microbiology 23:105–111. https://doi.org/10.1016/J.FM.2005.03.002

Juneja VK, Bari ML, Inatsu Y, et al (2007) Control of *Clostridium perfringens* spores by green tea leaf extracts during cooling of cooked ground beef, chicken, and pork. Journal of Food Protection 70:1429–33.

Juneja VK, Call JE, Marmer BS, Miller AJ (1994) The effect of temperature abuse on *Clostridium perfringens* in cooked turkey stored under air and vacuum. Food Microbiology 11:187–193. https://doi.org/10.1006/FMIC.1994.1022

Juneja VK, Call JE, Miller AJ (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 2:203–218. https://doi.org/10.1111/j.1745-4581.1993.tb00290.x

Juneja VK, Dwivedi HP, Yan X (2012) Novel natural food antimicrobials. Annual Review of Food Science and Technology 3:381–403. https://doi.org/10.1146/annurev-food-022811-101241

Juneja VK, Fan X, Peña-Ramos A, et al (2006) The effect of grapefruit extract and temperature abuse on growth of *Clostridium perfringens* from spore inocula in

marinated, sous-vide chicken products. Innovative Food Science and Emerging Technologies 7:100–106. https://doi.org/10.1016/j.ifset.2005.09.004

Juneja VK, Friedman M (2007) Carvacrol, cinnamaldehyde, oregano oil, and thymol inhibit *Clostridium perfringens* spore germination and outgrowth in ground turkey during chilling. Journal of Food Protection 70:218–222. https://doi.org/10.4315/0362-028X-70.1.218

Juneja VK, Marks H, Huang L, Thippareddi H (2011) Predictive model for growth of *Clostridium perfringens* during cooling of cooked uncured meat and poultry. Food Microbiology 28:791–795. https://doi.org/10.1016/j.fm.2010.05.013

Juneja VK, Marmer BS (1996) Growth of *Clostridium perfringens* from spore inocula in sous-vide turkey products. International Journal of Food Microbiology 32:115–23.

Juneja VK, Thippareddi H, Friedman M (2006) Control of *Clostridium perfringens* in cooked ground beef by carvacrol, cinnamaldehyde, thymol, or oregano oil during chilling. Journal of Food Protection 69:1546–51.

Kang S, Jang A, Lee SO, et al (2003) Effect of organic acids on microbial populations and *Salmonella* Typhimurium in pork loins. Asian-Australasian Journal of Animal Sciences 16:96–99. https://doi.org/10.5713/ajas.2003.96

King AM, Glass KA, Milkowski AL, Sindelar JJ (2015) Impact of clean-label antimicrobials and nitrite derived from natural sources on the outgrowth of *Clostridium perfringens* during cooling of deli-style turkey breast. Journal of Food Protection 78:946–953. https://doi.org/10.4315/0362-028X.JFP-14-503

Knabel SJ (1989) Effects of polyphosphates and heat on selected Gram-positive bacteria important in foods. Retrospective Theses and Dissertations. 9141. https://lib.dr.iastate.edu/rtd/9141

Krawczyk AO, de Jong A, Omony J, et al (2017) Spore heat activation requirements and germination responses correlate with sequences of germinant receptors and with the presence of a specific *spoVA* ^{2mob} operon in foodborne strains of *Bacillus subtilis*. Applied and Environmental Microbiology 83:. https://doi.org/10.1128/AEM.03122-16

Labbe RG, Garcia S (2001) Guide to Foodborne Pathogens. John Wiley & Sons, Ltd (10.1111), New York, New York.

Li L, Valenzuela-Martinez C, Redondo M, et al (2012) Inhibition of *Clostridium perfringens* spore germination and outgrowth by lemon juice and vinegar product in reduced NaCl roast beef. Journal of Food Science 77:M598–M603. https://doi.org/10.1111/j.1750-3841.2012.02922.x

Long NHBS, Gál R, Buňka F (2011) Use of phosphates in meat products. African Journal of Biotechnology 10:19874–19882. https://doi.org/10.5897/AJBX11.023

Lorencová E, Vltavská P, Budinsk P, Koutn M (2012) Antibacterial effect of phosphates and polyphosphates with different chain length. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering 47:2241–2245. https://doi.org/10.1080/10934529.2012.707544

Lucera A, Costa C, Conte A, del Nobile MA (2012) Food applications of natural antimicrobial compounds. Frontiers in Microbiology 3:287. https://doi.org/10.3389/fmicb.2012.00287

Ludwig W, Schleifer K-H, Whitman WB (2009) Revised road map to the phylum *Firmicutes*. In: Systematic Bacteriology. Springer New York, pp 1–13.

Majou D, Christieans S (2018) Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats. Meat Science 145:273–284.

Mascaretti OA (2014) Bacterial pathogenesis. in: bacteria versus antibacterial agents. American Society of Microbiology, pp 33–42.

McClane BA, McDonel JL (1980) Characterization of membrane permeability alterations induced in vero cells by *Clostridium perfringens* enterotoxin. Biochimica et Biophysica acta 600:974–85. https://doi.org/10.1016/0005-2736(80)90499-x

Miwa N, Nishina T, Kubo S, et al (1998) Amount of enterotoxigenic *Clostridium perfringens* in meat detected by nested PCR. International Journal of Food Microbiology 42:195–200. https://doi.org/10.1016/S0168-1605(98)00082-8

Miwa N, Nishina T, Kubo S, Honda H (1997) Most probable numbers of enterotoxigenic *Clostridium perfringens* in intestinal contents of domestic livestock detected by nested PCR. Journal of Veterinary Medical Science 59:557–560. https://doi.org/10.1292/jvms.59.557

Molle V, Fujita M, Jensen ST, et al (2003) The Spo0A regulon of *Bacillus subtilis*. Molecular Microbiology 50:1683–1701. https://doi.org/10.1046/j.1365-2958.2003.03818.x

Monk JD, Beuchat LR, Doyle MP (1995) Irradiation inactivation of food-borne microorganisms. Journal of Food Protection 58:197–208. https://doi.org/10.4315/0362-028X-58.2.197

Montville TJ, Matthews KR, Kniel KE (2012) Food Micro Intro Clostridium Chapter. In: Food Microbiology An Introduction, 3rd edn. ASM Press, Washington, D.C., pp 160–169.

Moskowitz M, Deverell MW, Mckinney R (1956) Protection against *Clostridium perfringens* type A toxin by a metal-chelating compound. Science 123:1077–1078.

Painter JA, Hoekstra RM, Ayers T, et al (2013) Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. Emerging Infectious Diseases 19:407–415. https://doi.org/10.3201/eid1903.111866

Paredes-Sabja D, Gonzalez M, Sarker MR, Torres JA (2007) Combined effects of hydrostatic pressure, temperature, and pH on the inactivation of spores of *Clostridium perfringens* type A and *Clostridium sporogenes* in buffer solutions. Journal of Food Science 72:. https://doi.org/10.1111/j.1750-3841.2007.00423.x

Paredes-Sabja D, Shen A, Sorg JA (2014) *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. Trends in Microbiology 22:406–416.

Ray B, Bhunia A (2008) Fundamental Food Micro Clostridium Chapter. In: Fundamental Food Microbiology, 4th edn. CRC Press, Boca Raton, FL, pp 315–326.

Rea MC, Clayton E, O'Connor PM, et al (2007) Antimicrobial activity of lacticin 3147 against clinical *Clostridium difficile* strains. Journal of Medical Microbiology 56:940–946. https://doi.org/10.1099/jmm.0.47085-0

Reddy Velugoti P, Rajagopal L, Juneja V, Thippareddi H (2007) Use of calcium, potassium, and sodium lactates to control germination and outgrowth of *Clostridium perfringens* spores during chilling of injected pork. Food Microbiology 24:687–694. https://doi.org/10.1016/j.fm.2007.04.004

Redondo-Solano M, Valenzuela-Martinez C, Cassada DA, et al (2013) Effect of meat ingredients (sodium nitrite and erythorbate) and processing (vacuum storage and packaging atmosphere) on germination and outgrowth of *Clostridium perfringens* spores in ham during abusive cooling. Food Microbiology 35:108–115. https://doi.org/10.1016/j.fm.2013.02.008

Regan CM, Syed Q, Tunstall PJ (1995) A hospital outbreak of *Clostridium perfringens* food poisoning--implications for food hygiene review in hospitals. The Journal of Hospital Infection 29:69–73.

Rood JI, Adams V, Lacey J, et al (2018) Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe 53:5–10. https://doi.org/10.1016/j.anaerobe.2018.04.011

Rouger A, Tresse O, Zagorec M (2017) Bacterial contaminants of poultry meat: sources, species, and dynamics. Microorganisms 5:50. https://doi.org/10.3390/microorganisms5030050

Ruhr E, Sahl HG (1985) Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrobial Agents and Chemotherapy 27:841–845. https://doi.org/10.1128/AAC.27.5.841

Russell JB, Diez-Gonzalez F (1997) The effects of fermentation acids on bacterial growth. Advances in Microbial Physiology 39:205–234. https://doi.org/10.1016/S0065-2911(08)60017-X

Sánchez-Plata MX, Amézquita A, Blankenship E, et al (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 68:2594–605.

Sarker MR, Akhtar S, Torres JA, Paredes-Sabja D (2015) High hydrostatic pressure-induced inactivation of bacterial spores. Critical Reviews in Microbiology 41:18–26.

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30:3875–3883.

Scallan E, Hoekstra RM, Angulo FJ, et al (2011) Foodborne illness acquired in the United States--major pathogens. Emerging Infectious Diseases 17:7–15. https://doi.org/10.3201/eid1701.091101p1

Scannell AGM, Ross RP, Hill C, Arendt EK (2000) An effective lacticin biopreservative in fresh pork sausage. Journal of Food Protection 63:370–375. https://doi.org/10.4315/0362-028X-63.3.370

Setlow P (2014) Germination of spores of *Bacillus* species: what we know and do not know. Journal of Bacteriology 196:1297–305. https://doi.org/10.1128/JB.01455-13

Setlow P (2003) Spore germination. Current Opinion in Microbiology 6:550–556.

Setlow P, Johnson EA (2019) Food Micro Fundamentals and Frontiers Spore Chapter. In: Doyle MP, Diez-Gonzalez F, Hill C (eds) Food Microbiology Fundamentals and Frontiers, 5th edn. ASM Press, Washington, D.C., pp 23–63.

Shane SM, Koetting DG, Harrington KS (1984) The occurrence of *Clostridium perfringens* in the intestine of chicks. Avian Diseases 28:1120–4.

Shelef LA (1994) Antimicrobial effects of lactates: A review. Journal of Food Protection 57:445–450. https://doi.org/10.4315/0362-028X-57.5.445

Shin JM, Gwak JW, Kamarajan P, et al (2016) Biomedical applications of nisin. Journal of Applied Microbiology 120:1449–1465.

Singh A, Korasapati NR, Juneja VK, Thippareddi H (2010) Effect of phosphate and meat (pork) types on the germination and outgrowth of *Clostridium perfringens* spores during abusive chilling. Journal of Food Protection 73:879–887. https://doi.org/10.4315/0362-028X-73.5.879

Singh M, Thippareddi H, Wang L, Balamurugan S (2019) Food Micro Fundamentals and Frontiers Meat and Poultry Chapter. In: Doyle MP, Diez-Gonzalez F, Hill C

(eds) Food Microbiology Fundamentals and Frontiers, 5th edn. ASM Press, Washington, D.C., pp 125–177.

Skjelkvale R, Uemura T (1977) Experimental diarrhoea in human volunteers following oral administration of *Clostridium perfringens* enterotoxin. Journal of Applied Bacteriology 43:281–286. https://doi.org/10.1111/j.1365-2672.1977.tb00752.x

Smedley JG, Fisher DJ, Sayeed S, et al (2004) The enteric toxins of *Clostridium perfringens*. Reviews of Physiology, Biochemistry and Pharmacology 152:183–204. https://doi.org/10.1007/s10254-004-0036-2

Smith AM, Dunn ML, Jefferies LK, et al (2018) Inhibition of *Clostridium perfringens* growth during extended cooling of cooked uncured roast turkey and roast beef using a concentrated buffered vinegar product and a buffered vinegar product. Journal of Food Protection 81:461–466. https://doi.org/10.4315/0362-028X.JFP-17-274

Stephenson K, Hoch JA (2002) Evolution of signaling in the sporulation phosphorelay. Molecular Microbiology 46:297–304.

Talukdar PK, Udompijitkul P, Hossain A, Sarker MR (2017) Inactivation Strategies for *Clostridium perfringens* spores and vegetative Cells. Applied and Environmental Microbiology 83:. https://doi.org/10.1128/AEM.02731-16

Taormina PJ, Dorsa WJ (2004) Growth potential of *Clostridium perfringens* during cooling of cooked meats. Journal of Food Protection 67:1537–47. https://doi.org/10.4315/0362-028x-67.7.1537

Tirado C, Schmidt K (2001) WHO surveillance programme for control of foodborne infections and intoxications: preliminary results and trends across greater Europe. In: Journal of Infection. W.B. Saunders Ltd, pp 80–84.

Titball RW, Naylor CE, Basak AK (1999) The *Clostridium perfringens* α-toxin. Anaerobe 5:51–64.

Tiwari BK, Valdramidis VP, O' Donnell CP, et al (2009) Application of natural antimicrobials for food preservation. Journal of Agricultural and Food Chemistry 57:5987–6000. https://doi.org/10.1021/jf900668n

Udompijitkul P, Paredes-Sabja D, Sarker MR (2012) Inhibitory Effects of Nisin Against *Clostridium perfringens* Food Poisoning and Nonfood-Borne Isolates. Journal of Food Science 77:M51–M56. https://doi.org/10.1111/j.1750-3841.2011.02475.x

Unlu M, Ergene E, Unlu GV, et al (2010) Composition, antimicrobial activity and in vitro cytotoxicity of essential oil from *Cinnamomum zeylanicum Blume (Lauraceae*). Food and Chemical Toxicology 48:3274–3280. https://doi.org/10.1016/j.fct.2010.09.001

Uzal FA, Freedman JC, Shrestha A, et al (2014) Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. Future Microbiology 9:361–377.

Uzal FA, Vidal JE, McClane BA, Gurjar AA (2010) *Clostridium perfringens* toxins involved in mammalian veterinary diseases. The Open Toxinology Journal 2:24–42.

Valenzuela-Martinez C, Pena-Ramos A, Juneja VK, et al (2010) Inhibition of *Clostridium perfringens* spore germination and outgrowth by buffered vinegar and lemon juice concentrate during chilling of ground turkey roast containing minimal ingredients. Journal of Food Protection 73:470–6.

van Immerseel F, de Buck J, Pasmans F, et al (2004) *Clostridium perfringens* in poultry: An emerging threat for animal and public health. Avian Pathology 33:537–549.

Wang G, Paredes-Sabja D, Sarker MR, et al (2012) Effects of wet heat treatment on the germination of individual spores of *Clostridium perfringens*. Journal of Applied Microbiology 113:824–836. https://doi.org/10.1111/j.1365-2672.2012.05387.x

Yang S-C, Lin C-H, Sung CT, Fang J-Y (2014) Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Frontiers in Microbiology 5:. https://doi.org/10.3389/fmicb.2014.00241

Appendix A

Antimicrobial Screening and Pure Culture pH Measurements

Table A1. *C. perfringens* strain 8239 antimicrobial screening data in FTG media. Individual OD measurements were taken after 24 h and were done three times independently and three replicates each.

Ingredient	Concentration (%)	Optical density (OD_{600}) of individual wells									Average	STDEV
LV	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.692	0.785	0.815	0.643	0.712	0.681	0.633	0.719	0.780	0.718	0.06
	1.0	0.870	0.693	0.701	0.805	0.767	0.677	0.577	0.658	0.638	0.710	0.09
	1.5	0.640	0.712	0.716	0.696	0.645	0.668	0.655	0.619	0.760	0.679	0.05
	2.0	0.797	0.567	0.634	0.649	0.518	0.670	0.546	0.499	0.621	0.611	0.09
	2.5	0.565	0.542	0.584	0.549	0.602	0.571	0.357	0.528	0.596	0.544	0.07
	3.0	0.213	0.237	0.207	0.224	0.199	0.217	0.302	0.116	0.124	0.204	0.06
LVNF	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.864	0.778	0.713	0.638	0.727	0.804	0.709	0.692	0.538	0.718	0.09
	1.0	0.789	0.715	0.956	0.728	0.725	0.788	0.833	0.782	0.488	0.756	0.124
	1.5	0.675	0.854	0.672	0.637	0.679	0.613	0.680	0.491	0.405	0.634	0.127
	2.0	0.592	0.754	1.078	0.594	0.605	0.772	0.507	0.516	0.461	0.653	0.191
	2.5	0.597	0.742	1.258	0.649	0.533	0.569	0.613	0.379	0.494	0.648	0.250
	3.0	1.210	1.108	1.179	0.335	0.311	0.303	0.310	0.312	0.247	0.591	0.432
VCE	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.496	0.589	0.442	0.621	0.712	0.764	0.131	0.072	0.061	0.432	0.276
	1.0	0.067	0.069	0.070	0.080	0.078	0.079	0.075	0.076	0.077	0.072	0.004
	1.5	0.076	0.069	0.070	0.080	0.078	0.079	0.075	0.076	0.077	0.076	0.004
	2.0	0.076	0.084	0.075	0.080	0.080	0.082	0.080	0.089	0.079	0.081	0.004
	2.5	0.084	0.074	0.078	0.083	0.083	0.082	0.080	0.081	0.082	0.081	0.003

	3.0	0.083	0.083	0.084	0.092	0.091	0.089	0.084	0.085	0.083	0.086	0.004
VLJC	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.677	0.836	0.366	0.831	1.054	0.741	0.533	0.314	0.507	0.651	0.242
	1.0	0.143	0.311	0.469	0.858	0.102	1.240	0.400	0.471	0.387	0.487	0.357
	1.5	0.091	0.088	0.091	0.085	0.096	0.103	0.096	0.098	0.091	0.093	0.006
	2.0	0.096	0.101	0.095	0.108	0.101	0.099	0.107	0.101	0.107	0.102	0.005
	2.5	0.096	0.099	0.098	0.101	0.105	0.105	0.110	0.114	0.106	0.104	0.006
	3.0	0.117	0.114	0.117	0.119	0.125	0.119	0.123	0.121	0.117	0.119	0.003
VC	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.852	1.297	0.504	0.894	0.801	0.725	0.673	0.713	0.789	0.805	0.217
	1.0	1.168	0.694	0.895	0.884	0.771	0.762	0.607	0.740	0.679	0.800	0.166
	1.5	1.014	0.804	0.911	0.640	0.762	0.767	0.605	0.664	0.511	0.742	0.157
	2.0	0.625	0.628	0.573	0.856	0.773	0.674	0.730	0.533	0.635	0.670	0.101
	2.5	0.503	0.579	0.867	0.648	0.607	0.609	0.651	0.435	0.509	0.601	0.123
	3.0	0.471	0.843	0.699	0.462	0.656	0.574	0.437	0.480	0.564	0.576	0.135
DV	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.838	0.787	0.742	0.712	0.737	0.835	0.637	0.701	0.690	0.742	0.067
	1.0	0.962	0.639	0.647	0.690	0.802	0.756	0.593	0.578	0.530	0.689	0.134
	1.5	0.724	1.296	0.732	0.652	0.660	0.549	0.525	0.474	0.419	0.670	0.259
	2.0	0.394	0.495	0.558	0.449	0.471	0.468	0.418	0.439	0.416	0.456	0.049
	2.5	0.533	0.919	0.073	0.506	0.426	0.637	0.355	0.359	0.346	0.462	0.233
	3.0	0.371	0.441	0.406	0.355	0.432	0.434	0.378	0.382	0.352	0.395	0.035

Table A2. *C. perfringens* strain 10240 antimicrobial screening data in FTG media. Individual OD measurements were taken after 24 h and were done three times independently and three replicates each.

Ingredient	Concentration (%)	Optical density (OD_{600}) of individual wells									Average	STDEV
LV	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.657	0.646	0.474	0.686	0.539	0.783	0.808	0.488	0.330	0.601	0.156
	1.0	0.659	0.892	0.798	0.820	0.840	0.714	0.631	0.366	0.416	0.682	0.186
	1.5	0.454	0.658	0.799	0.646	0.890	0.586	0.559	0.529	0.480	0.622	0.145
	2.0	0.737	0.502	0.709	0.492	0.561	0.501	0.255	0.253	0.552	0.507	0.168
	2.5	0.468	0.519	0.513	0.569	0.622	0.678	0.148	0.367	0.360	0.472	0.200
	3.0	0.265	0.303	0.283	0.232	0.254	0.266	0.227	0.156	0.235	0.247	0.042
LVNF	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.768	0.661	0.830	0.644	0.860	0.469	0.829	0.517	0.898	0.720	0.154
	1.0	0.707	0.771	0.758	0.783	0.787	0.973	0.417	0.192	0.479	0.652	0.241
	1.5	0.712	0.696	0.786	0.994	0.839	0.844	0.765	0.347	0.877	0.762	0.180
	2.0	0.669	0.800	0.676	0.773	0.710	0.502	0.398	0.247	0.371	0.572	0.200
	2.5	1.346	0.607	0.478	0.648	0.659	0.649	0.196	0.364	0.573	0.280	0.316
	3.0	0.369	1.347	0.434	0.400	0.432	0.407	0.349	0.273	0.372	0.487	0.326
VCE	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.403	0.284	0.491	0.617	0.814	0.598	0.098	0.081	0.102	0.388	0.265
	1.0	0.076	0.076	0.076	0.073	0.073	0.072	0.081	0.081	0.082	0.077	0.004
	1.5	0.079	0.080	0.080	0.077	0.075	0.076	0.082	0.082	0.083	0.079	0.003
	2.0	0.084	0.083	0.083	0.079	0.081	0.079	0.086	0.086	0.087	0.083	0.003
	2.5	0.083	0.084	0.084	0.080	0.080	0.079	0.088	0.088	0.088	0.084	0.004
	3.0	0.090	0.091	0.070	0.087	0.091	0.085	0.091	0.092	0.090	0.087	0.007
VLJC	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.638	1.234	0.987	0.372	0.625	0.975	0.420	0.212	0.441	0.656	0.340
	1.0	1.518	0.946	1.456	0.879	1.273	1.010	0.289	0.156	0.151	0.853	0.290
	1.5	0.138	0.102	0.396	0.135	0.144	0.300	0.105	0.089	0.101	0.168	0.107
	2.0	0.103	0.106	0.108	0.101	0.104	0.098	0.107	0.113	0.125	0.107	0.008
	2.5	0.106	0.108	0.113	0.568	0.104	0.103	0.109	0.110	0.112	0.159	0.153

	3.0	0.128	0.129	0.130	0.119	0.122	0.124	0.127	0.401	0.121	0.156	0.092
VC	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.577	0.883	0.710	0.917	0.935	0.749	0.244	0.484	0.651	0.683	0.226
	1.0	0.568	0.751	0.675	1.113	0.545	0.671	0.542	0.341	0.537	0.638	0.213
	1.5	0.679	0.665	0.815	0.711	0.532	0.787	0.461	0.346	0.299	0.588	0.188
	2.0	0.764	0.581	0.392	0.547	0.627	0.686	0.255	0.479	0.474	0.534	0.155
	2.5	1.005	0.679	0.946	0.533	0.615	0.529	0.279	0.221	0.423	0.581	0.268
	3.0	0.421	0.418	0.395	0.389	0.355	0.419	0.188	0.399	0.190	0.353	0.095
DV	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.648	0.623	0.668	0.482	0.556	0.576	0.114	0.327	0.388	0.498	0.189
	1.0	0.563	0.752	0.567	0.542	0.706	0.922	0.532	0.403	0.383	0.597	0.171
	1.5	0.907	0.580	0.858	0.841	0.486	0.612	0.335	0.492	0.435	0.616	0.206
	2.0	0.483	0.740	0.518	0.804	0.386	0.835	0.175	0.205	0.378	0.503	0.246
	2.5	0.467	0.533	0.885	0.384	0.627	0.569	0.190	0.164	0.392	0.468	0.223
	3.0	0.888	0.508	1.007	0.599	0.410	0.405	0.405	0.338	0.317	0.542	0.247

Table A3. *C. perfringens* strain 8238 antimicrobial screening data in FTG media. Individual OD measurements were taken after 24 h and were done three times independently and three replicates each.

Ingredient	Concentration (%)	Optical density (OD_{600}) of individual wells									Average	STDEV
LV	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.875	0.691	0.790	1.020	0.898	1.138	0.776	0.918	0.987	0.899	0.137
	1.0	0.773	0.812	0.868	0.881	0.857	0.900	0.841	0.861	0.748	0.838	0.051
	1.5	0.776	0.758	0.786	0.923	0.905	0.788	0.855	0.781	0.810	0.820	0.060
	2.0	0.721	0.694	0.715	0.598	0.726	0.673	0.811	0.602	0.690	0.692	0.065
	2.5	0.596	0.653	0.607	0.604	0.577	0.692	0.640	0.688	0.617	0.630	0.041
	3.0	0.300	0.325	0.154	0.301	0.397	0.317	0.209	0.121	0.318	0.271	0.090
LVNF	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	1.024	0.928	0.896	0.513	0.877	0.984	0.837	0.880	0.928	0.874	0.147
	1.0	0.892	0.841	0.799	0.894	0.968	1.184	0.938	0.755	0.921	0.910	0.123
	1.5	0.788	0.761	0.857	0.840	0.902	0.763	0.879	0.799	0.751	0.816	0.056
	2.0	1.136	1.074	0.990	0.876	0.775	0.849	0.790	0.792	0.747	0.892	0.141
	2.5	0.920	0.793	0.653	0.727	0.566	0.763	0.891	0.578	0.617	0.723	0.130
	3.0	0.461	0.473	0.241	0.569	0.591	0.487	0.574	0.675	0.621	0.521	0.127
VCE	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.618	0.647	0.640	0.944	0.964	1.019	0.420	0.916	0.886	0.784	0.206
	1.0	0.080	0.081	0.082	0.076	0.077	0.076	0.083	0.086	0.086	0.081	0.004
	1.5	0.086	0.094	0.085	0.081	0.080	0.081	0.088	0.088	0.087	0.086	0.004
	2.0	0.084	0.083	0.084	0.083	0.083	0.084	0.087	0.088	0.089	0.085	0.002
	2.5	0.088	0.088	0.088	0.086	0.085	0.086	0.092	0.092	0.092	0.089	0.003
	3.0	0.096	0.095	0.096	0.094	0.093	0.094	0.093	0.094	0.093	0.095	0.002
VLJC	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.858	0.483	1.520	1.036	1.008	1.141	0.713	0.712	0.613	0.898	0.316
	1.0	1.520	1.000	1.110	1.555	1.534	1.026	0.685	0.646	0.639	1.122	0.394
	1.5	0.112	0.100	0.109	0.099	0.093	0.098	0.292	0.106	0.108	0.124	0.063
	2.0	0.106	0.107	0.109	0.106	0.105	0.107	0.115	0.107	0.114	0.107	0.003

	2.5	0.111	0.111	0.114	0.111	0.111	0.101	0.119	0.112	0.114	0.112	0.005
	3.0	0.126	0.127	0.125	0.123	0.127	0.120	0.131	0.135	0.126	0.127	0.004

VC	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	0.927	1.037	1.087	1.276	1.022	0.919	0.620	0.816	0.798	0.945	0.190
	1.0	1.151	0.933	1.067	1.086	1.020	1.000	0.969	0.778	0.967	0.997	0.190
	1.5	1.317	0.853	0.861	0.914	0.900	1.009	0.648	0.673	0.898	0.897	0.195
	2.0	0.787	0.745	0.744	0.719	0.751	0.700	0.730	0.828	0.657	0.740	0.049
	2.5	0.673	0.971	0.680	0.805	0.771	0.701	0.604	0.758	0.760	0.747	0.104
	3.0	0.580	0.643	0.639	1.317	0.752	0.796	0.711	0.650	0.776	0.763	0.220
DV	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	1.060	0.866	0.853	1.108	1.008	0.925	1.003	0.973	0.988	0.976	0.084
	1.0	0.749	0.906	0.931	0.752	0.795	1.022	0.823	0.707	0.772	0.829	0.103
	1.5	0.792	1.139	1.024	0.846	0.758	0.658	0.673	0.658	0.668	0.802	0.174
	2.0	0.540	0.550	0.675	0.635	0.614	0.660	0.536	0.589	0.598	0.600	0.051
	2.5	0.695	0.617	0.587	0.689	0.726	0.605	0.560	0.491	0.547	0.613	0.078
	3.0	0.400	0.489	0.369	0.408	0.412	0.549	0.467	0.385	0.376	0.428	0.061

Table A4. pH measurements of natural antimicrobial ingredients in FTG media. Measurements were done three times independently and three replicates each.

Ingredient	Concentration (%)	pH values of samples									Average	STDEV
	Replicate #	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
FTG media		6.73	6.79	6.79	6.91	6.91	6.85	6.81	6.85	6.82	6.83	0.058
VCE	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	6.73	6.66	6.67	6.62	6.64	6.67	6.64	6.66	6.67	6.66	0.031
	1.0	6.18	6.21	6.20	6.11	6.14	6.13	6.21	6.18	6.15	6.17	0.037
	1.5	5.87	5.88	5.89	5.81	5.83	5.85	5.87	5.89	5.86	5.86	0.027
	2.0	5.77	5.75	5.78	5.78	5.77	5.77	5.76	5.77	5.78	5.77	0.010
	2.5	5.70	5.69	5.70	5.68	5.69	5.71	5.69	5.68	5.65	5.69	0.017
	3.0	5.66	5.67	5.65	5.67	5.66	5.64	5.64	5.62	5.66	5.65	0.016
LVNF	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	6.33	6.30	6.31	6.28	6.31	6.30	6.31	6.32	6.30	6.31	0.014
	1.0	6.08	6.06	6.06	6.09	6.11	6.08	6.08	6.06	6.05	6.07	0.019
	1.5	5.85	5.85	5.84	5.85	5.85	5.84	5.85	5.85	5.84	5.85	0.005
	2.0	5.76	5.75	5.75	5.73	5.76	5.75	5.74	5.75	5.75	5.75	0.009
	2.5	5.66	5.67	5.66	5.68	5.67	5.66	5.67	5.68	5.65	5.67	0.023
	3.0	5.63	5.63	5.62	5.6	5.62	5.63	5.64	5.63	5.64	5.63	0.012
VLJC	**Replicate #**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
	0.5	6.34	6.33	6.34	6.34	6.30	6.32	6.33	6.35	6.32	6.33	0.015
	1.0	6.27	6.27	6.28	6.27	6.26	6.26	6.29	6.28	6.29	6.27	0.011
	1.5	5.90	5.91	5.91	5.92	5.92	5.91	5.90	5.92	5.93	5.91	0.010
	2.0	5.79	5.79	5.78	5.77	5.78	5.79	5.78	5.77	5.78	5.78	0.008
	2.5	5.70	5.71	5.71	5.73	5.72	5.71	5.72	5.73	5.71	5.72	0.010
	3.0	5.67	5.66	5.67	5.66	5.64	5.65	5.66	5.67	5.66	5.66	0.010

LV	Replicate #	1	2	3	1	2	3	1	2	3		
	0.5	6.33	6.34	6.34	6.32	6.3	6.31	6.32	6.33	6.33	6.32	0.013
	1.0	6.13	6.14	6.14	6.11	6.13	6.12	6.12	6.11	6.14	6.13	0.012
	1.5	5.85	5.84	5.85	5.84	5.85	5.84	5.85	5.84	5.83	5.84	0.007
	2.0	5.75	5.77	5.77	5.76	5.77	5.75	5.77	5.75	5.76	5.76	0.009
	2.5	5.72	5.72	5.71	5.71	5.72	5.7	5.71	5.72	5.71	5.71	0.007
	3.0	5.69	5.68	5.68	5.69	5.69	5.68	5.67	5.68	5.68	5.68	0.007
DV	Replicate #	1	2	3	1	2	3	1	2	3		
	0.5	6.54	6.53	6.53	6.54	6.55	6.54	6.53	6.54	6.54	6.54	0.007
	1.0	6.2	6.21	6.2	6.22	6.2	6.21	6.21	6.23	6.21	6.21	0.010
	1.5	6.1	6.11	6.11	6.1	6.1	6.11	6.11	6.1	6.11	6.11	0.005
	2.0	6.05	6.04	6.02	6.04	6.04	6.05	6.05	6.04	6.03	6.04	0.010
	2.5	5.99	5.99	5.98	5.99	5.99	5.98	5.99	5.99	6	5.98	0.006
	3.0	5.98	5.99	5.98	5.97	5.98	5.98	5.98	5.98	5.99	5.98	0.006
VC	Replicate #	1	2	3	1	2	3	1	2	3		
	0.5	6.43	6.41	6.43	6.4	6.44	6.43	6.4	6.42	6.42	6.42	0.014
	1.0	6.08	6.1	6.1	6.09	6.08	6.11	6.08	6.09	6.09	6.09	0.011
	1.5	5.85	5.86	5.85	5.86	5.88	5.88	5.84	5.85	5.86	5.86	0.014
	2.0	5.63	5.72	5.71	5.69	5.71	5.7	5.7	5.68	5.71	5.69	0.027
	2.5	5.66	5.68	5.66	5.69	5.68	5.67	5.67	5.69	5.68	5.68	0.011
	3.0	5.62	5.64	5.62	5.64	5.66	5.67	5.6	5.63	5.63	5.63	0.021

Appendix B

Growth Curve, Endospore, and Vegetative Cell Experiment Data

Table B1. *C. perfringens* growth curve data. Measurements were taken three times independently.

Strain	Time (h)	Optical density (OD_{600})			Average	STDEV
8239						
	0	0.08	0.089	0.09	0.086	0.006
	2	0.520	0.55	0.51	0.527	0.021
	4	1.48	1.54	1.52	1.513	0.031
	6	1.54	1.59	1.57	1.567	0.025
	8	1.53	1.54	1.56	1.543	0.015
	24	1.49	1.50	1.51	1.500	0.010
10240						
	0	0.08	0.089	0.09	0.086	0.006
	2	0.39	0.49	0.40	0.427	0.055
	4	1.41	1.36	1.33	1.367	0.040
	6	1.47	1.44	1.49	1.467	0.025
	8	1.51	1.48	1.50	1.497	0.015
	24	1.44	1.49	1.44	1.457	0.029
8238						
	0	0.08	0.089	0.085	0.085	0.005
	2	0.55	0.59	0.49	0.543	0.050
	4	1.56	1.51	1.43	1.500	0.066

	6	1.59	1.58	1.49	1.553	0.055
	8	1.55	1.52	1.50	1.523	0.025
	24	1.40	1.42	1.50	1.440	0.053

Table B2. Mode of action endospore experiment data. Experiments were done three times independently.

Strain	Ingredient	Concentration (%)	Incubation (pre/post)	Microbial counts (Log CFU/mL)			Average	STDEV
8239	**VCE**							
		1.0	Pre	4.760	4.860	4.900	4.826	0.065
		1.5	Pre	4.820	4.851	4.960	4.829	0.073
		1.0	Post	3.826	4.114	4.233	4.058	0.209
		1.5	Post	2.602	2.574	2.439	2.432	0.087
	VLJC							
		1.5	Pre	4.816	4.816	4.810	4.814	0.004
		2.0	Pre	4.752	4.875	4.836	4.821	0.063
		1.5	Post	1.700	1.000	1.000	1.233	0.404
		2.0	Post	1.400	1.000	1.000	1.099	0.230
10240	**VCE**							
		1.0	Pre	4.686	4.648	4.752	4.681	0.061
		1.5	Pre	4.653	4.667	4.778	4.700	0.068
		1.0	Post	1.477	2.000	1.176	1.551	0.417
		1.5	Post	1.301	1.000	1.176	1.159	0.168
	VLJC	1.5	Pre	4.756	4.648	4.740	4.715	0.058
		2.0	Pre	4.763	4.767	4.556	4.700	0.121

		1.5	Post	3.002	2.940	2.686	2.876	0.168
		2.0	Post	2.993	2.789	2.477	2.753	0.260
8238	**VCE**							
		1.0	Pre	5.574	5.512	5.439	5.508	0.067
		1.5	Pre	5.686	5.423	5.380	5.496	0.165
		1.0	Post	1.602	1.477	1.176	1.418	0.219
		1.5	Post	1.000	1.000	1.301	1.100	0.174
	VLJC	1.5	Pre	5.290	5.538	5.423	5.417	0.124
		2.0	Pre	5.580	5.477	5.398	5.485	0.091
		1.5	Post	1.778	1.000	1.000	1.259	0.449
		2.0	Post	1.000	1.000	1.000	1.000	0

Table B3. Mode of action vegetative cell experiment data. Experiments were performed three times independently.

Strain	Ingredient	Concentration (%)	Incubation (pre/post)	Microbial counts (Log CFU/mL)			Average	STDEV
8239	**VCE**							
		1.0	Pre	5.512	6.439	6.607	6.632	0.552
		1.5	Pre	4.732	6.462	6.813	6.207	0.997
		1.0	Post	1.000	1.000	1.000	1.000	0
		1.5	Post	1.000	1.000	1.000	1.000	0
	VLJC							
		1.5	Pre	5.176	6.525	6.813	6.322	0.775
		2.0	Pre	5.312	6.380	6.810	6.324	0.704
		1.5	Post	1.000	1.000	1.813	1.247	0.386
		2.0	Post	1.000	1.000	2.505	1.824	0.958
10240	**VCE**							
		1.0	Pre	5.519	5.512	6.462	6.015	0.547
		1.5	Pre	5.455	5.415	6.431	5.964	0.576
		1.0	Post	1.000	1.000	1.000	1.000	0
		1.5	Post	1.000	1.000	1.000	1.000	0
	VLJC	1.5	Pre	5.585	5.658	6.964	6.269	0.776
		2.0	Pre	5.686	5.744	6.810	6.262	0.633
		1.5	Post	1.176	1.176	1.954	1.501	0.776
		2.0	Post	1.000	1.000	2.591	1.745	0.919

8238	**VCE**							
		1.0	Pre	6.699	6.799	6.881	6.805	0.078
		1.5	Pre	6.677	6.803	6.760	6.759	0.058
		1.0	Post	1.000	1.000	1.000	1.000	0
		1.5	Post	1.000	1.000	1.000	1.000	0
	VLJC	1.5	Pre	6.653	6.672	6.942	6.756	0.162
		2.0	Pre	6.677	6.775	6.695	6.715	0.052
		1.5	Post	1.000	1.000	1.653	1.218	0.377
		2.0	Post	1.000	1.000	2.789	1.528	0.915

Table B4. Sporulation efficiency experiment data. Experiments were performed three times independently.

Strain	Treatment (%)	Pre/Post heat shock	Microbial counts (CFU/mL × $10^{7)}$			Average	STDEV
8239							
	Positive control	Pre	3.60	9.20	3.45	5.42	3.28
		Post	0.300	0.385	0.210	0.298	0.086
	VCE (0.05%)	Pre	3.40	3.70	2.30	3.13	0.737
		Post	0.090	0.100	0.029	0.073	0.038
	VCE (0.1%)	Pre	4.30	4.10	2.45	3.62	1.02
		Post	0.007	0.004	0.004	0.005	0.002
	VLJC (0.5%)	Pre	1.03	0.220	3.30	1.52	1.60
		Post	0.006	0.0005	0.017	0.008	0.008
	VLJC (0.75%)	Pre	7.45	2.09	5.65	5.06	2.73
		Post	0.000006	0.0005	0.0003	0.0003	0.0003

10240							
	Positive control	Pre	2.15	1.67	1.05	1.62	0.551
		Post	0.255	0.695	0.955	0.140	0.100
	VCE (0.05%)	Pre	0.049	0.081	0.053	0.061	0.018
		Post	0.000004	0.000005	0.000010	0.00006	0.00003
	VCE (0.1%)	Pre	0.045	0.076	0.050	0.057	0.017
		Post	0.000002	0.000006	0.000001	0.00003	0.00003
	VLJC (0.5%)	Pre	0.230	7.45	0.905	2.86	3.99
		Post	0.0094	0.0090	0.0005	0.0063	0.0050
	VLJC (0.75%)	Pre	0.550	0.565	0.840	0.652	0.163
		Post	0.00089	0.00075	0.00028	0.00064	0.00032
8238							
	Positive control	Pre	2.50	0.805	3.15	2.15	1.21
		Post	0.485	0.057	0.325	0.289	0.216
	VCE (0.05%)	Pre	0.123	0.285	1.90	0.769	0.983
		Post	0.00081	0.00118	0.0230	0.0083	0.013

	VCE (0.1%)	Pre	0.0265	0.0475	2.15	0.741	1.22
		Post	0.00008	0.00009	0.00087	0.00035	0.00045
	VLJC (0.5%)	Pre	0.975	0.690	2.50	1.39	0.973
		Post	0.00245	0.00465	0.00028	0.0025	0.0022
	VLJC (0.75%)	Pre	0.770	1.85	0.790	1.14	0.618
		Post	0.00002	0.00059	0.00003	0.00021	0.00033

Appendix C

Chicken Experiment Data

Table C1. Chicken water activity measurements were taken three times independently and three replicates per trial.

Treatment	Concentration (%)	Water activity of chicken samples									Average	STDEV
	Replicate #	1	2	3	1	2	3	1	2	3		
Control		0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.990	0.002
VCE	1.0	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.989	0.002
VCE	3.0	0.982	0.982	0.982	0.982	0.982	0.982	0.982	0.982	0.982	0.986	0.002
VLJC	1.5	0.987	0.987	0.987	0.987	0.987	0.987	0.987	0.987	0.987	0.988	0.002
VLJC	3.0	0.984	0.984	0.984	0.984	0.984	0.984	0.984	0.984	0.984	0.986	0.002

Table C2. Chicken pH measurements were taken three times independently and three replicates per trial.

Treatment	Concentration (%)	pH values of chicken samples									Average	STDEV
	Replicate #	1	2	3	1	2	3	1	2	3		
Control		6.36	6.33	6.31	6.14	6.16	6.16	6.25	6.24	6.23	6.24	0.079
VCE	1.0	6.20	6.18	6.18	6.00	6.04	6.03	6.08	6.06	6.05	6.09	0.075
VCE	3.0	6.12	6.13	6.13	5.95	5.95	5.94	6.00	5.98	5.95	6.02	0.085
VLJC	1.5	6.15	6.16	6.03	6.00	5.99	6.14	6.10	6.07	6.16	6.09	0.069
VLJC	3.0	6.11	6.10	6.11	6.00	5.95	5.96	6.05	6.03	6.00	6.03	0.062

Table C3. Defective chilling cooling profiles.

Cooling time from 54.4 °C to 4.0 °C			
Elapsed time (h)	**6.5 h (°C)**	**12 h (°C)**	**18 h (°C)**
0	54.40	54.40	54.40
1	37.84	48.05	47.60
2	25.68	38.48	41.70
3	18.59	30.82	36.60
4	13.46	24.68	32.20
5	9.74	19.77	28.30
6	7.05	15.83	25.00
6.5	4.89		
7		12.68	22.10
8		10.15	19.60
9		8.13	17.40
10		6.51	15.60
11		5.21	13.90
12		4.18	12.50
13			11.30
14			10.20
15			9.30
16			8.50
17			7.10
18			4.95

Table C4. Extreme abusive storage (42 °C) experiment data. Three independent trials.

Incubation time (h)	Treatment (%)	Microbial counts (Log CFU/mL)			Average	STDEV
0	Positive control	3.744	3.498	3.398	3.496	0.178
	VCE 1.0	3.352	3.332	3.380	3.347	0.026
	VCE 3.0	3.455	3.455	3.431	3.426	0.044
	VLJC 1.5	3.290	3.470	3.332	3.373	0.079
	VLJC 3.0	3.371	3.580	3.322	3.420	0.112
10	Positive control	6.519	6.423	6.618	6.520	0.097
	VCE 1.0	5.119	5.395	5.129	5.214	0.157
	VCE 3.0	4.282	4.169	4.200	4.216	0.059
	VLJC 1.5	4.720	4.964	4.672	4.785	0.156
	VLJC 3.0	3.989	4.431	4.455	4.291	0.262
24	Positive control	5.947	6.789	6.544	6.427	0.433
	VCE 1.0	4.059	5.244	5.212	4.838	0.675
	VCE 3.0	4.009	4.991	4.247	4.416	0.513
	VLJC 1.5	4.667	5.013	4.906	4.862	0.177
	VLJC 3.0	4.154	4.810	4.291	4.418	0.346

Table C5. Abusive storage experiment positive control data. Three independent trials.

Ingredient	Temperature (°C)	Time (day)	Microbial counts (Log CFU/g)			Average	STDEV
Positive control	4	0	3.312	3.677	3.151	3.380	0.269
	4	1	3.352	3.389	3.562	3.435	0.112
	4	4	3.279	3.361	3.439	3.360	0.080
	4	7	3.301	3.189	3.079	3.190	0.111
	4	10	3.242	3.141	3.212	3.198	0.052
	4	13	3.064	3.477	3.407	3.316	0.221
	4	16	3.109	3.531	3.623	3.421	0.274
	12	0	3.312	3.677	3.151	3.380	0.269
	12	1	3.312	3.380	3.332	3.341	0.269
	12	4	3.256	3.342	3.256	3.284	0.050
	12	7	3.947	3.854	3.771	3.857	0.088
	12	10	4.447	4.267	5.332	4.682	0.570
	12	13	4.987	4.648	5.519	5.051	0.439
	12	16	4.796	4.628	5.845	5.090	0.659
	16	0	3.312	3.677	3.151	3.380	0.269
	16	1	3.322	3.695	3.653	3.557	0.269
	16	4	4.498	4.447	4.204	4.383	0.204
	16	7	5.651	5.551	5.449	5.550	0.157
	16	10	5.643	5.491	5.380	5.505	0.132
	16	13	5.884	6.029	5.940	5.951	0.074
	16	16	5.643	6.000	5.985	5.876	0.202

Table C6. Abusive storage experiment VCE 1.0% data. Three independent trials.

Ingredient	Temperature (°C)	Time (day)	Microbial counts (Log CFU/g)			Average	STDEV
VCE (1.0)	4	0	3.352	3.447	3.207	3.335	0.121
	4	1	3.312	3.556	3.455	3.441	0.123
	4	4	3.352	3.112	3.672	3.379	0.281
	4	7	3.122	3.179	3.267	3.189	0.073
	4	10	3.484	3.380	3.312	3.392	0.087
	4	13	3.267	3.011	3.267	3.182	0.148
	4	16	3.322	3.267	3.498	3.363	0.121
	12	0	3.352	3.447	3.207	3.380	0.121
	12	1	3.362	3.342	3.677	3.460	0.188
	12	4	3.397	3.006	3.491	3.359	0.257
	12	7	2.799	2.736	2.602	2.713	0.101
	12	10	2.380	2.922	1.653	2.410	0.497
	12	13	2.380	2.415	2.498	2.431	0.061
	12	16	2.580	2.267	2.519	2.455	0.166
	16	0	3.352	3.447	3.207	3.380	0.121
	16	1	3.455	3.597	3.389	3.480	0.188
	16	4	3.204	3.342	3.491	3.346	0.257
	16	7	3.146	3.254	3.695	3.365	0.101
	16	10	2.255	2.978	1.653	2.295	0.497
	16	13	2.130	3.313	2.550	2.604	0.061
	16	16	2.114	3.004	2.845	2.654	0.166

Table C7. Abusive storage experiment VCE 3.0% experiment. Three independent trials.

Ingredient	Temperature (°C)	Time (day)	Microbial counts (Log CFU/g)			Average	STDEV
VCE (3.0)	4	0	3.389	3.352	3.677	3.473	0.178
	4	1	3.112	3.455	3.690	3.441	0.123
	4	4	3.230	3.093	3.810	3.379	0.281
	4	7	3.312	3.462	3.484	3.189	0.073
	4	10	3.322	3.484	3.462	3.392	0.087
	4	13	3.332	3.484	3.519	3.182	0.148
	4	16	3.550	3.301	3.290	3.363	0.121
	12	0	3.389	3.352	3.677	3.473	0.178
	12	1	3.512	3.431	3.760	3.568	0.178
	12	4	3.217	3.107	3.544	3.290	0.171
	12	7	2.775	3.093	3.525	3.131	0.227
	12	10	3.171	2.829	2.580	2.860	0.377
	12	13	2.525	3.092	2.932	2.850	0.297
	12	16	2.491	2.643	3.290	2.700	0.292
	16	0	3.389	3.352	3.677	3.473	0.178
	16	1	3.531	3.352	3.816	3.567	0.234
	16	4	3.267	3.398	3.886	3.517	0.326
	16	7	2.991	2.756	3.854	3.200	0.578
	16	10	2.398	3.021	3.484	2.968	0.545
	16	13	2.000	2.957	3.662	2.873	0.835
	16	16	2.796	2.968	3.491	3.085	0.362

Table C8. Abusive storage VLJC 1.5% experiment. Three independent trials.

Ingredient	Temperature (°C)	Time (day)	Microbial counts (Log CFU/g)			Average	STDEV
VLJC (1.5)	4	0	3.279	3.638	3.505	3.474	0.182
	4	1	3.217	3.431	3.778	3.476	0.283
	4	4	3.470	3.175	3.591	3.412	0.214
	4	7	3.556	3.252	3.505	3.438	0.163
	4	10	3.613	3.550	3.580	3.581	0.031
	4	13	3.313	3.447	3.322	3.300	0.160
	4	16	3.574	3.290	3.332	3.399	0.153
	12	0	3.279	3.638	3.505	3.474	0.182
	12	1	3.290	3.371	3.519	3.393	0.116
	12	4	3.255	3.322	3.041	3.206	0.147
	12	7	2.342	3.009	2.929	2.760	0.364
	12	10	2.732	3.431	1.477	2.547	0.990
	12	13	2.512	2.677	2.484	2.558	0.104
	12	16	2.342	3.217	2.929	2.830	0.446
	16	0	3.389	3.352	3.677	3.474	0.182
	16	1	3.230	3.667	3.519	3.472	0.222
	16	4	3.332	3.498	3.550	3.460	0.114
	16	7	2.728	3.405	3.230	3.121	0.351
	16	10	1.778	2.826	2.968	2.524	0.650
	16	13	2.312	3.215	3.009	2.845	0.473
	16	16	3.842	2.860	2.908	3.204	0.553

Table C9. Abusive storage VLJC 3.0% experiment. Three independent trials.

Ingredient	Temperature (°C)	Time (day)	Microbial counts (Log CFU/g)			Average	STDEV
VLJC (3.0)	4	0	3.613	3.653	3.568	3.611	0.043
	4	1	3.470	3.484	3.810	3.588	0.192
	4	4	3.371	3.148	3.756	3.425	0.308
	4	7	3.498	3.179	3.574	3.417	0.210
	4	10	3.255	3.423	3.470	3.383	0.113
	4	13	3.407	3.332	3.352	3.364	0.038
	4	16	3.322	3.477	3.371	3.390	0.079
	12	0	3.613	3.653	3.568	3.611	0.043
	12	1	3.602	3.352	3.775	3.576	0.212
	12	4	3.371	3.255	3.243	3.290	0.071
	12	7	2.470	2.854	2.699	2.674	0.193
	12	10	2.695	2.760	2.332	2.596	0.230
	12	13	2.512	2.477	2.740	2.576	0.143
	12	16	2.204	1.903	2.820	2.309	0.467
	16	0	3.613	3.653	3.568	3.611	0.043
	16	1	3.312	3.362	3.628	3.434	0.170
	16	4	3.243	3.207	3.658	3.369	0.251
	16	7	2.525	2.989	3.658	3.057	0.570
	16	10	1.544	2.900	3.447	2.631	0.980
	16	13	1.978	3.072	3.423	2.824	0.754
	16	16	1.778	2.987	3.267	2.677	0.791

Table C10. Defective chilling 6.5 h experiment data. Three independent trials.

Treatment (%)	Time (h)	Microbial counts (Log CFU/g)			Average	STDEV
Positive control	0	3.491	3.407	3.550	3.483	0.072
	6.5	4.031	3.863	3.869	3.921	0.095
VCE (1.0%)	0	3.690	3.519	3.796	3.668	0.140
	6.5	3.182	3.066	3.085	3.111	0.062
VCE (3.0%)	0	3.602	3.398	3.796	3.646	0.272
	6.5	3.051	3.011	3.353	3.138	0.187
VLJC (1.5%)	0	3.352	3.352	3.866	3.524	0.297
	6.5	3.029	3.248	3.416	3.231	0.194
VLJC (3.0%)	0	3.470	3.322	3.911	3.568	0.306
	6.5	3.302	2.971	3.760	3.344	0.396

Table C11. Defective chilling 12 h experiment data. Three independent trials.

Treatment (%)	Time (h)	Microbial counts (Log CFU/g)			Average	STDEV
Positive control	0	3.398	3.455	3.151	3.335	0.162
	12	4.900	4.792	5.205	4.966	0.214
VCE (1.0%)	0	3.095	3.322	3.207	3.208	0.114
	12	3.173	2.097	3.124	2.798	0.608
VCE (3.0%)	0	3.525	3.574	3.677	3.592	0.077
	12	3.326	2.447	3.244	3.006	0.486
VLJC (1.5%)	0	3.484	3.352	3.505	3.447	0.083
	12	3.124	2.267	3.109	2.833	0.490
VLJC (3.0%)	0	3.686	3.531	3.568	3.595	0.081
	12	3.397	2.146	3.288	2.944	0.693

Table C12. Defective chilling 18 h experiment data. Three independent trials.

Treatment (%)	Time (h)	Microbial counts (Log CFU/g)			Average	STDEV
Positive control	0	3.462	3.447	3.151	3.353	0.176
	18	7.057	7.315	7.212	7.186	0.182
VCE (1.0%)	0	3.342	3.638	3.207	3.396	0.221
	18	5.066	4.643	4.799	4.855	0.299
VCE (3.0%)	0	3.498	3.352	3.677	3.509	0.163
	18	3.380	3.322	3.756	3.351	0.041
VLJC (1.5%)	0	3.519	3.585	3.505	3.536	0.043
	18	4.699	5.479	5.633	5.089	0.551
VLJC (3.0%)	0	3.519	3.653	3.568	3.580	0.068
	18	3.736	3.806	4.350	3.771	0.049