Fate of Spore-forming Pathogens in High and Reduced Moisture, Processed Meat and Poultry Products Subjected to Post-Packaging Pasteurization

by

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ABSTRACT

Spore-forming bacteria can survive lethality schedules directed to destroy vegetative pathogens and rapid cooling is necessary to inhibit germination and growth during stabilization and storage of reduced moisture processed meat and poultry products. Post-drying heat treatments are implemented to control microbial surface contaminants and can potentially control germinated spores that might grow during product cooling. This study was designed to assess the fate of spore-forming pathogens when subjected to post-packaging pasteurization after lethality and stabilization schedules on reduced- and high-moisture, shelf-stable meat and poultry products, and to evaluate the effect of water activity (a_W) of meat and poultry products on spores survival, germination and growth.

A cocktail of *C. perfringens* spores was used to inoculate reduced- and high-moisture meat and poultry products before exposure to a lethality process. Different incremental stabilization schedules (time 0, 1, 2, 3 h) were evaluated to determine spore survival and germination rates with and without subsequent exposure to post-packaging pasteurization (77 °C 6 min).

It was found that viability of germinated spores was significantly decreased ($p > 0.05$) when extended stabilization rates were evaluated in reduced- and high-moisture products. However, higher levels of reduction were achieved when the a_W of the product was higher (a_W: 0.98 ± 0.1). On the other hand, post packaging pasteurization applied after short-term stabilization schedules had no effect on inactivation of spore survivors. Nonetheless, after lethality treatment, cooling and post-packaging pasteurization spore levels were lower in both reduced- and high-moisture meat and poultry products. Moreover, 1-log reduction was observed after processing and prolonged storage at room

temperature (23±2°C) and abusive temperatures (37°C). In addition, morphological changes were observed in *C. perfringens* colonies which might indicate the effect of the pasteurization on stressing the cells and further reducing their viability in cooked meat and poultry products.

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CHAPTER I

LITERATURE REVIEW

Introduction

Microorganisms need water to survive as well as to grow (*91, 92*). Depending on the water activity (a_W), food products may be classified as high moisture foods (HMF), intermediate moisture foods (IMF), and low moisture foods (LMF). High moisture foods have a_W above 0.85 and below this value are intermediate and low moisture products (*73*). Above 0.85, foods might require refrigeration or other barriers to control the growth of pathogens; however, depending on other factors such as packaging, additives, pH and ingredients, some food products with a_W higher than 0.85 can still be shelf stable (*68*, *73*). For instance, meat and poultry jerky is a ready-to-eat (RTE) product that is considered shelf-stable. For this product, the U.S. Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) recommends an upper limit of 0.85 under aerobic conditions but a limit of 0.91 under anaerobic conditions (*31*).

In the present study, the fate of spore-forming pathogens was evaluated in a jerky-like product with a a_W of 0.91± 0.01. This is a product with an a_W that is within the limit of what is recommended by the FSIS guidelines to be a shelf-stable RTE product. Moreover, even though it is a RTE, the a_W is above 0.85. Therefore, considering the characteristics of the product used in this project, it was referred as a reduced moisture meat or poultry strip.

When producing ready-to-eat shelf stable products, spore-forming pathogens become one of the biggest challenges since they are not eliminated with conventional lethality schedules but might need a combination of different heat treatments,

stabilization and additives in the product (*79*, *81*). The objective of this study was to evaluate post-packaging pasteurization (PPP) as a post-drying treatment to control spore-forming pathogens in reduced moisture products that have been subjected to a lethality and stabilization schedule. Furthermore, this study attempted to evaluate the effect of a_W of meat and poultry products on spore survival and growth. The following literature review focused on meat and poultry reduced moisture products, FSIS regulation and guidelines, spore-forming pathogens and strategies to control these microorganisms in food processing.

Water activity and food

Water activity $_{(aw)}$ of a food product refers to the availability of water to the organism in a food matrix and it plays an important role in the identification of the food product, especially regarding its shelf life (*22*, *64*). When the a_W is high, there is more opportunity for the microorganisms to survive and affect the quality and safety of the products (*28*). According to the International Commission on Microbiological Specifications for Foods (ICMSF), food can be classified into three categories by its water content (moist foods, intermediate moisture foods and low-moisture foods) (*26*).

High moisture foods have a_W above 0.85 and require refrigeration or another barrier to control foodborne pathogens; meanwhile intermediate-moisture foods and low moisture foods do not require refrigeration to control pathogens, but they differ in shelf-life due to the growth of spoilage microorganisms (*26*, *32*). The microbiological stability of these type of foods may depend on factors other than a_W, such as pH, chemical preservatives, heat treatments, or combination of these, even though the reduced a_W is of major importance (*26*).

Low moisture food products

Low moisture foods are foods that are naturally low in moisture or are produced from higher moisture foods through drying or dehydration processes (*96, 100*). Lowering the a_W of food is among the oldest and the most common forms of food preservation, since a decreased a_W inhibits the growth of most bacteria, yeasts, and molds (*10, 61*).

Low moisture foods are usually referred to as food items that have an a_W less than 0.85. This group includes cereals and grains, dried protein products, spices and dried herbs, nuts, dried fruits and vegetables, and seeds among others (*93, 100*). The low a_W of these foods contributes to a long shelf-life since it is more difficult for microorganisms to utilize water in the food matrix (*10, 100*). Nonetheless, the increase in foodborne illness outbreaks related to LMF have been changing the reputation of LMF as safe and changing the misperception that these foods were not of concern from a microbiological food safety perspective (*10, 92, 100*).

Low-moisture foods such as cereals, seeds, nuts and jerky have been recently linked to outbreaks (*2, 101*). As an example, beef jerky has been linked to multiple outbreaks of salmonellosis and *Escherichia coli* O157:H7 infection over the past 40 years (*9*). Therefore, since the consumption of shelf-stable and ready-to-eat-products is increasing due to the convenience of purchasing and consuming the products, it is necessary to implement strategies to ensure the safety of these type of foods and change the perception of low moisture foods as naturally safe (*42*).

Meat and poultry Jerky

Meat and poultry jerky are some of the most consumed low moisture products and consumption trends are increasing in the market. These products have a high demand since

they have high protein value and can be stored at room temperature (*20, 98*). However, shelf-stable jerky can also be identified as an IMF considering that FSIS has established an a_W critical limit of 0.85 or lower for products stored under aerobic conditions, and 0.91 or lower if the product is vacuum packaged (*30*).

Meat or poultry jerky is a RTE, dehydrated product that is considered shelf-stable, which means that it does not require refrigeration after proper processing (*30, 62*). According to USDA, the standard of identity for beef jerky requires a moisture-to-protein-ratio value of $\leq 0.75{:}1$ and has a shelf life or approximately 2 years when it has been vacuum packaged and stored at room temperature (*30*). This is a very popular RTE product in the United States (US) and Canada, where it is available in nearly every convenience store, gas station, supermarket, and variety shop (*20*).

Beef and chicken jerky are the result of what is known hurdle technology, which includes multiple control factors as temperature, a_W and preservatives in the preparation. In general, the production of jerky consists of slicing a whole muscle or ground beef, then marinating it with spices and nitrites, drying and packing of the final product (*20*, *27*). Multiple control factors can be combined in order to obtain the stability and sensory characteristics of the final product (*27*). These factors can include the type of meat, the spice composition, length of curing and drying and the use of preservatives (*14*, *51*).

Meat jerky industry has proven to be resilient over the five years preceding 2018, since IBISWorld estimates that revenue for the Meat Jerky Production industry has grown in each of the past five years, including a 3.0% increase in 2018. This industry with a small-scale nature, low prices and innovative flavors and content, compared to red meat have led

revenue to grow at an estimated annualized rate of 3.4% to $1.3 billion over the five years to 2018 alone (*41*).

On the other hand, the jerky manufacturing process and the inherent characteristics of the final product provide barriers to microbial survival and growth (*27*, *45*). Indeed, FSIS recognizes that most jerky-type products have different intrinsic factors, such as sodium nitrite, indigenous microflora, and salt concentration, that can serve as barriers to prevent microorganisms growth (*30*). However, due to the high variability of processing times and temperatures, processing environment relative humidity, and methods to control humidity used by small and very small plants, it is necessary to train processors and asses quality and safety of the product (*60*).

Furthermore, due to the lack of processing knowledge and equipment capabilities, there is a need for lethality studies in this area in addition to education on jerky process control and food safety for processors (*15*, *60*). On the other hand, one of the strategies for innovation in this industry is the increase of a_W of the products in order to improve consumer acceptability and flavors; however, producing more moist products would need implementing more controls to prevent foodborne pathogens.

Outbreaks related to jerky meat products

Due to the discrepancies in the jerky manufacturing processes regarding time and temperature of cooking and drying, there have been several outbreaks associated with jerky consumption in different countries. These outbreaks can be caused by cross contamination during jerky processing, molding, cutting and packaging; furthermore, most of jerkies are made in small companies, and these have difficulties with food hygiene management (*1*, *34*).

Some of the first reported outbreaks took place in New Mexico between 1966 and 1995 with 250 illnesses and 8 reported gastroenteritis outbreaks due to ingestion of contaminated meat jerky (*17*, *23*). Primarily implicated was a locally produced jerky and the outbreaks could have been caused by failure during processing to reach a temperature sufficient to kill bacteria. Organisms that were isolated from samples included *Staphylococcus aureus* and several types of *Salmonella* (Thompson, Cerro, Montevideo, Kentucky, Typhimurium, and Newport) (*23*). In 1995, an outbreak of *Escherichia coli* O157:H7 occurred in Oregon, involving home-processed deer jerky and it was concluded that this traditional home-drying jerky process was insufficient to kill *E. coli* O157:H7 (*23*).

In addition, in 1996, the first and only reported outbreak of botulism involving type F in the US occurred in California from home-prepared venison jerky. In this outbreak, out of 20 people who ate the product 3 developed botulism (*67*). In 2003 at least 22 cases of salmonellosis were attributed to consumption of commercially produced beef jerky in New Mexico where 20% of the jerky lots tested positive for *Salmonella* (*1*). Additionally, in 2012 there was an outbreak linked to consumption of turkey jerky in Minnesota. Hoffman Town & County Meat Market issued a voluntary recall of all whole-muscle turkey jerky (*19*).

Regulation: Compliance guidelines for jerky production

Following a 2003 salmonellosis outbreak from *Salmonella* Kiambu in beef jerky produced in New Mexico with a recall of nearly 9,797 kg of product, (FSIS) published the first version of the "Compliance Guideline for Meat and Poultry Jerky Produced by Small and Very Small Establishments" (*30*, *60*). The objective of this guideline was to help jerky

manufacturers identify critical steps to ensure safety and eliminate foodborne pathogens through verifying lethality and drying conditions for jerky (*60*).

One potential cause of the 2003 *Salmonella* Kiambu outbreak in jerky was the very slow drying process under low humidity conditions which allowed the bacterium to become resistant to heat. A_W has been recognized as one of the primary factors influencing the thermal resistance of pathogens in LMF. Several studies relate thermal resistance of pathogens to a_W of LMF at room temperature (*20*, *92*).

Experimental evidence supports that the principal cause of thermal inactivation of bacterial cells under high moisture conditions is irreversible destabilization of ribosomes; however in LMF it has been hypothesized that desiccation of bacterial cells sharply reduces molecular mobility and help stabilize ribosomal units against irreversible damage due to thermal energy in low-moisture environments. For instance, bacterial spores exhibit higher thermal resistance than vegetative bacterial cells, attributed to the lower flexibility of bacterial protein structure (*82*, *92*).

Considering this, the FSIS compliance guideline emphasizes the importance for high levels of humidity during jerky manufacturing process (*30*). This guideline proposes 8 general processing steps for jerky production that includes strip preparation, marination, antimicrobial interventions, surface preparation, lethality or "cooking time," drying, post-drying heat step and handling. Although an establishment may not include all these steps in the process, the lethality treatment and drying should be used to ensure the safety of the product (*30*).

The lethality treatment refers to the process to destroy pathogens and make the product safe following a combination of critical operational parameters which includes

time and temperature and relative humidity. Drying is the process in which water is removed from the product to a level to prevent the growth of microorganisms, especially toxigenic microorganisms such as *Staphylococcus aureus* (*66*).

According to the ICMSF, the a_W limit for *Staphylococcus aureus* growth is 0.83 under aerobic conditions and 0.90 under anaerobic conditions. This is why FSIS recommends an upper limit of 0.85 under aerobic conditions or 0.91 under anaerobic conditions, since FSIS acknowledges that jerky has other factors such as nitrites and salt that prevents microorganism's growth (*30*).

Producers of jerky and any RTE product must control food safety hazards and implement Hazard Analysis and Critical Control Point (HACCP) (*80*). Moreover, they need to achieve lethality of pathogens in the product but also stabilize it to inhibit the growth of spore-forming bacteria such as *Clostridium botulinum* and *Clostridium perfringens* (*30*). Stabilization processes may include cooling and hot-holding, drying and fermentation or acidification (*31*).

Food Safety Inspection Service has designed a guideline to help small meat and poultry establishments that produce heat-treated RTE products and not RTE products that stabilize their products by cooling or hot-holding in order to understand the regulatory requirements associated with stabilization. This Compliance guideline defines stabilization as the process of preventing or limiting the growth of spore-forming microorganisms capable of producing toxins in the product or in the human intestine after consumption. However, products with $pH \leq 4.6$ before cooling or products with $a_W < 0.93$ (such as jerky) before cooling are not covered by this guideline (*31*).

Despite that, considering that product innovation has been the primary driver of revenue of jerky (*41*) and that the market is trying to innovate new flavors and texture while increasing a_W, it is important to evaluate the safety of the products and test for spore-forming pathogens since they represent one of the main hazards in products which include stabilization processes in their manufacturing.

Moreover, recent outbreaks of *Salmonella* in beef jerky and *E. coli* O157:H7 in venison jerky support the fact that there are a variety of preparation methods and drying procedures, and there is a concern about the safety of processed meat products made in the home (*27*, *35*). Additionally, discrepancies in temperatures used in the drying procedures for jerky indicate the need for investigation (*35*).

Proper lethality treatments of meat and poultry jerky should destroy pathogens present in the product; nonetheless, when there are spore-forming bacteria (which survive cooking treatments) it is important to verify the stabilization process in order to prevent growth of these microorganisms. Since all producers of RTE products must ensure that they stabilize the product to inhibit the growth of spore-forming bacteria, it is necessary to consider the critical operation parameters that affect the growth of spore-forming bacteria such as *C. perfringens* and *C. botulinum* during cooling of meat and poultry products (*38*, *74*). These parameters include product time/temperature profile, pH, salt concentration, ingoing sodium nitrite concentration, the type and concentration of phosphates, the type and concentration of organic acid salts, and a_W (*31*).

Among spore-forming bacteria, *C. botulinum* is of greatest concern since it is able to produce potentially deadly neurotoxins; therefore, it is critically important to control *C. botulinum* spore germination and organism growth in food products. While not the most

dangerous *C. perfringens*, spore-forming bacteria, is of concern due to its rapid growth rate, so it is a good indicator of food safety and is often used as the target organism during stabilization to demonstrate growth of all spore-formers including that *C. botulinum* is limited to acceptable levels (*31*, *89*).

A limited number of *C. perfringens* illnesses have been associated with food products inspected by FSIS. Only a limited number of outbreaks related to commercially produced meat and poultry products have been reported in the U.S., likely due to good controls in the commercial setting. There was one outbreak associated with *C. perfringens* from a commercially produced RTE turkey loaf product (*31*).

Spore-forming pathogens

Spore-forming bacteria are of special concern in the food industry because it is not always possible to apply enough heat during food processing to kill the spores (*3*, *55*). Bacterial spores are dormant structures of a cell that are able to survive environmental conditions that a bacterium normally would not be able to resist. They are much more resistant to heat, chemicals, irradiation and desiccation than vegetative cells (*3*, *90*). The extraordinary resistance to such stresses makes spores of importance and concern because they are not killed by many antimicrobial treatments including traditional cooking (*30*, *76*).

Thermal processing of meat and poultry products is generally sufficient to destroy vegetative cells, but the spores may survive and multiply during cooling when the conditions favor their growth (*58*). Besides, with the destruction of vegetative cells from Clostridia as well as other microorganisms, there is little competition for the spores

germination. Also anaerobic, non-refrigerated conditions facilitate multiplication of these microorganism (*31*, *74*).

Meat and poultry products can become contaminated with spore-forming Clostridia during the slaughter/dressing process as well as a result of cross-contamination in the processing environment when insanitary conditions are present. Moreover, spices and herbs can be a source of contamination in raw formulated cooked meat and poultry products (*31*). The main food poisoning spore-formers are *C. botulinum*, *C. perfringens* and *Bacillus cereus* (*11*). For the meat industry specifically, *C. perfringens* might become a special problem due to its rapid rate of growth; however, this bacterium mainly causes food poisoning through food that is served in restaurants, and hospitals of homes for elderly people (*3*, *29*).

Clostridium botulinum

Clostridium botulinum is the most important spore-forming pathogen due to the potent neurotoxin that it produces (*11*, *63*). The strains of *C. botulinum* are divided into seven types, A-G, based on the serological specificity of the toxins. Types A, B, E and F have been implicated in human food-borne botulism (*11*, *54*, *86*). Moreover, despite that the ability to produce the botulinum neurotoxin is confined to the genus *Clostridium*, especially to *C. botulinum*, it is recognized that *C. botulinum* contains four distinct genetic and physiological groupings. Group I (proteolytic *C. botulinum*) strains produce one or sometimes two toxins of type A, B or F; Group II (nonproteolytic *C. botulinum*) strains produce toxins of type B, E, or F; Group III strains produce toxins of type C or D; and Group IV strains produce toxin of type G (*84*).

Botulism is a rare but severe neurological disorder that is characterized by a flaccid paralysis (*83*). Three major types of botulism occurs in humans, foodborne botulism, infant/intestinal (adult) botulism and wound botulism (*12*). Foodborne botulism is an intoxication caused by consumption of pre-formed toxin. Botulism was initially linked to consumption of blood sausage. In Belgium in 1895, there was an outbreak associated to home-made raw salted ham and it was concluded since then that it had been produced by an anerobic bacterium during the salting process. After this, there have been several cases in Europe and in North America related to the use of canning and bottling processes to extend shelf-life (*77*).

Clostridium botulinum causes human illness when people ingest a potentially deadly neurotoxin that this bacterium produces in the food (*47*). This neurotoxin can cause muscle paralysis and suffocation with as little as 1 ng of toxin per kg of body weight after 12 to 36 h of ingestion. Therefore, it is critically important to control this microorganism in food products (*31*).

Foodborne botulism outbreaks have been associated with temperature abuse of products intended to be stored chilled, such as vacuum-packed fish. As such, it is necessary to properly cook and cool food products in order to prevent the spores germination or the cells to form the toxins and cause the illness (*78*).

Clostridium perfringens

Clostridium perfringens is one of the most common types of foodborne illness in the US (*57*). It is a concern to the food industry, particularly the retail food service industry, and has been implicated in several large outbreaks (*50*). According to Centers for Disease Control and Prevention (CDC) there is an estimated of 1 million cases of *C. perfringens*

gastroenteritis each year (*16*). *Clostridium perfringens* is a Gram-positive, rod-shaped bacterium, widely distributed in nature and commonly found in fresh meat and poultry products (*97*). Growth limiting conditions for *C. perfringens* and *C. botulinum* are shown in Table 1.

Clostridium perfringens is an important anaerobic, spore-forming bacterium that produces a foodborne illness when foods containing a large population of spores or vegetative cells are ingested (*97*). This bacterium is widely distributed in nature and it is commonly found in the environment and in the intestines of animals and humans as a member of the normal flora (*87*).

Table 1. Limiting conditions for *Clostridium* growth (Adapted from FDA (*26*)).

Pathogen	Min a_w (using salt)	Min pH	Max pH	Min T° (°F/°C)	Max T° (°F/°C)	O_2 Req
Clostridium botulinum, type A, and proteolytic types B and F	0.93	4.6	9	50 10	118.4 48	Obligate Anaerobe
Clostridium botulinum, type E, and nonproteolytic types B and F	0.97	5.0	9	37.9 3.3	113 45	Obligate Anaerobe
Clostridium perfringens	0.93	5.0	9	50 10	125.6 52	Obligate Anaerobe

The infective dose of *C. perfringens* isn't accurately known; however, a concentration around 10^6 CFU or larger produces enough enterotoxin to cause disease (*97*). This bacterium is responsible for the rare but severe foodborne necrotic enteritis. *Clostridium perfringens* enterotoxin (CPE) has been shown to be the virulence

factor responsible for causing the symptoms of *C. perfringens* type A food poisoning (*13, 71*). The USDA-FSIS reported *C. perfringens* prevalence rates of 1% for steers and heifers and 2.7% for cows and bulls (*46*). As a consequence, a wide variety of processed meat products have been implicated in different foodborne outbreaks involving *C. perfringens.*

The ability of *C. perfringens* to grow at elevated temperatures, produce spores, and reproduce rapidly contributes to its role as an important cause of foodborne illness (*43*). This microorganism has been reported as one of the bacteria most commonly isolated from ready-to-serve and pasteurized foods; moreover, uncured meat and meat products are considered to be the favorable substrates for its growth (*5*).

Although *C. perfringens* vegetative cells are not able to survive the regular heat-processing schedules that are employed in the meat industry, the bacterium's spores can survive (*97*). Heat-activated spores can germinate and grow rapidly if thermal processing deviations occur (*97*). The U.S. Food and Drug Administration (FDA) Division of Retail Food Protection has recognized that inadequate cooling is a major food safety problem and established a recommendation that all food be cooled from 60 to 21ºC (from 140 to 70 ºF) in 2 h and from 21 to 5 ºC (from 70 to 41 ºF) in 4 h (*97*). *Clostridium perfringens* is one of the fastest-growing bacteria, with a generation time of about 10 min under optimal conditions compared to *E. coli* that can double every 20 min (*97*). Therefore, they can grow very fast to hazardous levels in the final cooked products (*44*).

Several studies examining models of *C. perfringens* growth in high moisture meat and poultry products have been published; however, there are few investigations regarding *C. perfringens* growth in reduced moisture meat and poultry products. Therefore, more

research is needed to control and prevent growth of *C. perfringens* spores in shelf-stable meat and poultry products.

C. perfringens sporulation and spore germination

Clostridium species have the ability to form metabolically dormant spores that are extremely resistant to environmental stresses (*76*). *Clostridium* spores are the morphotype of persistence and dissemination that are able to survive to different chemical and physical agents (*72*). *Clostridium perfringens* vegetative cells can easily be killed by introducing various abusive physical conditions, but their spores are difficult to inactivate with these treatments (*94*). As a consequence of this resistance, spores are agents of food spoilage and food-borne gastrointestinal diseases (*76*).

The sporulation process in spore-forming bacteria is initiated as a result of the integration of a wide range of environmental and physiological signals induced from changes in cell density, quorum sensing, Krebs cycle, and nutrient starvation (*56*, *75*). In many *Clostridium* species, the decision to enter sporulation is regulated by several orphan histidine kinases that can phosphorylate the master transcriptional regulator Spo0A and trigger a cascade of reactions to prepare the cell to produce the spore (*75*).

Clostridium perfringens initiates the process of sporulation through an asymmetrical division of its cytoplasm membrane, which results in a small compartment (termed the forespore), and a large compartment (termed the mother cell), each with a complete genome. As sporulation progresses with a series of morphological and biochemical changes, the forespore becomes the mature spore that is eventually released to the environment upon lysis of the mother cell (*56*). These spores are able to resist to

different environmental stresses; nonetheless, to cause disease or food spoilage, these spores must return to active growth through a process that is called germination (*72*).

Spore germination occurs when species-specific environmental cues, termed germinants, are sensed by specific germinant receptors. After that, a series of irreversible reactions are triggered which lead to the degradation of the spore's peptidoglycan cortex, rehydration of the spore and resumption of the metabolism.

Finally, the outermost spore layers are opened to release the nascent cell to environment (*72*). Spores often germinate in temperature-abused foods, followed by rapid multiplication of the resultant vegetative cells. After the food is ingested, many vegetative cells are killed by exposure to the low pH of the stomach, but others will survive and pass into the small intestines. In the intestines, the cells sporulates and during this process form the enterotoxin that causes the food poisoning (*56, 72*).

Factors affecting spore germination

Dormant spores must first go through germination and then outgrowth to be converted to vegetative cells and be able to cause the illness (*56, 76*). Spore germination may be affected by different environmental conditions, such as nutrients, pH and a_W. Germinants may include amino acids, monosaccharides, nucleosides, salts, organic acids and bile salts and they are specific for each specie. However, the germinant specificity might correspond to their environmental niche of survival. For instance, spores of *C. perfringens* type A food poisoning isolates are able to germinate in the presence of co-germinants Na^+ and P or K^+ ions which are present in meat and processed meat products (*72*).

Moreover, germination, growth, and also toxin production will also depend on environmental factors or food characteristics such as pH and a_w (*25*, *37*). For instance, vinegar and lemon juice concentrate, have been demonstrated to be able to control germination and outgrowth of *C. perfringens* spores in ground turkey roast without other conventional meat processing ingredients like nitrite and/or nitrate and phosphates during extended chilling times (*99*). Therefore, pH affects growth of vegetative cells but also the opportunity for spore's germination.

Clostridium. perfringens grows in a pH range of 5.0 and 9.0 with an optimum between 6.0 and 7.0, then decreases in pH may influence *C. perfringens* spore germination and outgrowth (*99*). Regarding a_W of the food, spore's germination occurs at levels as low as those that allow growth of vegetative cells. The minimum value of a_W for the growth of *C. perfringens* in 0.93; which means that this is the required value for the spores to germinate (*24*).

Strategies to control spore-forming pathogens in food products

Since spores are inherently more resistant than vegetative cells, the methods for controlling these microorganisms need to be chosen carefully (*4*, *11*). While cooking meat and poultry products can destroy pathogen's vegetative cells, bacteria like *C. perfringens* and *C. botulinum* form spores that may survive cooking (*31*). These spores can grow into vegetative cells during cooling because they do not have to compete with other bacteria and temperature is also in the danger zone for them to grow. This is shown in Figure 1 which explains the process of spore formation, germination and outgrowth that occurs in meat and poultry products after the lethality treatment. Therefore, the best

control to stabilize heat-treated products is rapid cooling to reduce the amount of time spores have to germinate then grow into cells (*31*).

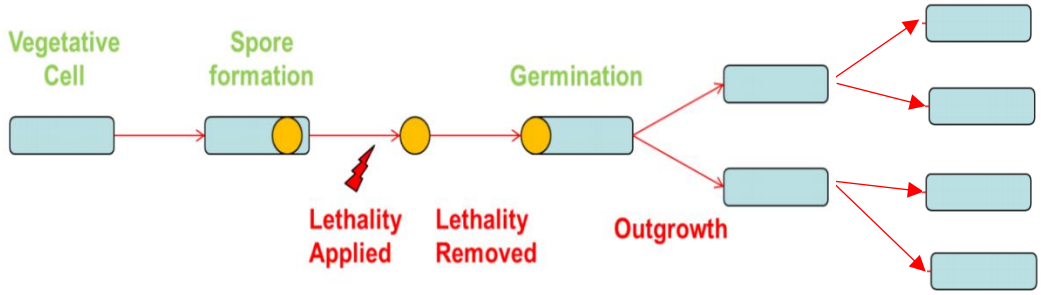

Figure 1. Spores formation, germination and outgrowth. Adapted from FSIS, 2017 (*31*).

The FSIS draft compliance guidelines for RTE meat and poultry products state that such products should be cooled at a rate sufficient to prevent more than a 1-log increase of *C. perfringens* cells (*97*). *Clostridium perfringens* levels can significantly increase during cooling of cooked meat and poultry products (*46*). When heated food is cooled down, it passes temperatures that are suitable for spore germination and consequently the growth of vegetative cells (*8*). Current strategies to control *C. perfringens* involve rapid cooling of meat and poultry products after thermal processing (*25*). According to the USDA guidelines, cooked uncured meat and poultry products should be cooled from 54.5 to 26.78 ºC within 1.5 h and from 26.7 to 4.48 ºC within 5 h. Meanwhile, for cooked cured meat and poultry items the guidelines require the cooling from 54.4 to 26.78 ºC within 5 h and from 26.7 to 7.28 ºC within 10 h (*46*).

These bacteria are also of particular concern in minimally processed foods, such as *sous vide* cooked products, due to the ability of surviving the mild heat treatment (*8*). Furthermore, considering that the growth of these spore-forming bacteria depends on

many factors, it is necessary to combine different factors to control their growth. These factors might include heat treatment, storage temperature and gaseous atmosphere and intrinsic parameters such as pH, salt and other additives (*8*).

The use of additives such as nitrites and nitrates in meat and poultry products is one of the factors that help to control spore-forming pathogens. Nitrites and nitrates in the manufacturing of meat products, which is commonly expressed as ‘‘curing’’, meant in the beginning the use of salt which lowered the a_W and inhibited the growth of spoilage microorganisms (*36*, *70*).

Nitrate and nitrite have been used in processed meat products to enhance color, flavor and storage (*33*). They are commonly used additives in cured meats because of their technological contribution to the oxidative stability of lipids and to the typical flavor and color development. Furthermore, they play a key role on the inhibition of the Gram-positive spore-forming anaerobic bacteria as *Clostridium botulinum*. Such inhibition is the result of the interaction between nitric oxide derived from nitrite and clostridial iron–sulphur proteins, such as ferredoxin and ferredoxin-pyruvate oxidoreductase, resulting in a rapid decrease of intracellular ATP and pyruvate accumulation (*37*, *53*).

Besides, nitrite has been demonstrated to be able to control other foodborne pathogens such as *Listeria monocytogenes, Achromobacter, Aerobacter*, *Escherichia*, *Flavobacterium*, and *Micrococcus* spp.(*53*, *59*). On the other hand, synergistic inhibitory activity of sodium nitrite and sodium chloride against *C. perfringens* has been demonstrated in media and meat. Combined inhibitory effects of these chemical compounds with other parameters such as pH against *C. perfringens* may explain the lack

of illnesses attributable to cure meats. However, there have been some cases of outbreaks, for example, *C. perfringens* traced to ham in Ohio and Virginia (*95*).

Despite the efficacy of sodium nitrite in meat products to prevent pathogens growth, many studies have been conducted to examine the use of natural additives as a result of the increase in consumer demand for nitrite-free safe meat products. For instance Lee et al. (*52*) evaluated the combined effect of high pressure and vinegar addition on the control of *C. perfringens* and quality in nitrite-free emulsion-type sausage. The study showed that the addition of 1% (w/v) of vinegar and pressure treatment at 500 MPa (four cycles and each for 3 min) can replace sodium nitrite to inhibit growth of *C. perfringens* in emulsion-type sausages.

Processing technologies to control spore-formers in low-moisture foods

Other strategies beside additives that are used to control spore-forming pathogens include thermal treatments. For instance, the safety of LMF may be improved by controlling the pathogens during food manufacturing and by preventing recontamination and cross-contamination of the final products. Moreover, treatments may be necessary in order to eliminate the pathogens in a contaminated low-moisture product.

Several thermal and nonthermal pasteurization technologies, such as steam, moist and dry air, radiofrequency, X-ray, electron beam, propylene oxide, and use of plant extracts have been reported to improve LMF (*7*, *18*, *21*, *43*, *49*, *53*, *92*). However, these technologies have been mainly studied with pathogens' vegetative cells, but in the case of spore-forming pathogens a combination of different technologies or strategies might be needed.

For instance, *C. perfringens* vegetative cells can easily be killed by introducing various abusive physical conditions, but their spores are difficult to inactivate with these treatments. However, studies have shown that either the manipulation of physical conditions or combined treatment with two or more stress factors can be able to inactivate the spores (*94*). Considering that the cooling practices are not always followed strictly, the elimination of *C. perfringens* spores through processing is an alternative strategy. The thermal inactivation of *C. perfringens* spores in foods has been reported in several works (*25, 40*). High pressure processing (HPP) is a commercial food preservation technology with less adverse effects on food quality compared with conventional thermal processes alone and is typically applied to inactivate spoilage/pathogenic microorganisms and to extend food shelf-life (*6, 85*).

Preservation of foods with this technology would enhance the spore inactivation compared to thermal processing alone at the same temperature. Besides the uses of HPP, together with the application of Good Manufacturing Practices, including rapid cooling, is a good alternative to the traditional methods for producing safe processed meat and poultry products with enhanced sensory and nutritional quality (*25, 94*).

Post-Packaging Pasteurization (PPP)

Current efforts in food safety of meat and poultry products have included both the reduction of microorganism on raw meat at slaughter and processes to control potential pathogens that may be acquired as incidental contaminants (*48, 69*). Moreover, in the case of spore-forming pathogens, some common lethality schedule cannot eliminate the spores then other treatments are needed. Some strategies as post-package or post-process

heat treatments have been reported to control surface contaminants as *Listeria monocytogenes* (*69*).

However, few studies have been conducted to use post-packaging or post process heat treatments to control spores of pathogens as *C. perfringens*. Furthermore, little literature is available that adequately addresses submerged water pasteurization of ready-to-eat meat and poultry products.

Hungwei et al. (*39*) proposed PPP as an intervention for RTE products to control pathogens after lethality treatments. They decided to patent the intervention that consisted of heating for a time and at a temperature effective to kill, inactivate or reduce bacteria to safe levels using a water bath that provides pressure in excess of atmospheric pressure. Nonetheless, there is not literature available showing the effectivity of the treatment.

Therefore, even though PPP seems as a promising technology to control pathogens in products that have been subjected to a lethality schedule, there is a need of research and studies regarding the efficacy of this treatment for both vegetative and spores of pathogens in meat and poultry products.

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CHAPTER II

FATE OF SPORE-FORMING PATHOGENS IN HIGH AND REDUCED MOISTURE, PROCESSED MEAT AND POULTRY PRODUCTS SUBJECTED TO POST-PACKAGING PASTEURIZATION

Introduction

With rapid urbanization and socioeconomic change, there has been an increase in demand for convenient RTE meat and poultry products. To prepare shelf-stable and microbiologically safe RTE meat products requires different strategies and technologies such as reducing a_W, vacuum packaging and a combination of heat treatments might be implemented (*3, 18*).

Meat and poultry jerky provide high protein value and consumption trends are increasing in the market since they can be stored without refrigeration (*8, 26*). For these products FSIS has established an a_W critical limit of 0.85 or lower for products stored under aerobic conditions, and 0.91 or lower if the product is vacuum packaged (30). However, due to the high variability of processing times and temperatures, processing environment relative humidity, and methods to control humidity used by small and very small plants, it is necessary to conduct lethality studies and asses quality and safety of the product (*20*).

Currently, one of the strategies for developing new beef and poultry jerky products is the increase of a_W of the product to improve consumer acceptability and flavor; therefore, implementing additional hurdles is of high importance to prevent foodborne pathogens. Traditional jerky products are the result of what is known as hurdle technology, which includes heat treatments, reduction of a_W and additives in the preparation (*8, 13*). In general, the production of jerky consists of marinating and slicing

whole muscle meat or poultry, followed by a lethality schedule, drying and packing of the final product (*13*).

Post-drying heat steps might be included to reduce surface contaminants such as *L. monocytogenes* when cooling, handling or packing the product (*22*). Few studies have been reported regarding the use of post-packaging pasteurization to control environmental pathogens as *L. monocytogenes* after cooking the products (*22*). However, very few studies have been conducted about the use of this technology to control spore-forming pathogens in shelf-stable RTE meat and poultry products.

Spore-forming bacteria are of special concern in the food industry because it is not always possible to apply enough heat during food processing to kill the spores. Bacterial spores are dormant structures of a cell that are able to survive environmental conditions that vegetative cells will not be able to resist such as heat, chemicals, irradiation and desiccation (*6, 24*).

Thermal processing of meat and poultry products is generally sufficient to destroy vegetative cells, but the spores may survive and multiply during cooling when conditions favor growth (*19*). Moreover, with the destruction of vegetative cells there is little competition for the spores to grow. Also anaerobic non-refrigerated conditions facilitate multiplication of the microorganism (*17, 23*).

The main food poisoning spore-forming pathogens are *Clostridium botulinum*, *Clostridium perfringens*, and *Bacillus cereus*. *C. botulinum* is the spore-former of greatest concern due the potent neurotoxin that it is able to produce, but *C. perfringens* might become a special problem in meat industry due to its fast rate of growth (*4, 21*).

We conducted this study under the assumption that if the spores of *C. perfringens*

are able to germinate during the cooling of the product, germinated spores can be inactivated with Post Packaging Pasteurization (PPP). The objectives of this study, therefore, were a) to evaluate the fate of spore-forming pathogens in a jerky-like product (a_W:0.91±0.01) that was subjected first to a lethality schedule (heat and drying), then to a cooling period and finally submitted to post-packaging pasteurization; b) to assess the effect of a_W of meat and poultry products on spore survival and growth, and c) to conduct shelf-life studies to evaluate the fate of surviving spores in the reduced-moisture products.

Materials and Methods

Meat and poultry products preparation

Meat and poultry samples were prepared at the Texas Tech University G. W. Davis Meat Science Laboratory. Denuded, cap-off beef top rounds (*semimembranosus)* were used to prepare beef strips and whole breast meat was used to prepare chicken strips. For beef samples, a proprietary brine solution containing roughly 53.8% water, 5.6% salt, 0.6% celery powder for cure, and 40.0% flavorings, seasonings, and spices was injected with a multi-needle injector (Wolf-Tec, Kingston, NY) at a rate of 133% of green weight into each muscle.

Immediately after injecting, muscles were placed in a vacuum tumbler (Koch Industries, Wichita, KS) and tumbled under vacuum (33.9 kPa) at a rate of 5 rpm for one hour. Top rounds were set overnight to allow for equilibration of the brine at 4 °C. Muscles were then sliced to 2.5-cm across the grain and 12-cm with the grain to create a uniform piece (2.5-cm width x 12-cm depth x natural length). Same procedure was

followed to prepare chicken samples; however, the meat breast was only vacuum tumbled with the marinade vacuum (67.7 kPa) and not injected.

Spores preparation

Three strains of *C. perfringens*, NCTC 8239, NCTC 10244, and NCTC 10388 were used in all inoculated meat and poultry strip studies. Stock cultures were maintained in cryoBeads (Key Scientific products, Stanford, TX, USA) at -20 °C at the International Center for Food Industry Excellence (ICFIE) laboratory.

The three *C. perfringens* strains were grown individually in 10-mL tubes containing fresh fluid thioglycolate medium, incubated at 37 °C for 24 h in an anaerobic chamber (Coy, Inc., Grass lake, MI, USA) with 10% CO_2, 10% H_2, 80% N_2. A 100-µL aliquot of each culture was added to separate 10-mL tubes of a fresh fluid thioglycolate medium and incubated for four h at 37 °C in order to keep the cells in the exponential growth phase. Upon incubation, the inoculated tubes were transferred to separate 1-L bottles of Duncan Strong medium with raffinose supplemented with caffeine (100 µg/mL). All bottles were then incubated at 37 °C for 18 h in the anaerobic chamber. Cultured solutions were centrifuged at 4 °C three times for 20 min at 5,000 x g, washed with sterile water and kept refrigerated at ≤7 °C. To enumerate the spores, every culture was heat-shocked at 80 °C for 20 min and plated with Tripticase-Sulfite-Cycloserine (TSC) agar using pour-plating and overlaid technique according to Harmon et al. (*7*).

Inoculum preparation

Spore cultures were kept in sterile water and refrigerated at 7 °C. The day of the experiment, a 3-mL aliquot of each culture was added to a Falcon tube and mixed with a vortex to obtain a spore cocktail of the three strains of *C. perfringens*.

Samples Inoculation

Reduced moisture products

Beef and chicken strips were standardized to weight 30 ± 3 g and were inoculated with the *C. perfringens* spore cocktail. Three-tenths of a microliter of the inoculum was added to each beef or chicken strip to yield a final concentration of 3 $\log_{10}$ UFC/g. The inoculated sample was hand-massaged and mixed in a stomacher (Seward, Bohemia, NY, USA) for 30 seconds at 230 rpm. Samples were then packed and taken to the Texas Tech University Microbial Pathogen Laboratory to be subjected to thermal processing.

High moisture products

For conducting high moisture experiments, beef and chicken strips were standardized to weight 10 ± 3 g and were inoculated with 0.1-µL of the *C. perfringens* spores cocktail following the same procedure described above for reduced moisture products.

Lethality treatment (thermal processing) of meat and poultry products

Reduced moisture products

Inoculated beef and chicken strips were taken to the Texas Tech University Microbial Pathogen Laboratory and placed into trays to be subjected to thermal processing following a standard heating cycle with humidity, followed by a drying cycle in a commercial smokehouse (Enviro-Pak, Clackamas, OR, USA). The cooking cycle of the products is shown in Table 2. After the smokehouse cycle was completed to reach an a_W of 0.91 ±0.01, every sample unit was vacuum packaged individually.

Table 2. Smokehouse cycle conditions for reduced moisture meat and poultry products.

Step	Time (min)	Dry Bulb (°C)	Wet Bulb (°C)	RH %	Exhaust	Steam
1	10	48.9	37.8	50	On	Off
2	10	60.0	51.7	65	On	On
3	10	71.1	65.6	78	On	On
4	10	79.4	71.1	70	On	On
5	30	85.0	76.6	71	On	On
6	30	82.2	60.0	35	On	On
7	40	82.2	60.0	35	On	On
8	10	76.6	55.6	35	On	On
9	10	76.6	55.6	35	On	On
10	10	71.1	51.1	35	On	On
11	30	71.1	51.1	35	On	On
12	open	71.1	51.1	35	Off	Off

High moisture products

Inoculated beef and chicken strips were vacuum packaged and then cooked in a programmable water bath (Thermo Scientific, Waltham, WA, USA) following the same cycle temperature values and time intervals of the smokehouse cycle that was used for reduced moisture product studies (Table 2). These products had an a_W of 0.99 ±0.01.

Product Cooling

For both studies (reduced moisture and high moisture), the products were cooled at room temperature (23±2 °C) for different periods of time (one, two and three h). The temperature of the products during the cooling was continuously recorded using temperature recorders (Temprecord^{TM}, Auckland, New Zealand).

Post-Packaging Pasteurization

After completing each cooling time, the products were subjected to PPP. The conditions for this heat treatment were 77 °C for 6 min. Once the product was pasteurized, it was cooled by submerging the product in ice water for 1 min.

Water activity and pH measurements

At each experiment repetition, 3 product samples were collected to measure a_W and pH. To evaluate a_W, every sample unit was cut in small pieces and collected into a container to be placed in the a_w instrument (Hygrolab, rotronic, Hauppauge, NY, USA). To measure pH, sample units were cut into small pieces, submerged and mixed in water using a stomacher (Seward, Bohemia, NY, USA) and the pH was measured using a pH meter (Mettler Toledo, Columbus, OH).

Sample collection for microbial analysis

Samples were taken at 8 different steps during the thermal processing schedule. Sampling was conducted at the beginning of the process to determine initial concentration of the spores in raw product, right after lethality treatment (cooking), after each cooling time interval (one, two and three h) and after pasteurizing the product (right after each cooling period of time). Ten samples were obtained in every step described above for reduced moisture products; meanwhile five samples were taken at every stage for high moisture products study. The experiment was repeated in triplicate for each type of product (beef and chicken) (See Appendix A).

Fate of C. perfringens during extended product storage

To evaluate the fate of surviving non-germinated *C. perfringens* spores in the products after processing, samples were stored at different temperatures and storage times. Reduced moisture beef strips were stored during two, four, seven, ten and twelve days at room temperature (23 ± 2 °C). Reduced moisture chicken strips were stored during seven, twelve and twenty-one days at room temperature and at 37 °C to simulate abusive temperature conditions. After completing the different storage times, the samples were analyzed to enumerate *C. perfringens* spores in the product.

Microbial Enumeration

Each sample was aseptically opened to transfer the meat to a filter stomacher bag. Sterile buffered peptone water (BPW) was added to obtain a 1:2 dilution and mixed in a stomacher (Seward, Bohemia, NY, USA). Serial dilutions were prepared in sterile BPW and pour-plated on TSC agar plates. Solidified plates were overlaid with an additional 5 mL of TSC and incubated for 24 h at 37 °C in an anaerobic chamber (Coy, Inc., Grass lake, MI, USA). Total *C. perfringens* populations were reported as CFU/g of samples.

Statistical Analysis

In order to evaluate the effect of the PPP treatment and cooling time on *C. perfringens* spores in the meat and poultry products, the *C. perfringens* counts were compared using ANOVA test of R (R T 386. 3.4.3., RStudio, Inc., Boston, MA, USA). ANOVA was conducted in a factorial design 3*2, with three different levels of cooling times (1, 2 and 3 h after cooking) and 2 different steps in the process (before and after PPP).

Samples taken right after lethality treatment (cooking) were considered as the control to compare the counts. The initial concentration was evaluated to determine the attachment of the spores in the inoculum, but this was not included in the statistical analysis. Whenever there was an interaction between time and treatment in ANOVA main effects, then a Tukey test was conducted to compare among the different combinations of treatments and cooling times.

The results were represented using boxplots in order to evidence the distribution of the data and evaluate the behavior of the spores during the thermal processing. This representation is helpful to observe all the data points, the normal distribution of the data and the outliers. Besides, it is possible to evidence the differences among the treatments and times.

Results

Effect of post-packaging pasteurization on C. perfringens spores inoculated on reduced moisture beef strips

The levels of *C. perfringens* spores in reduced moisture, heat-treated beef strips are shown in Table 3 and represented in Figure 2. *Clostridium perfringens* spores must germinate before developing into actively growing vegetative cells; therefore, the effect of PPP treatment is dependent on the presence of germinated cells to be effective. Since spores have not develop into vegetative cells, PPP shows minimal inactivation effect due to the spore-resistance to heat.

Table 3 shows the initial *C. perfringens* spore concentration, spore numbers after the product was thermally processed for lethality in the smokehouse and spore concentration after cooling the product at different time intervals (1 hour, 2 h and 3 h). To

evaluate the effect of PPP on the reduction of the spore numbers after cooling, the inoculated product was evaluated with and without PPP application. In spore form, PPP treatment has no effect in the reduction of *C. perfringens*; however, if the spores enter germination stages during cooling, then the second thermal treatment will be effective to reduce vegetative cell counts (Figure 2).

Table 3. *Clostridium perfringens* spore counts on reduced moisture beef strips after lethality schedule, stabilization (cooling) and post-packaging pasteurization.

Time	n	Treatment (PPP)*		
		Before PPP	**After PPP**	**Reduction**
Initial Concentration	30	3.47±0.17	----	----
After lethality schedule	30	3.11 ± 0.64^{ax}	----	----
1 h cooling	60	3.07 ± 0.81^{axy}	2.70 ± 0.64^{bxy}	0.37
2 h cooling	60	3.21 ± 0.80^{ax}	2.83 ± 0.69^{by}	0.38
3 h cooling	60	2.76 ± 0.58^{ay}	2.43 ± 0.59^{bx}	0.33

**C. perfringens* spores counts are expressed as mean log CFU/g. a-d Means within a row with no common letter differ significantly ($p < 0.05$; n=30). x-y Means within a column with no common letter differ significantly ($p < 0.05$; n=30).

When the concentration level of spores after the lethality schedule is compared with the counts after cooling for 3 h and a subsequent post-package pasteurization, there is a statistically significant difference ($p < 0.05$) of approximately 0.6 log. In the analysis of variance table (Table 4) it is shown that the treatment (PPP) has a significant effect on the reduction of *C. perfringens* spores once the product is cooled down for at least 1 hour to allow for some spores to germinate and become vulnerable ($p < 0.05$). Table 3 shows an approximately 0.3 log reduction when applying the PPP treatment.

Table 4. Analysis of variance (ANOVA) of *C. perfringens* counts on reduced moisture beef strips that were subjected to post-packaging pasteurization.

Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)*
Time	3	7.487	2.495	5.249	**0.0016**
Treatment	1	5.885	5.884	12.379	**0.0005**
Time: Treatment	2	0.026	0.013	0.027	**0.9729**

*** Pr= *p* value**

As shown in Table 3 the spore counts that do not share same letters are significantly different ($p < 0.05$). For instance, cooling the product for three h and pasteurizing it results in different counts than the initial spore concentration in the product and the spore concentration after it has been cooked in the smokehouse, which confirms the premise that allowing the product to cool down and subjecting it to PPP has a deleterious effect in *C. perfringens* spore concentration. Finally, there was a reduction of approximately 1 log after the beef strips were heat-treated in the smokehouse, cooled for 3 h and subjected to PPP. Figure 2 shows the differences between the trend lines of the process with and without PPP, providing evidence of the effect that PPP has on reducing *C. perfringens* spores that might had germinated in the product.

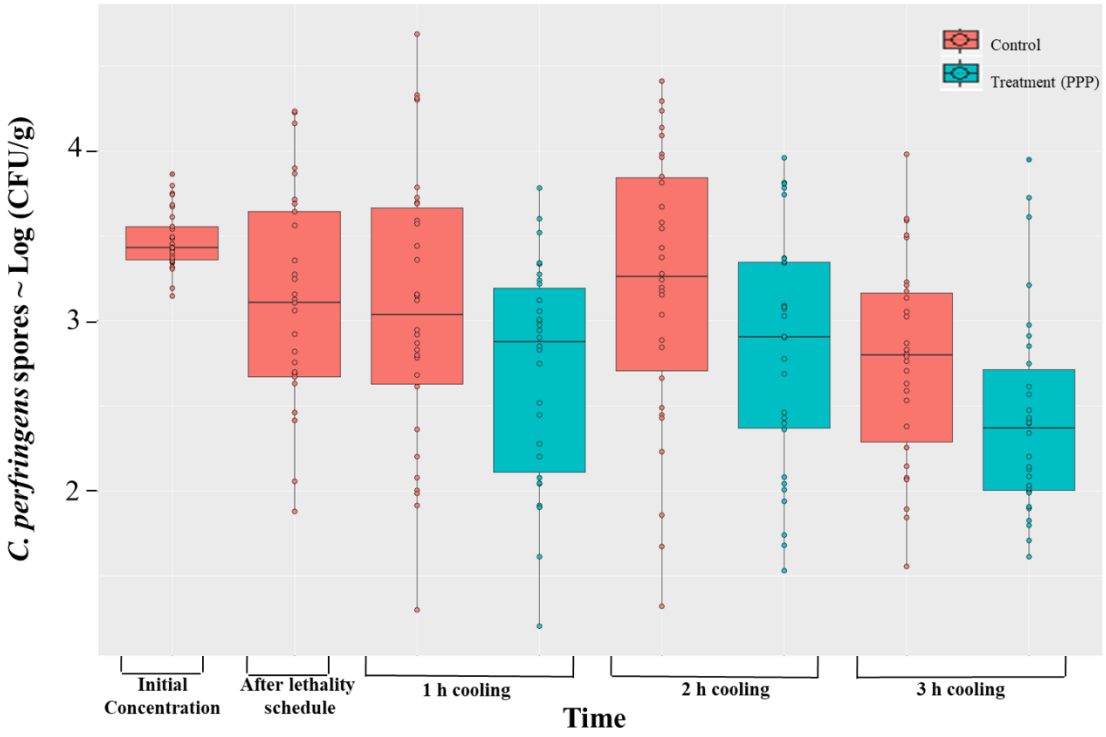

Figure 2. *C. perfringens* spores' levels on reduced moisture beef strips submitted to lethality treatment and post-packaging pasteurization.

Effect of post-packaging pasteurization on C. perfringens spores inoculated on reduced moisture chicken strips

Results for the evaluation of the effect of PPP in reduced moisture chicken strips inoculated with *C. perfringens* spores are shown in Table 5 and represented in Figure 3. There are no major differences when comparing the different cooling times with the initial spore concentration after cooking, which may suggest that the spores did not germinate and/or grow during cooling.

However, Table 6 shows the results of ANOVA test to verify if the treatment (Post-Packaging Pasteurization) had an effect on spore reduction. There is statistical evidence that the PPP treatment has an effect on the spores counts ($p<0,05$). Nonetheless,

the reduction observed was only around 0.2 ± 1 log when subjecting the product to PPP after cooling.

Table 5. *C. perfringens* spores counts on reduced moisture chicken strips after lethality schedule, stabilization and post-packaging pasteurization (PPP).

Time	n	Treatment (PPP)*		
		Before PPP	After PPP	Reduction
Initial Concentration	30	2.88±0.26	----	----
After lethality schedule	30	2.39±0.55	----	----
1 h cooling	60	2.36±0.59[a]	2.19±0.50[b]	0.17
2 h cooling	60	2.32±0.59[a]	2.10±0.55[b]	0.22
3 h cooling	60	2.30±0.58[a]	1.98±0.43[b]	0.32

**C. perfringens* spores counts are expressed as mean log CFU/g. a-d Means within a row with different superscripts differ significantly ($P < 0.05$; n=30).

Furthermore, when comparing the initial concentration with the number of spores after cooling for 3 h and subjecting the product to PPP, there is a reduction of approximately 1 log, which shows that the lethality schedule and further pasteurization influenced *C. perfringens* spore viability (Figure 3).

These results are very similar to the ones that were observed with beef strips. This shows that in reduced moisture meat and poultry products, despite the potential effect of PPP on *C. perfringens* cells viability, the reductions are minimal, and this may be due to the minimal levels of spore's germination.

Table 6. Analysis of variance (ANOVA) of *C. perfringens* counts on reduced moisture chicken strips that were subjected to post-packaging pasteurization.

Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)*
Time	3	0.593	0.197	0.682	**0.5633**
Treatment	1	4.040	4.040	13.949	**0.0002**
Time:treatment	2	0.276	0.091	0.317	**0.8123**

*** Pr= *p* value**

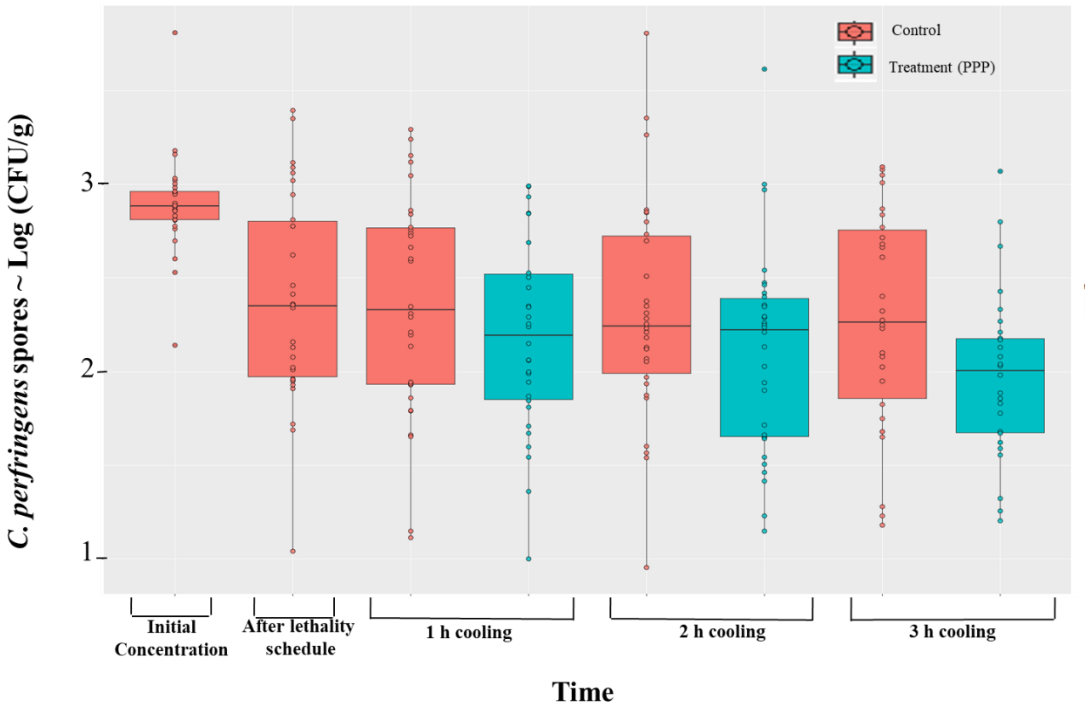

Figure 3. *C. perfringens* spores' levels on reduced moisture chicken strips submitted to lethality treatment and post-packaging pasteurization.

Effect of post-packaging pasteurization on C. perfringens spores inoculated on high moisture beef strips

The effect of post packaging pasteurization on *C. perfringens* spores in high moisture beef strips after cooling the product once they have been subjected to heat treatment are shown in Table 7 and represented in Figure 4. Up to 1 log reductions are

observed after cooling the product and subjecting it to PPP in all of the cooling times evaluated. However, there is no higher reduction when cooling the product for longer intervals.

Moreover, there is a reduction of around 1.3 log CFU/g s in the spore concentration after the two heat treatments (cooking and PPP). Table 8 shows that there is a statistically significant effect of the PPP treatment on spore concentration ($p<0,05$). Moreover, Figure 4 shows the differences between the trend lines when the product is subjected to PPP, indicating significant spore counts reduction.

Table 7. *C. perfringens* spores counts on high moisture beef strips after lethality schedule, stabilization and post-packaging pasteurization.

Time	n	Treatment (PPP)*		
		Before PPP	**After PPP**	**Reduction**
Initial Concentration	15	3.96±0.41	----	----
After lethality schedule	15	3.89 ± 0.43^{ay}	----	----
1 h cooling	30	3.79 ± 0.58^{axy}	2.91 ± 0.66^{b}	0.88
2 h cooling	30	3.75 ± 0.49^{axy}	2.90 ± 0.62^{b}	0.85
3 h cooling	30	3.64 ± 0.64^{ax}	2.69 ± 0.85^{b}	0.95

**C. perfringens* spore counts are expressed as mean log CFU/g. a-d Means within a row with no common letter differ significantly ($p < 0.05$; n=30). x-y Means within a column with no common letter differ significantly ($p < 0.05$; n=30).

Table 8. Analysis of variance (ANOVA) of *C. perfringens* counts on high moisture beef strips that were subjected to post-packaging pasteurization.

Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)*
Time	3	5.22	1.741	4.484	**0.0055**
Treatment	1	16.60	16.598	42.743	**3.43e-09**
Time:treatment	2	0.04	0.018	0.047	**0.9539**

*** Pr= *p* value**

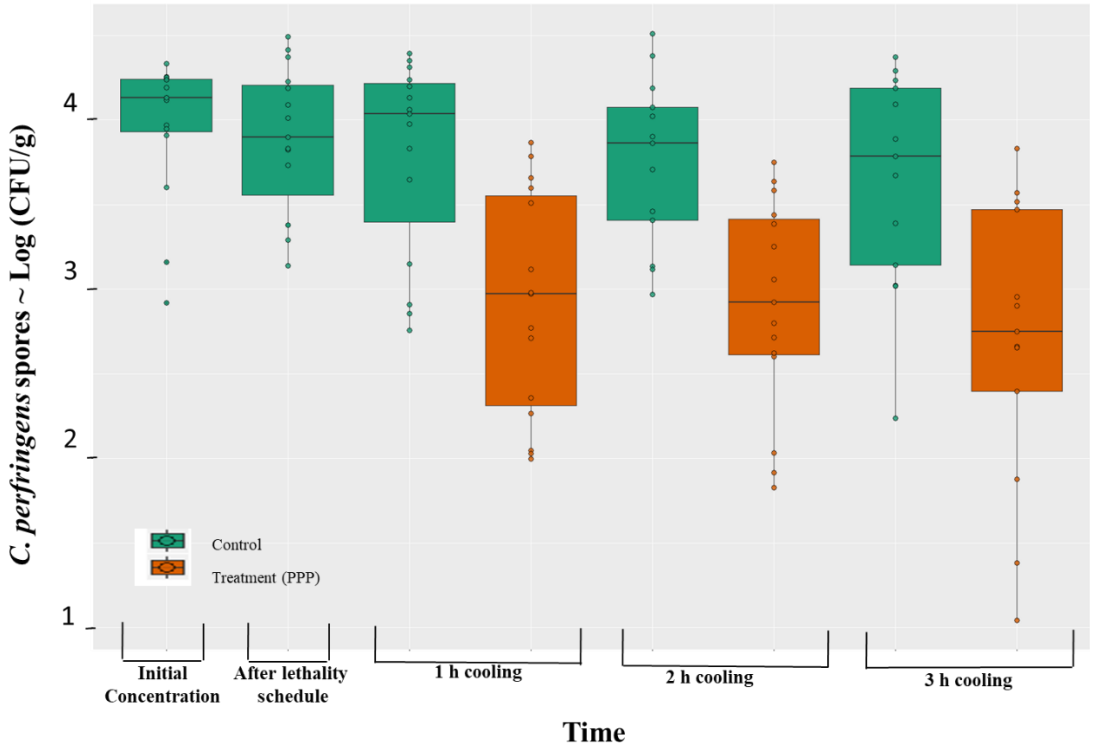

Figure 4. *C. perfringens* spores' levels on high moisture beef strips submitted to lethality treatment and post-packaging pasteurization.

Effect of post-packaging pasteurization on C. perfringens spores inoculated on high moisture chicken strips

The effect of post packaging pasteurization on *C. perfringens* spores in high moisture chicken strips after cooling the product once they have been subjected to heat treatment are shown in Table 9 and represented in Figure 5. A 1.68 log reduction is obtained after cooling the product for 1 hour and subjecting to PPP. Furthermore, 2.2 and 2.09 log reductions are obtained after cooling the product for 2 h and 3 h, respectively.

After cooling the product for 2 or 3 h, it is possible to reduce most of the spores that were originally present in the product. This shows that time and treatment, both, have an effect on the reduction of the spores when high moisture is provided, and this is also confirmed in Table 10 ($p<0.05$). The trends of the process observed in Figure 4, show

that there is a clear difference when subjecting the product to PPP in *C. perfringens* spores counts.

Table 9. *C. perfringens* spores counts on high moisture chicken strips after lethality schedule, stabilization and post-packaging pasteurization.

Time	n	Treatment (PPP)*		
		Before PPP	**After PPP**	**Reduction**
Initial Concentration	15	3.16±0.44	----	----
After lethality schedule	15	2.84 ± 0.30^{ax}	----	----
1 h cooling	30	2.79 ± 0.32^{ax}	1.10 ± 0.80^{by}	1.69
2 h cooling	30	2.76 ± 0.46^{ax}	0.56 ± 0.60^{bx}	2.20
3 h cooling	30	2.78 ± 0.42^{ax}	0.69 ± 0.79^{bxy}	2.09

* *C. perfringens* spore counts are expressed as mean log CFU/g. a-d Means within a row with no common letter differ significantly ($p < 0.05$; n=30). x-y Means within a column with no common letter differ significantly ($p < 0.05$; n=30).

Table 10. Analysis of variance (ANOVA) of *C. perfringens* counts on high moisture chicken strips that were subjected to post-packaging pasteurization.

Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)*
Time	3	6.64	1.660	5.409	**0.000455**
Treatment	1	3.56	3.556	11.589	**0.000875**
Time:treatment	2	23.13	5.783	18.849	**2.58e-12**

*** Pr= *p* value**

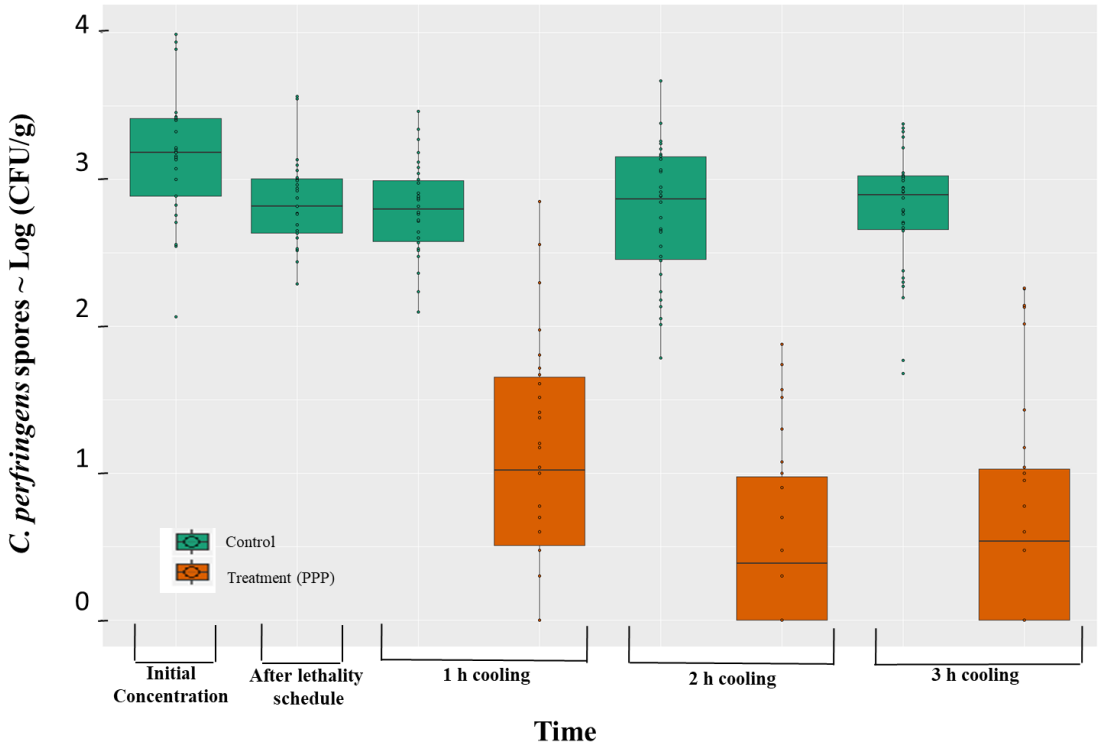

Figure 5. *C. perfringens* spores' levels on high moisture chicken strips submitted to lethality treatment and post-packaging pasteurization.

Microbiological shelf-life studies of reduced moisture meat and poultry products

Table 11 and Table 12 show the results of microbiological shelf-life study after evaluating *C. perfringens* spore levels when storing the products. After storing the product for different intervals of time, there is approximately 1 log reduction in reduced moisture beef and chicken products.

This shows that the characteristics of the product (low a_W and additives) contributes to the prevention of *C. perfringens* growth and reproduction. However, the results for reduced chicken strips shelf-life experiments show that there is a difference when storing the product at room temperature and when this is challenged at abused temperature.

When comparing results of the storing the product at room temperature and treating it with PPP, the value before and after the treatment are similar, which suggests that *C. perfringens* was in its spore state. Nonetheless, when comparing the values at abused temperature there are some differences, which might indicate that the spores were able to germinate at this temperature and therefore were eliminated with PPP. Enumerating *C. perfringens* with and without PPP is helpful to understand weather the cells are active or are in the spore form.

Table 11. Microbiological shelf-life studies of reduced moisture beef strips inoculated with *C. perfringens* spores

Time of storage (days)	n	Initial [ ]*	*C. perfringens* counts*	Reduction*
2	15	2.36	1.05	1.31
4	5	2.57	1.40	1.17
7	3	2.57	1.71	0.86
10	5	2.57	2.1	0.47
12	3	2.57	1.68	0.89

*Counts are expressed as mean log CFU/g

Table 12. Microbiological shelf-life studies of reduced moisture chicken strips inoculated with *C. perfringens* spores

Time of storage (days)	n	Initial [ ]*	Temperature			
			Room T° (25°C)		Abuse T° (37°C)	
			Control**	PPP**	Control**	PPP**
7	7	1.90	1.11	1.38	1.16	0.32
12	3	1.69	0.20	0.30	0.77	0.34
21	7	1.69	0.78	0.45	0.39	0.08

*Initial [ ]: Initial *C. perfringens* spores concentration before storing ** Counts are expresses as the mean Log CFU/g *C. perfringens* spores with and without pasteurizing

C. perfringens colony morphological changes after lethality treatments

Morphological changes were observed in the size of the colonies after the products were subjected to the heat treatments. Once the products were inoculated, the initial concentration was tested, and the colonies were black with rode shape. However, after cooking the product, stabilizing and pasteurizing, the morphology of the colonies changed. Black rounded colonies were observed but a noticeable change in the size of the colonies was observed. Figure 5 shows the differences between the colonies before and after the heat treatments.

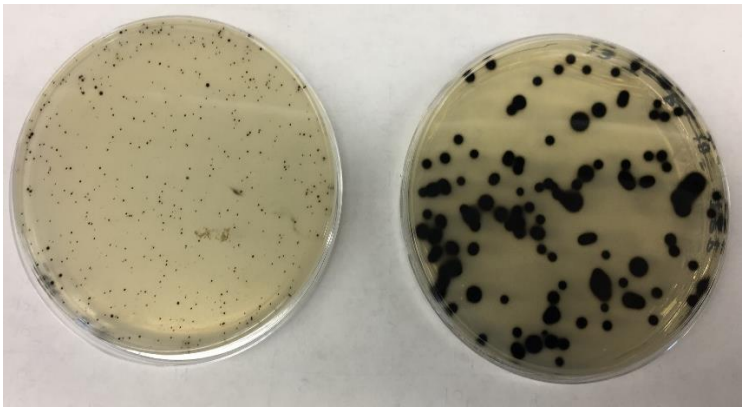

Figure 6. *C. perfringens* colonies in raw chicken strips (left) and *C. perfringens* colonies in heat treated chicken strips (right).

Water activity and pH measurements

Water activity and pH value of reduced moisture products and high moisture products are shown in Table 13 and Table 14 respectively. Reduced moisture products had an a_W of approximately 0.91±0.1 while high moisture product values were around 0.99±0.1. Moreover, pH values for both reduced and high moisture meat and poultry products were approximately 6.1±0.1.

Table 13. Water activity values for reduced and high moisture meat and poultry products

Day	n	Reduced Moisture product*		High Moisture product*	
		Beef	Chicken	Beef	Chicken
1	3	0.910	0.905	0.981	0.987
2	3	0.908	0.911	0.991	0.990
3	3	0.920	0.928	0.982	0.989

*Values are expressed as mean of water activity measurements

Table 14. pH values for reduced moisture meat and poultry products

Day	n	Reduced Moisture product	
		Beef	Chicken
1	3	6.17	6.13
2	3	6.19	6.01
3	3	6.19	6.07

*Values are expressed as mean of pH measurements

Discussion

The effect of post-packaging pasteurization (PPP) after cooling reduced and high moisture meat and poultry products was evaluated in this investigation as a post drying heat treatment to control survivable *C. perfringens* cells. This study has shown that PPP has an effect on reducing *C. perfringens* active cells. However, if the spores do not go into germination phase before PPP is applied, then the effect of this heat treatment is not observed.

Reduced moisture beef and chicken strips inoculated with *C. perfringens* spores showed a reduction of 0.7 (beef) and 0.4 (chicken) log CFU/g s when comparing the concentration of the spores right after lethality with the concentration after cooling and subjecting to PPP. Moreover, while comparing each cooling time interval (one, two and

three h) with and without the treatment, there was a reduction of *C. perfringens* spores of approximately 0.3 log CFU/g s for beef strips and 0.2 log CFU/g s for chicken strips.

The statistical analysis (ANOVA) shows that there is significant effect of the treatment in the reduction of spores. Nonetheless, because the level of reduction observed was minimal, it might be possible that the conditions of the product did not allow the spores to germinate; therefore, there were not enough vegetative cells to be eliminated with the post packaging pasteurization.

Vegetative cells can be easily eliminated through cooking the product; however, the spores can survive and persist after treating them with heat. Different authors have reported high heat-resistance of *C. perfringens* spores when cooking meat and poultry products. For instance, the most heat-resistant C. perfringens spores were reported to survive for 1 h at 100 °C (*11*).

Byrne and other authors conducted a study in 2016 to evaluate the thermal inactivation of *Bacillus cereus* and *Clostridium perfringens* vegetative cells and spores in pork luncheon roll. They found that D-values for *C. perfringens* vegetative cells ranged from 0.9 min (65 °C) to 16.3 min (55 °C), but they ranged from 2.2 min (100 °C) to 34.2 min (90°C) for *C. perfringens* spores. The authors mentioned that the high temperature heating might cause changes in the meat color and texture, and the formation of undesirable flavors in the products (*11*). Therefore, using these high temperatures affect the quality of the products.

Moreover, they found that a mild cook of 70°C for 1.3 min would achieve a 6 log CFU/g reduction of *C. perfringens* vegetative cells, but a reduction in spores would require the pork luncheon meat to be heated for 36 s at 110 °C (*5*). Therefore, in the

present study, the temperature used in the PPP (77 C) is not enough to destroy the spores but it does reduce the active germinated spores. This means that in the present study, *C. perfringens* spores could germinate in the product and be eliminated with further pasteurization; however, the additives in the product could have prevented the germination of the spores and this is why only a minimal reduction was achieved.

Cooling down the product with further heat treatment is a strategy to allow spores to germinate in the product and then eliminate them with the post-packaging pasteurization treatment. Talukdar et al. (*25*) have reported germination-induced inactivation strategies to control spores in meat products.

These authors have discussed the significant progress that have been made in understanding the mechanism of *C. perfringens* spore germination, leading to the identification of suitable germinant that has led to the development of novel strategies that involve the induction of spore germination followed by subsequent killing of germinated spores with mild treatments. For instance, Akhtar et al. (*2*) studied the formulation of germinants in poultry meat to make the spores go into germination phase and further eliminate them with heat and pressure.

They found that the most efficient strategy to inactivate *C. perfringens* spores in poultry meat containing 50 mM of L-asparagine and KCl, was to follow a procedure combining different heat and pressure conditions. They first heat treated the product (80 degrees °C, 10 min), then cooled it down to 55 C in about 20 min and further incubation at 55 °C for 15 min for spore germination. After that, they inactivated the germinated spores by pressure-assisted thermal processing (586 MPa at 73 degrees C for 10 min). In this present study the products were cooked, then cooled down to allow spores to

germinate and then they were treated with post-packaging pasteurization (77 °C for 6 min).

The conditions of temperature to treat the product after cooling are similar to the study described above; however, those authors implemented other strategies as adding germinant and more heat steps process for the products. In this present study PPP had a significant effect reducing germinated spores; nonetheless the germination phase was influenced by the a_W and intrinsic characteristics of the product.

It was observed in this study that a_W of the product has a direct effect on spore germination. The results of reduced moisture meat and poultry are similar because with low aW the spores are not able to germinate and then the effect of PPP is not observed. However, there were slight differences between the controls and the treatments which shows that a small amount of the spores could germinate during the cooking process or during the cooling times and these were eliminated with PPP.

It is also possible that the spores could not germinate but that the double heat treatment impacted the spore concentration in the product. Furthermore, when comparing the concentration of *C. perfringens* spores after lethality with the different cooling time intervals, it is possible to observe that the spores could have germinated but they did not reproduce nor increase their numbers in the products. This also confirms that the characteristics of the products (additives, a_W or pH) are not the optimum for this bacterium to grow and reproduce.

Abbona and Stagnitta conducted a study in 2016 to evaluate the effects of nitrites on spores outgrowth and they found 1-log reduction in non-enterotoxigenic strains caused for the addition of ~0.3% $NaNO_2$ to brain heart infusion agar and 1-log reduction in

spore outgrowth for enterotoxigenic strains when the medium was supplemented with nearly 0.5% $NaNO_2$ (*1*).

In this study, there was not a reduction of *C. perfringens* spores during the cooling times that could have explained the effect of additives like nitrites in the product; nonetheless, spores were not able to germinate and reproduce in the product and this can be explained for the presence of nitrites in the meat and poultry strips.

Spores which have been damaged by heat are more sensitive to the effects of nitrite and the exposure to heat during the thermal processing could have injured the spores and germination will not occur if the damage was not repaired (*10*). To sum on, many factors such a temperature and additives could have prevent *C. perfringens* spores to germinate and the vegetative cells to grow. Besides, the combination of two heat treatments may affect spore survival which explain the final reduction when comparing initial spore levels in the products.

Moreover, according to the FDA Hazard Analysis and Risk-Based Preventive Controls for Human Food Guidance for Industry, 0.93 is the minimum a_W value for *C. perfringens* to grow in products using salt, therefore, the a_W of the product evaluated was not enough to allow *C. perfringens* to reproduce (*12*). The results of high moisture meat and poultry strips differ from what was obtained with reduced moisture strips.

It was possible to evidence that a_W is an important factor in spore germination. Higher levels of spore's reduction were achieved when the a_W of the product was higher thus allowing spores go into germination phage. Once the spores became active cells, PPP was effective and therefore higher reduction numbers were obtained.

The use of post-packaging pasteurization has been reported to control surface contaminants such as *Listeria monocytogenes;* however, few studies have been conducted to evaluate the effectivity of this treatment to control *Clostridium* spores. For instance, Muriana et al. (*22*) conducted a study to evaluate post-package pasteurization of ready-to-eat deli meats (turkey, ham and roast beef) by submersion heating for the reduction of *Listeria monocytogenes*. They evaluated 90.6 °C, 93.3 °C and 96.1 °C during 2-10 min and concluded that heating regimens of 2 min at 90.6 to 96.1 can provide 2-log reductions in most RTE deli meats.

On the other hand Cooksey et al. (*7*) conducted a study to evaluate post-packaging pasteurization to reduce *Clostridium perfringens* and other bacteria in precooked vacuum package beef loin chunks. For this study, the product was inoculated, and vacuum packaged, then it was submerged in 82C water contained in a steam jacketed kettle for 16 min. After pasteurization the chunks were cooled in ice and placed in refrigerated storage and samples were taken after 0, 7, 14, 28, 45, 65 and 85 days of storage.

Even though authors reported that high levels of reduction were achieves, this occurred after pasteurization and during the storage time, which might suggest that after pasteurization the spores germinated in the product and were eliminated either by temperature of refrigeration or the conditions of the product. In this present study when reduced moisture products were pasteurized, the initial concentration of spores remained almost the same (Table 3 and Table 5.) But for high moisture products the spore levels were reduced when cooling first the product and the pasteurizing.

Cooling down the product first may allow the spores to germinate and consequently they can be further eliminated through post-packaging pasteurization.

Besides, the double heat treatments combined with the additives in the product might affect spore survival in the products which was also observed in the microbial shelf-life studies.

Moreover, Cooksey et al. (*7*) reported that non pasteurized chunks retained a lower number (1.44 $\log_{10}/cm^2$) of the inoculum and this was decreased during storage time. Therefore, this confirms that the reduction might have been caused by the increase in populations of microflora which was also reported by the authors or by temperature of refrigeration. It has been suggested that *C. perfringens* does not grow well at refrigeration temperatures and it is not a good competitor against psychrophilic microflora.

Finally, interesting morphological changes were observed in *C. perfringens* colonies after the inoculated meat and poultry products were subjected to lethality schedule and post-packaging pasteurization (Figure 6). This behavior had been observed before with other spore-forming pathogens. For instance, Gola et al. (*15*) found that high pressures cause morphological changes such as lengthening and flattening at 300 Mpa for 5 min at 30° C and breaking at 500 Mpa for 5 min at 20°C in spores of *B. cereus* which ultimately resulted in complete spore inactivation or destruction.

Also, D'Incecco et al. (*9*) reported morphological changes in *Clostridium tyrobutyricum* when manufacturing and ripening cheese. They found that after severe heating (54°C) and acidification, highly-dense black spot appeared inside most of the cells and turned into a more complex oval structure. This is similar to what was observed in the present study, since *C. perfringens* colonies appeared bigger after heating the products which also had additives which might affect the bacterium growth and cause stress.

CONCLUSIONS

Despite the fact that the post packaging pasteurization (PPP) treatment had an deleterious effect in reduced-moisture products, low cell counts were observed due to the limited spore germination. However, viability of germinated spore-formers was significantly decreased ($p<0.05$) in high moisture products when different cooling times (1h, 2h, 3h) were evaluated prior to subjecting the product to PPP. This treatment had an effect on germinated *C. perfringens* spores but had no inactivation effect on spore survivors. Nonetheless, the combination of several strategies, such as different heat treatments and the use of additives in meat and poultry products might be a potential approach to control spore-forming pathogens.

Post-Packaging Pasteurization (PPP) is a post dehydration step to be used in Jerky-like strips production in order to control spore-forming organisms that could have been able to germinate during cooling of the product or during the thermal treatments. PPP provides an additional margin of safety, that in combination with low a_W, extend the shelf life of the product stored at room temperature by controlling pathogens that are not inactivated by conventional lethality treatments.

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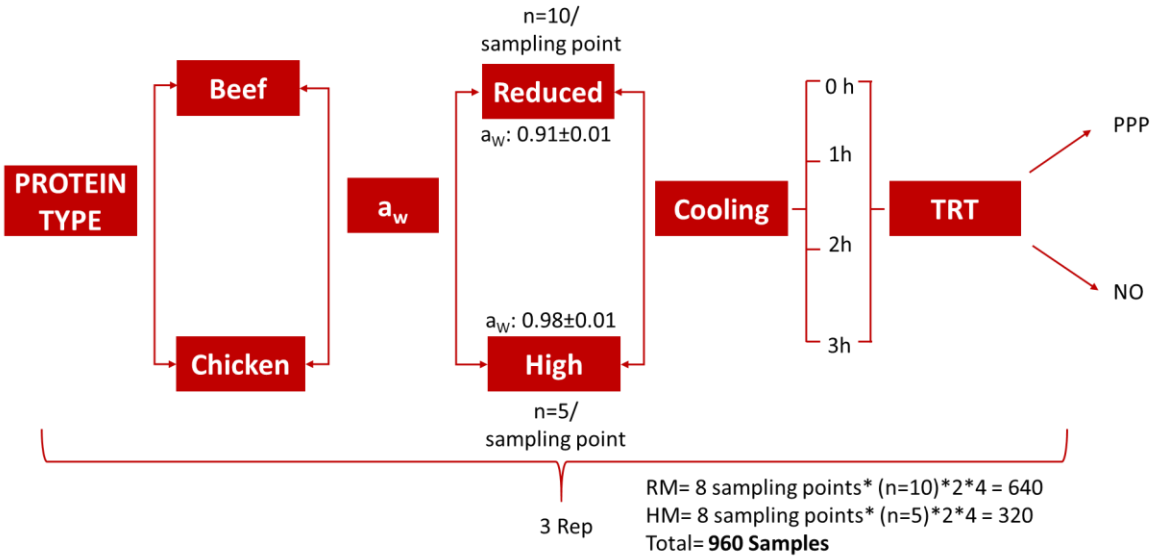
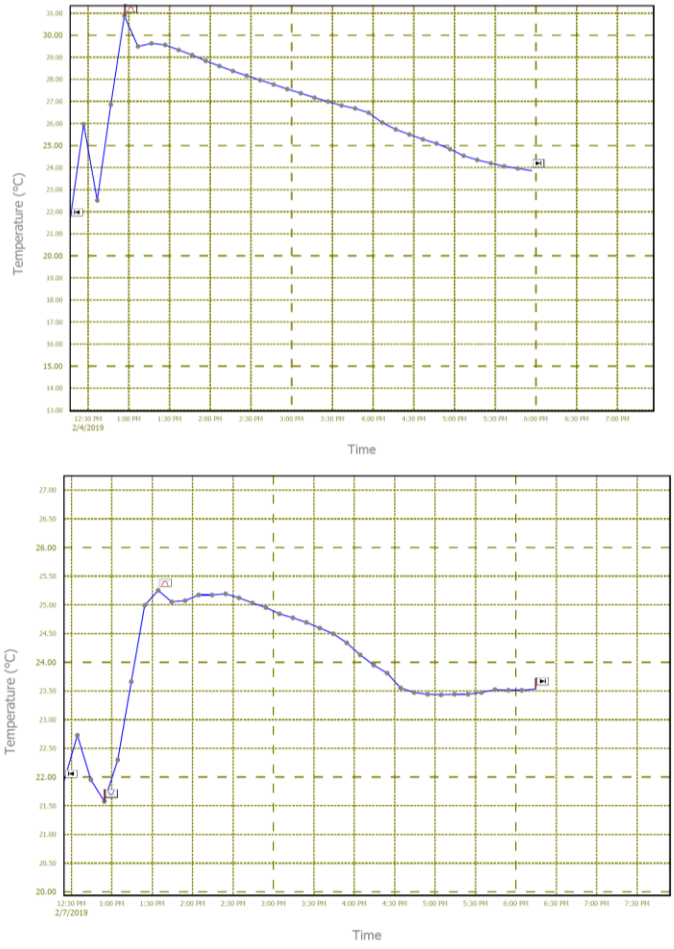
APPENDICES

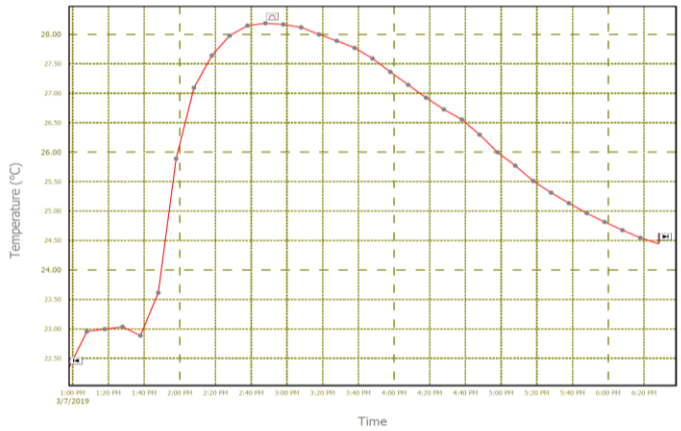
Appendix A: Experimental Design

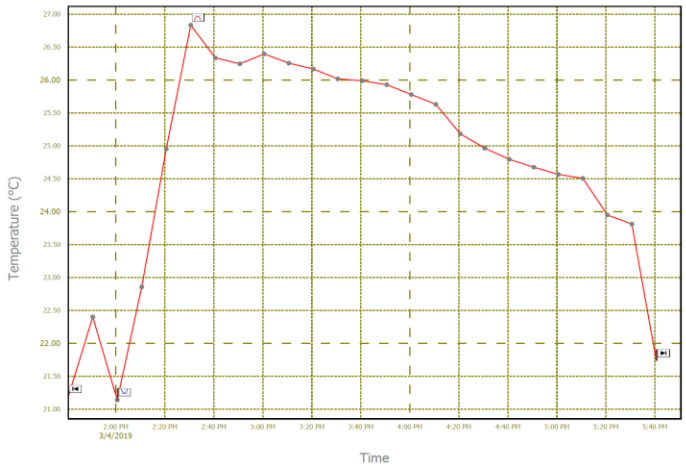


Appendix B: Temperature records of reduced-moisture beef strips during cooling

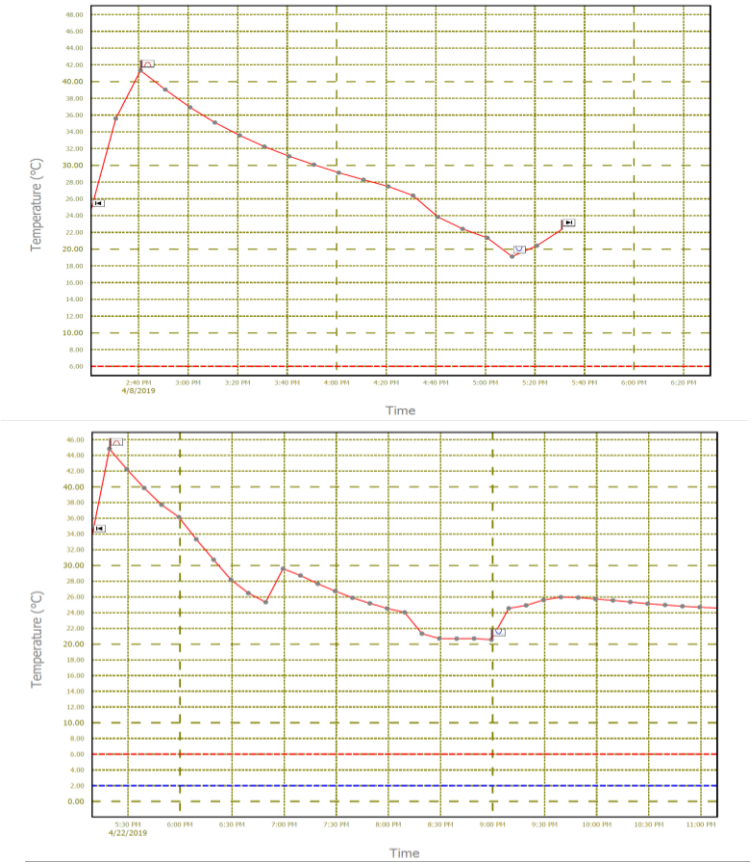


Appendix C: Temperature records of reduced-moisture chicken strips during cooling

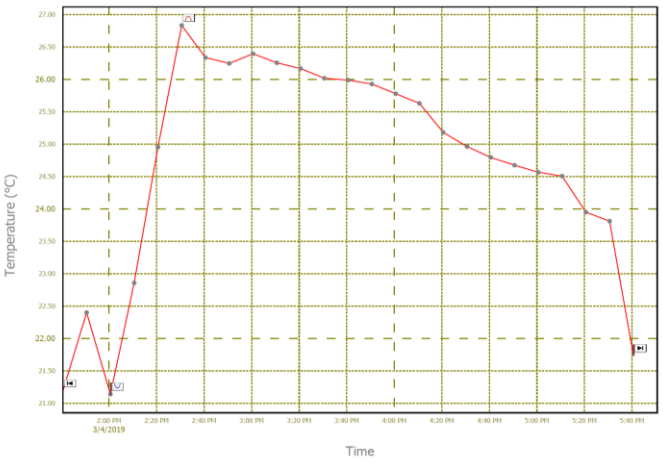


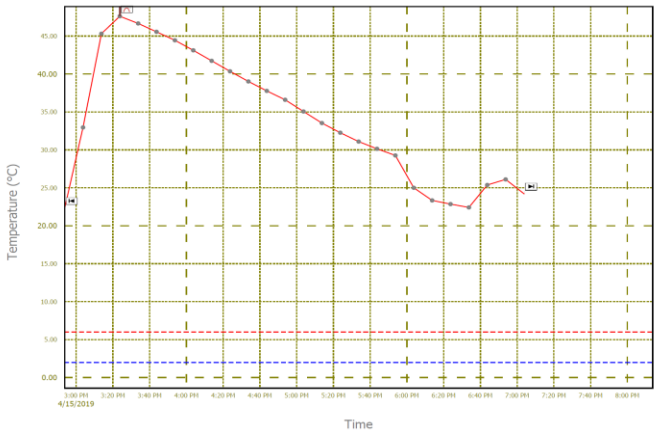


Appendix D: Temperature records of high-moisture beef strips during cooling



Appendix E: Temperature records of high-moisture chicken strips during cooling





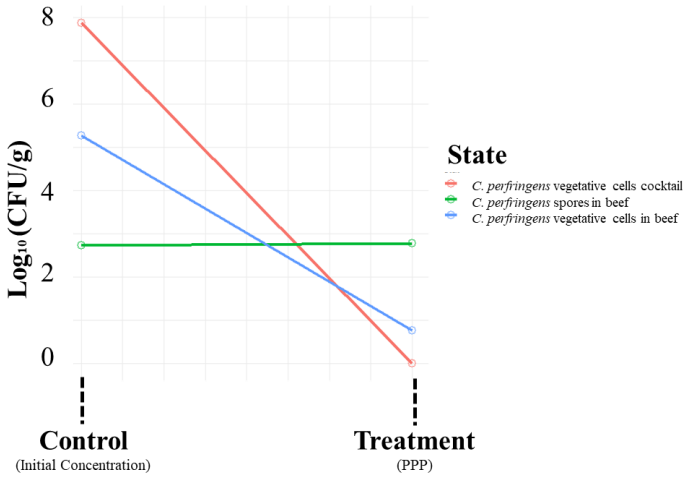
Appendix F: Tukey test results to evaluate the interactions between time and treatment (obtained in ANOVA) in high-moisture chicken strips

Variable	diff	lwr	upr	p adj
treatment-control	-1.1077	-1.2309	-0.9846	0

*Time:treatment	diff	lwr	upr	p adj
1:control-0:control	-0.061709	-0.516917	0.393499	0.99999
2:control-0:control	-0.091998	-0.547206	0.363209	0.99974
3:control-0:control	-0.079873	-0.535082	0.375334	0.99992
1:treatment-0:control	-1.760404	-2.215612	-1.305195	0.00000
2:treatment-0:control	-2.294902	-2.750110	-1.839693	0.00000
3:treatment-0:control	-2.165668	-2.620876	-1.710459	0.00000
2:control-1:control	-0.030289	-0.464313	0.403735	1.00000
3:control-1:control	-0.018164	-0.452188	0.415859	1.00000
1:treatment-1:control	-1.698694	-2.132718	-1.264670	0.00000
2:treatment-1:control	-2.233192	-2.667216	-1.799168	0.00000
3:treatment-1:control	-2.103958	-2.537983	-1.669934	0.00000
3:control-2:control	0.012124	-0.421899	0.446148	1.00000
1:treatment-2:control	-1.668405	-2.102429	-1.234381	0.00000
2:treatment-2:control	-2.202903	-2.636927	-1.768879	0.00000
3:treatment-2:control	-2.073678	-2.507693	-1.639645	0.00000
-4:treatment-3:control	0.380034	-0.075173	0.835243	0.19421
1:treatment-3:control	-1.680530	-2.114554	-1.246506	0.00000
2:treatment-3:control	-2.215028	-2.649052	-1.781004	0.00000
3:treatment-3:control	-2.085794	-2.519818	-1.651770	0.00000
2:treatment-1:treatment	-0.534497	-0.968522	-0.100473	0.00429
3:treatment-1:treatment	-0.405264	-0.839288	0.028760	0.09013
3:treatment-2:treatment	0.129233	-0.304790	0.563258	0.99457

*-4: Initial concentration, 0: After lethality schedule, 1: One hour cooling, 2: Two h cooling, 3: Three h cooling.

Appendix G: Preliminary results of the effect of Post-packaging pasteurization (PPP) on *C. perfringens* vegetative cells and spores



Evaluation of PPP on: 1. *C. perfringens* vegetative cells cocktail, 2. Inoculated beef strips with *C. perfringens* vegetative cells, and 3. Inoculated beef strips with *C. perfringens* spores (n=3).