AN ABSTRACT OF THE DISSERTATION OF

Maryam Alnoman for the degree of Doctor of Philosophy in Food Science and Technology presented on August 3, 2016.

Title: Inactivation of Clostridium perfringens in Meat Products

Abstract approved:

________________________________________
Mahfuzur R. Sarker

*Clostridium perfringens* type A strains are one of the main causative agents of gastrointestinal (GI) diseases in human and can cause both food poisoning (FP) and non-food-borne (NFB) diseases. Several factors contribute to the pathogenesis of *C. perfringens* type A strains including the wide presence of *C. perfringens* spores with highly resistant properties, production of *C. perfringens* enterotoxin (CPE), and the high growth rate of the bacterium. In addition to these intrinsic virulence factors, other external factors such as inadequate cooking, improper cooling, and abusive storage temperature of meat and poultry products can contribute to the majority of *C. perfringens* type A food poisoning outbreaks. The risk of contracting food-borne illnesses is usually reduced by commonly used food preservation method such as thermal processing. However, inactivating extremely heat-resistant spores is a major challenge and, thus, alternative strategies to conventional thermal processing technologies are in urgent demand.
In the first study, the effectiveness of potassium sorbate and sodium benzoate were evaluated as growth inhibitors of vegetative cells and spores of *C. perfringens* type A FP and NFB isolates, in both laboratory medium and chicken meat model system. Our study showed that although the permissive levels of sorbate and benzoate used in food products did not affect spore germination, the outgrowth of germinated spores was effectively arrested. Lowering the pH of the medium increased the inhibitory effects of sorbate and benzoate against germination of spores of NFB isolates, and outgrowth of spores of both FP and NFB isolates. Furthermore, sorbate and benzoate inhibited vegetative growth of *C. perfringens* isolates. Although sorbate and benzoate showed inhibitory activities against *C. perfringens* in the rich medium, no such effect was observed in cooked chicken meat stored under extremely abusive conditions. Therefore, caution should be taken when applying these organic salts to meat products to reduce or eliminate *C. perfringens* spores.

In the second study, the natural polymer chitosan with low, medium, and high molecular weight was examined to assess its inhibitory effect against *C. perfringens* FP isolates in a variety of aspects including spore germination, outgrowth, and vegetative growth in laboratory medium or chicken meat model system. Chitosan with all three different molecular weights at concentration of 0.1 mg/ml at pH 4.5 could inhibit the germination of spores of FP isolates. However, higher level (0.25 mg/ml) of chitosan was required to effectively arrest outgrowth of the germinated *C. perfringens* spores in rich medium. Furthermore, chitosan concentration of 1.0 mg/ml showed bacteriostatic activity against vegetative cells of *C. perfringens* strains in rich medium. Although chitosan showed strong inhibitory activities against *C. perfringens*
in laboratory medium, higher levels were required to achieve similar levels of inhibition for *C. perfringens* spores inoculated into chicken meat. Thus, from this study, it has been shown that the inhibitory effects of chitosan against spore germination, outgrowth, and vegetative growth was concentration dependent, and no major differences in effects of different molecular weights of chitosan were observed. Our results also contribute to a better understanding on the potential application of chitosan in cooked meat products to control *C. perfringens* contamination.

Bacterial spore germination is an essential step for the spores to restore their metabolic activities and lose their resistance properties. *C. perfringens* vegetative cells are easier to kill than the spore form. *C. perfringens* spores can initiate germination upon sensing a variety of compounds, termed germinants, via their cognate germinant receptors (GRs). In the third study, we identified 12 individual amino acids (aa) that triggered the germination of spores of *C. perfringens* NFB isolates in the presence of bicarbonate buffer. Unlike *C. perfringens* NFB isolates, spores of *C. perfringens* FP strain SM101 required potassium ions to germinate with all these amino acids. Surveying germination of spores of NFB isolates lacking one of the GR proteins with the newly identified aa germinants revealed that GerKC and GerAA play major roles in bicarbonate-aa germination. Furthermore, this study also supports the correlation of germination requirement of *C. perfringens* FP and NFB isolates and their environmental niches. Potassium ions are abundant in food environment and, thus, are required for FP spores to germinate. However, bicarbonate is a specific intestinal element and is essential for germination of NFB spores. In summary, these results should help developing a new spore-inactivation strategy
where spore germination will be induced by aa germinants followed by mild chemical treatments.

Collectively, this dissertation reports experimental results relevant to different inactivation approaches to control spores and vegetative cells of *C. perfringens* FP and NFB isolates in laboratory medium and chicken meat model system. These approaches include the incorporation of well-known GRAS-listed antimicrobial agents to food formulation, and the identification of nutrient compounds to trigger *C. perfringens* spore germination, which will allow spore-killing with mild treatments. Combination of these approaches should be effective to control the risk of *C. perfringens* associated food-borne and non-food-borne illnesses.
Inactivation of *Clostridium perfringens* in Meat Products

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APPROVED:

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Head of the Department of Food Science & Technology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Maryam Alnoman, Author
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CONTRIBUTION OF AUTHORS

Chapter 2. Dr. Pathima Udompijitkul, Dr. Daniel Paredes-Sabja, and Dr. Mahfuzur R. Sarker were involved with experimental design and manuscript preparation.

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Chapter 4. Dr. Pathima Udompijitkul and Dr. Mahfuzur R. Sarker were involved with manuscript preparation. Dr. Saeed Banawas constructed all germination receptor mutants used in this study and provided mutant spores.
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CHAPTER 1

General Introduction and Literature Review

1.1. Characteristics of the bacterium

*C. perfringens* is a gram-positive, rod-shaped, endospore-forming bacterium that causes a broad spectrum of human and animal diseases (Labbe, 1989; McClane et al., 2013). *Clostridium perfringens* (formerly known as *C. welchii*) was first recognized in the 1940s and 1950s as a cause of foodborne disease. It is one of the most widely distributed pathogenic microorganisms in nature owing to its spore, a highly resistant form of the bacteria (Labbe, 1989). *C. perfringens* is ubiquitous in soil, foods, water, dust, sewage, feces, and the intestinal tract of human and animals (McClane et al., 2013). *C. perfringens* is an anaerobic bacterium due to its failure to grow on agar plates continuously exposed to air; however, it can tolerate moderate exposure to air due to its modest oxidation-reduction potential (E<sub>H</sub>) that modify its surrounding environment by producing reducing molecules (Labbe, 1989; McClane et al., 2013; Montville et al., 2008). It is a mesophilic bacterium with an optimum growing temperature between 37 °C and 45 °C, but can grow at temperatures up to 50 °C. Growth rate of *C. perfringens* significantly decreases at temperatures below 15 °C and no or very little growth is observed at 6 °C or below. However, the spore form of this bacterium is cold and heat
resistant (Jay, 2005; McClane et al., 2013). The *C. perfringens* generation time is 7.3 minutes at optimal conditions (under 45 °C) making it more competitive than other similar types of microorganisms (Jay, 2005; McClane et al., 2013; Montville et al., 2008) (Montville and Mathews, 2008). The minimum water activity for supporting *C. perfringens* growth is between 0.93 to 0.97 (Jay, 2005; McClane et al., 2013). *C. perfringens* can grow optimally at neutral pH conditions (pH 6 to 7). However, the growth rate decreases in alkaline or acidic conditions (pH value ≤5 and ≥8.3).

*C. perfringens* virulence is largely attributed to its ability to produce at least 17 different toxins. Four of them are major toxins (alpha, beta, epsilon, and iota), which are used to classify *C. perfringens* into five toxinotypes (A-E) (Table 2.1) (Freedman et al., 2016; McClane et al., 2006). Each toxinotype of *C. perfringens* causes a variety of illnesses in both human and animals varying from gas gangrene to several entrotoxemias and enteric diseases (McClane et al., 2006; Petit et al., 1999; Rood, 1998).

**Table 1.1.** Toxintyping of *C. perfringens* (McClane et al., 2013; Petit et al., 1999)

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<td>Alpha</td>
<td>Beta</td>
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</tr>
<tr>
<td>A</td>
<td>+</td>
<td>−</td>
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<sup>a</sup>*C. perfringens* type

<sup>b</sup>+; produced; −, not produced
In addition to these four major toxins, *C. perfringens* strains can produce a number of gastrointestinal (GI) active toxins including: *C. perfringens* enterotoxin (CPE), beta-2 toxin (CPB2), necrotic enteritis B-like toxin (NetB) and TpeL. These toxins have received more attention because of their role in the pathogenesis of *C. perfringens*-associated diseases (Chen et al., 2015; Popoff and Bouvet, 2009). Two important foodborne diseases are caused by two different toxinotypes of *C. perfringens*. One of the diseases is the human necrotic enteritis (also known as Darmbrand or Pig-Bel) with low incidence and a high mortality rate caused by *C. perfringens* type C. Another is the *C. perfringens* Type A food poisoning (FP) associated with high incidence and a low mortality rate caused by the *C. perfringens* type A isolates (Lawrence, 1997; McClane et al., 2013; Smedley et al., 2005). FP is a common syndrome in developed countries with mild symptoms including diarrhea and abdominal pain. In contrast, human necrotic enteritis symptoms are more severe than FP symptoms, and include bloody and mucosal stools (Brynestad and Granum, 2002; Lindstrom et al., 2011).

1.2. CPE associated gastrointestinal GI diseases.

CPE is responsible for all symptoms associated with *C. perfringens* Type A GI disease (Sarker et al., 1999). Around 5% of all *C. perfringens* isolates produce CPE, and most of the CPE-positive strains belong to toxino-type A (Li et al., 2010a). *C. perfringens* type A isolates carry the enterotoxin gene (*cpe*) either on the chromosome (*C-cpe*) or on a large plasmid (*P-cpe*); and no isolates have been identified yet to have *cpe* on both chromosomal and plasmid DNA (Freedman et al., 2016). Around 70% of type A strains carrying chromosomal *cpe* gene are associated with food poisoning outbreaks (Tse et al.,
2011). However, 30% of type A strains carry its cpe on conjugative plasmids and are associated with non-foodborne (NFB) diseases including antibiotic-associated diarrhea (AAD), and sporadic diarrhea (SD) (Collie and McClane, 1998; Freedman et al., 2016). CPE protein produced by C. perfringens type A, type C and type D strains have the identical amino acid sequence (Billington et al., 1998; Li et al., 2010a).

1.2.1. C. perfringens type A food poisoning.

Clostridium perfringens type A food poisoning is currently ranked as the third most common foodborne illness in developed countries (Scallan et al., 2011b). Each year, approximately one million cases of this food poisoning are reported in the United States, which results in an estimated economic loss of ~$400 million annually (Hoffmann et al., 2012; Scallan et al., 2011b). CPE is responsible for the type A food poisoning that results in diarrhea and abdominal cramps (McClane et al., 2013). C. perfringens FP strains lacking CPE are avirulent in animal models and virulent can be restored by complementing the strain with functional cpe gene (Sarker et al., 1999).

There are several factors that are involved in the progression of C. perfringens type A food poisoning. These include the short generation time of the bacteria, and most importantly, the dormant spore form that can tolerate different food environmental stresses (McClane et al., 2013). These factors facilitate the survival of C. perfringens type A isolates in processed meat and poultry products that are most commonly implicated in C. perfringens FP outbreaks. Improperly cooked, cooled, and stored food items can activate these heat-resistant spores to germinate and outgrow into vegetative cells that leads to multiplication of high numbers of viable cells (~10^6 colony forming unit
(CFU)/g) in a short period of time. If the food is consumed with high number of *C. perfringens* cells, some of the viable cells could survive the stomach acidity and go into the GI tract, where they can initiate sporulation and release CPE upon cell lysis (McClane, 2007; McClane et al., 2013).

Growth and resistance characteristics of vegetative cells and spores of *C. perfringens* C-cpe type A isolates are remarkably different than the P-cpe and other *C. perfringens* isolates (McClane et al., 2013). C-cpe spores are 60-folds greater heat resistant at 100 °C, and also can survive under refrigeration, freezing, osmotic and nitrite stresses much better than other *C. perfringens* isolates. Moreover, vegetative cells of C-cpe isolates have higher maximum growth temperature (~53 °C), lower minimum growth temperature (~12 °C), and better survival under refrigeration and freezing than other isolates of *C. perfringens* (Labbe, 1989; Li and McClane, 2006a, 2006b; Sarker et al., 2000). These distinct characteristics facilitate their survival and dissemination in the food environment and explain the high incidence rate of *C. perfringens* type A C-cpe related FP (McClane et al., 2013).

1.2.2. *C. perfringens* non-food-borne human GI diseases

CPE-positive type A strains have also been reported to cause several non-food-borne (NFB) human gastrointestinal diseases, including about 5%–20% of all cases of antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD) (Carman, 1997; Collie and McClane, 1998; Mpamugo et al., 1995). Studies with animal model proved that CPE production is required for the pathogenicity of NFB strains (Sarker et al., 1999). The
detection of CPE from the feces of patient infected with *C. perfringens* NFB strains support that CPE is also essential for the NFB strains to cause the GI diseases in humans (Carman, 1997). NFB associated GI diseases develop from the small inoculum of P-cpe cells, that may result in the transfer of cpe containing plasmid to normal gut flora of cpe-negative *C. perfringens* strains. This leads to extensive *C. perfringens* colonization inside the gut followed by subsequent sporulation and CPE production (Lindstrom et al., 2011; Sparks et al., 2001). In alternative proposed theory, it has been hypothesized that spores repeatedly germinate and sporulate in the intestine, leading to greater cpe mobilization to normal flora of *C. perfringens* cells and also causing the progression of virulence in many intestinal disease-causing strains of *C. perfringens* (Carman, 1997; Freedman et al., 2016; Li and McClane, 2014). Even though, the route of P-cpe transmission is unclear, a recent review considered *C. perfringens* P-cpe strains to be a FP agent, and may be transmitted via foods due to the recent isolation of P-cpe isolates from food products, as well as the reported outbreaks of FP caused by P-cpe isolates (Lindstrom et al., 2011).

1.3. *Clostridium perfringens* enterotoxin (CPE)

1.3.1. Structure of CPE and its regulation

CPE is a single polypeptide consisting of 319 amino acids with a unique primary sequence conserved among *C. perfringens* type A, C, and D strains (Freedman et al., 2016; McClane et al., 2013). CPE protein has two domains: C-terminal CPE receptor-binding domain and the N-terminal oligomerization/membrane insertion domain (Briggs et al., 2011; Saitoh et al., 2015). The production and regulation of CPE are tightly controlled during *C. perfringens* sporulation and also dependent on several other global
regulators and some sporulation-specific sigma factors (Freedman et al., 2016; Harry et al., 2009; Li and McClane, 2010). There are four different sporulation-specific sigma factors involved in the process of sporulation in \textit{C. perfringens}, which are tightly regulated by the master regulator, Spo0A. Phosphorylated Spo0A activates the expression of many sporulation genes as well as \textit{cpe} (Talukdar et al., 2015). It has been shown that \textit{C. perfringens} mutant lacking Spo0A or SigF failed to sporulate and produce CPE (Huang et al., 2004; Li and McClane, 2010). SigF is an alternative sporulation-specific sigma factor that is required for \textit{C. perfringens} sporulation and activation of other sporulation-specific sigma factors including SigG, SigE, and SigK (Li and McClane, 2010). \textit{cpe} expression is also dependent on SigK, and SigE, but not SigG (Harry et al., 2009). Three \textit{cpe} regulatory promoters upstream of the coding sequence of \textit{cpe} contain consensus sequences similar to other sigma factor recognition sequences (Zhao and Melville, 1998). The first promoter (P1) contains sequences similar to SigK recognition sequences, while the second and third promoters (P2 and P3) demonstrate similarity to recognition sequences for SigE (Freedman et al., 2016; Talukdar et al., 2015; Zhao and Melville, 1998).

Another global regulatory protein involved in CPE production is the catabolite control protein A (CcpA). CcpA regulates the expression of carbon and nitrogen utilization genes found in Gram-positive bacteria (Richardson † et al., 2015; Varga et al., 2004). Inactivation of \textit{ccpA} in \textit{C. perfringens} type A strain SM101 significantly reduces sporulation and CPE production (Varga et al., 2004). However, CcpA-dependent regulation mechanism contributing to the sporulation and CPE production remains unidentified (Freedman et al., 2016). In addition to CcpA, the Agr-like quorum sensing
system of *C. perfringens* also regulates sporulation and CPE production (Li et al., 2011). Quorum sensing system positively governs Spo0A and other sporulation sigma factors in *C. perfringens* type A NFB strain F5603 (Li et al., 2011). A recently identified repressor protein named VirX negatively regulates sporulation and CPE production in *C. perfringens* type A SM101 strain. A *virX*-mutantion in SM101 strain results in higher levels of CPE production and an increase in *sigE*, *sigF* and *sigK* expression compared to wild-type strains (Ohtani et al., 2013; Talukdar et al., 2015).

1.3.2. CPE cytotoxic activity

The cytotoxic activity of CPE is initiated by the binding of CPE to claudin receptors present in the surface of host cells (Gunzel and Yu, 2013). This binding results in the formation of small complexes that interact to form a prepore on the plasma membrane surface (Robertson et al., 2007). A larger CPE complex (CH-1) is formed by the oligomerization of CPE which assembles into a β-barrel. This complex structure inserts into the membrane to form active pores that alter the plasma membrane permeability and cause calcium influx (McClane et al., 2006). Small dose of CPE causes low number of pores and modest calcium influx and results in apoptosis (Chakrabarti and McClane, 2005). However, larger CPE doses cause massive calcium influx that leads to necrotic cell death or oncosis (Chakrabarti et al., 2003). These morphologically damaged cells expose the basolateral surface of the intestine for further CPE access, and more CH-1 complexes are formed (Robertson et al., 2007). Additionally, a second larger complex (CH-2) is formed in the basolateral surface that will trigger the internalization of epithelial tight junction proteins (occludin and claudin) into the cytoplasm (Freedman et
al., 2016; Smedley et al., 2007).

In the human small intestine, histological damage involves extreme villus blunting, along with epithelial necrosis (Caserta et al., 2011; Fernandez Miyakawa et al., 2005; McDonel and Demers, 1982). CPE derivatives that have binding ability, but are not cytotoxic, fail to develop histologic damage in animal model (Smedley et al., 2008). In rabbit small intestines, CPE first damages villi tips, where claudin 4 (CPE receptor) abundantly existed, then is followed by the fluid and electrolyte loss associated with CPE-induced histologic damage (Smedley et al., 2008). A recent study established that CPE histological damage and fluid/electrolyte losses also occurred in the rabbit colon (Garcia et al., 2014). Moreover, the absorption of CPE through the intestines was detectable in CPE-challenged mouse small intestinal loops, suggesting that CPE can bind to the non-intestinal organs such as liver and kidneys and results in enterotoxemia. This actually explains the severity of the most recent C. perfringens type A FP outbreaks in psychiatric hospitals that have involved deaths (Caserta et al., 2011; Freedman et al., 2016).

1.4. Clostridium perfringens sporulation

Sporulation is a complex regulatory process that transforms a growing bacterial cells into metabolically dormant spores (Paredes et al., 2005; Talukdar et al., 2015). It has been speculated that unfavorable conditions such as nutrition depletion triggers sporulation process as an adaptive strategy to survive in the environment for long period of time (Higgins and Dworkin, 2012; Talukdar et al., 2015). The sporulation process has been extensively studied in Bacillus species, in particular Bacillus subtilis. In recent
years, sporulation has been also studied in *Clostridium* species and revealed that both *Bacillus* and *Clostridium* species have similar morphological changes during spore formation stages, with the only difference in the spore initiation stage (Dürre and Hollergschwandner, 2004; Dürre, 2011; Paredes et al., 2005; Stephenson and Hoch, 2002; Talukdar et al., 2015). Sporulation is initiated by a phosphorelay system, which uses a multi-component signal transduction pathway that results in the phosphorylation of the master sporulation regulator protein, Spo0A (Stephenson and Hoch, 2002). Inactivated *spo0A* in numerous *Clostridium* species inhibits sporulation and synthesis of other sigma factors (Dürre and Hollergschwandner, 2004; Huang et al., 2004; Talukdar et al., 2015). While Spo0A is conserved among *Bacillus* and *Clostridium* species, the phosphorelay system is absent or has not been identified in *Clostridium* species (Brown et al., 1994; Sauer et al., 1994). The absence of the phosphorelay system in *Clostridium* leads to the hypothesis that *Clostridium* Spo0A is either activated by direct phosphorylation from orphan histidine kinases or by an unknown phosphorelay system (Talukdar et al., 2015).

Phosphorylation of Spo0A results in the activation of a sporulation-specific sigma factor cascade that acts in both mother cell and forespore in a time dependent manner to facilitate the transition of cells to mature spores (Errington, 2003; Talukdar et al., 2015). These sporulation-specific sigma factors are conserved between different *Clostridium* and *Bacillus* species with differences in their expression pattern (Talukdar et al., 2015). In addition to regulation of sporulation process, sigma factors control the expression and production of some clostridial toxins such as CPE and TpeL as described in section 1.3.1 (Chen et al., 2015; Harry et al., 2009; Li and McClane, 2010; Talukdar et al., 2015).
1.5. Spore resistance properties

Spores are one of the most resistant forms of life. Spores exhibit increased resistance to a large number of treatments, including desiccation, freezing, thawing, elevated temperatures in either wet or dry states, UV, γ-radiation, and high pressure situations (Setlow, 2014b). Also, spores are highly resistant to toxic chemicals with a variety of effects including oxidizing agents, alkylating agents, aldehydes, halogens, acids, and bases (Setlow, 2014b). The spore is a multilayered structure inside of which the bacterial genome is deposited (McKenney et al., 2013; Talukdar et al., 2014). Spore structure plays a major role in spore resistance and each of the spore components contribute to its dormancy and sustainability in a variety of environmental stresses (Setlow, 2014a; Talukdar et al., 2015). In *C. perfringens* spores, the order of the layers from the outside to inside is coat, outer membrane (OM), cortex, germ cell wall, inner membrane (IM), and core. The coat and OM act as permeability barrier protecting the spore against peptidoglycan-lytic enzymes, biocidal chemicals and UV damage. Two peptidoglycan (PG) layers (cortex and germ cell wall) play roles in spore viability and heat resistance. The IM has unique properties such as high viscosity and impermeability, which are important in spore resistance to biocidal chemicals (Setlow, 2006, 2014b). The novel properties inside the spores’ core that result in the resistance properties of spores are the low water content, high levels of dipicolinic acid and associated divalent cations, and sporulation-specific proteins named small acid soluble proteins (SASPs) (Paredes-Sabja et al., 2008b; Paredes-Sabja et al., 2008c; Talukdar et al., 2014).

DNA in the spore core is guarded by a group of α/β-type SASPs that are
produced during sporulation (Paredes-Sabja et al., 2008a; Setlow, 2006; Talukdar et al., 2014)(Raju et al., 2006). Three SASP genes (ssp1, ssp2, and ssp3) have been identified in different C. perfringens FP and NFB strains (Raju and Sarker, 2007). C. perfringens strains with mutation in sasp genes demonstrated that α/β-type SASPs play a main role in mediating resistance of C. perfringens spores to UV radiation, moist heat and chemicals (Raju et al., 2006; Raju et al., 2007), but not to dry heat (Raju et al., 2006). Production of similar levels of SASPs by FP and NFB strains (Raju and Sarker, 2007) can not explain lower heat resistance specific to spores of NFB isolates (Sarker et al., 2000). However, the newly identified SASP 4 in both FP and NFB, has a single amino acid substitution at position 36 where aspartic acid in FP isolates is switched to glycine in NFB isolates (Koehler et al., 2008; Talukdar et al., 2015).

1.6. Bacterial spore germination

In spite of their dormancy and resistance, spores are able to monitor their surrounding environment and lose their dormancy and resistance properties under favorable growth conditions. This is a process called spore germination, which is followed by spore outgrowth and ultimately return to actively growing cells (Setlow, 2014a). Spore germination process is initiated by sensing the presence of strain-specific environmental signals, termed germinants (Olguin-Araneda et al., 2015). The complete germination process can be divided to different stages, stage I and stage II (Setlow, 2014a). In stage I, spore’s outer layers proteins facilitate the movement of germinant molecules. Then, germinant molecules interact with their cognate germinant receptor (GR) located in the spore’s inner membrane (Moir, 2006; Setlow, 2014a). These
germinant-GR interactions change the spore’s IM permeability leading to the release of different monovalent cations from the core and increase of spore core’s pH (Setlow, 2014a). Spore also releases most of its dipicolinic acid (DPA) and its associated divalent cations, primarily Ca\(^{2+}\) from its core, which is replaced with water from environment. Core hydration results in the decrease of moist heat resistance. These irreversible events will complete stage I of spore germination (Setlow, 2014a). In stage II, the hydrolysis of PG cortex is the hallmark event in which germinating spore takes more water, the core is expanded, and all metabolic activities are resumed. After the completion of stage II, all required macromolecules are synthesized to allow subsequent developmental process (spore outgrowth) and the germinating spores are converted into actively growing cells (Moir, 2006; Olguin-Araneda et al., 2015; Setlow, 2014a).

1.6.1. *C. perfringens* germinants and germination receptors

Knowledge about germination of *C. perfringens* spores gained significant understanding due to the identification of germinants and their cognate GR (Olguin-Araneda et al., 2015). Recent studies identified some nutrient germinants including a mixture of L-asparagine and KCl (AK) at pH 7.0, and different amino acids at pH 6.0 such as L-cysteine, L-serine, L-threonine, and L-asparagine. These nutrient germinants can function as universal germinants for both FP and NFB isolates. Other germinants like KCl, L-asparagine, all at pH 7.0, and L-glutamine, and sodium phosphate (NaPi) at pH 6.0, can induce germination of spores of various FP isolates, but not NFB isolates. However, L-alanine and L-valine at pH 7.0 can initiate germination of spores of some NFB isolates only (Paredes-Sabja et al., 2008d; Paredes-Sabja et al., 2009d; Udompijitkul
et al., 2014). The germinant mixture of L-asparagine, D-glucose, D-fructose, and potassium ion (AGFK), which is specific for B. subtilis spore germination, is also a potent germinant for C. perfringens type A SM101 spores (Paredes-Sabja et al., 2008d).

In the genome of C. perfringens SM101, four GRs encoded by a monocistronic gerAA, a bicistronic gerKA-KC operon, and an upstream located and oppositely-oriented gerKB were identified (Myers et al., 2006). GerAA, GerKA and GerKB protein(s) have been shown to play an auxiliary role during spore germination with all known germinants (Banawas et al., 2013a; Paredes-Sabja et al., 2008d; Paredes-Sabja et al., 2009b; Paredes-Sabja et al., 2009d; Udompijitkul et al., 2014). However, GerKC is the unique and most important GR for nutrient (i.e., K⁺, L-asparagine, L-cysteine and L- glutamine) and non-nutrient (i.e., Ca-DPA and dodecylamine) induced germination of SM101 spores (Banawas et al., 2013a; Udompijitkul et al., 2014). A recent work determined that GerKC subunit is present in the inner membrane of C. perfringens spores at a level of ~250 molecules/ spore (Banawas et al., 2013a).

1.6.2. C. perfringens cortex lytic enzymes

In addition to GRs, two cortex-lytic enzymes (SleC and SleM) and one serine protease (CspB) that belong to the subtilisin subfamily have been identified in the genome of C. perfringens strains (Miyata et al., 1995). SleC is synthesized during sporulation as an inactive form (pro-SleC), and activated by CspB early in germination and converted into active SleC (Koehler et al., 2013; Shimamoto et al., 2001). However, SleM is synthesized as a mature enzyme and does not require the activation process and is likely to degrade specific peptides generated by SleC during spore germination (Chen
et al., 1997). Genetic studies in *C. perfringens* SM101 have proved that spores lacking *sleC* are unable to degrade their PG cortex and complete germination, demonstrating that SleC is essential for cortex hydrolysis (Paredes-Sabja et al., 2009a). Spores that have lost their SleM are germinated as wild type spores, implying that SleM might play an auxiliary role in PG cortex hydrolysis (Paredes-Sabja et al., 2009a). Furthermore, spores with inactivated *cspB* are also unable to degrade PG cortex and activate pro-SleC into mature SleC (Olguin-Araneda et al., 2015; Paredes-Sabja et al., 2009c).

1.7. *C. perfringens* inactivation

One of the major challenges in the food industry is the inactivation of *C. perfringens* spores and vegetative cells. Several factors are involved in the resistance properties of *C. perfringens* spores and vegetative cells to different inactivation strategies. One of the most important reasons is the common presence of *C. perfringens* spores. *C. perfringens* spores are often found in the food processing facilities and can survive in different sections from entry point to the finished products. Another reason is the resistant properties of *C. perfringens* spores to various preservatives and inactivation treatments. Finally, *C. perfringens* vegetative cells can rapidly grow in a wide range of temperatures, and, thus, reach pathogenic burden. Each of these factors contributes to the food contamination and foodborne illnesses associated with *C. perfringens* (McClane et al., 2013; Wells-Bennik et al., 2016). However, the differences in spore resistant and vegetative cell growth types among different types of *C. perfringens* strains, especially between FP and NFB strains questions the effectiveness of the current inactivation methods (Augustin, 2011; Eijlander et al., 2011; McClane et al., 2013). As a result,
alternative strategies to common processing treatment (i.e. thermal) to inactivate *C. perfringens* and increase food safety and quality are in high demand (Novak and Juneja, 2002; Wells-Bennik et al., 2016).

Combinations of multiple preservation methods can lead to alternative approaches to inactivate *C. perfringens* spores and vegetative cells (Novak and Juneja, 2002; Wells-Bennik et al., 2016). For instance, combination of high hydrostatic pressure (HHP) (650 MPa), high temperature (75 °C) and low pH (4.75) inactivates spores of *C. perfringens* type A C-cpe and P-cpe isolates by 2.8 and 5.1 log reduction, respectively (Paredes-Sabja et al., 2007). This suggests that C-cpe isolates associated with food poisoning are more resistant to heat and pressure than P-cpe isolates, and thus require more abusive conditions for inactivation (Sarker et al., 2015). Another example is the combination of ozone treatment and thermal inactivation, which resulted in the inhibition of vegetative cells and spores of *C. perfringens* in a meat system (Novak and Yuan, 2004). A recent study used ultrasound technology to enhance thermal effects against *C. perfringens* in beef slurry (Evelyn and Silva, 2015). Combination of HHP and a natural antimicrobial such as nisin enhances HHP effects against *C. perfringens* spores in milk (Gao et al., 2011). The later study emphasizes the promising effects of incorporation of natural antimicrobial agents into food formulations to control spore forming pathogenic bacteria (Gao et al., 2011).

1.7.1. Natural antimicrobial compounds

Antimicrobial agents are mainly used to preserve food from natural spoilage and to ensure food safety by inhibiting growth or inactivating disease-causing
microorganisms (Solorzano-Santos and Miranda-Novales, 2012). The direct incorporation of antimicrobial compounds into food formulations provides advantages of low-capital cost, warrants the presence of active agents and maintains the antimicrobial activity until final consumption (Seman et al., 2008a). The antimicrobial properties of a variety of chemicals and natural food preservatives have received tremendous attention from researchers and the food industry for a long time to validate their inhibitory efficiency against food spoilage and foodborne pathogens on numerous types of food (Davidson et al., 2005). In addition to antimicrobial activity, some food preservatives also have a role in stabilizing the food color and enhancing the flavor (Davidson et al., 2005; Tompkin, 2005). For example, nitrite is a chemical preservative that has been shown to effectively inhibit spore germination and outgrowth of *C. perfringens* in meat products, specifically ham (Redondo-Solano et al., 2013). However, nitrite reacts with amines in meat proteins and forms a carcinogenic derivative named nitrosamine. This eliminates the use of such chemical agents and highlights the needs for alternative natural agents (Tompkin, 2005).

1.7.2. Organic acids and their salts

Organic acids and their salts are food additives that have been used to enhance food flavor and to prevent fungal spoilage (Doores, 2005). Owing to their classification as generally recognized as safe (GRAS) compounds, sorbate and benzoate are the most common and oldest organic acids used in food industries (van Melis et al., 2011). Even though benzoate has lower water solubility than sorbate, both agents are well-known as antifungal agents and widely used chemical antimicrobials because of their low cost, ease of incorporation into foods, colorless properties, and low toxicity (Chipley, 2005;
Stopforth et al., 2005). Different studies showed contradictory effects of sodium benzoate against *C. perfringens* growth in cooked sausage (Petaja et al., 1979; Tompkin et al., 1974).

Sodium lactate, a flavor enhancer used in meat and poultry products, delays spore germination and outgrowth of *C. perfringens* during storage at 19 and 25 °C in marinated cooked chicken products (Doores, 2005). Further experiments also indicated that 1.5% sodium lactate could retard growth of *C. perfringens* spores during storage at abusive temperature up to 40 h (Juneja, 2006). In order to reduce the risk of *C. perfringens* spore germination and outgrowth in thermally processed pork under the extended USDA-FSIS stabilization procedure, 2% calcium lactate and 3% sodium lactate or potassium lactate are required. At the same concentrations, calcium lactate is more effective than sodium or potassium lactates to control germination and outgrowth of 3 strain-mixtures of *C. perfringens* spores in injected chilled pork (Velugoti et al., 2007).

A previous study reported that a blend of vinegar and buffered lemon juice concentrate was highly effective in controlling growth from spores of *C. perfringens* in reduced NaCl roast beef during abusive exponential cooling (Li et al., 2012). Another study using ground turkey roast, reported that 2.5% buffered vinegar, and a blend of buffered lemon juice concentrate and 3.5% vinegar could inhibit *C. perfringens* spore germination and outgrowth to less than 1 log CFU/g increase during deviated chilling rate within 21 h (Valenzuela-Martinez et al., 2010).

1.7.3. Chitosan

Chitosan is an animal-derived natural antimicrobial (Davidson et al., 2013). It is a carbohydrate polymer derived from the deacetylation of chitin (poly-β-N-acetyl-D-
glucosamine), which is the main component in a crustacean’s shell, and the second most abundant biopolymer in nature after cellulose. Different production methods produced chitosan with different degrees of deacetylation as well as molecular masses (MM) reflecting its variation in properties (No and Meyers, 1995). The antimicrobial property of chitosan combined with its natural origin, nontoxic nature, and biodegradability make it an attractive choice for the food industry in a variety of products (Friedman and Juneja, 2010a; Kong et al., 2010; Su et al., 2009). Intensive research has focused on the antimicrobial activity of chitosan against vegetative cells of various foodborne pathogens (Su et al., 2009). The ability of chitosan to inhibit spore outgrowth is strongly influenced by its molecular weight and degree of acetylation (Friedman and Juneja, 2010a; Mellegard et al., 2011b). Previous study reported 3% chitosan mixed into ground beef or turkey resulted in 4 to 5 log reductions of C. perfringens spore germination and outgrowth during exponential cooling of cooked beef or turkey up to 18 h (Juneja et al., 2006a).

1.7.4. Nisin

Nisin is a microbial-derived antimicrobial agent which is also listed as GRAS to be used in food products. It is a 34 amino acid polypeptide produced by certain strains of Lactococcus lactis subsp. lactis and permitted to be used in various food products in more than 50 countries (Davidson et al., 2005). Owing to its natural source and effectiveness against broad-spectrum Gram-positive bacteria, nisin is an attractive choice as a food additive. Several reports demonstrated the inhibitory activity of nisin alone or in combination with other food preservation technologies such as heat and HHP in controlling spores of Clostridium spp. in a variety of food products (Gao et al., 2011;
Udompijitkul et al., 2012). A recent study reveals that the inhibitory effect of nisin against germination of spores of both FP and NFB isolates is negligible. Also, the observed inhibitory effect during spore outgrowth and vegetative cell growth of *C. perfringens* in laboratory conditions is not achievable in a meat model system (Udompijitkul et al., 2012).


Objectives of this study

This dissertation consists of three studies that will contribute to *C. perfringens* inactivation:

1. In the first study, the objectives were (i) to investigate the inhibitory effect of sorbate and benzoate against a collection of clinical isolates of *C. perfringens* FP and NFB GI disease, and (ii) to evaluate the efficacy of sorbate and benzoate as antimicrobial agents in food model system contaminated with *C. perfringens* spores during storage at extremely abusive temperature.

2. In the second study, the objectives were (i) to investigate the inhibitory effect of chitosan against germination, outgrowth and vegetative growth of spores of the enterotoxigenic *C. perfringens* type A isolates, and (ii) to evaluate the efficacy of chitosan as an antimicrobial agent to control the germination and outgrowth of *C. perfringens* spores in cooked meat products during storage at extremely abusive temperature.

3. In the third study, our objectives were (i) to investigate the role of bicarbonate with 20 individual amino acids or in combination in triggering germination of spores of various *C. perfringens* NFB isolates and one FP isolate, (ii) to identify and characterize new amino acid germinants for spores of NFB isolates, (iii) to compare the germinant selectivity between *C. perfringens* FP and NFB isolates; and finally (iv) to evaluate the role(s) of each of the GR proteins in the germination of *C. perfringens* NFB spores with the newly identified germinants combined with bicarbonate.
References


Velugoti, P.R., Bohra, L.K., Juneja, V.K., Thippareddi, H., 2007. Inhibition of germination and outgrowth of Clostridium perfringens spores by lactic acid salts during cooling of injected turkey. J. Food Prot. 70, 923-929.


Chapter 2

The Inhibitory Effects of Sorbate and Benzoate Against *Clostridium perfringens* Type A Isolates

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Abstract

This study evaluated the inhibitory effects of sorbate and benzoate against *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) disease isolates. No significant inhibition of germination of spores of both FP and NFB isolates was observed in rich medium (pH 7.0) supplemented with permissive level of sodium sorbate (0.3% ≈ 0.13 mM undissociated sorbic acid) or potassium benzoate (0.1% ≈ 0.01 mM undissociated benzoic acid) used in foods. However, these levels of sorbate and benzoate effectively arrested outgrowth of germinated *C. perfringens* spores in rich medium. Lowering the pH of the medium increases the inhibitory effects of sorbate and benzoate against germination of spores of NFB isolates, and outgrowth of spores of both FP and NFB isolates. Furthermore, sorbate and benzoate inhibited vegetative growth of *C. perfringens* isolates. However, the permissible levels of these organic salts could not control the growth of *C. perfringens* spores in chicken meat stored under extremely abusive conditions. In summary, although sorbate and benzoate showed inhibitory activities against *C. perfringens* in the rich medium, no such effect was observed in cooked chicken meat. Therefore, caution should be taken when applying these organic salts into meat products to reduce or eliminate *C. perfringens* spores.
2.1. Introduction

*Clostridium perfringens* is a Gram-positive, rod-shaped, spore-forming anaerobic bacterium with a prolific toxin-producing ability. Thus, it is a causative agent of wide variety of human and veterinary diseases (McClane, 2007). *C. perfringens* can be classified into 5 types (A-E) based on their abilities of producing a sub-set of four major lethal toxins (alpha, beta, epsilon, and iota) (McClane, 2007; Petit et al., 1999). Nevertheless, some *C. perfringens* strains (~ 5%), mostly type A, are able to produce another medically important toxin termed *C. perfringens* enterotoxin (CPE). CPE-producing *C. perfringens* is a major cause of *C. perfringens* type A food poisoning (FP) as well as non-food-borne (NFB) human gastrointestinal (GI) diseases such as antibiotic-associated diarrhea, sporadic diarrhea, and nosocomial diarrheal diseases (Lindström et al., 2011; Miyamoto et al., 2012; Sarker et al., 1999). Previous studies have shown that CPE encoding gene (*cpe*) can be located either on the chromosome or on a large plasmid; however, *C. perfringens* isolates associated with FP typically carry the *cpe* on their chromosome, whereas NFB human GI illnesses are related to isolates carrying *cpe* on their large plasmid (Collie and McClane, 1998; Sarker et al., 2000). *C. perfringens* type A FP is one of the most common reported food-borne illnesses in the United States and other developed countries and currently ranks as the second most reported bacterial food-borne disease outbreaks in the United States accounting for ~ 1 million cases per year (Hoffmann et al., 2012; Juneja et al., 2006b; Lynch et al., 2006b; Scallan et al., 2011a).

*C. perfringens* continues to be a concern to food industries due to its ability to form spores that are highly resistant to various preservative approaches commonly used to control spoilage and pathogenic microorganisms in food products (Li and McClane,
Moreover, a thermal treatment applied during food processing is generally not sufficient to kill spores and could act as an activation step for the surviving spores to germinate and outgrow to hazardous level thereafter (Thippareddi et al., 2003). Hence, alternative strategies to the conventional thermal processing technology to increase palatability of processed foods while meeting the bacterial spore inactivation standards are currently required. Several ingredients and combination of ingredients have been continually investigated for their antimicrobial effectiveness against spore-forming bacteria (Juneja and Thippareddi, 2004; Maldonado et al., 2013; Reddy Velugoti et al., 2007; Sabah et al., 2003; Thippareddi et al., 2003; Valenzuela-Martinez et al., 2010; Walker and Phillips, 2008; Yetim et al., 2006). The ingredient-based intervention such as incorporation of antimicrobial agents into food formulations offers low-cost advantages that may assure that active ingredients are present in foods and retain antimicrobial activity even after the package is opened (Seman et al., 2008b). Among the innovative food additives tested, organic acids and their salts are attractive options due to their various positive attributes including, broad-spectrum antimicrobial activity, toxicologically safe, heat stable at acidic pH, no effect on color or flavor, and long-history of successful usage as food preservatives (Chipley, 2005; Glass et al., 2007b; Mani-López et al., 2012; Wan Norhana et al., 2012).

In the United States, sorbate and benzoate are affirmed as Generally Recognized As Safe (GRAS) food preservatives with the permissible concentration of sodium benzoate used in foods is 0.1% in the USA, whereas up to 0.15 to 0.25% in most other countries (Chipley, 2005). The amount of sorbate typically used in food products is in the
range of 0.02% to 0.3% (Stopforth et al., 2005). Although these antimicrobial agents are traditionally used to control growth of molds in various food products (Chipley, 2005; Stopforth et al., 2005), their inhibitory activity against many Gram-positive bacterial pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* in media system as well as in meat products are also reported (Glass et al., 2007a; Tompkin et al., 1974). Despite the fact that an extensive study has been conducted to utilize sorbate as an antibotulinal agent in meat products (Stopforth et al., 2005), such study against enterotoxigenic *C. perfringens* is limited and inconclusive. For example, one previous study, which investigated the effect of potassium sorbate on the growth of *C. perfringens* in cooked sausage, claims no difference in the level of *C. perfringens* regardless of the presence of sorbate in the samples (Tompkin et al., 1974). However, a follow-up study reported successful inhibition of *C. perfringens* growth with potassium sorbate in cooked sausage (Petaja et al., 1979). Therefore, the detailed study on the effect of benzoate and sorbate on various aspects of *C. perfringens* life cycle, as well as their capability of controlling germination and outgrowth of *C. perfringens* spores in meat samples under extremely abusive storage condition are warranted.

The objectives of this study were to evaluate the effects of sorbate and benzoate against germination, outgrowth and vegetative growth of spores of the enterotoxigenic *C. perfringens*. Furthermore, the application of sorbate and benzoate as antimicrobial agents to control the growth of *C. perfringens* spores in cooked meat products during storage at extremely abusive temperature was also examined.
2.2. Material and methods

2.2.1. Bacterial strains and growth conditions

The *C. perfringens* isolates used in this study consist of four type A FP isolates (SM101, NCTC10239, E13, and FD1041) and four type A NFB isolates (NB16, B40, F5603, and F4969) (Sarker et al., 2000). All stock cultures were maintained in cooked meat medium (Difco, BD Diagnostic Systems, Sparks, MD, USA) and stored at -20 °C. The bacterial growth was revived by inoculating cooked meat culture into fluid thioglycollate (FTG) medium (Difco) and incubated overnight at 37 °C. TGY (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) broth was used for examining vegetative growth and spore outgrowth of *C. perfringens* strains (Paredes-Sabja et al., 2008d).

2.2.2. Spore preparation and purification

Spores of *C. perfringens* were prepared and purified as described previously (Akhtar et al., 2008; Paredes-Sabja et al., 2008c). Briefly, the stock cultures of *C. perfringens* were inoculated into 10 ml FTG and incubated overnight at 37 °C. The 0.4 ml of an overnight FTG culture was inoculated into a new 10 ml FTG medium and grew for 8 h at 37 °C. Next, 0.4 ml of 8-h FTG culture was inoculated into 10 ml Duncan-Strong sporulation medium (Duncan and Strong, 1968) and sporulation capacity was confirmed under phase contrast microscope after incubation at 37 °C for 24 h. Large amounts of spores were prepared by scaling up the aforementioned procedure. Spores were purified by repeated washing and centrifuging with sterile distilled water until the spore suspensions were > 99% free of sporulating cells, cell debris, and germinated spores.
Purified spores were suspended in sterile distilled water to obtain an optical density at 600 nm (OD_{600}) of ~ 6, and stored at -80 °C until use.

2.2.3. Preparation of antibacterial solutions

Stock solutions of sodium sorbate (SS), potassium sorbate (PS), and sodium benzoate (SB) (PS and SB were purchased from Alfa Aesar, Ward Hill, MA, USA containing 99% pure substance; and SS from Tokyo Chemical Industry Co., LTD) were prepared with sterile distilled water at a concentration of 10% (w/v). These solutions were then filter sterilized (0.45 μm, Millipore, Bedford, MA, USA) and stored at 4 °C. The stock solutions were used within two weeks of preparation. In order to obtain the desired present concentrations, the stock solutions were diluted in a specific medium used for the corresponding experiments. The concentration (mM) of undissociated acid from each salt was calculated using Henderson-Hasselbalch equation based on the pKa of sorbic acid (4.76) or benzoic acid (4.202) at each tested pHs (pH 5.5 - 7.0) and presented in Table S1.

2.2.4. C. perfringens spore germination in the presence of SS and SB

The purified spore suspensions in water at a final OD_{600} ~ 1.0 were heat activated at 80 °C for 10 min or 75 °C for 15 min for FP and NFB spores, respectively, prior to germination as previously described (Paredes-Sabja et al., 2008d; Paredes-Sabja et al., 2009d), and then cooled in water bath at room temperature for 5 min. The heat-activated spores were further incubated at 40 °C for 10 min before mixing with the pre-warmed Brain Heart Infusion (BHI) broth (Difco) (pH 5.5, 6.5 or 7.0) alone or supplemented with
various concentrations (0.1- 1.0%) of SS or SB. When *C. perfringens* spores were incubated with SB (0.1%), PS (0.3%), or SS (0.3%) in buffer (25mM Na$_2$HPO$_4$ at pH 7.0) for 60 min, no significant germination of spores of both FP and NFB isolates was observed with SB or SS. However, PS triggered germination of spores of only FP isolates (data not shown) due to the presence of K$^+$, a strong germinant for FP spores (Paredes-Sabja et al., 2008d). These preliminary results suggest that SS or SB is not a germinant for the tested *C. perfringens* spores (data not shown). Therefore, SS and SB were selected to evaluate the inhibitory effects of sorbate and benzoate, respectively, on *C. perfringens* spore germination. Spore germination was routinely monitored by measuring OD$_{600}$ of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), in which OD$_{600}$ is decreased by ~ 60% upon complete spore germination (Paredes-Sabja et al., 2008d). The extents of spore germination were also confirmed by phase-contrast microscopy in which germinated spores lose refractility and become phase dark spores. Germination rates were calculated as percentage loss of OD$_{600}$ relative to the initial value after 60 min of incubation in BHI with or without SS and SB. Results were expressed as percentage inhibition of spore germination compared with the control germination (without SS or SB) as described previously (Cortezzo et al., 2004). All germination experiments were performed at least twice with two different spore preparations for each strain.

### 2.2.5. *C. perfringens* spore outgrowth in the presence of PS and SB

A 0.2 ml spore suspensions of an OD$_{600}$ of ~ 6.0 were heat activated at 80 °C for 10 min for FP spores and at 75 °C for 15 min for NFB spores, and then cooled in a water
bath at room temperature before inoculating into the pre-warmed 10 ml TGY broth (pH 6.8, 6.0 or 5.5) alone or supplemented with PS (0.3%) or SB (0.1 to 0.3%). Cultures were incubated at 37 °C and the OD$_{600}$ was measured over time interval up to 180 min post-inoculation. The inhibition of spore outgrowth was calculated as % inhibition = (OD$_{600}$ increase in treatment/ OD$_{600}$ increase in control) x100 (Cortezzo et al., 2004). The experiments were performed at least in duplicate with two independent spore preparations.

2.2.6. *C. perfringens* vegetative growth in the presence of PS and SB

The inhibitory effects of PS and SB on growth of *C. perfringens* were determined in conditions supporting vegetative growth in TGY. A 0.4 ml of an overnight FTG culture was transferred into 10 ml TGY and grown for 3 h at 37 °C. Then, 0.4 ml of 3-h TGY culture was inoculated into fresh 10 ml TGY supplemented with various concentrations of PS or SB ranging from 0.1% to 1.5% (v/v). A control sample without PS or SB was included to compare the effects of PS or SB. The growth was monitored by measuring OD$_{600}$ hourly for up to 24 h. All growth experiments were performed independently at least in triplicate and values are given as means. In order to correlate the OD$_{600}$ reading results with the corresponding colony forming units per ml (CFU/ml), an aliquot of culture from each treatment was withdrawn at 0 and 6 h post-inoculation, and then serially diluted and plated onto BHI (Difco, BD Diagnostic Systems) agar. Plates were incubated anaerobically at 37 °C for 24 h before colonies were counted.
2.2.7. Growth of C. perfringens spores in cooked meat in presence of PS and SB

The ability of PS and SB to prevent germination and outgrowth of C. perfringens spores in meat during storage at abusive conditions was measured as previously described (Udompijitkul et al., 2012). Briefly, grounded chicken meat samples (10g/bag) were placed into the UV-sterilized plastic bag (5.5” W × 6”L) and then sealed, autoclaved and stored at -20 °C until use. Prior to each experiment, bags containing meat sample were thawed and appropriate amounts (0, 0.5, 1.0, 5.0% (w/w)) of PS or SB stock solution were added. Then 0.1 ml of 3-strain spore cocktails were mixed by manually massaging for 1 min after resealing bags. The 3-strain spore cocktails of FP (SM101, NCTC10239, and FD1041) and NFB (NB16, F4969, and F5603) isolates were prepared by combining approximately equal number of spores from each strain containing ~10⁸ spores per ml. Negative control was included in every replication to ensure that meat samples were free of naturally occurring bacteria. Samples were kept on ice before cooking at 80 °C for 15 min and cooled in water bath at room temperature for 20 min. For each treatment, one bag content was used to determine the initial population of C. perfringens in the meat, and the other sample was transferred to sterile petri dish and stored under anaerobic condition at 37 °C for 6 h. To determine populations of C. perfringens in the meat, each sample (10 g meat) was transferred into a stomacher bag, mixed with 90 ml 0.1% (w/v) peptone water, serially diluted, and then plated onto BHI agar plates that were incubated anaerobically at 37 °C for 24 h before counting the colonies. Results were expressed as CFU/g.
2.2.8. Statistical analysis

Data were analyzed by the analysis of variance procedure (ANOVA) using the statistical software SAS version 9.3 (SAS Inst. Inc., Cary, N.C., USA). ANOVA among treatments was performed and comparisons of mean values were established by Duncan’s New Multiple Range Test at the significant level of 0.05. In all figures, error bars represent the standard deviations.
2.3. Results and discussion

2.3.1. Inhibition of *C. perfringens* spore germination with SS and SB

Since germination of *C. perfringens* spores is a prerequisite step for the development of growing cells in food products, we first assessed the inhibitory effects of sorbate and benzoate on spore germination. To generalize our results, a variety of clinical isolates of the enterotoxigenic *C. perfringens* type A were included. Previous studies indicated that the chromosomal *cpe* isolates NCTC8239 and SM101 belong to the group I as analyzed by the multilocus sequence typing (MLST), while the plasmid *cpe* isolates F4969 and F5603 belong to diverse MLST clusters. (Deguchi et al., 2009; Miyamoto et al., 2012; Xiao et al., 2012). The incorporation of SS or SB at various concentrations in rich BHI broth (pH 7.0) inhibited the germination capabilities of spores of most tested FP and NFB isolates during 60 min of incubation at 40 °C. Nevertheless, the germination inhibition is dependent on concentrations of SS or SB and the significant effect was obtained with higher concentrations (Table 1). Interestingly, spore germination of NFB isolates was more susceptible to SB, as germination of spores of most tested NFB isolates was inhibited significantly with lower concentrations of SB as compared to the effect of SS at the same concentrations (Table 1). Collectively, our results indicate that the inhibition of *C. perfringens* spore germination is dependent on the concentration of SS or SB as well as the isolation sources of the strains. This is consistent with previous reports where high concentrations of PS was required to inhibit germination of spores of *Bacillus cereus* T or *Clostridium botulinum* 62A; and there are considerable variations in germination inhibition among strains of *C. botulinum* (Smoot and Pierson, 1981). Since *C. perfringens* spore germination is also dependent on germinant receptors (GRs) located
in the spore inner membrane (Banawas et al., 2013b), it is possible that SS inhibits *C. perfringens* spore germination via an inhibition of action of, or response to, GR as proposed for *Bacillus subtilis* (Cortezzo et al., 2004). Alternatively, as proposed for *B. cereus*, the undissociated sorbic acid that accumulates in spore’s inner membrane may inhibit *C. perfringens* spore germination via an interference of the signal transduction between GR and the ion/calcium–dipicolinic channels (van Melis et al., 2011).

As sorbate- and benzoate-induced inhibition of germination and outgrowth of spores of various spore-forming bacteria is dependent on pH (Blocher and Busta, 1985; Chipley, 2005; Seward et al., 1982), we examined the inhibitory effects of SS or SB at different pHs ranging from 5.5-7.0, which are the typical pHs found in meat, the most common food vehicle for *C. perfringens* type A FP (Carse and Locker, 1974; Luciano et al., 2008; Thomson, 1978; Udompijitkul et al., 2012). Our results demonstrated that pH did not significantly affect the germination inhibition capacity of SS, since all the tested pHs showed very limited efficacy of SS against spores of both FP and NFB isolates (Table 2). Although our results contradict to the previous findings with *C. botulinum* and *B. cereus* (Seward et al., 1982), it is possible that concentration of SS had a more pronounced effect than pH in inhibiting *C. perfringens* spore germination, at least in rich medium. On the other hand, pH and concentration of undissociated acid seemed to influence the specific inhibitory effect of SB against spores of NFB strains, NB16 and B40 (Table 2). The 0.1% SB (≈ 0.33 mM undissociated benzoic acid) in BHI adjusted to pH 5.5 exhibited an enhanced inhibitory effect, which was comparable to the effect of 0.25% SB (pH 7.0) (≈ 0.03 mM undissociated benzoic acid) against these particular NFB isolates (Table 1 and 2). This is consistent with an acidic pH of lemon juice concentrate.
improved the effectiveness of SB in controlling growth from spores of *Alicyclobacillus acidoterrestris* upon storage at 44 °C (Maldonado et al., 2013).

### 2.3.2. PS and SB block *C. perfringens* spore outgrowth

The inhibitory effects of SS and SB on germination of spores of *C. perfringens* isolates led us to hypothesize that these compounds might inhibit outgrowth of spores, as previously found with nisin (Udompijitkul et al., 2012). In rich TGY medium (pH 6.8), spores of FP strain SM101 and NFB strain F4969 were able to initiate outgrowth after incubation for ~90 and 110 min, respectively (Fig. 1). However, the presence of 0.3 % PS (≈ 0.18 mM undissociated sorbic acid) in TGY was sufficient to inhibit outgrowth of both SM101 and F4969 spores significantly (p <0.05) during the 3-h incubation. Although the outgrowth of F4969 spores blocked completely, a slight outgrowth was observed with SM101 spores after ~120 min of incubation in TGY supplemented with 0.3% PS (Fig 1A and C). In contrast, while 0.1% SB (≈ 0.02 mM undissociated benzoic acid) was able to block the outgrowth of F4969 spores (Fig. 1C), SM101 spores were able to initiate outgrowth after ~110 min of incubation in TGY supplemented with 0.1% SB (data not shown). However, a higher level of SB (0.3% ≈ 0.05 mM undissociated benzoic acid) completely arrested the outgrowth of SM101 spores (Fig. 1A). Similar results were observed with spores of other FP (NCTC10239) and NFB (NB16) strains (Fig. 1B and D). It should be noted that the concentrations PS and SB that successfully arrested spore outgrowth were relatively lower than those required for inhibiting spore germination. Our data are in consistent with the previous findings that the outgrowth of spores is the most susceptible stage for the inhibitory actions of benzoate and sorbate (Jay et al., 2005). The
longer outgrowth initiation period was observed in the presence of low concentrations of tested antimicrobials compared with control. These results are in line with the recent observation that the presence of 0.75 mM undissociated sorbic acid at pH 5.5 leads to the lengthening of lag time of *Bacillus cereus* ATCC14579 spores to resume outgrowth (den Besten et al., 2012).

Furthermore, lowering the pH of TGY medium (from 6.8 to 6.0 and 5.5) significantly increased the inhibitory effects of PS and SB against spore outgrowth; at pH 6.0, lower concentration of PS (0.1% ≈ 0.36 mM undissociated sorbic acid) or SB (0.05% ≈ 0.05 mM undissociated benzoic acid) was sufficient to inhibit significantly the outgrowth of spores of both FP SM101 and NFB F4969 isolates (Table 3). Unlike SM101 spores, outgrowth of F4969 spores had a higher resistance to sorbate and benzoate at pH 5.5 after 3-h post inoculation. Surprisingly, when we extended the outgrowth incubation time to 24 h, a complete inhibition of F4969 spores’ outgrowth was observed (data not shown). Collectively, these results suggest that PS or SB at a permitted concentration used in foods effectively arrested outgrowth of germinated spores of tested FP and NFB isolates and the pH as well as the concentration of undissociated acid of these food preservatives plays an important role in controlling *C. perfringens* spore outgrowth in rich medium.

2.3.3. PS and SB inhibit the growth of *C. perfringens* vegetative cells

Next, we examined the vegetative growth of *C. perfringens* isolates in TGY medium supplemented with various concentrations of PS and SB. *C. perfringens* strains SM101 and F4969 were selected as representative strains for FP and NFB isolates,
respectively. Results demonstrated that the incorporation of PS or SB at the lowest concentration tested (0.1% ≈ 0.06 and 0.02 mM undissociated sorbic and benzoic acids, respectively) resulted in a lower biomass as compared to cultures grown in the absence of PS or SB for the entire incubation period (Fig. 2). Increasing the concentrations of PS and SB in TGY broth resulted in higher inhibitory effects on the growth of SM101 and F4969 cells. Interestingly, the SB inhibition was approximately two-fold more effective than PS inhibition; especially against the FP strain SM101.

In order to validate the growth-inhibitory effect of PS or SB observed by measuring OD\textsubscript{600}, C. perfringens cell populations in TGY media with or without PS and SB was determined at 0 h and 6 h post-inoculation by plating aliquots of diluted cultures onto BHI agar (Fig. 3). There was no significant increase in SM101 viable cell counts during 6 h growth in TGY supplemented with the maximum allowable concentration of PS (0.3%) used in the foods (FDA, 2013; Islam et al., 2002). However, treatment with the permitted level of SB (0.1%) resulted in an increase of SM101 viable cell counts to a similar level of the culture grown without SB (Fig. 3A). Surprisingly, a combination of permitted levels of PS (0.3%) and SB (0.1%) resulted in only 0.5 log CFU/ml higher than those of PS- and SB- free media (control treatments). On the other hand, the total viable counts of F4969 after 6 h growth in the presence of PS (0.3%) alone or combined with SB (0.1%) were decreased by 0.5 and 1.0 log CFU/ml, respectively. However, addition of 0.1% SB alone resulted in an elevation of F4969 viable counts to a similar level to the control without SB (Fig. 3B). Microscopic observation revealed an aggregation of long filamentous cells in cultures containing PS or SB (Fig. 4). This unique characteristic of cell deformation could explain an increase in OD\textsubscript{600} observed during the growth in Fig. 2.
and was also observed when *C. botulinum* type E cells were grown in the presence of sorbate and these aberrant-growing cells were lysed more frequently than the normal cells (Seward et al., 1982).

To generalize our findings, additional strains of FP and NFB isolates were examined for their vegetative growth characteristics in TGY medium supplemented with PS and/or SB (Fig. 5). Two additional FP isolates (E13 and FD1041) showed similar growth patterns as SM101 in the presence of PS or SB alone, regardless of concentrations tested. Unlike SM101, the combination of 0.3% PS and 0.1% SB had significant (*p* < 0.05) growth inhibition on other tested FP isolates. In case of NFB isolates, vegetative growth of strain B40 was completely inhibited with 0.3% PS alone and in combination with 0.1% SB, whereas strain NB16 showed a similar growth-inhibition trend as F4969 grown in presence of PS and/or SB. Previous studies also demonstrated that the enterotoxigenic *C. perfringens* FP and NFB isolates differ in their resistance to a variety of food preservatives such as salt, nitrite, polyphosphates, and nisin (Akhtar et al., 2008; Li and McClane, 2006a; Udompijitkul et al., 2012). It has been suggested that the differences in antimicrobial susceptibility among the bacterial strains could be associated with the isolation sources as well as the distinct composition and/or the distribution of fatty acids in bacteria’s cytoplasmic membrane (Maldonado et al., 2013). Moreover, the degree of cell hydrophobicity might also play a role in determining the sensitivity of cells to antimicrobial compounds (Chaibi, 1997). Although not yet proven, these factors are likely to account for the inter-strain variations in growth response to PS and SB between *C. perfringens* FP and NFB isolates as observed in this study.
2.3.4. Control of germination and outgrowth of *C. perfringens* spores in cooked meat products

To validate the ability of PS and SB to arrest growth from spores of *C. perfringens* in cooked chicken meat during storage at abusive conditions, a previously described meat model system contaminated with *C. perfringens* spores was employed (Udompijitkul et al., 2012). Results indicate that PS and SB concentrations that successfully inhibited the growth of *C. perfringens* in laboratory media, failed to control germination and outgrowth of spores in cooked chicken meat during 6 h period of storage under anaerobic condition at 37 °C (Fig. 6). Meat samples containing concentrations of ≤ 1% of PS or SB exhibited an increase of ~2 logs in *C. perfringens* viable cell counts after 6 h of incubation at 37 °C, which is similar to those observed in control meat samples without PS or SB. However, increasing the amount of PS and SB to 5% resulted in more than 1 log reduction in *C. perfringens* viable cell counts. This antimicrobial effect of PS and SB observed in meat samples against *C. perfringens* spores was comparable for both FP and NFB isolates. Although the inhibitory concentrations of PS and SB against *C. perfringens* growth in cooked chicken meat in the current study was much higher than the permissible level of PS and SB in meat products (FDA, 2013), similar concentration have been successfully used as dipping solutions to control *Listeria monocytogenes* on sliced pork bologna (Samelis et al., 2005) and beef franks (Uhart et al., 2004; Wan Norhana et al., 2012). Furthermore, a 0.05% (w/v) SB in combination with other organic acid is able to rapidly destroy the contaminated yeast in the seasoned olives prepared from fresh olives during storage at room temperature (23 ± 2 °C). In the same study, PS exhibits a general inhibitory effect against yeast in both seasoned olive prepared from fresh fruits
and fruits stored in 11% NaCl brine, whereas SB and PS lack inactivation capabilities towards lactic acid bacteria, especially in seasoned olive prepared from stored fruits (López et al., 2006). Although PS can extend the shelf-life vacuum-packed raw chicken meats and lower the number of mesophilic aerobic bacteria under refrigeration storage, an effective concentration of 5% is much higher than the maximum allowable level, and thus these results are in line with our findings (Kolsarici and Candogan, 1995). Our results are also in agreement with the previous report in which sorbate at most practical concentrations could not control growth from spores of *C. botulinum* type E in fresh fish stored at elevated temperature (Seward et al., 1982). One possible explanation is that, absorption of organic acid salts by meat results in the decrease of the antimicrobial activities of PS and SB upon their application in meat samples (Samelis et al., 2005). Moreover, the pH of meat samples might also plays role in maintaining efficacy of benzoate and sorbate in a food system. According to the weak-acid theory, the amount of undissociated acid present is primarily responsible for an antimicrobial activity of weak-acid preservatives such as sorbic acid and benzoic acid, and it was suggested that the inhibitory capacity of dissociated sorbic acid is 10 to 600 times lower than that of an undissociated form (Krebs et al., 1983; Stopforth et al., 2005; Stratford and Anslow, 1998). In the current work, pHs of cooked chicken samples without preservative and with the maximum tested level of PS and SB (5%) were approximately 6.0, 6.9, and 6.8 respectively. As the pHs of meat samples used in this study, regardless of an addition of antimicrobial agents, were higher than the pKa of sorbic acid (pKa 4.76) and benzoic acid (pKa 4.202), this could lead to the dissociation of these weak organic acids rendering them being ineffective antimicrobials in meat products. Nevertheless, it is important to
note that the storage condition tested in this study was extremely abused in order to simulate the worst case scenario that could happened if cooked meats are kept under warm temperature supporting the optimum growth of *C. perfringens* for a certain period of time before consumption. Collectively, these results suggest that high level (5%) of PS or SB exhibits inhibitory effect against *C. perfringens* spores in chicken meat most likely by affecting the germinated spores and inhibiting further outgrowth under abusive temperature condition.

In conclusion, besides their well-recognized antifungal properties, benzoates and sorbates also exert their inhibitory effects against the food-borne spore-forming pathogen *C. perfringens* in a variety of aspects including spore germination and outgrowth, and vegetative growth of both FP and NFB isolates. Despite their effectiveness in laboratory medium, PS and SB failed to control germination and outgrowth of *C. perfringens* spores inoculated into cooked chicken meat stored in improper condition for an extended period. Thus, cautions need to be taken when applying these organic salts into food formulations of meat products in order to reduce or eliminate bacterial spores, especially enterotoxigenic *C. perfringens* spores.
Acknowledgements

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**Table 2.1: Inhibition of spore germination by SS or SB**

<table>
<thead>
<tr>
<th>Inhibitor&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Concentration (% v/v)</th>
<th>Undissociated acid (mM)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Inhibition of germination (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td></td>
<td></td>
<td></td>
<td>FP strains</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>SM101 NCTC 10239 E13</td>
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<tr>
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<sup>a</sup> spores were germinated in BHI broth containing antimicrobial agents at various concentrations and percentage of inhibition of germination was calculated as described in Material and methods. Values are the average of duplicate experiments from two different spore preparations. The standard deviations from these experiments were less than 17% of the mean.

<sup>b</sup> inhibitors were SS, sodium sorbate and SB, sodium benzoate.

<sup>c</sup> amount of undissociated acid was calculated as described in Material and methods.

<sup>d</sup> negative value indicates higher germination rate than the control.
Table 2.2: Effect of pHs on spore germination inhibition in the presence of SS and SB

<table>
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<tr>
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<th>Undissociated acid (mM)&lt;sup&gt;c&lt;/sup&gt;</th>
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<th>NCTC10239</th>
<th>E13</th>
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</table>

<sup>a</sup> Spores were germinated in BHI broth supplemented with SS or SB adjusted to different pHs and percentage of inhibition of germination was calculated as described in Material and methods. Values are the average of duplicate experiments from two different spore preparations. Standard deviations from these experiments were less than 14% of the mean.

<sup>b</sup> Inhibitors were SS, sodium sorbate and SB, sodium benzoate.

<sup>c</sup> Amount of undissociated acid was calculated as described in Material and methods.

<sup>d</sup> Negative value indicates higher germination rate than the control.
Table 2.3: Effect of pHs on outgrowth of *C. perfringens* spores in TGY supplemented with PS and SB

<table>
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<th>Inhibitor&lt;sup&gt;b&lt;/sup&gt; % (v/v)</th>
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<th>pH 6.0</th>
<th>pH 5.5</th>
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<tr>
<td></td>
<td>UA&lt;sup&gt;c&lt;/sup&gt; (mM)</td>
<td>SM101 (FP)</td>
<td>F4969 (NFB)</td>
</tr>
<tr>
<td>0.1% PS</td>
<td>0.06</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>0.2% PS</td>
<td>0.12</td>
<td>65</td>
<td>ND</td>
</tr>
<tr>
<td>0.3% PS</td>
<td>0.18</td>
<td>75</td>
<td>74</td>
</tr>
<tr>
<td>0.05% SB</td>
<td>0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.1% SB</td>
<td>0.02</td>
<td>65</td>
<td>85</td>
</tr>
</tbody>
</table>

<sup>a</sup> spore were heat activated and inoculated into TGY medium supplemented with PS and SB at different pHs and inhibition was assessed after 3 h of incubation as described in Material and methods. Each value represents the average of duplicate experiments from two different spore preparations. All standard deviations of these experiments were less than 30% of the mean.

<sup>b</sup> inhibitors were PS, potassium sorbate, and SB, sodium benzoate.

<sup>c</sup> UA, “undissociated acid”. The amount of undissociated acid was calculated as described in Material and methods.

<sup>d</sup> ND, “Not determined”.
Fig. 2.1. Effects of PS and SB on *C. perfringens* spore outgrowth. Spores of *C. perfringens* FP strains SM101 (A) and NCTC10239 (B); and NFB strains F4969 (C) and NB16 (D) were heat-activated and inoculated into TGY broth containing PS or SB. Outgrowth of spores was measured as described in Material and methods. Symbols represent: filled diamonds, TGY only; open circles, TGY plus PS (0.3% ≈ 0.18 mM undissociated sorbic acid); open triangles, TGY plus SB (0.1% ≈ 0.02 mM undissociated benzoic acid); and Open diamonds, TGY plus SB (0.3% ≈ 0.05 mM undissociated acid). Error bars represent the standard deviation of the mean.
Fig. 2.2. Effect of PS and SB on vegetative growth of C. perfringens. Vegetatively growing (3-h TGY grown culture) cells of FP strain SM101 (A and B) and NFB F4969 (C and D) were inoculated into TGY medium containing various concentrations of PS (A and C) or SB (B and D) and growth was monitored by measuring OD$_{600}$ at hourly intervals. Symbols represent: filled diamonds, no PS or SB; filled squares, 0.1 % PS ($\approx$ 0.06 mM undissociated sorbic acid) or 0.1 % SB ($\approx$ 0.02 mM undissociated benzoic acid); filled triangles, 0.3% PS ($\approx$ 0.18 mM undissociated sorbic acid) or 0.3% SB ($\approx$ 0.05 mM undissociated benzoic acid); cross marks, 0.5% PS ($\approx$ 0.30 mM undissociated sorbic acid) or 0.5% SB ($\approx$ 0.09 mM undissociated benzoic acid); filled circles, 0.75% PS ($\approx$ 0.45 mM undissociated sorbic acid) or 0.75% SB ($\approx$ 0.13 mM undissociated benzoic acid); open squares, 1.0% PS ($\approx$ 0.6 mM undissociated sorbic acid) or 1.0% SB ($\approx$ 0.17 mM undissociated benzoic acid); and open circles, 1.5% PS ($\approx$ 0.9 mM undissociated sorbic acid) or 1.5% SB ($\approx$ 0.26 mM undissociated benzoic acid). Error bars represent the standard deviations from these experiments.
Fig. 2.3. Effects of PS and SB on viable counts of *C. perfringens* vegetative cells. Numbers of colony forming unit per ml (CFU/ml) of *C. perfringens* type A FP strain SM101 (A) and NFB strain F4969 (B) were determined before and after 6 h of exponential growth in TGY medium containing various concentrations of PS or SB. Bars represent: black bars, no PS or SB; gray bars, 0.3 % PS (≈ 0.18 mM undissociated sorbic acid); white bars, 0.1% SB (≈ 0.02 mM undissociated sorbic acid); and white bars with black diagonal strips, 0.3% PS plus 0.1% SB (≈ 0.2 mM undissociated benzoic acid). Error bars represent standard deviation from the mean.
Fig. 2.4. Cell morphology of *C. perfringens* vegetative cells grown in TGY medium containing PS or SB. Phase-contrast photomicrographs of cells of SM101 grown for 6 h in TGY medium alone (A) and TGY containing 0.3% PS (≈ 0.18 mM undissociated sorbic acid) (B) or 0.1% SB (≈ 0.02 mM undissociated benzoic acid) (C). Representative fields were photographed at 1000x magnification.
Fig. 2.5. Effects of PS and SB on vegetative growth of a collection of enterotoxigenic *C. perfringens* type A FP (A-C) and NFB (D-F) isolates. Vegetatively growing cells (3-h TGY grown culture) of strains SM101 (A); E13 (B); FD1041 (C); F4969 (D); NB16 (E); and B40 (F) were inoculated into TGY medium supplemented with various concentrations of PS or SB. Bars represent: black bars, no PS or SB; gray bars, 0.3% PS (≈ 0.18 mM undissociated sorbic acid); white bars, 0.1% SB (≈ 0.02 mM undissociated benzoic acid) and white bars with black diagonal stripes, 0.3% PS plus 0.1% SB (≈ 0.2 mM undissociated acid). Growth was monitored by measuring OD$_{600}$ at 6 and 24 h post-inoculation. Error bars represent standard deviation from the mean.
Fig. 2.6. Effects of PS and SB on growth of *C. perfringens* spores in cooked meat products. Spores of 3-strain cocktail of *C. perfringens* FP (A) or NFB (B) isolates were inoculated into cooked chicken samples containing various concentrations of PS or SB as indicated and CFU formed by survival spores were determined by plating onto BHI agar and incubated anaerobically at 37°C for 24 h. Black bars, initial CFU and white bars, viable CFU in cooked chicken samples after 6 h of anaerobic incubation at 37 °C. The amount of undissociated acid in 1% PS, 5% PS, 1% SB, and 5% SB were 3.62, 18.11, 1.09, and 5.44 mM, respectively. Error bars represent standard deviation from the mean.
Chapter 3

Chitosan inhibits enterotoxigenic Clostridium perfringens type A in growth medium and chicken meat

Maryam Alnoman, Pathima Udompijitkul, and Mahfuzur R. Sarker

Submitted to:
Food Microbiology
Abstract

_Clostridium perfringens_ is a spore-forming bacterium and a major cause of bacterial food-borne illness. Alternatives to common treatment processes used in the food industries are needed to reduce the number of spore contamination and thereby reducing the potential of foodborne diseases. In this study, we evaluated the inhibitory effects of chitosan against _C. perfringens_ type A food poisoning isolates. All tested chitosans inhibited germination of spores of all tested FP isolates in germinant solution containing 0.1 mg/ml chitosan at pH 4.5. However, higher level (0.25 mg/ml) of chitosan was required to effectively arrest outgrowth of the germinated _C. perfringens_ spores in rich medium. Furthermore, chitosan (1.0 mg/ml) showed a bacteriostatic activity against vegetative cells of _C. perfringens_ in rich medium. Although chitosan showed strong inhibitory activities against _C. perfringens_ in laboratory medium, higher levels were required to achieve the similar inhibition upon _C. perfringens_ spores inoculated into chicken meat. In summary, the inhibitory effects of chitosan against spore germination, outgrowth, and vegetative growth was concentration dependent, and no major difference in effects of different molecule weight chitosan was observed. Our results contribute to a better understanding on the potential application of chitosan in cooked meat products to control _C. perfringens_ contamination.
3.1. Introduction

*Clostridium perfringens* is a Gram-positive, anaerobic, spore-forming bacterium causing histotoxic and gastrointestinal (GI) diseases in humans and animals (McClane, 2007). *C. perfringens* type A producing *C. perfringens* enterotoxin (CPE) is the causative agent of *C. perfringens* type A food poisoning (FP), which currently ranks as the 2nd most commonly reported bacterial food-borne outbreaks in the United States affecting nearly 1 million cases annually and results in economic loss of over $309 millions (Hoffmann et al., 2012; Lynch et al., 2006b; Scallan et al., 2011b). This pathogenic bacterium remains concern to food industry due to its ability to produce metabolically dormant spores that are highly resistant to various stresses related to food preservation approaches, such as moist heat, extreme pH, osmotic, nitrite, prolong low temperature storage, and high pressure processing (Li and McClane, 2006a, 2006b; Paredes-Sabja et al., 2007; Sarker et al., 2000). These resistance properties of spores of FP isolates facilitate their survival in processed meat and poultry; products most commonly implicated in *C. perfringens* FP outbreaks (McClane 2007). Once conditions are favorable, these heat-resistant spores can germinate and outgrow into vegetative cells reaching high viable cell numbers (~10^6 colony forming Unit (CFU)/g). After food is consumed, viable cells that survived stomach acidity will initiate sporulation in the GI tract, releasing CPE upon cell lysis and causing GI illnesses (McClane 2007).

Currently, the food industry is interested in developing bacterial spore-inactivation strategies alternative to conventional thermal processing technologies to meet the consumers’ demand for natural, minimally processed and organic products. Alternative technologies are applications of natural antimicrobial agents in foods in order
to maintain food safety, food quality, extended shelf life by controlling microbial spoilage and foodborne pathogen contamination. Chitosan is a natural carbohydrate polymer derived from the deacetylation of chitin, a main component of exoskeletons of crustaceans, insects, and cell wall of fungi. Different production methods are used to produce chitosan with different chemical properties, which potentially reflect variations in the antimicrobial activity (No and Meyers, 1995). Chitosan shows a broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, and fungi. Numerous factors could affect chitosan’s antibacterial activities; most significantly among others are chitosan’s molecular weight (Mw), degree of deacetylation (DD), physical state and pH (Kong et al., 2010). Most of the commercially available chitosan is shrimp-derived and is used as a food additive or preservative, and as an active packaging material (Friedman and Juneja, 2010b; Kong et al., 2010; Su et al., 2009). It has been approved as a food additive in Korea’s and Japan’s generally-recognized-as-safe (GRAS) list (Kong et al., 2010), and recently a GRAS notice inventory list showed that shrimp-derived chitosan is under FDA evaluation to be added to GRAS list in the United States (FDA, 2014). Intensive research has been focused on the application of different forms of chitosan including films, coats, and nanochitosans, in different food categories (Friedman and Juneja, 2010b; Su et al., 2009). However, information on chitosan’s effect on bacterial spores, especially C. perfringens spores and its possible application in poultry products is limited. Moreover, there are gaps on the fundamental knowledge of whether chitosan inhibits initiation of spore germination or prevents outgrowth of germinated spores, which are essential basis in developing an effective strategy to control C. perfringens in food products.
The objectives of this study were to examine the effects of chitosan against germination, outgrowth and vegetative growth of spores of the enterotoxigenic *C. perfringens* type A. Furthermore, the application of chitosan as an antimicrobial agent to control the germination and outgrowth of *C. perfringens* spores in cooked meat products during storage at extremely abusive temperature was also examined.

3.2. Material and methods

3.2.1. Bacterial strains and growth conditions

The *C. perfringens* isolates used in this study consist of three type A FP isolates (SM101, NCTC10239, and E13) (Sarker et al., 2000). All stock cultures were maintained in cooked meat medium (Difco, BD Diagnostic Systems, Sparks, MD, USA) and stored at -20 °C. The bacterial growth was revived by inoculating cooked meat culture into fluid thioglycollate (FTG) medium (Difco) and incubated overnight at 37 °C. TGY (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) broth was used for vegetative growth and spore outgrowth of *C. perfringens* strains as previously described (Paredes-Sabja et al., 2008d).

3.2.2. Spore preparation and purification

Spores of *C. perfringens* were prepared and purified as described previously (Akhtar et al., 2008; Paredes-Sabja et al., 2008c). Briefly, the stock cultures of *C. perfringens* were inoculated into 10 ml FTG and incubated overnight at 37 °C. Then, 0.4 ml of an overnight FTG culture was inoculated into a new 10 ml FTG medium and grew for 8 h at 37 °C. Next, 0.4 ml of 8-h FTG culture was inoculated into 10 ml Duncan-
Strong sporulation medium (Duncan and Strong, 1968) and sporulation capacity was confirmed under phase contrast microscope after incubation at 37 °C for 24 h. Large amounts of spores were prepared by scaling up the aforementioned procedure. Spores were purified by repeated washing and centrifuging with cold sterile distilled water until the spore suspensions were > 99% free of sporulating cells, cell debris, and germinated spores as determined by phase contrast microscope. Purified spores were suspended in sterile distilled water to obtain an optical density at 600 nm (OD\textsubscript{600}) of ~ 6, and stored at -80 °C until use.

### 3.2.3. Preparation of chitosan solutions

Three stock solutions of chitosan with different molecular weight (M\textsubscript{w}) including low (50-190 kDa), medium (190-310 kDa), and high (310-375 kDa) with 75-85% deacetylation were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Chitosan stock solutions were prepared with 1% (v/v) acetic acid at a concentration of 10 mg/ml according to the previously described protocol (Cruz-Romero et al., 2013; Li et al., 2010b). These solutions were then filter sterilized (0.45 μm, Millipore, Bedford, MA, USA) and stored at 4 °C. The stock solutions were used within a month of preparation. In order to obtain the desired concentrations, the stock solutions were diluted in a specific medium used for the corresponding experiments.

### 3.2.4. C. perfringens spore germination in the presence of chitosan

The purified spore suspensions in water at a final OD\textsubscript{600} ~ 1.0 were heat activated at 80 °C for 10 min prior to germination as previously described (Paredes-Sabja et al.,...
2008d; Paredes-Sabja et al., 2009d), and then cooled in water bath at room temperature for 5 min. To examine chitosan’s ability to induce C. perfringens spore germination, heat-activated spores were incubated with various concentrations (0.1, 0.5 or 1 mg/ml) of low-, medium-, or high-Mw chitosan (suspended in 1% acetic acid and adjusted to pH 6.0). To test the inhibitory effect of chitosan on spore germination, heat-activated spores were incubated with pre-warmed 100 mM potassium chloride (KCl) in 25 mM Tris-HCl (pH 6.0) alone or supplemented with different Mw chitosan (0.1 mg/ml). To test effect of pH on spore germination inhibition, the final pH of germinant solution with or without chitosan were adjusted to the desired pH ranging from 3.5 to 6.5. Spore germination was routinely monitored by measuring OD$_{600}$ of the spore-germinant solutions (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), in which OD$_{600}$ is decreased by ~ 60% upon complete spore germination (Paredes-Sabja et al., 2008d). The extents of spore germination were also confirmed by phase-contrast microscopy in which germinated spores lose refractility and become phase dark spores. Germination rates were calculated as percentage loss of OD$_{600}$ relative to the initial value after 60 min of incubation in KCl with or without chitosan. Results were expressed as percent inhibition of spore germination compared with the control germination (without chitosan) as described previously (Alnoman et al., 2015; Cortezzo et al., 2004). All germination experiments were performed in triplicates with three different spore preparations for each strain.
3.2.5. Dipicolinic acid (DPA) release in the presence of chitosan

The release of DPA during *C. perfringens* germination was performed as previously described (Paredes-Sabja et al., 2008d; Udompijitkul et al., 2014). Briefly, spores of SM101 (OD$_{600}$ of 1.5) were heat activated at 80 °C for 10 min, and then incubated with low Mw (LMW) chitosan (0.1 mg/ml), 100 mM KCl or combination of 100 mM KCl and 0.1 mg/ml LMW chitosan at 37 °C at two different pHs 6.0 and 4.5. The 1 ml aliquots of germinating solutions were taken at various time periods and centrifuged for 3 min at 13,200 rpm in microcentrifuge tube, and DPA in the supernatant fluid was determined by measuring absorbance at 270 nm ($A_{270}$). The total spore DPA content was evaluated by boiling 1 ml aliquot of germinating spores for 60 min, centrifuging for 5 min in a microcentrifuge, and measuring the $A_{270}$ of the supernatant fluid. Our previous study indicated that ~90% of the material absorbing at 270 nm contained DPA in *C. perfringens* (Paredes-Sabja et al., 2008d).

3.2.6. *C. perfringens* spore outgrowth in the presence of chitosan

A 0.3 ml spore suspensions of an OD$_{600}$ of ~ 6.0 were heat activated at 80 °C for 10 min, and then cooled in a water bath at room temperature before inoculating into the pre-warmed 10 ml TGY broth (pH 6.0) alone or supplemented with different Mw chitosans at different concentrations (0.05, 0.1 and 0.25 mg/ml). The final pH of the medium containing highest concentration of chitosan changed by 0.2 unit. Cultures were incubated at 37 °C and the OD$_{600}$ was measured over time interval for up to 180 min post-inoculation. Spore outgrowth rates were calculated as percentage of increase in OD$_{600}$, and the inhibition of spore outgrowth was calculated as % inhibition = (OD$_{600}$ increase in
The inhibitory effects of chitosan on growth of *C. perfringens* were determined in conditions supporting vegetative growth in TGY broth. A 0.2 ml of an overnight FTG culture was transferred into 10 ml TGY and grown for 3 h at 37 °C. Then, 0.2 ml of 3-h TGY culture was inoculated into fresh 10 ml TGY (pH 6.0) supplemented with various concentrations of chitosan ranging from 0.5 to 2.0 (mg/ml). The final pH of the medium containing the highest concentration of chitosan tested changed from 6.0 to 5.0. A control sample without chitosan was included to compare the effects of chitosan. The growth was measured by enumerating colony forming unit (CFU)/ml of each treatment at 0, 3, 6 and 24 h post-inoculation. For CFU/ml counting, aliquots of diluted cultures were plated onto Brain Heart Infusion (BHI) (Difco, BD Diagnostic Systems) agar plates, incubated anaerobically at 37 °C for 18 h, and colony counted. All growth experiments were performed independently at least in triplicate and values are given as means.

3.2.8. *Growth of C. perfringens spores in cooked chicken meat in the presence of chitosan*

The ability of chitosan to prevent germination and outgrowth of *C. perfringens* spores in meat during storage at abusive conditions was measured as previously described (Alnoman et al., 2015; Udompijitkul et al., 2012). Briefly, grounded chicken meat samples (10g/bag) were placed into the UV-sterilized plastic bag (5.5” W × 6”L) and
then sealed, autoclaved and stored at -20 °C until use. Prior to each experiment, bags containing meat sample were thawed and appropriate amounts (0, 0.5, 1.0, 2.0, 3.0 (mg/g)) of LMW chitosan stock solution were added. Then 0.1 ml of 3-strain spore cocktails were mixed by manually massaging for 1 min after resealing bags. The 3-strain spore cocktails of FP (SM101, NCTC10239, and FD1041) isolates were prepared by combining approximately equal number of spores from each strain containing ~10⁸ spores per ml. Negative control was included in every replication to ensure that meat samples were free of naturally occurring bacteria. Samples were kept on ice before cooking at 80 °C for 15 min and cooled in water bath at room temperature for 15 min. For each treatment, one bag content was used to determine the initial population of \textit{C. perfringens} in the contaminated meat, and another two samples were transferred to sterile petri dish and stored under anaerobic condition at 37 °C for 6 and 18 h. pHs of chicken samples without chitosan and with highest tested concentration of chitosan (3 mg/ml) were 6.0 and 5.5, respectively. To determine populations of \textit{C. perfringens} in the meat, each sample (10 g meat) was transferred into a stomacher bag, mixed with 90 ml 0.1% (w/v) peptone water, serially diluted, and then plated onto BHI agar plates that were incubated anaerobically at 37 °C for 18 h before counting the colonies. Results were expressed as CFU/g.

\textbf{3.2.9. Statistical analysis}

Data were analyzed by the analysis of variance procedure (ANOVA) using the statistical software SAS version 9.3 (SAS Inst. Inc., Cary, N.C., USA). ANOVA among treatments was performed and comparisons of mean values were established by Duncan’s
New Multiple Range Test at the significant level of 0.05. In all figures, error bars represent the standard deviations.
3.3. Results and discussion

3.3.1. Chitosan do not induce germination of spores of *C. perfringens*

When *C. perfringens* SM101 spores were incubated with 0.1 mg/ml of low-, medium-, or high-Mw chitosan (suspended in 1% acetic acid and adjusted to pH 6.0) for 60 min at 37 °C, no change in OD<sub>600</sub> decrease, i.e., no germination was observed (Fig. 1). Phase contrast microscopy indicated that > 90 % spores are phase bright (data not shown). However, under similar experimental condition, SM101 spores germinated slightly with higher concentrations (0.5 and 1.0 mg/ml) of low-, medium-, or high-Mw chitosan. Phase-contrast microscopy confirmed that only ~ < 20% spores became phase bright with higher concentrations of all tested Mw chitosans (data not shown). These data indicate that chitosan, irrespective of Mw, do not induce germination of spores of FP strain SM101 at pH 6.0.

3.3.2. Chitosan inhibits *C. perfringens* spore germination

As expected from our previous studies (Paredes-Sabja et al., 2008d), when SM101 spores were incubated with 100 mM KCl at pH 6.0, ~50% OD<sub>600</sub> decrease was observed (Fig. 2A), indicating significant germination. However, similar level of OD<sub>600</sub> decrease was observed with SM101 spores incubated with germinant solution of 100 mM KCl plus 0.1 mg/ml chitosan (low-, medium-, or high-Mw) at final pH of 6.0, indicating no inhibition of spore germination (Fig. 2A). This is as consistent with a previous study (Mellegard et al., 2011a) where no inhibition of *B. cereus* spore germination was observed with 0.5 mg/ml chitosan-HCl at pH 6.0.
As chitosan’s activity is pH dependent (Kong et al., 2010), we examined the inhibitory effects of LMW chitosan (0.1 mg/ml) at different pHs ranging from 3.0 to 6.5. We found that inhibition of KCl-induced germination of SM101 spores was only achieved at pHs 4.5 (Fig. 2B and data not shown). Acidic pH alone was not the sole inhibitor for spore germination, as SM101 spores successfully germinated with KCl pH 4.5 to 6.5 (Fig. 2B and data not shown). Moreover, the presence of acetic acid alone (at the same concentrations used to dissolve chitosan) exhibited inhibitory effect on KCl-induced spore germination at pH 4.5; nevertheless, the extent of germination inhibition was significantly less than those observed with chitosan incorporation, regardless of their Mw. No significant difference (p > 0.05) on spore germination inhibition was observed with varying chitosan’s Mw (Fig. 2B).

When we extended germination inhibition experiments on three different FP strains including SM101 with 0.1 mg/ml of different Mw chitosan, 89-99% inhibition of C. perfringens spore germination was observed with spores of all tested strains (Table 1). Further decreasing the concentration to 0.05 mg/ml could inhibit spore germination up to 74-96% depending on strains and chitosan’s Mw (Table 1). Collectively, these data indicate that chitosan and acetic acid should have synergistic effect on inhibition of KCl-induced germination of spores of C. perfringens FP isolates at pH 4.5 regardless of Mw of chitosans being assayed. Our results are consistent with an earlier study that reported a stronger antimicrobial activity of chitosan at pH 5.5 than at pH 6.5 against five species of foodborne pathogens including: Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica, Listeria monocytogenes, and Salmonella Typhimurium (Wang, 1992).
3.3.3. Chitosan inhibits DPA release from spore core

To further validate the germination-inhibition capacity of chitosan, another measurable event (spore core’s DPA release) in the spore germination was monitored. Spores of various species in the genera *Bacillus* and *Clostridium* generally release DPA from the spore core during an early step of spore germination (Olguin-Araneda et al., 2015; Setlow, 2014a). Therefore, we also examined the effects of chitosan on DPA release during KCl-induced germination of SM101 spores at pH 4.5 or 6.0. As expected, when SM101 spores incubated with 100 mM KCl at 37°C for 60 min, most (~80-85%) of the spore core’s DPA was released at pH 4.5 or 6.0 (Fig. 3), indicating complete spore germination. However, approximately 20 and 40% DPA was released when SM101 spores were incubated with chitosan (0.1 mg/ml LMW) at pH 4.5 and 6.0, respectively, indicating no significant germination as previously observed with OD$_{600}$ decrease (Fig. 1 and 3). However, while ~80% DPA-release was observed upon incubation of SM101 spores with 100 mM KCl supplemented with chitosan (0.1 mg/ml LMW) for 60 min at pH 6.0, DPA release decreased to ~ 45% when similar germination experiment was performed at pH 4.5 (Fig. 3). Collectively, these results indicate that chitosan (0.1 mg/ml LMW) can inhibit KCl-induced DPA release from SM101 spore core at pH 4.5 which further supports that chitosan can inhibit KCl-induced germination of *C. perfringens* spores at pH 4.5. Our finding that *C. perfringens* SM101 spores released most DPA upon incubation with 100 mM KCl plus 0.1 mg/ml chitosan-acetate at pH 6.0 is consistent with *B. cereus* spores’ DPA release when incubated with 50 mM L-alanine plus 0.5 mg/ml chitosan-HCl at pH 6.0 (Mellegard et al., 2011a).
3.3.4. Chitosan inhibits C. perfringens spore outgrowth

Previous study (Mellegard et al., 2011a) reported that chitosan (≤ 0.5 mg/ml) was able to inhibit B. cereus spore outgrowth when suspended in growth medium (pH 6.0) containing 25 mM L-alanine and 2.5 mM inosine as a germinant. Therefore, the inhibitory effect of chitosan against C. perfringens spore outgrowth was assessed after inoculation of C. perfringens spores into rich growth medium. SM101 spores were initiated outgrowth after incubation in rich TGY medium (pH 6.0) for ~ 60 min and 37°C (Fig. 4). However, the incorporation of 0.25 mg/ml LMW-, MMW, or HMW-chitosan into TGY significantly delayed SM101 spore outgrowth until ~120 min, and significant inhibition of spore outgrowth as compared to control was observed after 180 min of incubation at 37°C (Fig. 4 and Table 2). Moreover, lower concentrations (0.05 and 0.1 mg/ml) of chitosan resulted in a slight inhibition of outgrowth rate as compared to control. Unlike the effect of chitosan concentration, no significant effect of chitosan’s Mw was observed (p > 0.05) (Fig. 4 and Table 2). When similar outgrowth experiments were performed using spores of two additional FP strains (NCTC10239 and E13), slightly less % inhibition of spore outgrowth compared to SM101 was observed with all tested chitosans (Table 2), suggesting that chitosan-induced spore outgrowth inhibition is strain specific. The observed lower extent of chitosan-induced inhibition of spore outgrowth compared to that of germination inhibition, especially in the case of HMW chitosan (Table 1 and 2) might be due to the lower susceptibility of the outgrowing spores to chitosan than germinating spores. Another possible explanation for this phenomenon is that chitosan might lose some antimicrobial activity due to competitive interaction of existing medium cations with negatively charged bacterial cell wall (Kong et al., 2010).
In sum, our data demonstrate that chitosan-induced inhibition of spore outgrowth depends on concentrations, but not Mw of chitosan (Table 2). However, previous studies with *B. cereus* spores suggested that chitosan’s effect is dependent on fraction of acetylation and Mw (Mellegard et al., 2011a). This discrepancy correlates with the previous study by Eaton et al. (2008), which suggested the strong dependency of chitosan’s activity on target microorganisms and its Mw (Eaton et al., 2008).

### 3.3.5. Chitosan inhibits the growth of C. perfringens vegetative cells

As our results showed that higher concentration of chitosan was required to inhibit spore outgrowth in TGY rich medium, we examined the vegetative growth of *C. perfringens* FP strain SM101 in TGY medium supplemented with various concentrations of chitosan. Results demonstrated that the incorporation of 0.5 mg/ml of chitosan to TGY gave log CFU/ml similar to that of control samples during the entire period of sampling (Fig. 5). However, increasing chitosan level to 1 mg/ml in TGY almost arrested the vegetative growth, as only a very slight elevation on log CFU/ml was obtained after 24 h incubation. Interestingly, more higher levels of chitosan (1.5 and 2.0 mg/ml) reduced the 3-h culture populations by ~2 log CFU/ml and their effects lasted up to 6 h of incubation. In contrast, longer incubation for up to 24 h resulted in 0 and 3 log CFU/ml reduction from initial counts in presence of 1.5 and 2.0 mg/ml LMW chitosan, respectively (Fig. 5). The vegetative cell growth resumption of *C. perfringens* SM101 upon longer incubation in TGY supplemented with chitosan suggest the bacteriostatic effect of LMW chitosan at lower concentrations on *C. perfringens* cells. Phase-contract microscopy revealed an
aggregation of long filamentous cells in SM101 cultures grown in TGY supplemented with 0.5 mg/ml LMW chitosan (Fig. 6). Furthermore, severe aggregation and clumping of SM101 cells was observed when grown in TGY plus 1.0 mg/ml chitosan (data not shown). These cell deformation characteristics could explain no elevation of cell numbers in TGY supplemented with higher concentrations of chitosan because of blockage of cell division. Collectively, vegetative growth of *C. perfringens* was suppressed by LMW chitosan in a concentration-dependent manner; with 1.0 mg/ml LMW chitosan acts as a bacteriostatic agent and 2.0 mg/ml as a bactericidal agent. The MMW and HMW chitosan showed inhibition of *C. perfringens* vegetative growth similarly as LMW chitosan (data not shown). Similar results were found with two additional FP strains, E13 and NCTC10239 (data not shown).

This study revealed that the higher concentrations of chitosan was required to inhibit *C. perfringens* vegetative cell growth relative to spore outgrowth which is resemble the inhibitory effects of chitosan on *B. cereus* spore outgrowth and vegetative cells (Tsai et al., 2006). Similar to our findings, previous study (No et al., 2002) reported the minimum inhibitory concentration (MIC) of 28 kDa chitosan to be >1 mg/ml against *B. cereus* and *E. coli*. However, a noticeably lower MIC values of 0.1 and 0.003 mg/ml to control *B. cereus* and *E. coli*, respectively, were also reported (Mellegard et al., 2011b). These variations on MICs might presumably be linked to different characteristics of chitosan preparations used in each study (Mellegard et al., 2011b).

The precise mechanism of inhibition of chitosan towards *Clostridium* spp. has not been established. However, chitosan was reported to cause damage to the cell membrane
of Gram-positive bacteria (Vårum and Smidsrød, 2005). In vegetative cells of *B. cereus* ATCC 14579, chitosan (Mw 28.4 kDa and degree of acetylation 0.16) induced a significant efflux of K⁺ indicating an increase in cell membrane permeability and membrane damage (Mellegard et al., 2011b).

### 3.3.6. Chitosan blocks *C. perfringens* growth in cooked chicken meat products

To examine the inhibitory effects of chitosan on *C. perfringens* spore growth in cooked chicken meat during storage at abusive conditions, we employed our previously described meat model system (Akhtar et al., 2008; Alnoman et al., 2015; Udompijitkul et al., 2012). Our data (Fig. 7) demonstrated an increase of ~3 log CFU/ml viable cell count in *C. perfringens* spore-contaminated cooked chicken meat incubated anaerobically for 18 h at 37 °C. However, in the presence of chitosan in cooked chicken meat at a concentration of 1 mg/g resulted in 1 log CFU/ml decrease in *C. perfringens* viable cell count (Fig. 7). Moreover, increasing the amount of chitosan in cooked chicken meat to ≥ 2 mg/g resulted in ~ 3 log CFU/ml reduction in *C. perfringens* viable cell count compared to the control sample after 18 h storage under anaerobic condition at 37 °C (Fig. 7). Collectively, these data illustrate the inhibitory effect of chitosan against the growth of contaminating *C. perfringens* FP spores in meat samples under abusive storage conditions.

Previous study reported 3% (30 mg/g) of chitosan (degree of acetylation = 0.14) mixed into ground beef or turkey resulted in 4 to 5 log reductions of *C. perfringens* spore germination and outgrowth during exponential cooling of cooked beef or turkey up to 18 h (Juneja et al., 2006a). However, in the current study, with 10-fold lower concentration
of chitosan (3 mg/g) we obtained 3 log CFU/ml reductions in *C. perfringens* spore growth in cooked chicken meat under extremely abusive storage condition. One possible reason for this discrepancy might be the physical state and chemical characteristics of chitosan, as we used LMW chitosan solution dissolved in acetic acid; however, Juneja et al. (Juneja et al., 2006a) used chitosan-glutamate applied as a solid state into meat products. Another possible reason might be food matrices; we used fat-free ground chicken, while Juneja et al. (Juneja et al., 2006a) used ground beef (25% fat) and turkey (7% fat).

### 3.4. Conclusions:

The present study demonstrated, for the first time, the inhibitory effects of chitosan against *C. perfringens* FP isolates in a variety of aspects including spore germination, outgrowth, and vegetative growth in laboratory medium and chicken meat model. At pH 6.0, all tested chitosans affected the outgrowth, but not the initiation of germination, of *C. perfringens* spores. However, significant inhibitions of spore germination and DPA release were observed with LMW-, MMW- or HMW-chitosan at pH 4.5. Since results obtained in this study revealed no significant differences in the inhibitory properties of three different Mw chitosans against *C. perfringens*, the use of LMW chitosan, which also exhibited better solubility, was encouraged (Chang et al., 2015; Friedman and Juneja, 2010b; Tsai et al., 2006). We also tended to use LMW chitosan especially where higher concentrations were tested to avoid the high viscosity of chitosan solution. The LMW chitosan, albeit at higher concentration, showed the bacteriostatic and bactericidal activity against vegetative cells of the enterotoxigenic *C.*
Despite the fact that the previous study showed the effectiveness of chitosan in controlling *C. perfringens* in beef and turkey, we were able to control growth of *C. perfringens* spores inoculated into cooked chicken meat stored in improper condition for an extended period by using relatively lower concentration of LMW chitosan. It is noteworthy that our meat challenge study was performed with relatively high spore inoculum, and cooked meat being stored at extreme abusive conditions in order to simulate the worst-case scenario promoting the rapid growth from spores. Even under these extreme conditions, the tested chitosan was still quite effective in controlling spore germination and outgrowth in the cooked chicken meat despite lacking the sporicidal effect. Therefore, an alternative use of chitosan as a food preservative to control growth of *C. perfringens* spores in cooked meat products should be considered. However, further studies with different types of meats as well as under practical conditions typically applied in food industries are necessary to establish the effective use of chitosan for the safety of food products.

**Acknowledgements**

This work was supported by a grant from the Agricultural Research Foundation of Oregon State University, and by a Department of Defense Multidisciplinary University Research Initiative (MURI) award through the U.S. Army Research Laboratory and the U. S. Army Research Office under contract number W911NF-09-1-0286 (all to MRS); MA was supported by a fellowship from Taibah University (Saudi Arabia).
References


Tsai, G.J., Tsai, M.T., Lee, J.M., Zhong, M.Z., 2006. Effects of chitosan and a low-molecular-weight chitosan on *Bacillus cereus* and application in the preservation of cooked rice. J. Food Prot. 69, 2168-2175.


Table 3.1: Inhibition of *C. perfringens* spore germination by different Mw chitosan at pH 4.5\(^a\)

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Inhibition of germination (%)(^b)</th>
<th>SM101</th>
<th>NCTC10239</th>
<th>E13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LMW(^c)</td>
<td>MMW(^c)</td>
<td>HMW(^c)</td>
<td>LMW</td>
</tr>
<tr>
<td>0.01</td>
<td>8</td>
<td>16</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
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<td>43</td>
<td>50</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>0.05</td>
<td>74</td>
<td>79</td>
<td>81</td>
<td>83</td>
</tr>
<tr>
<td>0.1</td>
<td>89</td>
<td>89</td>
<td>92</td>
<td>94</td>
</tr>
</tbody>
</table>

\(^a\) Spores were germinated with 100 mM KCl solution containing chitosan at various concentrations as indicated and percentage of inhibition of germination was calculated as described in Material and methods.

\(^b\) Values are the average of triplicate experiments from three different spore preparation. The standard deviations from these experiments were less than 10% of the mean.

\(^c\) LMW, MMW, HMW indicates low-, medium- or high-Mw chitosan, respectively.
Table 3.2: Inhibition of *C. perfringens* spore outgrowth by different Mw chitosan at pH 6.0\(^a\)

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Inhibition of outgrowth (%)</th>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SM101</td>
<td>NCTC10239</td>
<td>E13</td>
<td>LMW(^c)</td>
<td>MMW(^c)</td>
<td>HMW(^c)</td>
</tr>
<tr>
<td>0.05</td>
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<td>82</td>
<td>76</td>
<td>69</td>
<td>65</td>
<td>66</td>
<td>62</td>
</tr>
</tbody>
</table>

\(^a\) Heat-activated spores were inoculated into TGY medium supplemented with different Mw chitosan at various concentrations as indicated. Spore outgrowth was assessed after 3 h of incubation at 37°C and inhibition of outgrowth was calculated as described in Material and methods.

\(^b\) values are the average of triplicate experiments from three different spore preparation. The standard deviations from these experiments were less than 19 % of the mean.

\(^c\) LMW, MMW, HMW indicates low-, medium- or high-Mw chitosan, respectively.
Fig. 3.1. Germination of *C. perfringens* spores with chitosan. Spores of *C. perfringens* FP strain SM101 were heat activated and incubated with different Mw chitosans at concentrations of 0.1 mg/ml (black bars), 0.5 mg/ml (white bars), or 1 mg/ml (gray bars) at pH 6.0. Spore germination was measured as described in Material and methods. Error bars represent standard deviation from the mean of triplicate experiments with three independent spore preparations. LMW MMW, HMW indicates low-, medium- or high-Mw chitosan, respectively.
Fig. 3.2. Inhibitory effects of chitosan on *C. perfringens* spore germination. Heat-activated spores of *C. perfringens* FP strain SM101 were incubated with 100 mM KCl alone or 100 mM KCl supplemented with 0.01% acetic acid (chitosan’s solvent) or different Mw chitosan (0.1 mg/ml) at pH 6.0 (A) or pH 4.5 (B). Spore germination was measured as described in Material and methods. Error bars represent standard deviation from the mean of triplicate experiments with three independent spore preparations. LMW, MMW, HMW indicates low-, medium- or high-Mw chitosan, respectively.
Fig. 3.3. Effects of chitosan on DPA release during KCl-induced germination of *C. perfringens* spores. Heat-activated SM101 spores were germinated with 100 mM KCl (filled diamonds), 0.1 mg/ml LMW chitosan (filled triangles) or combination of both (filled squares) at pH 6.0 (A) or 4.5 (B). At various times as indicated, DPA release was measured as described in Material and methods. Error bars represent standard deviation from the mean of triplicate experiments with three independent spore preparations.
Fig. 3.4. Effects of chitosan on *C. perfringens* spore outgrowth. Spores of *C. perfringens* FP strain SM101 were heat activated and inoculated into TGY broth supplemented with LMW (A)-, MMW (B)- or HMW (C)-chitosan. Outgrowth of spores was measured as described in Material and methods. Symbols represent concentrations: diamonds, 0 mg/ml; circles, 0.05 mg/ml; triangles, 0.1 mg/ml; and cross, 0.25 mg/ml. Each data point represents an average of triplicate experiments with three independent spore preparations.
Fig. 3.5. Inhibitory effects of chitosan on *C. perfringens* vegetative cell growth. *C. perfringens* SM101 cells were inoculated into TGY (control) or TGY supplemented with various concentration of LMW chitosan as indicated and incubated at 37°C. Samples were collected after 0 h (black bars), 3 h (white bars), 6 h (gray bars), and 24 h (white bars with black diagonal stripes) incubation, serially diluted and plated onto BHI agar, and colony forming unit per ml (CFU/ml) determined. Error bars represent standard deviation from the mean of triplicate experiments performed independently.
Fig. 3.6. Cell morphology of *C. perfringens* SM101 grown in TGY medium supplemented with LMW chitosan. Phase-contrast photomicrographs of cells of SM101 grown for 24 h at 37°C in TGY medium alone (A) or TGY supplemented with 0.5 mg/ml LMW chitosan (B). Representative fields were photographed at 1000 magnification.
Fig. 3.7. Inhibitory effects of chitosan on growth of *C. perfringens* spores in cooked chicken meat products. Spores of 3-strain cocktail of *C. perfringens* FP isolates were inoculated into cooked chicken meat samples containing various concentrations of LMW chitosan as indicated and CFU formed by survival spores were determined by plating onto BHI agar and incubated anaerobically at 37°C for 24 h. Black bars, initial CFU, white bars, viable CFU in cooked chicken samples after 6 h, and gray bars, after 18 h of anaerobic incubation at 37 °C. Error bars represent standard deviation from the mean of two independent experiments with two different spore preparations.
Chapter 4

Bicarbonate and amino acids are cogerminants for spores of *Clostridium perfringens* type A non-foodborne isolates.

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Abstract

_Clostridium perfringens_ type A are the causative agents of _C. perfringens_ type A food poisoning (FP) and non-food borne (NFB) gastrointestinal (GI) diseases. Both FP and NFB isolates can form metabolically dormant spores, which through germination process can return to actively growing cells to cause diseases. Germinant requirement is vastly diverse between spores of FP and NFB isolates, which in turn might reflect the diversity of their environmental habitats. In this study, we identified 12 individual amino acids (aa) that triggered germination of spore of NFB isolates in the presence of bicarbonate buffer, unlike FP SM101 spores needed potassium ions to germinate with these amino acids. Surveying germination of spores of NFB isolates lacking one of the germinant receptor proteins with the newly identified aa germinants revealed that GerKC and GerAA play major roles in bicarbonate-amino acid germination. Furthermore, variation on germination requirement for _C. perfringens_ FP and NFB isolates is correlated with their environmental niches, where K⁺ element abundant in food environment is required for FP spores to germinated and bicarbonate an specific intestinal element is essential for NFB spores.
4.1. Introduction

*Clostridium perfringens* is a Gram-positive, anaerobic, rod-shaped, endospore-forming bacteria that can cause a variety of human and veterinary diseases due to its prolific toxin-producing capability (McClane, 2007). It can be classified into five toxinotypes (A to E) based on the production of four major toxins (α, β, ε, and ι toxins) (McClane, 2007). Another important toxin produced by *C. perfringens* type A is *C. perfringens* enterotoxin (CPE), which is the major virulence factor responsible for *C. perfringens* type A food poisoning (FP) and non-food borne (NFB) gastrointestinal (GI) diseases such as antibiotic-associated diarrhea and sporadic diarrhea (Abrahao et al., 2001; Asha and Wilcox, 2002; Borriello et al., 1984; Kobayashi et al., 2008; Lindstrom et al., 2011; Mpamugo et al., 1995). Even though only less than 5% of the global population of *C. perfringens* type A can produce CPE, *C. perfringens* type A FP still currently ranks as the third most commonly reported foodborne disease outbreaks in the United States with estimated annual cost of $309.4 millions (Buzby and Roberts, 1997; Hoffmann et al., 2012; Lynch et al., 2006a; McClane, 2007; Scallan et al., 2011b; Xiao et al., 2012). Interestingly, CPE-encoding gene (*cpe*) can be located either on the chromosome or on a large plasmid, and chromosomal *cpe* isolates are generally linked to FP, while the plasmid-borne *cpe* isolates are associated with NFB GI diseases (Collie et al., 1998; Cornillot et al., 1995; Sarker et al., 2000). Nevertheless, recent findings suggested that plasmid-borne *cpe* isolates also can be a causative agent for *C. perfringens* type A FP (Lahti et al., 2008; Tanaka et al., 2003).

In addition to toxin production, *C. perfringeans* can form metabolically dormant spores that are resistant to environmental stress conditions. Once conditions are favorable
for growth, these dormant spores can return to actively growing cells through the process of germination (Paredes-Sabja et al., 2011). Spore resistance properties and germination capability play critical roles in the onset of *C. perfringens*-associated diseases (Novak and Juneja, 2002). Spore germination initiated when germination receptors (GRs) recognize the specific nutrient, termed germinant. Germinant requirement is vastly diverse among species of spore-forming bacteria, which in turn might reflect the diversity of their environmental habitats. In case of *C. perfringens*, KCl, L-asparagine, and a mixture of L-asparagine and KCl (AK), all at pH 7.0, and L-glutamine, and sodium phosphate (NaPi) at pH 6.0, can induce germination of spores of various FP isolates; whereas, AK at pH 7.0, and L-cysteine, L-serine, L-threonine, and L-asparagine all at pH 6.0 are universal germinants for spores of both FP and NFB isolates (Paredes-Sabja et al., 2008d; Paredes-Sabja et al., 2009d; Udompijitkul et al., 2014). The binding of a germinant to its cognate GR(s) results in ions and Ca-DPA release from spore core, which followed by partial core hydration. To complete spore germination, hydrolysis of peptidoglycan cortex occur by cortex lytic enzymes (CLEs), which are activated by germination specific serine proteases (Csps) (Olguin-Araneda et al., 2015; Setlow, 2014a). Genetic studies in *C. perfringens* strain SM101 identified four GRs encoded by a monocistronic *gerAA*, and *gerK* locus containing a bicistronic *gerKA-KC* operon, and an upstream oppositely-oriented *gerKB* (Myers et al., 2006; Paredes-Sabja et al., 2008d). Previous studies (Paredes-Sabja et al., 2008d; Paredes-Sabja et al., 2009b) demonstrated that GerKA and/or GerKC are the main GRs, while our further study recently showed that GerKC is indeed the most important GR protein involved in *C. perfringens* spore germination with KCl, L-asparagine, AK, L-cysteine, L-glutamine, NaPi, and the
nonnutrient germinants, Ca-DPA and dodecylamine (Banawas et al., 2013a; Udompijitkul et al., 2014).

Carbon dioxide (CO$_2$) has been known to influence microbial growth for bacteria and fungi. CO$_2$ as gas or as sodium bicarbonate inhibits spore germination of bacilli and fungi; however; it promotes spore germination of clostridia (Durrell, 1924; Enfors and Molin, 1978; Hachisuka et al., 1956; Hambleton and Rigby, 1970; Wynne and Foster, 1948). Differences in Clostridium spore germination requirement for CO$_2$ or bicarbonate have been documented. Previous studies showed that sodium bicarbonate increases the rate and extent of germination of spores of non-proteolytic Clostridium botulinum types B, E, and F (Plowman and Peck, 2002) as well as proteolytic C. botulinum types A, B, and F (Alberto et al., 2003). However, a combination of bicarbonate, lactate and amino acid (alanine / cysteine) could induce optimum germination of spores of proteolytic C. botulinum types E and G (Ando and Iida, 1970; Ando, 1971; Takeshi et al., 1988). Moreover, bicarbonate combined with three structurally different groups of amino acids: small amino acid (L-alanine or glycine), a basic amino acid, and an aromatic amino acid, could induce maximum germination of Clostridium sordellii spores (Ramirez and Abel-Santos, 2009). Kato et al., (2009) analyzed the role of CO$_2$ in C. perfringens spore germination by using bicarbonate or weakly acidic pH (6.0-6.5). They noted that spores overproducing germination-specific proteases (GSPs) could germinate in presence of one of three germinants: bicarbonate, weakly acidic pH, or nutrient. However, wild-type spores required the combination of nutrient and bicarbonate or nutrient and weakly acidic pH to germinate. They proposed that differences in GSP level in spore affect the regulation of germination enzymes which is in turn affects spore germination (Kato et al.,
2009). Even though the aforementioned reports imply the association of bicarbonate with spore germination of several Clostridium spp., the detailed information concerning germination conditions, the presence of other nutrient germinants, and GR protein(s) that is responsible for recognizing bicarbonate-induced germination are limited. Upon acquiring this knowledge, a better understanding on factors affecting germination of C. perfringens NFB isolates and preventive measures could possibly be developed.

In this study, we investigated the role of bicarbonate in triggering germination of spores of various C. perfringens NFB isolates and the selected FP isolate alone or in combination with 20 individual amino acids. The current work is also able to identify and characterize new amino acid germinants for spores of NFB isolates. We have also evaluated the role(s) of each GR protein in the germination of C. perfringens NFB spores with the newly identified germinants combined with bicarbonate.

4.2. Materials and methods

4.2.1. Bacterial strains and growth conditions

C. perfringens strains used in this study consisted of a FP isolate SM101 (an electroporatable derivative of the type A FP isolate NCTC8798) (Zhao and Melville, 1998), and four strains of type A NFB isolates including NB16 (Sarker et al., 2000), B40, F4969, and F5603 (Collie et al., 1998). The GR-mutant derivatives of C. perfringens F4969 used in this study are as follows SB106 (gerKC), SB106(pSB18) (gerKC mutant complemented with wild type gerKA-KC), SB103 (gerAA), SB103(pSB23) (gerAA mutant complemented with wild type gerAA), and SB110 (gerKA) (Banawas et al., unpublished data). All of the strains were maintained in cooked-meat medium (Difco,
Becton Dickinson, Spark, MD) and stored at -20°C. *C. perfringens* cultures were revived by growing in 10 ml fluid thioglycolate broth (FTG) (Difco) for 18-24 h at 37 °C.

### 4.2.2. Spore preparation and purification

The sporulating cultures of *C. perfringens* were prepared by a previously described method (Paredes-Sabja et al., 2009d; Udompijitkul et al., 2012). Briefly, 0.4 ml aliquots of overnight FTG culture were transferred into new 10 ml FTG and incubated at 37 °C for ~ 8 h. Then, 0.4 ml of actively growing cells were transferred into 10 ml of freshly prepared Duncan-Strong (DS) sporulation medium (Duncan and Strong, 1968) and incubated overnight at 37 °C. The presence of spores was confirmed by phase-contrast microscopy. A large number of *C. perfringens* spores was obtained by scaling up the aforementioned procedure in 800 ml DS, and spores were purified by repeated washing with cold sterile distilled water until spore suspensions were > 99% free of cell debris, sporulating and germinating cells, as determined by phase-contrast microscopy. The spore suspensions were adjusted to a final optical density at 600 nm (OD$_{600}$) ~ 6.0 with sterile distilled water and stored at -80 °C until used.

### 4.2.3. Preparation of germinant solutions

All tested amino acid (L-isomer) solutions were prepared with 25 mM NaHCO$_3$ buffer (pH 7.0). The tested compounds were categorized into five groups, according to their side chain (R group) (Table 1) (Berg et al., 2006). All amino acids used in this study were purchased from Sigma (Sigma-Aldrich, Co., St. Louis, MO). To determine the
ability of bicarbonate to induce spore germination, we tested sodium and potassium bicarbonate (Sigma) at various concentrations ranging from 1-200 mM at pH 7.0.

To determine the effect of pH, alanine, threonine, and histidine were prepared at 100 mM with 25 mM NaHCO₃ buffer (pH 7.0) and adjusted to various pHs, ranging from pH 4.0 to 9.0 in 1.0 unit increments, with 1 M HCl or 1 M NaOH. The concentration effects of alanine, threonine and histidine prepared with 25 mM NaHCO₃ buffer (pH 7.0) were assessed at various concentrations ranging from 1-200 mM.

4.2.4. Spore germination

Spore suspensions were heat activated at 80 °C for 10 min for the FP strain, while NFB strains were treated at 75 °C for 15 min, and then cooled in a water bath at room temperature for 5 min, before being equilibrated at 37 °C for 10 min. These two isolates and GR-mutant strains were heat activated at different temperatures, since our previous study demonstrated that FP spores germinated better when heat activated at 80 °C for 10 min, and spores of NFB isolates germinated better with heat activation at 75 °C for 15 min (Paredes-Sabja et al., 2008d). These heat-activated spores (to a final concentration at OD₆₀₀ of ~ 1.0) were mixed with pre-warmed germinant solution, and the change in OD₆₀₀ was routinely monitored at 37 °C in a 96-well plate and measured by using Synergy™ MX multi-mode microplate reader (BioTek® Instruments, Inc., Winooski, VT). The total volume of spore suspension and germinant (0.2 ml) that carried on microplate was vigorously shaken before every OD₆₀₀ measurement. The decrease of OD₆₀₀ ~ 60% indicates complete spore germination as determined in our previous study (Paredes-Sabja et al., 2008d). The extent of germination was expressed as the percentage
of decrease in OD$_{600}$ relative to initial value. The maximum rate of spore germination was expressed as a percentage of maximal loss of OD$_{600}$ per minute, relative to the initial value of spore suspension. Rate was determined by measuring the OD$_{600}$ of germinating cultures every 2.5 min, and the maximum slopes were calculated as previously described (Paredes-Sabja et al., 2009d).

### 4.2.5. DPA release

The release of DPA during nutrient-induced germination was performed as previously described (Paredes-Sabja et al., 2008d; Paredes-Sabja et al., 2009d). Briefly, spores of F4969 (parental strain) and GR mutant strains (OD$_{600}$ of 1.5) were heat activated at 75 °C for 15 min and then incubated with 100 mM threonine or histidine at 37 °C for 60 min. The 1 ml aliquots of germinating solutions were centrifuged for 3 min at 13,200 rpm in a microcentrifuge tube, and DPA in the supernatant fluid was determined by measuring absorbance at 270 nm ($A_{270}$) as described previously (Cabrera-Martinez et al., 2003; Paredes-Sabja et al., 2008d). The total spore DPA content was evaluated by boiling 1ml aliquot of germinating spores for 60 min and centrifuging for 5 min in a microcentrifuge. The $A_{270}$ of the supernatant fluid was measured. Our previous study indicated that ~ 90% of the material absorbing at 270 nm contained DPA in *C. perfringens* (Paredes-Sabja et al., 2009a).

### 4.2.6. Statistical analyses

Data were analyzed by the analysis of variance procedure (ANOVA) using the statistical software SAS version 9.3 (SAS Inst. Inc., Cary, N.C., USA). ANOVA among
treatments was performed and comparisons of mean values were established by Duncan’s New Multiple Range Test at the significant level of 0.05. In all figures, error bars represent the standard deviations.

4.3. Results

4.3.1. Ability of carbonate and bicarbonate to trigger germination of C. perfringens spores

We initially assessed the effect of bicarbonate and carbonate salts (Na and K) on germination of spores of the representative C. perfringens FP (strain SM101) and NFB (strain NB16) isolates. Our data show that incubation of spores of SM101 and NB16 with all tested concentrations of either sodium carbonate or sodium bicarbonate did not induce germination (Fig. 1A-B). On the other hand, potassium carbonate and bicarbonate at concentrations of 1-200 mM (pH 7.0) induced germination of spores of SM101, but not of NB16 (Fig. 1A-B). These results are consistent with previous findings that i) potassium ion is an strong germinant for FP isolate but not for NFB isolates (Alnoman et al., 2015; Paredes-Sabja et al., 2008d) and ii) bicarbonate, by itself, cannot trigger germination of spores of C. perfringens non-food-borne strain S40 (Kato et al., 2009). These results also suggest that bicarbonate might be a co-germinant for spores of C. perfringens.

4.3.2. Germination of C. perfringens spores with different amino acids in presence of bicarbonate buffer.

To identify which amino acid can induce germination of C. perfringens spores in
presence of bicarbonate buffer, solutions of L-amino acids prepared individually with 25mM NaHCO₃ buffer (pH 7.0) were tested with spores of FP strain SM101 and NFB strain NB16. Our microscopic observations revealed that 10-20% decrease in OD₆₀₀ correlated with 90-80% phase bright spores indicating negligible germination and 20-30% decrease in OD₆₀₀ correlated with 80-70% phase bright spore indicating partial germination. Considering at least 35% decrease in OD₆₀₀ as strong germination response, only L-cysteine was able to induce strong germination of SM101 spores. In contrast, 12 amino acid solutions in NaHCO₃ buffer triggered germination of NB16 spores (Table 1).

To clarify if observed germination responses were caused by the presence of bicarbonate or sodium ions, sodium bicarbonate was substituted by potassium bicarbonate under the same germination conditions. Interestingly, NB16 spores showed similar germination responses to 12 tested L-amino acids in the presence of potassium bicarbonate (Table 1). However, SM101 spores were able to germinate with 15 single amino acids supplemented with potassium bicarbonate, which could be attributed to the presence of potassium ion as a strong germinant for *C. perfringens* FP isolates (Paredes-Sabja et al., 2008d) (Table 1). These data support the role of bicarbonate as a co-germinant for NFB (NB16) spores in combination with any L-amino acids except proline, arginine, negatively charged (glutamic acid, aspartic acid), and aromatic (phenylalanine, tryptophan, tyrosine) (Table 1). However, FP (SM101) spores require potassium ions to enhance bicarbonate germination in present of L-amino acids except leucine, isoleucine, and aromatic amino acids (Table 1).
4.3.3. Effects of pH and concentration of amino acids on NFB spore germination

In order to identify the optimal conditions for amino acid-bicarbonate germination, pH and concentration of alanine (represent nonpolar, aliphatic amino acids), threonine (represent polar, uncharged amino acids), and histidine (represent positively charged amino acids) were varied in combination with 25 mM NaHCO₃ (Fig. 2 and 3). Our data showed that threonine (100 mM) induced significant germination at pH range 6.0 – 9.0 (Fig. 2C); however, alanine and histidine (100 mM) induced significant germination at pH 6.0 and 7.0 and no significant germination was observed below 6.0 or above 7.0 (Fig. 2A and E). The maximum rate of germination of NB16 spores with alanine, threonine, or histidine was observed at pH 7.0 (Fig. 2B, D, and F). Also, no significant difference (p > 0.05) in the rate of germination was detected with alanine or threonine at pH 6.0 as well as with threonine pH 8.0 comparing to pH 7.0-germination rate (Fig. 2D). According to these data, the subsequent germination experiments were performed with amino acids prepared with 25 mM NaHCO₃ adjusted to pH 7.0.

Variations in germinant concentration at pH 7.0 demonstrated that 10 mM alanine, 1 mM threonine and 5 mM histidine were sufficient to trigger significant germination of NB16 spore after 60 min (Fig. 3A, C, and E). However, the lowest concentration to achieve maximum rate of spore germination was 25 mM threonine and higher concentrations of 100 mM histidine and 200 mM alanine, all at pH 7.0 (Fig. 3B, D and F). Furthermore, no statistically significant difference (p > 0.05) was observed in the germination rate of alanine-induced germination at concentration range 50-400 mM (Fig. 3B and data not shown), and all tested concentration of threonine at 1-400 mM (Fig. 3D and data not shown) as well as 5 to 100 mM histidine (Fig. 3F). The extents of spore
germination were correlated with their germination rates. Collectively, these results suggest that L-alanine, L-threonine or L-histidine each in 25 mM NaHCO₃ (pH 7.0) is effective germinant for spores of NFB strain NB16.

4.3.4. Germination of spores from various NFB isolates with L-alanine, L-threonine and L-histidine in 25 mM NaHCO₃ at pH 7.0.

Next, germination experiments were extended to spores of three additional NFB C. perfringens isolates (B40, F4969, and F5603) to verify whether the observed results were strain-specific. As shown in Fig.4, spores of all tested NFB isolates significantly (p < 0.01) germinated in the presence of L-threonine or L-histidine. However, the extent of L-alanine-triggered F5603 spore germination was similar to the results observed with NB16 spores (Fig. 2 and 3) and lower germination extents were observed with B40 and F4969 isolates. These data clearly demonstrate that L-threonine and L-histidine in presence of NaHCO₃ (pH 7.0) act as strong germinants for all tested NFB isolates.

4.3.5. Germination of spores from various NFB isolates with other amino acids at pH 7.0

Our results (Table 1) suggested that a variety of amino acids belong to three different R-groups including nonpolar-aliphatic, polar-uncharged, and positively charged group in combination with NaHCO₃ adjusted to pH 7.0 could be potential germinants for C. perfringens NFB spores. Therefore, germination of NFB spores was examined with all amino acids from nonpolar-aliphatic group and polar-uncharged group as well as L-lysine at pH 7.0. Strikingly, nonpolar-aliphatic amino acids including: L-valine, L-glycine, L-
methionine, L-leucine, and L-isoleucine induced considerable germination of spores of all tested NFB isolates (Table 2), which is in contrast to our previous finding that only L-valine or L-alanine (pH 7.0) in Na$_2$HPO$_4$ buffer could trigger NFB spore germination (Paredes-Sabja et al., 2008d). Furthermore, polar-uncharged amino acids including: L-serine, L-cysteine, L-glutamine, and L-asparagine trigger germination responses of the majority of tested NFB spores (Table 2). Unlike these data, previous studies identified L-asparagine and L-glutamine in phosphate buffer (pH 7.0) as FP-specific germinants (Udompijitkul et al., 2014). Also, L-lysine (pH 7.0) induced considerable germination of spores of all tested NFB isolates (Table 2). Overall, these data suggest the important role of bicarbonate in amino acid-germination response of NFB spores. Thus, bicarbonate acts as a co-germinant for NFB spores in presence of amino acids including nonpolar-aliphatic, polar-uncharged, and positively charged amino acids, except L-arginine.

4.3.6. Role of GRs in threonine- and histidine-triggered germination

In order to gain insights into the mechanism of threonine- or histidine- induced germination in the presence of bicarbonate, the germination of spores of wild-type F4969 and its derivatives carrying mutations in GR genes gerKC, gerAA, and gerKA were assayed using 100 mM threonine or histidine in bicarbonate buffer at pH 7.0. Our data show that gerKC spores did not germinate in presence of threonine or histidine, and gerAA spores germinated extremely poorly as compared to wild-type and other GR mutant spores with threonine or histidine. In contrast, significant germination was observed with gerKA spores incubated with threonine or histidine for 60 min (Fig. 5A and D). These results suggest that GerKC and GerAA, but not GerKA, play major role in
threonine and histidine germination. Importantly, the germination defect of gerKC or gerAA spores was almost corrected by complementation of each mutant with its corresponding wild-type gene (Fig. 5A and D). Collectively, these results indicate that GerKC and GerAA are the main GR proteins involved in the recognition of threonine and histidine when combined with bicarbonate buffer pH 7.0.

As upon binding of germinants to their cognate GR, DPA is released from spore core, we also examined the effects of GR mutations on DPA release during threonine- and histidine-induced germination (Fig. 5C and F). As expected, wild-type F4969 spores released ~95% of their DPA after 60 min of incubation with threonine or histidine (Fig. 5C and F). Strikingly, the amount of DPA released by gerKC spores incubated with these germinants was significantly lower than wild type and other GR mutants and complementation spores, which was consistent with the lower germination extent of gerKC spores. Unlike gerKC spores, gerAA spores released the majority of their DPA during threonine- or histidine-induced germination, which was inconsistent with germination extent of gerAA spores observed. Spores of each strain of gerKA and complementation of gerKC and gerAA released comparable amount of DPA to those of wild-type spores, which was in agreement with each strain’s extent of germination observed (Fig. 5A and D). These results suggest that GerKC is mainly involved in DPA release during C. perfringens spore germination with threonine or histidine, while GerAA have minor role and GerKA have no role.
4.3.7. Role of GRs in other amino acid-triggered germination

Since we have demonstrated that other amino acids belong to nonpolar-aliphatic group, polar-uncharged group, and positively charged group in combination with NaHCO$_3$ buffer (pH 7.0) can efficiently induce germination of *C. perfringens* NFB spores, it is of interest to determine which GR protein(s) is responsible for sensing these germinants, Table 3 showed that spore lacking *gerKC* and *gerAA* exhibited no germination or significantly less germination than spores of wild type and *gerKA* spores, and spores of *gerKC* or *gerAA* complemented strain was only partially restored the defect in germination-phenotypes, indicating that GerKC and GerAA are required for normal germination of *C. perfringens* NFB spores at pH 7.0 with nonpolar-aliphatic amino acids and polar-uncharged amino acids as well as lysine in bicarbonate buffer.

4.4. Discussion

The present work is the first detailed study on bicarbonate requirement during spore germination of a collection of CPE-producing *C. perfringens* FP and NFB isolates and the role of GR proteins involved in NFB spore germination with newly identified amino acid-bicarbonate germinants. Spores of NFB isolates required the presence of bicarbonate to trigger germination with 12 individual amino acid at pH 7.0, in contrast to spores of FP strain SM101 that need the combination of potassium ions with bicarbonate to germinate with 15 single amino acids at pH 7.0. These newly identified amino acid germinants were not able to trigger germination of NFB spores in the absence of bicarbonate as our previous study recognized 2 amino acids (alanine or valine) as NFB germinants and only asparagine was specific for FP isolates in combination with sodium
phosphate buffer at pH 7.0 (Paredes-Sabja et al., 2008d). In addition, the mixture of asparagine and KCl (AK) at pH 7.0 is a universal germinant for both FP and NFB isolates of *C. perfringens*. Thus, bicarbonate increases the number of amino acid germinants that can be recognized by GR proteins of NFB isolates. This information supports the possible role of bicarbonate to activate GRs and render them to be more responsive to amino acids (Plowman and Peck, 2002). Fourier transform infrared analysis revealed that sodium bicarbonate reacts with proteins inside the spore of *Bacillus stearothermophilus* leading to structural changes and the generation of CO$_2$ which is in turn either inhibits spore germination in aerobic species or facilitates spore germination of anaerobic species (Cheung et al., 1998).

The detailed mechanism of CO$_2$ in spore germination is largely unknown, earlier studies reported that CO$_2$ could incorporate into spore membrane lipid bilayer and change the membrane permeability (Enfors and Molin, 1978) A recent study showed that GSP-overproducing *C. perfringens* spores germinated without CO$_2$ in the presence of bicarbonate, nutrients, or weakly acidic pH and proposed that bicarbonate interacts with outer layer of *C. perfringens* spores and results in increase in GSP to activate cortex lytic enzyme SleC.

It is worth noting that the differences in germination requirement between *C. perfringens* FP and NFB spores exist and this issue has been demonstrated in our previous studies (Paredes-Sabja et al., 2008d; Paredes-Sabja et al., 2009d; Udompijitkul et al., 2014). The current work demonstrates the specificity in germination response of NFB isolates, uniquely having bicarbonate as a co-germinant for the newly identified amino acid germinants. The requirement of FP spore germination with potassium ions
has been reported (Paredes-Sabja et al., 2008d), and this work provides additional evidence to this fact with bicarbonate- amino acid germinants. The diversity in germination requirements between spores of *C. perfringens* FP and NFB isolates could be related to bacterial adaptation to the environmental niches specific for each isolate. Since *C. perfringens* FP incidents are generally associated with consumption of cooked meat and poultry- products, it suggests that *C. perfringens* FP isolates have adapted to conditions encountered in meats. Furthermore, the bicarbonate requirement for germination of spores of NFB isolates suggests that the spores can germinate inside the digestive tract, especially in the small intestine where sodium bicarbonate released from the pancreas to maintain pH homeostasis (Kivela et al., 2005). Another example of adaptation of NFB isolates to their host environment is the ability of NFB spores to germinate in the presence of intestinal epithelial cells and macrophages (Paredes-Sabja and Sarker, 2011, 2012). The host-adapted germination capability could provide an advantage to *C. perfringens* NFB isolates to cause wide spectrum of GI diseases in human and animals through small inoculum of NFB cells followed by conjugative transfer of *cpe* plasmid to non pathogenic *C. perfringens* in the normal gut (Brynestad et al., 2001; Freedman et al., 2016; Lindstrom et al., 2011).

Surprisingly, bicarbonate demonstrated the strongest capacity as a co-germinant at pH 7.0 as reflected by the extremely low concentrations of L-threonine (1 mM) required to trigger NFB spore germination, followed by L-histidine at 5 mM. One possible explanation is the aforementioned changes in spore proteins caused by bicarbonate that lead to the remarkable GR activation with such low amount of threonine and it might account for the wider pH germination range (pH 6-9) of spores with threonine (Fig. 2C).
It is interesting to note that *gerAA* and *gerKC* germination rate with threonine did not restore by complementation of each gene unlike the histidine germination phenotype that was restorable with complementation (Fig. 5D and E). Furthermore, spore lacking GerAA protein was not able to germinate normally since germination rate and extent was significantly lower than wide type spores, but the DPA level released from *gerAA* spores was similar to wide type level for both threonine- and histidine-triggered germination. It is likely that defects on GerAA lead to changes in the initial stage of germination such as increasing leakage of Ca-DPA from spore core, but might also cause inferiority in the downstream germination stage. This assumption needs further investigation to clearly identify the role of GerAA in *C. prefringens* NFB spore germination.

Our recent studies demonstrated that GerKC is the key GR protein involved in amino acid triggered germination of spores of FP strain SM101 (Banawas et al., 2013a; Paredes-Sabja et al., 2008d; Udompijitkul et al., 2014). In this study, for the first time, we identified GerKC and GerAA are the main GR proteins for germination of spores of NFB strain F4969 with amino acid-bicarbonate by demonstrating that *gerKC* and *gerAA* spores did not germinate with these germinants and the germination defect was restored by complementing the *gerKC* or *gerAA* spores with wild-type *gerKA-KC* or *gerAA*, respectively. The findings from our current work provide further evidence supporting that GerKC is indeed the key GR protein involved in amino acid-triggered germination of spores of FP SM101 and NFB F4969 isolates.

In conclusion, the important findings of the current work are the following: (i) bicarbonate is a co-germinant for amino acid-triggered germination of spore of NFB isolates; (ii) bicarbonate and potassium ion are required to trigger germination of spores
of FP isolates with amino acids; (iii) GerKC and GerAA are the major GR proteins involved in bicarbonate-amino acid germination of spores of NFB strain F4969.

Acknowledgements

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Table 4.1: Germination of spores of *C. perfringens* representative FP and NFB stains with different amino acids at pH 7.0

<table>
<thead>
<tr>
<th>Germinant</th>
<th>Nonpolar, aliphatic</th>
<th>Polar, uncharged</th>
<th>Positively charged</th>
<th>Negatively charged</th>
<th>Aromatic</th>
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<tbody>
<tr>
<td></td>
<td>Ala</td>
<td>Val</td>
<td>Gly</td>
<td>Met</td>
<td>Leu</td>
</tr>
<tr>
<td>FP SM101</td>
<td>25 mM NaHCO$_3$</td>
<td>25 mM KHCO$_3$</td>
<td>25 mM NaHCO$_3$</td>
<td>25 mM KHCO$_3$</td>
<td>25 mM NaHCO$_3$</td>
</tr>
<tr>
<td>% Decrease in OD$_{600}$ after 60 min$^b$</td>
<td>20</td>
<td>12</td>
<td>9</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

$^a$ All amino acids were tested at 100 mM in 25 mM buffer, pH 7.0, except for tyrosine and tryptophan that were tested at 3 mM.

$^b$ Values are the average of at least duplicate experiments from two different spore preparations. The standard deviations from these experiments were less than 18% of the mean.
**Table 4.2:** Germination of spores of *C. perfringens* NFB stains with other amino acids

<table>
<thead>
<tr>
<th>Germinant</th>
<th>% Decrease in OD$_{600}$ after 60 min$^b$</th>
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<tbody>
<tr>
<td></td>
<td>F4969</td>
</tr>
<tr>
<td><strong>Nonpolar, aliphatic</strong></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>44</td>
</tr>
<tr>
<td>Gly</td>
<td>45</td>
</tr>
<tr>
<td>Met</td>
<td>45</td>
</tr>
<tr>
<td>Leu</td>
<td>48</td>
</tr>
<tr>
<td>Ile</td>
<td>47</td>
</tr>
<tr>
<td><strong>Polar, uncharged</strong></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>45</td>
</tr>
<tr>
<td>Cys</td>
<td>44</td>
</tr>
<tr>
<td>Gln</td>
<td>45</td>
</tr>
<tr>
<td>Asn</td>
<td>46</td>
</tr>
<tr>
<td><strong>Positively charged</strong></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>54</td>
</tr>
</tbody>
</table>

$^a$ All amino acids were tested at 100 mM in 25 mM NaHCO$_3$ buffer, pH 7.0.

$^b$ Values are the average of at least duplicate experiments from two different spore preparations. The standard deviations from these experiments were less than 13 % of the mean.
### Table 4.3: Germination of spores of C. perfringens GR mutant strains with other amino acids

<table>
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<tr>
<th>Germinant&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Decrease in OD&lt;sub&gt;600&lt;/sub&gt; after 60 min&lt;sup&gt;b&lt;/sup&gt;</th>
<th>F4969 (wild type)</th>
<th>gerKC complement</th>
<th>gerKC complement</th>
<th>gerAA complement</th>
<th>gerAA complement</th>
<th>gerKA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nonpolar, aliphatic</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
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<td>11</td>
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</tr>
<tr>
<td>Gly</td>
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<td>1</td>
<td>40</td>
<td>14</td>
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<td>42</td>
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<tr>
<td>Leu</td>
<td></td>
<td>48</td>
<td>0</td>
<td>34</td>
<td>11</td>
<td>20</td>
<td>42</td>
</tr>
<tr>
<td>Ile</td>
<td></td>
<td>47</td>
<td>2</td>
<td>36</td>
<td>13</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td><strong>Polar, uncharged</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td></td>
<td>45</td>
<td>13</td>
<td>34</td>
<td>17</td>
<td>21</td>
<td>39</td>
</tr>
<tr>
<td>Cys</td>
<td></td>
<td>38</td>
<td>12</td>
<td>30</td>
<td>7</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Gln</td>
<td></td>
<td>45</td>
<td>4</td>
<td>29</td>
<td>3</td>
<td>13</td>
<td>36</td>
</tr>
<tr>
<td>Asn</td>
<td></td>
<td>46</td>
<td>0</td>
<td>37</td>
<td>8</td>
<td>19</td>
<td>39</td>
</tr>
<tr>
<td><strong>Positively charged</strong></td>
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<td></td>
<td>54</td>
<td>1</td>
<td>40</td>
<td>7</td>
<td>14</td>
<td>26</td>
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</tbody>
</table>

<sup>a</sup> All amino acids were tested at 100 mM in 25 mM NaHCO<sub>3</sub> buffer, pH 7.0.

<sup>b</sup> Values are the average of at least duplicate experiments from two different spore preparations. The standard deviations from these experiments were less than 19% of the mean.
Fig. 4.1. Germination of *C. perfringens* spores with bicarbonate. Spores of *C. perfringens* FP strain SM101 (A), or NFB strain NB16 (B) were heat activated and incubated with different concentrations of sodium bicarbonate (white bars), potassium bicarbonate (black bars), sodium carbonate (gray bars) or potassium carbonate (white bars with horizontal strips) at pH 7.0. Spore germination was measured as described in Material and methods. Error bars represent standard deviation from the mean of triplicate experiments with three independent spore preparations.
Fig. 4.2. Effects of pHs on germination of *C. perfringens* spores. Heat-activated NB16 spores were incubated with 100 mM L-alanine (A and B), L-threonine (C and D) or L-histidine (E and F) adjusted to various pHs. The extents of germination (A, C, and E) after 60 min of incubation at 37 °C and maximum rates of germination (B, D, and F) were calculated as described in Material and methods. Error bars represent standard deviations from the mean of triplicate experiments with three independent spore preparations.
Fig. 4.3. Effects of amino acid concentrations on germination of *C. perfringens* spores. Heat-activated spores of strain NB16 were incubated with various concentrations of L-alanine, L-threonine, or L-histidine (in 25 mM NaHCO₃, pH 7.0) and extents of germination (A, C, and E) after 60 min of incubation at 37 °C and maximum rates of germination (B, D, and F) were calculated as described in Material and methods. Error bars represent standard deviations from the mean of triplicate experiments with three independent spore preparations.
Fig. 4.4. Germination of spores from various *C. perfringens* NFB isolates. Heat-activated spores of NFB isolates (B40, F4969, and F5603) were incubated with 25 mM NaHCO$_3$, pH 7.0 (black bars) alone or 25 mM NaHCO$_3$ (pH 7.0) plus each of 100 mM alanine (gray bars), 100 mM threonine (white bars), or 100 mM histidine (white bars with black horizontal strips) at 37 °C for 60 min, and OD$_{600}$ was measured as described in Material and methods. Data represents percentage decrease in OD$_{600}$ of the initial values and error bars indicate standard deviations from the mean of triplicate experiments with three independent spore preparations.
Fig. 4.5. Germination of spores of *C. perfringens* GR-mutant strains. (A and C) Heat-activated spores of strain F4969 (wild type) (filled diamonds), SB106 (gerKC mutant) (filled squares), SB106(pSB18) (gerKC mutant complemented with wild-type gerKA-KC) (open squares), SB103 (gerAA mutant) (filled circles), SB103(pSB23) (gerAA mutant complemented with wild-type gerAA) (open circles), and SB110 (gerKA mutant) (cross marks) were incubated with 100 mM L-threonine (in NaHCO₃, pH 7.0) (A) or 100 mM L-histidine (in NaHCO₃, pH 7.0) (C) at 37 °C, and the OD₆₀₀ was measured. (B and D) DPA release during *C. perfringens* spore germination with L-threonine (in NaHCO₃, pH 7.0) (B) or L-histidine (in NaHCO₃, pH 7.0) (D). Error bars indicate standard deviations from the mean of at least duplicate experiments with two independent spore preparations.
Chapter 5

General Conclusion

The enterotoxin-producing *C. perfringens* type A isolates can cause FP and NFB GI disease in humans and other animals. *C. perfringens* pathogenesis develops initially by the extremely resistant spores that can survive the food processing treatment and afterwards these spores germinate to become active cells and rapidly multiply to reach a hazardous level that can contaminate food items. In general, meat and poultry products not properly cooked and/or stored are the main source of *C. perfringens* type A associated FP outbreaks. However, the physiological differences and the differences in diseases progression between *C. perfringens* FP and NFB strains emphasize the importance of developing an effective inactivation method to control and prevent *C. perfringens* associated GI illnesses. In this dissertation, the efficacy of several natural and chemical antimicrobial agents against the growth and survival of *C. perfringens* vegetative cells and spores were studied in an effort to provide new information and to develop effective inactivation strategies in food items, especially in meat products.

In the first study, the inhibitory effects of commonly used preservatives (sorbate and benzoate) against *C. perfringens* type A FP and NFB disease isolates were evaluated both in laboratory conditions and in a meat model system under abusive conditions. Results from this study indicated that, besides their well-recognized antifungal properties, sorbates and benzoates also exert their inhibitory effects against the food-borne spore-forming pathogen *C. perfringens* in a variety of aspects including spore germination and outgrowth, and vegetative growth of both FP and NFB isolates. Despite their effectiveness in laboratory medium, potassium sorbate and sodium benzoate with
permissive level failed to control germination and outgrowth of *C. perfringens* spores inoculated into cooked chicken meat stored in improper conditions for an extended period. Thus, proper attention needs to be paid when applying these organic salts into food formulations, especially in meat products, in order to reduce the potential risk of bacterial spores, especially enterotoxigenic *C. perfringens* spores.

In the second study, we demonstrated the inhibitory effects of chitosan against *C. perfringens* FP isolates during various stages of its life cycle in both laboratory medium and a chicken meat model system. At pH 6.0 condition, chitosan with three different molecular weights (MW) affected the *C. perfringens* spore outgrowth, but not the initiation of spore germination. However, significant inhibition of spore germination and DPA release were observed with chitosan at pH 4.5. Although the results obtained from this study revealed no significant differences in the inhibitory properties of three different MW of chitosan against *C. perfringens* spores and vegetative cells, the use of low molecular weigh (LMW) chitosan was encouraged because it exhibited better solubility than other two MW of chitosan. The LMW chitosan, albeit at higher concentration, showed bacteriostatic and bactericidal activity against vegetative cells of the enterotoxigenic *C. perfringens* type A isolates.

To further improve the spore inactivation strategies, induction of spore germination prior to inactivation by mild treatments could be an alternative approach. Based on this idea, in our third study, we identified compounds contributing to maximal germination of spores of CPE-producing *C. perfringens* type A NFB isolates. In addition, GR proteins responsible for sensing these newly identified nutrient germinant were also characterized. We identified 12 individual amino acids that could trigger germination of
spores of *C. perfringens* NFB isolates in the presence of bicarbonate buffer. However, *C. perfringens* FP spores required potassium ions for the germination with these 12 amino acids and could not germinate with a bicarbonate buffer system. Surveying the germination of spores of *C. perfringens* NFB GR mutant isolates with these newly identified amino acid germinants revealed that GerKC and GerAA play major roles in the bicarbonate-amino acid germination system.

In conclusion, this dissertation provides new information about the efficacy of different natural and chemical food preservatives in the inactivation of *C. perfringens* spores and vegetative cells. It also gives better understanding of the biology of *C. perfringens* spores and their response to different types of germinants under different conditions. Collectively, this dissertation delivers new insights about the effective inactivation strategies against *C. perfringens* spores and vegetative cells and renders the importance of further research on the interaction between *C. perfringens* and food and food related items.
Appendices

Appendix A: Scientific work published or in preparation.


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