AN ABSTRACT OF THE THESIS OF

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Title: Inactivation Strategy for *Clostridium perfringens* Spores Adhered onto Stainless Steel Surfaces

Abstract approved:

Mahfuzur R. Sarker

Clostridium perfringens is a spore-forming pathogenic bacterium that causes a variety of diseases in human and animals. *C. perfringens* type A isolates produce enterotoxin (CPE) causing food poisoning (FP) and non-food-borne (NFB) gastrointestinal (GI) diseases including antibiotic-associated diarrhea and sporadic diarrhea. *C. perfringens* type A food poisoning currently ranks as the second most commonly reported bacterial foodborne outbreaks in the United States. *C. perfringens* has the ability to form metabolically dormant spores in the environment that are resistant to various lethal factors such as, moist heat, dry heat, UV radiation, nitrate, pH-induced stress, prolonged frozen storage, and high pressure processing. These spore resistant properties allow the survival of spores against the preservative approaches that are applied in food manufacturing plants. Thus, the cross-contamination of *C. perfringens* spores from food contact surfaces into finished products might increase the consumer health risk.

In this work, *C. perfringens* type A isolates were evaluated for their ability to survive on stainless steel (SS) chips under aerobic conditions. *C. perfringens* spores adhered onto SS chips and remained viable up to 48 h in aerobic conditions while vegetative cells died within 30 minutes of exposure to aerobic environment. Further, we determined the surface hydrophobicity of *C. perfringens* cells and spores and its correlation to the adhesion onto SS chips. Results showed that spores are more hydrophobic than vegetative cells, and this hydrophobicity is related to the presence of the spore outer coat. Lastly, we applied a modified Clean-in-Place (CIP) procedure on *C. perfringens* spores adhered onto SS chips as an inactivation strategy to control the contamination level of adhered *C. perfringens* spores. Our results demonstrated that CIP wash steps are able to inactivate *C. perfringens* spores from SS chips after treating with sodium hydroxide (NaOH).

Collectively, our current findings contributes to food industry in order to enhance food safety by lowering the potential cross-contamination of *C. perfringens* into food products, thereby helping reducing the risk of *C. perfringens*-associated food poisoning outbreaks.

Inactivation Strategy for *Clostridium perfringens* Spores Adhered onto Stainless Steel Surfaces

by

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Dean of the Graduate School

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

Yasmeen S. Alzubeidi, Author

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Dr. Mahfuzur R. Sarker as a major professor provided the guidance, supervision and laboratory facilities needed for the research work presented in this thesis.

Dr. Mahfuzur R. Sarker and Dr. Pathima Udompijtikul were involved in the experimental design, and preparation of the manuscript in chapter 3.

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DEDICATION

I would like to dedicate this work to my father Suleiman Ali Alzubeidi, who has been a source of encouragement and inspiration to me throughout my study, and for providing me assistance to pursue the highest degree of education. To my lovely mother Fatimah Alyafie who spent her days and years taking care of me, raising me to become a better person, and praying for me to succeed in my studies. I love you.

Inactivation Strategy for *Clostridium perfringens* Spores Adhered onto Stainless Steel Surfaces

CHAPTER 1

Introduction

Clostridium perfringens is defined as a Gram-positive, anaerobic, rod-shaped bacterium. It is a non-motile pathogen that forms endospores. *C. perfringens* is considered to be the most commonly reported pathogenic bacterium that belongs to *Clostridium* genus. More pathogenic bacterium belong to *Clostridium* genus such as *C. botulinum, C. tetani, C. difficile,* and other industry related organisms, such as *C. acetobutylicum* and *C. thermocellum* (Hatheway, 1990). In the 1940's and 1950's *C. perfringens* was first recognized as a causative agent of foodborne disease. Later on, *C. perfringens* was found to cause human gas gangrene and two different foodborne diseases, i.e., *C. perfringens* type A food poisoning and enteritis necroticans (McClane, 2007). *C. perfringens* type A isolates that produce *C. perfringens* enterotoxin (CPE) are the causative agent of *C. perfringens* type A food poisoning, which is estimated to be the second most commonly reported pathogenic bacteria that causes food-borne diseases in the United States (Grass et al., 2013; McClane, 2007). It estimated to cause nearly one million cases of foodborne illnesses annually and results in economical loss of $309.4 million per year (Hoffmann et al., 2012; Lynch et al., 2006; McLinden et al., 2014; Scallan et al., 2011). Also, *C. perfringens* type A strains

are recognized as the cause of non-food-borne (NFB) human gastrointestinal (GI) diseases, such as antibiotic-associated and sporadic diarrheas (Borriello et al., 1984; Collie and McClane, 1998; Lindström et al., 2011). Inactivating *C. perfringens* bacteria in food industry is a major challenge to food manufacturers due to its ability to form dormant spores that become extremely resistant to lethal treatments such as hydrostatic pressure, temperature, pH stress, heat, chemicals, nitrite, osmotic, and prolonged frozen storage (Li and McClane, 2006a, b; Paredes-Sabja et al., 2007; Paredes-Sabja et al., 2008; Sarker et al., 2000; Udompijitkul et al., 2013). These resistant properties of *C. perfringens* against various treatments commonly applied in manufacturing plants makes it very difficult to eliminate or control contamination of food products.

The cross-contamination of pathogenic organisms from contaminated food contact surfaces into finished products in food processing plants during food product handling or food preparation is one of the leading causes of food-related GI diseases (Kusumaningrum et al., 2003; Ryu et al., 2004). When *C. perfringens* spores attach to food contact surfaces (i.e., stainless steel, glass, and plastic), it enhances the resistance to disinfectants and becomes a continuous source of cross contamination of pathogen onto food products, thus affecting the quality, shelf life, and safety of the consumer (Hornstra et al., 2007). Among *C. perfringens* type A FP outbreaks, contamination of equipment accounted for 15% of the total cases (McClane, 2007). One of the important characteristics of the microorganisms is the ability to attach onto surfaces,

which allow them to survive under stressful environments. Adhesion of pathogenic microorganisms onto surfaces can act as an initial stage for developing biofilms, and allowing microbial transmission to finished products, and subsequently leading to consumer health risk (Boulané‐Petermann, 1996; Frank, 2001). Several studies suggest that bacterial surface characteristics such as cell surface hydrophobicity, surface charge, and the presence of particular surface structures play an important role on bacterial adhesion on surfaces. Although adhesion factors have been extensively studied in *Bacillus* species, *Escherichia coli*, *Salmonella typhimurium*, *Lysteria monocytogenes*, and *Staphylococcus aureus* (Dickson and Koohmaraie, 1989; Escobar-Cortés et al., 2013; Faille et al., 2007; Gilbert et al., 1991; Parkar et al., 2001; Rönner et al., 1990; van Loosdrecht et al., 1987; Wiencek et al., 1991), such information is much less available for *Clostridium* species. Therefore, understanding the surface hydrophobicity of *C. perfringens* cells and spores and its relation to the adherence of this organism on SS chips as a model of food contact surfaces, would lead to development of a strategy to prevent or minimize the adhesion of microorganism on surfaces.

To control bacterial contamination, a system called Clean-in-Place (CIP) has been successfully applied in food manufacturing plants, which is an automated method of cleaning and disinfecting the surface of large and fixed equipment without disassembly. The CIP procedure aims to remove any undesired organic and inorganic fouling layers in a closed system using chemical, physical, and thermal aspects

(Stanga, 2010). It is known that surface attached or biofilms-associated spores, which enhance resistance against crucial procedure of the CIP regime (Faille et al., 2001). In several studies, CIP has been applied on biofilms of *Streptococcus thermophilus* and *Bacillus* species (Bremer et al., 2006; Flint et al., 1999; Parkar et al., 2004) rather than single organism. The effectiveness of the standard CIP regime on biofilms did not result in significant reduction in viable cells due to many factors influencing the effectiveness; however, adding a sanitizer in the CIP regime was more effective at reducing the adhered biofilm than the standard CIP regime (Bremer et al., 2006; Dufour et al., 2004). The effect of the CIP regime on *C. perfringens* had never been reported; therefore a study of the effect of the CIP system on adhered *C. perfringens* onto SS surfaces is required. In this work, we focused on studying the adhesion and the survival rate of *C. perfringens* spores and cells of FP and NFB isolates and its relation to surface hydrophobicity, as well as the effect of a modified CIP system on *C. perfringens* spores adhered onto SS surfaces.

Objective of this study

C. perfringens is known to firmly adhere to wide variety of materials commonly found in food manufacturing plants, and is easily transmitted to finished products and affecting quality of food, shelf life, and consumer health risk. Therefore, understanding the behaviors of *C. perfringens* spores and cells adhered onto SS surfaces as well as factors affecting their adhesion could provide valuable information towards developing an effective inactivation procedure.

The objectives of this research are:

- Determine the viability of *C. perfringens* FP and NFB isolates adhered onto SS surfaces under aerobic conditions at different temperatures.
- Measure the surface hydrophobicity of vegetative cells and spores from various *C. perfringens* FP and NFB isolates
- Evaluate the effectiveness of the widely used CIP procedure in removing *C. perfringens* from a model food contact surfaces.

CHAPTER 2

Literature Review

2.1. Bacterium characteristics

Clostridium perfringens is a Gram positive, rod-shaped, nonmotile, spore-forming, anaerobic bacterium. *C. perfringens* is ubiquitous and present normally in many environmental sources like soil, water, wastewater as well as an inhabitant of humans and animals intestinal normal flora. Although *C. perfringens* is anaerobic bacterium and produces no colony on agar plate in the presence of oxygen, it is considered moderately aerotolerant (Brynestad and Granum, 2002; McClane, 2007). The growth of *C. perfringens* in food is influenced by many environmental factors, i.e., temperature, pH, water activity (a_w), and oxidation reduction (E_h) (McClane, 2007). Vegetative forms of *C. perfringens* can grow at temperature range between 20 °C and 50 °C, with an optimal growth temperature from 43 °C to 45 °C for most strains (Brynestad and Granum, 2002; Novak et al., 2005). At lower temperatures, *C. perfringens* growth rate notably decrease for all strains. However, spore of *C. perfringens* are more resistant than vegetative cells to cold temperature, and all *C. perfringens* strains do not grow at 6 °C. Once contaminated frozen food products are warmed improperly, spores can germinate and multiply rapidly causing *C. perfringens* type A FP disease (McClane, 2007). *C. perfringens* isolates show sensitivity towards the pH, with optimal growth at pH values of 6.0 to 7.0. Besides, *C. perfringens* strains

have lower growth rate at pH $\leq$ 5 and $\geq$ 8.3 (Labbe and Juneja, 2006; McClane, 2007). Under favorable conditions of other environmental factors, *C. perfringens* needs the minimum water activity of 0.93 to grow and requires very low oxidation-reduction potential (E_h) in order to support growth. It was suggested that most common food products have acceptable level of E_h for *C. perfringens* to initiate growth (Labbe and Juneja, 2006; McClane, 2007).

Pathogenicity of *C. perfringens* is also attributed to several factors. First, *C. perfringens* strains have the ability to produce at least 15 different toxins. However, an individual isolate produces only a certain toxin or combination of toxins (Petit et al., 1999). Second, *C. perfringens* is able to grow faster in meat-based systems and can proliferate and multiply rapidly in less than 15 minutes causing contamination. Third, *C. perfringens* is able to form highly resistant spores that survive under environmental stresses such as radiation, low temperature, heat, chemicals preservative, and high hydrostatic pressure (McClane, 2001; Paredes-Sabja et al., 2007; Sarker et al., 2000). Lastly, *C. perfringens* spores have the ability to survive in inadequately cooked food or in improperly heated food during food services (McClane, 2007)

2.2 Major toxins produced by *C. perfringens*

C. perfringens isolates are classified into five toxino-types (A, B, C, D, and E), depending on the expression of four major toxins (alpha, beta, epsilon, and iota) (Table 2.1).

Table 2.1: *C. perfringens* toxin typing (McClane, 2007; Petit et al., 1999)

	Toxin Expressed[b]			
Type[a]	**Alpha**	**Beta**	**Epsilon**	**Iota**
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

[a] *C. perfringens* type,

[b] + expressed; - not expressed

Alpha toxin

All *C. perfringes* types produce abundant alpha toxin. It has a molecular weight of 43-kDa. The alpha toxin encoding gene (*plc*) is located on the chromosome of *C. perfringens* (Ohtani et al., 2002). It contains of two domains, N-domain and C-domain, one exhibits phospholipase activity, and the other partly responsible in binding to membrane. It was suggested that the activity of N-domain is affected by C-domain (Sakurai et al., 2004). Alpha toxin causes tissue damage and lyses the blood cells and epithelial cells by degradation of phosphatidylcholine and sphingomyelin followed by membrane disruption when large amount is expressed (Sakurai et al., 2004). The ability of alpha toxin to lyse blood cells has been used in the reverse CAMP (Christie, Atkins, Munch-Peterson) test to diagnose the presence of alpha toxin as an identification of *C. perfringens*. Alpha toxin causes gas gangrene by damaging tissues, hepatic toxicity, and myocardial disfunction (Murray et al., 1998).

Beta toxin

C. perfringens type B and C produce extracellular beta toxin. Beta toxin is encoded by *cpb* gene, which is located on the large plasmid of *C. perfringens* (Hatheway, 1990). Beta toxin is a pore forming toxin and it is suggested that the formation of cation-selective pores is responsible of the toxin lethality (Nagahama et al., 2003). Inactivation of beta toxin occurs in GI tract by trypsin. Also, beta toxin causes necrotic enteritis or pigbel disease in human and domesticated livestock, which is associated with necrosis of the intestine. The symptoms of pigbel disease are diarrhea and abdominal pain (Songer, 1996). Administrating beta-toxoid is a cure for the disease (Songer, 1996).

Epsilon toxin

C. perfringens type B and D animal isolates produce the epsilon toxin. It is encoded by *etx* gene, which is located on a large plasmid of *C. perfringens* (Songer, 1996). Epsilon toxin is classified as Category B bioterror agent, after botulinum and tetanus neurotoxins (Rood, 1998). Epsilon toxin causes fatal diseases such as enterotoxaemia (sudden death syndrome) in lamb, goat, horses, and rarely in adult cattle, which results in neurological disorder and sudden death. Other diseases include dysentery in newborn lambs (Petit et al., 2003; Rood, 1998). When a large amount of the toxin is produced, it facilitates its absorption in the intestinal mucosa causing increases in vascular permeability, elevation of blood pressure, and kidney necrosis (Petit et al., 2003).

Iota toxin

C. perfringens type E isolates are characterized by their ability to produce the binary iota toxin. It has comparable structure and activity to *C. spiroforme* toxin (Perelle et al., 1993; Songer, 1996). Iota toxin is composed of two independent subunits: Ia, exhibit ADP-ribosyltrasferase activity that causes globular skeletal muscle and nonmuscle actin and induces cell death; and Ib, required for translocating Ia subunit into the host cell (Perelle et al., 1993). These subunits are encoded by *iap* and *ibp* and located on a large plasmid, and their toxic activity is turned on only when combined. This toxin causes sporadic diarrhea in calves and lambs (Barth et al., 2004; Rood, 1998).

***Clostridium perfringens* enterotoxin (CPE)**

CPE is the most important virulence factor for pathogenesis of *C. perfringens* food poisoning and non-food borne GI diseases in humans (Sarker et al., 1999). About ~ 5% of *C. perfringens* type A produce this medically important CPE. It is encoded by *cpe* gene and can be located on either the chromosome or large plasmid (McClane, 2007). The chromosomal copy of *cpe* is carried by *C. perfringens* type A FP isolates (Collie and McClane, 1998; Novak et al., 2005), whereas the plasmid copy of *cpe* is carried by *C. perfringens* isolates from non-food-borne GI diseases (i.e., antibiotic-associated or sporadic diarrhea) (Lahti et al., 2008). CPE is a heat-labile protein

(inactivate at 60°C for 10 min) with a molecular mass of 35-kDa, and is sensitive to pH values of < 6 or >8 (Labbe and Juneja, 2002; McClane, 2007).

The expression of CPE is regulated during bacterial sporulation. CPE is released when *C. perfringens* cells grown under sporulation-inducing condition; the mother cell lyses and releases mature spores and CPE (Duncan et al., 1972; Labbe and Rufner, 1980). *C. perfringens* strains often secret large amounts of CPE in the intestinal lumen, whereas CPE was not detected in the vegetative growth (McClane, 2007; McClane et al., 2006). The mechanism of regulation of sporulation and CPE production is not fully understood at the molecular level. It was hypothesized that *Spo0A*, a master regulatory protein that initiate sporulation in *C. perfringens*, plays an important role in producing CPE and in forming heat resistant endospore. This was proven by *spo0A* gene knock-out studies, which indicated that in the absence of *spo0A*, the *C. perfringens spo0A* knockout mutant wasn't able to form spores or produce CPE, showing a direct correlation between sporulation and CPE production (Huang et al., 2004). The major sigma factors that regulate sporulation of *B. subtilis* found to be encoded in *C. perfringens*. In a previous study, it was suggested that for CPE synthesis only SigF, SigE, and SigK are necessary, whereas for spore formation, all sigma factors (SigF, SigE, SigK, and SigG) are required (Harry et al., 2009; Li and McClane, 2010). CPE acts as pore forming protein with cytotoxic activity that binds to its protein receptor via its C-terminal portion in the host epithelial cells which results in formation of ~90 kDa small complex (Fujita et al., 2000; McClane et al., 2006).

This complex binds to different proteins to form a large complex of ~ 155 kDa which stimulates plasma membrane permeability and ion influx in mammalian cells, causing damage to the small intestine. Eventually induce diarrhea, acute abdominal pain, and nausea; vomiting and fever are rare (Cabrera-Martinez et al., 2003; McClane, 2001, 2007; Songer, 1996).

CPE toxicity increases by removal of the first 45 N-terminal amino acids and the activity of CPE increases by three fold in the presence of trypsin or chymotrypsin. These results suggest a similar activation of CPE may undergo in intestine during GI disease; thus, enhancing the toxicity of the CPE (Brynestad and Granum, 2002; McClane, 2001).

2.3 CPE associated GI diseases

C. perfringens isolates that produce CPE are considered to be one of the most important causative agent for human GI disease i.e., *C. perfringens* type A FP and *C. perfringens* type A NFB diseases. Importantly, most of *C. perfringens* type A strains that carry a chromosomal *cpe* gene (C-*cpe*) cause FP GI disease, while strains that carry a plasmid copy of the *cpe* gene (P-*cpe*) cause NFB human GI disease including ~20% of antibiotic-associated diarrhea (AAD), and sporadic diarrhea (SD) (Collie and McClane, 1998; Lindström et al., 2011; Sarker et al., 2000; Sparks et al., 2001). Interestingly, *C. perfringens* FP isolates possess higher resistance properties against environmental stresses (such as heat, osmotic induced stress, nitrite, pH, and

prolonged frozen storage) than *C. perfringens* NFB isolates (McClane et al., 2006; Sarker et al., 1999). C-*cpe* isolates are associated with food poisoning in food processing plants, which may be related to the phenotype of *C. perfringens* FP isolates that are able to grow rapidly and survive at wider ranges of temperature than P-*cpe* isolates (Deguchi et al., 2009; Xiao et al., 2012).

2.3.1 *C. perfringens* type A FP

C. perfringens type A is currently ranked as the second most reported bacterial cause of foodborne outbreaks in the United States, accounting for almost 1 million illnesses per year (Grass et al., 2013). *C. perfringens* type A FP illnesses cost is estimated to be $309.4 million annually (Buzby and Roberts, 1997). *C. perfringens* type A illnesses are often related to dishes containing raw meat or poultry. Since spores are commonly found in soil and water, during slaughter operation of animals, spores tend to transmit to and contaminate raw products (Juneja and Thippareddi, 2004; Juneja et al., 2006). Importantly, the application of heat treatment on contaminated meat by the meat industry activates *C. perfringens* spores to germinate but not kill them (Paredes-Sabja et al., 2008; Thippareddi et al., 2003). Upon activation and spore survival and during improper handling, cooling and storage, *C. perfringens* spores germinate and rapidly proliferate to high levels (10^6 CFU/g). When a person ingests this contaminated food, some vegetative cells survive stomach acidity, enter into small intestine where they proliferate, sporulate, and produce CPE.

Once the mother cell lyses, the mature spores and CPE releases in the intestinal lumen, and the released CPE binds to the epithelial cells of the intestine. This leads to intestinal tissue damage and initiates fluid loss (diarrhea). One important aspect of controlling contamination during processing and handling and eliminating *C. perfringens* spores from food is cooling and reheating food properly prior consumption and selecting high quality food sources. Most *C. perfringens* type A FP disease is considered to be mild and self-limiting and last for about 12 to 24 hours. Symptoms are mainly diarrhea and severe abdominal pain but in rare cases vomiting and fever might be observed (McClane, 2007; McClane et al., 2006). Antibiotics are not recommended since the disease is self-limiting and keeping an individual hydrated would be necessary (Labbe and Juneja, 2002)

2.3.2 Antibiotic-associated diarrhea and sporadic diarrhea

C. perfringens isolates that produce CPE have been reported to cause ~20% of AAD and SD illnesses in humans. In 1984, 11 patients were diagnosed with AAD caused by *C. perfringens* following ingestion of antibiotics (Asha and Wilcox, 2002). These incidents are considered non-food borne illnesses. It was implicated that AAD is developed after exposure to antibiotics (i.e., penicillin, cephalosporins, trimethoprim or cotrimoxazole), whereas SD proposed to be developed independently after exposure to any antimicrobial drugs. It was suggested that a small number of P-*cpe* strains are able to cause ADDs and SDs, as the *cpe* plasmid can be transferred to *cpe*-negative *C.*

perfringens strains that already present in the gut as normal microbiota (Heikinheimo et al., 2006; Lindström et al., 2011; Sparks et al., 2001). Until today, the transmission route of P-*cpe* is still unknown. Furthermore, some cases of AAD and SD are caused by *C. perfringens* P-*cpe* strains and were isolated from food products (Lahti et al., 2008; Miki et al., 2008; Nakamura et al., 2004), suggesting that development of AAD and SD in some cases might also transmit via food, and thus consider as food poisoning (Lahti et al., 2008). However, these FP illnesses may not be as severe as in traditional CPE food poisoning and their food vehicle often unknown (Lindström et al., 2011). Elderly and people who take antibiotics for long terms are more susceptible to AAD and SD and treatment for the most cases of AAD and SD is needed by restoring fluid/electrolyte balance therapy (McClane et al., 2006).

2.4. Spore formation

Bacterial spore formation of *Bacillus* species has been widely studied, especially in *Bacillus subtilis* (Errington, 2003; Piggot and Hilbert, 2004). Studies reported that *Clostridium* species spore formation is similar to spore formation of *Bacillus* species. (Durre and Hollergschwandner, 2004). The sporulation process takes place through seven stages (Hitchins and Slepecky, 1969; McDonnell, 2007; Piggot and Coote, 1976). Stage 0 is the normal growing vegetative cell, followed by stage I and II where the DNA is remodeled into an axial filament, while the cell undergoes asymmetric division. This process forms two compartments; a smaller compartment

called the pre-spore and a large mother compartment, which separated by a septum within a cell. Stage III called the engulfment where the formation of a free protoplast when the mother cell engulfs the pre-spore to form fore-spore surrounded by inner and outer fore-spore membrane. Synthesis of spore cortex occurs in stage IV, leading to deposition of primordial germ cell wall and cortex between the inner and outer membrane surrounding the fore-spore. Stage V is entered during formation of the spore coat, a complex structure of protein outside the surface of fore-spore. Stage VI is also termed spore maturation, a time period in which spore acquires resistance characteristics against heat, UV radiation, and chemicals. The coat becomes denser with no morphological changes. In the final stage VII, the mother cell lyses and releases the mature spore structure in the environment. This mature spore structure protects the dormant microorganism until spore find favorable conditions once again for vegetative cell growth. When dormant spores are reactivated, it undergoes germination and outgrowth (Errington, 2003; Leggett et al., 2012; Piggot and Coote, 1976).

2.5 Spore structure

The structure and chemical composition of spore differs from those of vegetative cell (Fig. 2.1). The differences mostly accounts for spore resistance features against environmental stresses (Setlow, 2014).

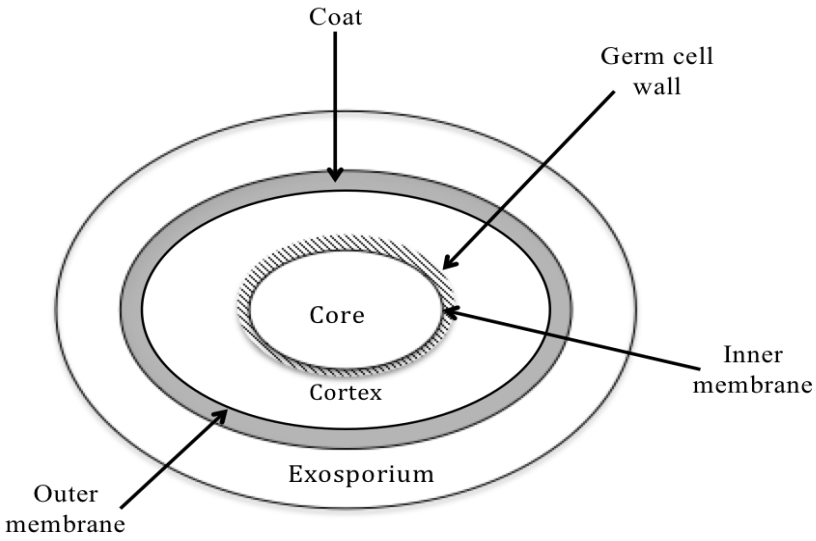

Fig. 2.1 The structure of typical bacterial spore. The layers are not drawn to scale, and sub-layers may present in the coat and exosporium. In many species the exosporium layer is absent. Modified from Setlow (2014)

Starting from the outside to inside layers are in order of: exosporium, coat, outer membrane, cortex, germ cell wall, inner membrane and central core. The spore structure of *Clostridium* spp. is similar to the spore structure of *Bacillus* ssp., except that in some of *Clostridium* spp. the outermost structure is the coat. Also, in some of the *Bacillus* spp., the exosporium is absent or greatly reduced in size (Henriques and Moran, 2007; Lai et al., 2003; Todd et al., 2003). The exosporium consists mainly of proteins (43-52% of dry weight), including some glycoproteins. The function of these proteins is still unknown but it was suggested that they have a role in adherence and hydrophobic interaction of the spores (Koshikawa et al., 1989). The coat structure is composed of several layers of > 50 spore-specific proteins. It plays a role in

protecting the spore from reactive chemicals and lytic enzymes. Since, in some species, this is the outermost layer, it might be also responsible for the spore hydrophobicity (Koshikawa et al., 1989; Kutima and Foegeding, 1987; Wiencek et al., 1990). Under the spore coat lies the outer membrane, which is essential structure during spore formation (Leggett et al., 2012; Piggot and Hilbert, 2004). However, the function of the outer membrane remains unknown and doesn't act as significant permeability barrier in dormant spore. The cortex structure is similar to the cortex of a growing cell, which is composed of peptidoglycan (PG) with several spore-specific modifications (Popham, 2002; Warth and Strominger, 1972). The cortex is important in attaining spore dormancy and resistance characteristics. Also, the cortex is essential for reducing the water content of the spore core and maintaining its dehydrated environment. During spore germination, the cortex is degraded, leading to core expansion and outgrowth (Leggett et al., 2012; Setlow, 2014). Just under the cortex another PG structure comes, which is the germ cell wall; this structure probably identical to PG of a growing cell wall. After germination and the outgrowth this structure becomes the cell wall of a growing cell. There is no role for germ wall in spore resistance properties (Leggett et al., 2012; Setlow, 2014). Unlike the outer spore membrane, the inner membrane of spore has a very low permeability to small molecules that plays a major role in protecting the spore core DNA from damage. The inner membrane is significantly compressed, in which the lipid composition are largely immobile. This lipid composition becomes fully mobile upon germination.

However, the lipid composition of the spore inner membrane is very similar to the plasma membrane of growing cell, but completely different protein composition than the growing cell membrane (Cowan et al., 2003; Cowan et al., 2004; Leggett et al., 2012; Setlow, 2006). The innermost layer is the core that contains most spore enzymes, DNA, and RNA. The core has low water content (27-55% of wet weigh), which play a major role in spore resistance to heat and some chemicals. Another important factor that is likely important in spore's enzymatic dormancy is pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA), which is present at 5-15% in the spore core. The third group of molecules that play a major role in spore resistance are α/β-type small acid-soluble proteins (SASP). These comprise 3-6% of total spore protein and involved in saturation of the spore DNA. Each of those factors contributes to the spore resistance characteristic against UV radiation, heat, and chemicals (Leggett et al., 2012; Setlow, 2014).

2.6 Spore germination

Under unfavorable growth conditions, vegetative cells of *Bacillus* spp. and *Clostridium* spp. initiate the sporulation process to become dormant spores (Piggot and Hilbert, 2004; Setlow, 2014). During dormancy, the spore surveys the surrounding environments until favorable growth conditions are present. A dormant spore must undergo germination, and then outgrowth processes, in order to return to life as an actively grown cell (Moir, 2006; Setlow, 2014). The presence of specific nutrients, termed germinant, in the environment, initiate dormant spore germination. There are

number of germinate agents such as amino acids, sugar, or purine nucleosides that trigger spore germination (Setlow, 2003; Setlow, 2014). Spore germination is initiated by interaction between germinant molecules and their cognate germinant receptor (GR) that is located in the spore's inner membrane (Hudson et al., 2001; Paidhungat and Setlow, 2001). Upon initiation of germination, the spore undergoes a series of different biophysical and biochemical events.

I. The spore core releases monovalent cations, H^+, and Zn^{2+}, which lead to pH elevation of the spore core's from ~6.5 to 7.7. This is an essential change for spore metabolism once the core's water content is low enough for enzyme action.
II. Following ion release, the spore core's large DPA, along with divalent cations, (predominantly Ca^{2+}) are also released.
III. The hydration level of the spore core is increased as the released DPA is replaced with water. This results in decreased moist heat resistance. However, the hydration increase in the core does not favor protein motion or enzyme action (Cowan et al., 2003; Setlow, 2003).
IV. Germination actually begins with the hydrolysis of spore cortex peptidoglycans by cortex-lytic enzymes.
V. The spore core takes up more water, causing swelling of the spore core and expansion of the germ cell wall.

The germination process is then complete. After this step, the protein mobility

resumes, allowing enzyme action and normal metabolism in the core. Then, macromolecular synthesis follows that event in later development of spore outgrowth process that converts germinated spore into a growing cell (Paidhungat and Setlow, 2002; Setlow, 2003).

2.7. Bacterial adhesion to surfaces and its relation to hydrophobicity

Bacterial adhesion onto surfaces has been studied for many years, and it has significant implications for food safety. Microorganisms have the ability to adhere firmly onto surfaces commonly found in manufacturing plants such as stainless steel (SS), plastic, and glass (Chae and Schraft, 2000; Chia et al., 2009; Frank, 2001). The adhered organisms have the potential to transmit to food products, which is considered a serious problem for food industries. Once microorganisms adhere to surfaces, they become highly resistant to disinfecting chemicals, which subsequently affect the quality of food, shelf life, and consumer risk of foodborne diseases (Andersson et al., 1995; Austin and Bergeron, 1995; Frank, 2001). Microbial characteristics leading to attachment and later release from surfaces are critical for their survival; in fact, microbial attachment to surfaces is probably the first stage of surviving in natural environment and a type of protection against environmental stresses. Because, the attached organism can detach from surfaces and transmit to finished food products, it is important to study the mechanism of attachment and inactivating procedures for attached spores (Boulané‐Petermann, 1996; Frank, 2001).

Adhesion to surfaces is influenced and enhanced by cell surface charges, hydrophobicity, and presence of particular surface structures (Gilbert et al., 1991; van Loosdrecht et al., 1987). In *Pseudomonas* spp., the adhesion to SS was due to hydrophobic interaction between the bacteria and the surface (Vanhaecke et al., 1990). Many studies have been conducted to study the mechanism of microbial attachment (Bitton and Marshall, 1980; Fletcher, 1996; Klavenes et al., 2002; Mafu et al., 1990; Marshall et al., 1971). The microbial attachment to surfaces needs 5 to 30 seconds and occurs in two stages; reversible attachment followed by irreversible attachment. The weak interaction between the substratum and bacteria is referred to as the reversible attachment, which involves van der waal attraction forces, electrostatic forces, and hydrophobic interactions. During this stage, shear forces such as rinsing can easily remove attached bacteria (Kumar and Anand, 1998; Marshall et al., 1971). Over time, reversible attachment become irreversible, when bacteria produce extracellular polymers (Sutherland, 1982), which bridge the gap between the bacteria and substratum (Boulané‐Petermann, 1996; Bower et al., 1996; Dawson et al., 1981). Several studies suggest that irreversible attachment occurs from 20 min to 4 h post contact with substratum (Lundén et al., 2000; Mafu et al., 1990; Sorongon et al., 1991). However, in the case of attachment to SS surface, the attachment to substratum takes less than one minute and rapidly increases with time. In contrast to reversible attached microbes, removal of irreversible attached cells require strong chemicals, heat, sanitizers, or application of enzymes (Bower et al., 1996).

Hydrophobicity has been identified as a dominant factor for bacterial adhesion to surfaces (Escobar-Cortés et al., 2013; Faille et al., 2007; Peng et al., 2001). Hydrophobicity of bacterial surface is determined by different methods, such as bacterial adherence to hydrocarbon (BATH), hydrophobic interaction chromatography (HIC), and the salt aggregation test (Mozes and Rouxhet, 1987; Rönner et al., 1990; Rosenberg et al., 1980). Cells with hydrophobic characteristic are more adherent to surfaces than hydrophilic cells, and most bacteria are likely to adhere to hydrophobic surfaces (Pringle and Fletcher, 1983; van Loosdrecht et al., 1987). In *Bacillus* spp., a strong correlation has been observed between spore hydrophobicity of exosporium-positive species and the adhesion. Also, similar observation was demonstrated with *Clostridium* spp. (Andersson et al., 1998; Paredes-Sabja and Sarker, 2012). More likely, attachment of spores is greater than the attachment of vegetative cell due to the spore's high hydrophobicity and the surface coverage hair-like structure (Bower et al., 1996).

Equally important, the outer layer of spore structure considered playing a role in spore adhesion due to its large collection of proteins (Doyle et al., 1984; Matz et al., 1970; Takumi et al., 1979). Several researchers have correlated the presence of an exosporium with spore hydrophobicity in several *Bacillus* species (Kjelleberg, 1984; Kutima and Foegeding, 1987). When the exosporium of spores of *B. cereus* strain T and *B. megaterium* QMB1551 are removed by chemical treatment, a decrease in the hydrophobicity was observed compared with normal spores of the same strains

(Koshikawa et al., 1989; Kutima and Foegeding, 1987).

2.8 Spore inactivation

Spore forming pathogens lead to challenges in developing countries due to their ability to survive during food processing and production. *C. perfringens* is one of the major pathogens with significant implications in the food industry, due to its ability to form spores that are resistant to various preservation approaches such as, moist heat, osmotic, nitrite, pH, prolonged frozen storage, and high pressure processing (Li and McClane, 2006a, b; Sarker et al., 2000). When spores encounter suitable conditions, they germinate, and subsequently proliferate in food during cooling and storage (McClane, 2007). An alternative strategy, referred to as the "Clean-in-Place system", has been developed in order to inactivate harmful bacteria by using alkaline detergent, rinsing, acid detergent and using sanitizers if needed. Use of the Clean-in-Place system increases food safety, shelf life, and leads to better food quality for consumer (Frank and Chmielewski, 2001; Leclercq-Perlat et al., 1994).

2.9 Disinfectant chemicals

Food processing plants use a variety of cleaning chemicals to sanitize food contact surfaces in order to inactivate spore activity or minimize the harmful bacteria. Many chemicals have been used to disinfect the adhered bacteria on SS surfaces. In a recent study, typical disinfectant agents such as ethanol, iodophores, Quarternary Ammonium Compounds have been used to sanitize SS surfaces. Results showed no

inhibitory effect of these disinfectants at maximum acceptable levels on *C. perfringens* spores adhered onto SS (Udompijitkul et al., 2013). Furthermore, various factors accompanied the effectiveness of disinfectant chemicals such as, temperature, chemical concentration, pH, characteristics of the surface being cleaned, microbial density, exposure time, and solution flow pressure (Dufour et al., 2004; Husmark and Rönner, 1992; Lelievre et al., 2001). Thus, understanding the characteristics of the chemical agent as well as the effect of this agent on pathogenic bacteria is essential in order to select the most suitable agent for the selected cleaning application.

2.9.1 Sodium hydroxide (NaOH)

NaOH is widely available and inexpensive and is one of the powerful surfactants among other alkaline solutions. Thus, using NaOH as a detergent in manufacturing plants is economical and it provides high level of hygiene. Solutions of NaOH have a pH of ≥ 9. Since NaOH is highly basic, most bacterial growth is restricted at that pH. Low concentrations of NaOH, lead to an inhibitory effect; whereas at high concentrations, it has a bactericidal effect. Depending on concentration, contact time, and temperature, NaOH has the ability to inactivate bacteria and yeast (Committee, 1996; McDonnell, 2007; Tilley, 1946). In manufacturing plants, NaOH is used as a routine cleaning agent of surfaces and equipment; however, it is considered as an aggressive chemical to inactivate microorganisms. NaOH has been known as an effective antimicrobial agent, including efficacy, low cost, ease to disposal. Moreover, NaOH is highly corrosive agent to SS

and skin and should be handled with caution (Committee, 1996; Troller, 2012).

2.9.2 Nitric acid (HNO_3)

Nitric acid was discovered between 12^{th} and 13^{th} century A.D. and for many years has been very important chemical that has used for industrial purposes (Stern et al., 1960). It is a colorless liquid that contains 50-65% of HNO_3 in water; solutions of HNO_3 have a pH of < 3. It's characterized by a unique odor that fades with increasing water. HNO_3 is a very dangerous chemical, as the high concentration of this may destroy organic tissues, cause skin burns, and inhaling the red fumes coming out of any reaction with the acid may easily cause deadly pneumonia. Therefore, care must be taken during handling (Miles, 1961). HNO_3 is formulated as detergent that can remove soiling, staining, and scaling (Troller, 2012).

2.10 Clean-in-Place (CIP)

A satisfied standard of hygiene is one of the most critical aspects to increase the safety of the consumer and quality of products. A proper cleaning and sanitization procedure is also required for high quality production (Chisti, 1999; Tamime, 2009). CIP is a very common procedure in food, dairy, brewery and beverage processing plants for sufficient chemicals cleaning in closed system. It involves piping connected to tanks, valves, connections, and pumps that distributes cleaning detergents remotely with high pressure and low volume throughout the plant (Troller, 2012).

CIP can be defined as: ”The cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator. The process involves jetting or spraying of surfaces or circulation of cleaning solutions through the plant under conditions of increased turbulence and flow velocity.” (NDA Chemical Safety Code, 1985(Romney, 1990)).

Since the 1950’s, CIP has been introduced to the industries, especially in dairy industries needed for a frequent, rapid and consistent cleaning. In recent years, CIP has been accepted by the pharmaceutical, biotechnology, and other processing operations (Chisti, 1999; Flint et al., 1997; Stewart et al., 1996). In general, tightly connected equipment in the plant is cleaned automatically using chemicals, physical processes, and thermal aspects. The CIP process involves a series of cleaning and rinsing cycles as follows:

I. Pre-rinsing with water to remove loosely adhered substances from the surface.
II. Alkaline cleaning to remove any remaining soil on the plant surface with heated solution.
III. Rinse out the alkaline solution with water at ambient temperature for preventing disruption with the following step.
IV. Acid cleaning to removing more of the remaining soil especially inorganic residues with heated solution.

V. A final rinse with water at ambient temperature. In some cases a final step of adding sanitizer is also applied (Chisti, 1999; Stanga, 2010).

The CIP system cleaning is achieved via different processes: physical action of velocity flow, chemical action of cleaning agent, and high temperature of the cleaning solution (Chisti, 1999). The CIP system is mostly dependent on chemical actions, which are selected for their ability to lift organic and inorganic residues. The most common cleaners used are alkaline (sodium hydroxide, NaOH) and acid (nitric acid, HNO_3) cleaners. The alkali wash step primarily removes protein and fats, while the acid washing step mainly removes mineral deposits and helps to remove alkaline traces from the plant surfaces (Bremer et al., 2006). There are few factors that should be taken into consideration for the bactericidal efficacy of the solutions: including concentration, temperature, and contact time. These factors vary among microorganisms. For example, 0.5% of NaOH at 120 °F for 16 min is sufficient to remove 25% of *B. subtilis* spores, whereas 1.66% of NaOH at 150 °F for 1 min is sufficient to remove 25% of *B. subtilis* (Romney, 1990). Moreover, industries have been using CIP for three primary reasons. First, it is an automated system and repeatable which reduces the chances of errors during manual cleaning. Second, it reduces labor costs and minimizes the use of water and detergents required. There also is no labor needed for disassembly of equipment and reducing the material cost used for cleaning and the cost of getting rid of detergent waste. Finally, it increases plant and product safety by cleaning cytotoxic products form the plants, and personnel have

much less contact with hazardous material (Stewart et al., 1996; Tamime, 2009; Troller, 2012).

CHAPTER 3

Inactivation of *Clostridium perfringens* Type A Spores Adhered onto Stainless Steel Surfaces by a Clean-In-Place Procedure

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Abstract

The cross-contamination of the enterotoxigenic *Clostridium perfringens* spores from contaminated food-contact surfaces onto finished food product is one of the leading causes of food-related GI diseases caused by *C. perfringens*. This is mostly due to the high resistance of *C. perfringens* spores to various disinfectants commonly used to decontaminate the food-contact surfaces in the food industry. In this study, we aimed to understand the mechanism of attachment of *C. perfringens* spores onto stainless steel (SS) surfaces and then validate the effectiveness of a simulated Clean-in-Place (CIP) regime in decontamination of SS surfaces. Our results demonstrated that spores of all tested *C. perfringens* isolates were adhered firmly onto SS surfaces and survived up to 48 h under aerobic conditions at ambient and refrigerated temperatures. The spores carrying intact spore-coat were more hydrophobic than the decoated spores, which might possibly explain the low hydrophobicity of vegetative cells. These results suggest a correlation between spore coat components and the adhesion onto surfaces. The effectiveness of the CIP cleaning agents showed a complete reduction of adhered spores onto SS surface after treating with 1% NaOH as compared to control surface, suggesting that 1% NaOH enhances the inactivation of *C. perfringens* spores adhered onto SS surfaces. Collectively, our current findings might contribute towards developing a strategy to control cross-contamination of *C.*

perfringens spores into food products, which will help reducing the risk of *C. perfringens*-associated food poisoning outbreaks.

3.1 Introduction

Clostridium perfringens is a Gram-positive, anaerobic, rod shaped bacterium. It is a non-motile pathogen that produces prolific toxins causing a wide verity of gastrointestinal (GI) diseases in humans and animals (McClane, 2007). *C. perfringens* can be classified into 5 types (A through E) based on the production of four major lethal toxins (α, β, ε, and ι toxins) (McClane, 2007; Petit et al., 1999). However, approximately 5% of C. *perfringens* type A are able to produce *C. perfringens* enterotoxin (CPE), which is an important factor for most cases of *C. perfringens* type A food poisoning (FP) as well as non-food borne (NFB) GI diseases such as antibiotic-associated diarrhea, sporadic diarrhea and nosocomial diarrheal diseases (Grass et al., 2013; Kobayashi et al., 2009; Lindström et al., 2011; Miyamoto et al., 2012; Sarker et al., 1999). In previous studies, genotyping of CPE-positive *C. perfringens* isolates reveal that CPE-encoding gene (*cpe*) can be either chromosomal- or a plasmid- borne. The chromosomal *cpe* isolates are generally associated with FP due to their high resistance to food-related preservatives such as heat, low temperature, NaCl, and nitrite, whereas the plasmid-borne *cpe* isolates are associated with NFB GI diseases and show lower resistance to environmental insults than those of the chromosomal *cpe* isolates (Collie and McClane, 1998; Li and McClane, 2006a, b; Li and McClane, 2008; Miyamoto et al., 2012; Raju and Sarker, 2007; Sarker et al., 2000). *C. perfringens* type A FP is currently ranked as the second most reported bacterial foodborne illness outbreaks in United States causing ~1 million cases per

year, and estimated cost of $309.4 million loss annually (Bennett et al., 2013; Grass et al., 2013; Hoffmann et al., 2012; Lynch et al., 2006; Sarker et al., 1999; Scallan et al., 2011; Xiao et al., 2012).

Spores of *C. perfringens* type A exhibit higher resistance to various lethal factors such as heat, osmotic stress, chemicals, prolonged frozen storage and high pressure processing than vegetative cells (Li and McClane, 2006a, b; Paredes-Sabja et al., 2007; Sarker et al., 2000). These resistant properties allow spores to survive against various preservative approaches applied in the food industry in which spores remain in the dormancy, and only resume growth once the favorable conditions are achieved (McClane, 2007; Paredes-Sabja et al., 2008). Adherence of microorganisms onto surfaces commonly found in manufacturing plants such as SS, glass, or plastic could act as a source of product contamination, eventually leading to the occurrence of foodborne disease outbreaks. Understanding the mechanisms of adhesion of harmful bacteria onto food contact surfaces is critical in order to develop effective measures to decontaminate, or at least minimize, microbial contamination onto food contact surfaces thereby reducing the risk of foodborne illnesses (Ortega et al., 2010; Peng et al., 2001; Ryu et al., 2004; Simmonds et al., 2003; Tauveron et al., 2006). Bacterial adherence to surfaces has been related to cell surface hydrophobicity and relative surface charge, as well as the presence of particular surface structures (Escobar-Cortés et al., 2013; Faille et al., 2007; Peng et al., 2001; Rönner et al., 1990; van Loosdrecht et al., 1987; Wiencek et al., 1990). Some reports suggest that the presence of particular

structures such as the outer coat or exosporium in *B. cereus* group and *C. difficile* can further enhance the adhesion process to solid surfaces (Doyle et al., 1984; Faille et al., 2007; Husmark and Rönner, 1992; Joshi et al., 2012; Tauveron et al., 2006). However, hydrophobic interaction is considered to be an important factor contributing to bacterial adhesion to solid surfaces (Faille et al., 2007; Husmark and Rönner, 1992; van Loosdrecht et al., 1987). The adherence of *Bacillus* spores and *Stapylococcus epidermidis* to solid surfaces is correlated with hydrophobicity and cell-surface negative charges (Gilbert et al., 1991; Koshikawa et al., 1989; Rönner et al., 1990). Moreover, the spores of some *Clostridium* species can be highly hydrophobic and have the ability to adhere firmly on surfaces encountered in manufacturing plants (Craven and Blankenship, 1987; Husmark and Rönner, 1992; Simmonds et al., 2003; Wiencek et al., 1990). These spores' characteristics can lead to the cross-contamination of pathogenic bacteria from contaminated food contact surfaces into finished products during food processing and handling (Andre et al., 2012; Bae and Lee, 2012; Kusumaningrum et al., 2003). Adhered pathogenic bacteria on surfaces and materials are more resistant to various disinfectants used in the food industry and could serve as a continuous source of product contamination affecting their quality, shelf-life, and safety of the consumer (Andrade et al., 1998; Andre et al., 2012; Das et al., 1998; Frank and Koffi, 1990; Holah, 2003; Hornstra et al., 2007; Kreske et al., 2006; LeChevallier et al., 1988). A recent study on *C. perfringens*, demonstrates that

commonly used disinfecting agents showed limited inhibitory effect towards *C. perfringens* spores adhered onto SS surfaces (Udompijitkul et al., 2013).

Food processing industries successfully use Clean-in-place (CIP) procedure to clean and disinfect the surface of large and fixed equipment without disassembling. The general CIP regime involves cleaning with alkaline solution (NaOH) followed by acid solution (HNO_3) in order to control bacterial contamination and remove organic and inorganic residues (Bremer et al., 2006; Romney, 1990; Stanga, 2010). However, the effectiveness of CIP regimes differ in eliminating adherence pathogenic bacteria to surfaces (Austin and Bergeron, 1995; Dufour et al., 2004; Faille et al., 2001), depending on number of factors, i.e., the concentration of cleaning solutions, treatment duration, temperature of the solutions, and the characteristics of the surface being cleaned (Boulange-Petermann et al., 2004; Lelievre et al., 2001; Stewart et al., 1996). Nevertheless, the effectiveness of the CIP procedure is debated in the literatures as some adhered bacteria are resistant to CIP (Bénézech et al., 2002; Blel et al., 2007; Faille et al., 2002; Le Gentil et al., 2010), and other adhered bacteria are decreased after applying the CIP procedure (Bremer et al., 2006; Faille et al., 2001; Hornstra et al., 2007; Parkar et al., 2004). So far, the detailed study regarding the application of CIP against *C. perfringens* spores adhered to the model food contact surface is lacking.

The purpose of this study was to 1) determine the viability of *C. perfringens* vegetative cells and spores adhered onto SS surfaces under different temperatures. 2)

Measure the surface hydrophobicity of vegetative cells and spores (intact and decoated) from various *C. perfringens* isolates. 3) Evaluate the effectiveness of the CIP procedure in removing *C. perfringens* spores from the model food contact surfaces.

3.2 Material and methods

3.2.1. Bacterial growth conditions

The bacterial strains examined in this study included 4 C. *perfringens* type A FP isolates (SM101, E13, NCTC10239, and NCTC8239) and 2 NFB isolates (F4969 and NB16) (Sarker et al., 2000). All isolates were maintained at -20 °C in a cooked meat medium (Difco, BD Diagnostic Systems, Sparks, Md., U.S.A.). Each strain was retrieved by inoculating 0.1 ml of cooked meat culture into fluid thioglycollate medium (FTG) (Difco), and incubating at 37 °C overnight. Vegetative cell cultures of *C. perfringens* were grown in a TGY (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) broth (Kokai-Kun et al., 1994).

3.2.2 Spore preparation and purification

Sporulating cultures of *C. perfringens* were prepared by using previously described method (Akhtar et al., 2008). Briefly, 0.1 ml of *C. perfringens* stock cultures were inoculated into FTG and grown overnight at 37 °C. Then 0.4 ml of the FTG cultures were transferred to a fresh 10 ml FTG medium and incubated for 8 to 12 h at 37 °C. 0.4 ml of actively growing cultures were then inoculated into 10 ml of Duncan Strong (DS) sporulation medium (1.5% protease peptone, 0.4% yeast extract, 0.1% sodium thioglycolate, 0.5% sodium phosphate dibasic [Na_2HPO_4; anhydrous], 0.4% soluble starch) (Duncan and Strong, 1968) and incubated for 24 h at 37 °C. Spore formation in DS culture was observed and confirmed by phase-contrast microscopy.

The preparation of large amounts of *C. perfringens* spores was accomplished by scaling up the aforementioned procedure. Spore cultures were purified by repeated washing with sterile distilled water through centrifuging (800 rpm, 15 min) until spore suspension was > 98% free of vegetative cells, cell debris, and germinated spores. The purified free spores were suspended in sterile distilled water and adjusted to a final optical density at 600 nm (OD_{600}) of ~6 using SmartspecTM 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) and stored at -20 °C until used (Paredes-Sabja et al., 2008).

3.2.3. SS surface preparation and adhesion of spores onto SS chip

SS chips (300 series, no. 4 finish) were purchased and prepared as 2 × 3 inches size from The Home Depot (Corvallis, OR) for adhesion and survival experiments. Prior to use, the surface of each chip was cleaned with 1% (w/v) Alconox® (VWR International, West Chester, PA) followed by rinsing with distilled water, drying, and then wrapping individually with aluminum foil. The SS chips were sterilized in the autoclave at 121 °C for 20 min and stored at room temperature (RT) until used (Udompijitkul et al., 2013). Purified spore or vegetative cell suspensions of *C. perfringens* at OD_{600} of ~6 were prepared in a suspension of 0.1 ml. Spore suspensions were heat-activated at 80 °C for 10 min or 75 °C for 20 min for FP and NFB spores, respectively, and then cooled in a water bath at room temperature for 5 min. Spore and cell adhesion to SS chips were assessed by inoculating 0.1 ml of heat-activated spores

or vegetative cells onto sterilized SS chip and spread with a sterile bent glass rod (Udompijitkul et al., 2013). All chips were contaminated under Class II biosafety cabinet (Labconco, Kansas, MO, USA.) and dried for 60 min to promote adherence. For every trial of adhesion experiment, two chips were included. After drying, SS chips were placed in sterilized plastic bags and stored under aerobic conditions at both room temperature (20 ± 2 °C) and refrigeration temperature (4 ± 1 °C).

The number of total viable cells and dormant spores was determined after 0, 1, 3, 6, 10, 24 or 48 h storage at both temperatures. The contaminated chips were aseptically transferred to sterile petri dishes and the surface of every individual chip was entirely dried by swabbing with 4 sterilized cotton swabs (Puritan Medical Products Company LLC, Guilford, ME) and soaked in 10 ml of 25 mM Na_2HPO_4 buffer adjusted to pH 7.5 and mixed vigorously with a vortex mixer (Vortex Genie2, Model G-560, Scientific Industries Inc., NY) for 1 min (Ortega et al., 2010). 1 ml of the spore suspension was then subjected to heat shock at 75 °C for 20 min in order to enumerate population of non-germinated spores and another 1 ml with no heat shock for population of total count of spores and germinated spores. The number of viable *C. perfringens* cells was assessed by serially diluting aliquots from swabs, plating onto Brain Heart Infusion (BHI) agar (Difco, BD Diagnostic Systems), and counting colonies after 24 h anaerobic incubation at 37 °C.

3.2.4. Scanning electron microscopy

Contaminated stainless steel chips were prepared as described in section 3.2.3. The chips were sputter coated with gold palladium at a 10-15 nm thickness and imaged on a Quanta Dual Beam Scanning Electron Microscope (FEI Co.) at the Oregon State University Electron Microscopy Facility (Corvallis, OR).

3.2.5. Attachment of spores onto SS surfaces

A set of chips were artificially contaminated as described in section 3.2.3 and dried for 1 h under Class II biosafety cabinet to promote adherence. The chips were transferred to a sterile petri dish containing 25 ml of 25 mM Na_2HPO_4 buffer at pH 7.5 and subjected for shaking with a shaker (Barnstead Lab-Line, Melrose Park, IL, USA) at 100 rpm for 10 min. Under the biosafety cabinet, the entire surface of the SS chips was swabbed as described in section 3.2.3 in order to enumerate the number of the remaining attached spores on the surfaces. In addition, the number of removed or detached spores from the SS chips was enumerated by plating the washed buffer itself after serial dilution.

3.2.6. Determination of cell surface hydrophobicity

Two different methods were used to assess the relative cell surface hydrophobicity of *C. perfringens* spores and vegetative cells. The bacterial adhesion to hydrocarbons (BATH) assay was performed as previously described (Sorongon et al., 1991; Wiencek et al., 1990). Briefly, spore and cell suspensions were prepared by

suspending spores in distilled water, and vegetative cells in 25 mM Na_2HPO_4 buffer (pH 7.5) at OD_{600} of 0.8 to 1.0 for a total volume of 3 ml and incubated in 35 °C water bath for 15 min. Various volumes of hexadecane (Avantor Performance Materials. Inc., Center Valley, PA, USA) (0.2, 0.6, or 1.0 ml) were added to each spore or cell suspension and agitated vigorously for 1 min with a vortex mixer. The mixture was left for 15 min to allow separation of the hexadecane and the aqueous phases. After separation, the aqueous phase was carefully removed and the OD_{600} was measured. The results were expressed as percent hydrophobicity of the suspension, calculated by the formula: $100[(A_i - A_f)/A_i]$, where A_i and A_f are the optical density of the initial suspension and the final optical density of the aqueous phase after partition, respectively. The percentage hydrophobicity was obtained as the average percent decrease of the initial OD_{600} for the different volumes of hexadecane in each trial. All surface hydrophobicity measurements for spores and vegetative cells were carried out in triplicates.

The hydrophobic interaction chromatography (HIC) assay was performed based on a previously published method (Ismaeel et al., 1987; Wiencek et al., 1990). Sepharose CL-4B (Sigma Aldrich) columns were prepared in short-tip glass Pasteur pipettes (7 mm diameter) plugged with glass wool and packed to a height of 20 mm with Sepharose. Spore suspensions of various strains were prepared by centrifuging twice and suspending in NaCl buffer to a final volume of 5 ml upon adjusting spore concentration to OD_{600} ~0.3 to 0.6, and then incubated at 35 °C for 15 min. Prior to

passing the suspension, columns were flushed with 10 ml of 4 M NaCl containing 20 mM $NaPO_4$ buffer (pH 6.8) in order to reduce charged particles of the Sepharose CL-4B thereby allowing hydrophobic interaction to occur. The 5 ml spore suspension was added to the top of the column and allowed to pass through the gel. After passing through the column, the eluent was collected, OD_{600} measured, and spore hydrophobicity percentage was calculated with formula used in BATH assay by taking an average of triplicate trials.

3.2.7. Spore decoating treatment

Purified *C. perfringens* spores at an OD_{600} of ~20 were decoated as described (Paredes-Sabja et al., 2009). Briefly, 1 ml of 50 mM Tris-HCl (pH 8.0), 1% (w/v) Sodium dodecyl sulfate, 50 mM Dithiothretiol, and 8 M Urea was added to the spore pellet and incubated at 37 °C for 90 min. After incubation, decoated spores were washed 10 times with sterile distilled water in order to isolate clear spores. BATH assay was preformed as described above in section 3.2.6. in order to validate the removal of the coat, spores were prepared for transmission electron microscopy (TEM) using sorvall ultramicrotome MT-2 for ultrasectioning. Sections were placed on the 300 mesh Cu grid and imaged with a Titan TEM made by FEI Co in Hillsboro, OR.

3.2.8. Application of CIP procedure on **C. perfringens** *spores*

The CIP procedure employed in this work was described by Bremer (Bremer et al., 2006) with some modifications in order to evaluate whether the CIP wash steps were effective in removing adhered *C. perfringens* spores onto SS surfaces. We selected two representative FP (SM101 and NCTC8239) and 2 NFB (F4969 and NB16) isolates to validate the modified CIP regime. The SS chips (2 × 3 inches) were prepared and inoculated with spores as described in section 3.2.3. for every trial, four chips were incorporated, one chip as a control "no CIP" (spore inoculation; dry for 1 hour) was treated with only distilled water at RT, and one chip as a second control to examine the effect of high temperature treatment (spore inoculation; dry for 1 hour) was treated with distilled water at 65 °C. The remaining SS chips (spore inoculation; dry for 1 hour) were treated with the caustic step (1% (w/v) NaOH at 65 °C) or the caustic and acid steps of a CIP regime (1% (w/v) NaOH at 65 °C and 1% (w/v) HNO_3 at 65 °C). After inoculating and drying, the SS chips were CIP placed in a sterile beaker containing of 1% (w/v) of NaOH solution at 65 °C and shaken by using a shaker bath (Orbit Shaker Bath, Lab-Line Inst, Inc., Melrose Park, IL, PA) for 10 min. The SS chips were then transferred to another sterile beaker containing sterile cold distilled water and shaken for 5 min, followed by treatment with 1% (w/v) of HNO_3 at 65 °C and shaken for 10 min. Finally, the treated chips were soaked, with shaking in sterile cold distilled water for 5 min. After each water-soaking step, SS chips were removed to determine the number of viable cells recovered after each step as described in 3.2.3. The experiments with the two control chips were performed simultaneously

following the CIP steps, but using sterile distilled water as the cleaning agent at two different temperatures (RT and 65 °C). In order to determine the number of viable cells, the SS chips were transferred to a sterile petri dish for bacterial enumeration by the technique described in section 3.2.3. Each test step was performed in a separate sterile beaker with 125 ml of solutions and shaking at 150 rpm. The experiment was performed in triplicate.

3.2.9. Statistical analysis

The analysis of variance procedures were performed using the statistical software SAS version 9.3 (SAS Inst. Inc., Cary, N.C., USA), and multiple comparisons of mean values were established by Tukey's test at the significant level of 0.05. Error bars in all experiments represent the standard deviations.

3.3. Results

3.3.1. Survival of C. perfringens *on SS chips*

Spores of all tested strains survived on SS chip surfaces at RT and 4 °C for up to 48 h (Fig. 3.1 and Table 3.1, 3.2). The spore concentration remains approximately unchanged for up to 48 h of storage time, indicating that during this time, the heat-activated spores maintained their dormancy, and only minor spore population germinated as reflected by the similarity in CFU counts obtained for dormant spores (Gray bars; Fig. 3.1) and total cells (Black bars; Fig. 3.1). Moreover, the adherent surviving spores on the SS chips throughout the storage times and under both conditions reached ~10^4 log CFU/cm^2 to 10^5 log CFU/cm^2 for all FP and NFB isolates tested. The final number of adhered spores onto SS chips was relatively similar (~5 $\log_{10}$ CFU/cm^2) among all strains ($p > 0.05$) at the end of storage period. However, the survival rate for NFB spores was slightly greater than FP spores ($P > 0.05$) at RT and 4 °C (Table 3.1, 3.2). In contrast, vegetative cells of both FP and NFB isolates did not survive on the SS chip after 30 min of aerobic incubation at RT or 4 °C (data not shown). This could be attributed to a couple of factors such as aerobic conditions and loss of some resistance traits to survive in dry conditions (Hornstra et al., 2007; Wiencek et al., 1990).

The adherence of *C. perfringens* spores onto SS chips was further confirmed by scanning electron micrographs of SS chips contaminated with spores of FP strain SM101 and NFB strain F4969 (Fig. 3.2). The adherence of *C. perfringens* spores

appeared to take place in single layer clusters attached together by extracellular materials (Fig. 3.2A-D). Collectively, our results demonstrate that spores of the *C. perfringens* FP and NFB isolates are capable of surviving on the model food contact surface for up to two days.

3.3.2 Attachment of C. perfringens *spores onto SS surface*

To test whether *C. perfringens* spores can adhere firmly to SS surfaces, spore-contaminated SS chips were subjected to soaking in buffer (25 mM Na_2HPO_4 pH 7.0) and shaking (100 rpm for 10 min) before enumeration of CFU. SS chips were inoculated with ~10^7 CFU/ml of *C. perfringens* spores of each particular FP and NFB isolates in order to yield the initial spore contamination level of ~6 $\log_{10}$ CFU/cm^2 of SS after 1 h drying. As shown in Fig. 3.3, after the detachment procedure, a significant number of spores of each tested strains (~ >5 $\log_{10}$ CFU/cm^2) remained attached to SS chips. The remaining spores represented the population that firmly attached to SS surface. Furthermore, a fraction of loosely adhered spores were detached from the chips, as ~3-4 log CFU/cm^2, depending on strains, could be recovered from the soaking buffer. Collectively, these results highlight the persistence characteristics of the enterotoxigenic *C. perfringens* spores once attached onto the food contact surface and it is consistent with the fact that approximately 15% of food-related *C. perfringens* outbreaks have been linked to cross-contamination from dirty surfaces and equipment (McClane, 2007).

3.3.3. Spore and vegetative cell surface hydrophobicity

As bacterial adhesion to solid surfaces has been linked to cell surface hydrophobicity, we next examined the hydrophobicity of vegetative cells and spores of *C. perfringens* FP and NFB isolates using the BATH assay. Our findings indicated that, for each strain, spores had significantly higher affinity to the hexadecane than vegetative cells ($p < 0.05$). Spores of strain SM101 exhibited the highest hydrophobicity (~89%) than spores of all tested strains (Fig. 3.4), whereas spores of NB16 and E13 strains exhibited the lowest hydrophobicity (~ 64%). Vegetative cells of four of six tested strains exhibited significantly low hydrophobicity (< 20%) with the exception of NCTC10239 and NB16 (Fig. 3.4).

Next, we employed HIC with Sepharose CL-4B to measure the relative spore surface hydrophobicity. It was observed that spores tended to adhere to Octyl-sepharose when passed through the column. Results obtained form HIC showed that spores of most tested strains also exhibited high hydrophobicity of >70% (Fig. 3.5), while strain E13 exhibited lowest hydrophobicity (55%). The percent hydrophobicity obtained from HIC was slightly lower than those obtained from BATH assay, but this difference was not statistically significant ($p > 0.05$). The reduced level of spore hydrophobicity measured by HIC assay might be a result of spore adhering to Sepharose gel matrix; thus, they did not pass through into the eluent. Overall, these results clearly show that *C. perfringens* spores are hydrophobic in nature and the degree of spore hydrophobicity is strain-dependent.

3.3.4. Hydrophobicity of decoated Spores

Previous studies suggested that spore structures such as, spore coat or exosporium might play a role in adherence of spores to solid surfaces (Doyle et al., 1984; Faille et al., 2002; Koshikawa et al., 1989). In order to determine the relationship between spore coat and the hydrophobicity, we examined the surface hydrophobicity of decoated spores of SM101, NCTC8239, F4969, and NB16 using the BATH assay and compared this to the surface hydrophobicity of intact spores. Results showed that intact spores had higher affinity to hexadecane than decoated spores. The surface hydrophobicity of decoated spores varied among strains, but all strains showed ~ 40% lower in percent hydrophobicity as compared to the values obtained from intact spores (Fig. 3.6). The transmission electron micrographs in Fig. 3.7 confirmed that the spore decoating treatment employed in this work was able to remove the spore outer coat in the representative *C. perfringens* FP strain SM101. Fig. 3.7A clearly shows the presence of outer coat layer in the intact spores, whereas this layer was absent in spores subjected to the decoating treatment (Fig. 3.7B). Therefore, the lack of spore coat could likely explain the partial reduction in degree of spore hydrophobicity thereby exhibiting the role of this particular spore's structure or its composition in the establishment of hydrophobic characteristics.

3.3.5. Effectiveness of modified CIP procedure on removing **C. perfringens** *spores*

The effect of a modified CIP procedure on bacterial removal was determined against four representative *C. perfringens* FP (SM101 and NCTC8239) and NFB isolates (F4969 and NB16).

The bacterial numbers for each strain was standardized against the "no CIP" control chips and compared with chips treated either with NaOH or with NaOH + HNO_3 (Fig. 3.8). The initial population of adhered spores onto the SS surfaces was ~ 6 $\log_{10}$ CFU/cm^2. The control chips "no CIP" exhibited small reduction of ~1 $\log_{10}$ CFU/cm^2 after applying CIP wash steps using only distilled water at RT and this could be likely caused by the removal via the physical force. Since CIP procedure depends on; chemical, physical, and thermal factors, it is imperative to evaluate whether applying only heat, would have any effects on the adhered *C. perfringens* spores. The CIP cleaning steps were followed using distilled water at 65 °C. This regime resulted in similar reduction of spores as "no CIP" control chips indicating that heat alone did not provide superior effect over water cleaning on removing adhered spores. Most importantly, effect of CIP cleaning steps, NaOH + HNO_3 at 65 °C, on adhered spores of *C. perfringens* strains on the SS chips resulted in a complete inhibition ($p < 0.05$) in numbers of remaining survived cells. All tested strains exhibited a reduction of ~ 5 $\log_{10}$ CFU/cm^2 after applying the CIP wash steps on contaminated chips. Hence, these results suggest that the CIP agents and procedure used in this work was highly effective against *C. perfringens* spores adhered onto the SS chips. Consequently, the effect of each step of CIP regimen on adhered *C. perfringens* spores was as followed.

The effectiveness of NaOH alone in reducing the number of adhered spores was also compared to the control chips. Interestingly, there was no survival spores found following this particular step; thus, complete inhibition of attached spores onto chips was obtained with this treatment. Collectively, the data from this work show a strong impact of caustic wash NaOH towards the adhered *C. perfringens* spores onto the SS surfaces, and thus suggest that this modified CIP procedure can effectively apply to decontaminate and prevent cross-contamination from SS surfaces.

3.4. Discussion

In the food industry, adherence of microorganisms to surface found in manufacturing plants such as stainless steel, glass, and plastic has been controversial to food manufacturers from the viewpoints of controlling biofilm formation, maintaining quality of the products, ensuring safety of the consumer, and concerning over the emergence of microbial resistance to cleaning and sanitizing procedures (Andre et al., 2012; Simmonds et al., 2003). The focus of this study is to acquiring knowledge on the adherence of the enterotoxigenic *C. perfringens* spores onto SS surfaces as well as evaluating the effectiveness of typical CIP agents in removing adhered spores.

Our current results demonstrated that spores of *C. perfringens* type A were able to maintain their survivability and remain firmly attached on SS surfaces under a given set of aerobic conditions (RT and 4 °C) up to 48 h. The adhesion extent of *C. perfringens* spores onto SS surfaces was found to be about 10^4 log CFU/cm^2, which is in agreement with previously found adhesion extent of *S. typhimurium*, *S. enteritidis* and *L. monocytogenes* to SS surfaces (Bae et al., 2012; Casarin et al., 2014; Chia et al., 2009). A similar adhesion extent of 10^2 log and 10^3 log CFU/cm^2 was also found when *L. monocytogenes* and *Salmonella* spp., respectively, were adhered to plastic and glass surfaces (Chae and Schraft, 2000; Stepanović et al., 2004).

Many researchers proposed that the event of bacterial adhesion to surfaces known to take place in two stages, an initial reversible attachment which is a weak

interaction between the bacteria and substratum, followed by a time dependent irreversible adhesion resulting from the anchoring of appendages and/or the production of extracellular polymers (Chmielewski and Frank, 2003; Frank, 2001; Marshall et al., 1971). However, in our study, when soaking and shaking the spore-contaminated SS surfaces in the buffer, a portion of spores (~ $3 \log_{10}$ CFU/cm^2) were detached from the SS surfaces, while the majority remained attached to the surfaces. These findings suggest that, some spores were loosely attached to the SS, whereas others attached more firmly to the SS and could not detach easily after soaking in buffer with shaking at 100 rpm for 10 min. The rational for variation in attachment capacity among spore population within the same strain is still unclear and this could be attributed to a single-cell difference in spore's surface characteristics interacting with the inanimate surface. Furthermore, our results are consistent with previous findings in which the adhered cells of *S. epidermidis* and *E. coli* to SS could be removed by 82% and 35%, respectively, after receiving a whirlpool rinsing treatment (Ortega et al., 2008, 2010).

According to the literatures, the significance of the surface hydrophobicity is positively correlated to the adhesion capability of bacteria onto surfaces (Gilbert et al., 1991; Hogt et al., 1983; Husmark and Rönner, 1992; van Loosdrecht et al., 1987; Wiencek et al., 1991). In the current study, spores of all six *C. perfringens* isolates exhibited a significantly higher hydrophobicity ($p < 0.05$) than the vegetative cells (Fig. 3.3). Similar observation was found when the hydrophobicity of *Bacillus* and

Clostridium spp. was measured by BATH assay used in this study and results reported that spores were ~ 67% - 80% more hydrophobic than the vegetative counterparts (Craven and Blankenship, 1987; Koshikawa et al., 1989), whereas *B. subitils* 168 spores exhibited the lowest hydrophobicity of ~13% among the other spores of *Bacillus* spp. (Doyle et al., 1984). The earlier studies exhibited that vegetative cells of *Bacillus* and *Clostridium spp*. tend to have lower affinity to hexadecane as measured by BATH assay (Doyle et al., 1984; Koshikawa et al., 1989; Wiencek et al., 1990), which is in consistent with our results where vegetative cells of most tested isolates showed percentage hydrophobicity of < 20%, excluding strains NCTC10239 and NB16 that had higher level of hydrophobicity of ~ 50% (Fig. 3.3). As the controversial observation regarding degree of hydrophobicity and spore attachment capacity has been reported (Simmonds et al., 2003), we also measured *C. perfringens* spore hydrophobicity using an alternative method HIC assay. We found good correlation between the results obtained with BATH and HIC assays ($P > 0.05$); there was a slight variation in the percentage hydrophobicity of the corresponding strains but the index number for the strains was relatively consistent between the two methods.

It has been suggested that the presence of the outer coat or the exosporium contributes to the hydrophobicity of the surfaces and enhance bacterial adhesion to organic (human adenocarcinoma cells) or inorganic (stainless steel) surfaces (Doyle et al., 1984; Joshi et al., 2012; Paredes-Sabja and Sarker, 2012). Studies on several *Bacillus* species found that the outer coat or the exosporium of the bacterial spores

possess significant amounts of proteins which play a role in the establishment of hydrophobicity unlike the vegetative cells that lack surface proteins (Doyle et al., 1984; Kjelleberg, 1984; Matz et al., 1970). In this study, when we removed the spore coat by chemical treatments, the decoated *C. perfringens* spores exhibited significantly decreased hydrophobicity ($p < 0.05$) as compared to intact spores, suggesting that spore coat may play a role in spores' hydrophobicity. Our result is in agreement with other studies with *B. cereus* T and *B. megaterium* ATCC 12872 in which a decreased adherence to hexadecane was observed when the spore exosporium was removed by chemical treatments (Koshikawa et al., 1989; Kutima and Foegeding, 1987). Our results in conjunction with previous findings support that the spore outer coat influences the hydrophobic interactions, which may influence in a reduction of spore adhesion onto SS surfaces. Therefore, knowledge of the structural properties of *C. perfringens* spore coat proteins will increase our understanding of the coat-specific protein that affects the hydrophobicity and adhesion to solid surfaces.

CIP regime has been implemented by many food manufacturing plants as cleaning system to eliminate or inactivate biofilms formed by various food-related bacteria, such as *Streptococcus thermophilus* and *Bacillus* spp. (Flint et al., 1999; Parkar et al., 2004). Since the CIP system was difficult to mimic in our laboratory, a modified CIP regime that included cleaning conditions of sodium hydroxide followed by nitric acid at temperature of 65 °C was employed. Throughout this study, every cleaning treatment was performed in a shaker water bath with temperature control in

order to maintain similar condition as food manufacturer's CIP regime. It was found that after alkaline (1% NaOH) and acid (1% HNO_3) treatments, the number of bacterial spores were completely reduced to undetectable limit as compared to controls chips. However, very surprising result is that *C. perfringens* spores could not survive through an alkaline (1% NaOH) treatment. These findings were consistent over the course of 6 trials, each with all four strains tested (SM101, NCTC8239, F4969, and NB16). Thus, these results strongly indicate that caustic agent at typical concentration and temperature used in the food industry can be successfully applied to remove spores of *C. perfringens* type A attached onto SS surfaces. In contrast to our findings, the previous studies showed no significant effects of treatments with caustic and acidic agents against dairy biofilm developed onto SS surfaces (Bremer et al., 2006; Dufour et al., 2004; Flint et al., 1999). It is important to note that our experiment was conducted against attached spores of a single bacterial strain rather than bacterial biofilms, which is usually more resistant to cleaning and disinfecting agents (Faille et al., 2001; Flint et al., 1997; Peng et al., 2002).

In conclusion, our study demonstrates the following findings: 1) *C. perfringens* type A spores adhered firmly onto the SS steel surfaces and survived under aerobic conditions at refrigerated and ambient temperatures up to 48 h of storage, unlike the vegetative cells which exhibited high sensitivity to aerobic conditions; 2) Spores exhibited higher level of hydrophobicity than vegetative cells, and the hydrophobicity degree demonstrated a positive correlation to spore adhesion capacity onto SS

surfaces; 3) The spore outer coat played an important role in the hydrophobicity of the spores; 4) The CIP cleaning agents successfully eliminated contaminated *C. perfringens* spores from the SS chips, and 1% NaOH was sufficient to decontaminate all attached spores from the SS chips in 10 min.

Finally, since bacteria harbored in the biofilm is generally more resistant to cleaning and sanitizing regimes than planktonic cells, it is tempting to investigate whether the typical CIP procedure employed in the food industry is efficient in reducing or eliminating *C. perfringens* that embedded in biofilm matrix. Therefore, further studies are desired to evaluate the relationship between the spore structure and adhesion strength onto surfaces and the effectiveness of CIP system in removing *C. perfringens* biofilms from a variety of food contact surfaces.

Figures

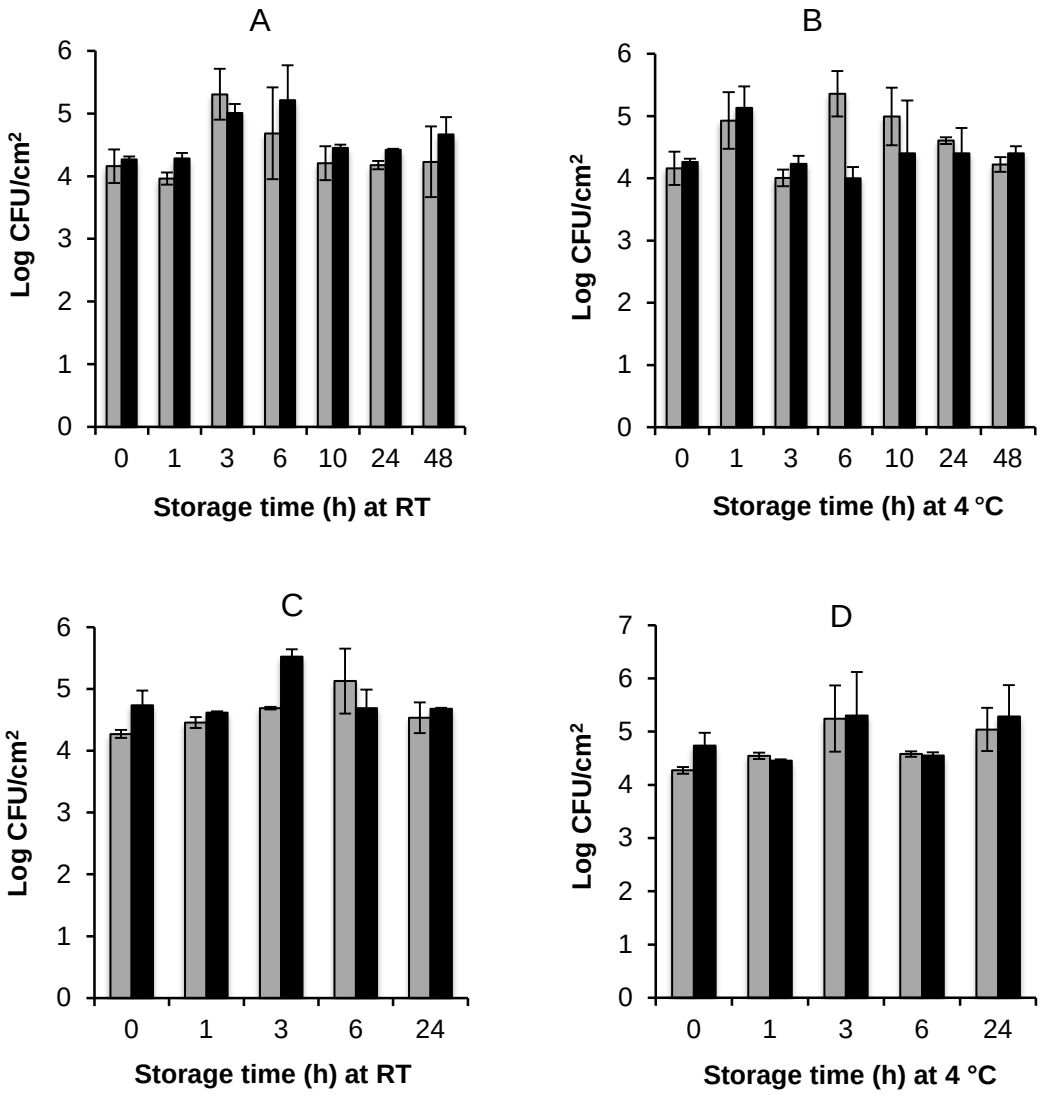

Fig. 3.1. Survival of *C. perfringens* spores onto SS chips at RT and 4 °C. Spores of FP strain SM101 (A, B), and NFB strain F4969 (C, D) were inoculated onto SS chips and dried for 1 h. Survival was determined after different time point of incubation by swabbing and plating technique as described in Material and methods. Heat-treated spore suspension (grey bars) are the enumeration of the remaining dormant spores, non-heat treated spores (black bars) are the enumeration of total viable cells. Data were average of triplicate trials and error bars represent standard deviation.

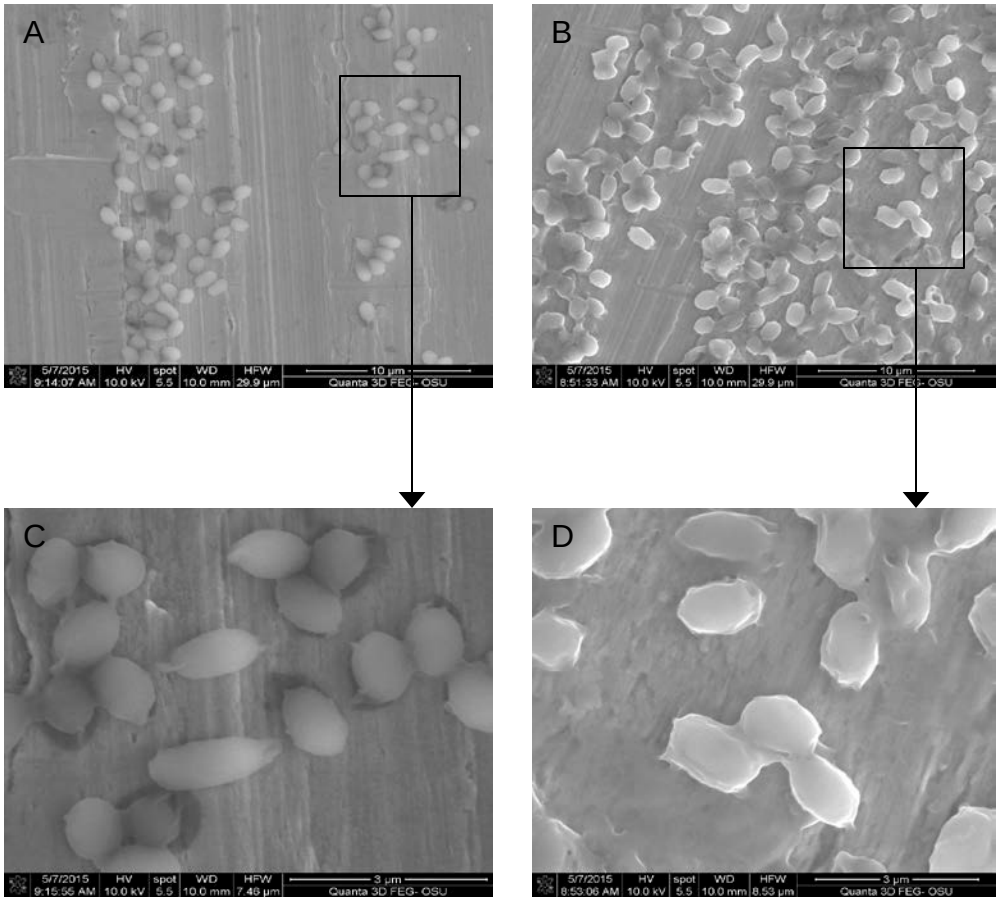

Fig. 3.2. Scanning electron micrographs of *C. perfringens* spores attached onto SS chips. The SS chips were contaminated with spores of SM101 (A) and F4969 (B) and dried in aerobic conditions then analyzed by SEM. The magnified images of attached spores of SM101 (C) and F4969 (D) showing that the attachment occurs in a single layer of clusters.

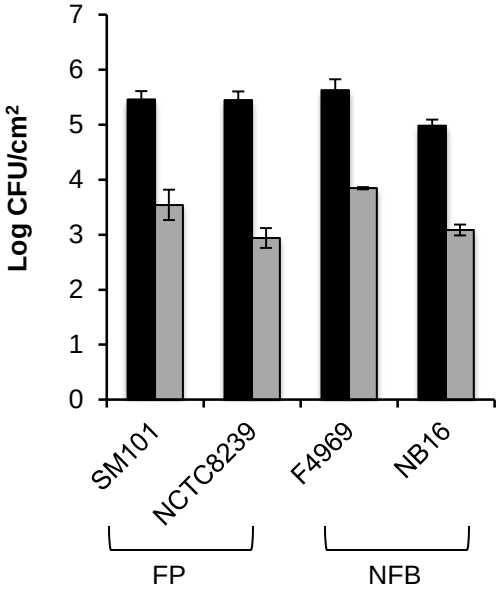

Fig. 3.3. Attachment of *C. perfringens* onto SS chips. Contaminated SS chips with spores of FP isolates (SM101, NCTC8239) and NFB isolates (F4969, NB16) were subjected for soaking and shaking procedure as described in Material and methods. The detached spores (grey bar) were counted by plating the buffer onto BHI agar, firmly attached spores onto SS surface (black bar) were counted by swabbing the surface and plating onto BHI agar. Data were the average of triplicate trials and error bars represent standard deviation.

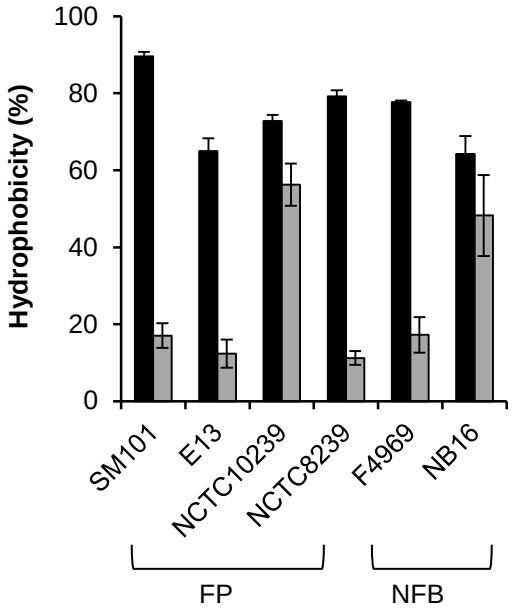

Fig. 3.4. Hydrophobicity of *C. perfringens* spore and vegetative cell as measured by BATH assay. Different concentrations of hexadecane were added to suspensions of *C. perfringens* spores (black bars) and vegetative cells (grey bars) of FP strains SM101, E13, NCTC10239, and NCTC8239; NFB strains F4969, and NB16 and left for 15 min for partition. After partition forms by hexadecane, the aqueous phase was carefully measured and the percent hydrophobicity is the percent decrease of OD_{600} in the aqueous phase after partition. Data are the average of different concentrations of hexadecane and error bars indicate the standard deviation of triplicates trials.

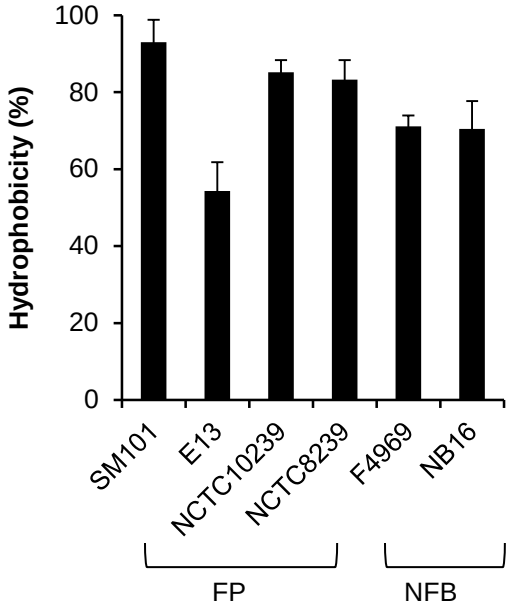

Fig. 3.5. Hydrophobicity of *C. perfringens* spore as measured by HIC. Spore of *C. perfringens* FP strains SM101, E13, NCTC10239, and NCTC8239; NFB strains F4969, and NB16 were added to Sepharose columns prepared in pipettes plugged with glass-wool as described in Material and methods. The percent hydrophobicity was measured by the percent decrease of OD_{600} in the eluent after passing through Sepharose gel matrix in the column. Data are the average of triplicate trials and the error bars are the standard deviations from the mean.

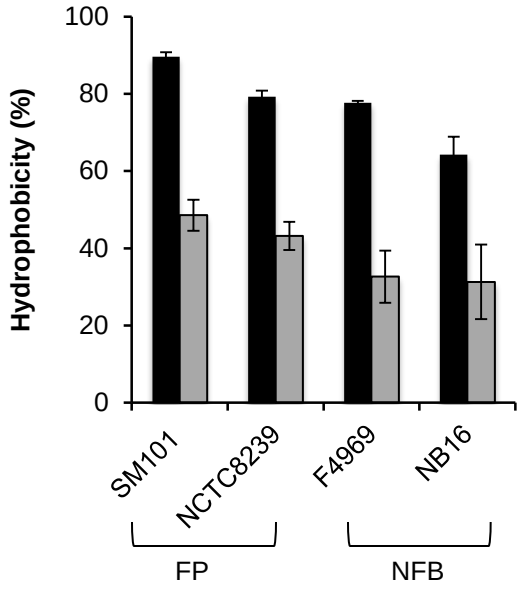

Fig. 3.6. Hydrophobicity of intact and decoated *C. perfringens* spores. Spores of FP isolates SM101, E13, NCTC10239, and NCTC8239; NFB isolates F4969, and NB16 were decoated by chemical treatment as described in Material and methods. Intact spores (black bars) and decoated spores (grey bars) were assessed for the hydrophobicity characteristic by BATH assay. The percent hydrophobicity is the percent decrease of OD_{600} in the aqueous phase after partition from the hexadecane. Data were the average of different concentrations of hexadecane and error bars indicate the standard deviation of triplicates trials.

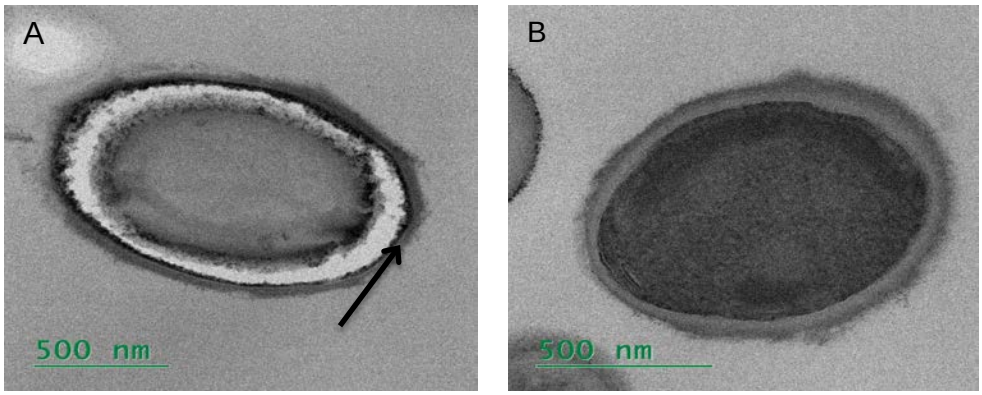

Fig. 3.7. Transmission electron microscopy images, showing ultrastructure of normal intact spores (A) and decoated spores (B) of SM101.The outer coat (indicated by arrow) was removed after applying decoating treatment on normal spores. Sorvall Ultramicrotome MT-2 was used for ultrathin sectioning, sections were placed in the 300 mesh Cu grid and imaged with a Titan TEM made by FEI Co. in Hillsboro, OR.

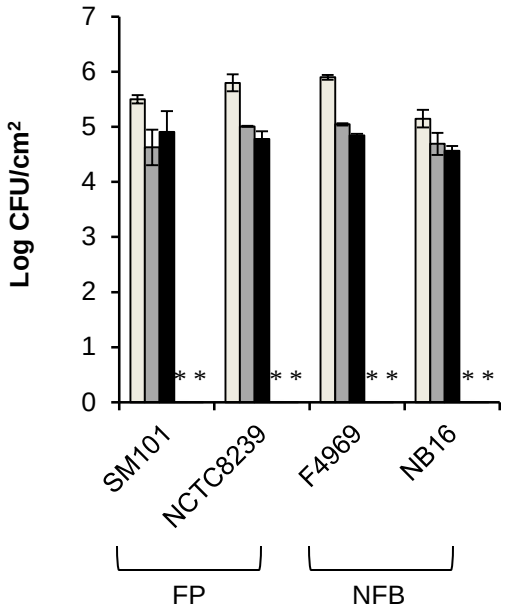

Fig. 3.8. Effect of CIP regime on *C. perfringens* spores. Contaminated SS chips of FP isolates (SM101, NCTC8239) and NFB isolates (F4969, NB16) were subjected to CIP caustic and acidic agents. The populations of spores adhered onto SS chips initially (white bars), control chips "No CIP" (grey bars) treated with distilled water at RT, second control chip (black bars) treated with distilled water at 65 °C, chips treated with only NaOH (horizontal bars), and CIP applied chips (black and white bars) treated with NaOH + HNO_3 were examined as described in Material and methods. Asterisks indicate no survival on stainless steel surfaces could be detected after treatment with NaOH only or NaOH followed by HNO_3. Data are average of triplicate trials and error bars represent standard deviation.

Tables

Table 3.1. The number of dormant spores (heat-treated) and total cell count (non heat-treated) of various strains of the enterotoxigenic *C. perfringens* adhered on stainless steel surfaces at room temperature

Strain	0 h		1 h		3 h		6 h		24 h	
	NHS[a]	HS[b]	NHS[a]	HS[b]	NHS[a]	HS[b]	NHS[a]	HS[b]	NHS[a]	HS[b]
NCTC8239	4.53±0.08	4.5±0.09	4.39±0.19	4.27±0.17	4.60±0.16	5.25±0.61	4.59±0.25	4.53±0.56	4.45±0.1	4.44±0.4
NCTC10239	4.42±0.11	4.35±0.05	4.89±0.33	4.41±0.08	4.88±0.43	4.85±0.48	4.56±0.1	4.39±0.15	4.38±0.02	4.08±0.13
E13	4.63±0.46	4.48±0.36	4.36±0.22	4.13±0.19	4.20±0.27	3.99±0.27	4.48±0.21	4.20±0.42	4.45±0.1	4.18±0.07
NB16	4.41±0.08	5.05±0.35	4.85±0.40	4.12±0.21	4.50±0.1	5.09±0.51	4.48±0.02	4.30±0.24	4.38±0.13	4.67±0.01

The mean ± standard deviation of the heat-activated spore inoculum was between 6.81±0.48 and 6.26±0.07 $\log_{10}$ CFU/ml, which was used to artificially contaminate stainless surfaces.

[a] is mean ± standard deviation of the number of total viable cells in $\log_{10}$ CFU/cm^2 that was inoculated and dried on stainless steel surfaces for 1 h (the same stainless steel coupons used to examine the dormant spore counts). The number of total viable cells was determined immediately (0 h) and after indicated periods obtained from plating swabbing samples as described in Material and Methods.

[b] is mean ± standard deviation of the number of heat-activated spores in $\log_{10}$ CFU/cm^2 that was inoculated and dried on stainless steel surfaces for 1 h. The number of dormant spores was determined immediately (0 h) and after indicated period after receiving heat treatment (75 °C, 20 min) to inactivate the germinating and vegetative cells as described in Material and methods.

Table 3.2. The number of dormant spores (heat-treated) and total cell count (non heat-treated) of various strains of the enterotoxigenic *C. perfringens* adhered on stainless steel surfaces at 4 °C

Strain	0 h		1 h		3 h		6 h		24 h	
	NHS[a]	HS[b]	NHS[a]	HS[b]	NHS[a]	HS[b]	NHS[a]	HS[b]	NHS[a]	HS[b]
NCTC8239	4.53±0.08	4.5±0.09	5.47±97	4.23±0.11	5.54±0.73	4.77±0.34	4.53±0.31	4.43±0.31	4.31±0.13	4.04±0.17
NCTC10239	4.42±0.11	4.35±0.05	4.66±0.12	4.46±0.12	4.33±0.07	4.08±0.12	4.72±0.06	4.65±0.05	4.56±0.06	4.04±0.25
E13	4.63±0.46	4.48±0.36	4.39±0.21	4.24±0.39	4.48±0.27	4.33±0.18	4.43±0.08	4.18±0.08	4.5±0.15	4.52±0.04
NB16	4.41±0.08	5.05±0.35	4.48±0.12	4.59±0.31	4.93±0.64	4.29±0.06	4±0.13	4.60±0.07	4.2±0.16	4.38±0.20

The mean ± standard deviation of the heat-activated spore inoculum was between 6.81±0.48 and 6.26±0.07 $\log_{10}$ CFU/ml, which was used to artificially contaminate stainless surfaces.

[a] is mean ± standard deviation of the number of total viable cells in $\log_{10}$ CFU/cm^2 that was inoculated and dried on stainless steel surfaces for 1 h (the same stainless steel coupons used to examine the dormant spore counts). The number of total viable cells was determined immediately (0 h) and after indicated periods obtained from plating swabbing samples as described in Material and Methods.

[b] is mean ± standard deviation of the number of heat-activated spores in $\log_{10}$ CFU/cm^2 that was inoculated and dried on stainless steel surfaces for 1 h. The number of dormant spores was determined immediately (0 h) and after indicated period after receiving heat treatment (75 °C, 20 min) to inactivate the germinating and vegetative cells as described in Material and methods.

CHAPTER 4

Conclusion

Clostridium perfringens is a Gram-positive, anaerobic and endospore forming bacterium that has the ability to produce at least 15 toxins. *C. perfringens* type A is one of the most important human GI pathogen that causes food poisoning, antibiotic-associated diarrhea, sporadic diarrhea, and gas gangrene. This pathogenic bacterium is ubiquitously found in the environment, as highly resistant spores allowing them to survive under harsh conditions. Besides, under favorable growth conditions spores are able to germinate and grow rapidly into actively grown cells causing disease in human and animals. *C. perfringens* is a major concern to food industry due to its ability to adhere to surfaces commonly encountered in food processing plants and resistance to various lethal factors and disinfectants applied. Inactivating dormant spores has been very challenging to food industries; therefore understanding the mechanism of *C. perfringens* spore adhesion to surfaces and developing an effective strategy to inactivate the dormant spore is essential.

In this study, we evaluated the adhesion and survival of *C. perfringens* type A FP and NFB isolates onto SS chips as a model food contact surface, and its relation to surfaces hydrophobicity of *C. perfringens*. Also, we examined the efficacy of a modified CIP procedure to decontaminate *C. perfringens* type A FP and NFB isolates adhered onto SS chips. Results showed survival of *C. perfringens* spores on SS chips

up to 48 h under aerobic conditions unlike the vegetative cells that showed no survival rate on SS chips under aerobic conditions. Furthermore, *C. perfringens* spores exhibited significantly higher surface hydrophobicity than vegetative cells. However, removing the spore outer coat resulted in decrease in the surface hydrophobicity of *C. perfringens* spores, suggesting a role of outer coat in adhesion to surfaces. The CIP wash steps shown to be effective on removing the adhered spores onto SS chips. After applying NaOH wash step, no survival spores could be detected from the SS chips, which indicate the sensitivity of *C. perfringens* spores to NaOH solution. Collectively, our current study provide valuable results that help developing a strategy to control cross-contamination of *C. perfringens* spores into food products, which should reduce the risk of *C. perfringens*-associated food poisoning outbreaks.

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