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Clostridium difficile: A Study on its Potential as a Food-borne Pathogen and Strategies for Controlling its Transmission

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***Clostridium difficile*: A Study on its Potential as a Food-borne Pathogen and Strategies for Controlling its Transmission**

Genevieve Flock, Ph. D

University of Connecticut, 2017

Clostridium difficile is a gram-positive, spore-forming, anaerobic, nosocomial pathogen affecting hospitalized patients taking long-term antibiotics. The bacterium is responsible for over 500,000 cases and 29,000 deaths annually (Lessa et al., 2015), with ~ \$3 billion in health-care costs (O'Brien et al., 2007). There has been the emergence of a new hypervirulent strain of *C. difficile* known as North American Pulsotype 1 (NAP1), which is responsible for severe infections. Further, an increase in the incidence of *C. difficile* infections outside of hospital environments (Hensgens et al., 2012; McDonald et al., 2006; Rupnik et al., 2009) is observed. It is reported that 32% of all *C. difficile* illnesses in the United States are community associated *C. difficile* infections, especially in individuals with no recent hospitalization (Lessa, 2013). *C. difficile* has been isolated widely from the environment in many countries, especially from soil and water. The persistence of *C. difficile* in the environment and isolation of hypervirulent *C. difficile* from food animals and foods suggest a potential role for foods as a source of *C. difficile* infection in the community.

In this dissertation, the prevalence of *C. difficile* in ready-to-eat lettuce collected from retail stores in Connecticut was investigated. The results showed a prevalence rate of 23% in ready-to-eat lettuce, with the isolates being toxigenic and resistant to multiple antibiotics. The survival of *C. difficile* in ready-to-eat lettuce bags packaged with modified atmosphere packaging (MAP) conditions was also studied. *C. difficile* spores were found to remain viable under MAP, but did not increase in number over 10 days of refrigerated storage.

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As the second objective, the viability of *C. difficile* spores in ground beef subjected to chilling, freezing and cooking was investigated. Results revealed that chilling for a week or freezing for 12 weeks did not affect the survival of *C. difficile* spores ($p > 0.05$). Moreover, cooking ground beef to the USDA recommended internal temperature (71.1°C) decreased spore counts only by 1 log CFU/g compared to uncooked controls.

Since no previous research investigated the viability of *C. difficile* spores in any fermented meat product, the effect of acidity (fermentation) and cooking on the viability of *C. difficile* spores in ready-to-eat, fermented pork summer sausage over a period of 60 days at 4°C was determined. Results indicated that *C. difficile* spores can survive the acidity and cooking in fermented pork summer sausage, underscoring the need for effective intervention strategies to control *C. difficile* contamination of pork carcasses.

C. difficile spores can persist on hospital surfaces and equipment for prolonged periods of time, thereby serving as a potential source of infection. Commonly contaminated hospital surfaces and equipment include, floors, toilets, windowsills, bedrails, bedside-tables, thermometers, blood-pressure cuffs, and intravenous catheters (Dubberke et al., 2008; Gerding et al., 2008; Guerrero et al., 2012). *C. difficile* spores are resistant to most of the commonly used physical and chemical disinfectants, including household dilutions of sodium hypochlorite (Edwards et al., 2016; Fawley et al., 2007; Gerding et al., 2008). Moreover, sodium hypochlorite is not suitable for sanitizing some medical surfaces and devices. Therefore, as the fourth objective, the efficacy of a novel disinfectant, namely octenidine hydrochloride (OH), for reducing *C. difficile* spores on stainless steel surfaces was evaluated. Application of OH (5%)

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directly on stainless steel surfaces or wiping with OH decreased populations of *C. difficile* spores significantly compared to controls.

In infected humans, sporulation of *C. difficile* vegetative cells occurs in the colon, and newly formed spores shed in the feces can potentially contaminate hospitals and healthcare facilities, leading to new infections through the fecal-oral route (Barbut, Menuet et al. 2009). Therefore, it is critical to control *C. difficile* sporulation in the human gastrointestinal tract for controlling the infection and its transmission. Aspirin (acetylsalicylic acid) is a non-steroidal anti-inflammatory drug widely used in medicine (Soni 2005). In an observational study of adults diagnosed with *C. difficile* infection, aspirin was identified as a factor that reduced mortality (Saliba, Barnett-Griness et al. 2014), although the mechanism behind this is not known. Therefore, as the final objective, the effect of aspirin on *C. difficile* toxin production, spore production, spore germination and spore outgrowth was investigated. Although aspirin had no effect on *C. difficile* toxin production, it reduced spore production and spore outgrowth significantly in *C. difficile* ($p < 0.05$). Results justify follow-up studies in an appropriate animal model to validate the anti-sporulation effect of aspirin against *C. difficile*.

***Clostridium difficile*: A Study on its Potential as a Food-borne Pathogen and Strategies for
Controlling its Transmission**

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**Bachelors of Science, University of Connecticut, 2012
Masters of Science, University of Connecticut, 2015**

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at the University of Connecticut**

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APPROVAL PAGE

Doctor of Philosophy Dissertation

***Clostridium difficile*: A Study on its Potential as a Food-borne Pathogen and Strategies for Controlling its Transmission**

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The surface of each disc was inoculated with 100 ul of *C. difficile* spores (ATCC BAA 1805) with 1.7×10^6 spores. The discs were then subjected to treatments for 10 min including, control, ethanol control, 1%, 2%, 3%, 4%, or 5% OH. The dilutions were pour-plated with BHIT agar supplemented with CDMN and incubated anaerobically for 48 h after which colonies were enumerated.

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List of Abbreviations

BHI	Brain heart infusion
CA-CDI	Community-associated <i>Clostridium difficile</i> infection
CDC	Center for Disease Control and Prevention
CDMNT	<i>Clostridium difficile</i> moxalactum norfloxacin supplemented with 0.1% sodium taurocholate
CDI	<i>Clostridium difficile</i> infection
CLSI	Clinical and Laboratory Standards Institute
MAP	Modified atmosphere packaging
MIC	Minimum inhibitory concentration
MDR	Multi drug resistant
OH	Octenidine hydrochloride
REL	Ready-to-eat lettuce
RT-qPCR	Real-time quantitative polymerase chain reaction
SAS	Statistical analysis software
SIC	Sub-inhibitory concentration
PBS	Phosphate-buffered saline
PCA	Plate count agar
USDA	United States Department of Agriculture

Chapter I
Introduction

Clostridium difficile is an anaerobic, spore-forming bacterium, which is transmitted through the fecal-oral route. *C. difficile* infection (CDI) can be mild to life-threatening, and outbreaks are predominantly observed in healthcare settings (Stanley et al., 2013; Weese, 2010). In the United States, over 500,000 cases of CDI are reported each year, costing the economy an estimated 3 billion for treatment (Ghose et al., 2007; Lessa et al., 2015). Although toxins are the major virulence factors responsible for the pathogenesis of *C. difficile* infection, sporulation, spore germination and spore outgrowth are critical for transmission and relapse of CDI (Akerlund et al., 2008; Hookman & Barkin, 2009; Sunenshine & McDonald, 2006). Transmission of *C. difficile* spores occurs through shedding in the feces of infected individuals and asymptomatic carriers, which leads to contamination of the hospital environment (Riggs et al., 2007; Weber et al., 2010). Relapse of CDI is a major concern, and is observed in 20-30% of reported cases with risk increasing to 60% after 2 or more recurrences (Barbut et al., 2000; Kelly & LaMont, 2008; Louie et al., 2011). Moreover, CDI patients can shed *C. difficile* spores for up to four weeks post recovery (Sethi et al., 2010).

C. difficile spores can survive in the abiotic environment such as on hospital surfaces and equipment for up to 5 months, thereby serving as a potential source of infection. In addition, spores are resistant to most of the commonly used physical and chemical disinfectants, including household dilutions of sodium hypochlorite (Edwards et al., 2016; Fawley et al., 2007; Gerding et al., 2008). A ten percent solution of bleach is effective against *C. difficile* spores (Edwards et al., 2016), but it is not recommended for routine use due to its potential to cause respiratory distress in healthcare workers and patients, and corrosion on hospital equipment (Keward, 2013). Therefore, there is a need for safe and effective disinfectants, which can be used routinely to reduce CDI transmission in hospitals.

Susceptible individuals undergoing long-term antibiotic therapy have disrupted gut microbial flora, which provides an opportune environment for *C. difficile* spores to germinate and cause infection (Evans & Safdar, 2015; Sun et al., 2011). *C. difficile* spores germinate in the intestine, where bile acid acts as a germinant, and vegetative cells produce toxins in the colon, leading to severe diarrhea. In addition, sporulation of vegetative cells occurs in the colon, which produce new spores that are shed in the feces to continue the infectious cycle. However, *C. difficile* transmission and relapse can be prevented by intervening with *C. difficile* sporulation, germination and/or outgrowth.

Although *C. difficile* is acquired primarily from nosocomial settings, there has been an increase in community-associated *C. difficile* infections (CA-CDI), especially in low risk individuals outside of hospital settings. Community-associated infections occur in the community in individuals with no hospitalization within the past 12 weeks or infections that occur within 48 h of hospital admission (Lessa, 2013). Community-associated infections now account for 32% of all CDI cases, suggesting the possibility of an additional *C. difficile* source that can cause CDI in non-hospitalized patients (Hensgens et al., 2012; Lessa, 2013; McDonald et al., 2006; Rupnik et al., 2009). *C. difficile* is widely detected in the gut of most domestic and wild animals, including pets (Álvarez-Pérez et al., 2015; Himsworth et al., 2014; Medina-Torres et al., 2011). Shedding of *C. difficile* spores in the feces of infected or asymptomatic carrier animals has been identified as a source of environmental contamination (Rodriguez-Palacios et al., 2006; Rodriguez-Palacios et al., 2014b). Research in several countries has identified the presence of *C. difficile* spores in a variety of food animals, meat, water, and the environment (Bakri et al., 2009; Costa et al., 2012; Houser et al., 2011; Thitaram et al., 2016; Xu et al., 2014). However, the effect of refrigeration, freezing, cooking and acidity on the viability of *C. difficile*

spores in meat products has not been investigated. In addition, limited information exists on the prevalence of *C. difficile* in fresh produce in the US. Although researchers in Europe have isolated *C. difficile* in ready-to-eat lettuce (Bakri et al., 2009; Eckert et al., 2013), US data are limited to a single publication in vegetables (Rodriguez-Palacios et al., 2014a). Potential contamination of fresh produce with *C. difficile* spores from soil, water and animal waste in agricultural fields, along with the absence of cooking before consumption, increase the foodborne disease risk associated with *C. difficile* in fresh produce.

Hypothesis:

Foods such as ready-to-eat lettuce are a potential source of multi-drug resistant *C. difficile* spores. In addition, *C. difficile* spores can survive chilling, freezing and cooking temperatures in meat products such as ground beef, and can withstand the high acidity and cooking in ready-to-eat fermented products such as summer sausage. Further, the new generation disinfectant, octenidine dihydrochloride, is effective in killing *C. difficile* spores on stainless steel surfaces, whereas the commonly prescribed, non-steroidal anti-inflammatory drug, aspirin, exerts potent anti-sporulation effects against *C. difficile*.

The specific objectives of this dissertation are to:

1. Determine the prevalence of *C. difficile* in ready-to-eat lettuce (REL) in Connecticut, and study the effect of modified atmosphere packaging (MAP) on the viability of *C. difficile* spores in REL.
2. Determine the effect of chilling, freezing and cooking on the viability of *C. difficile* spores in ground beef.
3. Study the effect of acidity and cooking on the viability of *C. difficile* spores in fermented pork summer sausage.

4. Determine the efficacy of octenidine hydrochloride (OH) in reducing *C. difficile* spores on stainless steel surfaces.
5. Investigate the effect of acetylsalicylic acid (aspirin) on *C. difficile* sporulation and spore germination *in vitro*.

Chapter II

Review of Literature

Clostridium difficile is a spore-forming anaerobic bacterium which causes a serious toxin-mediated infection in the gastrointestinal tract (Hookman & Barkin, 2009; Smits et al., 2016). The mild symptoms of the disease include fever and abdominal pain, and more severe infections lead to toxic megacolon, sepsis and shock (Rupnik et al., 2009). Some infected individuals do not show symptoms but play a role in *C. difficile* transmission, and are classified as asymptomatic carriers (Galdys et al., 2014; Hensgens et al., 2012). A study on the incidence of *C. difficile* in a healthy human population in Pennsylvania found a carriage rate of 6.6% underscoring the potential for asymptomatic carriers to be a reservoir for *C. difficile* (Galdys et al., 2014). There has been a rise in community-associated CDI in individuals without the common risk factors of antibiotic therapy, recent healthcare exposure or advanced age (Dumyati et al., 2012; Lessa, 2013). The increase in community-associated *C. difficile* infection (CDI) along with the isolation of *C. difficile* isolates in animals and the environment suggests that food could be a potential source for community-associated human infections.

1. Morphology and biochemical characteristics

Clostridium difficile is an anaerobic bacillus measuring 3-5 µm in length. During stationary phase after 72 h of growth, terminal spores bulging from one end of the bacillus can be visualized with Gram staining (Postma et al., 2015). Flagella allow motility of *C. difficile*, but the role played by flagella in adherence to the host mucosa is unclear. However, studies involving *C. difficile* mutants, lacking flagella-building components showed increased adherence of the bacterium to cells (Janoir, 2015). *C. difficile* can be grown in non-selective microbiological media such as brain heart infusion (BHI) or blood based media for pure culture experiments. The bacterium is a strict anaerobe, which grows optimally under the gaseous conditions of 80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37°C. *C. difficile* colonies are flat, non-hemolytic, range from opaque

to milky white in color, and most often have irregularly shaped edges resembling a fried egg shape (Wren, 2010). Colonies have a characteristic horse manure smell, which is a preliminary identification characteristic of *C. difficile* (Delmée, 2001). To enhance growth, sodium taurocholate can be added to the medium, which improves germination and sporulation of *C. difficile* (Wren, 2010). For the detection of *C. difficile* in prevalence studies, selective media such as *Clostridium difficile* moxalactam norfloxacin (CDMN) broth containing selective antibiotics and 7% horse blood can be used (Mooyottu et al., 2015). An L-Proline-Aminopeptidase test can be used along with selective media for initial identification of *C. difficile*. The L-proline-aminopeptidase uses p-dimethylaminocinnamaldehyde as the substrate, and produces a dark pink color in the presence of -proline-beta-naphthylamide, thereby suggesting *C. difficile* (Fedorko & Williams, 1997). *C. difficile* produces spores, which are highly resistant to high temperature, desiccation, nutrient deprivation and other environmental stresses.

2. Epidemiology of *C. difficile*

Annually in the US, an estimated 500,000 cases of CDI are reported, which results in ~ \$3 billion as healthcare and treatment costs (Lessa et al., 2015). In the last decade, CDI has become more difficult to treat as a result of increased resistance to antibiotics and from the emergence of new hypervirulent strains (Postma et al., 2015; Valiente et al., 2014; Zhanel et al., 2015). A strain known as North American Pulsotype 1 (NAP1) emerged in the last 10 years, and is among the most common *C. difficile* strains in healthcare settings (Lessa et al., 2015). The NAP1 strain belongs to the ribotype 027 and toxin type III (Valiente et al., 2014). Along with increased infections, relapse of infection is a major concern in CDI-recovered patients, and is observed in 20-30% of the reported cases (Barbut et al., 2000; Gerding et al., 2015). In addition, the mortality associated with CDI in the US has increased from 5.7 per million to 23.7 per million

from 1999 to 2004 (Redelings et al., 2007), and the incidence rate among hospitalized patients doubled from 2001 to 2010 (Reveles et al., 2014). In 2011, 29,000 deaths were reported nationwide from CDI (Lessa et al., 2015). Further, *C. difficile* has shown an increasing resistance to antibiotics such as fluoroquinolones (Drudy et al., 2007), clindamycin, erythromycin, metronidazole (Qiong & Haihui, 2015) and vancomycin (Kelly & LaMont, 2008). *C. difficile* was recently classified as an “urgent threat” in terms of antibiotic resistance by the Centers for Disease Control (CDC) (Evans & Safdar, 2015). Also, *C. difficile* has surpassed *Staphylococcus aureus* with the highest percentage of hospital-acquired infections (Magill et al., 2014). The increase in *C. difficile* infections in the community adds weight to the classification of *C. difficile* as an urgent threat.

3. *C. difficile* pathogenesis

The most important predisposing factor for *C. difficile* infection is long-term antibiotic treatment. Antibiotic therapy can disrupt gut microbial communities and allow colonization of opportunistic pathogens such as *C. difficile* (Perez-Cobas et al., 2014). The main mode of transmission for CDI is through the fecal-oral route (Hookman & Barkin, 2009). *C. difficile* spores are shed in the feces and can remain viable in the environment and on hospital surfaces for up to 5 months. (Siani et al., 2011). Spores are metabolically inactive structures that protect the genetic material, and allow survival in the acidic stomach. Once spores reach the intestine, germination occurs and does so more readily in the absence of normal intestinal flora, when individuals are undergoing long-term antibiotic treatment. The presence of bile salts such as sodium taurocholate initiates spore germination (Wheeldon et al., 2011). Following germination, vegetative cells grow and multiply in the intestine and produce two potent toxins known as toxin A (TcdA) and toxin B (TcdB).

The *C. difficile* toxins are glucosyltransferase proteins, which have four functional domains (Solomon, 2013). The catalytic domain inactivates Rho family GTPases by glycosylation, which disrupts Rho GTPase function in maintaining the cytoskeleton integrity. Also, inactivation of Rho GTPases disrupts F-actin regulation and inhibits proper functioning of epithelial cell tight junctions (Rupnik et al., 2009). The cysteine protease domain is involved in autocatalytic cleavage of the toxins through action with cellular inositol hexaphosphate kinase 1 (InsP6) (Pruitt & Lacy, 2012). The next domain is responsible for the entry of the toxin into the cell cytoplasm and the last domain allows receptor binding. Breakdown of tight junctions through the action of the four domains causes fluid leakage and ultimately leads to diarrheal symptoms. The specific effects of *C. difficile* toxin A are cell rounding and apoptosis in monocytes, which leads to host immune response in secretion of proinflammatory cytokines (El Feghaly et al., 2015; Monaghan, 2015; Solomon, 2013). Proinflammatory cytokines such as IL-8 attract neutrophils which cause tissue damage upon the release of lytic enzymes. In more serious CDI cases, additional cytokines involved in inflammatory response such as IL-12 and IL-1 β are released, which further damages epithelial tissue leading to a debris build-up known as a pseudomembrane. This condition known as pseudomembranous colitis is characterized by raised yellow plaques that persist during severe CDI. In the most severe cases, uncontrolled cytokine release and diarrheal fluid loss can lead to shock and even death (Knight & Surawicz, 2013).

4. *C. difficile* infection

Annually in the United States a high number of *C. difficile* infections are reported, which clinically manifest as asymptomatic, mild, moderate or severe. (Lessa et al., 2015; McDonald et al., 2006). Asymptomatic CDI patients do not show clinical signs of infection, but can transmit the disease through shedding of *C. difficile* spores in feces. Early studies showed high

asymptomatic carriage of *C. difficile* in neonatal infants (61%), but only a small number developed CDI (Holst et al., 1981). Mild clinical symptoms of CDI are fever and abdominal pain. As the infection progresses to moderate CDI, watery diarrhea with 10 to 15 bowel movements per day is characteristic (Kelly & LaMont, 1998). In addition, patient blood samples show leucocytosis with $\geq 15,000$ – $20,000$ white blood cells/mm³. As CDI becomes more severe, abdominal pain increases due to ileo-colonic dilation and toxic megacolon can develop (Knight & Surawicz, 2013). Also, toxins can be detected in the stools of patients, and blood tests detect substantial leukocytosis ($> 50,000/\mu\text{L}$). Pseudomembranous colitis manifesting raised yellow plaques from the release of inflammatory cells and necrotic epithelial debris can also develop. An endoscopy is utilized clinically to visualize plaques. Severe cases of pseudomembranous colitis can lead to sepsis, and if perforations develop at the mucosal level renal failure and death can occur in the most severe cases (Sailhamer et al., 2009; Solomon, 2013).

Recurrent CDI is observed in 25-30% of individuals (Gerding et al., 2015). Subsequent infections can occur from ingesting spores from surfaces or objects contaminated from spore shedding by patients themselves or by other infected individuals or asymptomatic carriers. Recurrent CDI infections can be severe and debilitating to patients.

5. Community-associated CDI

The Infectious Disease Society of America classifies community-associated *C. difficile* infections (CA-CDI) as infections that occur in the community with no hospitalization within the past 12 weeks or infections that occur within 48 h of hospital admission (Lessa, 2013; McDonald et al., 2007). An overview study observing the risk of antibiotics in documented CA-CDI cases observed that antibiotics do increase the risk of CA-CDI; specifically clindamycin poses the greatest risk, and fluoroquinolones and cephalosporins also increase the risk (Deshpande et al.,

2013). A population study observed that CA-CDI affected younger individuals than hospital-acquired CDI, with a median age of 50 and 72, respectively (Khanna et al., 2012). In addition, the study noted a high percentage of individuals requiring hospital treatment and incurring severe and recurrent infections as a result of CA-CDI. The incidence of CA-CDI was investigated in Monroe County, New York, and was found to be responsible for 18% of total CDI cases within the county over a period of one year (Dumyati et al., 2012). Also, it was observed that CA-CDI patients were younger and had fewer classical risk factors for CDI compared to patients in the hospital-acquired CDI group. Investigators also observed that 21% of these patients reported visiting a health-care facility with a family member within 12 weeks before CDI onset. Therefore, minor exposure to health-care settings or contact with individuals under long-term hospital care could increase the risk of CA-CDI.

6. *C. difficile* toxins

The gene encoding region for *C. difficile* toxins is located in the pathogenicity locus (PaLoc), which varies by strain type (Neyrolles et al., 2011; Rupnik et al., 2005). The region encodes for high molecular weight toxins, which are classified as exotoxins and are a part of the large clostridial toxins family (Popoff & Bouvet, 2009). *C. difficile* toxins are the major virulence factors involved in CDI disease manifestation and include an enterotoxin for toxin A (TcdA) and a cytotoxin for toxin B (TcdB). TcdA is 308 kDa and TcdB is 270 kDa with similarities in their amino acid sequence and function. Both toxins have an A subunit, which has catalytic activity and a B subunit responsible for delivery of the A subunit to the host cell (Davies et al., 2011). The A subunit is involved in inactivating Rho family GTPases by glycosylation (Voth & Ballard, 2005). The B subunit is divided into three parts involved in delivery of the A subunit; receptor-binding, pore-formation and autoproteolysis. Overall, toxins

A and B act together through binding and internalization, pore formation, autoproteolysis and finally inactivation of the Rho GTPases (Pruitt & Lacy, 2012). Experiments have shown that TcdB has more prolific cell rounding capacity than TcdA, with a fold difference of 100-10,000. Also, strains with TcdA⁻TcdB⁺ cause similar infection conditions to TcdA⁺TcdB⁺. TcdA⁺TcdB⁻ strains have been found to be nonpathogenic to hosts, indicating the importance of toxin B (Sambol et al., 2000). In addition, binary toxins (CdtA and CdtB) are also produced by many toxigenic *C. difficile* strains (Gerding et al., 2014). CdtA modifies actin by enzymatic ADP-ribosyltransferase activity and CdtB binds to the host cell and aids in translocating CdtA. The ADP-ribosyltransferase activity causes depolymerization of the actin cytoskeleton, which leads to microtubule production on epithelial cells aiding in *C. difficile* attachment (Gerding et al., 2014; Hemmasi et al., 2015). Experiments with strain TcdA⁻TcdB⁻ but positive for binary toxins still cause enterocyte damage, indicating the importance of binary toxins in CDI (Gerding et al., 2014).

7. Regulation of *C. difficile* toxin production

Toxin A and toxin B are encoded by genes *tcdA* and *tcdB*, respectively, and are released from vegetative *C. difficile*. The Paloc region contains the toxin genes and encodes for regulatory proteins such as TcdR, TcdC and a holing-like protein, TcdE (Neyrolles et al., 2011). TcdR is an alternative sigma factor that is a positive regulator of toxin production and induces transcription of *tcdA* and *tcdB* (Bouvet & Popoff, 2008). The negative regulator of toxin production is TcdC, which interferes with the ability of TcdR to recognize *tcdA* and *tcdB* promoters (Dupuy et al., 2008). The *tcdC* gene is expressed during the exponential growth phase, earlier in the growing cycle than *tcdA*, *tcdB* and *tcdR* genes. Moreover, it has been identified that high toxin producing strains such as NAP1 have a mutation in *tcdC*, hence negative regulation of toxin production is

disrupted (Carter et al., 2011). Lastly, TcdE is required for secretion of *C. difficile* toxins (Govind et al., 2015).

The genes *codY* and *ccpA* are also involved in *C. difficile* toxin production, which are located outside of the Paloc region (Dineen et al., 2010). CodY represses toxin production in *C. difficile*, and is classified as a global regulator of virulence genes in many Gram-positive bacteria (Lobel et al., 2012). During nutrient deprivation, branched-chain amino acids are present which are shown to increase the binding affinity of *codY* to the *tcdR* promoter to suppress toxin production (Dineen et al., 2007). The *ccpA* gene codes for a protein functioning as a pleiotropic regulator involved in carbon catabolite repression. CcpA identifies rapidly metabolizable carbohydrate sources such as glucose and acts as the link between nitrogen and carbon pathways (Antunes et al., 2011). Therefore, both *ccpA* and *codY* are involved in sensing nutrient availability. In addition, the toxin genes (*tcdA* and *tcdB*) and the positive and negative regulators (*tcdR* and *tcdC*) are regulated by CcpA. Interestingly, CcpA is also involved in repressing sporulation through acting on Spo0A, which will be described further in subsequent sections (Antunes et al., 2011; Pettit et al., 2014).

8. *C. difficile* and the microbiome

An important predisposing factor for *C. difficile* infection is the disruption of normal gastrointestinal flora, collectively known as the microbiome. Disruption of the microbiome is typically associated with long-term exposure to antibiotics (Britton & Young, 2014). It has been demonstrated in studies with mouse models that antibiotics such as a single dose clindamycin predispose to CDI susceptibility (Buffie et al., 2012). Exposure to antibiotics disrupts the microbiome for prolonged periods, even after the treatment regimen is complete (Dethlefsen et

al., 2008). The known classes of antibiotics which have been shown to predispose patients to CDI are cephalosporins, clindamycin, fluoroquinolones and penicillins (McFarland, 2008). Fidaxomicin is a narrow spectrum antibiotic that was recently approved for CDI treatment ,and has shown less disruption of microflora. In addition, fidaxomicin treatment is associated with a reduced rate of CDI recurrence compared to treatment with vancomycin (Louie et al., 2011). This highlights the importance of the microbiome in CDI prevention and protection against recurrent infections. Fecal transplant from healthy individuals for re-establishing a healthy microbiome in CDI patients will be discussed in the “Treatments for CDI in humans” section of this review.

It has been observed that the composition of the gut microbiome becomes less diverse in elderly individuals. The protective classes of bacteria such as *Bifidobacterium* and *Firmicutes* decrease significantly in the elderly and the pathogenic bacterial class known as *Proteobacteria* increase (Biagi et al., 2010; Claesson et al., 2011). These alterations in the microbiome of the elderly can lead to gut dysbiosis and plays a role in predisposition to CDI (Mariat et al., 2009).

9. Environmental isolation of *C. difficile*

A pivotal study which isolated *C. difficile* from the environment was conducted in Wales, from a total of 2580 environmental samples, and identified positive isolates from soil, river water, sea water and animal feces (al Saif & Brazier, 1996). An environmental sampling in Zimbabwe found positive hyper-virulent *C. difficile* isolates in soil (37%), water (6%) and animal fecal samples (4.3%) (Simango, 2006). Environmental *C. difficile* isolates were obtained in Slovenia from puddle water (14.4% of n=104) and soil (36.7% of n=79), with diverse ribotypes suggesting high genetic diversity of *C. difficile* in the environment (Janezic et al.,

2016). *C. difficile* is also present in rivers and was found to be present in 68% of rivers in a recent study conducted in Slovenian rivers (Zidaric et al., 2010). A large study of 786 *C. difficile* isolates obtained from humans, animals and the environment were analyzed by polymerase chain reaction (PCR) to determine the ribotype and toxin grouping for each sample (Janezic et al., 2012). It was determined that samples in the environment contained the highest percentage of non-toxigenic strains (30.8%), while samples from humans and animals were more similar and contained a lower percentage of non-toxigenic strains (6.5% and 7.7%, respectively). Overall the study found that many *C. difficile* strains from humans are present in both animals and the environment. Therefore, the ubiquitous presence of *C. difficile* in the environment could be a source for community-associated outbreaks.

Sludge and wastewater samples in Switzerland and Slovenia were tested for presence of *C. difficile*, and 13 ribotypes with 85% toxigenic and 32 ribotypes with 3.3% toxigenic were identified, respectively, in each study (Romano et al., 2012; Steyer et al., 2015). Ribotypes 010 and 014 were most prevalent in Slovenia and 078 in Switzerland, which are similar to ribotypes isolated from humans, animals and water sources. A similar study in Canada identified a high prevalence of *C. difficile* in raw sludge (92%) and river sediments (60%), where processed waste water is released (Xu et al., 2014).

10. *C. difficile* in animals

Healthy domestic animals have been found to shed *C. difficile* in their fecal material. Positive *C. difficile* isolates are shed more readily in young animals such as calves and piglets but adult animals on farms and prior to slaughter also test positive for *C. difficile* (Keel et al., 2007; Rodriguez et al., 2012; Silva et al., 2015). Sampling data from 1500 neonatal piglets over 5 years

indicated that 67% of litters and 35% of individual pigs were positive for toxigenic *C. difficile* (Songer & Anderson, 2006). Another study in the US isolated *C. difficile* ribotype 078 from 83% of neonatal pig samples and 94% of calf samples (Keel et al., 2007). In the Netherlands, identical 078 *C. difficile* strains were isolated from pigs and humans with similar susceptibility profiles to antimicrobials (Debast et al., 2009). A study in Brazil identified positive isolates in dogs, foals, piglets and calves, with strains similar to human isolates, illustrating the potential for zoonotic transmission of *C. difficile* (Silva et al., 2015). *C. difficile* was detected in pets such as dogs and cats at high prevalence rates with confirmed shedding of spores in feces (Álvarez- Pérez et al., 2015; Koene et al., 2012; Riley et al., 1991). In addition, a recent study in 2013 confirmed that carcass contamination of pigs and cattle occurs in slaughter plants as observed in 7% of carcasses (Rodriguez et al., 2013). A US study in 875 food animals identified a prevalence rate of < 0.6% in poultry, cattle and swine (Rodriguez-Palacios et al., 2014b). Overall the prevalence rate of *C. difficile* in food animals and foods has been varied over the past decade (Costa et al., 2012; Lund & Peck, 2015), but there is a potential for *C. difficile* as a foodborne pathogen.

C. difficile has also been detected in wild animals such as raccoons, which could play a role in transmitting the pathogen. A study isolated *C. difficile* strains from 12% of raccoon feces tested, and five of the 19 strains identified were human strains. Raccoons were tested on multiple occasions and were observed to not be positive for *C. difficile* more than once, suggesting that raccoons could transmit the pathogen to sites such as farms (Bondo et al., 2015). *C. difficile* has been detected in other wild animals such as feral pigs, elk, and in an ocelot that was treated with antibiotics (Arroyo et al., 2005; Silva et al., 2013; Thakur et al., 2011). Also, migrating European barn swallows captured at different locations for fecal sample collection were positive for *C. difficile* (Bandelj et al., 2014). Many studies isolated ribotype 078 from wild animals, which

suggests a potential reason for the increased incidence of CDI in community-associated CDI.

However, to date, transmission of *C. difficile* between species has not been confirmed, but could occur from contact with feces by humans or other animals through the fecal-oral route.

11. Animal-derived foods as a source of *C. difficile*

The rise in community-associated *C. difficile* infections has suggested food as a possible source of *C. difficile* infection. In addition, genotypically similar or identical strains of *C. difficile* strains implicated in human infections have been identified in foods and food animals. An initial study of *C. difficile* contamination in retail meat showed a 21% prevalence rate from 53 ground beef samples, and a 14% prevalence rate from 7 ground veal samples obtained from stores in Canada (Rodriguez-Palacios et al., 2007). A follow-up study with a larger number of samples indicated lower prevalence rates of 6.7% out of 149 ground beef samples and 4.6% out of 65 veal chop samples (Rodriguez-Palacios et al., 2009). Overall, the prevalence of *C. difficile* in ground beef in the US has ranged between 0 to 44% (Houser et al., 2011; Songer et al., 2009). On the other hand, the prevalence rate of *C. difficile* in chicken and poultry products has ranged from 0 to 12.5% (Harvey et al., 2011a; Weese et al., 2010).

There are limited studies on the prevalence of *C. difficile* in pre-cooked ready-to-eat meats (RTE). One study in Texas focused on the prevalence of *C. difficile* in pork used to make ready-to-eat sausage, and identified the bacterium in 9.5% of 234 samples tested (Harvey et al., 2011b). A study of both uncooked and ready-to-eat retail meats, found a higher percentage of positive *C. difficile* isolates in RTE meats with isolates found in 62.5% of pork braunschweiger compared to 50% in ground beef (Songer et al., 2009). A more recent study identified toxigenic *C. difficile* isolates in 3 of 15 ready-to-eat pork products, braised skin and braised colon products (Wu et al., 2017).

12. *C. difficile* in vegetables

The prevalence of *C. difficile* in fresh produce has been investigated in fewer studies compared to that in meat. The incidence of *C. difficile* in vegetables would pose a greater risk since many are not cooked prior to consumption. In France, 60 ready-to-eat lettuce (REL) samples and 44 vegetables, including broccoli, carrots and beets were tested for *C. difficile*. The overall prevalence rate was 2.9%, with two samples of REL and one pea sprout sample testing positive (Eckert et al., 2013). A study in Scotland found *C. difficile* in 7.5% of 40 REL samples, identifying ribotypes 001 and 017 (Bakri et al., 2009). The isolated strains were resistant to moxifloxacin and clindamycin. The presence of *C. difficile* in vegetables could be from farm workers or environmental sources such as contaminated water, soil or fertilizer. A study conducted in ready-made salads for foodservice in Iran isolated *C. difficile* in 5.66% (6/106) of samples tested (Yamoudy et al., 2015). The only prevalence study in fresh produce in the US was conducted in Ohio, and 2.4% of 125 vegetable samples tested positive for *C. difficile*. One of the *C. difficile* strains was resistant to moxifloxacin and clindamycin. This US study found toxigenic *C. difficile* isolates in ready-to-eat vegetables such as pepper and lettuce, but did not investigate the prevalence in REL (Rodriguez-Palacios et al., 2014a). The majority of prevalence studies in vegetables did not quantify the level of *C. difficile* contamination, but many studies assumed that strains were isolated from spores instead of vegetative cells. The infectious dose of *C. difficile* is not confirmed, but a low level of *C. difficile* spore contamination could likely cause infection, especially in susceptible individuals (Lund & Peck, 2015).

13. Isolation of *C. difficile* from hospital environment

C. difficile spores are shed in the feces of infected individuals, which serves as a source of new and recurrent infections. Studies have indicated that *C. difficile* spores can survive in the hospital environment on surfaces and inanimate objects for up to 5 months (Hasan et al., 2011; Kim et al., 1981; Owens, 2006). Also, healthcare workers contribute to *C. difficile* transmission by touching contaminated surfaces or individuals, and directly spreading the pathogen (Boyce, 2007; Guerrero et al., 2012). *C. difficile* spores have been commonly isolated from hospital rooms on floors, bedpans, bed rails and furniture (Kim et al., 1981; Weber et al., 2013). *C. difficile* contamination in the rooms of CDI patients ranged from 2.9-75% (Weber et al., 2010). In addition, spores have been isolated from equipment shared between patients such as blood pressure cuffs and stethoscopes (Fekety et al., 1981; Gerding et al., 2008). There is a high transfer rate of *C. difficile* spores from healthcare workers' gloved hands after contact with patients, inanimate objects, and reverse transfer is also observed (Guerrero et al., 2012). *C. difficile* spores are also present in the air space surrounding patients (Roberts et al., 2008). Sharps containers are re-used in 50% of healthcare facilities and were investigated as a potential source of *C. difficile*. The data obtained from 604 hospitals found that hospitals which used single-use containers had significantly lower rates of *C. difficile* infections in their facilities (Pogorzelska-Maziarz, 2015). Therefore, it is accepted that there is a high rate of contamination of the hospital environment with *C. difficile* spores, which plays a critical role in CDI transmission.

14. Hospital disinfectants against *C. difficile*

C. difficile spores can survive on hospital surfaces for several months (Barbut et al., 2009; Fawley et al., 2007). Patients infected with *C. difficile* shed between 10^4 and 10^7 spores per gram of feces (Mulligan et al., 1984). Transmission and relapse of infection occurs from

ingestion of *C. difficile* spores from the environment. Commonly used hospital cleaning agents, such as quaternary ammonium-based and other surfactant-based detergents, do not kill *C. difficile* spores, and may potentially increase the sporulation capacity of *C. difficile* (Gerding et al., 2008). Currently, the only disinfectant recommended for use during *C. difficile* outbreaks is 1:10 sodium hypochlorite solution with 10 min of contact time (Cohen et al., 2010; Edwards et al., 2016; Fawley et al., 2007). Sodium hypochlorite has been shown to decrease the incidence of *C. difficile* infection in cross-over studies in hospitals (Wilcox et al., 2003). However, high concentrations of sodium hypochlorite are associated with skin irritation, respiratory distress in healthcare workers and patients, and corrosion of hospital equipment and surfaces (Keward, 2013). In addition, the 10% sodium hypochlorite solution must be prepared daily in order to achieve an effective concentration, which requires additional compliance measures for hospital staff (Dubberke et al., 2008).

Alternative disinfection methods using automated technology have recently emerged such as hydrogen peroxide vapor and ultraviolet-C emission (Anderson et al., 2013; Shapey et al., 2008). Specialized equipment is required for these disinfection techniques and hospital rooms need to be vacated during the procedure, making these processes lengthy and not-cost effective. In addition, the automated disinfection system with hydrogen peroxide showed statistically similar effectiveness to disinfection with sodium hypochlorite, indicating the need for continued research in disinfection procedures (Mosci et al., 2017). Currently, disinfection with 10% sodium hypochlorite disinfection is deemed as most effective against *C. difficile* spores, but is associated with health risks, equipment damage and risk of using ineffective concentrations.

15. *C. difficile* spores

C. difficile spores are dormant structures which perpetuate survival in the environment and allow transmission from host to host. The metabolic dormancy of *C. difficile* spores allows resistance to oxygen, disinfectants, antibiotics and common heat treatments (Ali et al., 2011; Baines et al., 2009). This resistance is due to low water content of spores (25-60% of wet weight), high amount of dipicolonic acid and saturation of DNA with protective α/β soluble proteins (Paredes-Sabja et al., 2014). The saturation of the spore-core containing DNA is protected by an impermeable inner membrane comprised of phospholipids, which prevent the passage of water. The next layer is the germ cell wall, which becomes the cell wall in the vegetative state. The cortex layer is made of peptidoglycan with every second muramic acid residue converted to muramic- δ -lactam (MAL) to reduce crosslinking. The MAL residues allow lytic enzymes to hydrolyze the spore cortex during germination due to less crosslinking compared to the cell wall layer. The proteinaceous coat helps with spore resistance to stresses (Permpoonpattana et al., 2011). Finally, the exosporium layer is a loose-fitting layer with hair-like projections similar to *B. anthracis* (Henriques & Moran, 2007). This layer plays a role in hydrophobicity, which affects the ability of spores to attach to surfaces (Joshi et al., 2012). Five proteins that are located in the outer layers of *C. difficile* spores are CotA, CotB, CotCB, CotD, and CotE, but only CotA is needed for the assembly of the exosporium (Permpoonpattana et al., 2011). The proteins CotD and CotE have demonstrated antioxidant activity to reduce H₂O₂ toxicity through conversion to water and oxygen (Permpoonpattana et al., 2011). In addition, a newly identified protein in the exosporium known as *Clostridium difficile* exosporium cysteine-rich protein (CdeC) was found to be expressed during sporulation, and is required for the correct assembly and development of the spore coat and exosporium. By using a spore $\Delta cdeC$ knockout

mutant, researchers found that mutants lacking an exosporium layer were more sensitive to ethanol and heat treatment, indicating that CdeC is necessary for exosporium development and resistance characteristics (Barra-Carrasco et al., 2013). Also, the $\Delta cdeC$ mutants attached more readily to intestinal epithelial cells suggesting the protein's function in wild type spores in decreasing adherence, potentially so they can be more readily excreted in the feces.

16. Sporulation of *C. difficile*

Sporulation of *C. difficile* typically occurs during the stationary phase or death phase after 72 h of anaerobic incubation. Sporulation of *C. difficile* is a virulence factor which perpetuates the bacterium by the production of survival structures known as spores that can persist in infected hosts and in the environment. Being a strict anaerobe, spore formation also allows *C. difficile* to be protected from the toxic effects of oxygen (Sorg & Sonenshein, 2008). Sporulation is initiated by various environmental stresses such as nutrient deprivation and other stress factors which are not fully understood (Higgins & Dworkin, 2012). This process occurs at the time of egestion in the host, followed by shedding of spores in the feces.

The genes involved early in the sporulation process of *C. difficile* include *spoOA*, *sigH* and *codY*. The gene *sigH* is a transition phase sigma factor involved in the transcription of *spoOA* to initiate sporulation (Saujet et al., 2014). The gene *spoOA* is identified as the master regulator of sporulation (Babakhani et al., 2012). In a recent study, it was illustrated that a $\Delta spoOA$ *C. difficile* strain was unable to survive and perpetuate in a mouse model with the absence spore formation (Deakin et al., 2012). There are five orphan histidine kinases (CD1352, CD1492, CD1579, CD1949, and CD2492), which are required to phosphorylate the master regulator of sporulation, Spo0A, for sporulation to proceed. CD1492, CD1579, and CD2492 are

located in the *codY* gene, and are classified as SpoOA-specific (Underwood et al., 2009). In particular, the histidine kinase CD 1579 has been shown to directly transfer the phosphoryl group to Spo0A for initiation, but autophosphorylation has not been proven with the other 4 kinases. However, CD1492 and CD2492 have also been shown to have conserved genome regions for autophosphorylation.

The sporulation process in *Bacillus subtilis* and *C. difficile* are similar, but vary at the level of regulation by the RNA polymerase sigma factors (σ^F , σ^E , σ^G , and σ^K). In *C. difficile* sporulation, there are more deviations between gene expression and morphogenesis of the forespore being engulfed by the mother cell. The two species share compartment-specific activation with σ^F and σ^G involved in forespore activity, and σ^E and σ^K activity with the mother cell (Fimlaid et al., 2013). Moreover, σ^F is necessary for post-translational activation of σ^G , but is not necessary for proteolytic activation of σ^E . A difference in *C. difficile* is the lack of necessity for σ^F to activate σ^E , but the forespore protein SpoIIR is required for σ^E activation (Saujet et al., 2014). The σ^E expression has been shown to be dependent on SpoOA, the master regulator. Also, σ^E plays a role in the transcription of genes such as *spoIIID*, which encodes for a regulator in the mother cell. The factors σ^E , σ^G and *spoIIID* are needed for σ^K production and activation, but σ^K does not require proteolytic activation (Paredes-Sabja et al., 2014). Signaling regulation of sporulation leads to spore cell formation with asymmetric division into a small forespore and a larger mother cell. The forespore is the portion that will become the spore. The mother cell engulfs the forespore and initiates the cortex, coat and exosporium layers to form. In *C. difficile*, SpoIVA is required for localizing the spore coat around the forespore and acts directly with a newly characterized protein, CD3567 (Putnam et al., 2013). Therefore, this interaction is required for the beginning stages of spore layer assembly. In summary, the known

factors of the spores include SpoIVA and CD3567, which are proteins involved in localization at the basement layer. The factors involved in inner coat, outer coat and crust formation are not yet known for *C. difficile*. CdeC is the protein required for exosporium development and assembly. Lastly, the *BclA1* glycoproteins spanning out through the exosporium layer important in *C. difficile* spores. Research suggests that the lower degree of regulation of sporulation and check points in *C. difficile* may be an adaptation for surviving in the gut of infected hosts (Saujet et al., 2014).

17. Germination of *C. difficile*

When initiation of spore germination occurs, a phase bright spore changes to a phase dark spore as germination receptors are activated by specific bile salts (Sorg & Sonenshein, 2008; Wax et al., 1967). Studies have shown that taurocholate, a derivative of cholate, acts as a germinant and L-glycine acts as a co-germinant (Sorg & Sonenshein, 2008). A 1 min exposure time to 0.1% taurocholate showed improved spore recovery compared to media without taurocholate indicating its activity as a germinant (Sorg & Sonenshein, 2008). The interaction between the germinant and co-germinant causes enzymatic reactions that ultimately release Ca^{2+} and dipicolinic acid from the spore core (Setlow, 2003). The spore becomes rehydrated and the cortex degrades, ultimately leading to vegetative growth. The CotE spore coat protein described in the spore section also has chitinase activity observed at the time of germination, which involves the breakdown of components into nutrients. The presence of germinants allows more prolific germination, but some *C. difficile* strains have been shown to germinate in media without taurocholate, indicating that the process is highly complex. *C. difficile* has different germination receptors than *B. subtilis*, and one of these has recently been identified as CspC. This serine protease acts as a bile salt germination receptor and induces the release of Ca-dipicolonic acid

upon bile salt interaction. Following bile salt interaction, CspC activates CspB, which then activates SleC which presumably leads to cortex hydration. Next, ions and Ca-dipicolonic acid are thought to be released, which allows spore core hydration ending in spore outgrowth to a vegetative cell (Paredes-Sabja et al., 2014).

18. Treatments for CDI in humans

18.1 Antibiotics

Medical management of *C. difficile* infection is a challenge in hospital settings since the treatment of *C. difficile* is frequently complicated by relapse of infection.

Metronidazole administered orally is prescribed for treating mild to moderate CDI as 500 mg 3 times a day for a 10 day regimen. For more severe CDI, and for pregnant or lactating women with CDI, the suggested antibiotic is oral vancomycin at 125 mg 4 times a day for a 10 day regimen. A higher dose of oral vancomycin is prescribed for most severe CDI cases at 250-500 mg 4 times a day for a 10 day regimen. Also, vancomycin can be administered rectally if conditions exist, where oral administration would not allow sufficient delivery (Knight & Surawicz, 2013). Treatment for initial CDI with 10 to 14 days of metronidazole or vancomycin have cleared infections in 50% of patients (Longo et al., 2015).

In case of recurrent CDI, the same antibiotic treatment prescribed could be re-prescribed. For a second recurrence, oral vancomycin should be prescribed with an extended “pulsed” course involving the use of intermittent high and low doses every 2 to 3 days. Pulsed dosing has shown effectiveness in clinical trials involving patients with recurrent CDI and showed a lower recurrence rate of 31% for pulsed dose compared to 40-50% for conventional dosing (McFarland et al., 2002). A recently identified antibiotic, fidaxomicin, was shown effective against CDI and prevented recurrent infections more effectively than vancomycin

(Housman et al., 2015; Zhanel et al., 2015). The antimicrobial activity of fidaxomicin is through inhibition of RNA polymerase to prevent transcription. The antibiotic also produces a bactericidal compound OP-1118, which acts against *C. difficile* for up to 10 h after antibiotic administration. Clinical trials showed that 200 mg of fidaxomicin two times daily for 10 days was as effective as 125 mg of vancomycin four times daily for 10 days. Also, this trial indicated the ability of fidaxomicin to prevent recurrent CDI more effectively than vancomycin (Zhanel et al., 2015).

18.2 Probiotics

C. difficile is the primary cause of antibiotic-associated diarrhea (Johanesen et al., 2015). Therefore, a preventative treatment which can be administered before antibiotics or concurrently with antibiotics could reduce the frequency of recurrent CDI. Probiotics are defined as “live microorganisms that when administered in adequate amounts confer a health benefit on the host” (Sanders, 2008). The protective effect of probiotics is through anti-inflammatory signalling in the gut. Clinical trials with live bacterial species (*Saccharomyces boulardii*, *Lactobacillus acidophilus* and *Lactobacillus casei*) have been shown effective in preventing recurrent CDI (Johnson et al., 2012). Probiotics are primarily prescribed along with antibiotic treatments in order to maintain desirable microfloral populations classically eliminated during antibiotic regimens. Clinical trials with probiotics have been more effective as a secondary prevention against recurrent CDI as compared to a primary treatment. A clinical trial including children taking antibiotics for ear or respiratory infections were randomly assigned *S. boulardii* treatment or placebo. The results showed significantly less incidence of antibiotic-associated diarrhea in the probiotic group (3.4%) compared to the placebo group (17.3%) (Kotowska et al.,

2005). However, this lower incidence of antibiotic-associated diarrhea cannot be linked to preventing *C. difficile* infection.

A meta-analysis of numerous controlled trials of primary CDI prevention in patients taking antibiotics showed a significant reduction in CDI (Crow et al., 2015). A study focusing on CDI prevention included patients with CDI assigned to one of two groups with vancomycin plus *S. boulardii* or vancomycin plus a placebo. The administration of probiotic showed a significant decrease in CDI recurrence at a rate of 16.7% for the *S. boulardii* group and 50% for the placebo group (Surawicz et al., 2000). Another experiment included patients taking antibiotics assigned to a group with a probiotic mixture drink (*Lactobacillus casei*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) or a placebo drink. There was no incidence of CDI in the group administered the probiotic compared to a 17% incidence of CDI in the placebo group (Hickson et al., 2007). A recent study conducted in high risk patients undergoing antibiotic therapy in the nephrology and transplantation wards of a hospital showed that daily administration of *Lactobacillus plantarum* 299v prevented CDI compared to the observation period when probiotics were not administered (Kujawa-Szewieczek et al., 2015). A recent meta-analysis of controlled trials investigating the effectiveness of probiotics as a secondary treatment for CDI found the highest significance for *S. boulardii* (Crow et al., 2015). However, the overall data are still limited in CDI patients and additional experiments are needed to evaluate the safety of probiotics for immunocompromised patients.

18.3 Immunization

Protection from mortality from CDI has been demonstrated in animals administered toxoids TcdA and TcdB (Siddiqui et al., 2012). The toxoids have been shown to confer an immune challenge, which allows natural production of IgG antitoxins. Hamsters administered

with the toxoid vaccine showed 100% protection from mortality when challenged with a lethal A-/B+ *C. difficile* strain (Siddiqui et al., 2012). The safety of toxoid A and B parenteral vaccine was tested in healthy adults and found to be safe, producing an antibody response in over 90% of patients tested (Kotloff et al., 2001). An international clinical trial with Health Authorities from numerous countries (ClinicalTrials.gov number [NCT01887912](https://clinicaltrials.gov/ct2/show/study/NCT01887912)) is currently investigating a *C. difficile* toxoid vaccine (Cdiffense vaccine) in subjects characterized as at risk for CDI (Longo et al., 2015). Injections containing the toxoids or a placebo will be administered in 3 doses on days 0, 7 and 30, and the safety and immunogenicity will be evaluated after each injection. A total of 15,000 participants will be included in the study and follow up assessments will be conducted three years after vaccination. The study is currently ongoing until 2017, and the results are expected to help determine if the vaccine has long term immunogenic effects, besides studying the potential for risk reduction of CDI. It is predicted that vaccination will not prevent *C. difficile* colonization, but it may lessen the severity of infection (Longo et al., 2015).

18.4 Fecal transplantation

The microbial population in the colon plays an important role in preventing colonization by pathogenic bacteria, including *C. difficile*. Antibiotic treatments disrupt the diversity of the microbiota and long-term administration predisposes patients to CDI. In addition, it takes at least 12 weeks for the colonic microbiota to re-establish following an antibiotic regimen (Dethlefsen et al., 2008; Jernberg et al., 2007). During this recovery period, recurrent *C. difficile* infections can occur. Fecal transplantation is a treatment to prevent relapse of CDI, where fecal material from healthy donors is used to replace important microflora, particularly in the *Bacteroidetes* and *Firmicutes* phyla. The procedure was first used in 1958, but has recently been deemed as a safe and viable treatment option for recurrent CDI (EISEMAN et al., 1958;

Kassam et al., 2013). Patients with recurrent CDI that receive oral or rectal fecal transplantation treatments and stop antibiotic therapy show 90% success in treatment (Kassam et al., 2013). A randomized trial using vancomycin followed by fecal transplantation or vancomycin alone showed greater efficacy against CDI for the combination treatment (van Nood et al., 2013). A randomized double-blind clinical trial involving 219 patients with recurrent CDI used treatment of frozen or fresh fecal transplant. Both fecal transplantation treatment types showed efficacy in reducing recurrence of CDI (Lee et al., 2016). Studies investigating the efficacy of fecal transplantation in treating primary CDI are currently underway (Schenck et al., 2015).

18.5 Bacteriophage treatment

The use of bacteriophages has recently been approved for use in foods to prevent foodborne disease outbreaks (Hagens & Loessner, 2010). This highlights the safety of bacteriophages as antimicrobials. Bacteriophages can be used as an alternative treatment to target *C. difficile* in the gastrointestinal tract and prevent colonization. However, it has been difficult to isolate bacteriophages that are strictly virulent against *C. difficile* (Hargreaves & Clokie, 2015). Bacteriophages act by attaching to receptors on *C. difficile*, inserting their DNA, which allows phage replication and eventually causes lysis of *C. difficile* cells. A study to identify a phage effective against *C. difficile* was conducted and a specific four-phage combination (phiCDHM1-phiCDHM2-phiCDHM5-phiCDHM6) was identified as most effective in lysing *C. difficile* and preventing resistant clones. The bacteriophage combination was delivered to hamsters and *C. difficile* colonization was reduced significantly (Nale et al., 2015). Therefore, this treatment method could be effective in treating CDI in humans. Novel phage tail-like particles (PTLP) have been identified, which are similar to bacteriophages, but do not require DNA transmission into the target cell. In an experiment, PTLP were isolated from CDI

patient tissue samples and were found to act specifically against *C. difficile* cells (Sangster et al., 2015). However, additional studies are necessary to confirm the efficacy of this treatment.

18.6 Nontoxigenic strains for controlling recurrent CDI

The non-toxigenic *C. difficile* strain VP20621 was evaluated for safety in healthy individuals between 18-45 years of age. Test subjects received vancomycin and then oral suspensions of the nontoxigenic strain spores or a placebo. The VP20621 strain was found to be safe, able to colonize the gastrointestinal tract, and recovered in the stools of all patients administered the treatment dose (Villano et al., 2012). A follow-up study was completed to determine the efficacy of the nontoxigenic *C. difficile* strain M3 (VP20621; NTCD-M3) in preventing recurrent CDI. A double-blind study was conducted in individuals with CDI that had been treated with metronidazole, vancomycin or both antibiotics. The patients were administered a placebo treatment or a treatment of varying nontoxigenic strain spore counts daily for 7 days. The nontoxigenic M3 *C. difficile* strain was found to be safe, had the ability to colonize the gastrointestinal tract of patients and significantly reduced the rate of recurrent CDI (Gerding et al., 2015).

18.7 Alternative treatment

Aspirin (acetylsalicylic acid) is a well-known non-steroidal anti-inflammatory drug (NSAID). Aspirin is the most widely used drug in the field of medicine (Soni, 2005). The US Preventative Services Task Force recommends daily low dose aspirin ($\leq 30\text{mg}$) for the prevention of cardiovascular disease as a preventative for recurrent cardiovascular events (Ittaman et al., 2014; US Preventive Services Task Force, 2009). Low dose aspirin has been shown to reduce the aggregation of platelets which prevents blood vessel obstruction and reduces the risk of heart attack and stroke (Dippel et al., 2004). Therefore, it is readily prescribed to

patients as a daily medication. Aspirin was recently identified as a factor that reduced mortality from CDI in a clinical observational study of adults diagnosed with CDI (Saliba et al., 2014). However, the mechanism of action involved in reduced CDI mortality by aspirin is unknown, but could potentially act through reduced toxin production, sporulation or spore outgrowth of *C. difficile*.

In summary, the isolation of genetically similar and identical strains of *C. difficile* in the environment, food animals and humans indicates the potential for food to be a source of CDI (Debast et al., 2009; Silva et al., 2015). *C. difficile* has been detected in retail meats, especially ground beef, but its survival during storage, cooking and acidic conditions has not been previously investigated. Moreover, US prevalence studies of *C. difficile* in vegetables, especially in ready-to-eat lettuce, are limited. Further, *C. difficile* is widely transmitted in hospital settings and relapse frequently occurs from re-ingestion of spores. *C. difficile* spores can survive in the abiotic environment such as on hospital surfaces and equipment for extended periods of time. Therefore, effective disinfection of hospital equipment and surfaces could control transmission of CDI.

This dissertation hypothesized that *C. difficile* is potentially present in ready-to-eat lettuce, where it can survive under modified atmosphere packaging conditions. Also, *C. difficile* spores in ground beef will survive the effect of chilling, freezing and cooking, and the effect of acidity in fermented pork summer sausage. In addition, it was hypothesized that octenidine hydrochloride will be effective in reducing *C. difficile* spores on stainless steel surface, and acetylsalicylic acid (aspirin) will decrease *C. difficile* sporulation and germination.

The specific objectives of this dissertation are to:

1. Determine the prevalence of *C. difficile* in ready-to-eat lettuce (REL) in Connecticut, and study the effect of modified atmosphere packaging (MAP) on the viability of *C. difficile* spores in REL.
2. Determine the effect of chilling, freezing and cooking on the viability of *C. difficile* spores in ground beef.
3. Study the effect of acidity and cooking on the viability of *C. difficile* spores in fermented pork summer sausage.
4. Determine the efficacy of octenidine hydrochloride (OH) in reducing *C. difficile* spores on stainless steel surfaces.
5. Investigate the effect of aspirin on *C. difficile* sporulation and spore germination *in vitro*.

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Chapter III

Prevalence of *C. difficile* in ready-to-eat lettuce (REL) in Connecticut, and the effect of modified atmosphere packaging (MAP) on the viability of *Clostridium difficile* spores on REL

Abstract

Clostridium difficile is a nosocomial pathogen with significant public health implications in humans. However, recent trends have shown increased *C. difficile* infections in communities outside of hospital environments. Additionally, *C. difficile* has been detected in water, soil, food animals, animal feces and foods such as meat and vegetables. Fresh produce is grown in contact with soil and water and may not be cooked prior to consumption. Therefore, there is an increased risk of contamination of fresh produce with *C. difficile*. This study was conducted to investigate the prevalence of *C. difficile* in ready-to-eat lettuce (REL) and to determine the survival of *C. difficile* spores on REL under modified atmosphere packaging (MAP) conditions.

One hundred and fifty samples of REL were collected from geographically distant, retail stores in Connecticut and tested for the presence of *C. difficile*. Lettuce samples (25 g) were mixed with *C. difficile* moxalactum norfloxacin broth (25 ml) with 0.1% (w/v) sodium taurocholate (CDMNT), and 1 ml aliquots were alcohol-shocked and pour-plated with CDMNT agar followed by 48 h of anaerobic incubation at 37°C for enrichment. Positive isolates were confirmed by Gram staining, L-proline aminopeptidase test, PCR targeting *C. difficile* housekeeping *tpi* gene, and toxin A and B genes. Further, the isolates were characterized by ribotyping and antibiotic resistance. A follow-up study was conducted to investigate the effect of MAP conditions (2% O₂, 5% CO₂ and 93% N₂) on the survival of *C. difficile* spores on REL under refrigeration. REL samples were inoculated with hyper-virulent *C. difficile* spores at 2.0 log CFU/g and 4.0 log CFU/g, and refrigerated under MAP conditions for 10 days. Enumeration of *C. difficile* on selective CDMNT agar was done on days 0, 1, 3, 5, 7 and 10.

Thirty-five REL samples out of 150 (23%) tested positive initially for *C. difficile*, and all isolates were found to contain the genes encoding for toxin A and B. However, none of the positive

REL yielded any *C. difficile* by direct plating. In addition, all isolates were found to belong to the ribotype 027. Twenty of the isolates were tested for sensitivity to ten clinical antimicrobials and were found to be multi-drug resistant. Results showed that *C. difficile* spores remained viable on REL under MAP conditions throughout 10 days of storage. Although the results suggest the presence of *C. difficile* on REL and the viability of *C. difficile* spores under MAP storage conditions, the potential role of REL in community-acquired *C. difficile* infection in humans requires additional investigation.

1. Introduction

Clostridium difficile is a gram-positive, spore-forming, anaerobic, nosocomial pathogen affecting hospitalized patients taking long-term antibiotics. However, a new trend has shown an increase in community-associated *C. difficile* infections (CA-CDI), especially in individuals who are not taking long term antibiotics and are not residing in hospital settings (Hensgens et al., 2012; Lessa, 2013). The source of infection of CA-CDI is currently not known, but food could be a potential source of the pathogen. An additional trend in the epidemiology of *C. difficile* is the emergence of a new hypervirulent strain belonging to PCR ribotype 027, which is reported in major outbreaks in Europe, Canada, Australia, and the United States (Cassir et al., 2016; He et al., 2013; Kuijper et al., 2006; Miller et al., 2010).

C. difficile is present in the environment and has been detected in water from lakes, rivers and wastewater effluent, in addition to various soil types in both farms and densely populated areas (al Saif & Brazier, 1996; Janezic et al., 2016; Steyer et al., 2015; Zidaric et al., 2010). *C. difficile* has also been isolated from the feces of livestock such as cattle and pigs, and from foods such as meat, shellfish and vegetables (Costa et al., 2012; Eckert et al., 2013; Janezic et al., 2012; Pasquale et al., 2011; Rodriguez-Palacios et al., 2007). *C. difficile* strains isolated from the environment and foods have been found genotypically similar or identical to strains implicated in CA-CDI cases (Debast et al., 2009; Silva et al., 2015). Therefore, food is increasingly being investigated as a potential source of CA-CDI, although no foodborne outbreaks have been reported to date (Gould & Limbago, 2010). Studies in Scotland, France, Canada, Iran and in the US (Ohio) have detected *C. difficile* in vegetables and ready-made-salads at rates of 7.5% (3/40), 3.33% (2/60), 4.5% (5/111), 5.66% (6/106) and 2.4% (3/125) respectively (Bakri et al., 2009; Eckert et al., 2013; Rodriguez-Palacios et al., 2014; Yamoudy et al., 2015). Further, the strains isolated from these studies above were genotypically similar to strains implicated in human infections, such as

ribotypes 001, 014, 027 and 078 (Eckert et al., 2013; Metcalf et al., 2010), thereby strengthening the potential role of fresh produce as a source of CA-CDI.

Lettuce is the dominant salad green consumed in the U.S. Head lettuce can be either field packed for bulk sale or transported to commercial facilities for further processing to ready-to-eat lettuce (REL) (AMRC, 2014). Ready-to-eat lettuce is commercially packed under MAP conditions of (2-5% O₂, 5% CO₂, 90% N₂, balance N₂), which reduces aerobic spoilage bacteria, thereby increasing shelf life of the product. However, lettuce is a respiring vegetable that decreases the oxygen level under MAP conditions, which has been shown to reach an anaerobic state after 7 days of storage (Hempel et al., 2013). Therefore, MAP conditions could potentially support the persistence of anaerobic pathogens such as *C. difficile*, since previous research has revealed that MAP conditions with low oxygen favor spore outgrowth and toxin production in *Clostridium botulinum* (Kasai et al., 2005; Newell et al., 2012). Currently little information is available on the viability characteristics of *C. difficile* on vegetables under MAP conditions. Therefore, this study was undertaken to determine the viability of *C. difficile* spores on commercially available REL under MAP conditions.

2. Materials and Methods

2.1 Sample collection and detection of C. difficile in REL

A total of 150 samples of REL were procured from geographically distant retail stores (n=18) in Connecticut. All procured samples were brought to the laboratory on ice and processed within 24 h. The detection of *C. difficile* was performed according to a previously published protocol with modification for a higher detection limit (Weese et al., 2010). Twenty-five grams of lettuce was separately added to 25 ml of *C. difficile* moxalactam-norfloxacin broth (Oxoid, Hampshire, UK) supplemented with 0.1% sodium taurocholate (CDMNT) (Sigma-Aldrich, Inc.,

St. Louis, MO, USA), and was subjected to stomaching for 1 min. After stomaching, 1 ml of broth was pour-plated in duplicate in CDMNT agar, while the remaining broth-sample mixture was incubated for enrichment in a Whitley A35 anaerobic workstation (Microbiology International, Frederick, MD, USA) at 37°C for 48 h. After incubation, a 5 ml aliquot of the enriched sample was subjected to alcohol shock by adding 5 ml of 100% anhydrous ethanol for 1 h to eliminate vegetative *C. difficile*. The broth was subsequently centrifuged at 4000 x g for 10 min, and the pellet was resuspended in 0.5 ml of sterile phosphate buffered saline (PBS, pH 7.0) and pour-plated in CDMNT agar. The direct pour plates and enrichment pour plates were incubated anaerobically at 37°C for 48 h. Presumptive *C. difficile* colonies were subcultured on 7% horse blood agar and follow up identification of *C. difficile* was done based on growth in CDMNT agar, Gram's staining, and colony morphology characteristics. The identity of *C. difficile* was further confirmed by L-proline aminopeptidase reaction (ProDisk, Remel, Lenexa, KS, USA) and PCR targeting *tpi*, a species-specific housekeeping gene in *C. difficile* (Lemee et al., 2004).

2.2 Antibiotic susceptibility tests

The isolated *C. difficile* strains were tested for susceptibility to 10 antimicrobials (ampicillin, cefoxitin, ciprofloxacin, clindamycin, erythromycin, metronidazole, moxifloxacin, rifampicin, tetracycline and vancomycin) using E-test (bioMérieux, Marcy l'Etoile, France) on Brucella blood agar supplemented with hemin and vitamin K, according to the manufacturer's instructions. The plates were incubated for 48 h at 37°C under anaerobic conditions, after which the minimum inhibitory concentration (MIC) values were determined. The MIC breakpoints for clindamycin, metronidazole, moxifloxacin and vancomycin were compared with those established by the CLSI (Clinical and Laboratory Standards Institute (CLSI), 2007), European Committee for Antimicrobial Susceptibility Testing (EUCAST) and British Society of antimicrobial

chemotherapy (BSAC) for *C. difficile*. For assessing the susceptibility to ampicillin, cefoxitin, ciprofloxacin, clindamycin, erythromycin, rifampicin and tetracycline the epidemiological cutoff values (ECOFF) recently established by the EUCAST obtained from the EUCAST MIC distribution database were utilized (EUCAST, 2015). *C. difficile* toxigenic strain ATCC 1870 was used as the control. The *C. difficile* isolates were considered multidrug resistant (MDR) if they were resistant to three or more classes of antimicrobial agents (Schwarz et al., 2010; Thitaram et al., 2016).

2.3 Toxin gene detection and ribotyping

A Chelex resin-based DNA extraction kit (InstaGene Matrix, Bio-Rad, France) was used for extracting the genomic DNA from *C. difficile* isolates. A multiplex PCR was carried out to detect the genes-encoding toxins TcdA and TcdB using previously published primers (Table 1) (Antikainen et al., 2009). PCR amplification was done using HotstarTaq (Qiagen, Germantown, MD, USA) PCR master mix, with thermocycler conditions: 10 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, with a final extension of 5 min at 72°C.

PCR ribotyping was performed using a previously published protocol (Bidet et al., 1999). The amplification reactions with designed primers were performed using HotstarTaq PCR master mix (Qiagen). The thermocycler conditions were 10 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, with a final extension of 5 min at 72°C. The PCR products using the toxin gene primers were electrophoresed for 2 h at 75 V in 0.5X TBE using 1% agarose gel (MidSci, St.Louis, MO, USA) stained with 0.5 µg/ml ethidium bromide, and analyzed under UV light using the ChemiDoc MP Imaging System (Biorad, Hercules, CA, USA). The PCR products using the ribotyping primers were electrophoresed for 10 h at 75 V in 0.5X TBE using 3% agarose gel stained with 0.5µg/ml ethidium bromide, and analyzed under UV light using the

ChemiDoc MP Imaging System. The presence of bands from the toxin gene PCR and ribotype profiles were analyzed with GelCompar II image analysis software (version 5.1; Applied Maths., Austin, TX, USA).

2.4 Spore preparation

C. difficile spores were prepared using a previously published protocol, with slight modifications (Sorg & Dineen, 2009). Briefly, single colonies of ATCC BAA 1870, 1805 and 1053 were separately inoculated into Brain Heart Infusion broth supplemented with 5% yeast extract (BHIS) (Oxoid) and cultured overnight at 37°C under anaerobic conditions. A 150 µl aliquot of overnight culture was gently spread to evenly distribute the culture onto BHIS agar in six-well plates, and incubated anaerobically for 7 days at 37°C in an anaerobic workstation (Microbiology Inc, USA) to allow sporulation. After 7 days, spores were harvested from the wells by flooding with 2 ml of ice cold sterile water. The spore suspension was heat treated at 60°C for 20 min to kill any vegetative cells, washed in dH₂O and centrifuged at 16,000 g for 5 min with five repeated washings and centrifugations to purify the spores. Spore suspensions were visualized under a microscope to ensure 90% sporulation prior to storage at –20°C.

2.5 Lettuce inoculation

Spore suspensions generated from hypervirulent *C. difficile* strains ATCC BAA 1870, 1805 and 1053 were diluted in sterile water to obtain 2 log CFU/ml and 4 log CFU/ml inoculations. An inoculum containing spores from the three *C. difficile* strains was prepared for each inoculation level. Then 25 g of pre-cut fresh romaine lettuce was spot inoculated with 1 ml of inoculum in droplets followed by drying under a laminar flow biosafety cabinet for 30 min (Chua et al., 2008; Oliveira et al., 2010). Using sterile tongs, the inoculated lettuce was aseptically transferred to vacuum sealable pouches with multilayer films designed for modified atmosphere packaging

(Winpak, Winnipeg MB, Canada) and were flushed with a gaseous mixture of 2% O₂, 5% CO₂ and 93% N₂ using an UltraSource ultra vac (UltraSource, Kansas City, MI, USA). Inoculated and control MAP bags were immediately stored at 4°C for 10 days.

2.6 Enumeration of *C. difficile* and total bacteria on lettuce

The *C. difficile* populations on control and inoculated lettuce samples were enumerated on days 0, 1, 3, 5, 7 and 10 of storage. Lettuce (25 grams) was diluted with 25 ml of sterile PBS and was stomached for 2 min. The samples were serially diluted and pour-plated in duplicate on CDMNT agar (Weese et al., 2010; Zhang et al., 2009). Plates were incubated at 37°C anaerobically for up to 5 days before counting colonies. The samples were enriched by transferring 1 ml of the PBS-lettuce to 9 ml of CDMNT broth for anaerobic incubation at 37°C for 48 h (Weese et al., 2010), followed by pour-plating in duplicate on CDMNT agar, if no *C. difficile* was recovered by direct plating. At each sampling time point, controls, 2 log CFU/ml and 4 log CFU/ml samples were spread-plated in duplicate on plate count agar (PCA) for incubation at both 37°C and 4°C to enumerate the total bacteria on the lettuce. The PCA plates were incubated at 37°C and 4°C, as previously reported and counts were combined for the two temperature incubations to obtain a total bacteria count for each sample (Akbas & Ölmez, 2007).

2.7 Statistical analysis

The required sample size for the prevalence study was determined using the PROC-POWER procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A sample size of 150 samples was required to allow the detection of a difference in prevalence between 3% versus 14% with an alpha = 0.05 and power = 0.80 (two-tailed test). All MAP experiments had duplicate samples for each treatment, and the study was repeated three times. The data were analyzed using

the PROC-MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Differences between the means were considered significantly different at $p < 0.05$.

3. Results and Discussion

A report from the US Centers for Disease Control and Prevention indicated that fresh produce is responsible for the highest number of foodborne infections, accounting for ~ 46% of the 9 million foodborne infections annually in the US (Painter et al., 2013). The high rate of foodborne outbreaks linked to fresh produce may be due to the greater potential for pre-harvest contamination from contaminated soil, irrigation water, improperly composted manure, farm workers, or fecal run-off from domestic or wild animals (Buck et al., 2003; Mukherjee et al., 2007). Hypervirulent *C. difficile* strains have also been isolated from soil, water, animal feces and asymptomatic human carriers, thereby underscoring that they can potentially serve as sources of *C. difficile* contamination on fresh produce (Himsworth et al., 2014; Janezic et al., 2016; Rodriguez-Palacios et al., 2013; Xu et al., 2014). The isolation of *C. difficile* from REL could be of greater concern than isolation from other foods since it is consumed raw. Moreover, REL is packaged under modified atmosphere packaging (MAP) with limited oxygen, which favors the persistence of anaerobic pathogens such as *C. difficile*. Previous research has revealed that MAP conditions with low oxygen favor spore outgrowth and toxin production by *C. botulinum* (Kasai et al., 2005; Newell et al., 2012). Moreover, potential temperature abuse of MAP-stored REL during transport and retail storage could promote *C. difficile* spore germination and growth.

The prevalence rate of *C. difficile* in REL in Europe was found to be 2.9% in France (Eckeert et al., 2013) and 7.5% in Scotland (Bakri et al., 2009; Eckert et al., 2013). The only prevalence study in vegetables conducted in the US did not include REL (Rodriguez-Palacios et al., 2014). In this study, we investigated the prevalence of *C. difficile* in 150 samples of REL from

different retail stores in Connecticut. Presumptive colonies of *C. difficile* on selective CDMNT agar were initially isolated from 35% of REL samples. Further testing of the presumptive colonies with the L-proline aminopeptidase activity test decreased the number of positive samples to 30%. However, PCR characterization to detect the housekeeping *tpi* gene, toxin genes, and ribotyping pattern identified *C. difficile* in 23% (35/150) of REL samples. All 35 isolates were identified as toxigenic by the presence of toxin genes *tcdA* and *tcdB*. Also, the ribotyping pattern of all 35 *C. difficile* isolates matched the banding pattern of ribotype 027 (Fig. 1). Interestingly, all the REL samples that tested positive for *C. difficile* were only by enrichment, and none of the samples yielded colonies by direct plating.

We screened 20 of the 35 positive *C. difficile* isolates for resistance to ten antibiotics (Table 2) typically used in hospitals (Mooyottu et al., 2015). All of the isolates were found to be resistant to ciprofloxacin (MIC > 32 µg/ml), erythromycin (MIC > 256 µg/ml) and moxifloxacin (MIC > 32 µg/ml) (Table 2). Six of the isolates were found to be resistant to vancomycin (MIC > 2 µg/ml). Moreover, the isolates were not susceptible to cefoxitin at the highest concentration (MIC > 256 µg/ml). Also, the isolates were not resistant to clindamycin, metronidazole, rifampicin or tetracycline (except for one isolate which was resistant to metronidazole), with MIC values for clindamycin, metronidazole, rifampicin or tetracycline below the ECOFF values of (16 µg/ml), (2 µg/ml), (0.004 µg/ml) and (0.25 µg/ml), respectively. The MIC of ampicillin was found to be > 1.5 µg/ml in five of the isolates, which is not the defined MIC for *C. difficile*, but is considered the MIC breakpoint for other Gram-positive anaerobes.

According to the Clinical and Laboratory Standards Institute (CLSI), bacterial isolates are considered to be multi-drug resistant when exhibiting resistance to more than three groups of antibiotics. American studies involving food animals, meat, and vegetables have reported

resistance of *C. difficile* isolates to multiple classes of antibiotics (Harvey et al., 2011; Rodriguez-Palacios et al., 2013), especially with isolation of multidrug resistant *C. difficile* from food animals and meat products (Peláez et al., 2013; Pirs et al., 2013; Thitaram et al., 2016). Similarly, our results indicated that *C. difficile* isolates from REL samples were multi-drug resistant, exhibiting resistance to ciprofloxacin, erythromycin and moxifloxacin. The presence of antimicrobial resistance in *C. difficile* isolated from ready-to-eat vegetables has been identified in several prevalence studies, where Bakri et al. (2009) and Rodriguez-Palacios et al. (2014) isolated *C. difficile* strains resistant to moxifloxacin and clindamycin from Scotland and Ohio, respectively. The *C. difficile* strains isolated in our study from REL were similarly resistant to moxifloxacin, but were not resistant to clindamycin.

We conducted a follow-up study to determine the viability of *C. difficile* spores on REL under MAP conditions used by the fresh produce industry (2% O₂, 5% CO₂ and 93% N₂). Two inoculation levels of 2.0 log CFU and 4.0 log CFU of *C. difficile* spores per gram of lettuce were used in the survival study. Enumeration of viable *C. difficile* spore populations on selective CDMNT agar on days 0, 1, 3, 5, 7 and 10 of refrigeration revealed that spores remained viable on REL samples at both inoculation levels throughout storage (Fig. 2). The viability of *C. difficile* on foods, including vegetables under modified atmosphere conditions has not been previously investigated, although Jobstl et al. (2010) isolated *C. difficile* from a raw meat product packaged under MAP conditions. This isolate was positive for the toxin producing genes, *tcdA* and *tcdB* and was resistant to the antibiotics clindamycin and moxifloxacin (Jobstl et al., 2010).

Respiration of produce results in the decrease of oxygen content in a modified atmosphere package, which could lead to an anaerobic environment during storage. The level of oxygen in commercial control MAP conditions (5% O₂, 5% CO₂ and 90% N₂) showed lack of oxygen after

7 days of storage (Hempel et al., 2013). In addition, temperature abuse can speed up the respiration rate of produce and lead to faster development of anaerobic conditions in MAP products, which could be a concern for anaerobic pathogens (Francis et al., 1999). *Clostridium botulinum*, like *C. difficile*, is an organism that has been isolated from the environment, including agricultural soils in numerous countries (Huss, 1980), and has historically been studied for growth and toxin production on vegetables packaged under MAP. Since the commercial gas mixture for MAP contains low oxygen, which can be consumed by respiring produce, a potential concern for *C. difficile* to grow and produce toxins exists if the packaging became anaerobic. A study on *C. botulinum* in MAP packaged produce showed that toxins were produced by the bacterium at temperatures of 15°C or higher, but growth and toxin production were controlled at temperatures less than 5°C (Austin et al., 1998). The study showed that *C. botulinum* can survive in produce packaged under MAP conditions, but will only grow and produce toxins under temperature abuse conditions. Interestingly, these observations could be valid for *C. difficile* too, and results from our study indicated that *C. difficile* spores remained viable at 4°C under MAP conditions, although temperature abuse was not a focus here.

In conclusion, this study isolated *C. difficile* spores from 23% (35/150) of REL samples procured from various retail stores in Connecticut. Although none of the positive samples yielded any countable *C. difficile* populations by direct plating, the results are important, especially in light of increasing REL consumption, particularly by vulnerable members of the population, including the elderly, immunocompromised, and convalescent people in hospitals and nursing homes. Therefore, further research is needed to determine if *C. difficile* spores could germinate and grow in REL under temperature abuse conditions. Additionally, the potential role of REL in community-acquired *C. difficile* infection in humans requires additional investigation.

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Table 1. Primers used for PCR and Ribotyping.

Oligo name	Gene	Primer Sequence (5'-3')	Size (bp) /position
TPI-F ¹	<i>tpi</i>	AAAGAAGCTACTAAGGGTACAAA	230
TPI-R ¹		CATAATATTGGGTCTATTCCTAC	
TA1 ²	<i>tcdA</i>	ATGATAAGGCAACTTCAGTGG	624
TA2 ²		TAAGTTCCTCCTGCTCCATCAA	
TB1 ²	<i>tcdB</i>	GAGCTGCTTCAATTGGAGAGA	412
TB2 ²		GTAACCTACTTTCATAACACCAG	
NK 104 ²	<i>tcdB</i>	GTGTAGCAATGAAAGTCCAAGTTTACGC	205
NK 105 ²		CACTTAGCTCTTTGATTGCTGCACCT	
	<i>16S</i> ³	CTGGGGTGAAGTCGTAACAAGG	21 (1445–1466)
	<i>23S</i> ³	GGTACCTTAGATGTTTCAGTTC	20 (1-20)

(Lemee et al., 2004)¹, (Antikainen et al., 2009)², (Bidet et al., 1999)³

Fig 1. Ribotyping pattern of *C. difficile* isolates from lettuce.

The gel image shows multiplex polymerase chain reaction amplification products representing ribotype banding patterns. Lane 1, 2: *C. difficile* ATCC 027, Lane 3: *C. difficile* ATCC 078, Lane 4: *C. difficile* ATCC 001, Lane 5, 6, 7, 8, 9: Representative REL isolates of *C. difficile*, Lane 10: 100 bp DNA ladder, Lane 11, 12, 13, 14, 16, 17, 18: Representative REL isolates of *C. difficile*, Lane 19: Negative control, Lane 20: *C. difficile* ATCC 038.

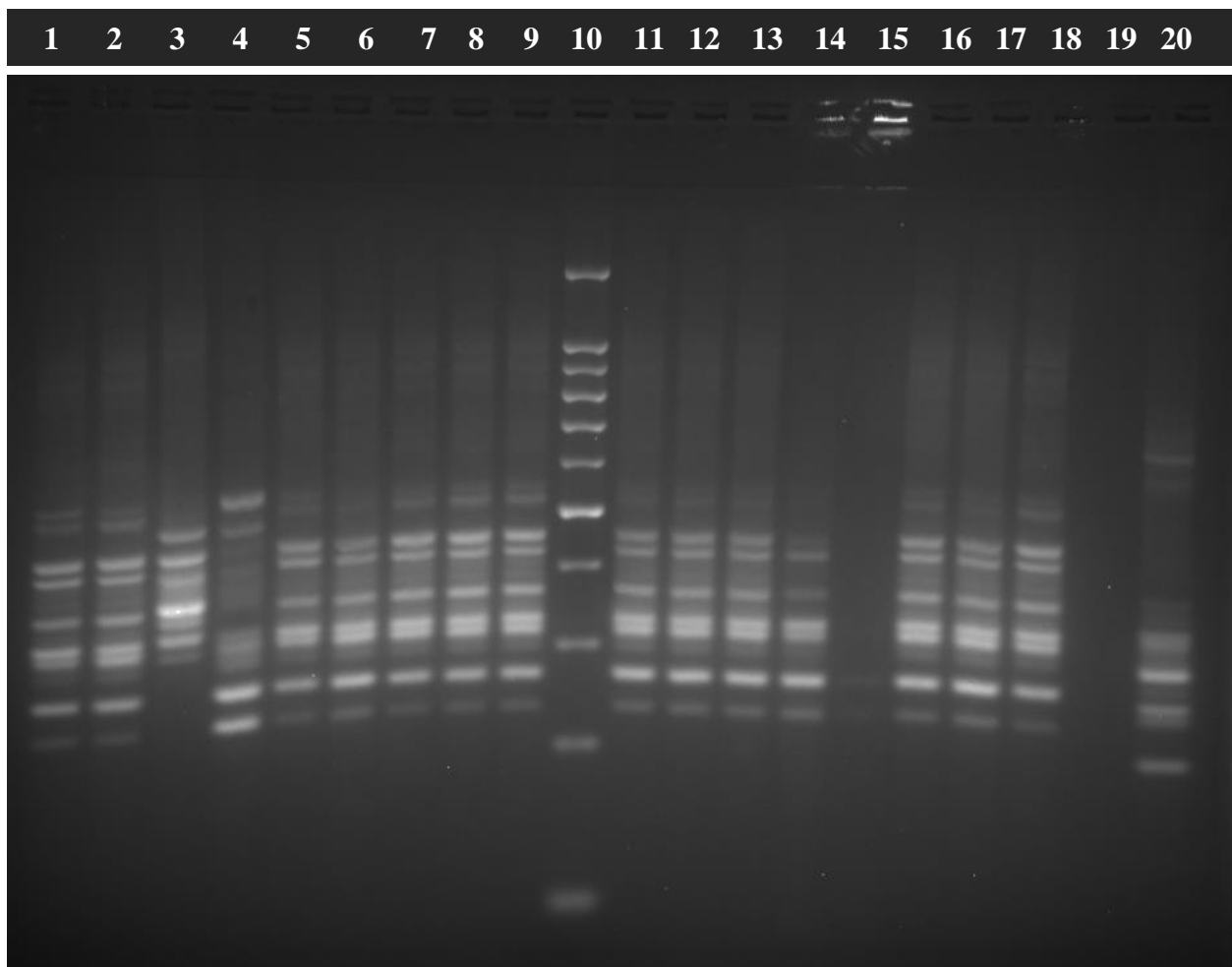


Table 2. Antibiotic sensitivity of *C. difficile* isolates to 10 clinically used antimicrobials

Antibiotic	MIC (ATCC 1870) µg/ml	CLSI break point µg/ml	EUC AST ECO FF	Minimum Inhibitory Concentration (MIC) µg/ml of lettuce isolates									
			(µg/ ml) <	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8	Isolate 9	Isolate 10
Ampicillin	1	-	-	0.75	1.25	1.5	2	1	0.75	1	1.5	1	0.75
Cefoxitin	>256	-	-	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Ciprofloxacin	>32	-	-	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Clindamycin	0.5	-	16	2	2	8	4	4	0.016	3	2	0.75	1.5
Erythromycin	>256	-	2	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Metronidazole	0.19	2	2	2	3	3	1.5	3	0.38	0.38	0.5	2	0.25
Moxifloxacin	>32	-	4	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Rifampicin	0.002	-	0.00 4	0.002	0.003	0.003	0.002	0.002	0.003	0.002	0.002	0.002	<0.002
Tetracycline	0.25	-	0.25	0.25	0.5	0.5	0.19	0.19	0.125	0.25	0.064	0.064	0.25
Vancomycin	>256	>2	2	1	1.5	1	1	1.5	1	2	2	1.5	0.75

Antibiotic	MIC (ATCC 1870) µg/ml	CLSI break point µg/ml	EUC AST ECO FF	Minimum Inhibitory Concentration (MIC) µg/ml of lettuce isolates									
			(µg/ ml) <	Isolate 11	Isolate 12	Isolate 13	Isolate 14	Isolate 15	Isolate 16	Isolate 17	Isolate 18	Isolate 19	Isolate 20
Ampicillin	1	-	-	2	2	0.75	1.5	2	1	1.5	2	0.5	1.75
Cefoxitin	>256	-	-	>256	>256	>32	>32	>32	>32	>32	>32	>32	>32
Ciprofloxacin	>32	-	-	>32	>32	>256	>256	>256	>256	>256	>256	>256	>256
Clindamycin	0.5	-	16	6	3	0.5	0.75	1.5	1	0.75	0.125	0.016	1
Erythromycin	>256	-	2	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Metronidazole	0.19	2	2	2	0.5	0.75	0.5	1.25	0.38	1.5	0.5	1	0.38
Moxifloxacin	>32	-	4	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Rifampicin	0.002	-	0.00 4	0.002	0.002	<0.002	0.003	0.002	0.002	0.002	0.002	<0.002	0.002
Tetracycline	0.25	-	0.25	0.19	0.125	0.125	0.094	0.25	0.125	0.38	0.19	0.064	0.125
Vancomycin	>256	>2	2	0.75	2	3	2	2	3	1.5	1.5	1	1.5

Fig 2. Viability of *C. difficile* spores on REL under MAP during refrigerated storage.

C. difficile spores (ATCC BAA 1870, 1805 and 1053) at three inoculation levels; 0 log CFU/ml (control), 2 log CFU/ml and 4 log CFU/ml. The *C. difficile* populations on lettuce for control and inoculated samples were enumerated on days 0, 1, 3, 5, 7 and 10 by pour-plating CDMNT agar with anaerobic incubation at 37°C for 48 h.

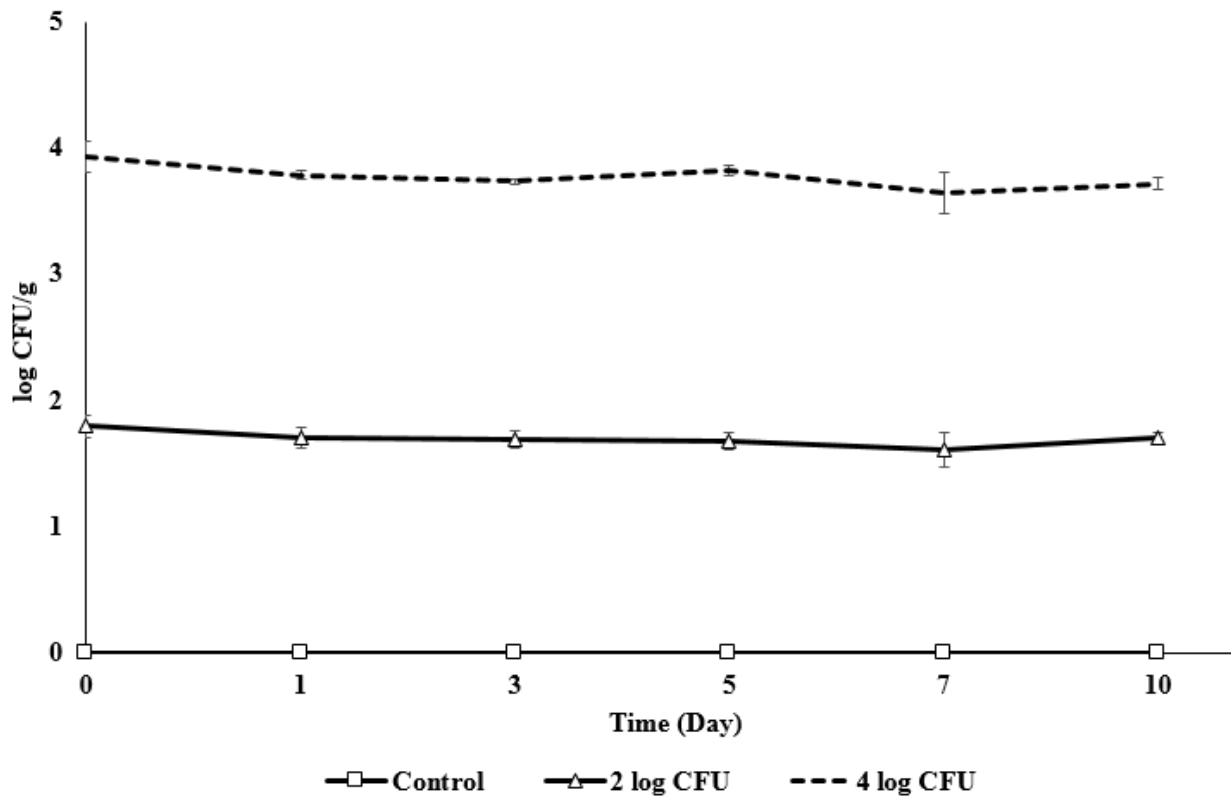
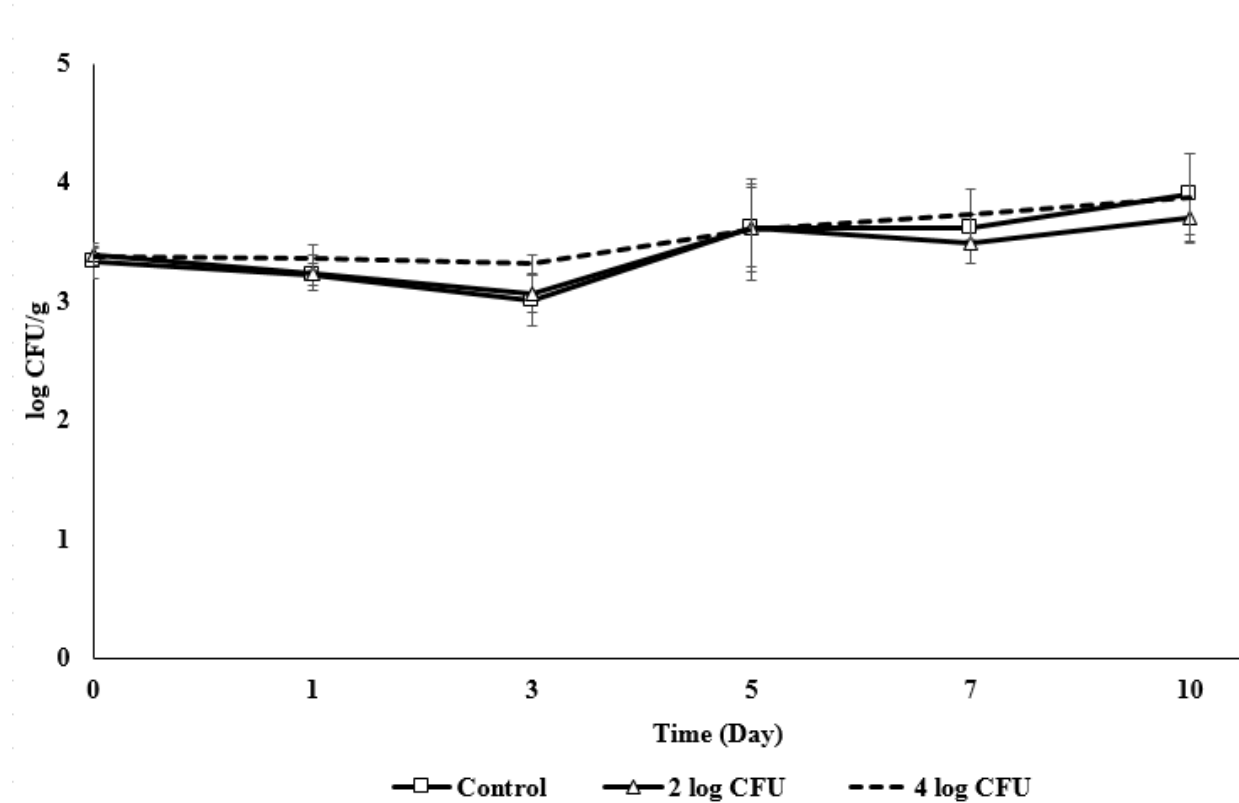


Fig 3. Total bacteria on REL inoculated with *C. difficile* under MAP.

C. difficile spores (ATCC BAA 1870, 1805 and 1053) at three inoculation levels; 0 log CFU/ml (control), 2 log CFU/ml and 4 log CFU/ml. Samples were serially diluted in PBS and spread plated on PCA (plate count agar) to enumerate the total bacteria on lettuce on days 0, 1, 3, 5, 7 and 10. PCA plates were incubated at 37°C and 4°C and counts were combined to determine total bacteria.



Chapter IV

The effect of chilling, freezing and cooking on survivability of *Clostridium difficile* spores in ground beef

Abstract

Clostridium difficile is an anaerobic pathogen that causes serious infections in hospital settings. However, recent trends have shown an increased incidence and severity of *C. difficile* infections outside of hospital environments. In addition, human toxigenic *C. difficile* strains have been isolated from food animals and animal-derived foods including ground meat, which suggests potential foodborne transmission of *C. difficile*. Prevalence studies observing *C. difficile* in ground beef in the US have isolated *C. difficile* in a range of 0 to 44%. This study investigated the effect of chilling, freezing and the USDA recommended cooking temperature on survival of *C. difficile* spores in ground beef.

A three-strain *C. difficile* spore suspension was inoculated at two levels (3 log CFU/g and 5 log CFU/g) in ground beef. Inoculated samples were separately subjected to chilling (4°C) for 1 week, freezing (-18°C) for 12 weeks, or cooking (71.1°C), after which viable spores were enumerated at different time intervals. Similarly, total bacteria in ground beef was enumerated at each time point by treatment.

Chilling for 1 week did not significantly affect the survival of *C. difficile* spores at both levels of inoculation ($p > 0.05$). Similarly, freezing for 12 weeks did not significantly affect the survival of *C. difficile* spores at both levels of inoculation in ground beef ($p > 0.05$). Freezing also did not affect the viability of total bacteria in ground beef ($p > 0.05$). Cooking ground beef to the USDA recommended temperature (71.1°C) decreased spore survival in ground beef inoculated with 3 log CFU/g and 5 log CFU/g by 1 log CFU/g compared to uncooked controls ($p < 0.05$).

Our results suggest that *C. difficile* spores can survive chilling, freezing and cooking in ground beef. The results warrant development of intervention strategies to control *C. difficile* contamination of bovine carcasses.

1. Introduction

Clostridium difficile is a spore-forming, nosocomial pathogen which causes a toxin-mediated enteric disease in humans. The pathogen is responsible for over 500,000 cases and 29,000 deaths annually (Lessa et al., 2015), with \$3 billion as health-care costs (O'Brien et al., 2007). Although traditionally a nosocomial pathogen, recent trends have shown an increase in the incidence and severity of *C. difficile* infections outside of hospital environments (Hensgens et al., 2012; McDonald et al., 2006; Rupnik et al., 2009). It is reported that 32% of all *C. difficile* illnesses in the United States are classified as community-associated *C. difficile* infections, especially in individuals with no recent hospitalization (Lessa, 2013). This leads to concerns on the potential sources of community associated *C. difficile* infections (Eyre et al., 2013). In addition, toxigenic *C. difficile* strains have been isolated from foods and food animals which match human strains, suggesting food as a potential source of *C. difficile* (Lessa et al., 2015; Thitaram et al., 2016).

The principal reservoir for *C. difficile* is the gastrointestinal tract of humans and warm-blooded animals (Cato et al., 1986). In a USDA-APHIS study on the prevalence of *C. difficile* in US cow-calf operations, *C. difficile* was isolated from 188 (6.3 percent) of 2,965 fecal samples from apparently healthy beef cows. The study also reported that 76 of the 173 beef operations (43.9 percent) yielded at least one positive sample. In a recent study by Bandelj et al. (2016), *C. difficile* was detected with an average prevalence of 10% and 35.7% in cows and calves, respectively (Bandelj et al., 2016). In another study conducted in the United States, *C. difficile* was isolated with a prevalence rate of 25% in stool specimens collected from 253 diarrheic calves (Hammitt et al., 2008). Likewise, in a study conducted in Canada, *C. difficile* was detected in calves with a prevalence rates as high as 49% (Weese, 2010). In addition, human hypervirulent *C. difficile* strain ribotype 027 has also been isolated from calves (Rodriguez-Palacios et al., 2006).

If cattle are a source of *C. difficile*, beef could serve as a potential transmission route of the pathogen from animals to humans. Results from several studies indicate that the overall prevalence of *C. difficile* in ground beef in the US ranges from 0 to 44%, with a minimum of 23% of positive isolates classified as toxigenic (Harvey et al., 2011; Houser et al., 2011; Limbago et al., 2012; Mooyottu et al., 2015; Songer et al., 2009).

Clostridium difficile produces highly resistant spores, which can persist for long periods in the environment (Fekety et al., 1981). Rodriguez-Palacios et al. (2016) observed that spores of 20 different strains of *C. difficile* survived the USDA-recommended meat cooking temperature of 71°C for 2 h in phosphate-buffered saline (PBS, pH 7). In addition, spores of hypervirulent *C. difficile* strain 078 were reported to be resistant to sub-boiling temperatures (Rodriguez-Palacios et al., 2016). This raises a significant food safety concern since heat tolerance studies with other spore-formers such as *C. perfringens* suggest that heat resistance can be much higher in complex food matrices such as ground meat than in buffer. Therefore, it is critical to understand *C. difficile* susceptibility to common antimicrobial hurdles applied in foods, which are critical for developing appropriate control measures. The objective of this study was to investigate the effect of chilling, freezing and cooking on the survival of *C. difficile* spores in ground beef.

2. Materials and Methods

2.1 Spore preparation

Clostridium difficile spores were prepared using a previously published protocol, with slight modifications (Sorg & Dineen, 2009). Three hyper virulent *C. difficile* isolates (ATCC BAA 1805, 1803 and 1053) were grown in brain heart infusion broth supplemented with 5% yeast extract (BHI) (Difco, Sparks, MD, USA) in a Whitley A35 anaerobic work station (Microbiology Inc., USA) in the presence of 80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37°C for 24 h. The three-strain *C. difficile* culture (150 µl) was inoculated onto 6-well BHI agar plates followed

by gentle rotation to evenly distribute the culture. After 7 d of incubation, when 90% sporulation was visualized under a microscope, the spores were harvested from the wells. For collecting spores, the wells were gently washed with 1 ml of sterile ice-cold water, after which the spore suspension was transferred to tubes and subjected to centrifugation (14,000 rpm for 5 min at 4°C). The supernatant was removed and the pellet was resuspended in 2 ml of sterile water. Centrifugation and resuspension were repeated five times to remove cellular debris. The resuspended spores were heat shocked at 60°C for 20 min to kill any remaining vegetative cells. The spores were enumerated by serial dilution and plating on BHI agar supplemented with 0.1% (w/v) sodium taurocholate (BHIS) (Sigma-Aldrich, Inc., St. Louis, MO, USA), followed by 48 h of anaerobic incubation.

2.2 Sample preparation, chilling and freezing of ground beef inoculated with *C. difficile*

Ground beef samples were prepared according to a previously published method (Juneja et al., 2013). Ten grams portions of 90% lean fresh ground beef were transferred to low oxygen transmission Whirl-Pak™ bags (Nasco, Ft. Atkinson, WI, USA). *C. difficile* spores were added to ground beef to obtain 3 log CFU/g or 5 log CFU/g inoculation levels. Following the addition of 100 µl of spore inoculum, each ground beef sample was hand-mixed for 1 min and subjected to stomaching for 1 min. The ground beef sample was flattened to a thickness of ~ 2 mm and bags were sealed with a Foodsaver (Sunbeam Products Inc., Boca Raton, FL, USA). Ground beef samples were either stored at 4°C for 7 days or frozen at -18°C for 12 weeks.

2.3 Enumeration of *C. difficile* spores and total bacteria in ground beef

Clostridium difficile spores were enumerated weekly from the frozen samples, and on days 0, 1, 3, 5 and 7 of storage in refrigerated meat (Weese et al., 2010). Frozen ground beef samples were removed from the freezer and thawed for 1 hr. Ten grams of ground beef were mixed with

20 ml of sterile PBS, and stomached for 1 min. The meat-PBS mixture was serially diluted and 1 ml portions were pour-plated in duplicate with *C. difficile* moxalactam-norfloxacin (Oxoid, Hampshire, UK) agar supplemented with 0.1% (w/v) sodium taurocholate (CDMNT) and 7% horse blood (Quad Five, Ryegate, MT, USA). In addition, 1 ml of the undiluted PBS-beef homogenate was added to 9 ml of CDMNT broth for enrichment. The CDMNT agar plates and enrichment broth were incubated anaerobically at 37°C for 48 h. After incubation, an aliquot of the broth was subjected to alcohol shock by adding 100% anhydrous ethanol for 1 h to eliminate vegetative bacteria. The broth was centrifuged at 4000 ×g for 10 min, and the pellet was resuspended in 0.5 ml of PBS and pour-plated in CDMNT agar. The CDMNT agar plates were incubated anaerobically at 37°C for 48 h. In addition, the total bacteria in ground beef was enumerated by plating 100 µl of the serial dilutions of the PBS-beef mixture on tryptic soy agar (TSA) with aerobic incubation at 37°C for 24 h (Dourou et al., 2011).

2.4 Sample preparation, and cooking of ground beef inoculated with *C. difficile*

Twenty-five gram portions of 90% lean ground beef were weighed. *C. difficile* spores were prepared and 100 µl added to each 25-gram portion of ground beef to obtain an inoculation level of 3 log CFU/g or 5 log CFU/g. Following this, the ground beef was hand-mixed for 1 min, and formed into patties (Amalaradjou et al., 2010). The patties were cooked individually in a double-sided George Foreman Lean Mean Grilling Machine (Salton Inc., Columbia, MO, USA) until the USDA recommended internal temperature for ground beef of 71.1°C was reached. A standard meat thermometer (Acutuff Model 34 Atkins 2 mm probe meat thermometer, Koch Supplies) was used to continuously monitor the internal temperature of patties. Each cooked patty was immediately transferred to a Whirl-PakTM bag containing 50 ml of chilled CDMNT broth, followed by hand-mixing and stomaching for 1 min. Uncooked patties were also transferred to Whirl-PakTM

bags containing chilled CDMNT broth and subjected to stomaching for 1 min. After stomaching, 1 ml of the meat homogenate was pour-plated in duplicate CDMNT agar plates. Enrichment and enumeration of samples was performed as previously described.

2.5 Statistical analysis

The freezing and chilling experiments included triplicate samples and the study was repeated two times. The cooking experiment had triplicate samples and the study was repeated three times. The data were analyzed using the PROC-GLM procedure of SAS v. 9.4 (SAS Institute Inc., Cary, NC, USA). Differences between the means were considered significantly different at $p < 0.05$.

3. Results & Discussion

C. difficile spores have been detected in water, soil, and animal feces (al Saif & Brazier, 1996; Rodriguez-Palacios et al., 2011), thereby potentially contaminating cattle hides and carcasses during slaughter. Further, isolation of toxigenic *C. difficile* in beef cattle and ground beef raises a concern on the potential meat-borne transmission of the pathogen to humans, especially in light of the increasing number of community-associated *C. difficile* infections. However, there is a paucity of information on the viability characteristics of *C. difficile* in meat, or the effect of various meat processing treatments on *C. difficile* spores. Therefore, this study investigated the effect of chilling, freezing and cooking on the viability of *C. difficile* spores in ground beef.

Enrichment of uninoculated ground beef in CDMNT broth and subsequent plating on CDMNT agar did not yield any *C. difficile*, thereby indicating the meat was devoid of the pathogen. The survival of *C. difficile* spores during chilling and freezing was determined by a standard quantitative method for enumeration on selective CDMNT agar (Weese et al., 2010). The effect

of chilling on *C. difficile* spores in ground beef is depicted in Figure 1. Results indicated that chilling for 1 week did not significantly affect the survival of *C. difficile* spores at either inoculation level ($p > 0.05$). In ground beef inoculated with 3 log CFU of *C. difficile* spores/g of meat, a mean spore population of ~ 2.50 log CFU/g was recovered on day 0, which remained the same throughout storage (Fig. 1). Similarly, in ground beef samples inoculated with 5 log CFU of *C. difficile* spores/g of meat, ~ 4.5 log CFU/g of spores were recovered on day 0, which did not increase or decline during the entire storage period. However, the total bacteria in ground beef samples inoculated with the two levels of *C. difficile* spores gradually increased from ~ 5.0 log CFU/g on day 0 to 7 log CFU/g on day 7 ($p < 0.05$) (Fig. 2). This increase in total bacteria could be attributed to the growth of psychrotrophic spoilage bacteria, which constitute the dominant microflora in meat (Venkitanarayanan et al., 1996), however, this increase in psychrotrophic bacteria had no inhibitory effect on *C. difficile* spores.

Freezing ground beef for 12 weeks did not significantly affect the viability of *C. difficile* spores at either inoculation level ($p > 0.05$) (Fig. 3). Spore counts of ~ 2.47 log CFU/g and 4.32 log CFU/g were recovered on day 0 from the samples inoculated with 3 log CFU/g and 5 log CFU/g, respectively, which remained the same throughout the storage period. In a very recent study, Deng et al. (2015) evaluated the viability of spores of two *C. difficile* strains in PBS and a meat model (in tube) during freezing, and reported a decreased spore viability in the meat model. In one of the strains, a reduction in spore viability of 0.4 log CFU/ml ($p < 0.05$) from 6.7 to 6.3 log CFU/ml was observed after 2 months of freezing, whereas no significant ($p > 0.05$) decrease in spore viability was identified in the second strain. The authors concluded that the meat matrix may provide protection to *C. difficile* spores at low temperatures (Deng et al., 2015). In contrast to these results, Lillard (1977) reported that freezing (-23°C) for 4 to 6 weeks significantly reduced

the incidence and levels of *C. perfringens* spores in ground chicken. Further, no change in aerobic bacteria was observed in our study during frozen storage ($p > 0.05$) (Fig. 4), which concurred with the findings of Lillard (1977).

The effect of cooking on the viability of *C. difficile* spores in ground beef is shown in Figure 5. At both low and high levels of inoculation, cooking beef patties to an internal temperature of 71.1°C decreased spore counts only by 1 log CFU/g compared to uncooked controls ($p < 0.05$) (Fig. 5). The USDA recommended internal cooking temperature of 71.1°C for ground beef targets vegetative cells of foodborne pathogens such as *E. coli* O157:H7 and *Salmonella enterica* (D'SA et al., 2000; USDA-FSI, 2006), but was ineffective in inactivating spores such as spores produced by *Bacillus cereus*. Spores are survival structures which are resistant to dry and wet heat because of the presence of protective exosporium layers in the inner and outer membranes (Setlow & Johnson, 2013). Moreover, the thick outer coating in spores protects the internal DNA against denaturation from heat, cold and other agents (Setlow, 2007). Previously, 71.1°C was found to be inadequate for killing the spores of *C. perfringens*, *C. botulinum* and *B. cereus* (Luu-Thi et al., 2014; Peck et al., 2011; Sanchez-Plata et al., 2005).

In summary, results from this study indicate that chilling and freezing of ground beef do not affect the survival of *C. difficile* spores. Further, cooking ground beef to the USDA recommended internal temperature of 71.1°C resulted in only marginal reduction in *C. difficile* spores. Therefore, effective intervention strategies should focus on control of *C. difficile* contamination of bovine carcasses to reduce food-borne transmission of the pathogen.

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Figure 1. Effect of chilling (4°C) on survival of *Clostridium difficile* spores in ground beef.

Two *C. difficile* spore inoculums were prepared from a 3 strain mixture (ATCC BAA 1805, 1803 and 1053) and inoculated at 2 levels (3 log CFU/g and 5 log CFU/g) in ground beef. *Clostridium difficile* was enumerated during refrigeration storage (4°C) on days 0, 1, 3, 5 and 7 by pour-plating on CDMNT agar with anaerobic incubation at 37°C for 48 h. Superscripts (a) and (b) denote no significant difference among means within an inoculation group.

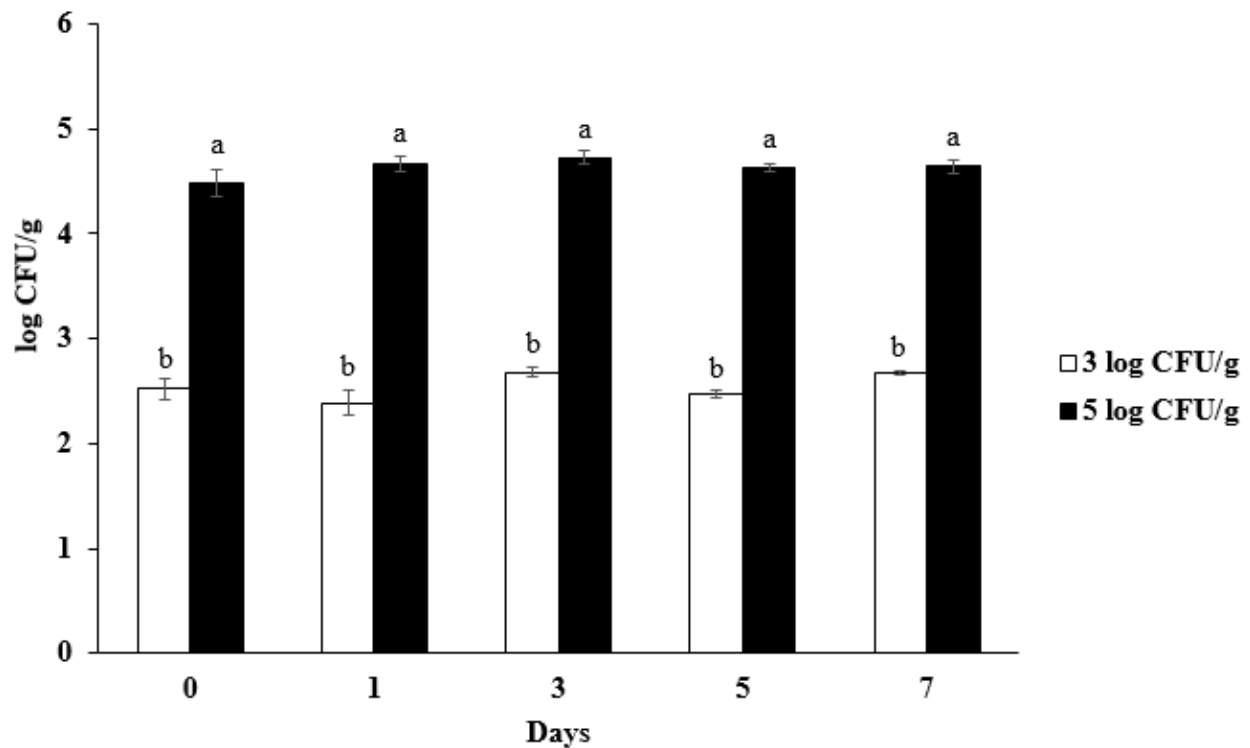


Figure 2. Viability of total bacteria in ground beef stored at 4°C for 7 days.

Viability of total bacteria in ground beef was determined on days 0, 1, 3, 5 and 7 by plating on tryptic soy agar. Superscripts (a, b, c, d, a', b', c' and d') denote a significant difference among means within an inoculation group.

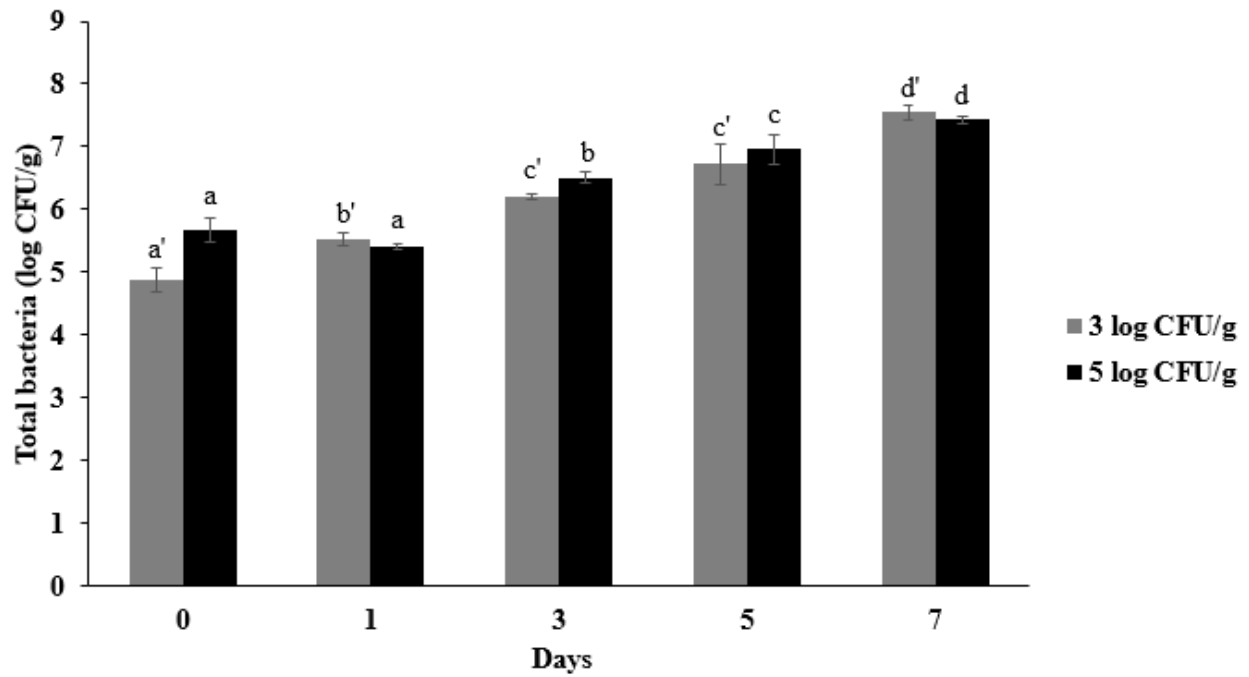


Figure 3. Effect of freezing (-18°C) on survival of *C. difficile* spores in ground beef.

Two *C. difficile* spore inoculums were prepared from a 3 strain mixture (ATCC BAA 1805, 1803 and 1053) and inoculated at 2 levels (3 log CFU/g and 5 log CFU/g) in ground beef. *Clostridium difficile* was enumerated during freezing storage on weeks 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 by pour-plating on CDMNT agar with anaerobic incubation at 37°C for 48 h. Superscripts (a) and (b) denote no significant difference among means within an inoculation group.

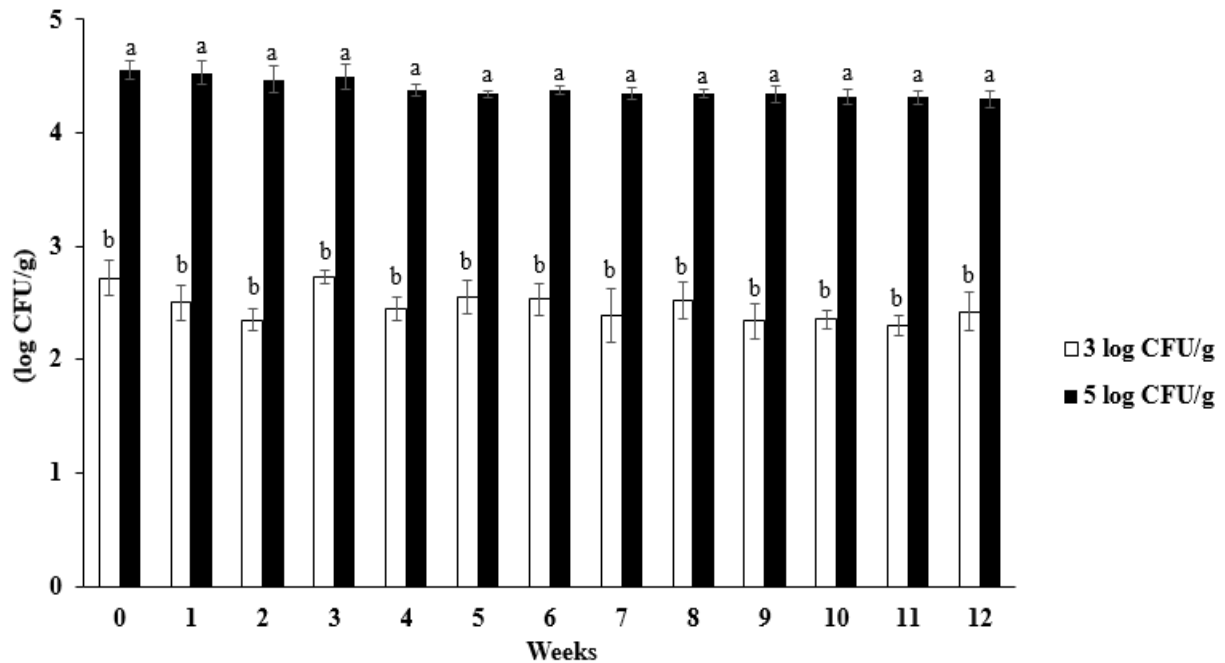


Figure 4. Total bacteria in ground beef stored at -18°C for 12 weeks

Total bacteria in ground beef were enumerated on weeks 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 of frozen storage by plating on tryptic soy agar. Superscripts (a) and (b) denote no significant difference among means within an inoculation group.

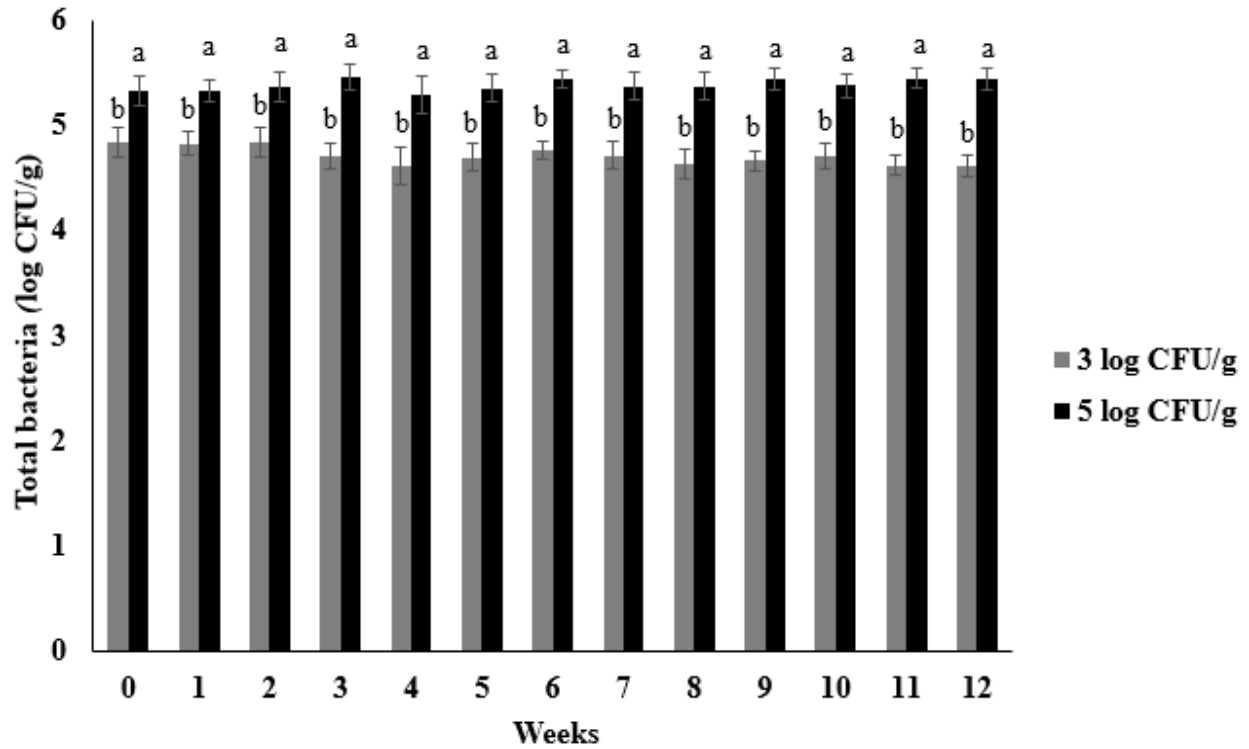
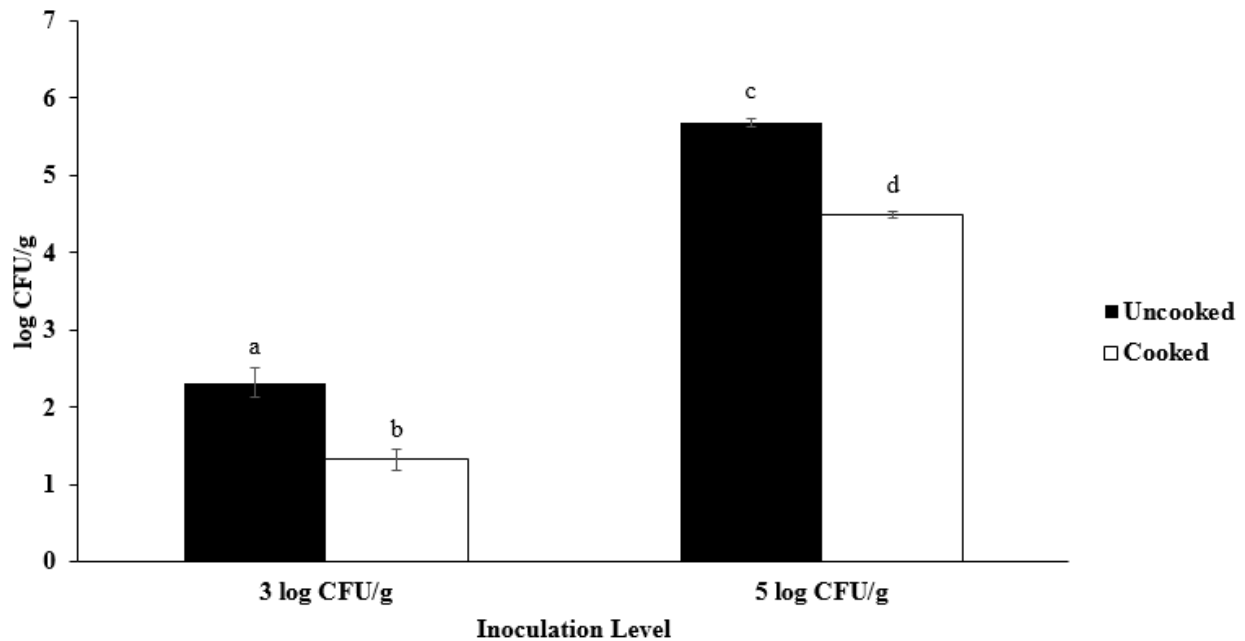


Figure 5. Effect of cooking (71.1°C) on *C. difficile* spores in ground beef patties

Ground beef patties were inoculated with a three-strain *C. difficile* spore mixture of 3 log CFU/g and 5 log CFU/g. The patties were cooked individually to the USDA recommended internal temperature for meat of 71.1°C. The cooked patties were immediately transferred to a Whirl-Pak™ bag containing 50 ml of chilled CDMNT broth, mixed and pour-plated for enumeration on CDMNT agar. Superscripts (a, b, c and d) denote a significant difference in the means among the treatments within an inoculation group.



Chapter V

Viability of *Clostridium difficile* spores in fermented pork summer sausage

Abstract

Clostridium difficile is a spore-forming pathogen, which is transmitted through the fecal-oral route, and causes a severe disease in humans. Hypervirulent strains of *C. difficile* have been isolated from a variety of food animals and meat, including pork. Pork summer sausage is a popular fermented and cooked/partially cooked meat product in the US, which is consumed cold. A lower cook temperature (internal temperature $\leq 57^{\circ}\text{C}$) can be used due to acidification of the product from bacterial fermentation. However, the effect of acidity and cooking on the viability of *C. difficile* spores in a fermented meat product has not been determined. Therefore, this study investigated the effect of fermentation and cooking on the survival of *C. difficile* spores in pork summer sausage.

Fermented pork sausages were prepared according to a commercially used recipe, where fresh ground pork (90% lean) was combined with salt (2.25%), spice mix, dextrose, starter culture (*Pediococcus acidilactici*, 7 log CFU/g), and inoculated with *C. difficile* spores (5 log CFU/g). Control unfermented sausages included all ingredients except the starter culture. The sausages were fermented at 37°C for ~ 12 h under 85% RH until the internal meat pH reached 5.0. Both fermented and unfermented sausages were either cooked to an internal temperature of 66.5°C for 45 min (cooked group) or were uncooked. The sausages were sealed in Whirl-PakTM bags, cooled and stored at 4°C . Viable *C. difficile* spores in sausages were enumerated at 1 h following inoculation, and on days 0, 1, 7, 14, 21, 30 and 60 of storage. Triplicate samples of sausages were included, and the study was replicated three times.

C. difficile spores remained viable in all treatment groups. *C. difficile* spore viability in control unfermented treatment was significantly different on day 0 from the fermented, fermented cooked and control unfermented cooked treatments ($p < 0.05$) however, there was no significant

difference among the latter three treatment groups throughout 90 days of storage ($p > 0.05$). On day 90 of storage, the unfermented control sausages yielded ~ 4.0 log CFU/g of *C. difficile* spores compared to ~ 3.5 log CFU/g recovered from fermented samples and the unfermented cooked control samples identifying spore viability in all treatment groups. Therefore, results suggest that *C. difficile* spores can survive the acidity and cooking typically encountered in fermented pork summer sausage, thereby highlighting the need for effective intervention strategies to control *C. difficile* contamination of pork carcasses, and its persistence in pork products.

1. Introduction

Clostridium difficile is a Gram positive, anaerobic nosocomial pathogen which causes a serious and potentially life-threatening toxin-mediated disease. The infection is characterized by abdominal pain and diarrhea, leading to more serious clinical manifestations such as colitis and toxic megacolon (Postma et al., 2015). Although traditionally regarded as a nosocomial infection, especially in those receiving antimicrobial therapy (Evans & Safdar, 2015; Hookman & Barkin, 2007; Lessa et al., 2015), a new epidemiological trend observed in *C. difficile* incidence is an increase in the number and severity of infections in humans, particularly those involving communities outside the hospital environment (Desai et al., 2016; Deshpande et al., 2013; Galdys et al., 2014). Further, *C. difficile* infections have been increasingly diagnosed in low risk individuals, who are younger with no history of receiving antibiotic treatment (Dumyati et al., 2012). Many investigators have reported the occurrence of *C. difficile* in a variety of food animals, and one of the reasons attributed to the increased reports of human *C. difficile* infections is the rise in isolation rates of the pathogen from animal reservoirs (Indra et al., 2009; Rupnik et al., 2008). *C. difficile* has been isolated from raw and ready-to-eat meats intended for human consumption at retail stores (Mooyottu et al., 2015; Rodriguez-Palacios et al., 2013; Rupnik & Songer, 2010). The detection of genotypically similar and identical *C. difficile* strains implicated from human infections in food animals and foods (Bauer & Kuijper, 2015; Rodriguez-Palacios et al., 2007; Weese, 2010) further strengthens the potential role of food as a source of community-associated *C. difficile* infection (Jhung et al., 2008; Kuijper et al., 2006; Songer et al., 2009).

C. difficile is a major cause of enteritis in neonatal pigs (Knight et al., 2015; Norén et al., 2014), and has been reported to cause significant mortality in suckling piglets (Songer & Anderson, 2006). *C. difficile* has also been isolated from live adult pigs at rates between 0 and

23%, with a high colonization rate in sows around the time of farrowing (Thakur et al., 2011; Weese, 2010). In a study involving 600 piglets with enteritis, Songer & Uzal (2005) observed that 35% of the animals were infected with *C. difficile* (Songer & Uzal, 2005). In another study, while examining 2000 piglets with enteritis, Songer & Anderson (2006) reported *C. difficile* in more than one-third of the animals, and concluded that *C. difficile* is potentially the most significant uncontrolled cause of neonatal diarrhea in pigs (Songer & Anderson, 2006). With swine being a potential reservoir of *C. difficile*, contamination of swine carcasses at the time of slaughter could lead to downstream contamination of pork and other ready-to-eat (RTE) pork products. A survey involving three sausage processing plants in Texas and five retail meat locations isolated *C. difficile* from 9.5% (23/243) of meat samples and swab samples collected over a five-year period (Harvey et al., 2011). Another prevalence study conducted in the US found that 62.5% of the RTE pork braunschweiger tested were positive for *C. difficile* (Songer et al., 2009). In addition, Wu et al. (2017) recently identified toxigenic *C. difficile* isolates in (3/15) ready-to-eat pork products, braised skin and braised colon, which were cooked to 100°C for 30 min (Wu et al., 2017). Thus, in light of *C. difficile* isolation in live pigs, processing facilities and retail pork products, there exists a potential risk of *C. difficile* contamination in RTE pork products.

Pork summer sausage is a popular fermented meat product in the US, and can be classified as cooked (internal temperature > 57°C), or uncooked to a lower temperature (internal temperature ≤ 57°C), since acidification due to fermentation is believed to kill any surviving pathogens in the product (Calicioglu et al., 1997; USDA FSIS, 2013). The starter culture, *Pedococcus acidilactici*, is commonly used in fermented sausage to produce lactic acid as a by-product of fermentation, which in turn lowers the product pH. If *C. difficile* spores could survive the pH and cooking temperature employed in fermented pork summer sausage, this RTE product could be a potential

source of the pathogen. Since, no previous research investigated the viability of *C. difficile* spores in any fermented meat product, the viability of *C. difficile* spores in RTE fermented pork summer sausage was determined.

2. Materials and Methods

2.1 Spore preparation

C. difficile spores were prepared using a previously published protocol with slight modifications (Sorg & Dineen, 2009). Briefly, single colonies of *C. difficile* ATCC BAA 1805, 1803 and 1053 were separately inoculated into Brain Heart Infusion broth supplemented with 5% yeast extract (BHIS) (Oxoid, Hampshire, UK), and cultured overnight at 37°C under anaerobic conditions. A 150 µl aliquot of overnight culture was gently spread to evenly distribute the culture onto BHIS agar (Oxoid) in six well plates and was cultured anaerobically for 7 days at 37°C in a Whitley A35 anaerobic workstation (Microbiology International, Frederick, MD, USA) to allow sporulation. After 7 days, spores were harvested from the wells by flooding 2 ml of ice cold sterile water. The spore suspension was heat treated at 60°C for 20 min to kill any vegetative cells, and was washed five times in sterile dH₂O by centrifuging at 16,000 g for 5 min. Spore suspensions were visualized under a microscope to ensure 90% sporulation prior to storage at -20°C.

2.2 Sausage preparation and fermentation

Fermented pork sausages were prepared according to a published protocol (Baccus-Taylor et al., 1993; Vignolo et al., 2010). Pork (90% lean) was hand-mixed with the addition of sterile deionized water, salt (2.25%), summer sausage spice mix (Sausage Maker, Buffalo, NY, USA), sodium erythorbate (Sigma-Aldrich, St. Louis, MO, USA), curing salt and dextrose (Sigma-Aldrich). Following the addition of *C. difficile* spores (5 log CFU/g of pork batter) and starter

culture, *P. acidilactici* at 7 log CFU/g (SAGA™ 200 Kerry Ingredients and Flavours, Rochester, MN, USA), ground pork was hand-mixed, and subsequently minced with a manual grinder (Weston, Strongsville, OH, USA) using a 3/8” grinding plate. A sausage formulation, including all ingredients except *P. acidilactici* was included as an unfermented control. The ground pork mixture was stuffed into synthetic casings (Nojax® Casings 13/16” provided by Viskase, Darien, IL, USA) using a jerky gun with a 10 mm stuffing horn (LEM, West Chester, OH, USA). Sausages were fermented at 37°C for ~ 12 h under 85% RH until the internal meat pH reached 5.0. A Caron humidity controlled incubator (Caron Products, Marietta, OH, USA) was utilized for fermentation to maintain 37°C and 85% RH. The fermented group of sausages were fermented to pH 5.0 and cooled by transferring to individual gas-impermeable Whirl-Pak™ bags and submerging in ice cold sterile deionized water, followed by sealing and storage at 4°C. One-half of the fermented sausages were immediately transferred to a drying oven (VWR, Radnor, PA, USA) set to 100°C for cooking to an internal temperature of 66.5°C for ~ 45 min, where temperature was continuously monitored using a digital thermocouple (Oakton Instruments, Vernon Hills, IL, USA) inserted internally in the sausage. Fermented cooked sausages were then cooled by transferring to individual gas-impermeable Whirl-Pak™ bags and submerging in ice cold sterile deionized water for refrigerated storage. The control unfermented group of sausages were also stored at 37°C for ~ 12 h under 85% RH, and subjected to cooking as before or uncooked, and subsequently stored in gas-impermeable Whirl-Pak™ bags at 4°C.

2.3 Bacterial enumeration

Three sausages were analyzed for pH, water activity (a_w), viable *C. difficile* spores and *P. acidilactici* populations at 1 h following inoculation, and on days 0, 1, 7, 14, 21, 30, 60 and 90 of refrigerated storage. Each sausage was removed from its casing, and both the casing and sausage

were together added to a Whirl- Pak™ bag containing 10 ml of phosphate-buffered saline (PBS pH 7.0), and subjected to stomaching for one min. After stomaching, 1 ml of the sausage homogenate was pour-plated in duplicate with *Clostridium difficile* moxalactam norfloxacin agar supplemented with 0.1% sodium taurocholate (CDMNT). The plates were incubated anaerobically at 37°C for 48 h. In addition, 1 ml of the homogenate was added to 9 ml of CDMNT broth and incubated for enrichment. Following enrichment, a 5 ml aliquot of the broth was subjected to alcohol shock by adding 5 ml of 100% anhydrous ethanol for 1 h to eliminate vegetative bacteria. The broth was subsequently subjected to centrifugation at 4000 x g for 10 min, and the pellet was resuspended in 0.5 ml of PBS and pour plated with CDMNT agar. The CDMNT agar plates were incubated anaerobically at 37°C for 48 h. Moreover, the population of *P. acidilactici* present in each sausage sample was enumerated at each time point by plating on de Man Rogosa and Sharpe agar (MRS) (Oxoid) with incubation at 37°C for 48 h (Casquete et al., 2011).

The pH and a_w of the fermented summer sausages were measured as described by Hristo et al. (2013) (Hristo et al., 2013). Briefly, pH was determined at 25°C by inserting a pre-calibrated pH meter probe (Thermo Fisher Scientific, Waltham, MA, USA) directly into three sausages from each treatment group. For measuring a_w at room temperature (25°C), a calibrated water activity meter (Rotronic, Hauppauge, NY, USA) was used and sausage samples from each treatment group were cut into small pieces and placed in a plastic sample cup. The bottom of the cups were completely covered with sausage sample, placed in the water activity meter and readings were recorded following humidity and temperature stabilization of the instrument.

2.4 Statistical analysis

All experiments included triplicate samples for each treatment, and the study was repeated three times. The data were analyzed using the PROC-GLIMMEX procedure of SAS

version 9.4 (SAS Institute Inc., Cary, NC, USA). Differences between the means were considered significantly different at $p < 0.05$.

3. Results and Discussion

Hypervirulent *Clostridium difficile* isolates have been detected in live pigs, pork sausage manufacturing plants, and retail pork products. Pork summer sausage is consumed cooked (internal temperature $> 57^{\circ}\text{C}$), or cooked to a lower temperature (internal temperature $\leq 57^{\circ}\text{C}$) since low pH resulting from fermentation is believed to kill pathogens (Calicioglu et al., 1997; USDA FSIS, 2013). However, the effect of heat and acidity on the viability of *C. difficile* spores in a fermented meat product has not been previously investigated. Therefore, this study determined the survival of *C. difficile* spores in pork summer sausage, which is a popular fermented RTE product in the US.

Results revealed that *C. difficile* spores remained viable under an acidic pH of 5.0 in fermented pork summer sausage during three months of refrigerated storage. *C. difficile* spores remained viable in all treatment groups. *C. difficile* spore viability in control unfermented treatment was significantly different at day 0 from the fermented, fermented cooked and control unfermented cooked treatments ($p < 0.05$) however, there was no significant difference among the latter three treatment groups throughout 90 days of storage ($p > 0.05$) (Fig. 1). On day 90 of storage, the control unfermented sausages yielded ~ 4.0 log CFU/g of *C. difficile* spores compared to ~ 3.5 log CFU/g recovered from fermented samples and the control unfermented cooked samples identifying spore viability in all treatment groups. *Pediococcus acidilactici* counts in the fermented group remained at ~ 7.5 log CFU/g throughout 90 days of storage, but declined in the fermented cooked group from day 21 reaching ~ 2 log CFU/g by the end of storage (Fig. 2). The pH of control unfermented sausage was 5.68 on day 0 and did not significantly change over 90

days of storage (Table 1). The pH of control unfermented cooked sausages was 5.73 on day 0, which significantly increased to 6.08 by the end of the storage period ($p < 0.05$). The pH of the unfermented sausage groups was consistent with agriculture standards for pork meat (Ministry of Agriculture, 2001). On the other hand, as expected, the pH of fermented sausages was ~ 4.8 on day 0, where it increased to 5.04 and 5.31 ($p < 0.05$) on day 90 in uncooked and cooked samples, respectively. The significant increase in pH observed in cooked fermented sausages is consistent with a previous study, where a significant increase in pH of cooked sausages from 4.75 to 4.84 was observed over 8 weeks of storage (Tremonte et al., 2005). Also, no significant difference in a_w was observed between control and fermented treatment groups during 90 days of storage ($p > 0.05$) (Table 2); the fermented cooked samples yielded the lowest a_w of 0.889 on day 90 of storage.

The USDA guidelines for meat fermentation are based on literature evaluating pH and temperature parameters for controlling pathogens such as *Escherichia coli* O157:H7 (USDA FSIS, 2013). A pivotal study on the fate of *E. coli* O157:H7 in fermented summer sausage identified that a 5-log reduction in pathogen counts can be achieved with fermentation to a final pH of 5.0, followed by heating to an internal temperature of 54°C for 30 min, and a 7-log reduction in counts with heating for 60 min at 54°C (Calicioglu et al., 1997). However, the results from this study revealed that *C. difficile* remained viable in pork summer sausage despite achieving the aforementioned pH and even higher cooking temperature. This could be attributed to the differences in the heat and acid resistance between the vegetative cells of *E. coli* O157:H7 and *C. difficile* spores. Although the effect of heat and acidity on the viability of *C. difficile* spores in meat has not been investigated, Rodriguez-Palacios et al. (2010) reported that *C. difficile* vegetative cells survived the USDA-recommended meat cooking temperature of 71°C for 2 hours in phosphate buffered saline (Rodriguez-Palacios et al., 2010). Thus, *C. difficile* spores are expected

to survive the acidity and cooking commonly employed in fermented pork summer sausage, thereby highlighting the need for effective intervention strategies to reduce spore contamination or viability in RTE meat products.

Many RTE meats, including fermented sausages, which are not heated, or heated to a lower internal temperature, contain preservatives to control spoilage bacteria and foodborne pathogens. Sodium nitrite is a preservative commonly used to control *C. botulinum* spores as well as spoilage bacteria in RTE cured meat (Hospital et al., 2016; Xi et al., 2011). However, a recent study reported that even vegetative cells of *C. difficile* are resistant to the USDA recommended levels of nitrite (200 ppm) and nitrate (500 ppm) in RTE meat products (Lim et al., 2015). The study also noted that the use of both preservatives together did not exert a synergistic effect against *C. difficile*. It is highlighted that this study was conducted with vegetative *C. difficile*, and spores being more resistant than vegetative cells to antimicrobials, it is logical to conclude that nitrites and nitrates would not kill *C. difficile* spores if present in RTE meat products.

The aforementioned results documenting the resistance of *C. difficile* spores to a commonly used preservative in RTE meat along with our results showing the survival of *C. difficile* spores under a pH of 5.0 and cooking temperature of 66.5°C for 45 min in fermented sausages, underscore the potential risk of the pathogen in RTE meat. Therefore, additional research is required to further investigate the prevalence of *C. difficile* in RTE meat and fermented meat products. Moreover, effective intervention strategies to control *C. difficile* in live pigs and pork carcasses are critical to reduce food-borne transmission of the pathogen.

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Figure 1. Viability of *C. difficile* spores in fermented pork sausage.

C. difficile strains were grown under anaerobic conditions for 10 days after which spores were harvested. Fermented pork sausages were prepared according to a commercially used recipe, where fresh ground pork (90% lean) was added with salt (2.25%), spice mix, dextrose, starter culture (*P. acidilactici*, 7 log CFU/g) and *C. difficile* spores (5 log CFU/g) strains (ATCC 1805, 1803 and 1053). Control unfermented sausages included all ingredients except the starter culture. The sausages were fermented at 37°C for 12 h under 85% RH until the internal meat reached pH 5.0. Both fermented and unfermented sausages were either cooked to an internal temperature of 66.5°C for 45 min or uncooked. Viable *C. difficile* spores in sausages were enumerated on CDMNT agar at 1 h and on days 0, 1, 7, 14, 21, 30 and 60 of storage at 4°C.

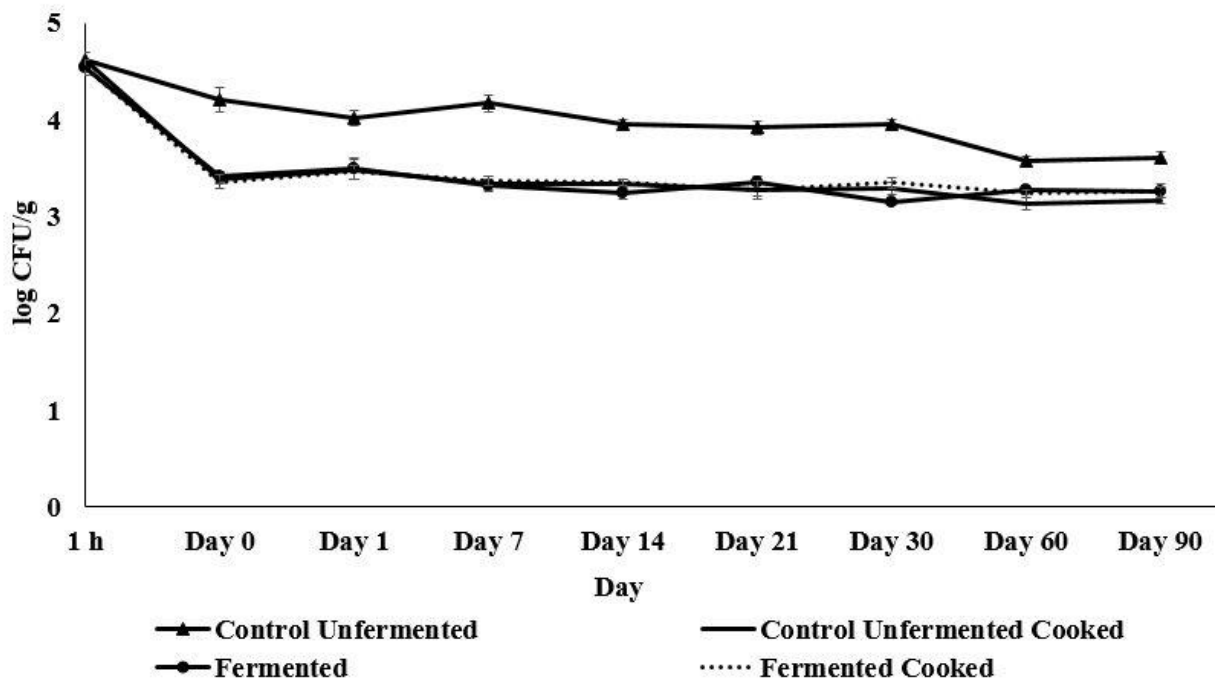


Figure 2. *Pediococcus acidilactici* populations recovered from fermented pork sausage during storage.

Fermented pork sausages were prepared according to a commercially used recipe, where fresh ground pork (90% lean) was added with salt (2.25%), spice mix, dextrose, starter culture (*P. acidilactici*, 7 log CFU/g) and *C. difficile* spores (5 log CFU/g). *P. acidilactici* counts were enumerated on MRS agar on days 0, 1, 7, 14, 21, 30 and 60 of storage at 4°C.

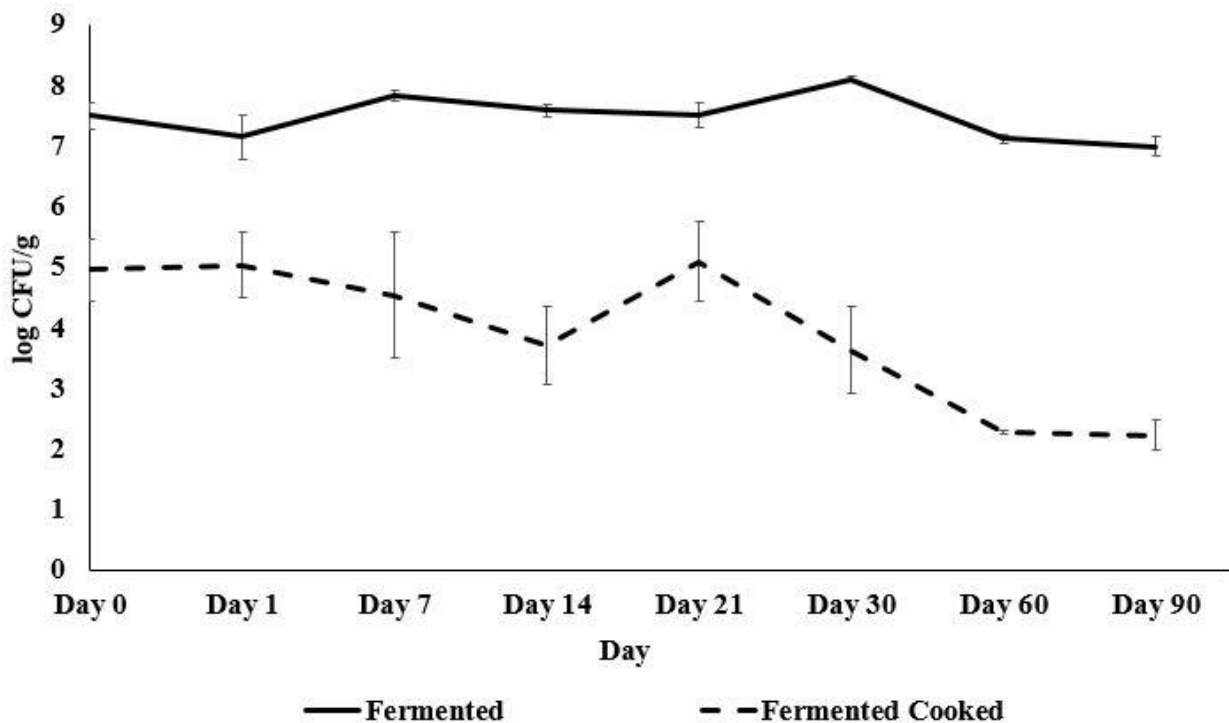


Table 1. Average pH of fermented and unfermented pork sausage with and without cooking.

	Control Unfermented	Control Unfermented Cooked	Fermented	Fermented Cooked
Day 0	5.68	5.73	4.80	4.81
Day 1	5.69	5.89*	4.92*	5.05*
Day 7	5.73	5.94*	4.74	5.02*
Day 14	5.81*	5.99*	4.65*	5.06*
Day 21	5.61	6.00*	4.72	5.15*
Day 30	5.58	6.10*	4.72	5.19*
Day 60	5.39*	6.19*	4.73	5.32*
Day 90	5.61	6.08*	5.04*	5.31*

* Significant difference between day 0 and sampling day within a treatment group ($p < 0.05$)

Table 2. Water activity measured at 25°C of fermented and unfermented pork sausage with and without cooking.

	Control Unfermented	Control Unfermented Cooked	Fermented	Fermented Cooked
Day 0	0.936	0.910	0.938	0.898
Day 1	0.938	0.941	0.944	0.907
Day 7	0.937	0.898	0.919	0.902
Day 14	0.935	0.908	0.919	0.883
Day 21	0.925	0.901	0.929	0.870
Day 30	0.926	0.900	0.887	0.886
Day 60	0.925	0.912	0.930	0.908
Day 90	0.929	0.905	0.935	0.889

*No significant difference ($p > 0.05$)

Chapter VI

Efficacy of octenidine hydrochloride (OH) in reducing *Clostridium difficile* spores on stainless steel surfaces

Abstract

Clostridium difficile is a spore-forming nosocomial pathogen which causes a toxin-mediated enteric infection in humans. The spores of *C. difficile* can survive on abiotic surfaces for several months, and act as a source of new and recurrent infections by a fecal-oral route. This study investigated the sporicidal efficacy of octenidine hydrochloride (OH), a new generation disinfectant, against *C. difficile* spores on stainless steel surfaces. Suspensions containing 10^6 hyper-virulent *C. difficile* (ATCC 1870 and 1805) spores/ml were inoculated on stainless steel discs (16 mm diameter) and treated with 0%, 1%, 2%, 3%, 4% and 5% of OH in ethanol for 10 min. Viable attached spores were recovered from the discs and enumerated by pour plating. In addition, discs were inoculated with different levels of *C. difficile* spores/ml (10^5 , 10^4 , 10^3 and 10^2), and wiped with 1%, 3% and 5% of OH, followed by enumeration of residual spores on the discs and wipes. Octenidine hydrochloride decreased *C. difficile* spores on steel discs ($p < 0.05$). In *C. difficile* strains ATCC 1870 and 1805, 5% OH decreased spores by ~ 2.5 log CFU/ml compared to controls. Similarly, wiping with OH reduced *C. difficile* spores on stainless steel surfaces by up to 4 log CFU/disc compared to controls. Additionally, more than a 4 log reduction in residual spores was found on wipes treated with 5% OH ($p < 0.05$). The results suggest that OH could potentially be used as a disinfectant to reduce *C. difficile* spores on stainless steel surfaces.

1. Introduction

Clostridium difficile is a gram-positive, spore forming anaerobic pathogen, which causes a serious enteric disease in humans (Hookman & Barkin, 2009). Annually, over 500,000 cases of *C. difficile* infections (CDI) are reported in the United States, which incur ~ \$3 billion in healthcare and treatment costs (Lessa et al., 2015; Lucado et al., 2012). *Clostridium difficile* infections are transmitted through a fecal-oral route, and the majority of cases occur in healthcare facilities (McFarland et al., 1989). Ingested *C. difficile* spores germinate in the intestine of susceptible individuals and cause toxin-mediated colitis and diarrhea (Hookman & Barkin, 2009; Kuehne et al., 2011). Infected patients shed highly resistant spores in their feces and contaminate the environment. These spores can survive on abiotic surfaces for up to 5 months (Dubberke et al., 2008; Hasan et al., 2011; Kramer et al., 2006). Commonly contaminated hospital surfaces and equipment include; floors, call buttons, windowsills, bedrails, toilets, bedside-tables, thermometers, commodes, blood-pressure cuffs, and intravenous catheters (Dubberke et al., 2008; Gerding et al., 2008; Guerrero et al., 2012). In addition, transmission through hands can occur when healthcare workers or patients come in contact with surfaces contaminated with *C. difficile* spores (Guerrero et al., 2012; Weber et al., 2010). An observational study including 30 patients with *C. difficile* found a 50% transfer rate on gloved hands following health-care worker examination of patient skin sites (chest, hand, abdomen and arm). This study also observed greater than a 50% transfer rate to health-care workers gloved hands after touching hospital surfaces such as call buttons, bed rails and tables (Guerrero et al., 2012). Therefore, it is critical for hospitals to establish a routine and effective disinfection procedure against *C. difficile* spores to control transmission. Commonly used hospital disinfection agents, such as quaternary ammonium-based and other surfactant-based detergents do not kill *C. difficile* spores, and may increase sporulation

capacity (Gerding et al., 2008; Kelly & LaMont, 2008; Nerandzic & Donskey, 2016). Currently, the only disinfectant recommended for use during *C. difficile* outbreaks is 1:10 sodium hypochlorite solution with 10 min of contact time (Cohen et al., 2010; Fawley et al., 2007). This high concentration can cause skin irritation and respiratory distress in healthcare workers and patients, and lead to corrosion of hospital surfaces and equipment (Barbut et al., 2009; Keward, 2013). Thus, there is a critical need for a safe and effective alternative disinfectant that can be used routinely against *C. difficile* in hospitals.

Octenidine hydrochloride (OH) is a bispyridinamine compound that has two active cation centers which bind to negatively charged components such as cardiolipin in bacterial cell membranes (Brill et al., 2006; Harke, 1989). Since human cell walls do not contain cardiolipin, OH does not bind to eukaryotic cells, which makes the compound safe for use on skin and wounds (Harke, 1989; Lademann et al., 2012). The compound has a broad spectrum of activity against Gram-positive and Gram-negative bacteria (Bailey et al., 1984; Hubner et al., 2010; Lademann et al., 2012; Sedlock & Bailey, 1985). It is used as an antiseptic on skin and wounds which identifies its safety as a routine disinfectant (Hirsch et al., 2009; Lademann et al., 2012; Tietz et al., 2005). Further, OH has proven effective in reducing the number of bacterial pathogens on wounds such as *Acinetobacter baumannii*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Proteus mirabilis* (Lademann et al., 2012; Selçuk et al., 2012; Sopata et al., 2008). Further, OH is used as a mouthwash, and was shown to eliminate plaque-forming microorganisms, including *Streptococcus mutans* (Bailey et al., 1984; Beiswanger et al., 1990; Dogan et al., 2008; Rohrer et al., 2010). In addition, OH showed antimicrobial effectiveness against planktonic cells and biofilms of *Listeria monocytogenes*, *Staphylococcus aureus* and multi-drug resistant *A. baumannii* (Amalaradjou & Venkitanarayanan, 2014; Amalaradjou et al., 2009; Junka et al., 2014; Narayanan

et al., 2016). Moreover, it has been observed that the development of bacterial resistance against OH is minimal, and based on animal studies, OH is neither carcinogenic nor mutagenic (Al-Doori et al., 2007; Hirsch et al., 2009). In this study, we investigated the efficacy of OH for reducing *C. difficile* spores on stainless steel surfaces for its potential use as a routine disinfectant in hospitals to reduce CDI transmission.

2. Materials and Methods

2.1 Spore preparation

Clostridium difficile spores were prepared using a previously published protocol with slight modifications (Sorg & Dineen, 2009). Two hypervirulent *C. difficile* isolates (ATCC BAA 1870 and 1805) were grown in brain heart infusion broth (BHI) supplemented with 5% yeast extract (Difco, Sparks, MD, USA) in a Whitley A35 anaerobic work station (Microbiology Inc., Frederick, MD, USA) in the presence of 80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37°C for 24 h. The cultures were inoculated onto 6-well BHI agar plates by adding 150 µl of each culture separately followed by gentle rotation to evenly disperse the culture. After 7 days of incubation, a loopful of colony was taken for Gram staining. When 90% sporulation was visualized under a microscope, the spores were harvested from the wells as follows.

The wells of the 6-well plate were flooded and gently washed with 1 ml of sterile ice-cold phosphate-buffered saline (PBS). Following washing, the spore suspension was transferred to tubes for sedimentation by centrifugation (14,000 rpm for 5 min, 4°C). The supernatant was removed with a pipette and the pellet was resuspended in 2 ml of sterile PBS. Centrifugation and resuspension were repeated 5 times to remove cellular debris. The resuspended spores were heat-shocked at 60°C for 20 min to kill any remaining vegetative cells. The spores were enumerated by

serial dilution and plating on brain heart infusion agar supplemented with 0.1% (w/v) sodium taurocholate (BHIT) (Thermo Fisher Scientific, Pittsburg, PA, USA). The plates were incubated anaerobically for 48 h and colonies enumerated. The spore solution was divided into aliquots and diluted to 1.0×10^7 spores/ml. The spore stock was then stored at -80°C .

2.2 Sporicidal efficacy of OH against C. difficile on stainless steel surfaces

This test procedure was based on the American Society of Testing Materials (ASTM) E2197 – 11 method for assessing the effect of a treatment on bacterial contamination (Boyce et al., 2011). Stainless steel discs (16 mm diameter) were placed in a 12-well plate. The surface of each disc was inoculated with 100 μl of spore solution containing $\sim 10^6$ spores. The inoculum was air dried at room temperature for 1 h. The disc treatments were as follows; an untreated control of *C. difficile* inoculation alone, an ethanol control, and 5 treatments with 1%, 2%, 3%, 4%, or 5% OH. The treatments were added at a volume of 1 ml to fully submerge the disc and incubated at room temperature for 10 min. The discs were transferred aseptically with sterilized forceps to 50-ml tubes containing 5 ml PBS and glass beads. The tubes were vortexed for 2 min and sonicated for 2 min to recover spores from the disc surface. The solution was serially diluted 3 times and 1 ml of each dilution was added to duplicate empty petri plates. The dilutions were pour-plated with BHIT agar supplemented with *C. difficile* moxalactum norfloxacin (CDMN) and incubated anaerobically for 48 h after which colonies were enumerated.

2.3 Efficacy of OH wipes in reducing C. difficile spores on stainless steel surfaces

The wiping experiments were conducted using a previously published protocol with modification (Siani et al., 2011). Wipes (Kimberly Clark™ WypAll™ X60 Wipers) (Kimberly Clark, Irving, TX, USA) were cut (4x4 cm) and sterilized by autoclaving. Stainless steel discs (16

mm diameter) were attached to petri plates and inoculated with 100 μ l of spore suspension containing $\sim 10^5$ spores. The experiment was repeated with 100 μ l of lower spore inoculations in log increments; i.e., 10^4 , 10^3 and 10^2 . The inoculum was air-dried at room temperature for 1 h. The treatments of 1%, 3%, and 5% OH were applied on disk surfaces and allowed to incubate for 10 min, followed by immediate wiping. A wipe was pinned to a sterile rubber stopper attached to a stirring rod of a rotating overhead electric drill (Fig. 1). Wipes were rotated mechanically with an electric drill for 10 s at 60 rpm with a downward weight of 500 g. The wipe was stamped on the four quadrants of a BHIT agar plate supplemented with CDMN and 7% horse blood. The discs were pour-plated with BHIT agar supplemented with CDMN. The plates were incubated anaerobically for 48 h at 37°C after which bacterial colonies were enumerated.

2.4 Statistical analysis

All experiments were carried out in duplicate and the study was repeated three times. The data were analyzed using one-way ANOVA. Differences between the means were considered significantly different at $p < 0.05$.

3. Results

To investigate the effect of OH on *C. difficile* spore survival and recovery on stainless steel surfaces, the inoculated discs were treated with varying concentrations of OH. The spores were recovered from the discs after each treatment and enumerated by dilution and plating. In addition, the residual spores on the discs after spore recovery were also determined by pour-plating the discs in BHIT agar supplemented with CDMN. OH significantly reduced the number of spores of *C. difficile* strain ATCC BAA 1870 recovered from the stainless steel discs ($p < 0.05$). The treatment with 1% OH reduced spores by 1 log CFU/ml; 2%, 3% and 4% OH reduced spores by 2 log

CFU/ml, and 5% OH reduced spores by 2.5 log CFU/ml compared to controls (Fig. 2). Similarly, OH significantly reduced *C. difficile* strain ATCC BAA 1805 spores recovered from stainless steel discs ($p < 0.05$). The 1% and 2% OH treatments reduced spores by 0.5 log CFU/ml; 3% and 4% OH reduced spores by 1 log CFU/ml and 5% OH reduced spores by about 2 log CFU/ml compared to controls (Fig. 3). In addition, there were no residual spores present on the surface of the discs treated with OH, indicated by absence of colonies after pour-plating the discs with BHIT agar supplemented with CDMN. However, untreated and ethanol-treated discs contained several residual spores, as indicated by the presence of colonies after pour-plating the discs with BHIT agar supplemented with CDMN.

In the wiping experiment stainless steel discs were inoculated with varying concentrations of *C. difficile* spores in log increments. The discs were then treated with different concentrations of OH and wiped with a dry wipe after 10 min of contact time. The sporicidal efficacy of each treatment was expressed in log reduction by observing complete absence of spores at a particular inoculation level. In addition, after each wiping, the wipe was stamped onto the four quadrants of a BHIT agar plate supplemented with CDMN in order to estimate the spore survival on the wipe. *C. difficile* spores were inoculated at the level of 10^5 , 10^4 , 10^3 and 10^2 onto the disc surface and treated with OH at 1%, 3% and 5% levels for 10 min followed by wiping. The results indicated that 5% OH completely inactivated spores from the discs inoculated with 10^4 , which suggests a 4-log CFU/disc reduction in *C. difficile* (ATCC BAA 1870) spores (Table 1). However, several colonies appeared on discs with control treatments (untreated control and ethanol control). Treatments with 1% OH and 3% OH resulted in a reduction of 3-log CFU/disc in spore counts (Table 1). In addition, the agar plates stamped with wipes containing 5% OH showed a 4-log reduction in spore counts (Table 2), whereas in 3% and 1% OH treatments, a 2 log reduction in

spore counts was observed. However, colonies were observed in all plates in the untreated control and ethanol control groups (Table 2).

4. Discussion

Approximately 10%–25% of hospitalized patients and 4%–20% of residents in long-term care facilities are colonized with *C. difficile* (Lessa et al., 2015; Simor et al., 2002). The shedding of spores to the hospital environment by infected individuals is the major cause of *C. difficile* transmission in healthcare facilities (Hookman & Barkin, 2009). *C. difficile* spores are extremely resistant to physical and chemical disinfectants, and can reside on surfaces for several months (Brill et al., 2006; Fawley et al., 2007; Hasan et al., 2011). *C. difficile* has been isolated from the rooms of infected patients in healthcare settings with a range of 2.9% to 75% (Weber et al., 2010). Further, in healthcare environments, *C. difficile* contamination has been identified in 49% of rooms occupied by CDI patients compared to 29% of rooms occupied by asymptomatic CDI carriers suggesting shedding of *C. difficile* (McFarland et al., 1989; Sethi et al., 2010). In addition, equipment shared between patients such as bed-side tables, blood pressure cuffs and other surfaces such as floors and toilets can be contaminated with *C. difficile* spores, thereby serving as a potential source for acquiring the infection (Dubberke et al., 2008). A transmission rate above 50% was observed after healthcare workers touched hospital surfaces such as call buttons and bed rails, exemplifying the requirement for disinfection to prevent CDI transmission (Guerrero et al., 2012). Therefore, it is critical to disinfect hospital rooms daily with an effective and safe antimicrobial against *C. difficile* spores.

Our results indicate that OH significantly reduced *C. difficile* (strains ATCC BAA 1870 and ATCC BAA 1805) spores on stainless steel discs compared to controls ($p < 0.05$) (Fig. 2 and 3). In addition, wiping with 5% OH treatment removed up to 4 log *C. difficile* spores from the

stainless steel disc surface (Table 1), thereby suggesting the potential for OH to be utilized as a surface wipe. Previously, it was reported that wiping with non-sporicidal agents alone removed 2.9 log of *C. difficile* spores, while wiping with a sporicidal agent yielded a greater reduction of 3.9 log (Rutala et al., 2012). However, these researchers did not determine the population of residual spores on the wipes. The efficacy of OH for reducing spores was generally found to increase with OH concentration, since the contact time was constant. For example, in the first experiment, where inoculated discs were immersed with various treatments, reductions in spore counts on discs increased with increased OH concentration, with 5% treatment leading to the maximum decrease of ~ 2.5 log CFU/ml (Fig. 2 and 3). Similarly in the wiping study, 5% OH was most effective, reducing the spore population by 4 log CFU/disc, followed by 3% OH with a 2 log CFU/disc reduction (Table 1). Likewise, the residual spore population in the wipes were also lowest in the 5% treatment, followed by 3% and 1% treatment groups (Table 2).

In conclusion, the results of this study suggest the potential use of OH as a disinfectant on hospital stainless steel surfaces to reduce *C. difficile* spores. This study represents the first report investigating the efficacy of OH for killing *C. difficile* spores on a surface. Although the mechanisms behind the sporicidal effect of OH are not known, OH exerts its antimicrobial effect against bacterial cells by binding to the negatively charged bacterial cell envelope, and disrupting the functions of the cell (Brill et al., 2006). Octenidine hydrochloride is stable within a wide pH range of 1.6 to 12.2, and is not sensitive to hydrolysis from light, harsh chemical or physical conditions (Harke, 1989). Thus, the safety and stability of OH make it an ideal disinfectant for routine use in hospitals.

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Fig. 1. The wiping apparatus to test the effect of octenidine hydrochloride wiping on *C. difficile* spores inoculated on stainless steel discs.

A wipe was pinned to a sterile rubber stopper attached to a stirring rod of a rotating overhead electric drill. Wipes were rotated mechanically with the drill for 10 s at 60 rpm with a downward weight of 500 g. A weigh balance was placed under the drill to monitor the 500 g of weight applied.

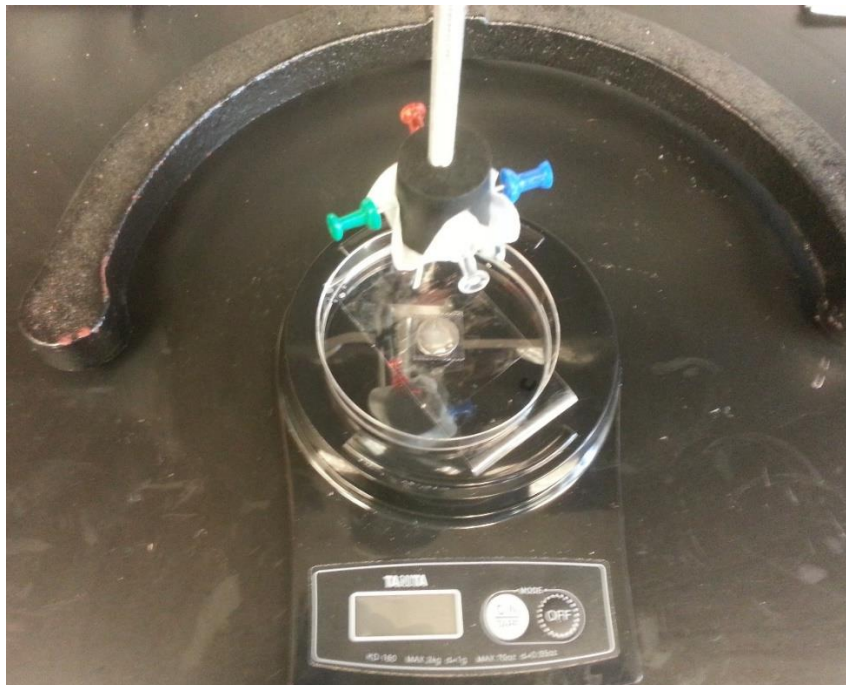


Fig. 2. The effect of octenidine hydrochloride on *C. difficile* spores (ATCC BAA 1870) inoculated on stainless steel discs.

The surface of each disc was inoculated with 100 ul of *C. difficile* spores (ATCC BAA 1870) with 1.7×10^6 spores. The discs were then subjected to treatments for 10 min including, control, ethanol control, 1%, 2%, 3%, 4%, or 5% OH. The dilutions were pour-plated with BHIT agar supplemented with CDMN and incubated anaerobically for 48 h after which colonies were enumerated. * indicates treatments with significant difference at $p < 0.05$.

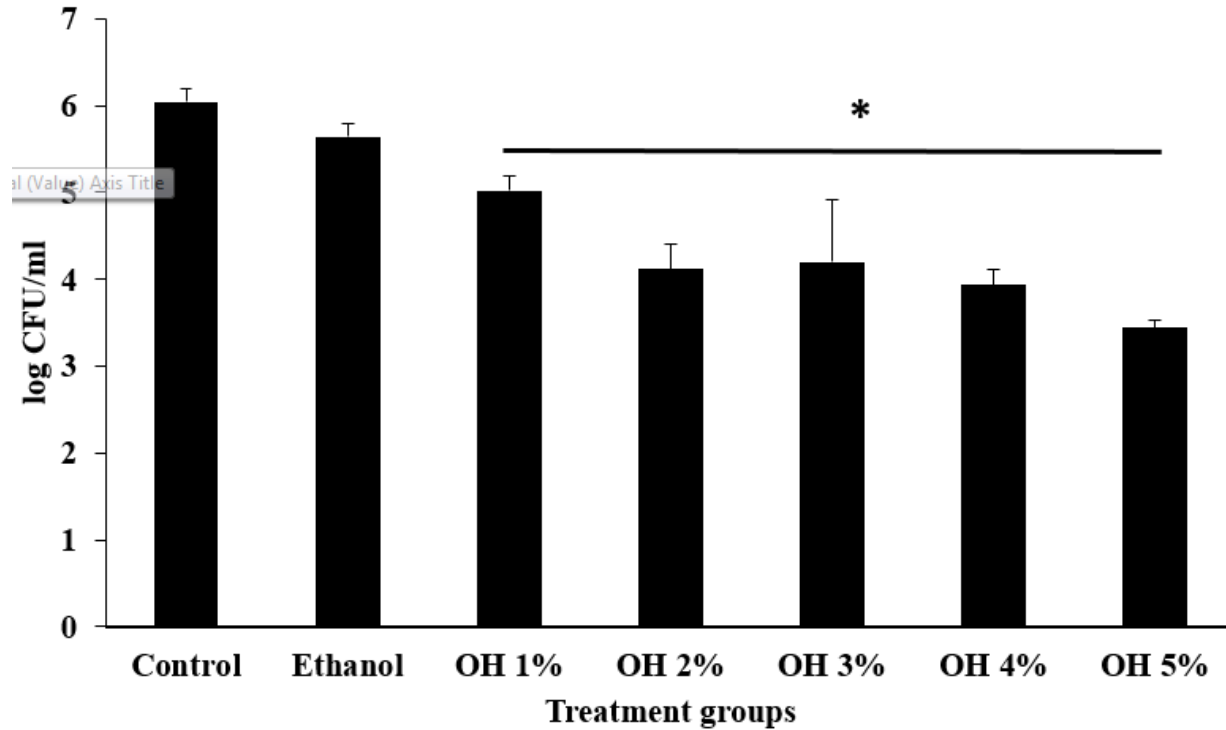


Fig. 3. The effect of octenidine hydrochloride on *C. difficile* spores (ATCC BAA 1805) inoculated on stainless steel discs.

The surface of each disc was inoculated with 100 ul of *C. difficile* spores (ATCC BAA 1805) with 1.7×10^6 spores. The discs were then subjected to treatments for 10 min including, control, ethanol control, 1%, 2%, 3%, 4%, or 5% OH. The dilutions were pour-plated with BHIT agar supplemented with CDMN and incubated anaerobically for 48 h after which colonies were enumerated. * indicates treatments with significant difference at $p < 0.05$.

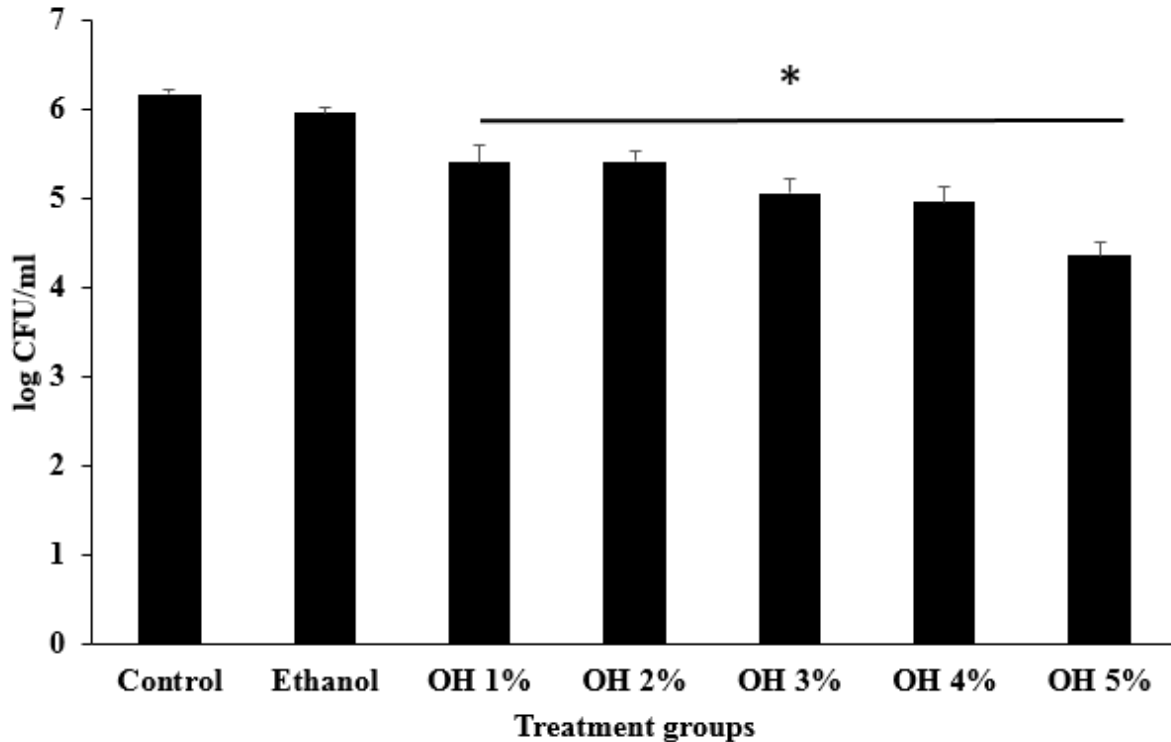


Table 1. Recovery of *C. difficile* spores from stainless steel discs after wiping with OH.

Stainless steel discs were inoculated with 100 µl of spore solution containing concentrations of 10^5 , 10^4 , 10^3 and 10^2 CFU spores/disc. The treatments [untreated control, ethanol control, 1% OH, 3% OH, and 5% OH] were applied on the disk surface and allowed to act for 10 min and were immediately wiped. The discs were pour-plated with BHIT agar supplemented with CDMN and incubated anaerobically for 48 h at 37°C. The (+) represents the presence of *C. difficile* colonies on the BHIT plate recovered from stainless steel discs.

	Control	Ethanol	1% OH	3% OH	5% OH
10^5 CFU/disc	+	+	+	+	+
10^4 CFU/disc	+	+	+	No colonies	No colonies
10^3 CFU/disc	+	+	No colonies	No colonies	No colonies
10^2 CFU/disc	+	+	No colonies	No colonies	No colonies

Table 2. Recovery of residual *C. difficile* spores from wipes after wiping stainless steel discs.

Stainless steel discs were inoculated with 100 µl of spore solution containing concentrations of 10^5 , 10^4 , 10^3 and 10^2 CFU spores/disc. The treatments [untreated control, ethanol control, 1% OH, 3% OH, and 5% OH] were applied on the disc surface and allowed to act for 10 min followed by immediate wiping. Wipes were mechanically rotated with an electric drill for 10 s at 60 rpm with a downward weight of 500 g. The wipe was stamped on the four quadrants of a BHIT agar plate supplemented with CDMN and 7% horse blood and incubated anaerobically for 48 h at 37°C. The (+) represents the presence of *C. difficile* colonies on the BHIT plate recovered from wipes.

	Control	Ethanol	1% OH	3% OH	5% OH
10^5 CFU/disc	+	+	+	+	+
10^4 CFU/disc	+	+	+	+	No colonies
10^3 CFU/disc	+	+	+	+	No colonies
10^2 CFU/disc	+	+	No colonies	No colonies	No colonies

Chapter VII

**Aspirin reduces sporulation and spore-outgrowth in hyper-virulent *Clostridium difficile* in
*vitro***

Abstract

Clostridium difficile is a spore-forming nosocomial pathogen that causes a serious enteric disease in humans. Treatments that can decrease *C. difficile* toxin and spore production could significantly reduce the severity, transmission and relapse of *C. difficile* infections (CDI). This study investigated the efficacy of a commonly used non-steroidal anti-inflammatory drug (NSAID) and cardio-protective medication, namely aspirin (acetylsalicylic acid), in reducing *C. difficile* toxin synthesis, spore production, germination and spore outgrowth.

Two *C. difficile* isolates (hyper-virulent stains ATCC BAA 1805 and 1870) were grown separately with or without sub-inhibitory concentrations (SIC) i.e. 0.05% (2.7 mM) and 0.1% (5.5 mM) of aspirin. Total viable counts (TVC) and spore counts (after heat shock at 60°C for 20 min) were determined after 72 h of incubation. In addition, the effect of aspirin on toxin production was determined. Real-time qPCR was performed to determine the effect of aspirin on *C. difficile* sporulation genes. To determine the effect of aspirin on *C. difficile* germination and spore outgrowth, *C. difficile* spores were grown in germination medium with or without the SIC or minimum inhibitory concentration (MIC) of aspirin and the optical density at 600 nm was recorded.

Aspirin SIC significantly reduced sporulation in two hyper-virulent strains of *C. difficile* and down-regulated genes involved in spore production ($p < 0.05$). In addition, the highest SIC of aspirin reduced spore outgrowth ($p < 0.05$), whereas the MIC completely inhibited outgrowth ($p < 0.05$). Results suggest that aspirin could potentially be used to control transmission and relapse of *C. difficile* by reducing spore production and spore outgrowth.

1. Introduction

Clostridium difficile is a spore-forming anaerobic bacterium, which causes a serious enteric illness in humans, predominantly in healthcare settings (Weese 2010, Stanley, Bartlett et al. 2013). Annually in the United States, more than 500,000 cases of *C. difficile* infection (CDI) are reported, costing ~ \$3 billion in healthcare and treatment costs (Ghose, Kalsy et al. 2007, Lessa, Mu et al. 2015). *C. difficile* has the highest incidence among all nosocomial pathogens reported globally (McDonald, Owings et al. 2006, Hookman, Barkin 2009, Stanley, Bartlett et al. 2013, Riggs, Sethi et al. 2007). The predominant predisposing factor of *C. difficile* infection in humans is prolonged antibiotic therapy (Hookman, Barkin 2009), which results in the disruption of normal enteric microflora, thereby allowing the germination and outgrowth of *C. difficile* spores in the gut. *C. difficile* spores germinate in the intestine in the presence of bile acid that acts as a germinant, and colonize the colon, where vegetative cells produce toxins, leading to severe diarrhea. Further, sporulation of vegetative cells occurs in the colon, and newly formed spores shed in the feces can potentially contaminate hospitals and healthcare facilities leading to new infections through the fecal-oral route (Barbut, Menuet et al. 2009). Sporulation in *C. difficile* is a complex process regulated by a set of genes, including *spo0A*, which is the master regulator of sporulation (Saujet, Pereira et al. 2013). Although toxins are the major virulence factors responsible for the pathogenesis of CDI, sporulation, germination and spore outgrowth in *C. difficile* are critical for transmission and relapse of the infection in humans (Sunenshine, McDonald 2006, Akerlund, Persson et al. 2008). Relapse of infection is a major concern in CDI recovered patients, and is observed in 20-30% of reported cases (Barbut, Richard et al. 2000, Kelly, LaMont 2008). Therefore, it is critical to control *C. difficile* sporulation in the human gastrointestinal tract for controlling CDI and its transmission.

Acetylsalicylic acid, commonly known as aspirin, is a well-known non-steroidal anti-inflammatory drug (NSAID). Aspirin is the most widely used drug in medicine (Soni 2005). The US Preventative Services Task Force recommends a daily low dose of aspirin ($\leq 30\text{mg}$) for the prevention of cardiovascular disease as a preemptive measure for recurrent cardiovascular events (Ittaman, VanWormer et al. 2014, US Preventive Services Task Force 2009). Low dose aspirin has been shown to reduce the aggregation of platelets, thereby preventing blood vessel obstruction and reducing the risk of heart attack and stroke (Dippel, Van Kooten et al. 2004). Moreover, the anti-inflammatory effect of aspirin has been reported to reduce colonic inflammation and decrease the risk of colorectal cancer in long-term clinical trials (Chan, Giovannucci et al. 2005, Smith, Hutchison et al. 2015). In addition, aspirin was identified as a factor that reduced mortality in an observational study of adults diagnosed with CDI (Saliba, Barnett-Griness et al. 2014). However, the mechanistic basis behind the reduced CDI mortality linked to aspirin is unknown. Therefore, the objective of this study was to investigate the effect of aspirin on *C. difficile* toxin production, spore production, spore germination and spore outgrowth.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Clostridium difficile hypervirulent strains (ATCC BAA 1805 and 1870) were grown in brain heart infusion broth (BHI) supplemented with 5% yeast extract (Difco, Sparks, MD, USA) at 37°C for 24 h in a Whitley A35 anaerobic workstation (Microbiology International, Frederick, MD, USA) under a gaseous atmosphere of 80% nitrogen, 10% carbon dioxide and 10% hydrogen. The broth cultures were sedimented by centrifugation (3600 g, 15 min, 4°C), and the pellet was washed twice, and resuspended in sterile phosphate-buffered saline (PBS, pH 7.0), which was used as the inoculum for this study. The bacterial populations in the cultures were determined by serial

dilution (1:10 in PBS) and plating 0.1 ml portions of appropriate dilutions on duplicate BHI agar and *Clostridium difficile* moxalactum norfloxacin (CDMN) agar (Oxoid, USA) supplemented with 5% horse blood under strict anaerobic conditions for 24 h.

2.2 Spore preparation

C. difficile spores were prepared using a previously published protocol (Sorg, Dineen 2009). Single colonies of ATCC BAA 1805 and 1870 were inoculated separately into BHI supplemented with 5% yeast extract (BHIS), and cultured overnight at 37°C under anaerobic conditions as before. A 150 µl aliquot of overnight culture was gently rotated to evenly distribute the culture onto BHIS agar in six well plates and was cultured for 7 days at 37°C in an anaerobic workstation to allow sporulation. After 7 days, spores were collected by flooding wells with 2 ml of ice cold sterile deionized water (dH₂O) with gentle pipetting and mixing. The collected spore suspension was heat-treated at 60°C for 20 min to kill any vegetative cells and was washed five times in sterile dH₂O by centrifugation at 16,000 g for 5 min for purification. Spore suspensions were visualized under a microscope to ensure 90% sporulation prior to storage.

2.3 Determination of MIC and SIC of aspirin against *C. difficile*

The MIC and SIC of aspirin (Sigma-Aldrich, St. Louis, MO, USA) was determined based on a previously published protocol (Mooyottu, Kollanoor-Johny et al. 2014). Forty ml of BHI supplemented with 5% yeast extract was inoculated separately with ~ 5.0 log CFU of *C. difficile*, followed by the addition of 0.5 ml to 10 ml of aspirin working solution (400 mg aspirin dissolved in 10 ml of dimethyl sulfoxide, DMSO (222 mM aspirin)) with an increment of 0.5 ml. The cultures were incubated at 37°C for 24 h, and bacterial growth was determined by measuring the optical density at 600 nm and by plating on BHIS agar with incubation in an anaerobic workstation. The lowest concentration of aspirin that inhibited bacterial growth after 24 h of incubation was selected

as its MIC, and the two highest concentrations below the MIC that did not inhibit bacterial growth were selected as the SICs for this study. Duplicate samples were included and the experiment was replicated three times.

2.4 Effect of aspirin on *C. difficile* growth and sporulation kinetics

The effect of aspirin on *C. difficile* growth and sporulation kinetics was determined using a published protocol (Babakhani, Bouillaut et al. 2012). Tubes containing 40 ml of BHIS were inoculated separately with 5.0 log CFU/ml of each *C. difficile* isolate (ATCC BAA 1805 and 1870) with and without the SICs (0.05% and 0.1% w/v) of aspirin dissolved in DMSO. Separate control tubes were included for DMSO alone and pH (adjusted to the pH of aspirin treatments using 0.1 N HCl) and were incubated anaerobically at 37°C for 72 h. Sampling was done after 72 h of incubation for quantitation of spores (spores surviving heat shock of 60°C for 20 min) and total viable count (TVC) by serially diluting each sample in PBS and plating in duplicate on BHIS agar supplemented with 0.1% sodium taurocholate (Sigma Aldrich).

2.5 Effect of aspirin on *C. difficile* total toxin A and B

Tubes containing 40 ml of BHIS were inoculated with 5.0 log CFU/ml of each *C. difficile* isolate (ATCC BAA 1805 and 1870) with and without the different concentrations of aspirin dissolved in DMSO. As before, control tubes were included for DMSO alone and pH (adjusted to the pH of aspirin treatments using 0.1 N HCl). The tubes were incubated anaerobically at 37°C for 48 h and the culture supernatant was collected at 24 h and 48 h of incubation for total toxin A and B quantitation by ELISA. Quantification of toxin present in the culture supernatant was done using the Wampole Tox A/B II kit (TechLabs, Inc., Blacksburg, VA, USA), as described by Merrigan et al. (Merrigan, Venugopal et al. 2010). Purified toxin B (Sigma Aldrich) was used to plot a standard curve for estimation of total toxin concentration. The culture supernatants were

diluted and ELISA was performed according to manufacturer's instructions. The optical density was measured at 450 nm and measurements were compared with the linear range of the standard curve to estimate total toxin concentration.

2.6 Effect of MIC and SIC of aspirin on *C. difficile* spore germination and outgrowth

To assess the germination of *C. difficile* (ATCC BAA 1805 and 1870), 100 µl suspensions containing 5.0 log CFU spores/ml were added to the wells of a 12-well plate containing 1.9 ml of pre-warmed (temperature), pre-reduced (exposure to anaerobic environment) BHIS broth supplemented with 0.1% sodium taurocholate inside an anaerobic workstation. The SIC (0.1% w/v) and MIC (0.15% w/v) of aspirin were added to the treatment wells. BHIS broth without sodium taurocholate and dH₂O were included as controls. One well with resazurin (0.1 mg/ml) was included to indicate the maintenance of anaerobiosis within the sealed plate during reading. The plate was sealed inside an anaerobic workstation using a sealant. The optical density was measured at 600 nm for the spore -media mixture in each well using a Synergy plate reader (Biotek, VT, USA) at 37°C over a 24 h time period with readings taken at 10 min intervals, and was expressed as a percentage of the initial OD₆₀₀ (t/t₀). The spore germination was measured as the initial loss of OD₆₀₀ and spore outgrowth was measured by the increase in OD₆₀₀ followed by spore germination plotted against time (Allen, Babakhani et al. 2013, Paredes-Sabja, Bond et al. 2008).

2.7 Real time quantitative PCR (RT-qPCR)

To determine the effect of aspirin on *C. difficile* genes involved in spore production, total RNA was isolated during the stationary phase of growth from SIC-treated and control cultures (Blossom, McDonald 2007). The culture supernatant was separated by centrifugation at 3000 × g for 10 min at 4°C and the bacterial pellet was immediately used for RNA extraction. Total RNA was extracted using the Ambion RiboPure Bacteria RNA kit (Ambion Austin, TX, USA) according

to the manufacturer's instructions, followed by DNase I digestion using Turbo DNase I (Ambion). The cDNA was synthesized using the Bio-Rad iScript cDNA synthesis kit according to manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The expression of genes associated with spore production were analyzed by RT-qPCR using published primers (Babakhani, Bouillaut et al. 2012, Saujet, Monot et al. 2011) normalized against rpoC and 16s gene expression. Twenty μ l reactions were performed in triplicate using iTaq universal SYBR Green (Bio-Rad). The relative fold change in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak, Schmittgen 2001).

2.8 Statistical analysis

All experiments had duplicate samples and the studies were repeated three times. The data were analyzed using one-way ANOVA. Differences between the means were considered significantly different at $p < 0.05$.

3. Results and Discussion

3.1 Sub-inhibitory and minimal inhibitory concentrations of aspirin

The MIC of aspirin against *C. difficile* was found to be 0.15% w/v. The SICs (two highest concentrations below MIC) of aspirin were 0.05% w/v (2.7 mM) and 0.1% w/v (5.5 mM), which did not inhibit the growth of *C. difficile* isolates after 24 h of incubation at 37°C.

3.2 Effect of aspirin on *C. difficile* sporulation kinetics

The effect of the SICs of aspirin on *C. difficile* spore production is depicted in Fig. 1. In isolate BAA 1805, in the presence of aspirin at 0.1% and 0.05%, there was a significant decrease in spore count after 72 h of incubation compared to controls ($p < 0.05$) (Fig. 1A). Aspirin at 0.1% and 0.05% resulted in ~ 3.0 log CFU/ml and 2.0 log CFU/ml reduction in spore counts, respectively. However, spore count of all control groups (no aspirin control, pH control and DMSO control) were not significantly different. In addition, the total viable count (plated without

heat treatment) in aspirin-treated and control samples were not significantly different ($p > 0.05$). Similar results were observed with isolate BAA 1870, where addition of 0.1% and 0.05% aspirin brought about 3.5 log CFU/ml and 2.0 log CFU/ml reduction in spore counts, respectively (Fig. 1B). No differences in spore counts were observed among the control samples.

3.3 Effect of aspirin on C. difficile spore germination and outgrowth

The effect of SIC (0.1%) and MIC (0.15%) of aspirin on spore germination and outgrowth over a 24 h time period was determined. In control wells (no aspirin control, DMSO control and pH control), spore outgrowth was observed as indicated by an increase in the absorbance starting at 12 h, with continued increase over the 24 h duration (Fig. 2A and 2B). Aspirin at 0.1% delayed *C. difficile* spore outgrowth by 4 h, and significantly reduced spore germination compared to controls in both isolates ($p < 0.05$). On the other hand, aspirin at 0.15% was more effective in inhibiting *C. difficile* spore outgrowth, with only a slight increase in absorbance observed at 22 h of incubation for both isolates ($p < 0.05$) (Fig. 2A and 2B).

3.4 Effect of aspirin on C. difficile toxin production

The effect of aspirin on toxin production was investigated by collecting the supernatant at 24 and 48 h and quantifying total toxin A and B by ELISA. Results revealed that aspirin did not significantly affect the toxin production compared to controls at both 24 and 48 h of incubation ($p > 0.05$) (data not shown).

3.5 Effect of aspirin on C. difficile spore production genes

The effect of the SIC of aspirin on the expression of genes critical for sporulation in *C. difficile* is shown in Fig. 3A and 3B. Transcriptional analysis of *C. difficile* sporulation associated genes by RT-qPCR showed that aspirin significantly down-regulated ($p < 0.05$) the expression of *spo0A* by 8 fold in BAA 1805 and 15 fold in BAA 1870, and *spoIIA* by 25 fold and 35 fold in

BAA 1805 and BAA 1870, respectively. In addition, the expression of *spoIIR* was decreased by 72 and 13 fold for BAA 1805 and 1870, and that of *spoIIID* by 69 fold and 10 fold in *C. difficile* BAA 1805 and 1870, respectively.

4. Discussion

C. difficile has the highest incidence as a nosocomial pathogen, and recent surveillance data have shown that *C. difficile* has surpassed methicillin-resistant *Staphylococcus aureus* as the most frequently acquired hospital infection (Miller, Chen et al. 2010). Sporulation of *C. difficile* is a crucial event in CDI transmission, where spore germination and outgrowth are necessary for establishing infection in a susceptible patient. *C. difficile* sporulation occurs at the time of egestion resulting in fecal shedding of spores which can subsequently contaminate the environment. In addition, *C. difficile* spores survive on hospital surfaces due to their resistance to most physical and chemical disinfectants used in hospital settings (Setlow 2006). *Clostridium difficile* spores reach the gut of the affected individual through the fecal-oral route, germinate and outgrow to form vegetative cells which colonize the large intestine and produce potent toxins that are responsible for *C. difficile* associated diarrhea. Sporulation is an important virulence factor in *C. difficile* pathogenesis and transmission and agents that can reduce *C. difficile* sporulation and outgrowth could potentially reduce *C. difficile* infection in susceptible individuals (Johnson 2009, Maroo, Lamont 2006). Our data suggest that aspirin, a commonly used non-steroidal anti-inflammatory drug, which is also routinely prescribed to heart patients due to its anti-thrombotic cardio protective effect, can significantly reduce *C. difficile* spore production and spore outgrowth *in vitro*. This finding could potentially explain the clinical observation of reduced CDI morbidity in patients taking daily low-dose aspirin (Saliba, Barnett-Griness et al. 2014).

Our data demonstrate that SICs of aspirin significantly reduced spore production by *C. difficile* after 72 h of incubation ($p < 0.05$). Aspirin readily dissociates to salicylic acid in the gut, which can reduce the pH of the gut environment. In our experiments, the pH of BHI broth was 7.09, which decreased to 6.78 with the addition of aspirin. Hence, we included a pH control in the experiment in order to rule out the pH effect of aspirin in reducing *C. difficile* sporulation. However, spore production in pH control did not differ significantly from the negative control, indicating that the observed reduction in spore production was not due to the reduced acidity effect of aspirin. Similarly, a diluent control (DMSO) was also included, where spore production did not differ significantly from the negative control. However, a reduction in sporulation was observed in aspirin-treated samples by dilution and plating method ($p < 0.05$). In addition, our gene expression studies indicated that aspirin significantly down-regulated important genes responsible for *C. difficile* sporulation. Moreover, the germination experiments showed that the SIC of aspirin significantly reduced *C. difficile* spore outgrowth compared to controls ($p < 0.05$). More strikingly, aspirin at the MIC almost completely inhibited *C. difficile* spore outgrowth. These results suggest that vegetative cells from newly germinated spores are more sensitive to aspirin compared to vegetative *C. difficile* cells grown in broth culture. Interestingly, the SICs of aspirin did not increase toxin production (data not shown) as is typically observed with the use of antimicrobials against *C. difficile* such as antibiotics, vancomycin and metronidazole (Gerber, Walch et al. 2008).

The anti-inflammatory action of aspirin in humans and animals is well studied. Aspirin inhibits cyclooxygenase (COX) enzymes in the arachidonic acid pathway, which are required for the synthesis of pro-inflammatory cytokines such as prostaglandins. (Vane, Botting 2003). However, studies on the effect of aspirin on bacteria and bacterial virulence factors are very limited. Aspirin has been shown to exert antibacterial properties against Gram-negative bacteria

such as *Brucella* spp. and *Helicobacter pylori* by downregulation of outer membrane protein expression (Wang, Wong et al. 2003, Munoz-Criado, Muñoz-Bellido et al. 1996). Similarly, the antimicrobial effect of aspirin against *Staphylococcus aureus* by modulation of its virulence genes has also been reported (Kupferwasser, Yeaman et al. 2003). In our study, we observed a downregulation of *C. difficile* sporulation genes in the presence of aspirin in vitro. Nonetheless, taking into account the pathogenesis of CDI, an anti-inflammatory effect of aspirin could be an added advantage in CDI patients in reducing severe colitis symptoms.

Our results indicate preventive and therapeutic potential of aspirin in controlling *C. difficile* infection. Aspirin significantly reduced *C. difficile* spore production besides decreasing the outgrowth of *C. difficile* spores, but did not increase toxin production compared to controls. These findings collectively suggest the potential use of aspirin to control CDI in humans. However, follow up *in vivo* and clinical validations are required to confirm this.

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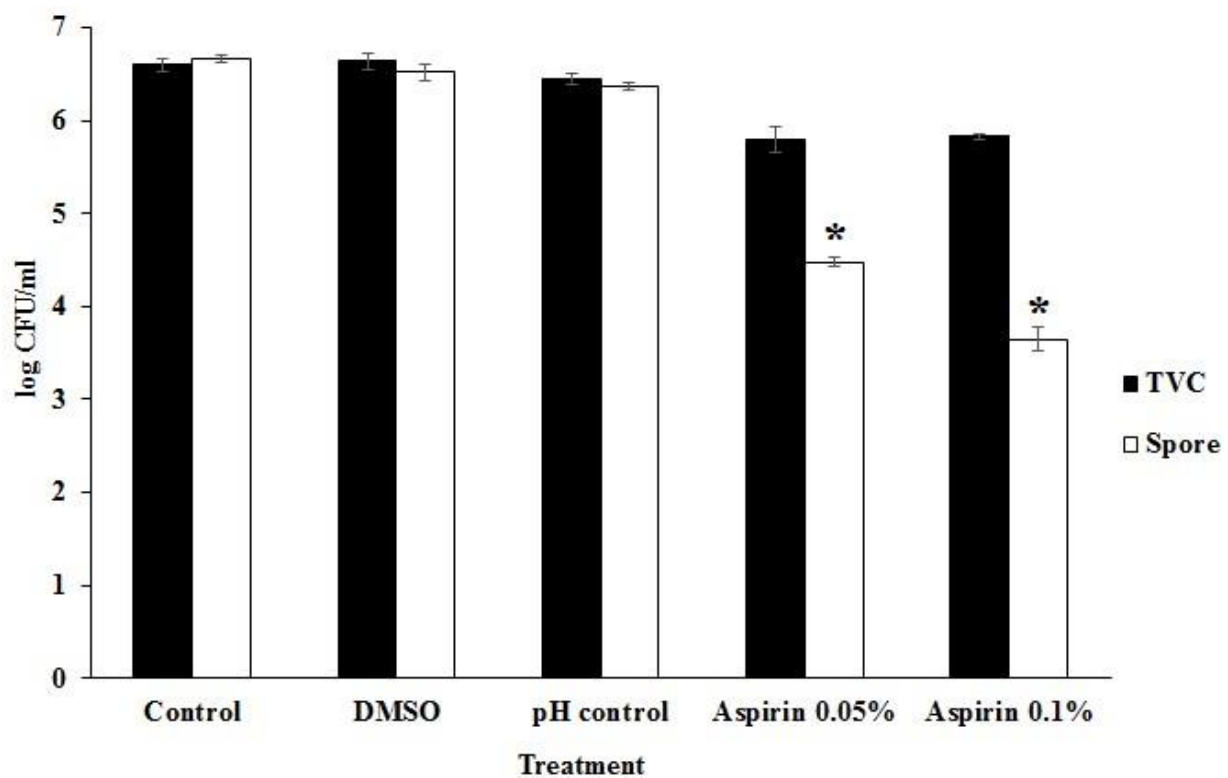
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Fig 1. Effect of SICs of aspirin (0.5 mg/l and 1.0 mg/l) on *C. difficile* BAA 1805 (1A) and 1870 (1B).

Growth and sporulation kinetics by quantitation of spores (survivors of incubation at 60°C for 20 min) and total viable count (TVC). * Treatments significantly differed from the respective controls ($p < 0.05$).

(A) *C. difficile* BAA 1805



(B) *C. difficile* BAA 1870

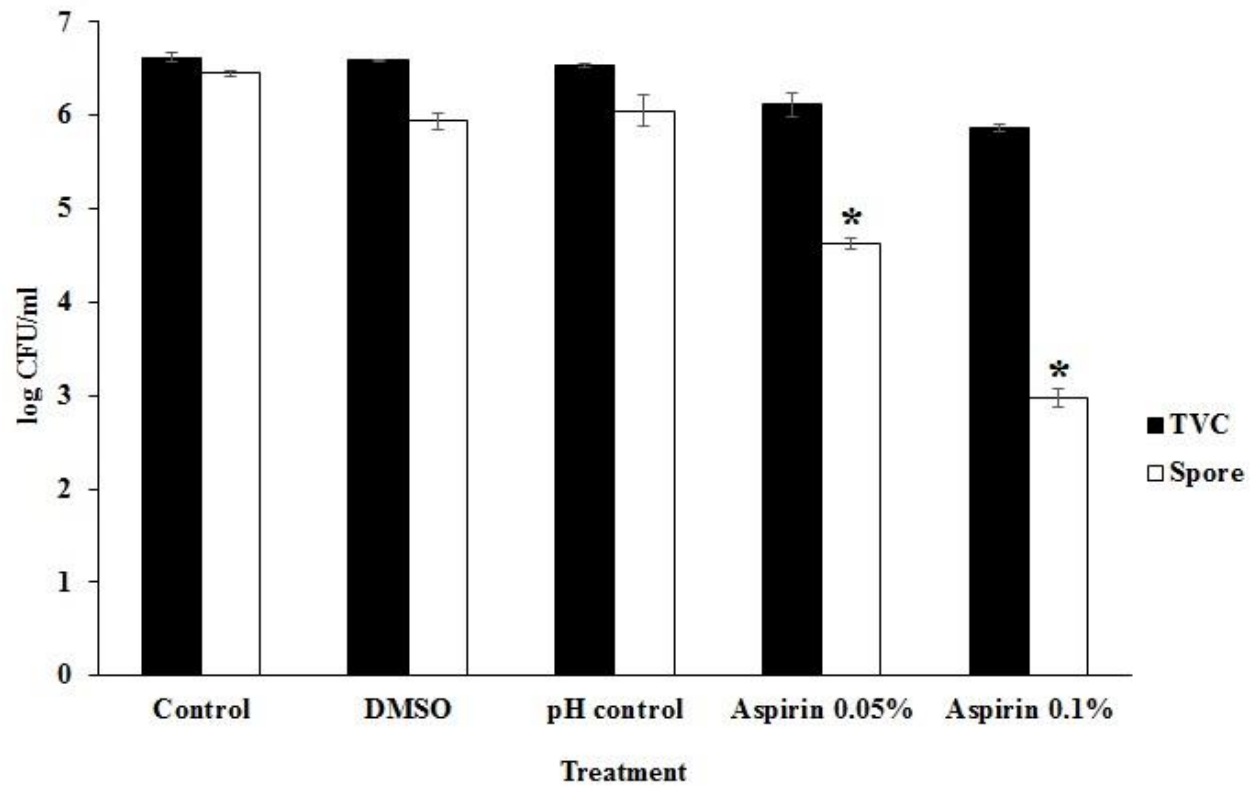
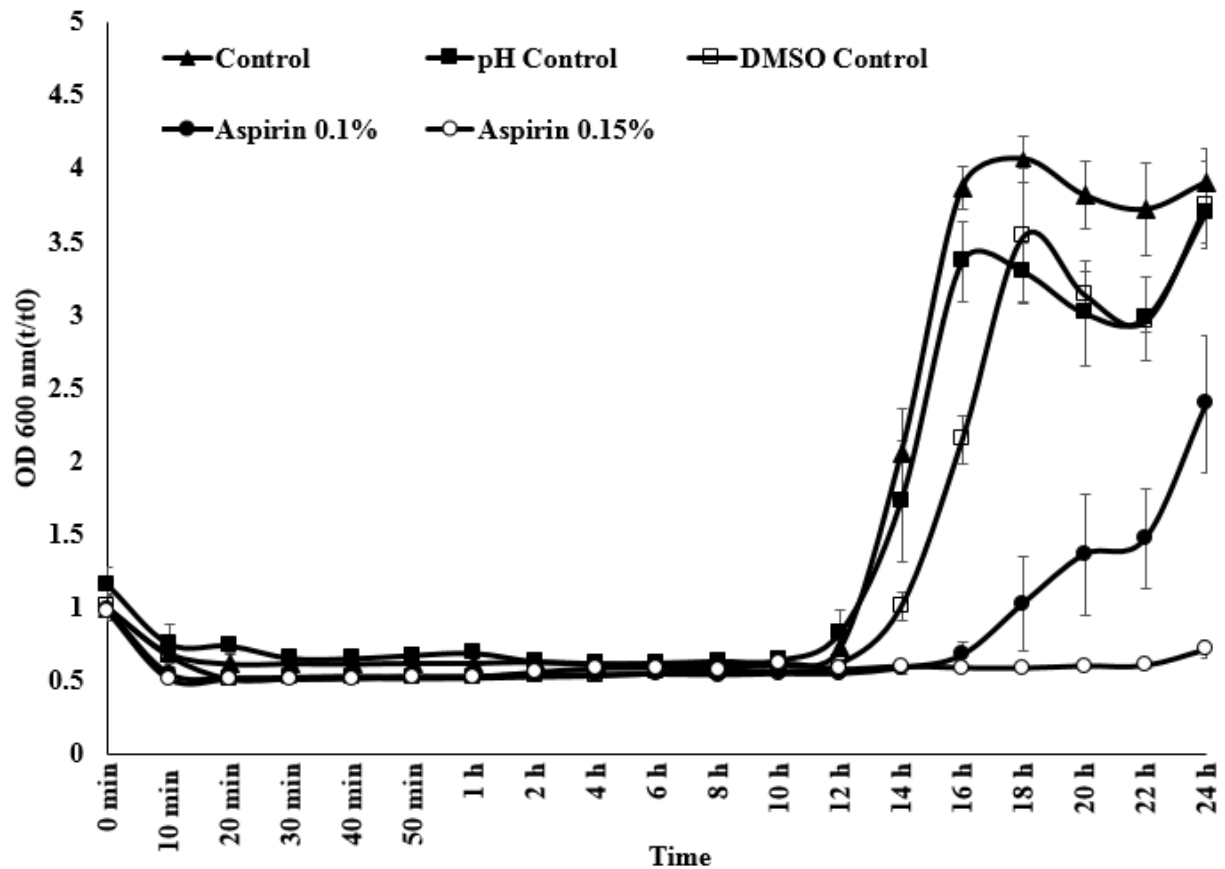


Fig 2. Effect of aspirin on germination and outgrowth of *C. difficile* BAA 1805 (A) and 1870 (B) spores.

Bacterial pellet was harvested at 24 h for RNA isolation and RT-qPCR was done for spore production genes. * Treatments significantly differed from the controls ($p < 0.05$).

(A) *C. difficile* ATCC BAA 1805



(B) *C. difficile* ATCC BAA 1870

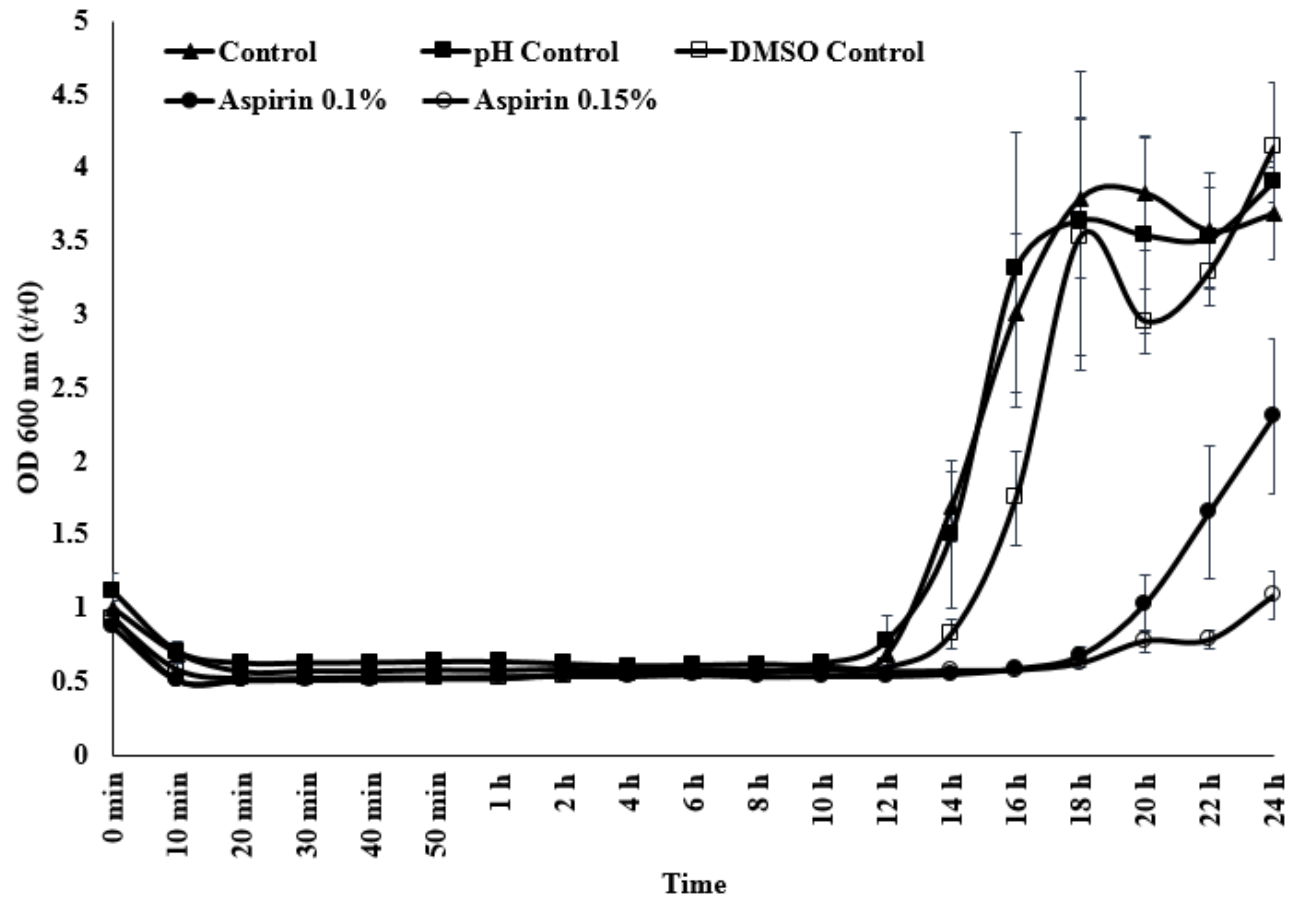
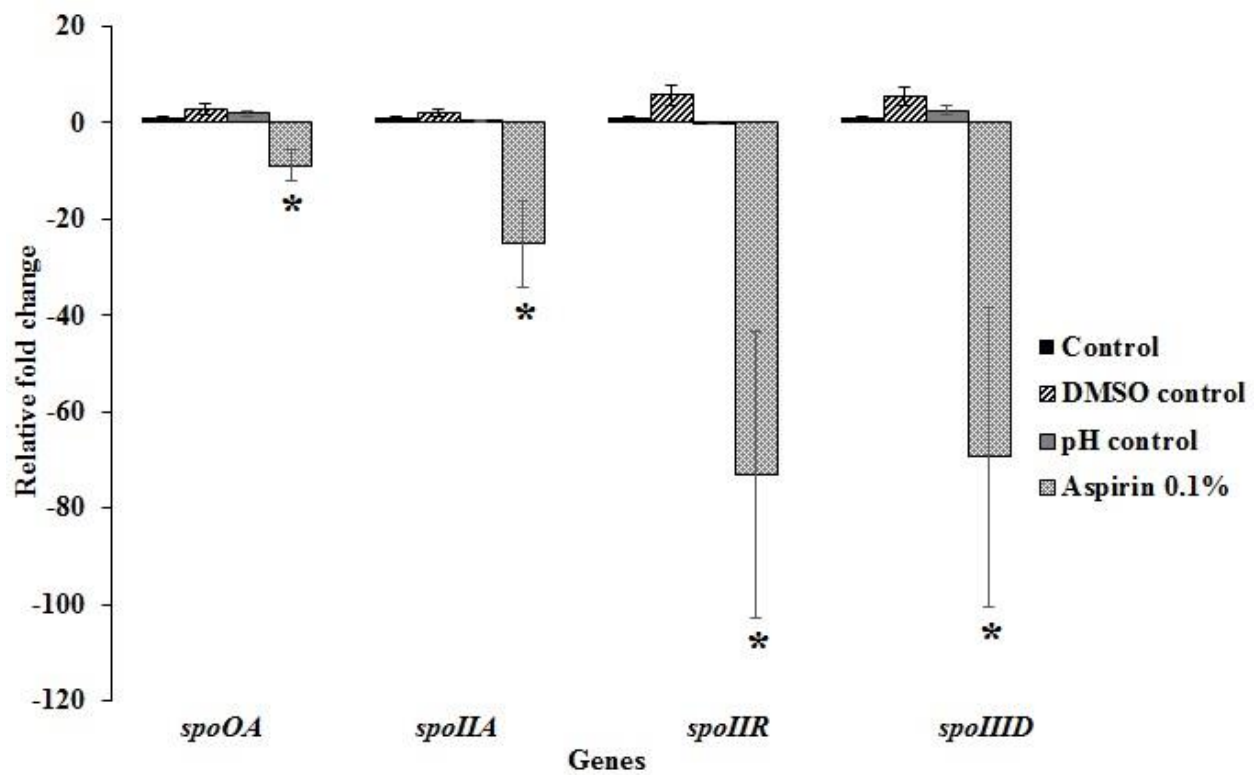


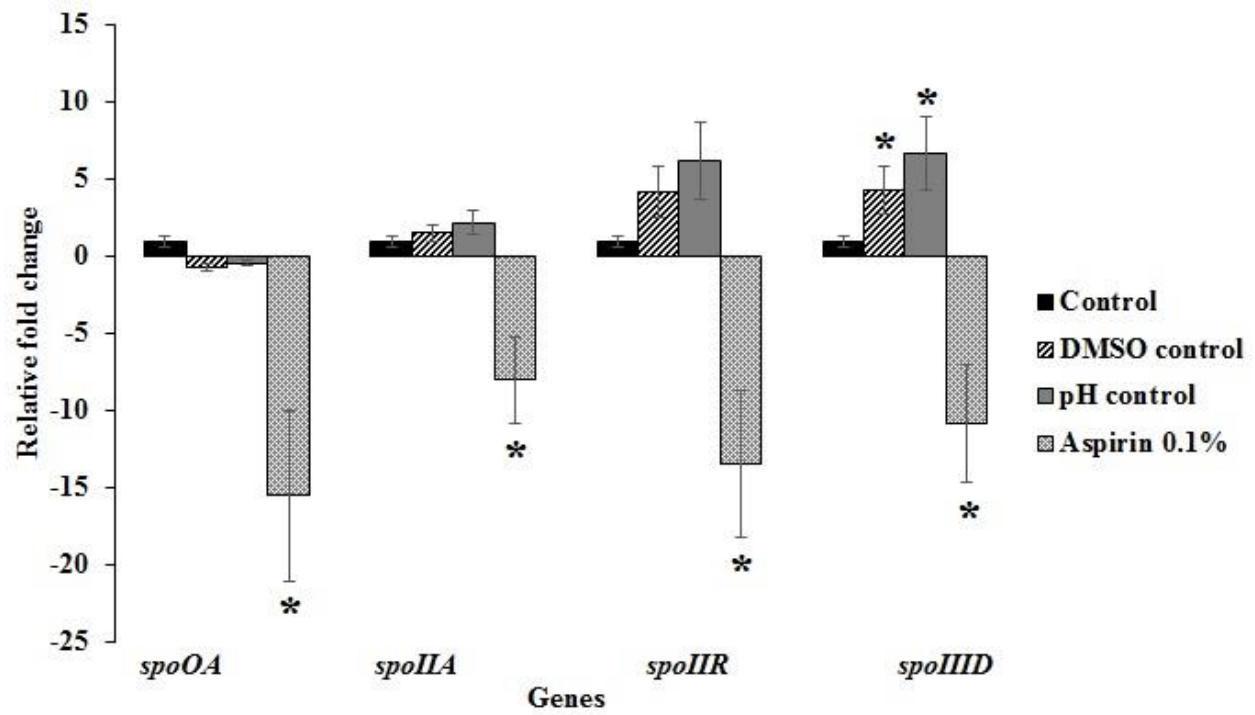
Fig 3. Effect of SIC of aspirin (1.0mg/l) on *C. difficile* ATCC BAA 1805 (A) and 1870 (B) sporulation regulatory genes.

The spore germination was measured as the initial loss of OD₆₀₀ and spore outgrowth was measured by recording the increase in OD₆₀₀ followed by spore germination. * Treatments significantly differed from the controls ($p < 0.05$).

(A) *C. difficile* ATCC BAA 1805



(B) *C. difficile* ATCC BAA 1870



Chapter VIII

Summary

Clostridium difficile causes a serious toxin-mediated infection in humans, primarily in nosocomial settings. The production of toxins is one of the most important virulence factors in *C. difficile*, however, sporulation, germination and spore outgrowth play a vital role in the transmission and relapse of *C. difficile* infection (CDI). Relapse of infection is a major concern in CDI patients, and is observed in 20-25% of cases. Spores can be transferred by the fecal-oral route, therefore, it is critical to control sporulation, germination and spore outgrowth in order to control transmission.

Hospital surfaces have been shown to be frequently contaminated with *C. difficile* spores, and can remain contaminated for an extended period, thereby serving as a source of infection. *C. difficile* spores are resistant to most commonly used disinfectants such as sodium hypochlorite. Therefore, it is critical to reduce control spore survival on surfaces. A novel disinfectant known as octenidine hydrochloride (OH) was investigated for its efficacy to decrease *C. difficile* spores on stainless steel surface. Results revealed that direct application of OH (5%) and wiping with OH reduced spore numbers on stainless steel surface ($p < 0.05$).

Transmission and relapse of CDI can also be reduced by controlling sporulation in the gut. Sporulation in *C. difficile* is regulated by *spoOA*, which is the master regulator of sporulation. Aspirin, a widely used non-steroidal anti-inflammatory drug was reported to reduce mortality in adults with CDI. This led to our investigation of aspirin at its sub-inhibitory concentration (SIC) and minimal inhibitory concentration (MIC) for reducing *C. difficile* sporulation, spore germination and spore outgrowth in vitro. The SIC of aspirin significantly reduced sporulation of *C. difficile* and down-regulated critical genes involved in spore production ($p < 0.05$). Also, the SIC of aspirin reduced spore outgrowth and the MIC completely inhibited outgrowth ($p < 0.05$).

A recent trend has identified increased *C. difficile* infections in community settings, totaling 32% of all cases. Community-associated infections occur in low risk patients who are comparably younger than traditional patients, and have no recent history of hospitalization or long term antibiotic treatment. In addition, *C. difficile* spores have been isolated from wild animals, food animals, animal-derived foods and the environment. *C. difficile* spores have also been isolated from fresh produce, but limited prevalence studies exist in the US. In our study, a 23% prevalence rate in REL (35/150) procured from various retail stores in Connecticut was observed. Additionally, *C. difficile* spores in REL packaged under MAP conditions (2% O₂, 5% CO₂ and 93% N₂) remained viable for 10 days of refrigerated storage.

Clostridium difficile spores were inoculated in ground beef and were subjected to chilling storage at 4°C for a week, freezer storage at -18°C for 12 weeks or cooking to the USDA recommended internal temperature of 71.1°C. At inoculation levels of 3 log CFU/g and 5 log CFU/g, *C. difficile* spores remained the same during chilling and freezer storage ($p > 0.05$). Cooking ground beef to the USDA recommended internal temperature of 71.1°C decreased spores by 1 log CFU/g for both inoculation level ($p < 0.05$). The results indicated that *C. difficile* spores can survive chilling, freezing and the USDA recommended cooking temperature for ground beef. Similarly, *C. difficile* spores remained viable in fermented summer sausage with low pH of 5.0 for 90 days of storage, and the survival was not significantly different from unfermented control sausages ($p > 0.05$). Therefore, if *C. difficile* spores were present in a commercial ground beef product or a fermented summer sausage product, they could survive typical storage and cooking conditions.