Prevalence of *Clostridium difficile* in retail meats from Minnesota and comparison of growth and survival of human and animal isolates

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ABSTRACT

Clostridium difficile is a Gram positive, anaerobic, spore-forming, rod-shaped bacterium that causes antibiotic-associated diarrhea in hospitalized and community-dwelling patients. Recent findings have suggested that this organism may be transmitted from animals to humans through the consumption of contaminated foods. Genotypic similarities have been found among *C. difficile* strains isolated from animals and humans. However, comparisons of the behavior of these two groups of strains at the physiological level have not been conducted in detail. This study sought to determine the prevalence of *C. difficile* in retail meats from Minnesota and to compare human and animal isolates by measuring the growth rate and their survival in meats during cold storage.

Meat samples were obtained from retail stores from Minnesota and consisted mainly of raw beef, pork, and poultry. These samples were analyzed for the presence of *C. difficile* by initial enrichment in *Clostridium difficile* moxalactam-norfloxacin (CDMN) broth, followed by ethanol shock, plating onto CDMN agar, and anaerobic incubation for 48 h at 37°C. Suspicious colonies were subjected to confirmation by colony morphology, Gram staining, and production of L-proline aminopeptidase.

Growth rate parameters were determined for a total of 35 *C. difficile* strains isolated from humans and animals. Optical density was measured during exponential growth on brain hearth infusion broth at 37°C anoxically. The average growth rates of the two groups were then compared. A subgroup of 5 human and 5 animal strains was

selected to assess the survival of *C. difficile* in meats during cold storage. *C. difficile* strains were inoculated onto ground meats to a level of approximately 10^5 CFU/g. Inoculated meats were stored at 4°C and -15°C for 5 and 20 days, respectively. *C. difficile* counts were determined at different times during storage by plating onto CDMN agar.

A total of 342 raw meat and poultry samples were collected from 5 different counties in central Minnesota from 25 retail stores. Twenty nine samples had CDMN-presumptive colonies, but none of them were confirmed as *C. difficile* C.I. 0-0.013% of meats positive for *C. difficile* at a 95% level. The average growth rate of 22 human strains at 37°C was 0.52± 0.25 h^{-1}, which was not significantly different from the value for 13 animal strains (0.62± 0.15 h^{-1}) (p>0.05). Counts of *C. difficile* strains inoculated on ground beef and chicken diminished during storage at 4°C and -15°C. Overall reduction in bacterial counts was not significantly different between human and animal strains.

These findings suggested that *C. difficile* was not commonly present in retail meats from Minnesota. *C. difficile* strains isolated from animals had similar growth rates at 37°C as the strains from human origin. *C. difficile* viable counts diminished over time during refrigeration and freezing of inoculated meats. Additionally, the methodology used in the present study was effective in recovering *C. difficile* from artificially inoculated meats.

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INTRODUCTION

Clostridium difficile is a Gram positive, anaerobic rod-shaped bacterium that has the ability to form endospores. This bacterium was identified as a human pathogen in 1978, and it is widely recognized as the main etiologic agent of antibiotic-associated diarrhea and pseudomembranous colitis. *C. difficile* infection (CDI) occurred traditionally in hospitalized patients of advanced age (65 years of age and older), who had received antibiotic treatment. However, the epidemiology of CDI has changed. Increased incidence and severity of nosocomial CDI has been reported in the U.S., Canada, and Europe. Recent estimates of the incidence of CDI have exceeded 300,000 cases per year in the United States (70). Furthermore, there are also increasing reports of CDI occurring in community settings, and in individuals previously considered at low risk of acquiring the disease.

Due to the increase in community-acquired CDI, additional modes of transmission have been proposed to better explain the epidemiology of *C. difficile* outside healthcare facilities (82). *C. difficile* has been isolated from different animal species, and a number of studies have analyzed the prevalence of this bacterium in food animals. Similarities in the genotypes of *C. difficile* strains isolated from food animals and those causing disease in humans have been reported (97), suggesting a zoonotic transmission of *C. difficile* with food of animal origin as a possible vehicle of transmission. Subsequently, research studies conducted since 2006 have reported the isolation of *C. difficile* from retail meats in different proportions.

Further information is needed regarding the presence of *C. difficile* in meat products, as well as the survival of this bacterium in food matrices if a foodborne acquisition is confirmed. In addition, *C. difficile* strains isolated from humans and animals have only been compared genotypically. However, information comparing the phenotype of these two groups of strains might be useful as part of the evaluation of zoonotic transmission of *C. difficile*. Therefore, the first objective of this study was to determine the prevalence of *C. difficile* in retail meats from the state of Minnesota. The second was to assess potential differences between *C. difficile* isolates of humans and animals, specifically regarding growth rate at 37°C. Finally, considering potential contamination of meats with *C. difficile*, to determine the survival of human and animal strains on meats during refrigeration and freezing.

CHAPTER 1

LITERATURE REVIEW

1. *Clostridium difficile*

1.1 Characteristics of the organism

Clostridium difficile is a pathogenic Gram-positive, anaerobic rod that forms subterminal spores. This bacterium is recognized as the main causative agent of antibiotic-associated diarrhea, particularly within the hospital environment. *C. difficile* belongs to the family *Clostridiaceae* and is included in cluster XIa of the genus *Clostridium* (73). Vegetative cells are 3 to 16 μm in length and can be motile due to the presence of peritrichous flagella. Optimal growth temperature ranges from 30° to 37°C although growth can also occur at 25° and 45°C.

C. difficile is predominantly saccharolytic, and it has a weak or absent proteolytic activity. It has the ability to ferment glucose, fructose, mannitol, and mannose (109). Fermentation products are organic acids, including acetic, butyric, isobutyric, valeric, isovaleric and isocaproic acids (73). In addition, hydrogen sulfide and hydrogen are also produced (90). Tests for production of indole, lecithinase, urease, and lipase are negative

for this organism (109). Upon tyrosine metabolism, *C. difficile* produces *p*-cresol, resulting in a characteristic horse manure-like odor of isolated colonies.

The genome of *C. difficile* strain 630 was sequenced in 2006 and consists of a chromosome of more than four million base pairs with a G+C content of 29%, and a plasmid. It was determined that the genome possesses genetic elements that are mobile, which can aid in the acquisition of antibiotic resistance and virulence factors (105).

1.2 Microbial ecology

Being an enteric pathogen, *C. difficile* is able to grow in the anaerobic environment of the intestinal tract when the colonic microbial flora is disrupted. However, approximately 3% of healthy individuals can carry this organism asymptomatically (73). In contrast, *C. difficile* and its toxins can be found in the feces of as many as 50% of neonates and infants younger than 1 year of age. Despite frequent colonization with *C. difficile*, infants do not present symptoms of disease and carriage rates diminish overtime (132).

The ability to form resistant spores allows *C. difficile* to be ubiquitous in the environment. Thus, it has been isolated from soil, hay, and water. In 1974, Hafiz isolated *C. difficile* from soil, sand, and from the stools of camels, horses, cows, and donkeys (45). A more extensive environmental study was conducted in South Wales, reporting recovery of *C. difficile* from river, sea, and lake water samples. Additional sources

included soil, swimming pools, raw vegetables, farm animals, cats, and dogs, and hospital-environment surfaces (2).

Spores of *C. difficile* are particularly persistent within the hospital environment. Hospitalized individuals who have acquired *C. difficile* infection (CDI) shed bacterial spores in their feces. Spores can therefore contaminate hospital rooms, and they have been found in fomites, beds, floors, frequently touched surfaces, and on the hands of health care personnel (75, 89). Furthermore, spores have been recovered from air surrounding symptomatic patients with CDI (12).

1.3 Sporulation

The formation of endospores allows bacteria to survive unfavorable environmental conditions. Spores of the genus *Clostridium* and *Bacillus* are known to resist both wet and dry heat treatments, UV and gamma radiation, as well as different antimicrobial chemicals (106). As a consequence, *C. difficile* spores can persist dormant for long periods of time, thus facilitating its transmission. In general, spores are formed during stationary phase in response to nutrient limitation or other stress conditions such as the presence of oxygen in the case of anaerobic bacteria.

The sporulation process has been widely studied in *Bacillus subtilis*. Sporulation is a very complex process that involves many components and genetic regulators. As sporulation begins, an asymmetric division of a vegetative cell produces a mother cell

and a small forespore. Then, the cell membrane of the mother cell engulfs the forespore and a thick layer of peptidoglycan is formed around the forespore. Finally, the mother cell lyses and the spore is released to the environment (112). The spore will allow the bacterial species to remain viable for long periods of time, but it is not capable of performing any metabolic activity.

C. difficile possesses similar genes to those of the sporulation cascade of *B. subtilis*, in particular downstream the sporulation regulator Spo0A. Nevertheless, the genes encoding the multicomponent phosphorelay system of sensory histidine kinases that trigger sporulation in *B. subtilis* are not present in *C. difficile* (84, 105). Underwood and collaborators (118) identified a putative sensor histidine kinase in *C. difficile* that directly phosphorylates Spo0A to initiate sporulation. However, the precise signals that induce sporulation in *C. difficile* remain unknown (118).

Different studies have been conducted to determine the sporulation rate in *C. difficile*. Researchers have reported an increased ability to form spores in epidemic strains implicated in severe outbreaks, in particular a hypervirulent strain classified as BI/NAP1/027 (1, 76, 123). In contrast, Burns and colleagues (2011) found no major differences in sporulation rates between a total of 28 BI/NAP1/027 and 25 non-BI/NAP1/027 strains (22). However, a high variability in the sporulation rate of different *C. difficile* strains was observed after 120 hours of incubation, irrespective of strain types (21, 22). The discrepancies between such studies have been attributed to different methodologies used and the number of strains tested.

C. difficile spores are metabolically inactive but in order to cause disease, cells must return to the vegetative state. The latter is achieved through a process called germination, in which spore-resistance properties are lost irreversibly when conditions for vegetative cell growth are appropriate (20). The germination process can be induced by different compounds, or germinants that are sensed by specific receptors located in the spore inner membrane. Specific germinants have been described for *B. subtilis*, such as glucose and L-alanine that bind to the receptors GerA, GerB, and GerK (112). However, germination components similar to those of *B. subtilis* have not been found in *C. difficile*.

In 1982, it was noted that addition of sodium taurocholate to selective culture media improved colony formation and recovery of *C. difficile* spores (133). A subsequent study demonstrated that bile salts, such as taurocholate, and glycine induced germination of *C. difficile* spores *in vitro* (112). Based on this observation, Sorg and Sonenshein suggested that *C. difficile* spores would germinate in the small intestine in the presence of bile salts. In addition to bile salts, Paredes-Sabja and coworkers found that *C. difficile* spores germinated in the presence of potassium chloride and sodium phosphate at pH 6.0 (85). In contrast, spores did not germinate when 20 aminoacids were tested individually as possible germinants.

1.4 Strain classification

Different methods have been used for typing *C. difficile* strains in order to better understand the epidemiology of the disease and investigate the occurrence of outbreaks. Among the main genotypic methods to study *C. difficile* isolates are PCR-Ribotyping (13, 81), pulsed field gel electrophoresis (PFGE) (40), restriction endonuclease analysis (REA), and toxinotyping (100).

PCR ribotyping is based on the amplification of the spacer region between the genes that encode for the 16S and 23S rRNA. Since this region has variations in *C. difficile*, different band patterns are obtained after electrophoresis with agarose gel. Thus, different strains can be identified with this technique (81). This methodology is mostly used in European laboratories. In 1999, Stubbs and colleagues generated a library of 116 PCR ribotypes of *C. difficile* strains isolated from a variety of sources in the United Kingdom (113). To date, the number of PCR ribotypes that have been identified is greater than 400 (65). The nomenclature of PCR ribotypes is in the form of three numbers.

The other molecular typing techniques frequently used are PFGE and REA. Typing by PFGE is done by digesting the bacterial DNA with a restriction endonuclease, usually *Sma*I (65). PFGE is mostly used in the United States and Canada as a standard method (57). The band patterns obtained are grouped into different North American Pulsotypes, often referred to as NAP. PFGE techniques have been modified to prevent degradation of DNA. Nevertheless, there are no standardized protocols for PFGE of *C. difficile* isolates (57). REA typing uses the restriction enzyme *Hin*dIII to digest the entire

cell DNA (28). After digestion, agarose gel electrophoresis is used to separate the DNA fragments. Although REA has a good discriminatory power, it is not widely used due to the difficulty of interpreting the resulting band patterns.

Rupnik and collaborators developed in 1997 a PCR-based method known as toxinotyping (101). Through this approach, specific regions of the genes encoding for toxins A and B are amplified and cut with different restriction enzymes to screen for polymorphisms (100). According to the patterns of changes in the toxin genes, strains of *C. difficile* can be categorized in a specific toxinotype. Toxinotypes are identified by roman numerals, with the exception of toxinotype 0. As many as 22 different toxinotypes of *C. difficile* have been reported (102).

Due to the different methods that have been used, strains of *C. difficile* can be identified by either one or more genotypic techniques. For example, an epidemic strain responsible for severe outbreaks of CDI is referred in the literature as NAP1/B1/027, as typed by PFGE, REA, and PCR ribotyping, respectively. However, since distinct standard techniques are used in Europe and North America, sharing data for strain identification is often challenging among different laboratories (65).

An additional method to study the relatedness between *C. difficile* isolates is multilocus variable-number tandem repeat analysis (MLVA). In this typing method, tandem repeats (*i.e.* sequences of DNA that are repeated multiple times) of different sizes that occur in the whole genome are amplified by PCR (103). Depending on the number of repeats obtained after capillary electrophoresis, clonal groups of strains can be recognized

and arranged in a minimum spanning tree (65). MLVA can be used to study outbreaks occurring in healthcare facilities and also the transmission routes of *C. difficile* strains.

1.5 Virulence factors

The main virulence factors involved in the onset of disease caused by *C. difficile* are two toxins known as toxin A (TcdA) and toxin B (TcdB). Toxin A is an enterotoxin and toxin B is a cytotoxin (116). Both toxins are among the group known as large clostridial toxins (103). TcdA has a size of 308 kDa, whereas TcdB is 270 kDa. Toxin production in *C. difficile* begins during stationary phase in response to nutrient limitation, for instance that of biotin (124, 134).

Toxins A and B are encoded in the genes *tcdA* and *tcdB*, respectively within the genome region known as the pathogenicity locus which has a size of approximately 20 kb (100). Additionally, three accessory genes are also encoded in this region namely *tcdC*, *tcdR*, and *tcdE*. The genes *tcdR* and *tcdC* encode positive and negative regulators for toxin production, respectively (103). The protein encoded in *tcdE* is thought to aid in the release of toxins A and B from the cell membrane of *C. difficile* (74).

TcdA and TcdB are glucosyltransferases that inactivate the family of Rho proteins. Rho proteins are low molecular weight GTPases that are involved in eukaryotic cell signaling and also regulate actin polymerization (116). When toxins A and B transfer a glucose unit to Rho proteins, these are inactivated. As a result, cell signaling is affected

and the actin cytoskeleton collapses (116, 124). Once the toxins are released inside the intestinal lumen, they enter the epithelial cells by endocytosis. Intoxicated cells get rounded and tight junctions are broken, which then results in cell death, fluid accumulation, and inflammation.

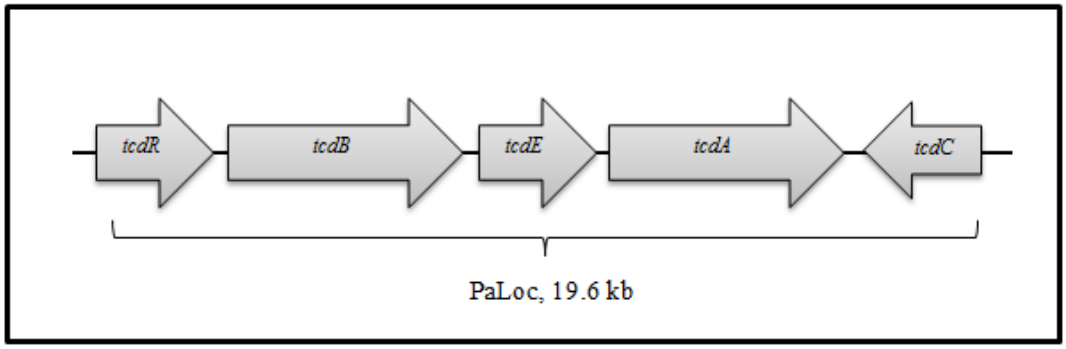

Figure 1.1 Schematic representation of the genes encoded in the pathogenicity locus (PaLoc) of *Clostridium difficile.*

Initial studies reported that toxin B alone was not toxigenic unless toxin A was present, generating a synergistic effect. However, Lyras concluded that toxin B can cause cytotoxicity and that it was lethal for hamsters by the use of isogenic mutants (71). In a similar way, Kuehne and collaborators reported in 2010 that *C. difficile* mutants producing either only toxin A or B were able to exert toxic effects *in vitro* and *in vivo* (66).

Some strains of *C. difficile* are also able to produce a third toxin, known as binary toxin or CDT. This toxin is an ADP-ribosyltransferase that has two subunits (CdtA and CdtB) and is similar to the iota toxin produced by *C. perfringens* (114, 124). CDT is encoded in a genomic region of 4.3 kb that contains the genes *cdtA*, *cdtB*, and the regulatory gene *cdtR*. This region is located separately from the pathogenicity locus (103). Although the effects of binary toxin are not well understood, this toxin is thought to enter colonic epithelial cells by endocytosis and prevent actin polymerization after a unit of ADP-ribosyl is transferred to actin monomers (107). *C. difficile* strains are often screened for the presence of the genes encoding this toxin.

Besides toxin production, *C. difficile* possesses several additional virulence factors. Once vegetative cells of *C. difficile* reach the colon, proteases break down the mucosal layer in the intestinal lumen, and the presence of flagella aids *C. difficile* cells to mobilize through the mucus. Attachment to epithelial cells is facilitated by adhesins and surface layer proteins (33). Strain 630 was found to possess a group of genes encoding for the production of extracellular polysaccharide, which can be related to the production of a capsule (105).

1.6 *Clostridium difficile* infection (CDI)

1.6.1 Disease characteristics

C. difficile was recognized as a pathogen in the 1970's. It was first described in 1935 by Hall and O'Toole, who isolated this bacterium from the feces of healthy neonates (47). In the 1960's, there were increasing reports of diarrhea and pseudomembranous colitis (PMC) occurring after antibiotic treatment with clindamycin (131). In 1978 *C. difficile* was identified for the first time as the causative agent in cases of PMC (10, 41). Since then it has been regarded as an important enteric pathogen, being particularly prevalent in hospitals.

There are a number of risk factors associated with acquiring *C. difficile* infection (CDI). Traditional risk factors for developing CDI were hospitalization, advanced age (65 years of age or older), suppressed immune system, and antibiotic therapy. A wide range of antimicrobial agents are implicated in CDI, including clindamycin, penicillins, cephalosporines and fluoroquinolones (83). Additional risk factors are cancer chemotherapy, surgery of the gastrointestinal tract, and tube feeding (15, 29). Some studies have described an association between the use of medications to suppress production of gastric acid, such as proton pump inhibitors and histamine type 2 blockers, and developing CDI in hospitalized patients receiving antibiotics (5, 135). However, a study conducted in 2012 in the Mayo Clinic revealed there was no association after adjustment for confounding variables (64).

Transmission of *C. difficile* occurs via the fecal-oral route (89). The organism is prevalent in hospitals and can be acquired by contact with infected patients, from contaminated surfaces, or transmitted through the hands of health care providers. The ingested spores are able to resist the acidic pH in the stomach. Upon reaching the small intestine, spores germinate into vegetative cells in the presence of bile salts. Next, toxins A and B exert their toxic effects on epithelial cells, ultimately causing fluid accumulation (63). Non-toxigenic strains can colonize, but will not cause disease. The toxins also cause an inflammatory response by activating production of cytokines and accumulation of neutrophils and monocytes (63, 89).

The main events involved in development of CDI are disruption of the colonic microbial flora, *C. difficile* colonization, toxin production, and progression of colitis. Antibiotic treatment alters the microbial flora in the colon and this provides an advantage for *C. difficile* to multiply and colonize. The typical symptoms of CDI are diarrhea, which can be mild or severe, and colitis. Abdominal pain, fever, and nausea may also occur (89). PMC is a condition that occurs when the infection is more advanced. Pseudomembranes are inflammatory lesions in the mucosa that appear as yellow plaques, and are composed of leukocytes, fibrin, necrotic cells and cell debris (73). Complications of the disease include toxic megacolon, colon perforation, peritonitis, and death (63).

Treatment for patients with mild CDI consists of suspending antibiotic use, if possible. If the disease is more severe, patients are treated with vancomycin or metronidazole (63). However, recurrence of the disease occurs in 5 to 40% of cases after

treatment (89). Recurrence is often caused by *C. difficile* spores that were not affected during treatment, if the patient received additional antibiotic treatment or by repeated acquisition of *C. difficile* from the environment.

1.6.2 Epidemiology

C. difficile is an important gastrointestinal pathogen, responsible for about 25% of cases of antibiotic-associated diarrhea (9, 63). The number of cases of CDI has increased considerably over the years. Data from 2009 indicated that the number of CDI in hospitalized patients were over 300,000 cases in the U.S., whereas in 1993 were only 85,000 infections (70). A study conducted in hospitals in Ohio reported an increase in mortality due to *C. difficile*, with 198 deaths in 2000 to almost 900 in 2006 (23). A recent study indicated that *C. difficile* was associated with 14,000 deaths per year in the United States between 2006 and 2007 (46). Most importantly, *C. difficile* is considered the main pathogen causing deaths due to gastroenteritis in the U.S., followed by norovirus (46).

In the last decade, similar changes in the epidemiology of *C. difficile* have been reported in Canada and Europe as increases in severity and number of cases of infection. For instance, in England and Wales the number of infections increased by more than 20,000 cases from 2000 to 2003 (67). In Canada, Pepin and collaborators noted important changes in the severity of CDI cases in hospitalized patients from 1991 to 2003 (87). A 10-fold increase in the number of infections was noted particularly for patients 65 years

of age or older. In addition, the proportion of CDI cases with complications including toxic megacolon, perforation, and colectomy increased from 7% in1991 to 18% in 2003.

Such epidemiologic changes are thought to be due to the emergence and spread of a hypervirulent strain type. This strain type is identified as group BI by REA, NAP1 by PFGE, ribotype 027 (BI/NAP1/027), and toxinotype III. Fifty percent of strains causing outbreaks from 2001 to 2005 in 8 different hospitals in the U.S. were found to be of the BI/NAP1/027 type (74). This strain type is thought to produce more toxins A and B since it has a deletion of 18 base pairs in the *tcdC* gene, which is a negative regulator for toxin production. In addition, strain type BI/NAP1/027 was resistant to fluoroquinolones and it was not prevalent among historic isolates before 2001 (74).

1.6.3 Community-acquired CDI

Further changes in epidemiology include the onset of CDI in groups previously considered at low risk of acquiring the infection. This would mean cases occurring in a younger population with no previous hospitalization or antibiotic use, and is referred as community-acquired CDI (CA-CDI). Cases of CA-CDI are defined as those that occur in patients who have not been hospitalized three months before the onset of symptoms, or in patients who develop diarrhea within 48 hours of admission to a hospital (38). Different studies have been conducted to analyze cases of CA-CDI, reporting incidences of 2.8 to 236 cases per 100,000 persons per year in North America and Europe (51). However, not

all studies have used the same definitions for CA-CDI, and the tests used to confirm infection by *C. difficile* have varied as well.

In 2005, 33 cases of CA-CDI, including 10 peripartum women, were identified in four states in a report from the Centers for Disease Control and Prevention (CDC) (24). In an attempt to better understand the epidemiology of CA-CDI, Otten and collaborators proposed a model for transmission of *C. difficile* in the community (82). The model included eight epidemiological states to classify patients as susceptible, colonized, diseased, and others including relapses and death. Furthermore, it was suggested that exposure to *C. difficile* in community settings might result from close contact with patients infected with CDI, environmental sources, or possible transmission from animal or food sources.

Aiming to study risk factors associated with acquisition of CA-CDI, Wilcox and colleagues conducted a case-control study in the United Kingdom (130). Fecal samples submitted to clinical laboratories from community patients were included in their study. The prevalence of CA-CDI was 2.1% from a total of 2,000 samples. The main risk factors were the use of antibiotics within 1 month of infection, hospitalization occurring 6 months prior to the onset of disease, and contact with infants of less than 2 years old. These risk factors were found to be significantly different when compared with controls. The use of proton pump inhibitors, however, was not different between patients with CA-CDI and controls.

A recent longitudinal study conducted interviews to patients with CA-CDI from 8 different states in the U.S. (26). Nearly 1,000 patients were interviewed and it was found that a large proportion of them (82%) had had healthcare exposures as outpatients within 3 months before the onset of infection. Healthcare exposures included visits to a physician or dentist office, or surgical procedures with no overnight stay at a hospital. Moreover, 64% of patients had used antibiotics mainly as treatment for ear infections as well as infections of the upper respiratory tract. The use of gastric acid suppressing medications was reported by 27% of all patients, but it was more common among those who did not use antibiotics. Further possible sources of infection were contact in the household environment with a person with CDI infection or with infants 1 year-old or younger.

Strains of *C. difficile* from community-dwelling patients have been classified into different types. In a study conducted by Limbago and collaborators in 2009 (68), 92 *C. difficile* isolates of CA-CDI cases were obtained from 9 different states in the U.S. A variety of 9 toxinotypes and 31 PFGE types were identified in their study. The NAP1 type was the most common PFGE profile, accounting for 21% of the total isolates. Fourteen of those isolates had a profile similar to the epidemic strain BI/NAP1/027. Similar results were reported by Chitnis and coworkers, as 22% of *C. difficile* isolates from patients with CA-CDI belonged to the NAP1 type (26). Overall, strains from that study were classified into more than 12 different PFGE profiles. Interestingly, NAP types previously isolated from animals and foods, *i.e.* NAP7 and NAP8 were not common among community isolates (26).

1.7 Culture methods

After *C. difficile* was identified as the causative agent of PMC and antibiotic-associated diarrhea in the 1970's, investigators were interested in developing adequate culture methods to further study this pathogen. These methods have improved the isolation and identification of *C. difficile* from fecal specimens thus aiding in the diagnosis of CDI. Moreover, culture of *C. difficile* is required for further molecular characterization of the isolates. Culture protocols developed for clinical samples have been adapted and used for the study of environmental and food samples.

In 1978, George and collaborators studied the susceptibility of *C. difficile* strains to 16 antimicrobial agents (42). They found a consistent resistance of the isolates to cycloserine and cefoxitin, and proposed their use as selective agents. In addition, cycloserine was found to inhibit the growth of other anaerobic bacteria present in fecal samples, such as *Bacteroides* spp., *Fusobacterium* spp., and other non-*C. difficile* clostridia. On a subsequent study, this group of researchers developed the culture medium cycloserine-cefoxitin-fructose agar (CCFA) for the selective and differential isolation of *C. difficile* (43). In their study, CCFA allowed for distinctive identification of *C. difficile* by its colony morphology after direct culture of fecal specimens. It was also shown that this medium was more efficient than clostrisel agar or reinforced clostridial agar for recovery of *C. difficile*.

CCFA was then modified by Wilson and coworkers, who substituted egg yolk in the original formulation by the bile salt sodium taurocholate (133). It was found that

addition of 0.1% of sodium taurocholate improved the recovery of spores as well as enhanced the growth of *C. difficile* colonies. A study conducted by Bliss and others compared CCFA versus taurocholate-added CCFA (TCCFA) for the examination of fecal specimens of hospitalized patients with diarrhea (14). TCCFA was reported to be better regarding sensitivity and more frequent *C. difficile* isolations than CCFA.

A further improvement as part of the culture method consisted of subjecting fecal samples to treatment with ethanol before culturing onto TCCFA (27). Ethanol was mixed on equivalent volumes with the samples to eliminate non-spore forming competing bacteria, thus allowing for the selection of *C. difficile* spores. Better recovery rates of *C. difficile* were achieved by the use of ethanol shock than performing direct culture of fecal specimens.

Aspinall and Hutchinson developed an additional selective medium in 1992 named *C. difficile* moxalactam norfloxacin (CDMN) (6). The same basal medium of CCFA was used, but the selective agents were replaced and cysteine hydrochloride and horse blood were added as a reducing agent and growth supplement, respectively. Gram-negative bacteria and strains of fecal streptococci were inhibited by norfloxacin, whereas moxalactam suppressed the growth of 17 strains of *Clostridium* spp. other than *C. difficile,* as well as *Bacteroides* spp. Furthermore, after comparison with CCFA, a greater number of *C. difficile* isolates were recovered from fecal samples on CDMN and with less contaminating bacteria than on CCFA.

Despite culture of fecal samples has a specificity and sensitivity greater than 90% for the detection of *C. difficile*, one of its disadvantages is the time required to obtain results, which ranges from 72 to 96 hours (89). Therefore, complementary and more rapid methods are also used as part of the diagnostic tests of CDI. Examples of additional methods are detection of *C. difficile* toxins in feces by enzyme immunoassays or cell cytotoxicity tests, and PCR (29).

1.8 *C. difficile* in Food Animals

C. difficile has been isolated from a variety of animal species, and particularly from domestic animals such as cats, dogs, and horses. *C. difficile* has been recognized as the cause of colitis in adult horses treated with antibiotics, and it has also been associated with diarrhea in foals usually less than 2 weeks old (11). A potential zoonotic transmission of *C. difficile* has been suggested since similar PCR ribotypes have been found in human and domestic animals, including dogs, cats, calves and horses (4). In order to further investigate animals as a possible source of human CDI, a number of recent studies have targeted food animals as well, and *C. difficile* has been isolated from pigs, cattle, and poultry.

1.8.1 Cattle and Calves

Investigations on the presence of *C. difficile* in cattle and calves are relatively recent. Most studies have been conducted in young animals and its detection has included isolation by culture or by testing the presence of its toxins. Cases in which the bacterium has been isolated from diarrheic animals have suggested that this pathogen is capable of causing disease in calves (48). Asymptomatic animals that test positive for *C. difficile* are thought to act as reservoirs.

Rodriguez-Palacios and coworkers reported the detection of *C. difficile* in 11% of healthy and diarrheic calves in Canada in 2006 (97). In contrast, a prevalence of 25% of *C. difficile* was found in diarrheic fecal samples from calves in the U.S. (48). The latter study also described intestinal lesions caused by toxins A and B in calves. Houser and collaborators detected *C. difficile* toxin genes in 28% of fecal samples of healthy calves (55). However, a lower prevalence was reported in Slovenia, with only 1.8% of calves positive for *C. difficile* (88). A study investigating the main PCR ribotypes in animal isolates identified ribotypes 078, 033, and 002 among 33 isolates obtained from calves (62). The vast majority of the calves' isolates were ribotype 078, but only 1 out of 23 (4%) human isolates that were also tested in the same study for comparison purposes belonged to ribotype 078.

The presence of *C. difficile* in older cattle has been reported to be lower than that in calves. Thitaram analyzed the feces of healthy cattle in 2011 and isolated *C. difficile* from 2.4% dairy cattle and 6.3% of cattle intended for beef production (117). In the same

year, Rodriguez-Palacios reported a prevalence of 1.8% *C. difficile* on the feces of cattle in different beef processing plants in the U.S. (92). Only 4 out of the 29 *C. difficile* isolates were toxigenic, and toxinotypes V and 0 were identified. In Switzerland, a prevalence of 6.6% was reported in cows, calves and goats, and in 21% of fecal samples collected from the farm ground (98). PCR ribotypes in those samples were 033, 066, 070, 003, 001, and 137.

1.8.2 Swine

C. difficile is an important bacterial pathogen in neonatal pigs. Cases of infection with *C. difficile* in newborn pigs have been described as watery diarrhea and colon edema affecting pigs of less than 10 days of age (110). *C. difficile* enteritis in piglets generates economic losses for swine producers as the disease results in animals with lower weights and death (7).

The prevalence of *C. difficile* in swine varies with the age of the animals. The bacterium was isolated from both healthy and diarrheic newborn piglets of less than 7 days of age, but not from 1 to 2 month old pigs in Spain (3). Similarly, Norman and collaborators found a higher recovery of *C. difficile* from farrowing piglets (50%) as compared with pigs at the finishing stage (3.9%) (80). Hopman demonstrated that *C. difficile* can be widely prevalent and rapidly transmitted in pig farrowing facilities. *C. difficile* was isolated from farrowing crates, and the bacterium was able to colonize

piglets after 48 hours of birth and sows within 5 days after giving birth (54). Isolation rates from fecal samples of piglets are between 25 to 52% in European countries and as high as 73% in the U.S. (3, 88, 115).

Most of the *C. difficile* isolates recovered from pigs have been classified as ribotype 078/ toxinotype V. A U.S. study identified four PCR ribotypes among various swine-origin *C. difficile* strains from different states, and more than 80% were 078 (62). Before 2001, Jhung and collaborators found a very low occurrence of toxinotype V affecting humans over a 17 year-period. On the other hand, between 2001 and 2006, the prevalence of this strain increased significantly in human infections (58). Furthermore, after analyzing ribotype 078 isolates from porcine and human origin by multilocus variable-number tandem-repeats, it was found that the isolates were genetically related and they could not be distinguished from each other (32).

1.8.3 Poultry

The prevalence of *C. difficile* in chickens has not been widely studied. Studies from the last 8 years have reported different isolation rates from fecal samples. A low isolation rate of 2 and 5% was found in Austria and the U.S., respectively (49, 56) for market age chickens. In contrast, Simango and Mwakurudza isolated *C. difficile* from the feces of 29% broiler chickens in Zimbabwe markets (108). Zidaric and coworkers found a higher prevalence in Slovenia, where 62% of fecal samples from one poultry farm were positive for *C. difficile* (136). The authors also suggested that colonization in chickens

could depend on the age of the birds. *C. difficile* was recovered from all 15-day-old chickens sampled, but the prevalence diminished to 40% for 18-week-old chickens.

Characterization of *C. difficile* isolates from chickens has shown that most strains are toxigenic. However, fecal samples have been obtained from healthy chickens in different studies, which suggests that the bacterium might not be pathogenic for chickens. The study conducted in Slovenia distinguished 14 PCR ribotype profiles and two toxinotypes (0 and IV) among 44 *C. difficile* isolates. PCR ribotypes 001 and 446 were identified by Indra from 3 isolates recovered in their study. From these, ribotype 001 has been commonly implicated in human infections (56). In contrast, isolates recovered by Harvey belonged to toxinotype V and NAP7 by PFGE (49). Typically, toxinotype V is the most common type for animal isolates (58).

1.9 *C. difficile* in foods

Although the role of *C. difficile* in some animal species is not fully understood, isolation of this pathogen from food animals and the increase in CA-CDI have generated the hypothesis that *C. difficile* could be transmitted via food. The first publication of *C. difficile* in food is from 1996, in which clostridial species were found to cause spoilage in vacuum-packed meats (17). One of the isolates was identified as *C. difficile* by microbiologic and biochemical tests. Since 2006, a number of studies have been conducted to investigate the prevalence of this bacterium in foods to determine the

likelihood of foodborne transmission to humans. Most of the foods tested have included raw meats and poultry, but there are a small number of studies with additional food items.

1.9.1 Meats

Initial studies on the prevalence of *C. difficile* on retail meats were conducted in the U.S. and Canada in the mid 2000's. After investigation of the prevalence of *C. difficile* in calves, Rodriguez-Palacios and co-workers recovered *C. difficile* from 20% of ground beef and veal samples in Canada (96). However, a lower prevalence of the bacterium was reported in a subsequent Canadian study (94). Songer and collaborators analyzed a variety of raw and cooked meats in Arizona, reporting an isolation rate of *C. difficile* in 42% of the samples (111). To date, this result represents the largest proportion of *C. difficile* found in meat products. Such findings created a desire to further study the presence of this pathogenic bacterium in different locations worldwide.

In the United States and Canada, prevalence rates have varied among laboratories. The isolation of *C. difficile* from meats in the U.S. has ranged from 0 to 42% (see Table 1.1). Harvey found 3 to 12% of chicken meats positive for *C. difficile*, and 9.5% of other meat types from production facilities and retail stores (49, 50). In contrast with results from Arizona (111), a study analyzing samples from eight states in the U.S. found no *C. difficile* from more than 1700 various retail meats (69). Canadian studies have reported an incidence of 6 to 12% mainly from beef, pork, and chicken samples at retail. In one of

these studies, Weese and collaborators also determined a low level of contamination by quantifying *C. difficile* (125). Ground beef had 20 to 240 spores per gram of meat, whereas pork had 20 to 60 spores per gram.

Studies conducted in different European countries have reported a lower occurrence of *C. difficile* in meats. In general, prevalence rates have been less than 10%. Initial studies only sampled ground beef from retail stores, from which the highest prevalence was 3% in Austria (59). When testing a wider variety of meats, De Boer isolated *C. difficile* only from lamb and chicken, but not from beef or pork samples (31). In contrast, none of the ground meats obtained from production plants in Switzerland were positive for *C. difficile* (52). Due to the low prevalence found in their studies, preliminary conclusions have been that meats may not be a significant source of *C. difficile*, and that additional sources of transmission should be investigated.

Although prevalence rates in meats have varied, studies in which *C. difficile* has been isolated have rendered toxigenic strains. Notably, strains classified as toxinotype III/ribotype 027, and toxinotype V/ribotype 078 have been reported only in North America (111, 125). Nevertheless, European isolates have shown a greater diversity of PCR ribotypes (31).

Table 1.1 Prevalence of *C. difficile* in meats across different countries.

Country	Positive samples/ Total	% Positive	Meat type(s)	Reference
United States	37/88	42	Beef, pork, turkey, processed meats	(111)
	4/32	12.5	Chicken	(49)
	23/243	9.5	Pork, beef, chicken	(50)
	0/1755	0	Beef, pork, turkey, chicken	(69)
	0/956	0	Beef	(61)
Canada	12/60	20	Beef, veal	(96)
	26/203	12.8	Chicken	(128)
	28/230	12	Beef, pork	(125)
	3/48	6.3	Beef, pork	(121)
	13/214	6.1	Beef, veal	(94)
Austria	3/100	3	Beef	(59)
Sweden	2/82	2.4	Beef, pork, reindeer, poultry, sheep, calf	(72)
France	2/105	1.9	Beef	(16)
Netherlands	8/500	1.6	Beef, pork, lamb, veal, chicken	(31)
Switzerland	0/46	0	Beef, pork	(52)

1.9.2 Vegetables

Isolation of *C. difficile* from foods other than meats is comparatively limited. In contrast with meats, the first report of *C. difficile* in vegetables was made in 1996. Besides isolating *C. difficile* from a variety of environmental sources in South Wales, the bacterium was also found in less than 3% of raw vegetables (2). Most of the positive samples were unwashed root vegetables, and the isolation rate from soil was 21% in the same study.

Over a decade later, *C. difficile* was recovered from 4.5% of root vegetables in Canada (78). Interestingly, three of the five isolates were of the toxigenic type 078/NAP7/toxinotype V. In Scotland, toxigenic ribotypes 017 and 001 were obtained from ready-to-eat (RTE) leafy green salads (8). The authors of that study emphasized that the presence of *C. difficile* in RTE products is of concern and could increase the risk of colonization with *C. difficile*. Although prevalence rates in vegetables are low, the source of contamination in vegetables could consist of soil, water, and/or manure (127).

1.9.3 Seafood

Incidence of *C. difficile* in seafood was relatively low, as indicated in the study by Metcalf and collaborators in 2011 (77). That study recovered *C. difficile* from approximately 5% of fresh and frozen fish and seafood obtained from retail stores in Canada. Five samples were positive for *C. difficile*, and consisted of scallop, salmon, perch, and two shrimp samples. Four of the five isolates were toxigenic and were classified as PCR ribotype 078, toxinotype V. In contrast, a higher prevalence was found

in Italy, as nearly 50% of bivalve mollusks were contaminated with *C. difficile* (86). Toxigenic and non-toxigenic strains were typed and classified into 20 different PCR ribotypes. Isolation of *C. difficile* from mollusks was related to the ability of these marine organisms to filter and accumulate microorganisms and other particles from seawater.

1.10 Bacterial foodborne pathogens present in meats

Foodborne pathogens that are present in the intestinal tract of food animals can be transmitted to humans through contaminated meats. Zoonotic transmission has been described for bacterial pathogens such as *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter* spp. and *Yersinia enterocolitica* (34). *Salmonella* and *Campylobacter* are among the top five pathogens causing foodborne disease in the U.S. (104). A common risk factor for acquiring an infection by these pathogens is consumption of undercooked meats.

Campylobacter spp. is frequently isolated from poultry meats, but it can also be present in beef and pork in smaller proportions (Zhao, 2001). In 2012, an estimated prevalence of 22% of *Campylobacter* spp. in chicken was reported by the Food Safety and Inspection Services (FSIS) from inspected meat production facilities in the U.S. Campylobacteriosis is a gastrointestinal infection manifested as diarrhea that is usually self-limiting. Complications of the disease are Guillain-Barre syndrome and reactive arthritis (79). Cases of *Campylobacter* infection occur after consumption of undercooked or mishandled poultry products, raw milk, or contaminated water. According to CDC

reports, the incidence of *Campylobacter* infections increased 14% from 2008 to 2012 (25).

Salmonella can be isolated from a variety of meat products, but it is more prevalent in poultry meats, particularly ground chicken and ground turkey (99). The prevalence of *Salmonella* in chicken from production facilities is estimated to be 24%, according to the FSIS (120). The serotypes most frequently isolated are Kentucky, Enteritidis, and Typhimurium. Infections with *Salmonella* can range from diarrheic disease or salmonellosis to typhoid fever as a result of ingestion of contaminated foods and not fully cooked meats and eggs (79). *Salmonella* is the main cause of bacterial foodborne illnesses and deaths in the U.S. (104).

Shiga toxin-producing *E. coli* (STEC) strains, in particular serotype O157:H7, are important foodborne pathogens that cause severe hemorrhagic diarrhea and hemolytic uremic syndrome (HUS) (79). STEC can be shed in the intestinal tract and feces of cattle, which are natural reservoirs of this pathogen. Approximately, 40% of foodborne outbreaks of *E. coli* O157:H7 are linked to ground beef as the vehicle (91). The FSIS report of pathogens in beef trims between 2005 and 2007 indicated an estimated positivity rate of 15.7% for generic *E. coli*, but only 0.39% for *E. coli* O157:H7 at the national level. In addition, the prevalence of O157 and non-O157 STEC in federal inspected plants producing ground beef was <1% in 2012 according to FSIS (119). The numbers of annual foodborne illnesses caused by non-O157 STEC are over 112,000 and 63,000 for O157 strains. However, cases of infection with O157 STEC require more hospitalizations and result in more deaths per year than non-O157 STEC strains (104).

The main animal reservoirs of *Yersinia enterocolitica* are pigs. *Y. enterocolitica* can be recovered from the tonsils and fecal samples of pigs that have reached market weight (129). This pathogen has been isolated from pork, chitterlings, and other pork products such as offal and pork tongue (37, 79). It was estimated that 155 cases of yersiniosis occurred in 2012 in the U.S. (25).

In order to reduce the prevalence of foodborne pathogens in meats, the FSIS ensures compliance with the Pathogen Reduction and Hazard Analysis and Critical Control Points (HACCP) system rule since the late 1990's (53, 120). Under this rule, meat and poultry facilities are regularly inspected for adequate hygienic practices and microbial tests are conducted frequently for the presence of relevant foodborne pathogens. It has been reported that the implementation of this rule has reduced the incidence of *Salmonella* in poultry meat (99).

CHAPTER 2

MATERIALS AND METHODS

2.1 Retail meats sampling

A total of 342 meat samples of different types were obtained from 25 retail stores and butcher shops in Minnesota from September, 2011 through August, 2012. Samples were collected on a weekly basis (8 to 10 samples per week on average) from 5 different counties in central Minnesota. Most of the samples (71%) were obtained from counties Hennepin and Ramsey in order to analyze retail meats mainly from the metropolitan area. The remainder samples were collected from counties Stearns, Benton, and Sherburne, which are among the area that the Minnesota Department of Health (MDH) monitors community-acquired CDI. Meat types included raw beef, pork, chicken, turkey, and lamb, either ground or cut in small pieces. Additionally, preserved meat products, such as summer sausage, braunschweiger (a smoked sausage made with pork liver), salami, and pepperoni were also sampled. Meat products were of organic and traditional types. After purchase, meat samples were transported in ice to the laboratory, kept at 4°C, and analyzed within 24 hours of receipt.

2.2 Detection of *Clostridium difficile* in retail meats

The detection protocol used in this study was based on the methods used by Weese in 2010 (128) with some modifications. Each meat sample (approximately 454 g) was processed aseptically by dividing it onto 4 quadrants. Small portions were taken from each of the quadrants to obtain a representative sample of 50 g, which was mixed with 50 ml of *Clostridium difficile* moxalactam norfloxacin (CDMN) broth (supplemented with 0.1% sodium taurocholate and previously stored under oxygen-free gases) and mixed in a stomacher (Lab-Blender 400®, Seward, London) for 2 min. The mixture was transferred inside an oxygen-free atmosphere, and 500 µl were pour-plated onto CDMN agar (Oxoid®, Basingstoke, UK) in duplicate. CDMN plates and the remaining meat-broth mixture were incubated anaerobically for 48 hours at 37°C. Incubations in oxygen-free atmosphere were conducted in an anaerobic vinyl glove box (Coy®, Ann Arbor, MI) using an anaerobic gas mixture of 10% hydrogen, 10% carbon dioxide, and 80% nitrogen. After incubation, 1 ml aliquots of cultures were subjected to ethanol shock for the selection of spores by addition of an equal volume of 95% ethanol. After vortex-mixing, they were incubated at room temperature for 1 hour. Subsequently, the mixtures were centrifuged at 4000 × g for 10 minutes. Supernatants were discarded and the pellets were re-suspended with 1 ml phosphate buffered saline (PBS), followed by pour plating as described earlier onto duplicate CDMN agar plates. CDMN agar plates were incubated as above. After incubation, suspicious colonies were subcultured onto 7% horse blood agar. Further confirmation included colony morphology, Gram staining, characteristic *p*-

cresol or “horse manure” odor, and the production of L-proline aminopeptidase (Pro Disk Remel®, Lenexa, KS).

A subset of eleven meat samples were randomly chosen to be inoculated with *C. difficile* strain 1035 (see Table 2.1) and serve as positive controls for the detection protocol. The meat samples consisted of six ground beef, two of each ground pork and chicken, and one ground turkey sample. Strain 1035 was grown in the oxygen-free gas mixture described above at 37°C overnight in brain heart infusion (BHI) broth supplemented with L-cysteine hydrochloride and yeast extract (BHIS). This overnight culture was used to inoculate 50 g of meat to a level of approximately 10^1 CFU/g. After inoculation, the samples were subjected to direct culture and enrichment as described above.

Additionally, a subset of 20 meat samples were analyzed using the protocol described by Songer in 2009 (111) for comparison purposes. Samples consisted of ground beef, pork, chicken and turkey. Briefly, 5 g of meat were mixed with 50 ml of pre-reduced BHI broth in duplicate. One set of samples were subjected to heat shock in a water bath at 80°C for 10 min. All meat-broth mixtures were incubated anaerobically at 37°C for 7 days. Next, aliquots of the enriched broths were mixed with ethanol (1:1 v/v) as described earlier. After centrifugation, the pellets were streaked onto duplicate cycloserin cefoxitin fructose agar (CCFA) plates and incubated for up to 72 hours anaerobically. CCFA plates were examined for the presence of suspicious colonies.

2.3 Strains of *C. difficile*

C. difficile strains used in this study were provided by the Minnesota Department of Health. Strains and their source of isolation are listed in Table 2.1. For simplicity, strains will be referred by the last 4 digits of their strain designation.

Table 2.1 *C. difficile* strains used in this study isolated from animals and humans.

Animal isolates, Toxinotype V	**Human isolates**[a]	
Strain designation	Strain designation	
M2007001035[bc]	M2012000389	M2012001256[c]
M2011038093	M2012000390	M2012001257
M2011038102	M2012000391	M2012001259[c]
M2011038107[c]	M2012000392	M2012001261
M2011039452	M2012000393	M2012001262
M2012002726	M2012000395	M2012001263
M2012002755	M2012001250	M2012001266
M2012002756	M2012001251	M2012001267
M2012002758	M2012001252[c]	M2012001269
M2012002760[c]	M2012001253[c]	M2012001270
M2012003706[c]	M2012001254[c]	M2012001271
M2012006007		
M2012007933[c]		

[a] Toxinotypes of human isolates were not available.
[b] Strain used as positive control as part of the detection protocol described in section 2.2.
[c] Strains used for determining survival of *C. difficile* in meats as indicated in section 2.5.

2.4 Growth rate determination

All strains listed in table 2.1 were used for determination of growth rates. Single colonies of each strain were inoculated onto 9 ml of pre-reduced BHI broth supplemented with L-cysteine hydrochloride and yeast extract (BHIS) and incubated overnight at 37°C in the oxygen-free gas mixture described above. One hundred microliters of the overnight cultures were then inoculated onto duplicate 9 ml BHIS broth tubes. Optical density was measured every hour during exponential phase at 600 nm on a spectrophotometer (Spectronic® 20 Genesys, Thermo Electron Corp., Madison, WI U.S.). Growth rates during exponential growth were determined by linear regression.

2.5 Survival in meats

Five *C. difficile* human isolates and five animal strains were randomly selected to be used in this part of the study (see Table 2.1). Individual strains were inoculated to samples of ground beef and ground chicken. Each strain was inoculated onto BHIS broth and incubated 24 h at 37°C anaerobically. Samples of 100 g of meat were inoculated with the 24-h cultures to a level of approximately 10^5 CFU per gram of meat. Inoculated meats were mixed for 2 min in a stomacher (Lab-Blender 400®) and stored at 4°C and -15°C. Meats stored under refrigeration were sampled at day 0 (after inoculation), and after 2 and 5 days of storage. Meats stored at -15°C were sampled at day 0 and every 5 days for up to 20 days of storage. For each sample taken, 1 g of meat samples were mixed with 9

ml peptone water, serially diluted and 0.1 ml aliquots were spread plated onto CDMN agar plates in duplicate. Agar plates were incubated for 72 h at 37°C anaerobically. *C. difficile* colonies were counted to determine CFU/g.

2.6 Statistical analysis

The Statistical Consulting Service at the University of Minnesota was contacted to assist with selection of statistical analyses for this study. The confidence interval for the proportion of meats contaminated with *C. difficile* was calculated with the use of GraphPad Software, a web-based calculator, at a 95% confidence level (44). Bacterial growth rates were reported as the average of two independent measurements for each of the strains tested, and the standard deviation of these samples was also calculated. In addition, the average growth rate of human and animal isolates was calculated. A t-test was conducted in order to compare the growth rate of these two groups and to identify potential differences between them. Each data point within survival studies in meats was the result of duplicate plating on CDMN agar. At least two independent experiments were conducted for each strain to calculate the average bacterial counts overtime and their standard deviations. Differences in survival of human and animal isolates were determined with a t-test comparing the average *C. difficile* counts of the two groups at each of the time points of storage conditions. t-tests were performed on Microsoft Excel 2010 at a significance level of $p < 0.05$.

CHAPTER 3

RESULTS

3.1 Prevalence of *C. difficile* in retail meats

A total of 342 retail meats were obtained from 5 counties in Minnesota (Table 3.1). Of these, 29 (8.5%) meat samples rendered presumptive *C. difficile* colonies (Table 3.2). Eleven of these isolates were recovered by direct culture only, but not after enrichment, which meant these isolates were probably non-spore formers. Overall, the colony morphologies observed after subculture onto non-selective agar were small and punctiform, round, and bright colonies of different colors, including white, white-gray, and beige. However, none of these colonies presented the characteristic *p*-cresol odor of *C. difficile* colonies.

Only six of the suspicious colonies produced L-proline aminopeptidase, which was observed as a slight pink coloration of the test disks. The majority of these isolates were Gram positive cocci, except for two which were Gram positive rods. However, these two isolates produced hemolysis on horse blood agar and *C. difficile* is non-hemolytic. The remainder presumptive isolates (n=23) had a negative test for L-proline aminopeptidase, which failed to confirm detection of *C. difficile*. Thirteen of these presumptive colonies were Gram positive cocci arranged in groups, and these colonies

were consistently isolated only after enrichment. Additionally, seven presumptive colonies were Gram positive rods and three more were Gram-positive round, elongated cells.

Strain 1035 was inoculated at a level of 10^1 CFU/g on a subset of 11 meat samples to be used as positive controls. Colonies with characteristic *C. difficile* morphology were recovered from all inoculated samples. The latter were small to medium size, round and irregular border, gray, nonhemolytic colonies with a distinctive *p*-cresol odor. Furthermore, L-proline aminopeptidase was detected in the isolates and cells stained as Gram positive rods. *C. difficile* strain 1035 was recovered on six occasions by direct culture as well as after enrichment. In contrast, 3 samples were culture-positive by direct culture only, and two more only post-enrichment.

Table 3.1 Meat samples tested for the presence of *C. difficile* and their geographical distribution.

Meat Type	County					Total
	Ramsey	Hennepin	Stearns	Benton	Sherburne	
Beef	30	21	29	4	3	87
Pork	43	15	17	9	4	88
Chicken	40	30	5	1	1	77
Turkey	26	2	3	1	0	32
Preserved	25	8	10	9	2	54
Lamb	3	0	1	0	0	4
Total	167	76	65	24	10	342

Table 3.2 Presumptive bacterial colonies isolated from meats subjected to confirmatory tests.

Meat type	Total [a]	Presumptive *C. difficile* [b]	Confirmed as *C. difficile* [c]
Beef	87	6	0
Pork	88	7	0
Chicken	77	6	0
Turkey	32	9	0
Preserved	54	1	0
Lamb	4	0	0
Total	**342**	**29**	**0**
95% C.I.[d]	**-**	**-**	**0-0.0134**

[a] Total number of meat samples included as part of the analysis (see Table 2.1)
[b] Bacterial colonies considered presumptive isolates by colony morphology on CDMN agar and subjected to confirmatory tests.
[c] Confirmatory tests: colony morphology after subculture onto horse blood agar, Gram staining, and Pro disk test.
[d] Confidence interval.

C. difficile was not isolated from meat samples analyzed with an additional detection protocol with and without heat shock before enrichment. After 7 days of incubation, enrichment broths appeared clear except for one sample of ground turkey and one sample of chicken gizzards that were enriched without heat shock. A subset of CCFA plates were streaked with aliquots of these two broths, but without performing ethanol shock. The colonies from these plates were small, round, raised, bright, and smooth, with a light pink color and lacked the characteristic *p*-cresol odor and stained as Gram positive cocci. In contrast, *C. difficile* strain 1035 streaked onto CCFA was small, irregular shape,

opaque, yellow and with a ground glass-like appearance. In addition, no bacterial colonies were recovered from any CCFA plates streaked after ethanol shock.

3.2 Comparison of growth rate between human and animal isolates

Optical density measurements were conducted in two replicate experiments for each strain. Growth rate was defined as the slope of the line obtained by plotting optical density versus time on a semi logarithmic scale during exponential growth. Tables 3.3 and 3.4 show the individual growth rates obtained for human (n=22) and animal (n=13) isolates, respectively. Growth rates of both groups of strains are distributed by ranges in table 3.5. The majority of strains, fourteen and seven human and animal isolates respectively, had a growth rate between 0.36 to 0.50 h^{-1}. The maximum growth rate obtained was 1.06 h^{-1} for the animal-origin strain 2726, and the minimum rate was 0.23 h^{-1} for strain 0390 which was a human isolate. On average, human isolates had a growth rate of 0.52±0.25 h^{-1}, whereas that of animal isolates was 0.63±0.15 h^{-1}. However, the difference between growth rates of human and animal isolates was not statistically significant (p=0.16) based on a t-test for difference in means.

Generation time was also calculated by dividing the natural logarithm of 2 by the growth rate of each of the strains. Accordingly, strain 0390 had the longest generation time of 2.96 h, and strain 2726 had the fastest generation time of 0.65 h among the strains

tested. The average generation time was 1.33 h and 1.09 h for human- and animal-origin strains, respectively (Figure 3.1).

Table 3.3 Individual growth rates of n=22 *C. difficile* human isolates grown on BHI broth at 37°C.

***C. difficile* strain**	**μ (h^{-1})[a]**	**SD[b]**	**R^{2}[c]**
0389	0.52	0.12	0.99
0390	0.23	0.03	0.98
0391	0.93	0.21	0.96
0392	0.47	0.06	0.98
0393	0.47	0.05	0.98
0395	0.44	0.02	0.99
1250	0.50	0.14	0.98
1251	0.34	0.10	0.93
1252	0.50	0.05	0.99
1253	0.48	0.10	0.98
1254	0.46	0.02	0.96
1256	0.46	0.01	0.96
1257	0.81	0.13	0.98
1259	0.69	0.00	1.00
1261	0.53	0.00	0.98
1262	0.43	0.15	0.99
1263	0.42	0.03	0.98
1266	0.86	0.07	0.97
1267	0.43	0.12	0.97
1269	0.50	0.11	0.98
1270	0.48	0.17	0.99
1271	0.47	0.11	0.98
Average	**0.52**	**0.16**	**-**

[a] μ, average growth rate (h^{-1}) of two independent experiments; slope of ln(OD_{600}) vs. time curve
[b] SD, standard deviation of the calculated growth rate
[c] R^2, R-squared values to estimate goodness of fit of data points to calculate μ by linear regression

Table 3.4 Individual growth rates of n=13 *C. difficile* animal isolates grown on BHI broth at 37°C.

***C. difficile* strain**	**μ (h^{-1})[a]**	**SD[b]**	**R^2[c]**
2760	0.46	0.13	0.99
3706	0.36	0.08	0.92
6007	0.50	0.17	0.99
7933	0.46	0.09	0.97
38102	0.43	0.04	0.98
38107	0.42	0.02	0.98
39452	0.62	0.14	0.99
1035	0.41	0.16	0.99
2726	1.06	0.24	0.96
2755	0.84	0.09	0.98
2756	0.79	0.08	0.98
2758	0.99	0.38	0.97
38093	0.91	0.25	0.97
Average	**0.63**	**0.25**	**-**

[a] μ, average growth rate (h^{-1}) of two independent experiments; slope of $\ln(OD_{600})$ vs. time curve

[b] SD, standard deviation of the calculated growth rate

[c] R^2, R-squared values to estimate goodness of fit of data points to calculate μ by linear regression

Table 3.5 Range of growth rates (μ) of *C. difficile* strains isolated from humans and from animals.

μ range (h^{-1})	**Number of *C. difficile* strains within range**	
	Human	Animal
0.20-0.35	2	0
0.36-0.50	14	7
0.51-0.65	2	1
0.66-0.80	1	1
0.81-0.95	3	2
0.96-1.10	0	2
Total	22	13
Mean μ (h^{-1})	**0.52 ± 0.25**	**0.63 ± 0.15**

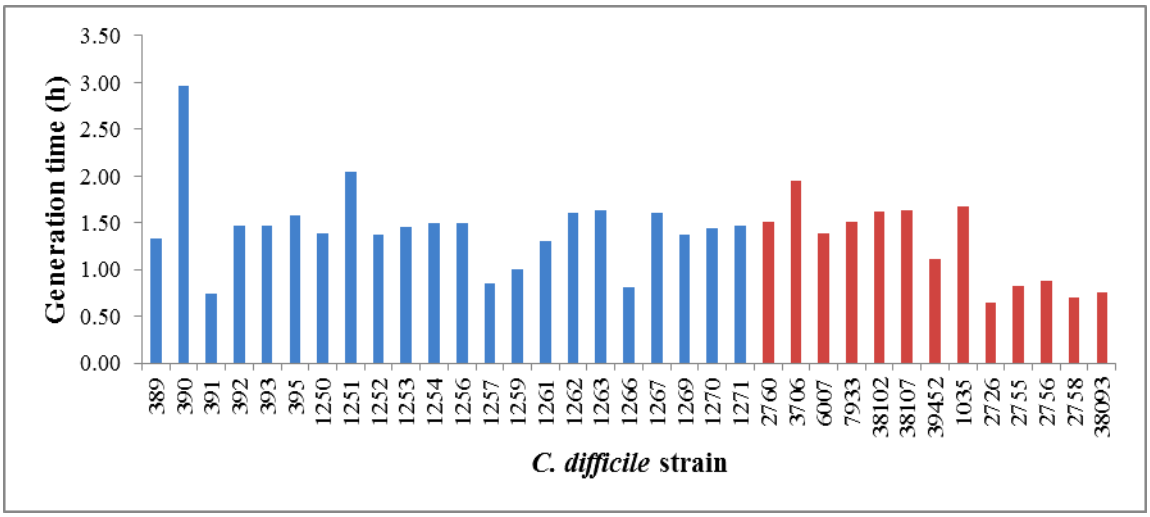

Figure 3.1 Generation time calculated from growth rate for human (blue bars) and animal (red bars) *C. difficile* isolates.

3.3 Survival of *C. difficile* strains in refrigerated and frozen meats

Counts of *C. difficile* strains inoculated on ground beef and ground chicken decreased overtime in most cases when stored at 4° and -15°C, regardless of strain origin. Inoculum levels for ground beef ranged from 4.7 to 5.6 log CFU/g, and from 3.5 to 5.6 log CFU/g for ground chicken. Variability was observed in the counts of *C. difficile* at different sampling time points between and within human and animal strains (Figures 3.2-3.5).

Reductions in the counts of *C. difficile* on ground beef stored at 4°C, ranged from 1.2 to 3.1 log CFU/g for human isolates, and from 1.5 to 2.8 log CFU/g for animal isolates (Figure 3.2). A slight increase in colony counts was observed on on day 5 for

strain 1252. The latter was attributed to variations on the counts obtained on individual trials rather than to bacterial growth. Human isolates had greater discrepancies in counts during these time courses than their animal counterparts. The survival plots of animal isolates showed a very similar pattern under this storage condition, although bacterial counts of strain 1035 on day 2 were apparently higher than the other four animal isolates. However, the standard deviation of *C. difficile* counts of strain 1035 was slightly larger.

Survival curves on refrigerated ground chicken were somewhat the opposite from ground beef since human-origin strains had more homogenous curves than did animal isolates (Figure 3.3). The count of most human isolates had reductions from 1.3 to 2.8 log CFU/g, except for strain 1252 with only 0.4 log CFU/g. However, the initial count at day 0 for strain 1252 was only 3.9 log CFU/g, whereas the rest of human strains were approximately 5 log CFU/g. Initial counts of animal strains were 3.5 to 5 log CFU/g on ground chicken at day 0. Strain 38107 had a smaller reduction overtime (0.4 log CFU/g) than the rest of animal strains. Strain 3706 had an initial increase in counts after 2 days of storage, but this was due to experimental variations.

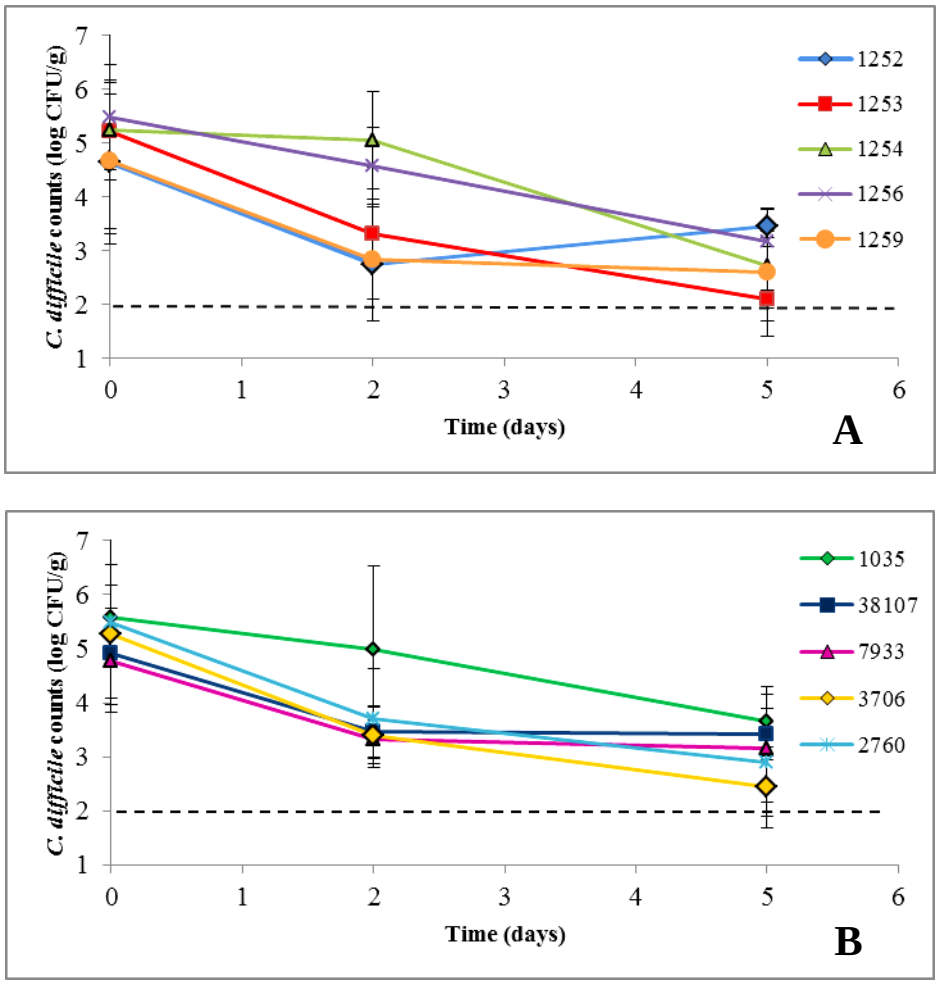

Figure 3.2 Survival of *C. difficile* isolates on ground beef stored at 4°C for 5 days. A: human strains; B: animal strains; dashed line: limit of detection.

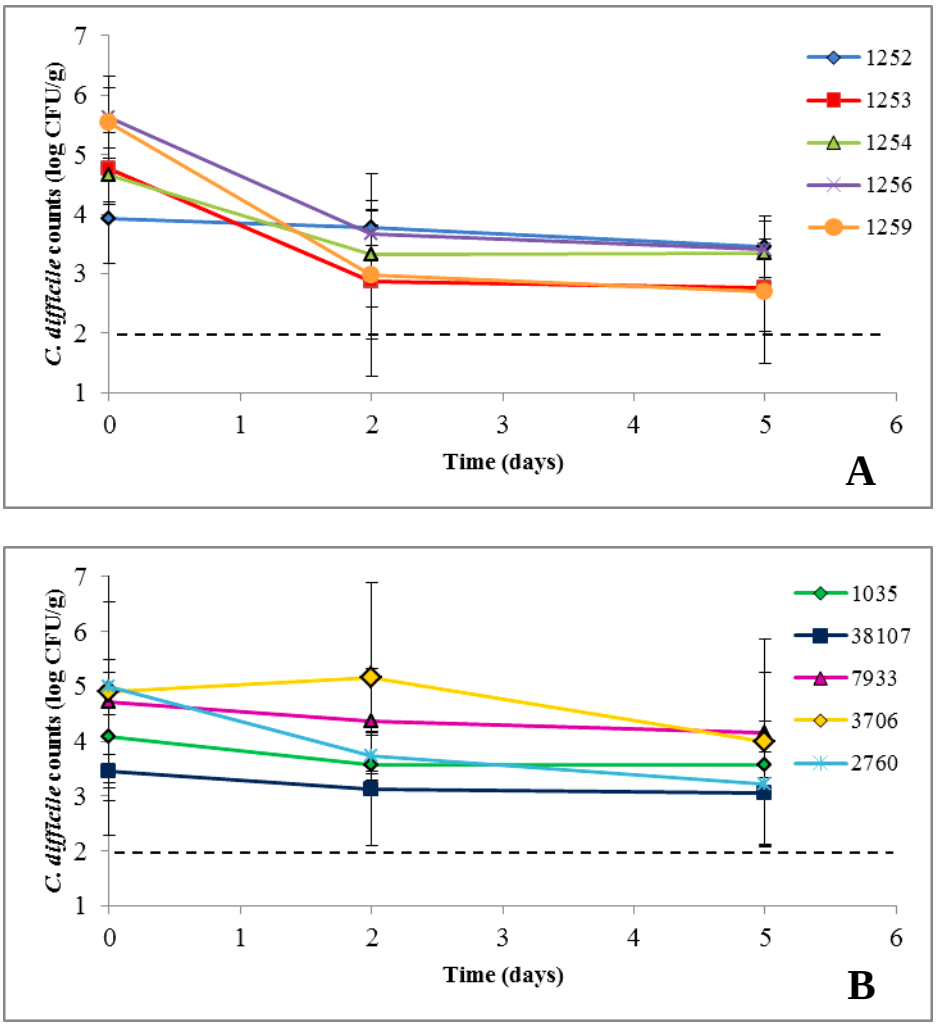

Figure 3.3 Survival of *C. difficile* isolates on ground chicken stored at 4°C for 5 days. A: human isolates; B: animal isolates; dashed line: limit of detection.

The survival of *C. difficile* strains in ground beef stored under freezing conditions was similar for animal and human isolates, since the overall count reductions were approximately 2 log CFU/g in both cases (Figure 3.4). Bacterial counts declined faster within the first 5 days of storage and were then less pronounced on the following sampling days. However, survival of strain 1035 was apparently different on sampling

days 5 and 10, but the final counts on day 20 were similar among the five animal strains. Strains 7933 and 2760 had an apparent increase in bacterial counts between days 5 and 10, but this was due to the different counts obtained in individual replicate experiments which accounted for larger standard deviations.

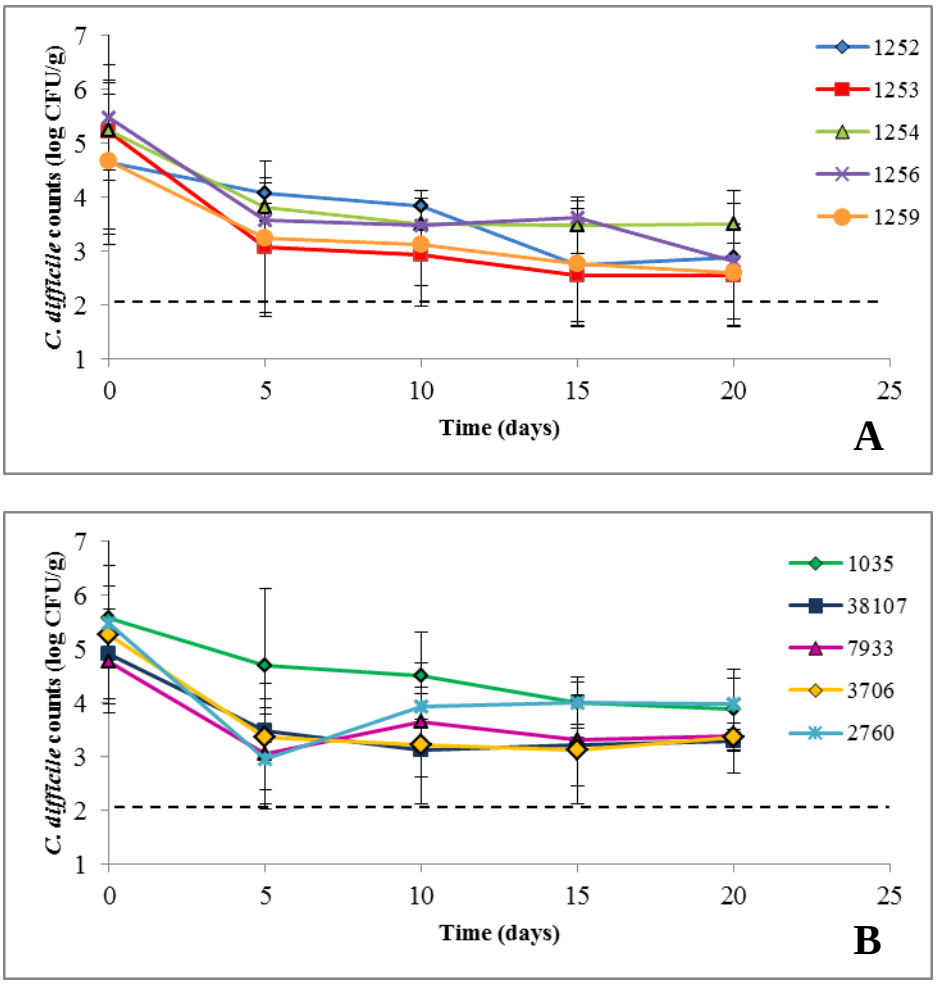

Figure 3.4 Survival of *C. difficile* strains on ground beef stored at -15°C for 20 days. A: human isolates, B: animal isolates; dashed line: limit of detection.

Survival in ground chicken at -15°C had also the same trend as ground beef, with *C. difficile* counts diminishing after 5 days of storage followed by more gradual changes in the next 15 days of storage. As shown in Figure 3.5, the group of human isolates had more variable bacterial counts overtime as compared with animal isolates. Reduction in the number of surviving *C. difficile* was from 0.4 to 2.8 log CFU/g. However, strain 1035 counts were relatively unchanged during 20 days of storage (Figure 3.5 B).

In general, *C. difficile* counts diminished over time on both ground beef and ground chicken stored at 4° and -15°C. Despite variations observed in *C. difficile* counts in inoculated meats, no significant differences were found between human and animal isolates. The mean *C. difficile* counts of human and animal isolates for each time point were compared using a t-test, but the difference in means was not statistically significant ($p<0.05$) (Tables 3.6, 3.7). However, significant differences were observed between two individual time points for frozen meats. The first was obtained on ground beef on day 20, and the second on ground chicken on day 15 with a *p*-value of 0.01 for both cases (Table 3.7). However, individual time point differences would not account for overall statistical significance on the survival of human and animal strains.

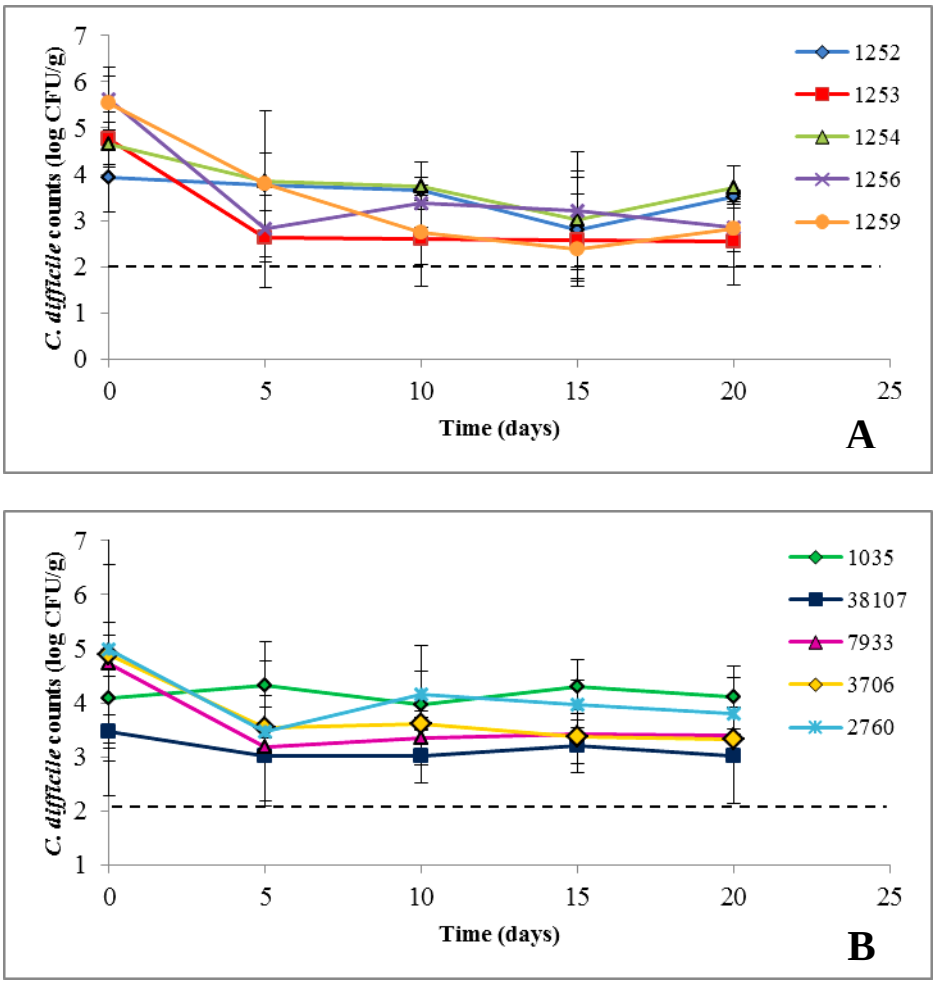

Figure 3.5 Survival of *C. difficile* strains on ground chicken stored at -15°C for 20 days.

A: human isolates, B: animal isolates; dashed line: limit of detection.

Table 3.6 Comparison of average *C. difficile* counts of human and animal isolates inoculated on meats stored at 4°C for 5 days.

Sampling day	Mean log CFU/g Human isolates	Mean log CFU/g Animal isolates	Difference in means	*p*-value
		Ground beef		
0	5.05	5.20	-0.15	0.52
2	3.71	3.78	-0.07	0.91
5	2.81	3.11	-0.30	0.37
		Ground chicken		
0	4.90	4.43	0.47	0.30
2	3.32	3.99	-0.67	0.13
5	3.13	3.60	-0.47	0.11

Table 3.7 Comparison of mean *C. difficile* counts of human and animal isolates inoculated on meats stored at -15°C for 20 days.

Sampling day	Mean log CFU/g Human isolates	Mean log CFU/g Animal isolates	Difference in means	*p*-value
Ground beef				
0	5.05	5.20	-0.15	0.52
5	3.56	3.52	0.04	0.91
10	3.37	3.69	-0.31	0.32
15	3.03	3.53	-0.50	0.12
20	2.87	3.58	-0.71	0.01*
Ground chicken				
0	4.90	4.43	0.47	0.30
5	3.37	3.51	-0.14	0.70
10	3.22	3.61	-0.39	0.25
15	2.80	3.65	-0.86	0.01*
20	3.08	3.52	-0.44	0.17

CHAPTER 4

DISCUSSION

4.1 Prevalence of *C. difficile* in retail meats

The increases in morbidity and severity of *C. difficile* infections, coupled with the occurrence of CDI in new population groups make this pathogen a great public health concern. It is important to understand the underlying causes of such epidemiological changes, mostly to help target measures for prevention. Investigation of additional transmission routes of this disease, such as foodborne contamination, is therefore relevant.

The main objective of this study was to investigate the prevalence of *C. difficile* in commercially-available meat products. Surprisingly, *C. difficile* was not isolated from any of the meats tested despite a large sample size was analyzed (n=342). Twenty nine of these samples rendered presumptive *C. difficile* colonies, but after confirmatory tests none of them had the characteristics of *C. difficile* regarding colony and cell morphology, and/or the production of L-proline aminopeptidase. Among these tests, the production of L-proline aminopeptidase is useful for the biochemical identification of *C. difficile* isolates. The activity of this enzyme is assessed through its reaction with L-proline-beta-naphthylamide present in the test paper disks, and further detection of the reaction product with *p*-dimethylaminocinnamaldehyde (35). The latter is observed as a dark pink

coloration of the test disks. In contrast, the L-proline aminopeptidase test of six of the suspicious isolates resulted in a slight pink coloration, whereas the rest of the isolates had a negative result. Most of the suspicious isolates were obtained from turkey meats, and more than half (59%) were Gram positive cocci.

These findings are contrary to initial research work conducted in Canada and the U.S., in which 20 and 42% of meats were found to be contaminated with *C. difficile*, respectively (96, 111). In contrast, relatively lower proportions of *C. difficile*-positive samples have been reported in Europe, the highest being 3% in ground beef (59). However, the organism was not isolated from any of the meats tested in Switzerland (52). Studies published in recent years support the findings of the present study. In 2012, Limbago and collaborators reported no isolation of *C. difficile* after culturing more than 1,700 meat samples collected from 9 different states in the U.S. (69). Similarly, 956 ground beef samples collected from seven geographical regions across the U.S. resulted negative for *C. difficile* in a report by Kalchayanand and coworkers in 2013 (61). Both studies have included meat samples obtained from the state of Minnesota. It should be noted the extensive sampling schemes used in the studies mentioned above regarding number of samples and their geographic distribution.

The reasons for different prevalence rates of *C. difficile* in meats reported to date among studies are unclear. However, comparisons are challenging due to differences in the methodologies that have been used, including sample sizes and culture procedures. Some studies have analyzed a variety of meats or only one type, which is the case for some European studies (16, 59) and one from the U.S. (61) that only included beef as

their samples. Sample sizes have been from 32 to 1,755, which can also account for the different percentages reported. Furthermore, there is no standard culture method to test for the presence of *C. difficile* on food. Most studies rely on an initial enrichment step with incubation times of 48 hours to 7, 10, or as many as 15 days. Enrichment broths and solid media have included diverse formulations, as well as different selective agents (see Table 4.1).

In an attempt to propose a standard methodology to test *C. difficile* on foods, Limbago and coworkers compared three culture protocols that had been used previously (69). In their study, samples of ground beef were inoculated with 100 *C. difficile* spores per gram, and were subjected to microbiologic analysis using three enrichment broths and subsequent culture after 1, 3 and 5 days of anaerobic incubation. In addition, enrichment broths were inoculated onto three different solid media. Among such methods, a protocol similar to the one described in this study proved to be useful for recovery of *C. difficile* after 3 and 5 days of enrichment. However, the standard protocol suggested by Limbago consisted of initial enrichment for 3 to 5 days on BHI broth, followed by ethanol shock and culture onto CCFA plates. Further efficacy of the method used in this study was supported by artificial inoculation of a subset of meats with approximately 10 CFU/g of meat with *C. difficile* strain 1035. Notably, *C. difficile* colonies were successfully recovered with and without enrichment, and the isolates were confirmed as *C. difficile*.

Considering a possible difference due to culture methodology, an additional method was used for comparison purposes. The latter consisted of a longer incubation time of the enrichment broth, *i.e.* 7 days versus 48 h, and the use of CCFA as selective

agar instead of CDMN agar. This culture protocol has been reported to be efficient for the recovery of *C. difficile* from meats (111). Although a small number of samples were tested under this protocol (n=20), the bacterium was not isolated from any of the meats.

Table 4.1 Comparison of culture media and incubation times used for the isolation of *C. difficile* from food samples.

Sample amount (g)	Enrichment media	Enrichment incubation time	Solid media	Solid media incubation time	Reference
4-5	CDMN[a] + 0.1% ST[b]	10-15 days	CDMN + 5% HB[c]	NS[n]	(96)
5	CDMN	10 days	Schädler and CCFA[d]	48-72 h	(59)
15	CDMN + 0.1% ST	7 days	Blood agar	48 h	(77)
25	CDMN and BHI[e]	10-12 days	FAA[g] + 5% HB and CDMN + 5% HB	48 h	(72)
1	BHIS[f] + 0.1% ST, heat shock	72 h	CCFA + ST	24-72 h	(111)
10	BHI, heat shock	7 days	CDSA[h]	24-72 h	(52)
5	BHI + 0.1% ST, cycloserine, cefoxitin	72 h	Columbia cysteine agar + ST, cycloserin, cefoxitin, 5% HB	48 h	(16)
10	BHI + ST	10 days	CDMN + 5% HB	48 h	(86)
5	CCFB[i] + 0.1% ST	15 days	CCFA	5 days	(50)
25	PBS[j] meat rinsate onto CDMN + 0.1% ST	48 h	CDMN	48 h	(125)
20	No enrichment, direct ethanol shock	NA[m]	CDMN	72 h	(121)
10	CCMB-TAL[k]	5 days	SBA[l]	NS	(30)

[a] CDMN, *Clostridium difficile* moxalactam norfloxacin media.

[b] ST, sodium taurocholate.
[c] HB, horse blood.
[d] CCFA, cycloserine cefoxitin fructose agar.
[e] BHI, brain heart infusion.
[f] BHIS, BHI broth supplemented with yeast extract and L-cysteine hydrochloride.
[g] FAA, fastidious anaerobe agar.
[h] CDSA, *Clostridium difficile* selective agar.
[i] CCFB, cycloserine cefoxitin fructose broth.
[j] PBS, phosphate buffered saline.
[k] CCMB-TAL, cycloserine cefoxitin mannitol broth with taurocholate and lysozyme.
[l] SBA, trypticase soy agar with 5% sheep blood.
[m] NA, not applicable.
[n] NS, not specified.

4.2 Comparison of growth rate between human and animal isolates

Determination of growth rate and generation time of *C. difficile* strains isolated from different hosts was conducted to test if these isolates may exhibit differences in these basic growth parameters at the physiological level. Growth rate is an important parameter of bacterial growth as it describes the number of microorganisms that are generated per unit of time under specific conditions (19). This rate is assumed to be constant during exponential phase. Generation time is related to growth rate as it defines the time required for a bacterial population to double in size.

In this study, some variations were observed in the growth rates of individual strains within the group of either human or animal isolates. Certain strains would grow apparently slower than others, such as strains 0390 (μ=0.23 h^{-1}) versus 0391 (μ=0.93 h^{-1}) within human isolates, and strains 3706 (μ=0.36 h^{-1}) versus 2726 (μ=1.06 h^{-1}) in the animal-origin group. Additionally, a larger proportion of animal strains had a growth rate greater than 0.6 h^{-1} than the human isolates (46% as opposed to 18%, respectively),

which would reflect shorter generation times for animal-origin isolates. Despite the apparent variability of individual strains, human- and animal-origin isolates did not differ significantly in their ability to grow after comparing the average growth rates of human and animal strains. Therefore, no strong evidence was found to consider that these two groups behave differently when grown at 37°C in oxygen-free conditions.

Generation times were 1.3 and 1.1 h on average for human and animal isolates, respectively, which indicated that doubling times of *C. difficile* cells at 37°C were relatively similar regardless of the strain origin. Other clostridial species are different in regards to growth, such as *C. perfringens* which has a generation time of less than 20 min. when grown between 33° to 49°C (18). Short generation times under a relatively wide range of temperatures make *C. perfringens* an important foodborne pathogen, since this bacterium can multiply rapidly in food matrices. Compared with *C. perfringens*, *C. difficile* growth was notably slower according to the results obtained in this study.

Other research studies have reported similarities in the growth of different *C. difficile* strains. Burns and collaborators aimed to assess potential differences in sporulation of a variety of *C. difficile* strains, but bacterial growth was also measured in their study. An initial report tested seven strains of the type BI/NAP1/027 and 8 non-BI/NAP1/027 strains. All strains were found to grow similarly after optical density was measured at 600 nm (21). A subsequent study compared the sporulation rate of a total of 53 *C. difficile* strains, 28 of which were of the type BI/NAP1/027 and 25 were non-BI/NAP1/027 strains (22). Bacterial growth was measured since differences in growth may influence the sporulation features of different strains. Although specific growth rates

were not determined, the authors found no difference in the measurements of optical density during the first 12 h of anaerobic incubation on BHIS broth. The latter may ultimately result in no major differences in growth rates between the isolates, which would be comparable with the findings of this study. The *C. difficile* isolates tested in Burns' study comprised eight different PCR ribotypes, including type 078 which has been isolated from animals. However, the origin of the strains, *i.e.* human or animal, was not specified.

Additional comparative studies have included strains of the type BI/NAP1/027 because of the reported increased virulence of this strain. Nevertheless, a smaller number of isolates have been tested. Merrigan and collaborators reported no major differences in the growth curves of eight different *C. difficile* isolates obtained from human CDI cases (76). Optical density was measured every hour for up to 18 h of anaerobic incubation. Vohra and coworkers analyzed 3 PCR ribotypes from human CDI's and two additional reference strains (122). These strains also exhibited similar growth curves of optical density overtime.

Comparisons between human and animal strains have been performed mainly based on genotypic characterization rather than phenotypic. High genotypic similarity has been reported for *C. difficile* strains isolated from animals and humans. In a study conducted in the Netherlands, porcine *C. difficile* isolates and strains isolated from hospitalized patients were found indistinguishable in regards to toxin production, antimicrobial resistance, and genotypic profiles (32). All isolates were classified as PCR ribotype 078/toxinotype V and high genetic similarity was found by MLVA, with five

porcine and human isolates sharing 100% homology. Furthermore, Keel and colleagues characterized *C. difficile* isolates from humans and 4 animal species by PCR ribotyping (62). Most human isolates shared the same ribotype profiles with those found in equine, canine, porcine and bovine isolates. However, the authors suggested that isolates with the same PCR ribotype could not be considered identical based solely on this approach.

4.3 Survival of *C. difficile* strains in refrigerated and frozen meats

Artificial inoculation of meats with *C. difficile* was performed to simulate a possible contamination of meats with this bacterium. Inoculated meats were stored at refrigeration and freezing temperatures to test if potential differences were found in the survival of human and animal strains under these conditions. Refrigerated meats were stored for up to 5 days at 4°C, whereas frozen meats were stored for 20 days at -15°C. Storage times were selected based on quality and safety recommendations by the USDA for cold storage of fresh ground meat products, which is 1 to 2 days in the refrigerator (40°F or 4.4°C) and up to 4 months in the freezer (0°F, or -17.7°C) (36).

Despite that ground beef and ground chicken samples were inoculated with the same *C. difficile* culture on individual experiments, initial counts on ground chicken were slightly lower than those of ground beef. *C. difficile* counts on ground chicken on day 0 reached 4.9 and 4.43 log CFU/g on average for human and animal strains, respectively. The lowest count was determined on the animal isolate 38107 with 3.46 log CFU/g. Initial counts on ground beef of human strains were 5.05 log CFU/g, and animal strains were 5.50 log CFU/g on average. The reason for this apparent variation between meat

types was not assessed. The inoculation level for meats used in this study was considerably greater than the actual counts of *C. difficile* reported in a Canadian study. Weese and collaborators sought to determine the levels of contamination of *C. difficile* in retail meats by direct culture without enrichment (125). Ground beef had *C. difficile* counts of 20 to 240 spores/g, whereas ground pork had 20 to 60 spores/g. Although the levels of contamination in meats were relatively low, the significance of such findings regarding foodborne transmission could not be determined since the infectious dose of *C. difficile* in human disease is not known.

Over the course of storage of refrigerated and frozen meats, *C. difficile* counts had a tendency to diminish, and this was similar for both human and animal strains. Variability in *C. difficile* counts was observed within individual trials, which was evidenced by the standard deviations shown in Figures 3.3 to 3.6, even though experimental conditions were kept consistent between replicate trials. On frozen meats, a reduction in bacterial counts was more evident initially after 5 days of storage, but it was then relatively maintained with no major changes. This initial reduction in counts might not be explained by a short exposure to air during sample preparation, since Jump and collaborators demonstrated that vegetative cells of *C. difficile* were able to survive for up to 6 hours when exposed to air on the moist surface of nutrient-free agar (60). Alternatively, the decline in numbers may just be an initial loss of viability by exposure to low temperatures, in particular to freezing.

Our findings indicated that *C. difficile* could be recovered from ground meats during cold storage, but the bacterial population declined over time. After comparison of

the bacterial counts at different time points during storage, human and animal isolates did not differ significantly in their survival when stored at 4°C or -15°C. Nevertheless, only 5 strains of each group were included in this part of the study.

Effective comparisons of the results reported here with other studies are limited since survival of *C. difficile* on food matrices during cold storage has not been studied in detail. However, researchers have assessed the survival of *C. difficile* strains during cold storage of fecal samples for its application on laboratory diagnostic tests. Freeman and Wilcox reported no major variation in the counts of *C. difficile* spores and vegetative cells on fecal samples stored at -20° and 4°C for a period of 56 days (39). Weese and collaborators inoculated human and animal strains of *C. difficile* on equine fecal samples that were then stored at 4°C for up to 12 days (126). *C. difficile* was recovered from only 29% of the inoculated samples after 72 h of storage, and recovery rates diminished even further after 5 days. Similarly, Rodriguez-Palacios and LeJeune tested the effect of refrigeration on the recovery of *C. difficile* spores suspended in phosphate buffered saline (PBS) (93). The authors also found that after refrigeration, recovery of *C. difficile* spores diminished significantly after 14 and 18 days of storage.

Additional studies by Rodriguez-Palacios and collaborators have investigated the survival of *C. difficile* during heat treatments and its implications for food safety. An initial study consisted of heating *C. difficile* spore suspensions on PBS at 71° and 85°C since these are temperatures recommended to cook meats (95). Twenty different strains that had been previously isolated from meats and food animals were used in their study. No significant difference was found when the survival of animal strains was compared

with that of meat-origin strains. All strains survived after heat treatment at 71°C for 120 min, and 90% of strains were inactivated after re-heating at 85°C for 10 min. In a second study, *C. difficile* spores were inoculated onto ground beef and gravy, which were heated at 61°, 71°, 85°, and 98°C for up to 30 min (93). Heat treatment at 61° and 71°C had minimum effect in reducing counts of *C. difficile* spores. *D*-values (*i.e*. time required to kill 90% of the bacterial population) calculated for ground beef were 46 and 47 min at 61° and 71°C, respectively, whereas the *D*-value at 85°C was 3.3 min. The authors suggested that meats should be heated at temperatures greater than 85°C to prevent ingestion of *C. difficile* in the event that meats are contaminated with spores.

CHAPTER 5

CONCLUSIONS

This study sought to investigate the prevalence of *C. difficile* on different types of retail meats in 5 counties in the state of Minnesota. An extensive sampling scheme was used, but none of the meats analyzed was found to be contaminated with this bacterium. Therefore, these results did not provide strong evidence to consider *C. difficile* as a common contaminant in meats at the retail level. Recent studies have not isolated this bacterium either from significantly larger numbers of samples from different geographical locations (61, 69), which validates the results of the present study. However, variable prevalence rates of *C. difficile* in meat products have been reported in the literature. Such discrepancies, along with the fact that no outbreaks of *C. difficile* infection have been linked to consumption of food, have prevented to reach a conclusion whether this bacterium could be considered an emerging foodborne pathogen.

The culture method used as part of this work efficiently recovered this microorganism from artificially inoculated samples. The use of an additional isolation method on a subset of meat samples further supported no isolation of *C. diffcile* from any of the meats tested. A standardized procedure to analyze food samples for the presence of *C. difficile* is needed, since some of the differences in results reported to date may be due to the use of different protocols. A uniform methodology would facilitate comparisons among laboratories.

This study also provided additional information on the behavior of different *C. difficile* strains at the physiological level. Specific parameters of generation time and growth rate were determined for various *C. difficile* isolates of human and animal origin grown at 37°C. It was found that the isolates had similar growth parameters overall, although strain-to-strain variations were noted. Based on this evidence, human and animal isolates did not differ significantly in their ability to grow.

Furthermore, subgroups of human and animal strains were inoculated onto samples of ground meats and subjected to challenge experiments under cold storage. It was found that counts of *C. difficile* in ground meats diminished overtime during refrigeration and freezing, regardless of strain origin, *i.e*. human or animal. This reduction in counts stresses the importance of processing food samples for detection of *C. difficile* preferably upon receipt, since prolonged storage at refrigeration or freezing could affect recovery of *C. difficile* overtime.

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APPENDIX

Table A.1 *C. difficile* counts (log CFU/g) of human isolates on ground meats during storage at 4°C.

Storage time (days)	***C. difficile* strain**				
	1252	**1253**	**1254**	**1256**	**1259**
Ground beef					
0	4.65	5.22	5.24	5.48	4.67
2	2.75	3.33	5.06	4.58	2.84
5	3.45	2.10	2.73	3.17	2.60
Ground chicken					
0	3.92	4.76	4.66	5.63	5.54
2	3.77	2.87	3.33	3.66	2.98
5	3.44	2.75	3.35	3.41	2.69

Table A.2 *C. difficile* counts (log CFU/g) of human isolates on ground meats during storage at -15°C.

Storage time (day)	***C. difficile* strain**				
	1252	**1253**	**1254**	**1256**	**1259**
Ground beef					
0	4.65	5.22	5.24	5.48	4.67
5	4.07	3.08	3.83	3.57	3.24
10	3.85	2.93	3.50	3.47	3.11
15	2.75	2.54	3.49	3.62	2.78
20	2.89	2.55	3.51	2.81	2.60
Ground chicken					
0	3.92	4.76	4.66	5.63	5.54
5	3.78	2.64	3.84	2.81	3.79
10	3.65	2.60	3.75	3.39	2.74
15	2.80	2.57	3.03	3.21	2.37
20	3.52	2.53	3.72	2.84	2.81

Table A.3 *C. difficile* counts (log CFU/g) of animal isolates on ground meats during storage at 4°C.

Storage time (days)	***C. difficile* strain**				
	1035	**38107**	**7933**	**3706**	**2760**
Ground beef					
0	5.58	4.92	4.78	5.26	5.49
2	4.99	3.47	3.33	3.39	3.72
5	3.66	3.41	3.15	2.43	2.89
Ground chicken					
0	4.08	3.46	4.72	4.90	4.99
2	3.57	3.13	4.37	5.17	3.74
5	3.57	3.06	4.16	4.00	3.22

Table A.4 *C. difficile* counts (log CFU/g) of animal isolates on ground meats during storage at -15°C.

Storage time (day)	***C. difficile* strain**				
	1035	**38107**	**7933**	**3706**	**2760**
Ground beef					
0	5.58	4.92	4.78	5.26	5.49
5	4.70	3.49	3.05	3.37	2.96
10	4.50	3.13	3.66	3.21	3.93
15	4.01	3.22	3.31	3.13	4.00
20	3.88	3.29	3.38	3.37	3.99
Ground chicken					
0	4.08	3.46	4.72	4.90	4.99
5	4.32	3.01	3.19	3.54	3.48
10	3.95	3.00	3.35	3.61	4.15
15	4.30	3.20	3.41	3.37	3.97
20	4.10	3.02	3.39	3.32	3.80