Determining the Growth Limiting Conditions and Prevalence of *Clostridium difficile* in Foods

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ABSTRACT

Community-acquired *Clostridium difficile* infections have recently been increasing in incidence and severity. Several studies have isolated *C. difficile* spores from livestock and retail meats, suggesting that food may play a role in transmission. No research has been done, however, on what food conditions might allow for the survival and/or growth of the bacterium. We therefore modelled the minimum thresholds for *C. difficile* growth under low pH, water activity (a_w), and temperature. We also sampled retail ground meats, cheese, and milk for the presence of *C. difficile* spores and subtyped food isolates for comparison with clinical strains. We found that *C. difficile* growth could be prevented by refrigeration temperatures. *C. difficile* spores were also detected for the first time in Canada in ground lamb, ground turkey, ground chicken, cheese and milk. The majority of these food isolates were genetically similar to epidemic strain NAP7/078, suggesting that food may not be a direct vector for *C. difficile* transmission, but could still be clinically relevant.

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LIST OF ABBREVIATIONS

AIC	Akaike's Information Criterion
BHI	Brain Heart Infusion
BHIS	BHI broth with yeast extract and cysteine HCl
BHIS + T	BHIS broth with sodium taurocholate
CDC	United States Centers for Disease Control and Prevention
CDI (CA-; CO-; HA-)	*C. difficile* infection (Community-acquired; Community-onset; Heath care-associated)
CDMN	*C. difficile* moxalactam norfloxacin
CDT	*C. difficile* transferase (aka Binary toxin)
CNISP	Canadian Nosocomial Infections Surveillance Program
ddH_2O	Distilled and deionized H_2O
EDTA	Ethylenediaminetetraacetic acid
G/NG	Growth / no growth
NAP	North American Pulsotype
NML	National Microbiology Laboratory
OD	Optical density
PaLoc	Pathogenicity locus
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMC	Pseudomembranous colitis
RAPD	Random amplification of polymorphic DNA
REA	Restriction Endonuclease Aanalysis
RFLP	Restriction Fragment Length Polymorphism
SAS	Statistical Analysis Software
SC	Schwarz Criterion
TBE	Tris-Borate EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic mean

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CHAPTER 1: INTRODUCTION TO *CLOSTRIDIUM DIFFICILE* AND ITS ASSOCIATED DISEASE

1.1 BACKGROUND AND PHYSICAL CHARACTERISTICS OF *C. DIFFICILE*

C. difficile was first isolated in 1935 from the stool of healthy neonates by Hall and O'Toole (47). They originally named the bacteria, *Bacillus difficilis,* to reflect the difficulty in their isolation and culture. The isolated bacteria were extremely pathogenic to hamsters and rabbits (47). Due in part to high asymptomatic carriage of *C. difficile* in human neonates (78), it was only in 1978, over 40 years later, that *C. difficile* was found to cause human disease in the form of antibiotic-associated pseudomembranous colitis [PMC] (15, 78). *C. difficile* is now known to cause mild to severe enteric infections, is highly associated with antibiotic treatment and is the leading cause of hospital-acquired infectious diarrhea (95).

C.difficile is a Gram-positive rod-shaped bacterium. Vegetative cells are slightly larger than most bacteria, measuring 0.5 to 2 μm wide by 3 to 17 μm long (50). Colonies are typically round, flat, off-white and have a "ground glass" appearance (31). Due to the production of various volatile metabolic products, *C. difficile* has a horse manure-like odour (85). The bacteria are strict anaerobes, and form oval, subterminal spores that can persist in a wide range of aerobic environments (47, 104).

Sporulation is induced by unfavourable environmental conditions, such as nutrient limitation. This process renders the cells metabolically dormant (144). The thickened cell wall of spores offers resistance to dessication, high or low temperatures, acidity and common disinfectants. Thus, contaminating spores are difficult to inactivate on environmental surfaces (49).

1.2 *C. DIFFICILE* INFECTION

1.2.1 Acquisition

The high resistance of *C. difficile* spores to harsh environments allows ubiquitous dissemination that contributes to pathogenic exposure (104). There are four broad categories listed as sources of acquiring *C. difficile* infection (CDI): environment-to-person, person-to-person, animal-to-person and consumption (107). Infected individuals may transfer the bacteria to animals, other people or the environment through faecal shedding. There has been one report of probable inter-species transmission of CDI from a patient to a hospital visitation dog (80), but zoonotic transmission has not been conclusively examined. While no cases of CDI transmission through food products are currently documented, several studies report *C. difficile* in raw and ready-to-eat foods, shedding light on this possibility (100, 101, 125, 141, 160, 161).

C. difficile enters the digestive system through a common route of faecal particle ingestion. Because vegetative cell survival is limited by the aerobic atmosphere and acidic conditions of the stomach, highly resistant *C. difficile* spores are most implicated in causing disease (38). Bile salts promote spore germination within the large intestine (144). For colonization, the resulting vegetative bacteria must penetrate the intestinal mucous layer and adhere to colonic epithelial cells. The bacterial exotoxins, toxin A and toxin B, can then act on host cells to cause symptomatic disease (154). Colonization by *C. difficile* is opportunistic and most commonly occurs after disruption of the normal colonic microflora, primarily from the host's use of broad-spectrum antibiotics. Antibiotics particularly associated with CDI include cephalosporins, clindamycin, ampicillin and amoxicillin (63). Other risk factors for infection include old age (>65 years), chronic comorbidity, admission to a hospital or

nursing home, long hospital stays, and prior chemotherapy (19, 108). Use of gastric-acid suppressants may also play a role by increasing the gastric pH (64).

There has been increased awareness that *C. difficile* can also be acquired outside of health care settings. Both health care associated [HA-] and community-acquired [CA-] CDI have been increasing in incidence and severity over the past decade (37).

1.2.2 Infection and diagnosis

CDI is the leading cause of health care-associated diarrhea (11). It was reported to be the second leading cause of acute gastroenteritis diagnosed in Austrian general practices, second only to norovirus (56). Inflammation of the colon (colitis), with or without pseudomembranes, can also occur. Pseudomembranous colitis, characterized by the formation of yellow pseudomembranous plaques on the colon, was the first disease attributed to *C. difficile* (15, 78). Fulminant colitis, the most severe form of CDI, can progress to a toxic megacolon or colon perforation, both of which are life-threatening (32).

Clinical diagnosis of CDI has relied on detecting *C. difficile* toxins in the stool of patients with unexplained diarrhea. Initial screening of these samples may involve an enzyme immunoassay test to detect toxin A and/or toxin B. Positive samples may then be further tested using a cell culture cytotoxicity assay, toxigenic culture, and/or polymerase chain reaction [PCR] assay for toxin genes (31).

1.2.3 Incidence

The incidence of CDI has reportedly been increasing around the world. From 1997 levels in Canada, the overall incidence of CDI increased 4-fold following a 2003 outbreak that began in Quebec (88). Quebec hospitals during that time recorded the CDI rate rising from 5.8 to 20.7 cases per 1000 discharges(39). In Austria, an almost 3-fold increase was observed from 2003 to 2007 (57), and a 4-fold increase was reported in Singapore from 2001

to 2006 (86). Approximately 500,000 CDI cases were reported in US hospitals and nursing homes in 2009 (110), almost twice the estimated number from 2003 (164). CDI is estimated to cost the US health care system as much as $3.2 billion per year (109). *C. difficile* carriage in neonates up to one year is high, but *C. difficile* infection in infants is disproportionately rare (94). This may be because infants have few intestinal cell receptors for the bacterial exotoxins (68). It is proposed that the eventual establishment of healthy gut flora displaces the original *C. difficile* prior to the development of these receptors. *C. difficile* was found to be asymptomatically carried in only 2 to 3% of the population over 1-year of age (16).

1.2.4 Treatment

Metronidazole and/or vancomycin are the current antibiotics of choice to treat CDI. These antibiotics must be used judiciously, however, to prevent further development of antibiotic-resistant strains (168). The novel resistance of one strain, NAP1/027, to fluoroquinolones was partially implicated in why this, now epidemic strain, has caused world-wide outbreaks. Following colonization, it is believed that two thirds of patients become asymptomatic carriers (122). Furthermore, up to 5-35% of patients develop at least one recurrent episode of CDI, even years after the first occurrence (9). Research continues on the purported efficacy of microbiological therapy to restore the normal microflora of patients with frequently recurring disease. Studies on faecal transplantation (45, 127, 167) and colonization with non-toxigenic *C. difficile* strains are currently underway (98, 135).

1.3 VIRULENCE OF *C. DIFFICILE*

1.3.1 Large clostridial toxins

C. difficile secretes two large clostridial toxins, enterotoxin A (TcdA, 308kDa) and cytotoxin B (TcdB, 270kDa). The toxins are closely-related, with 74% homologous amino

acid sequences (120). Following attachment, both toxins act on enteric cells by transferring a glucose moiety onto GTPases of the Rho family that are necessary for actin polymerization. Glycosylation renders the GTPases inactive, thus cytotoxically disrupting all actin-dependent processes. Loss of actin polymerization results in cytoskeletal morphology changes, the opening of tight junctions between enterocytes, increased fluid secretion and activation of host inflammatory responses, all of which compromises the integrity of the intestinal barrier (136). Researchers have revealed that either purified toxins A or B alone can induce the primary symptoms of CDI *in vivo*. Because similar symptomatic disease to A^+B^+ strains has been described from A^-B^+ variants, toxin B may have the highest impact on virulence (90). In contrast, another study using a gene knockout system found that an A^+B^- strain was virulent *in vitro* and in an *in vivo* hamster model, suggesting that the presence of either toxin is sufficient for *C. difficile* to be cytotoxic and pathogenic (74). No clinical infections have been reported from a naturally-occurring A^+B^- strain, however, this may be due to routine diagnostic procedures not always testing for toxin A (74). Non-toxigenic strains have been non-pathogenic in animal models (70, 74).

1.3.2 Pathogenicity locus

The large clostridial toxins are housed within the pathogenicity locus (PaLoc) of *C. difficile*. Also located within the PaLoc are the genes for TcdC, the negative regulator of toxins A and B production, TcdR, the positive toxin regulator, and TcdE, a protein involved in membrane disruption (130). There are common sequence variations of *tcdC* among strains which have been used to help differentiate strains. Particular deletions in *tcdC* are known to cause severely truncated TcdC proteins, leading to disregulated over- or under-production of toxin A and toxin B (24).

1.3.3 Binary toxin

Located outside of the PaLoc, some strains also have a 4.3 kb chromosomal locus containing the genes *cdtA* and *cdtB* which encode for binary toxin, also known as *C. difficile* transferase [CDT] (111, 145). This toxin was first characterized in 1988 (115) and is composed of enzymatic CDTa and cell-binding CDTb. *In vitro*, binary toxin is cytotoxic to eukaroyotic cells by acting as an actin-specific ADP-ribosyltransferase, however, its role is still not well understood *in vivo*. It may work in concert with toxin A and toxin B to contribute to disease (145). CDT may also induce the formation of microtubule protrusions which would improve the bacteria's potential for enteric cell adhesion. It was thought that the severity of CDI was more associated with the presence of toxins A and B than with the presence of binary toxin CDT (96). However, a Danish report comparing the outcomes of patients with CDI recently found that the risk of mortality was 60% higher in those infected with an $A^+B^+Cdt^+$ strain than an $A^+B^+Cdt^-$ type, irrespective of age, gender and geographic region (8).

1.4 HYPERVIRULENT *C. DIFFICILE* AND COMMUNITY-ACQUIRED *C. DIFFICILE* INFECTION

1.4.1 Epidemic outbreak from strain NAP1/027

A factor contributing to the increase in severity and incidence of CDI seems to be the emergence of a hypervirulent strain which is $A^+B^+CDT^+$. This strain is designated as type BI by restriction endonuclease assay, North American pulsotype 1 [NAP1] by pulsed-field gel electrophoresis [PFGE], 027 by ribotyping, and III by toxinotyping. It is commonly referred to as BI/NAP1/027 or NAP1/027. This strain was first characterized in 1984, however prior to 2000, it was attributed to less than 1% of CDI cases (93). From 2008-2009, surveillance of

CDI in Ontario, Canada found 20% of hospital cases attributed to NAP1/027. The emergence of NAP1/027 has now been associated with outbreaks across North America and Europe (14, 70, 158).

The first recognition of a severe CDI epidemic in Quebec, Canada, occurred in 2003. Province-wide outbreaks occurred from 2002-2005 that resulted in a 4.5-fold increase in the incidence of CDI as compared to 1991 and a nearly 5-fold increase in mortality (117, 118). A large percentage of outbreak cases from 2001-2003 were caused by NAP1/027 (88), some of which were shown to have a new resistance to fluoroquinolones (117). In addition, a truncation mutation in *tcdC* was thought to impair the negative regulation of toxin A and B production. An examination of one Quebec epidemic isolate found the strain produced 16x more toxin A and 23x more toxin B than control strains (158). There are conflicting reports on whether NAP1/027 produces more toxin than other strains (99, 158). However, there is agreement that it has increased sporulation activity which could promote its infectivity (3, 99).

1.4.2 Community-acquired *C. difficile* infection

For some time, the high incidence of *C. difficile* infection developed in hospitals overshadowed the fact that some cases of CDI were not acquired in a health care setting. Since hospitals and long-term care facilities are densely populated with patients on antibiotics with classic risk factors for CDI, the incidence of health care-associated CDI [HA-CDI] is understandably higher than in the community (37). However, an epidemiological study of CDI cases in a single hospital over one decade interestingly revealed significant changes in the diversity of infective strains (20). If the hospital environment was the primary reservoir, one would expect a low diversity of strains. As this was not the case, the diverse strains were presumed to be imported by patients and visitors

from the community (20, 131). Originally, strains producing binary toxin were thought to be particularly associated with CA-CDI, however this has been debated with the changing incidence of CDI (13, 151).

Unfortunately, studies have not always used the same definition for community-acquired CDI. The European Centre for Disease Prevention and Control, along with the US Centres for Disease Control and Protection [CDC], have now arbitrarily defined CA-CDI to be an onset of symptoms in the community or within 48 h of admission to a health care facility, with the stipulation that the patient was not discharged from a health care facility within the previous 3 months (75). Following these guidelines, the reported incidence and severity of CA-CDI increased in some developed countries (40, 61). It is estimated that CA-CDI accounts for approximately 20% of the CDI patients in North America (77, 91).

1.4.3 Ribotype 078 and community-acquired *C. difficile* infection

CA-CDI has been associated with NAP1/027 along with another $A^+B^+CDT^+$ strain which, since 2003, has been increasingly found to affect humans (133). By PFGE, ribotyping and toxinotyping, respectively, this strain has been designated NAP7 or 8/078/toxinotype V, subsequently referred to as 078. A nonsense mutation at the 185 position in *tcdC* (C184T) (101) causes this strain to produce a truncated negative regulator like NAP1/027. The truncation is thought to allow greater *in vitro* toxin A and B production, but less so than that produced by NAP1/027 (30, 61). This difference may help to explain why CDI from 078 has been associated with less severe disease than NAP1/027 (40).

Strikingly, while 078 is not as commonly linked to outbreaks as NAP1/027, 078 has been more frequently isolated from North American cases of CA-CDI with an uncharacteristic demographic of younger, previously healthy patients (40, 61). In Ireland, 33% of CA-CDI cases are attributed to this strain, while it only accounts for 13.7% of HA-

CDI (28). In the USA (87) and the Netherlands (40), 078 is the third most isolated strain from CA-CDI patients.

The incidence of disease from strain 078 in Canada increased from 0.5% to 1.6% during the years 2004 to 2008 (102). A Europe-wide surveillance study found an increase from 3% to 8% between 2005 and 2008. In the Netherlands from 2005 to 2007, one study found that the incidence of disease from NAP1/027 decreased from 27% to 1% while that from strain 078 rose from 3% to 13% (40). In addition, this latter 2007 study reported that infection by strain 078 had increased in morbidity and mortality (40).

1.5 *C. DIFFICILE* IN ANIMALS

1.5.1 Prevalence

The epidemiology of CDI in animals is still unclear. However, *C. difficile* is pathogenic to many species and has been particularly reported in young livestock (69). Spores shed from domestic and exotic animals may have the potential to cause zoonotic transmission to humans (57, 131, 142).

Young swine and, more recently, calves are considered to be important hosts of the bacteria. In swine, as high as 96% of neonatal piglets (1 - 7 days old) have been reported to be affected by *C. difficile* induced enteritis (140). As in humans, the prevalence of colonization seems to decrease with age, e.g., one study found a farm with a 50% prevalence in suckling pigs, 8.4% in weaned pigs and only 3.9% in grower-finisher pigs (106). *C. difficile* has also been implicated in causing enteritis in calves. One report from 2008 found a 25.3% prevalence in diarrheic cows (48), higher than a previous finding of 7.6% in 2006 (126). In both studies, some samples from non-diarrheic cows were positive for the bacteria

and, surprisingly, toxins, indicating that carriage of a toxin producing *C. difficile* type is not necessarily sufficient for pathogenesis.

While poultry have not exhibited disease symptoms, a large survey from Europe found 62.3% avian *C. difficile* carriers. Colonization was also age dependent, where the rate decreased with increasing age (169). Few studies have been done in less-developed countries, but an assessment of Zimbabwean broiler chickens found that 26% of faecal samples were positive for *C. difficile*. The ages of the chickens were not reported in that study (138).

It is important to note that there is a large variation in sampling methods between these studies, making the comparison of *C. difficile* prevalence difficult. Occasionally, only symptomatic animals were chosen for faecal testing. Since asymptomatic carriage is possible, the prevalence of the bacteria in animals is likely underreported. In healthy Austrian livestock, the prevalence of *C. difficile* was 3.3% in pigs, 4.5% in cows, and 5% in broiler chickens (ages not reported) (58).

1.5.2 Hypervirulent strains

Among the studies that have examined strain commonalities between animals and humans, indistinguishable hypervirulent strains have been found among bovine, porcine, poultry, and human samples (Table 1). The primary ribotypes currently found in pigs and cows are 078, one of the hypervirulent clinical strains, and 017 (68, 132). A North American wide study found an 83% and 94% prevalence of 078 in pigs and cattle, respectively. In contrast, canine, equine, and human strains from smaller geographic regions were highly diverse (68). Isolates from European poultry have also showed a high diversity of ribotypes. Most strains were $A^+B^+CDT^-$ and none were toxinotype V, the type corresponding with 078

(169). The relatively high prevalence of 078 in pigs and cattle may be because bovine and swine are more susceptible to colonization by this strain (68).

Finding indistinguishable strains in animals and humans in multiple countries around the world may be due to direct and/or indirect transmission between humans and animals, or a common environmental source (61). It is concerning that the hypervirulent strain 078 is simultaneously increasing in clinical incidence and veterinary prevalence (133). Comparison of 078 from pigs and humans by microarray-based comparative phylogenomic analysis suggested that this strain may have emerged in swine prior to occurring in humans (139). Determining the likelihood that the presence of *C. difficile* in animals may lead to disease in humans, still requires further investigation, including the prevalence and strain similarity of *C. difficile* between species. The particularly high prevalence of *C. difficile* in food animals also prompts the investigation of food as a transmission vector.

1.6 *C. DIFFICILE* IN FOODS

1.6.1 North American meat products

The first report of *C. difficile* in a food product was somewhat overlooked as an item of interest. The study identified multiple *Clostridium* spp., including one strain of *C. difficile* in blown-packs of retail meat (27). Several years later, a turkey-based dog food product was also found to contain *C. difficile* spores (162). It was a Canadian group in 2007 that first isolated *C. difficile* from food destined for human consumption (125). This study sparked the current world-wide investigation of food as a reservoir and possible transmission vector for *C. difficile*.

The Canadian study found a 20% prevalence of *C. difficile* in ground beef and veal from Ontario over a 10-month period (125). A subsequent American study found a high

Table 1. Overall prevalence and ribotypes of *C. difficile* isolated from food animals and meats.

			% of hypervirulent isolates	
Origin	**Country (Reference)**	**Prevalence (%)**	**078**[a]	**027**[a]
Calves	Canada (126)	15	26	12
	USA (48)	25	94	0
	Slovenia (112)	1.8	0	0
Cows	Austria (57)	4.5	0	0
Piglets	Canada (159)	95	94	0
	USA (166)	79	NT[b]	NT
	USA (68)	NA	83	0
	Slovenia (112)	52	0	0
Pigs	Austria (57)	3.3	50 (TT V)[c]	0
Poultry	Slovenia (169)	62.2	0	0
	Austria (57)	5	0	0
	Zimbabwe (138)	29	NT	NT
Beef	Canada (125)	20.8	0	17 (NAP1-r)[d]
	Canada (123)	6.7	86	7.1
	Canada (161)	12	100 (078/027)[e]	100 (078/027)
	USA (141)	42.4	NT	NT
	Sweden (1)	2.4	NT	-
	Austria (66)	0	-	
Pork	Canada (161)	12	71	7.1
	Canada (101)	1.8	0	43
	USA (141)	41.3	100 (078/027)	100 (078/027)
	Sweden (1)	0	-	-
	Austria (Beef & pork mix) (66)	3	NT	NT
Chicken meat	Canada (160)	12.8	100	0
	Netherlands (22)	2.7	0	0
	Sweden (1)	0	-	-
Turkey meat	USA (141)	44.4	100	0
Lamb meat	Netherlands (22)	6.3	0	0
	Sweden (Sheep) (1)	0	-	-
Veal	Canada (125)	14.3	0	100 (NAP1)[f]
	Canada (123)	4.6	0	66 (NAP1-r)

[a] Ribotype.
[b] NT: Not typed.
[c] TTV: Toxinotype V, corresponding toxinotype of 027.
[d] NAP1-r: PFGE profile similar to North American Pulsotype 1 (NAP1).
[e] 100 (078/027): 100% of isolates were 078 or 027.
[f] NAP1: North American Pulsotype 1, corresponding PFGE type of 027.

(42%) prevalence of *C. difficile* in various raw and prepared meat products from beef, pork and turkey over a period of 3 months (141). A more systematic, follow-up Canadian study was expanded to several provinces (Ontario, Quebec, Saskatchewan). Aiming to provide a more representative prevalence report of *C. difficile* in Canada, the authors found a 6.7% and 4.6% prevalence in ground beef and veal chops, respectively (123). In the first examination of Canadian chicken, 12.8% of whole piece samples were found to be positive (160). Of note, even if the overall prevalence was relatively low, a high proportion of isolates from all of these reports were associated with hypervirulent strains, the majority being similar to or indistinguishable from 078 and others to NAP1/027. In contrast to the reported diversity of *C. difficile* in poultry from Europe, all of the isolates from Canadian chicken meat were found to be 078 (160), however no other studies of chicken meat have been published.

1.6.2 European meat products

The prevalence of *C. difficile* in meat from Europe appears to be lower than in North America. In the Netherlands, there was a 1.6% (8/500) prevalence of *C. difficile* from various meat samples where only chicken and lamb were contaminated (22). Only two beef samples of 82 Swedish meat products (2.4%) were found to contain *C. difficile* (1). A similar 3% prevalence was found in Austrian beef and pork (66), although no positives had been found in an earlier Austrian report on meat (57). *C. difficile* was not found in various meat products from Switzerland (52).

The reported prevalence of hypervirulent strains from the North American and European studies are summarized in Table 1.

1.6.3 Other food products

There have been a few studies examining *C. difficile* in foods other than retail meats. There was a 2.3% prevalence reported in vegetables from South Wales, England (134) and

7.5% in ready-to-eat salads from Scotland (10). A convenience sampling of retail root vegetables in Guelph, ON resulted in finding a 4.5% prevalence (100). All of these isolates from vegetables were found to be toxigenic. The Scottish researchers ribotyped their three isolates and found two to be ribotype 017 and one 001 (10). In the Canadian study of vegetables, three out of the five positives samples were contaminated with 078 strains. It was noted that four of the positives were imported from China, but the origin of contamination could have been from either the source or distributing country (100). Contamination of vegetables may be from soil and water, both of which have been found to harbour spores (134).

1.6.4 Implications

The prevalence of *C. difficile* reported from various food types and countries is highly variable. This variation may be due in part to differences in geographical distribution, but is likely also influenced by the use of different sampling and detection methods. A true comparison of the prevalence of *C. difficile* in foods can only be done by standardizing these protocols. It remains clear, however, that *C. difficile* spores can be present in foods. Spores deposited in meats could come from an animal source tissue or faecal contamination during slaughter. Meat contact with the environment and/or food handlers can also introduce spore contamination during food processing (107).

The World Health Organization defines a foodborne illness to be a disease "*usually either infectious or toxic in nature, caused by agents that enter the body through ingestion of food*" (165). With this definition, it is difficult to determine whether a case of CDI may be foodborne, because there currently are no epidemiological guidelines to establish if a patient has acquired *C. difficile* from food. With most foodborne illnesses, there is a relatively short period from which a contaminated food is ingested to when symptoms occur. With *C.*

difficile on the other hand, the bacteria may not cause disease until the opportunity for colonization arises at a later date. This symptomatic colonization may also only occur in predisposed populations, a group that is currently not well defined for CA-CDI patients. Furthermore, it is possible that an individual may ingest and be colonized with *C. difficile* asymptomatically, then subsequently pass the bacteria to another individual resulting in symptomatic disease (159). Thus, it will be difficult to epidemiologically link food to CDI, but continued testing for *C. difficile* may clarify the role of food as a reservoir.

1.6.5 Risk of foodborne transmission

While the dose response for *C. difficile* in humans has not been established, intuitively, foods with low bacterial burdens should be less of a risk than those with higher levels (159). The *C. difficile* spore load in food has been enumerated in only one report on ground meats (161). The latter authors found that most samples had a low level of contamination. However, the distribution of spores may be non-homogenous, as a few of their samples had detectable spores only when using direct culture rather than enrichment. These samples were found to contain 20 spores / g (161). The environmental conditions under which *C. difficile* might grow in foods has not been tested, however. If the bacteria are able to multiply in a food, even a low initial spore level could result in colonization (159). To assess whether *C. difficile* has the potential to be a foodborne pathogen, the conditions that could support or limit *C. difficile* growth should be examined, along with an examination of its prevalence in foods.

1.7 OBJECTIVES AND HYPOTHESES

The objectives of this study are:

1) To assess the growth potential of *C. difficile* under the conditions representative of a typical food environment by determining its thresholds for growth in terms of minimum pH, water activity (a_w) and temperature; and
2) To examine regional retail food products for the presence of *C. difficile* spores; and
3) To assess the clinical relevance of strains isolated from foods by using strain typing assays to compare *C. difficile* strains isolated from food, humans and animals.

The hypotheses of this study are:

1) *C. difficile* is able to grow under the suboptimal pH, a_w, and temperature sometimes encountered in a food environment.
2) *C. difficile* spores are present in raw ground meats and retail cheeses.
3) Strains isolated from foods are indistinguishable from those infecting humans and animals, suggesting that these foods can act as a vector of *C. difficile* transmission.

CHAPTER 2: DETERMINATION OF *C. DIFFICILE* GROWTH THRESHOLDS

2.1 INTRODUCTION

2.1.1 Hurdle technology

The food industry is challenged to ensure the microbiological safety of foods while maintaining products that are desirable to consumer palates and nutritional needs (83). The main avenue to meeting these goals is the use of hurdle technology, which is a deliberate combination of preservative factors for safe food design (82). There are over 60 potential hurdles described in foods today, the most important of which include temperature, acidity (pH), and water activity (a_w) (81). Both high and low temperatures are used to prevent microbial growth, while a high pH has a more marginal effect on growth than low pH (72). A_w is the moisture content of a food that is available for the metabolic processes of microorganisms. Decreasing the a_w value from 1.000, corresponding to pure water, reduces the potential for microbial growth (82). Using hurdles, the initial population of food-spoilage or pathogenic microorganisms present in a food should not increase, and could decrease during storage in harsh environments (81).

2.1.2 Mechanism of function

While responding to the hurdles of a food environment, it is thought that microorganisms attempt to use every possible repair mechanism to re-establish homeostasis (72). Different hurdles may have different targets such as the cell membrane, DNA, enzymes and metabolic processes (81). One theory for how hurdles limit bacterial growth is that excessive energy demands lead to metabolic exhaustion, so that the cells have no more

energy to survive (81). For example, maintaining physiological pH in an acidic environment has been shown to cause metabolic exhaustion, because bacteria must actively pump protons from the cytoplasm to maintain homeostasis (72).

Hurdles may also affect key molecules, such as metabolic enzymes. These enzymes are only active within certain ranges of temperature and are limited by the environmental a_w which is dependent on the compatibility of humectant solutes. Beyond these tolerable extremities, biological pathways that support growth can be predictably inhibited (72, 81).

Another target of hurdles is the bacterial stress response. Response to the harsh environment can actually induce more bacterial resistance or even virulence, so it is important to realize that the improper use of hurdles may promote bacterial pathogenesis (81). The bacterial synthesis of stress response molecules, however, also contributes to metabolic exhaustion (81). Therefore, exposing bacteria to different hurdles simultaneously, can prevent or slow their growth, and/or potentially cause complete inactivation (81).

2.1.3 Combination of hurdles

The intensity of a hurdle determines whether it will be beneficial or detrimental to a food product. Cooling foods to inappropriately low temperatures might introduce ‘chilling injury’; a large addition of salt to lower a_w is not nutritionally desirable; and increasing the acidity of foods can make it unpalatable (83). Particularly today, hurdle technology must adapt to meet the increasing demands for nutritionally healthier foods that are minimally processed or that contain less fat and/or salt (83). Determining the thresholds for bacterial growth under hurdles is therefore important in order to formulate ingredients that will still result in safe foods. Since the hurdles can act on different processes to offset homeostasis, it is believed that the effect of combining hurdles on bacterial growth can be not only additive,

but also synergistic (81). Therefore, a combination of mild hurdles is generally used to make foods that are organoleptically and nutritionally acceptable, as well as safe.

2.1.4 Growth / no growth examination

As environmental conditions reach the extreme limits for bacterial growth, bacteria behave more variably (128). The probability of bacterial growth at these limiting conditions can be examined using replicate experiments. Primary data can be collected in a binary form: growth [1] and no growth [0] (128). These growth / no growth [G/NG] studies are useful to observe the possible synergism between multiple hurdles to best predict what food preservatives can be combined for optimal food safety and stability. G/NG boundaries have been determined for several foodborne pathogens including *Escherichia coli* (116), *Listeria monocytogenes* (157) and *Staphylococcus aureus* (155). These studies have been able to define the G/NG interface very closely. The typical growth limit for a_w is across 0.01 - 0.03 units, while for pH it is across 0.1 - 0.2 pH units (97). G/NG models have shown that the minimal conditions for growth are not necessarily intuitive. Instead of the highest bacterial tolerance of low a_w being at the bacteria's optimal growth temperature, *E. coli* is able to grow at the lowest a_w value of 0.948 between 25ºC and 30ºC, but only down to 0.951 at its optimal growth temperature of 37ºC (97).

The growth limits of other *Clostridium* spp. implicated in human pathogenesis and food-spoilage have also been examined (23, 50), including *C. botulinum* (42), *C. butyricum* (5) and *C. perfringens* (65). The reported growth thresholds for these bacteria and other common foodborne bacteria are listed in Table 2.

A model of the growth / no growth interface was first described by Ratkowsky and Ross in 1994 (119). The model variables affecting growth rate (such as temperature, pH and a_w) are incorporated into an equation to equal "logit (p)", a mathematical abbreviation for

Table 2. Examples of growth limiting conditions reported for common foodborne or food spoilage bacteria.

Pathogen	Individual Growth Limiting Condition			Reference
	pH	a_w	T (ºC)	
C. botulinum (proteolytic)	4.6	0.94	10-12	(89)
C. botulinum (nonproteolytic)	5.0	0.97	3.0	(89)
C. butyricum	4.1	0.95	10	(5)
C. perfringens	5.0	0.95	15	(67)
Bacillus cereus	5.0	0.93	4.0	(43)
Escherichia coli	4.6	0.95	2.5	(2, 62)
Listeria monocytogenes	4.8	0.95	1	(33, 62)
Staphylococcus aureus	4.0	0.86	7.0	(62)
Salmonella spp.	4.5	0.92	6.5	(2, 62)

logit (p) = ln [p/1-p)], where p is the probability of growth occurring. To form the data set, when growth is observed under a set combination of the variables, p is assigned a value of '1'. When growth is not observed, p is assigned a value of '0'. The experimental data is then fit to the equation by using software such as Statistical Analysis Software [SAS] (SAS Institute Inc., Cary, NC, USA). Using this software, coefficients to the equation are estimated with standard logistic regression procedures. After this model is determined, the interface of growth and no growth is predicted by setting $p = 0.5$, where there is a 50% chance that the organism will grow (119).

Since *C. difficile* has been found in food, determining whether food environments allow its growth will be useful in risk assessment. While it is suggested that foods have a low burden of spores (161), if a food supports bacterial growth, the bacterial burden could increase prior to consumption, likely increasing the chance of colonization and subsequent infection (159). This chapter describes the first systematic examination of the individual and combined effects of a_w, pH and temperature hurdles on the growth of vegetative *C. difficile*. Assessing the environmental thresholds of *C. difficile* growth can help define the clinical implications of finding *C. difficile* in food products, and help determine the intensity of environmental hurdles needed to minimize or inhibit cell growth.

In this chapter, we measured the growth of different *C. difficile* strains to examine the growth limiting hurdles, a_w, pH, and temperature, both individually and in combination. To maintain the consistency of other factors possibly influencing growth, in this preliminary study, we measured growth in laboratory media rather than complex foods.

2.2 METHODS

2.2.1 Bacterial strains and growth conditions

The characteristics, origin and source of bacterial strains used in these experiments are described in Table 3.

The *C. difficile* media used for optimal growth was described in the 2009 Current Protocols in Microbiology (143). Culture broth consisted of 3.7% (wt/vol) brain heart infusion [BHI] (Becton, Dickinson, and Company, Sparks, MD) with 0.5% (wt/vol) yeast extract (Becton, Dickinson, and Company) and 0.1% (wt/vol) cysteine HCl (Sigma-Aldrich, St. Louis, MO) [BHIS]. The pH of the BHIS broth was 7.2 and its water activity [a_w] value was 0.996. BHIS agar plates were used for colony isolation and contained 5.2% BHI agar, 0.5% (wt/vol) yeast extract, 0.1% (wt/vol) cysteine, and 0.1% (wt/vol) sodium taurocholate (Sigma-Aldrich) [BHIS+T]. All cultures were inoculated into pre-reduced BHIS broth and incubated in an atmosphere of 5% H_2, 10% CO_2, and 85% N_2 or in 3.5-liter anaerobe jars (Oxoid, Ottawa, ON) containing AnaeroGen packs for atmosphere production (Oxoid). Cultures were routinely grown at 35ºC.

The thresholds of *C. difficile* growth were measured in media with a range of pH, a_w and temperatures. The pH of BHIS broth was adjusted to final pH values ranging from 4.5 to 7.0 in 0.5 ± 0.2 increments using HCl and NaOH. The a_w of BHIS broth was adjusted with NaCl. When individually assessing the minimum a_w for *C. difficile* growth, BHIS broth was adjusted to a_w values of 0.973, 0.969, 0.966, 0.965, and 0.964. When multiple suboptimal conditions were combined, BHIS broth was adjusted to final a_w values ranging from 0.994 to 0.967 ± 0.01. The threshold temperatures were assessed by incubating the cultures in separate incubators at 4ºC, 10ºC, 15ºC, 25ºC, and 35ºC ±2ºC. The combined conditions

Table 3. Strains of *C. difficile* used in this study.

Strain	Toxigenic Profile	*tcdC* Δ[a]	Toxinotype	Pulsotype	Ribotype	Origin	Location	Source or reference
630	$A^+B^+Cdt^-$	No deletion	0	NC[b]	012	Human	Zurich, Switzerland	ATCC®
ATCC 9689	$A^+B^+Cdt^-$	No deletion	0	NC	NC	Human	Denver, CO, USA	ATCC®
BI-7	$A^+B^+Cdt^+$	18	NT[d]	NT	027	Human	Portland, OR, USA	NRC[c]
11ACD0028	$A^+B^+Cdt^+$	39	V	NAP7	078	Human	Montreal, QC, Canada	NML[e]
GrLa0912 22	$A^+B^+CdtB^+$	39	NT	NAP7	078/126-related	Ground lamb	Ottawa, ON, Canada	This study
GrCh1007 398	$A^-B^-CdtB^-$	No gene	NT	NC	067	Ground chicken	Ottawa, ON, Canada	This study
11ACD0001	$A^+B^+Cdt^-$	No deletion	NT	NT	NT	Human	Montreal, QC, Canada	NML[c]
11ACD0075	$A^+B^+Cdt^+$	18	III	NAP1	027	Human	Montreal, QC, Canada	NML[c]

[a] Base pair deletions in the *tcdC* gene amplicon. See Section 4.1.5.
[b] NC: Not characterized
[c] S. Logan, National Research Council, Ottawa, ON, Canada.
[d] NT: Not tested
[e] M. Mulvey, National Microbiology Laboratory, Winnipeg, MB, Canada.

tested are listed in Table 4. All media were filter-sterilized (0.22µm, Millipore, Billerica MA).

2.2.2 Individual suboptimal conditions

C. difficile strains 630 and ATCC 9689 were used to assess growth thresholds at individually varied suboptimal conditions of pH, a_w, and temperature. A single colony from each strain was picked from 2 day old BHIS+T plates that were streaked from frozen stock. The colonies were each subcultured into 5 mL of BHIS and incubated anaerobically overnight at 35ºC.

Seven tubes containing 10 mL of media with 0.5 pH unit increments from 4.5 to 7.0 or a_w values of 0.973, 0.969, 0.966, 0.965, and 0.964 were prepared. Media destined for low temperature incubation was maintained at room temperature and then transferred to their respective incubators after inoculation. Tubes were inoculated in triplicate with ~10^5 cfu / mL of overnight cultures and incubated anaerobically at 35ºC. The remaining un-inoculated tube was used as a reference blank for that condition. The optical density (600 nm; OD_{600}) of the cultures were measured daily using a Model 40 Cell Density Meter (Fisher Scientific, Ottawa, ON) for the first 20 days, then every 3 days over a 60-day period. An OD_{600} over 0.2, indicating early log phase, was recorded as growth (1, 3).

2.2.3 Combined suboptimal conditions

To assess growth thresholds with combined hurdles, an inoculum cocktail was prepared from the 630 and ATCC 9689 strains. The strains were subcultured from the frozen stock as above. Following individual overnight broth culture, 50 µL of each culture was transferred to 25 mL of BHIS broth and grown anaerobically overnight at 35ºC.

Table 4. The combined levels of pH, a_w, and temperature used to examine the growth of *C. difficile*.

pH values	A_w values	Temperature (ºC)
4.5	0.994; 0.989	15
	0.994; 0.989; 0.984; 0.979	25
	0.994; 0.989; 0.984; 0.979	35
5.0	0.994; 0.989; 0.984; 0.979; 0.975	15
	0.994; 0.989; 0.984; 0.979; 0.975, 0.971, 0.967	25
	0.994; 0.989; 0.984; 0.979; 0.975, 0.971, 0.967	35
5.5	0.994; 0.989; 0.984; 0.979; 0.975	15
	0.994; 0.989; 0.984; 0.979; 0.975	25
	0.994; 0.989; 0.984; 0.979; 0.975, 0.971, 0.967	35
6.0	0.994; 0.989; 0.984; 0.979; 0.975	15
	0.994; 0.989; 0.984; 0.979; 0.975, 0.971, 0.967	25
	0.994; 0.989; 0.984; 0.979; 0.975, 0.971, 0.967	35
6.5	0.994; 0.989; 0.984; 0.979; 0.975	15
	0.994; 0.989; 0.984; 0.979; 0.975	25
	0.994; 0.989; 0.984; 0.979; 0.975; 0.971; 0.967	35
7.0	0.994; 0.989; 0.984; 0.979; 0.975	15
	0.994; 0.989; 0.984; 0.979; 0.975	25
	0.994; 0.989; 0.984; 0.979; 0.975; 0.971; 0.967	35

Sterile, round-bottom 96-well plates (BD Biosciences, Mississauga, ON) were filled with 200 μL of medium per well with 30 wells per condition (Table 4). An additional 2 wells for each condition were filled and left without inoculum as negative controls. The outermost wells of the plates were filled with the non-inoculated controls or distilled and deionized H_2O [dd H_2O] to prevent evaporation. Anaerobically, the inner 60 wells were inoculated with 10 μL (~10^4 cfu / well) of the inoculum cocktail. The 96-well plates were sealed with sterile, transparent, air-tight, adhesive plate seals (Excel Scientific, Victorville, CA). The 96-well plates were incubated anaerobically at 15º, 25ºC, and 35ºC according to the combinations shown in Table 4. In a small number of conditions, media in a well evaporated over the period of incubation, slightly reducing the number of replicates. Growth was measured after one day and then weekly for 8 weeks using a 96-well plate reader (Tecan, Durham, NC). Sealed plates were removed from the anaerobic atmosphere for OD readings, but returned within 30 min. Prior to the OD_{600} reading, the 96-well plates were shaken thoroughly by inversion to resuspend any pellet and remove condensation build-up on the plate seal. If an individual well had an $OD_{600} > 0.2$, then bacterial growth was assumed to occur. At times, bacterial growth resulted in a cell aggregate that could not be resuspended to give a consistent OD_{600} reading. In this case, visualization of a dense cell aggregate was recorded as a grown well.

2.2.4 Model development

Growth interfaces were predicted using a linear logistic regression model fit to the observed G/NG data. A cut off point of $p = 0.5$, where p is the probability of growth, was used as the classification criterion that growth occurred. When 15 wells or more out of the 30 replicates demonstrated bacterial growth, it was plotted as growth and coded as “1”. When 1

to 14 wells showed bacterial growth, it was plotted as transitional growth. When no replicate wells demonstrated bacterial growth, it was plotted as no growth and coded as "0".

The observed data was fit to a second-order linear logistic regression equation, as developed by Valero et al. (155). The left-hand side term, log*it* (*p)*, is a mathematical abbreviation equal to ln (*p* / (1 - *p*)), where p is the probability of growth. This model is described as follows:

$$\text{log}it(p) = a_0 + a_1 * T + a_2 * \text{pH} + a_3 * b_w + a_4 * T * \text{pH} + a_5 * \text{T} * b_w + a_6 * \text{pH} * b_w + a_7 * T^2 + a_8 * \text{pH}^2 + a_9 * b_w^2 \qquad \textbf{Equation 1}$$

where a_0 - a_9 are the coefficients to be estimated, T is temperature and b_w is equal to $\sqrt{(1 - A_w)}$.

This model was fit to the experimental data using SAS 9.2 (SAS Institute Inc.) using the LOGISTIC procedure and the stepwise selection option to select the most significant term of the equation. From the model, predicted probabilities at p = 0.5 and 0.9 were calculated while maintaining temperature, pH or a_w values constant. These were then plotted in contour graphs.

2.2.5 Evaluation of model performance

Derived from SAS outputs, goodness-of-fit statistics and four predictive performance indexes were used to evaluate the estimation of the unknown coefficients in the log*it*(*p*) model.

The (Hosmer-Lemeshow) statistic was determined to indicate how adequately the model fit the data. A small value or a high corresponding *p*-value would indicate that the model fits the data well (46, 54). Pearson residuals were also calculated to provide

information about the model's lack of fit. This equation, as follows, measures the difference between observed and predicted events in terms of the number of observations:

$$e_i = \frac{r_i - (n_i * p_i)}{\sqrt{n_i * p_i * (1 - p_i)}}$$

where r_i is the number of times growth occurred at the i^{th} condition, n_i is the number of replicates tested, and p_i is the estimated probability of growth.

Predictive performance indices determine the agreement / disagreement between observed and predicted values. These measured indices included the percent concordant / discordant / tied and c, the concordance index. A perfect predictive power occurs if the concordance rate is 100%, discordance and tied rate is 0%, and if c = 1 (46, 54).

2.2.6 Combined suboptimal conditions with different strains

C. difficile strains BI-7, 11ACD0075, GrLa0912 22, and GrCh1007 398, were grown from frozen stock on BHIS+T agar for 2 days. A single colony from each strain was subcultured into tubes containing 5 mL of BHIS broth and incubated overnight anaerobically at 35ºC. The OD_{600} of each culture was adjusted to 1.80 - 1.90. A total of 50 µL of each culture was then inoculated separately into 10 mL of BHIS broth and incubated again overnight.

In total, 10 replicates per condition were assessed per strain (Table 4). As previously, 10 µL (~10^4 cfu / ml) of the overnight cultures were inoculated into the 96-well plates. Separate 96-well plates were used for each strain. The 96-well plates were incubated under anaerobic conditions. Growth was recorded as above (see Section 2.2.3).

2.3 RESULTS

2.3.1 Growth under a single hurdle

Two toxinotype 0 clinical strains, ATCC 9689 and *C. difficile* 630 were separately grown in media with an individual hurdle. The growth observations and time to detection are indicated in Table 5. Under the experimental conditions, the two strains exhibited growth and no growth at the same environmental thresholds, but at different rates. At otherwise optimal conditions, ATCC 9689 and 630 grew within 60 days in BHIS broth with an a_w value of 0.969, a pH of 5.0, or a temperature of 15ºC. Neither strain demonstrated growth at or below an a_w value of 0.966, pH of 4.5, or temperature of 10ºC during the observation period.

At an a_w value of 0.969, it took 16 days for ATCC 9689 to exhibit growth, 10 days longer than it took for strain 630. On the other hand, strain 630 took a little longer time to grow at a low pH value (5.5), reaching the threshold OD_{600} of 0.2 in 9 h, one hour longer than ATCC strain 9689. At 15ºC, strain 630 exhibited growth in 21 days, while strain 9689 took 16 days. The OD_{600} values of the cultures over time at different water activities, pH values or temperatures, are shown in Figures a1-a3 in Appendix A.

2.3.2 Predictive modelling of growth / no growth under multiple hurdles

A cocktail of the two toxinotype 0 strains examined previously (ATCC 9689 and *C. difficile* 630) were incubated in BHIS broth with a combination of pH, a_w and temperature hurdles. G/NG observations were recorded as before and the data was fitted to a polynomial logistic regression model.

The coefficients of the model were derived from the entire observed dataset using the outputs of SAS model fitting. This model showed that the quadratic terms in Equation 1 (Section 2.2.4) of temperature (T^2) and b_w (b_w^2), as well as the interaction terms of pH*b_w

Table 5. Time to detection for growth of *C. difficile* strains under a single hurdle

Hurdle[a]	Days to detection (OD_{600} > 0.2)	
	C. difficile strain	
Water activity	**9689**	**630**
0.973	4	4
0.969	16	6
0.966	NG[b]	NG
0.965	NG	NG
0.964	NG	NG
pH		
6.5	0.33	0.33
6.0	0.33	0.33
5.5	0.33	0.375
5.0	1	1
4.5	NG	NG
4.0	NG	NG
Temperature (ºC)		
35	0.33	0.33
25	1	1
15	16	21
10	NG	NG
4	NG	NG

[a] Except for the specified hurdle, the media had an a_w of 0.996, pH 7.0, and was stored at a temperature of 35ºC.
[b] No growth (NG) occurred within 60 days.

were not significant ($p > 0.05$). Thus, these were excluded from the equation. The other coefficients with their standard errors and *p*-values are shown in Table 6.

Through SAS, goodness-of-fit statistics and predictive performance indexes were calculated to quantify how well the predicted model fit with the observed data. These values (described in 2.2.5) are shown in Table 7. The *p*-value corresponding to the Homers-Lemershow statistic was reasonably high (0.841). The Pearson residuals of 93 out of the 99 (94%) conditions were between -1 and 1. Considering the number of observations, this indicated small differences between the observed data and predicted probabilities of the model. The predictive power indices thus indicate a high correspondence between the observed and predicted values. In total, 91% of the data was modelled correctly.

2.3.3 Observed and predicted effects of combining hurdles

The growth effect of combinations of temperature, pH, and a_w on *C. difficile* were assessed by experimental data and contour plots of the predicted model shown in Figure 1 - 3. The contour lines depict fixed probabilities of $p = 0.5$ and 0.9 according to the logistic regression model. Observed data is indicated by the points plotted. The proximity of the contour lines to the observed data depicts the logistic regression model's appropriate goodness-of-fit. The effects of each environmental factor are described below.

Temperature effect

C. difficile growth/no growth as a function of pH and a_w at the fixed temperatures of 15ºC, 25ºC and 35ºC are shown in Figure 1a-c. Both the predicted and observed results are shown. The model predicted no growth at low temperature (< 15ºC) when the pH is low (pH < 5.0), regardless of the a_w, which fit well with our observed data. The probability of growth increased at 15°C when a higher a_w and a pH between 5.0 and 7.0 was used. The harshest suboptimal condition in which growth was observed occurred at 15ºC with an a_w value of

Table 6. Estimated coefficients of the logistic regression for *C. difficile* growth in BHIS broth.

Coefficient[a]	**Estimate**	**SE**[b]	**df**[c]	***p*-value**
Intercept (a_0)	-178.4	38.6308	1	<.0001
Temperature (a_1)	1.1997	0.4165	1	0.0040
pH (a_3)	52.6272	11.3481	1	<.0001
b_w (a_4)	185.5	54.9569	1	0.0007
Temperature * pH (a_5)	0.2042	0.0638	1	0.0014
Temperature * b_w (a_6)	-16.1538	3.5613	1	<.0001
pH2 (a_7)	-4.6084	0.9535	1	<.0001

[a] Coefficients of Eq. 1 (Section 2.2.4)
[b] SE: Standard error
[c] df: Degrees of freedom

Table 7. Performance statistics for the polynomial logistic regression model of *C. difficile* growth. Statistics were based on the model described by the coefficients in Table 6 for Equation 1 (Section 2.2.4).

Statistic*	Value
Homer-Lemershow (p)	0.8409
c	0.98
% concordant	98
% discordant	1.9
% tied	0

* The meanings of statistical terms and their values are described in Section 2.2.5.

0.984 and pH value of 5.5.

The model predicted that tolerance to low a_w was dependent on temperature, and best at mildly acidic to neutral pH. The probability of growth was greatest at low pH and a_w levels when the temperature was high (T = 35ºC). For example, growth was predicted in 90% of cases at 15ºC when the pH was 6.0 and a_w was 0.992, while this occurred at the lower a_w of 0.981 at 35ºC (Figure 1a and 1c). As well, growth at an acidic pH value (4.5) was observed, but only at the higher temperatures of 25 and 35ºC. According to the model, at 25ºC, growth was predicted in 50% of cases at pH 4.5 when the a_w value was 0.993. At 35ºC, growth was predicted in 50% of cases at a lower a_w value of 0.989 (Figure 1b-c).

pH effect

There was no growth at pH 5.0 and 35ºC at an a_w value < 0.975. At this same pH and 25ºC, no growth was observed at an a_w value of 0.984 (Figure 1b-c).

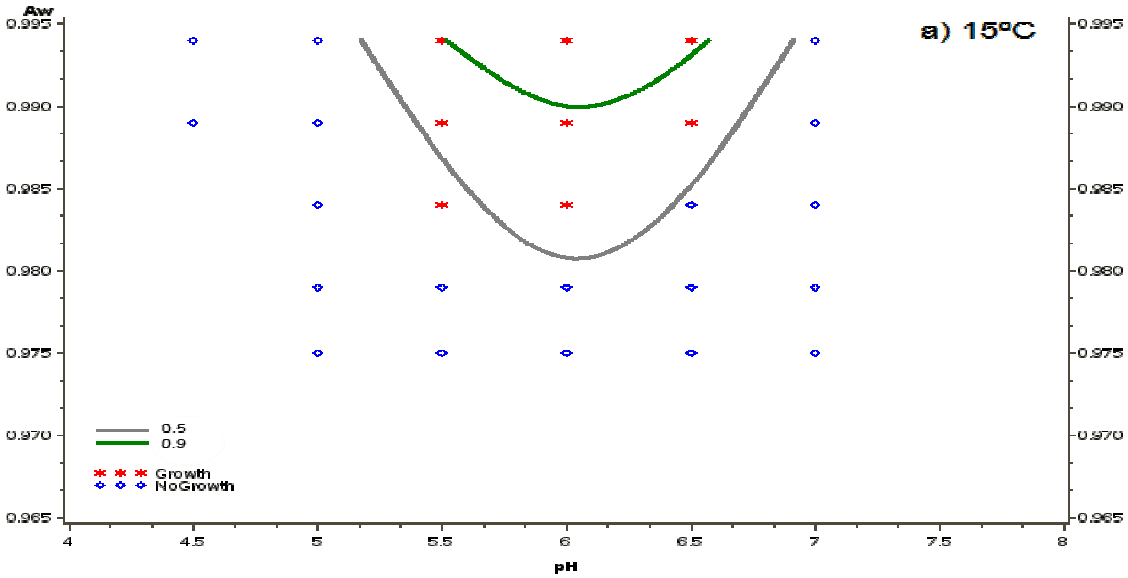
The observed and predicted results of *C. difficile* growth/no growth as a function of temperature and a_w at fixed pH levels of 5.0, 6.0 and 7.0 are shown in Figure 2a-c.

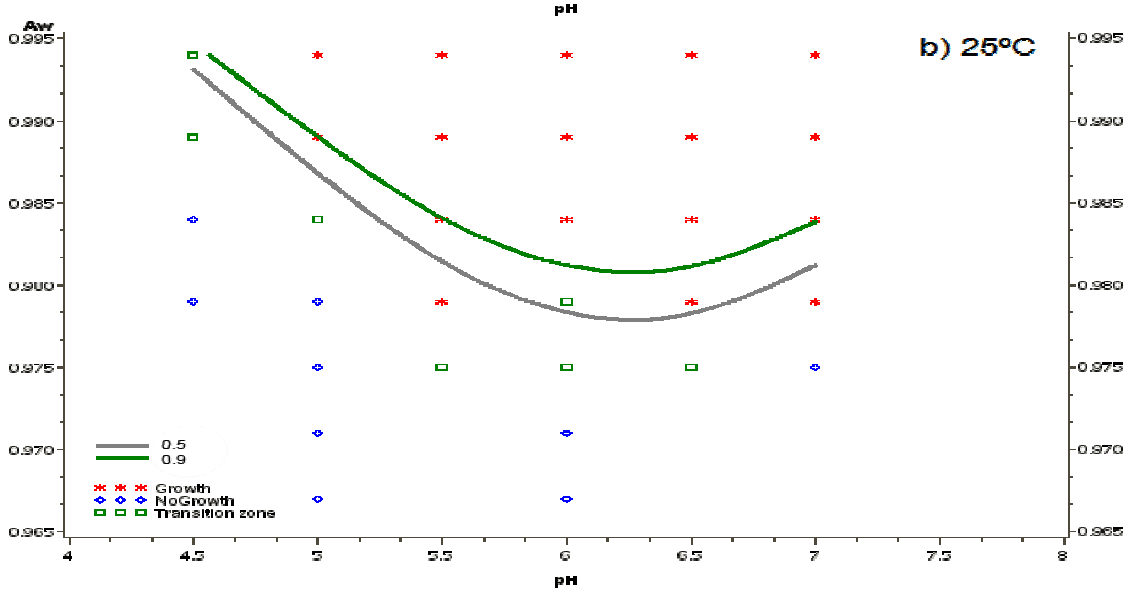
According to the model, the pH at which *C. difficile* has the greatest resistance to low temperatures occurs between 5.5 and 6.5. Figure 2b shows that growth at pH 6.0 was observed and predicted to occur in at least 50% of cases over a wide range of temperatures and low a_w values. The probability of growth at low a_w was best at moderate to high temperatures (T = 25 and 35ºC) and similar at either pH 6.0 or 7.0 (Figure 2b-c).

Water activity effect

Figure 1a-c shows that a small decrease from 1.000 in a_w would have the largest effect on growth when the pH was low. At nearly neutral pH, the probability of growth was less affected by the a_w.

Figure 1. Growth / no growth interfaces for *C. difficile* as a function of pH and a_w at fixed temperatures of a) 15 ºC, b) 25 ºC and c) 35ºC as predicted by the logistic regression model with fixed probabilities of 0.5 (grey curve) and 0.9 (green curve). Data points reflect observed growth: $p \geq 0.5$; transition zone: $0 < p < 0.5$; and no growth: $p = 0$.


a) 15°C
Aw
0.995
0.990
0.985
0.980
0.975
0.970
0.965
4
4.5
5
5.5
6
6.5
7
7.5
8
pH
0.5
0.9
Growth
NoGrowth


b) 25°C
Aw
0.995
0.990
0.985
0.980
0.975
0.970
0.965
4
4.5
5
5.5
6
6.5
7
7.5
8
pH
0.5
0.9
Growth
NoGrowth
Transition zone

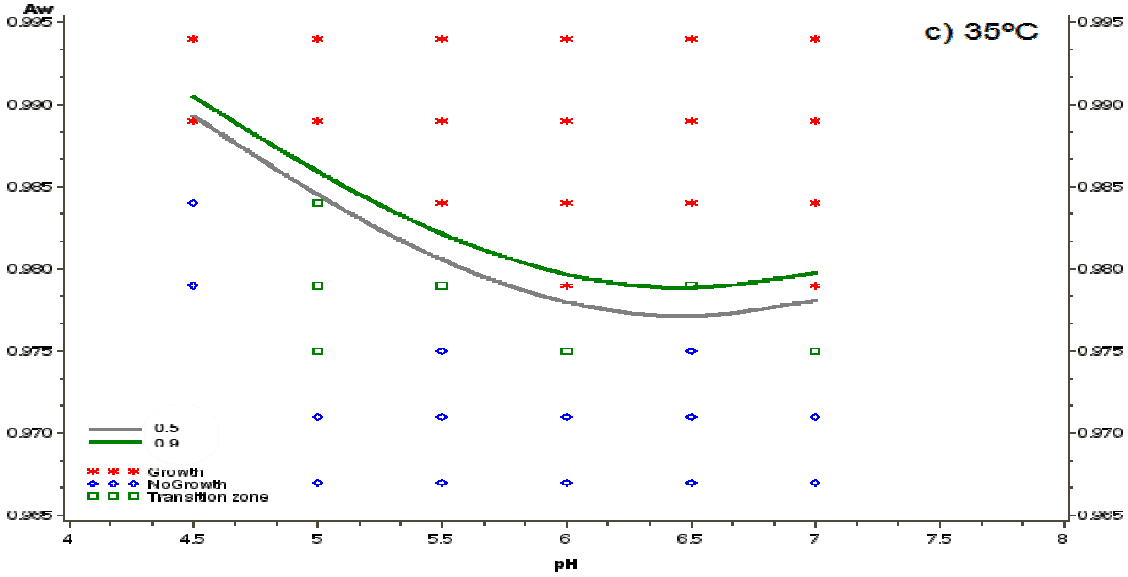
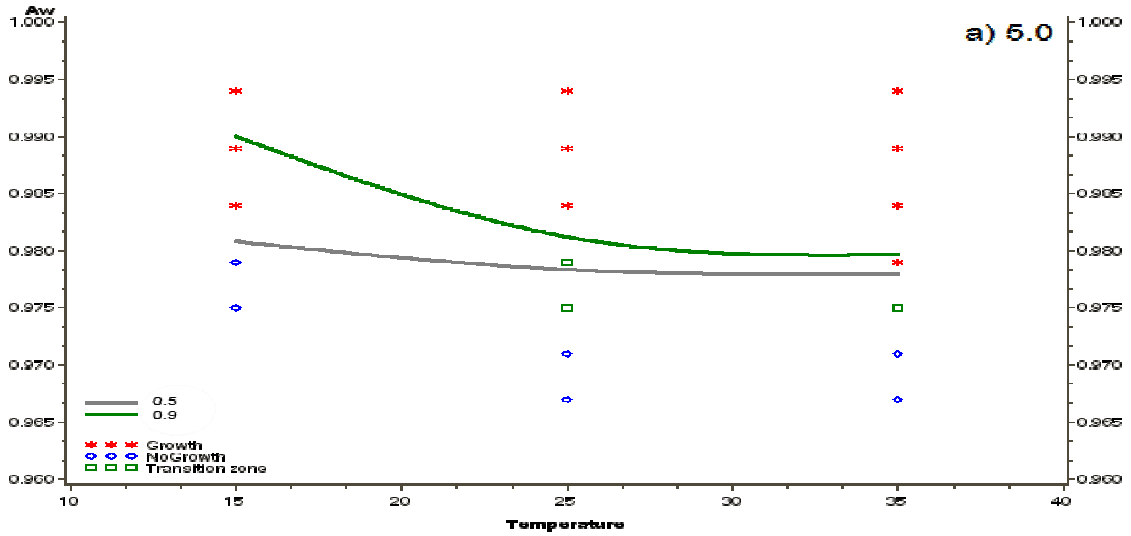
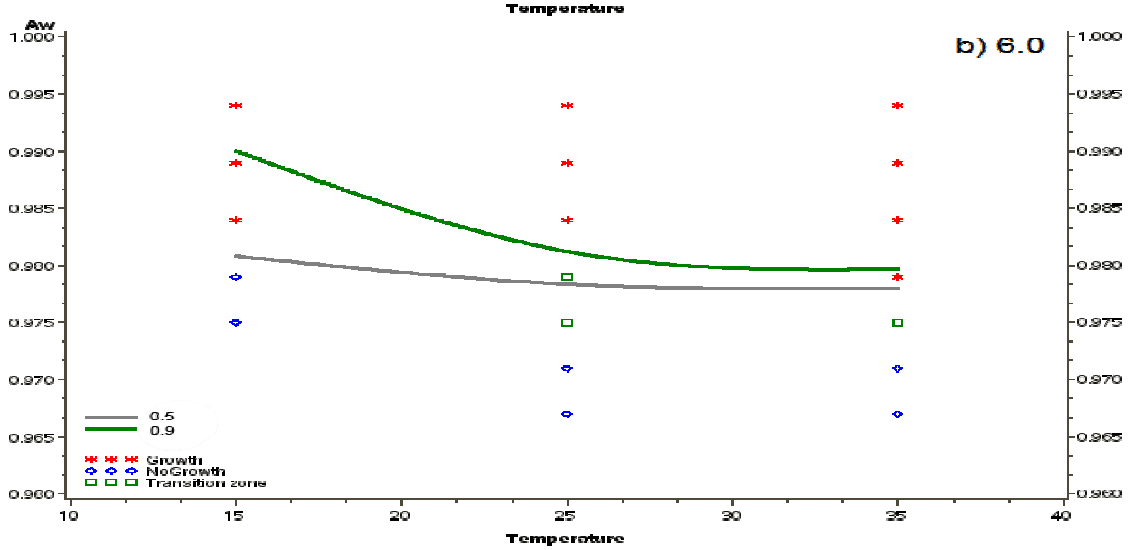
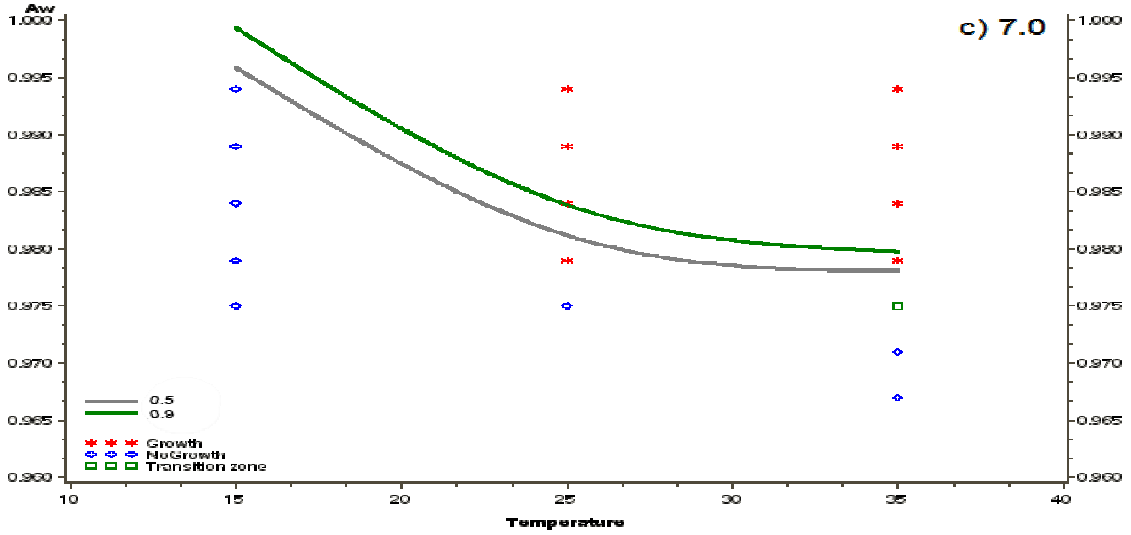

c) 35°C
Aw
0.995
0.990
0.985
0.980
0.975
0.970
0.965
4
4.5
5
5.5
6
6.5
7
7.5
8
pH
0.5
0.9
Growth
NoGrowth
Transition zone

Figure 2. Growth / no growth interfaces for *C. difficile* as a function of temperature and a_w at fixed pH levels of a) 5.0, b) 6.0 and c) 7.0 as predicted by the logistic regression model with fixed probabilities of 0.5 (grey curve) and 0.9 (green curve). Data points reflect observed growth: $p \geq 0.5$; transition zone: $0 < p < 0.5$; and no growth: $p = 0$.


a) 5.0
Aw
1.000
0.995
0.990
0.985
0.980
0.975
0.970
0.965
0.960
10
15
20
25
30
35
40
Temperature
0.5
0.9
Growth
NoGrowth
Transition zone


b) 6.0
Aw
1.000
0.995
0.990
0.985
0.980
0.975
0.970
0.965
0.960
10
15
20
25
30
35
40
Temperature
0.5
0.9
Growth
NoGrowth
Transition zone


c) 7.0
Aw
1.000
0.995
0.990
0.985
0.980
0.975
0.970
0.965
0.960
10
15
20
25
30
35
40
Temperature
0.5
0.9
Growth
NoGrowth
Transition zone

The observed and predicted results of *C. difficile* growth/no growth as a function of temperature and pH at fixed a_w values of 0.984, 0.989 and 0.994 are shown in Figure 3a-c. The model showed that growth was restricted by low a_w, particularly when the pH was outside of a pH between 5.5 and 6.5. For example, at high a_w (0.994) and temperature (35ºC), growth of *C. difficile* was predicted in 50% of cases at pH < 5.0 (Figure 3c). When the a_w value was 0.984, however, no growth was predicted at pH < 5.0, regardless of temperature (Figure 3a).

In Figure 3a-c, the distance between the contour lines of the predicted model widened as the a_w decreased. Therefore, around the growth thresholds, temperature changes were predicted to have a smaller effect on the probability of growth at low a_w than when the a_w was high.

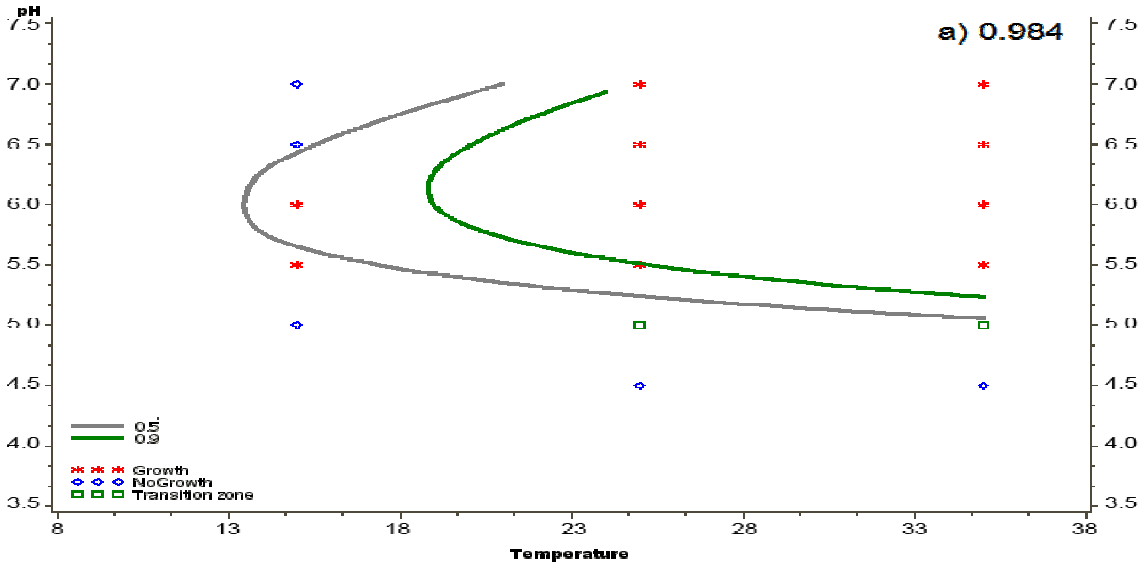
2.3.4 Comparison of growth tolerances between strains

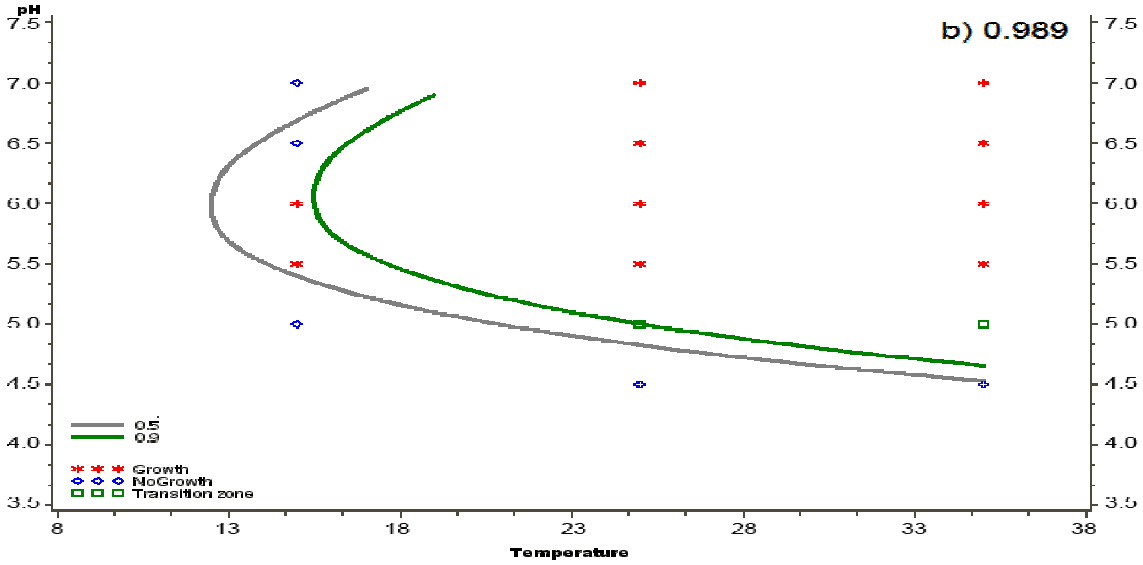
The environmental tolerance for the growth of more diverse *C. difficile* strains was assessed. Over 5 weeks, the growth limiting conditions for four monocultures of strains isolated from food and humans were examined as above. The two human isolates (BI-7 and 11ACD0075) were *C. difficile* 027 strains isolated from different individuals and geographic locations. One food isolate was a 078/126-like strain isolated from ground lamb. The other strain (GrCh1007398) was ribotype 067, a non-toxigenic strain isolated from ground chicken.

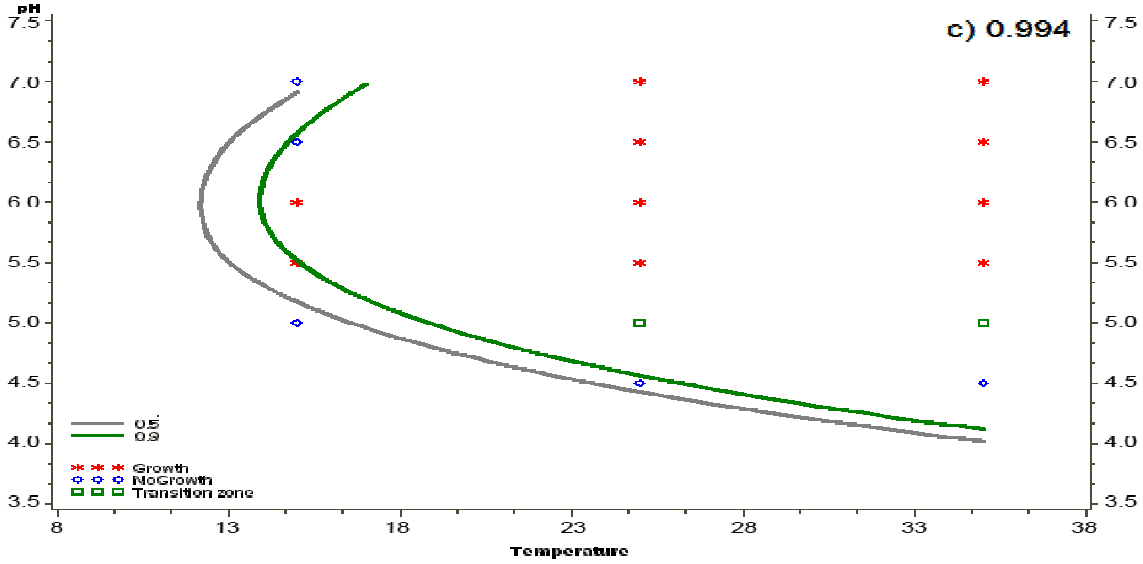
There were only marginal differences observed in the environmental tolerances of the different strains, and no clear trends of one strain's high tolerance or intolerance to hurdles were noticed. These results are depicted in Figure 4 and further described below.

All of the strains, except 11ACD0075, grew at pH 4.5, but only with a temperature of 35ºC. The minimum a_w value for the growth of all of the strains was 0.975 at 35ºC and this

Figure 3. Growth / no growth interfaces for *C. difficile* as a function of temperature and pH at fixed a_w values of a) 0.984, b) 0.989 and c) 0.994 as predicted by the logistic regression model with fixed probabilities of 0.5 (grey curve) and 0.9 (green curve). Data points reflect observed growth: $p \geq 0.5$; transition zone: $0 < p < 0.5$; and no growth: $p = 0$.


a) 0.984
pH
Temperature
0.5
0.9
Growth
NoGrowth
Transition zone


b) 0.989
pH
Temperature
0.5
0.9
Growth
NoGrowth
Transition zone


c) 0.994
pH
Temperature
0.5
0.9
Growth
NoGrowth
Transition zone

only occurred at a nearly neutral pH of 6.5 or 7.0. The harshest condition where growth was observed for all strains was at 15ºC, pH 5.5 and an a_w value of 0.989.

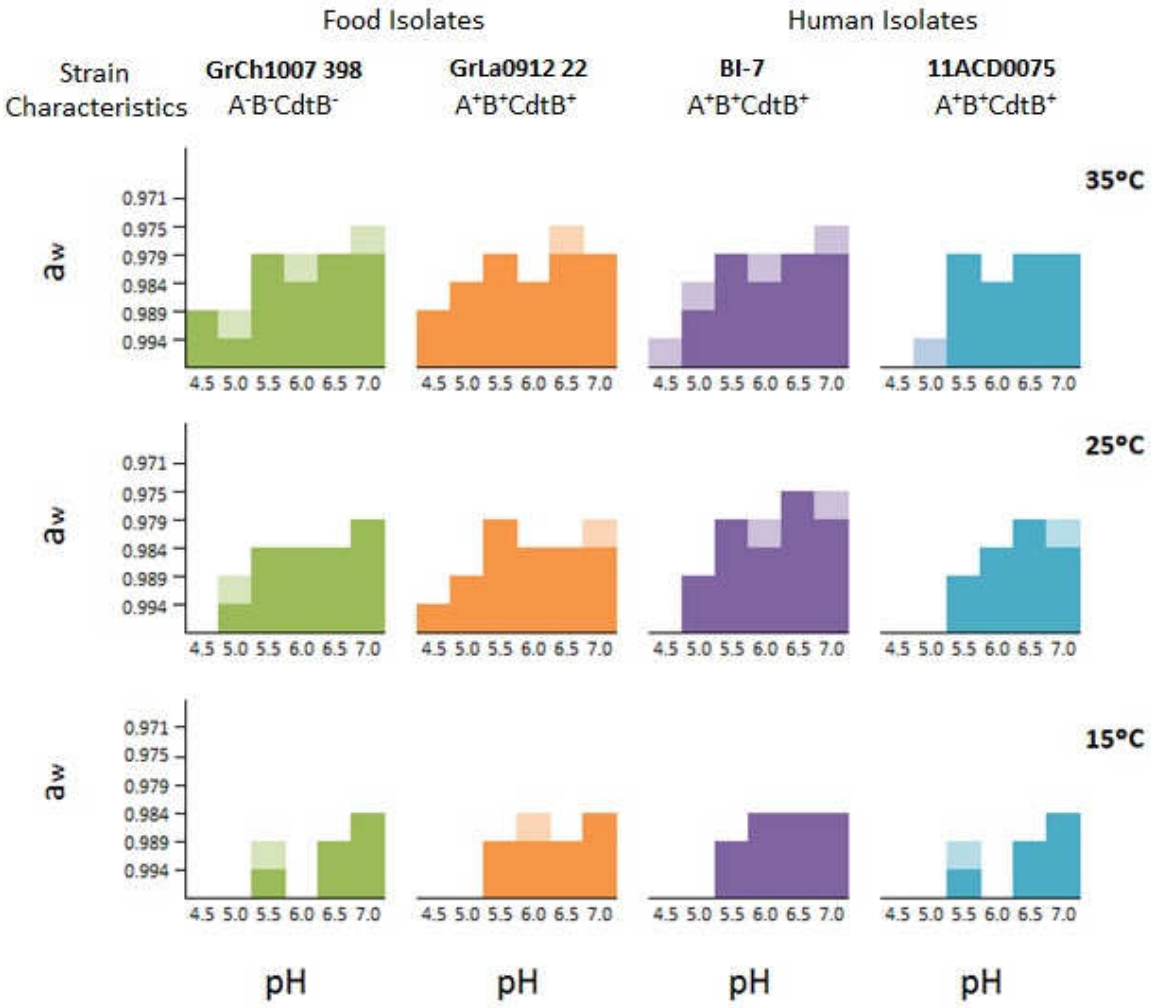
GrLa0912 22, a 078/126-like toxigenic isolate from food, was the only strain able to grow at pH 4.5 at 25ºC. *C. difficile* strain BI-7 had a slightly higher tolerance at 25ºC to low a_w; while the other three strains could grow down to an a_w value of 0.979 at pH 6.5 and 7.0, this human isolate (BI-7) grew at an a_w value of 0.975. The other ribotype 027 strain, 11ACD0075, had slightly less ability to grow under suboptimal conditions, particularly at a pH of 5.0 as compared to the other strains. However, at 15ºC, it was equally as tolerant as the avirulent food isolate, GrCh1007 398.

2.4 DISCUSSION

2.4.1 Genetically similar strains demonstrated similar environmental tolerance

The growth thresholds of *C. difficile* have never been assessed in a systematic fashion. While *C. difficile* spores are most often implicated in causing CDI, contaminating spores may be able to germinate in food and, as a result, increase the bacterial load and risk for *C. difficile* colonization following ingestion. Known germinants of *C. difficile* spores that are found in the gut include cholate, glycine and bile derivatives such as taurocholate (144). Lysozyme with thioglycollate are also germinants used *in vitro* (104). However, germination can also occur spontaneously. Thus, we assessed conditions that potential vegetative cells in food could grow under, first by individually varying three frequently encountered food hurdles, i.e., a_w, pH and temperature. The growth-limiting conditions were found to be the same between the two genetically similar strains of *C. difficile*, however, the time to detection varied slightly at conditions less favourable to growth. Some variability in environmental tolerance was expected between strains due to genetic variation (157). In a

Figure 4. Combined growth limiting conditions of a_w, pH, and temperature for different *C. difficile* strain monocultures. Cultures were grown in microplates with up to 10 replicates per condition. Growth was assessed over five weeks and measured by OD_{600}. If more than 50% of the replicates reached an $OD_{600} \geq 0.2$, this was recorded as growth (solid colour). If more than 0 but less than 50% of the replicates reached this threshold, it was recorded as transitional growth (faded colour).


Food Isolates
Human Isolates
Strain Characteristics
GrCh1007 398
A-B-CdtB-
GrLa0912 22
A+B+CdtB+
BI-7
A+B+CdtB+
11ACD0075
A+B+CdtB+
35°C
25°C
15°C
aw
0.971
0.975
0.979
0.984
0.989
0.994
4.5 5.0 5.5 6.0 6.5 7.0
pH

later experiment of this study, we assessed the growth of more genetically distant strain types.

It had been previously found in our lab that *C. difficile* growth was inhibited in media with an a_w value between 0.973 - 0.960 (unpublished data). Thus, we tested for growth in media within this range to more closely determine the limit. The pH values tested ranged from neutral (7.0) to acidic (4.0). The temperatures assessed represented those for optimal growth (35ºC), room temperature (25ºC), mild and moderate temperature abuse (10 and 15ºC respectively), as well as refrigeration temperatures (4ºC).

Turbidimetric analysis has been used frequently for testing growth / no growth boundaries, due to its ability to assess a wide variety of conditions efficiently (42, 155, 157, 163). With this type of study, since the resulting data is binary, growth [1] or no growth [0], recording OD without its relation to cell count is considered acceptable (36). As the first study to systematically examine the growth thresholds of *C. difficile*, we decided to use this method to obtain the best approximation of the bacterial growth limits. We acknowledge, however, that measuring growth by optical density is restricted because of the detection limit of approximately 10^7 cfu / mL (21). Thus, at harsher conditions than the thresholds we observed, the bacteria may have grown to a lower density than we could detect with our cell density meter. As the human dose response of *C. difficile* is not known, we cannot comment on the clinical risk of this possible low density of bacteria. Using OD measurements also does not differentiate live from dead cells. However, the initial inoculum used (10^4 - 10^5 cfu / mL) was below the limit of detection of the plate reader (data not shown), therefore by reaching a considerably higher OD than the limit of detection, 0.2 (21), growth was confirmed irrespective of the survival rate.

Bacterial growth under an individual hurdle was assessed in triplicate and it was found that as the media reached the limiting threshold for bacterial growth, there was greater variation in the OD values observed between replicates. These results were supported by a previous study with *Clostridium botulinum*, where growth was found to be less reproducible under growth limiting conditions (42).

2.4.2 Production and analysis of the logistic regression model for growth / no growth

For the assessment of growth under 99 different potential growth conditions, we inoculated the bacteria into 96-well microplates so that the growth could be assessed efficiently with an automated plate reader. To maintain the anaerobic environment needed for *C. difficile* growth, the microplates were inoculated anaerobically, covered with air-tight plate seals and then incubated anaerobically. The plate seals also limited media evaporation and allowed us to vigorously shake the plates prior to reading, in an attempt to resuspend the bacteria to get the most accurate OD readings.

To model a growth / no growth interface, we combined the two strains that had been first assessed individually. As described by Vermeulen et al. (157), it was expected that the hardiest strain would determine the growth boundaries. We used 30 replicates of each condition, so that we could observe conditions in which growth was not 100% probable. These conditions marked a transition zone and helped to better model a threshold interface. Particularly with decreasing temperature, the transition zone became more abrupt or non-existent. This observation has also been noted in studies using a large number of replicates for the growth assessment of *S. aureus* (155) and *L. monocytogenes* (39).

Because we followed a protocol similar to the G/NG study of *S. aureus* by Valero et al. (155), we also modelled the data similarly. Similar to their report, we found the terms T^2, b_w^2 and pH*b_w of Equation 1 to be non-significant. The performance statistics we obtained

were also similar, suggesting an appropriate goodness-of-fit. We also defined a cut-off point of $p = 0.5$ to determine growth or no growth. Notably, the polynomial logistic regression model would change with a different assumed probability level. The thresholds determined by a lower fixed probability may be desired if one would want to set a stricter limit for *C. difficile* growth in foods.

2.4.3 Assessment of growth under a combination of hurdles

We observed considerable additive or synergistic effects of combining hurdles on the growth of *C. difficile*. The bacteria could grow down to an a_w value of 0.969 in the optimal media at pH 7.0 and 35ºC (Table 5). It was predicted, however, that growth would be inhibited by an a_w value < 0.985 when combined with a pH < 5.0 at 35ºC, and any a_w at 15ºC and pH < 5.0 (Figure 1a-c).

Remarkably, with a combination of hurdles, we observed that the growth of *C. difficile* was most resistant to low temperature and a_w when in media between pH 5.5 and 6.5 (Figure 3a-c), considerably lower than the pH of 7.2 ± 0.2 used for making the optimal media (143). This experimental environmental tolerance is in accordance with the environmental niche of *C. difficile*. The bacteria colonize the human colon which has a slightly acidic to neutral pH of 5.6 to 7.3 (25). Like *E. coli*, it seems that *C. difficile* is most resistant to environmental conditions in suboptimal media (97). This finding should be taken into consideration when further assessing the growth potential of *C. difficile* in foods.

Because the growth boundaries of *C. difficile* have not been previously studied, the intervals of pH, a_w, and temperature that we tested were arbitrary. Due to time and materials, we were constrained to measuring growth at only three temperatures to maintain an anaerobic environment for *C. difficile.* Since our model showed good fit to the observed data, we would like to do future work with more conditions chosen in a systematic fashion. After

obtaining predicted growth thresholds, we can focus on obtaining more data where key changes in the growth of *C. difficile* occur. Examining smaller increments of the hurdles close to the conditions we found to be growth-limiting, may produce a better model of the growth interface for use in microbial risk assessments.

2.4.4 Little difference in tolerance for the growth of diverse *C. difficile* strains

We also tested four diverse strains of *C. difficile* to examine if there were strain- or origin-specific tolerances to the hurdles. We did not find considerable differences or trends in environmental tolerance among the different strains, between the monocultures and the strain cocktail, nor for the different origins of isolation. Two of the isolates were 027/NAP1 from humans and the other two were genotypically unrelated food isolates. These results suggest that the thresholds of growth for *C. difficile* may not be significantly affected by their environmental origin. This has been noted previously with *L. monocytogenes* (157). Thus, adjusting the hurdles in food beyond our observed thresholds may prevent the growth of a wide variety of *C. difficile* strains, including the most prevalent hypervirulent pathogens. Promisingly, as well, compared to other foodborne pathogens, the limiting conditions affecting growth are not very harsh, particularly for water activity. We observed that an a_w value of 0.971 prevented growth of *C. difficile* at any temperature or pH. This corresponds to a 5% salt concentration (92) and is similar to the a_w tolerance of nonproteolytic *C. botulinum* (Table 2). As shown in Table 2, other common foodborne pathogens have a considerably higher tolerance, being able to grow down to an a_w value of 0.950 or lower.

2.4.5 Assessment of growth thresholds considering risk in foods

It should be noted that the lowest pH value we tested was 4.5 and the lowest temperature was 15ºC. We observed transitional growth at some of the a_w, pH or temperature hurdle combinations at either of these levels. Therefore, the actual limiting pH and

temperature for *C. difficile* growth may be even lower. The thresholds we reported are likely dependent on other environmental factors as well, which we maintained constant by using laboratory media. Within a complex food environment, there may be other growth inhibiting or promoting factors which could alter our experimental growth boundaries. Further examination of the limiting factors for *C. difficile* growth will require validation of the model in food. In addition, the growth thresholds may change under the use of different products to adjust the hurdles. We used NaCl to adjust a_w, however, other humectants such as glycerol, sucrose, and fructose could also be used. These may have different effects on the growth supporting potential of media due to their distinct physical properties as solutes. For example, it has been found that using alternate humectants decreased microbial growth tolerance, but was dependent on the pH level (146). Organic acids have also been found to be more effective acidulants than HCl in inhibiting the growth of *L. monocytogenes* (34).

The average environmental conditions of refrigerated raw ground meat are normally a temperature of 2 to 8ºC, a pH > 5.5 and a_w value of 0.999 (59). At otherwise optimal conditions, we did not observe any growth of *C. difficile* below 15ºC (Table 5). Psychrotrophic bacteria are able to grow under 5ºC. It was reported by one group that one isolate of *C. difficile* could grow weakly at -1.5ºC. This was determined by observing an increase of 0.5 units of OD in peptone yeast extract glucose broth and/or colony growth on Columbia blood agar after one week (27). Our results do not agree with those findings that *C. difficile* may be psychrotrophic. The discrepancy may reflect the use of different media, the incubation time and/or the definition of "growth" used in our study. Our results suggest that at refrigeration temperatures, the recommended storage conditions for many foods, *C. difficile* will likely not grow. It is known, however, that temperature abuse occurs frequently prior to consumption, which could still put some foods at risk. We did observe

growth of *C. difficile* at 15ºC within the typical pH and a_w conditions of ground meat. Furthermore, cheeses like Brie and Camembert are ripened at 10-15ºC and often left at room temperature prior to consumption (35). Brie and Camembert cheese have a pH value > 5 and an a_w value of 0.980 and 0.982, respectively (35). According to our results, these cheeses could permit the growth of *C. difficile* at ambient temperature. At 15ºC and otherwise optimal conditions, it took at least 16 days for detectable growth of the *C. difficile* strain cocktail to occur (Table 5). Raw ground meats have a short shelf life of a few days, whereas that of cheese, smoked fish and vacuum-packed cured meats can be considerably longer. Further studies in this field should also take the "time to detection" into account to assess the potential for *C. difficile* outgrowth in foods.

CHAPTER 3: ISOLATION OF *C. DIFFICILE* FROM RETAIL FOODS

3.1 INTRODUCTION

3.1.1 Contamination of meats

To-date, zoonotic or foodborne transmission of *C. difficile* has not been established epidemiologically. However, the discovery of *C. difficile* strains in livestock and food that are indistinguishable from pathogenic human isolates suggests that interspecies transmission may occur through a food vector. A recent study showed that *C. difficile* spores can survive the minimal recommended temperature for cooking ground meats (71ºC) for at least 2 h (124). This demonstrates that it is likely that consumers are being exposed to viable toxigenic and even hypervirulent *C. difficile* such as 078, a strain that has been frequently isolated from food animals and products (Table 1). Increased vigilance in food production and preparation may therefore be required to limit the dissemination of *C. difficile* and its associated disease.

In this study, ground meat products from Ottawa, Canada, were tested for the first time. Previous studies from other regions have examined ground beef, pork, and veal, as well as whole pieces of chicken, for the presence of *C. difficile* (Table 1). Our study is the first in Canada to examine the prevalence of *C. difficile* in retail ground lamb, ground chicken and ground turkey. It has been reported that the isolation of *C. difficile* in Canadian ground meats was highest in the winter months of January and February (123). Our report also examines any trends in seasonality.

Similarly to other prevalence studies, enrichment culture was used to detect the bacteria. The one study that enumerated *C. difficile* spores in ground beef and pork found

low numbers of spores present in uneven distribution (161). In order to be able to compare results, we used similar protocols to other researchers for spore isolation and detection.

3.1.2 *Clostridium* spp. in cheese / milk

Several *Clostridium* spp., including *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum* and *C. sporogenes*, are considered as spoilage organisms for cheese (23, 50). Clostridial spores, due to their high resistance to heat, are not inactivated by pasteurization, which involves a 15 second incubation at 72ºC (35). Under suitable conditions, contaminating spores then may germinate to form vegetative cells that ferment lactic acid, producing carbon dioxide, hydrogen, butyric acid and acetic acid (148).The over-production of these by-products can cause late-blowing of the cheese packages and loss of product (148).
C. botulinum has also caused morbidity and mortality from the consumption of contaminated cheese (6, 153).

3.1.3 Sources of spore contamination in cheese

The teats of cows who eat low microbiological quality silage may become contaminated with clostridial spores (148). Spores of *C. tyrobutyricium* have been found in raw milk and likely come from cow teats during milk collection (148). The frequency of finding *C. difficile* spores in bovine faecal matter (Table 1) might also promote this method of dissemination into raw milk as well. Spores may additionally contaminate dairy products at any point during processing, as was found with *Bacillus cereus* (149).

During processing, milk undergoes a specialized centrifugation step called bactofugation to lower spore contamination. After bactofugation at approximately 9000 x g, the resulting sludge pellet can be removed and heat treated at 135ºC for 3 - 4 seconds to inactivate spores. The pellet may then be re-added to cheese milk to prevent yield loss (79). The size of the spores affects the effectiveness of this centrifugation step. Larger spores such

as *C. butyricum* are more effectively removed than smaller ones (148) and thus, a low number of spores may still be present following bactofugation. These contaminanting spores may persist in cheese for years. It was found that the number of *C. botulinum* spores artificially spiked into cheese decreased, remained equal or even increased over a 6-year incubation at 2 or 4ºC (44). Furthermore, commercial cheese brine is often reused several times, producing a nutrient-rich environment that could harbour spores and serve as another source of contamination. It was found that spores of *C. tyrobutyricum* and *C. sporogenes* could survive in 2% whey containing a 23% NaCl brine over 9 weeks at 15ºC, 8ºC or 4 ºC. However, *C. butyricum* and *C. beijerinckii* could not survive these conditions (148). Specific concentrations of salt in brine and/or ripening temperatures are used to prevent germination and growth of spores that cause late blowing. Without these precautions, even 5 to 10 spores of *C. tyrobutyricum* per litre could cause product loss (148).

There have been few studies of *C. difficile* contaminating dairy products. The two studies available to date on cow's milk did not find *C. difficile* within bactofugate or retail samples (55, 66). *C. difficile* has also not been reported in cheese before. Therefore, we examined semi-soft cheese for *C. difficile* contamination along with raw ground meats.

3.2 METHODS

3.2.1 Samples

All food samples were collected from four retail outlets in Ottawa, Ontario. A total of 725 samples of ground beef, chicken, lamb, pork, turkey and veal were collected between July 2009 and January 2011. Each month, 22 to 110 packages were obtained. Prior to processing, samples were either refrigerated at 4ºC for up to 7 days or frozen at -20ºC. In all, 146 cheese packages (Brie, Camembert, goat or sheep's milk cheese, surface-ripened, blue-

veined) were collected between March 2010 and September 2010. One-hundred cartons of retail dairy milk (Skim, 1%, 2%, and 3.25% milk fat; chocolate, strawberry, and vanilla flavoured; 5%, 10% and 35% cream) were similarly obtained between August 2010 and October 2010. Cheese and milk samples were stored at 4ºC up to 7 days after collection.

3.2.2 Ground meat sampling and enrichment culture

Enrichment broth cultures were used to detect the presence of *C. difficile*. Duplicate 25 g samples of ground meat were removed with a sterile scoop and added to sterile culture containers. *C. difficile* broth (125) which was used for culture enrichment, contained *C. difficile* agar base (CM0601, Oxoid), 0.1% (wt/vol) sodium taurocholate, and 5.5% *C. difficile* moxalactam norfloxacin [CDMN] selective supplement (Oxoid). The samples were placed into 250 mL of *C. difficile* broth. The meat was mixed by hand with the enrichment broth. Positive controls, which were run with each set of enrichment cultures, consisted of an additional 25 g of one of the ground meats spiked with 10 spores of *C. difficile* strain 11ACD0075 (Table 3) in duplicate. Cultures were incubated anaerobically at 35ºC for 72 h.

3.2.3 Cheese sampling and enrichment culture

Duplicate 25 g of cheese samples from each package (approximately 100 g) were added to 100 mL of *C. difficile* broth and 0.2% (wt/vol) of sodium citrate. One additional 25 g sample was spiked with 10 spores as a positive control with each set of enrichment cultures. Cheese mixtures were homogenized by a stomacher for 45 s in Stomacher™ bags (Seward, Worthing, West Sussex). The 100 mL mixtures were transferred into sterile culture tubes from the Stomacher™ bags using three 50 mL rinses of the bags with *C. difficile* broth containing 0.2% (wt/vol) sodium citrate. This made a total culture volume of approximately 250 mL. Cultures were incubated anaerobically at 35ºC for 72 h.

3.2.4 Milk sampling and enrichment culture

Duplicate 25 mL milk samples were taken from each carton and added to 225 mL of *C. difficile* broth with 0.2% (wt/vol) sodium citrate. One additional 25 mL sample from a milk carton was spiked with 10 spores as a positive control with each set of enrichment cultures. Cultures were incubated at 35ºC for 72h anaerobically.

3.2.5 Detection and isolation of *C. difficile*

After enrichment, the cultures were mixed and two 0.5 mL aliquots were removed. These aliquots were mixed 1:1 with 100% ethanol for 1 h at room temperature. Ethanol-shocked cultures were centrifuged at 1500 x g (Centrifuge 5424, Eppendorf, Mississauga, ON) for 10 min. Supernatants were discarded and pellets were resuspended in sterile water for plating onto *C. difficile* blood agar. The blood agar consisted of *C. difficile* agar base (Oxoid), 7% (vol/vol) laked horse blood (Oxoid), and 5.5% (wt/vol) CDMN supplement (Oxoid). These plates were incubated anaerobically at 35ºC for 72 h. Suspect colonies of *C. difficile* (flat, irregular, non-haemolytic and pale-white colonies) were subcultured on BHIS+T agar for 48 h and confirmed by characteristic odour, the activity of L-proline aminopeptidase (PRO-Disk, Remel, Lenexa, Kansas) and PCR amplification of the triose phosphate isomerase (*tpi*) gene fragment. For storage, a single colony from a positive isolate was subcultured overnight in 5 mL of BHIS broth at 35ºC anaerobically. A 1 mL aliquot of the culture was mixed 2:1 with 50% glycerol and stored at -80ºC.

3.2.6 Analysis of factors associated with the presence of *C. difficile* in foods.

The prevalence of *C. difficile* in foods was compared by type of food, season or month in SAS, using the complete data sets from the sampling of ground meats and cheese. The presence (positive outcome) and absence (negative outcome) of *C. difficile* in a food sample were coded as “1” and “0” respectively. To assess the seasonality of *C. difficile* in

ground meat, variables for season and month were assigned based on the month of the expiry date stated on the sample package. Seasons were coded as "Winter" (December, January, February), "Spring" (March, April, May), "Summer" (June, July, August), or "Fall" (September, October, November).

Descriptive analysis examined the consistency of the observations, as well as unlikely or missing values. Bivariate association between the type of meat, season and month to a positive outcome was analyzed using χ^2 (chi-square) analysis and evaluated for significance at $p \leq 0.05$. The bivariate association between the levels of each variable to the outcome was then evaluated using a simple logistic regression analysis. A significant association was determined at $\alpha = 0.05$. Significant variables by bivariate analysis were then considered in the multivariate logistic regression analysis. This was done using an unweighted forward stepwise selection in PROC LOGISTIC with SAS to evaluate an interaction between the main significant variables. The fit of the final model was assessed with the Homer and Lemershow goodness-of-fit statistic.

Fisher's exact test was used to determine the association between the type of cheese and the presence of *C. difficile*, since a low number of positive outcomes precluded the use of a likelihood ratio χ^2 test.

3.3 RESULTS

3.3.1 *C. difficile* isolation from ground meats

C. difficile was isolated from 93 (12.8%) of the 725 ground meat samples tested. All of the different meat types spiked prior to enrichment culture as positive controls resulted in

detectable *C. difficile* growth. Thus using enrichment culture, the detection threshold was 10 spores per 25 g of meat.

Clostridium difficile positive samples were found in 25/116 lamb (21.6%), 17/114 veal (14.9%), 18/147 (12.2%) chicken, 18/152 (11.8%) beef, 10/101 (9.9%) pork and 5/95 (5.3%) turkey samples. The highest proportion of meat contaminated with *C. difficile* was lamb (21.6%). Bivariate logistic regression analysis showed that the prevalence in lamb was significantly higher ($p < 0.05$) than all of the other meats except for veal ($p = 0.195$). The 14.9% prevalence of *C. difficile* in veal was the second highest among the meats, which was significantly different from that in turkey ($p = 0.03$), the least commonly contaminated meat. These results are summarized in Figure 5.

Meat samples were collected for over one year to assess trends in *C. difficile* seasonality. Likelihood ratio χ^2 derived from χ^2 analysis showed that associating the type of meat ($p = 0.0141$), season ($p < 0.001$), and month ($p < 0.001$) to the presence of *C. difficile* was valid. The monthly prevalence of *C. difficile* ranged from 7.3% to 40.9%. February, January and December months had the first (40.9%), second (22.9) and third (19.8) highest prevalence rates compared to the other months of the year. The prevalence in February was significantly higher than all of the other months, except for January ($p = 0.10$). The prevalence of *C. difficile* in meat in January was significantly higher than that found in the summer months (June, July, August), as well as March and October. In December, the prevalence of *C. difficile* in meats was only significantly higher than the prevalence found in the summer months. A summary of these results are shown in Table 8.

A multivariable logistic regression model was used to determine any statistical significance between the prevalence of *C. difficile* by season. The model revealed a

Figure 5. The percentages of retail ground meat samples by meat type that were positive for *C. difficile*. All positive samples of meat were confirmed positive after the isolation of *C. difficile* from enrichment broth culture. Numbers above the bars indicate the sample size.

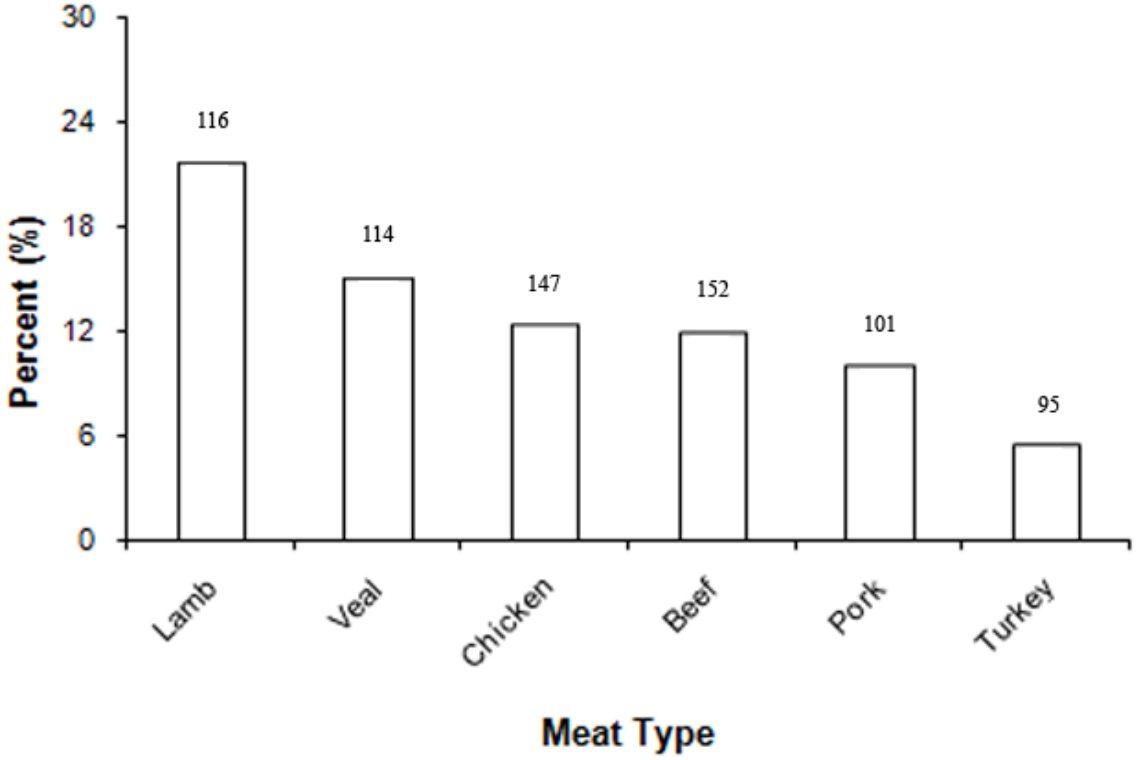

30
24
18
12
6
0
Percent (%)
116
114
147
152
101
95
Lamb
Veal
Chicken
Beef
Pork
Turkey
Meat Type

Table 8. The monthly incidence of *C. difficile* in meat samples obtained from Ottawa, Ontario retail stores.

				95% Confidence Limit	
Month	**Sample Size**	**Positive Samples**	**Incidence (%)**	**Lower**	**Upper**
Feb	22	9	40.9	18.6	63.2
Jan	70	16	22.9	12.8	32.9
Dec	81	16	19.8	10.9	28.6
Nov	71	12	16.9	8.0	25.8
April	40	5	12.5	1.8	23.2
Sept	46	4	8.7	2.3	17.2
Oct	58	5	8.6	11.8	16.1
March	58	5	8.6	1.2	16.1
July	84	7	8.3	2.3	14.4
May	38	3	7.9	0.01	16.9
June	110	8	7.3	2.3	12.2
August	47	3	6.4	0.001	13.6
Total	**725**	**93**	**12.8**	**10.4**	**15.3**

significantly higher proportion of *C. difficile* in meats obtained in the winter than the other three seasons ($p \leq 0.005$).

No significant difference existed between the prevalence rates in the spring, summer or fall (Figure 6).

3.3.2 *C. difficile* isolation from cheese and milk

C. difficile was isolated for the first time from various semi-soft cheeses, with a total of 14/146 samples (9.6%) being found positive. Positive samples were found in 3/19 (15.8%) Camembert, 3/31 (9.7%) blue cheeses, 3/34 (8.8%) Brie and 5/50 (10%) other semi-soft cheeses. *C. difficile* was not isolated from 12 goat milk cheese samples. By Fisher's exact test, no significant association was found between the type of cheese and the presence of *C. difficile* (Figure 7).

The cheeses tested were produced in 6 countries, namely Canada, Denmark, France, Germany, Italy and the USA. The highest proportion of positives (2/8; 25%) was from Italy. However, due to the small overall number of positive samples, the different proportions of *C. difficile* in cheese by country of origin, was not significant. Out of the 75 Canadian cheeses tested, only cheeses produced in the province of Quebec were positive for *C. difficile*. This difference in prevalence, however, was also not statistically significant (Appendix B, Figure b1).

Two pasteurized milk samples out of 100 (2.0%) contained *C. difficile*. This was the first report of *C. difficile* spores being present in pasteurized milk. None of the flavoured milk products or cream varieties were positive for *C. difficile*. The milk was produced in Canada, but the regional origin was not indicated by the manufacturers.

All of the positive control spiked cultures of dairy products resulted in detectable *C.*

Figure 6. Seasonality assessment of *C. difficile* in ground meats. All positive samples were categorized by season according to month of the meat sample expiry date. Seasons were divided as follows: Spring (March, April, May), Summer (June, July, August), Fall (September, October, November) and Winter (December, January, February). Numbers above the bars indicate the sample size.

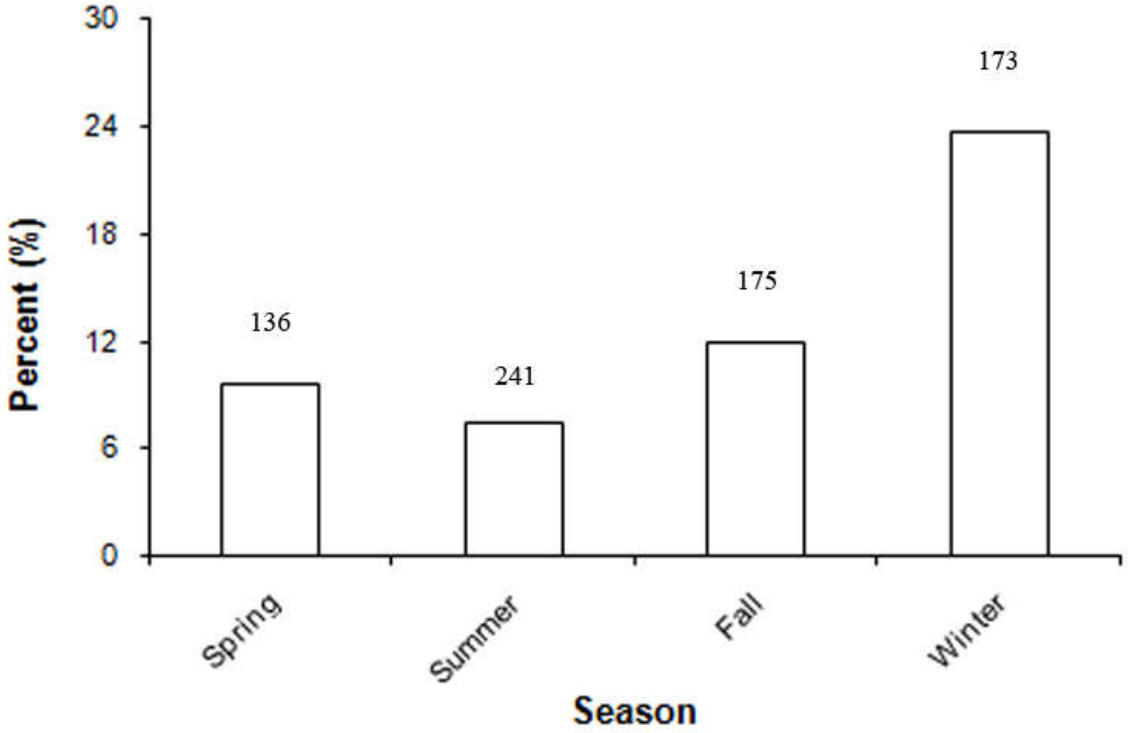
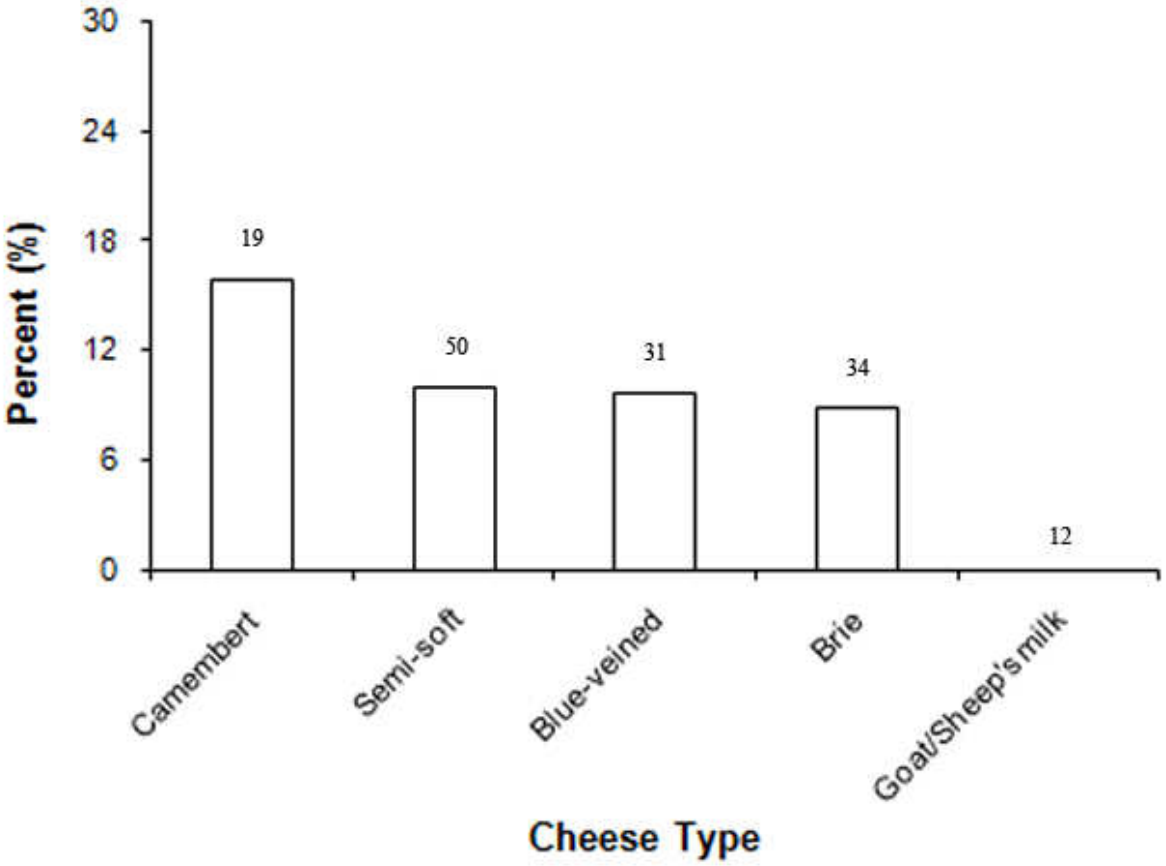

30
24
18
12
6
0
Percent (%)
136
241
175
173
Spring
Summer
Fall
Winter
Season

Figure 7. Percentage of retail cheese samples that were positive for *C. difficile* by cheese type. Samples of cheese were enriched in selective *C. difficile* broth for 72 h. Aliquots of enrichment cultures were plated onto *C. difficile* agar for 72 h to observe colony growth. The cheese was recorded as being positive after confirmation of *C. difficile* isolation from the enrichment culture. Numbers above the bars indicate the sample size.


30
24
18
12
6
0
Percent (%)
19
50
31
34
12
Camembert
Semi-soft
Blue-veined
Brie
Goat/Sheep's milk
Cheese Type

difficile growth. Similarly to meats, the detection threshold was 10 spores per 25 g of cheese or 25 ml of milk/cream.

3.4 DISCUSSION

3.4.1 *C. difficile* was found in various raw ground meats

To isolate *C. difficile* from meats and attain the most comparable results, we followed a protocol that was developed by Rodriguez-Palacios et al. (125) and adapted in several other related reports (1, 10, 22, 101, 123, 160, 161). A few modifications were made from the original methods to try to obtain a higher detection sensitivity. Instead of rinsing the food sample in buffer and then culturing the rinsate for enrichment (123, 125, 160, 161), the whole food sample was mixed with enrichment broth to prevent the loss of spores that might be strongly associated with or even within the tissues of meat (156, 161). In addition, we tested 25 g portions of the meat package in duplicate, a larger sample size than in most of the previous studies (52, 66, 125, 141). This was done to obtain a higher probability of detecting spores, since they may be non-homogenously distributed (161).

Various retail ground meats from Ottawa, Canada were examined for spores of *C. difficile*. This was the first Canadian study to determine the presence of *C. difficile* in ground chicken, ground lamb, and ground turkey. As well, number wise, it was also the largest sampling of ground meats for *C. difficile* contamination to-date. Overall, we found a 12.8% prevalence of *C. difficile* in ground meats. The isolation of *C. difficile* in meats was expected, as several ground meats had previously been tested in Canada (Table 1). Moreover, our prevalence rate was consistent with previous Canadian findings (125, 160, 161).

The first Canadian study on *C. difficile* in ground meats found an overall prevalence of 20% (125). While our overall prevalence of *C. difficile* in beef was lower, their sampling of ground veal revealed a 14.3% prevalence, which agrees with our findings of 14.9%. Our findings were very similar to a later report from the same group that examined a wider range of Canadian ground beef and pork in 2009 (123). By meat type, the latter researchers found a 12% prevalence of *C. difficile* in both beef and pork, as compared to our findings of 11.8% and 9.9%, respectively. In the one Canadian investigation that examined whole pieces of chicken meat, a 12.8% prevalence was found, which is very similar to our finding of a 12.2% prevalence in ground chicken (160). True comparisons between these studies cannot be done because of our modified isolation techniques and because we did not conduct random sampling. However, our study's agreement with previous results, suggests that ground meats are regularly contaminated with *C. difficile* spores.

Ground turkey had been tested in two reports from the USA (55, 141). Only in the study by Songer (141), was *C. difficile* detected. These authors found a much higher prevalence (≥ 42%) of *C. difficile* in each of the meats they tested than all other studies to date, including ours. Our results suggest that *C. difficile* is least likely to be recovered from turkey (5.3%) than the other retail ground meats tested. Thus, our reported prevalence of *C. difficile* in turkey meat warrants further examination and comparison to future studies.

Ground meat from sheep and poultry had been examined previously in Sweden and the Netherlands (1, 22). Like other North American studies, our report found a higher prevalence of *C. difficile* in meat than had been reported in these and other European studies (Table 1). The variability in the prevalence of contamination between regions could be due to regional differences in niches of the bacteria. The use of different sample sizes may also have affected the different prevalences observed. The study from Sweden only analyzed 7

packages of lamb and 2 packages of poultry and did not find any *C. difficile* spores (1). The study from the Netherlands tested only 16 packages of lamb and found one positive (6.3%) (22). On the other hand, these authors sampled a large number (257) of chickens, and still found a low (2.7%) prevalence. The use of different sampling methods and culture conditions can also affect the limit of bacterial detection and thus, the resulting prevalence. It has been noted previously that three different culture techniques of the same ground meat samples resulted in significant variation in detecting positive cultures (123). Future experiments should be done to validate and standardize protocols for selection, culturing and detecting *C. difficile* spores in ground meats and other foods.

3.4.2 Young animal meats had a higher prevalence of contamination

Interestingly, we found that the highest prevalence of *C. difficile* by meat type were in the young food animals, lamb (21.6%) and veal (14.9%). Although we collected our samples from stores in a narrow geographic region, these results are consistent with the findings that *C. difficile* shedding is inversely proportional to the age of the affected animals (106, 169). Thus, the age of the livestock may be a significant factor in the risk of meat contamination. Furthermore, this shedding hypothesis lends credence to the view that the spores contaminating meat originate from an animal source. Our study was the first to test retail lamb in Canada and our results suggest that it is the ground meat that is most likely to be contaminated with *C. difficile.* All of the lamb samples we examined originated from New Zealand livestock. Therefore, the country of origin may have also contributed to the higher prevalence. Further research should be conducted on lamb, veal and other meat products from young livestock and different regions for comparison.

3.4.3 *C. difficile* contamination of foods was more common in the winter

Our results also corroborate the trend, noted by Rodriguez-Palacios (123), that there was a higher prevalence of *C. difficile* contaminating ground meats in the winter as compared to the other three seasons (123). The authors also noted that *C. difficile* was isolated primarily in January and February over a 7-month period. Although we did observe a higher prevalence of *C. difficile* spores in January and February, we did not obtain sufficient positive outcomes by month to definitively find a significant trend re the prevalence of *C. difficile*. It is known that contamination of cheese with *C. tyrobutyricum* spores is much higher in the winter. This has been attributed to the animals being fed in the crowded indoors in the cold winter months, rather than being out grazing (35). Increased *C. difficile* contamination may also occur in this manner. In addition, there is less soil turn-over during the winter, and this may promote dispersion of *C. difficile* on the surface of the ground so that the organism is more likely to come into contact with and colonize the animals (134).

Epidemiologically, a clinical study from Wales noted a higher incidence of CDI during January and February of 2010 (105). In Quebec, Canada, analysis of HA-CDI during both epidemic and non-epidemic years demonstrated a marked seasonality, with the highest incidence in the winter. The authors attributed this to the increase of respiratory infections that occur in the winter time leading to increased antibiotic use during this season (39). There has also been an association of *C. difficile* infection with seasonal influenza due to antibiotic use following influenza activity. This report from hospitals in the USA found that CDI increased by 23% during the winter months as compared to summer levels. The authors cautioned, however, that the perceived high incidence may also be due to extravigilance for enteric infections like rotavirus that also peak in the winter, as well as hospital overcrowding, creating a higher environmental risk for contracting *C. difficile* (114). Our results suggest

that an increase in *C. difficile* in foods during the winter could also contribute to this heightened disease incidence.

3.4.4 *C. difficile* was a contaminant of cheese

This study confirmed the presence of *C. difficile* spores in semi-soft cheeses for the first time. Cheese was chosen as a potential reservoir for *C. difficile* because dairy products are pasteurized, but this method is ineffective for spore inactivation. Furthermore, several *Clostridium* spp. can be found in cheese and have been implicated in foodborne illness or food spoilage.

C. difficile has not been previously detected in dairy products. A study by Bourhis et al. (23) used PCR amplification for detection of *Clostridium* spp., including *C. difficile* in cheese. However, the limit of detection of their method (100 cfu / g) was much higher than ours of 10 spores / 25 g. Houser et al. (55) did not find *C. difficile* spores in pellets of pasteurized milk using a PCR method which was specific for *C. difficile*. A third report by Jöbstl et al. (66) analyzed bactofugate by culturing the concentrated spore solution collected from raw milk. The authors reported that the solution contained approximately 200 spores of various bacterial species within the sample volume. Thus, they noted the possibility that *C. difficile* spores could have been present, but at a level that was below their method sensitivity. All of these papers demonstrate that a standardized method should be used to further investigate the presence of *C. difficile* spores in dairy products. Our results demonstrate the need for future examination of cheeses for comparative analysis.

It is interesting that our prevalence of *C. difficile* in cheese of 9.6% was near the overall prevalence that we found in ground meats (12.8%). Several of these food items were purchased from the same stores and may reflect contamination from the retail source. In order to assess the likelihood of a common contamination source, sub-typing of the isolates

was done. This was conducted and discussed in the following chapter of this report. It is possible that the similar prevalence of *C. difficile* contamination in these diverse foods reflects the organism's ubiquitous dissemination in the farm environment.

It was interesting that out of our samples, none of the Canadian cheeses were contaminated, except for those produced in Quebec. There were 7 of 62 (11.2%) cheeses from Quebec that were contaminated with *C. difficile* spores and 0 out of 16 from other Canadian provinces (Appendix B, Figure b1). In one of the studies by Rodriguez-Palacios (123), after sampling retail meat from three provinces, the leading source of contaminated meat samples was also from Quebec. Both this study and ours, however, did not have a significantly different prevalence of *C. difficile* in foods by region. Further investigation with a larger sample size and more varied sampling will be needed to further examine the relationship between the geographical prevalence of *C. difficile* in cheese and the high clinical incidence of CDI in Quebec.

3.4.5 *C. difficile* in retail milk

To examine if the source of cheese contamination was potentially through milk, we also tested various retail pasteurized milk and cream samples for the presence of *C. difficile* spores. Interestingly, there was a very low prevalence of milk contamination, i.e., 2.0%. This could indicate that the source of cheese contamination was not from the milk, but may have occurred during the various stages of cheese production or processing. For example, to prevent a 3% loss in yield, the bactofugate pellet from raw milk can be re-added to the cheese milk after heat-treatment. This treatment has been reported to reduce the butyric acid-forming spore prevalence by 98% (79). Thus some spores, like *C. difficile*, a non-producer of butyric-acid, may be re-added to cheese.

CHAPTER 4: STRAIN CHARACTERIZATION OF *C. DIFFICILE* ISOLATES

4.1 INTRODUCTION

4.1.1 Typing methods

The varied nomenclature for *C. difficile* strain types reflects the diversity of typing assays that are employed globally. Complicating epidemiological investigation, research and hospital laboratories use different typing techniques depending on the sensitivity and rapidity required (71). Even among research laboratories, no standardized typing system or nomenclature currently exists. One reason for this is that certain typing methods are not comparable between labs without reference isolates to assign standardized types. This is the case for restriction endonuclease analysis [REA] and PCR-ribotyping. More comparable typing methods include PFGE and toxinotyping, however, they do not have sufficient discriminatory power to be used alone. Various typing methods have generally shown good agreement, but this does not necessarily infer identical strains (41). Therefore, several techniques are now used in tandem. These will be described below.

4.1.2 Serotyping

Serotyping is no longer commonly used as a typing technique, but it was the first method used to cluster different strains of *C. difficile*. It did so according to bacterial antigenic properties that were differentiated using an agglutination reaction with rabbit serum (152). Its discriminatory power was found to be significantly lower than genotypic typing methods, such as random amplified polymorphic DNA [RAPD] and PCR-ribotyping (29).

4.1.3 Random amplified polymorphic DNA [RAPD]

RAPD is an appealing PCR fingerprinting method because the target genome sequence does not need to be known prior to amplification. For PCR, two short (e.g., 10 bp) primers with arbitrary sequences are bound to random fragments. Amplification only occurs if the two primers bind in the correct directions close enough together. Polymorphisms in the genome lead to the appearance or non-appearance of bands between different strains. RAPD is still used due to its cost effectiveness and ease of use, however, compared to PFGE, band analysis is more difficult and its reproducibility is lower (29).

4.1.4 Restriction endonuclease analysis [REA]

REA involves the analysis of whole cell DNA with a restriction endonuclease such as HindIII. It has high discriminatory power, however, the use of a restriction enzyme on the whole genome generates hundreds of fragments (26). These complicated fingerprints make analysis and interlaboratory comparisons difficult. To simplify gel analysis, restriction fragment length polymorphism [RFLP] was added to the REA process after gel electrophoresis and Southern blotting. This allowed the bands to be probed for specific differences in restriction sites. Notwithstanding, REA and RFLP techniques alone or combined are laborious, so PCR amplification techniques are currently preferred (26).

4.1.5. Toxinotyping

Toxinotyping classifies strains by variations within the PaLoc (pathogenicity locus) (129). Six regions within the PaLoc (A1-A3 and B1-B3) are PCR amplified and then cut with restriction enzymes to see characteristic RFLPs. The restriction patterns are compared to the *C. difficile* reference strain VPI 10463, and strains with identical fingerprints are designated toxinotype 0. Toxinotypes correlate well with PCR-ribotypes and REA (76). This

method is highly reproducible, however, not as discriminatory as PCR-ribotyping or PFGE (76).

4.1.6 Toxigenic type characterization

Toxigenic type characterization using multiplex PCR was developed in order to have a more rapid and reliable method of characterizing isolates from stool samples. Multiplex PCR amplification of particular gene fragments allowed a one-step method to positively identify the presence of *C. difficile* and characterize its toxigenic type (84). The genes examined are species-specific housekeeping gene, *tpi*, and fragments of the toxin genes, *tcdA*, *tcdB* and *cdtB*, and *tcdC*, to identify characteristic deletions that could lead to a truncated negative regulator protein.

The hypervirulent *C. difficile* strains have characteristic deletions in *tcdC*. Using the primers developed by Lemée et al. (84), there is an 18-bp deletion in the *tcdC* gene for NAP1/027 and a 39-bp deletion for 078.

4.1.7 PCR-ribotyping

PCR-ribotyping has been used both in research and clinical laboratories. As a rapid and highly discriminatory technique, it is considered the gold standard typing method in the UK (147). The method involves the PCR amplification of intergenic regions bordering the 16S-23S genes which have highly variable lengths (26). Ribotypes are named by a three digit number which is chronological to the ribotype's discovery. Characterization of ribotypes requires the comparison of profiles to a large strain database so interlaboratory analysis is difficult, i.e., effective ribotyping can only be done by labs equipped with a large ribotype database.

4.1.8 Pulsed-field gel electrophoresis [PFGE]

PFGE is considered the gold standard typing method for many foodborne pathogens and is the standard typing method for *C. difficile* in North America. Inter-laboratory comparisons have been made possible with the use of standardized protocols and computer software for banding pattern analysis and clustering (60). Classically, patterns with greater than 80% similarity have been clustered into the same pulsotype (150). The standard North American pulsotype designation is a number preceded by "NAP". This nomenclature has been appropriate in a clinical setting where there are a relatively small number of relevant pathogenic strains. In light of there being several subtypes within these clusters and many genetically un-related strains that are less clinically relevant, reference laboratories with much larger strain collections are also using unique alphanumeric nomenclature for further classification. Standardization of subtyping nomenclature between reference databases has not been completed.

The PFGE protocol includes cutting the entire bacterial chromosome with rare-cutting restriction enzymes like SmaI, so that 6 to 11 large fragments of DNA are produced. Electrophoresis with alternating directions of current allows the resolution of these 10-700 kb fragments, resulting in clear fingerprints that are highly reproducible (73). *C. difficile* contains highly active endonucleases that promote DNA degradation, so some strains were originally untypeable by PFGE. Recent modifications of the protocol have helped to resolve this problem (4). The discriminatory power of PFGE is similar to REA (73) and PFGE profiles are easier to analyze than those from RAPD (76). Furthermore, there is good correspondence between strains typed by PFGE and PCR-ribotyping (71). However, PFGE is a more laborious technique that requires specific equipment (29, 71).

The aim of this chapter was to use genomic typing methods to profile the *C. difficile* food isolates described in Chapter 3 and to compare them to known isolates to assess their clinical relevance. Strains were characterized by toxigenic type, ribotyping and PFGE.

4.2 METHODS

4.2.1 Multiplex PCR

Multiplex colony PCR amplification was used for toxigenic typing. *C. difficile* toxin encoding genes *tcdA*, *tcdB*, the binding domain of CDT binary toxin, *cdtB*, toxin regulator, *tcdC*, and a *C. difficile* housekeeping gene, triose-phosphate isomerase (*tpi*), were amplified from a single colony template resuspended in 50 µL of Tris-Borate EDTA [TBE] buffer (Sigma-Aldrich, Inc., Oakville, ON). Two PCR master mixes were made using a Qiagen Long Range PCR kit (Qiagen, Mississauga, ON) and primer sets. The forward and reverse primers sequences (Sigma-Genosys, Sigma-Genosys, Sigma-Aldrich, Inc.) are shown in Table 9.

The 50 µL PCR reactions contained 5 µL of colony template, ddH_2O up to 50 µL, 1X Long Range PCR Master Mix (Qiagen), and the forward and reverse primer sets for particular gene fragments depending on the mixture. Master Mix 1 contained the forward and reverse primers for *tcdA*, *tcdB*, and *cdtB*. Master Mix 2 contained those of *tcdC* and *tpi*. A total of 100 nM of the forward and reverse primers were used per isolate, except for *tcdC*, for which 200 nM was used. PCR products were amplified in a thermocycler (Eppendorf) under the following conditions for both multiplex master mixes: 15 min at 93ºC followed by 30 cycles of 93ºC for 30 s, 57ºC for 1 min 30 s, and 68ºC for 1 min. Following the 30 cycles was a 7 min extension at 68ºC. Samples were held at 4ºC before gel electrophoresis analysis. The gene amplicons were separated by Qiaxcel capillary gel electrophoresis (Qiagen), or 2%

Table 9. Forward and reverse primers for *C. difficile* multiplex PCR.

Primer Set Name	Sequence (5'-3')	Product size(bp)	T_m (˚C)	Gene	Description
cdtB-F1 cdtB-R1	F*: TGGACAGGAAGAATAATTCCTTC R*: TGCAACTAACGGATCTCTTGC	582	68.2 68.9	*cdtB*	binary toxin subunit B
tcdA-F A3B	F: AGATTCCTATATTTACATGACAATAT R: ACCATCAATCTCGAAAAGTCCAC	420/150	65.0 70.0	*tcdA*	toxin A
tcdB-3 tcdB-4	F: AATGCATTTTTGATAAACACATTG R: AAGTTTCTAACATCATTTCCAC	329	63.6 63.9	*tcdB*	toxin B
tpi-F tpi-R	F: AAAGAAGCTACTAAGGGTACAAA R: CATAATATTGGGTCTATTCCTAC	230	66.4 66.4	*tpi*	triose phosphate isomerasae
Pal15 Pal16	F: TCTCTACAGCTATCCCTGGT3 R: AAAAATGAGGGTAACGAATTT	637-676	68.2 61.1	*tcdC*	negative regulator of PaLoc

* F= forward primer; R= reverse primer

agarose (Roche, Indianapolis, IN) gel electrophoresis. With Qiaxcel capillary gel electrophoresis, amplicons were detected by a photomultiplier and visualized by BioCalculator software (Qiagen). Agarose gel electrophoresis was run in 1X TBE buffer at 50V for 90 min to separate bands of the Master Mix 1 PCR products and for 180 min to separate bands of Master Mix 2 PCR products. Deletions in the *tcdC* amplicon were detected by agarose gel electrophoresis alongside three controls strains with a 0 bp deletion (11ACD0001), 39 bp deletion (11ACD0028), or 18 bp deletion (11ACD0075) (Table 3).

4.2.2 Pulsed-Field Gel Electrophoresis

For PFGE typing, isolates were grown from frozen stock overnight on BHIS agar. A pure colony was picked and inoculated into 5 mL of BHIS broth and incubated anaerobically overnight at 35ºC. A total of 1 mL of this culture was subcultured into 8 mL of BHIS broth and incubated for 6-8 h at 35ºC. Cells were collected by centrifugation at 29 000 x g (SA 600 rotor, Sorvall, Newport Pagnell, Buckinghamshire) for 5 min and the supernatant discarded. The cell pellet was resuspended in 500 µL of cell lysis buffer without enzymes, consisting of 1.0 M NaCl, 100 mM EDTA, 0.5% (wt/vol) Sarkosyl, 0.2% (wt/vol) deoxycholate, 0.5% (wt/vol) Brij 58, and 6 mM Tris-HCl.

PFGE plugs were made from melting 1% (wt/vol) Seakem gold agarose (Mandel Scientific, Guelph, ON) in 10 mM Tris-HCl, 1 mM EDTA and 1% (w/v) sodium dodecyl sulfate. The mixture was cooled to 55ºC, mixed in equal volumes with the cell suspension, and then left at room temperature to solidify in plug molds (Bio-Rad, Hercules, CA). Plugs were transferred into microfuge tubes and covered with 1 mL of cell lysis buffer plus the following enzymes: 20 µg/mL RNAse (Roche), 2.0 mg/mL lysozyme (Roche) and 12.5 U/mL mutanoylsin (Roche). These plugs were incubated at 37ºC overnight.

The lysis buffer was replaced with 1 mL of proteinase K solution containing 0.5 M EDTA, 1% (w/v) Sarkosyl, and 50 μg/mL of proteinase K (Boehringer Ingelheim, Burlington, ON). Following a 2-3 h incubation at 55ºC, the proteinase K solution was removed by aspiration and replaced with 1.4 mL of wash buffer rinse consisting of 10 mM Tris-HCl and 0.1 mM EDTA. Plugs were gently agitated by hand. Three additional washes with 1.4 mL wash buffer for 20 min each were completed at room temperature.

A fragment was cut from each plug and transferred into a new microfuge tube. The fragment was equilibrated for 10 min in 150 μL of 1X NEBuffer 4 (New England Biolabs, Inc., Pickering, ON). Following equilibration, the buffer was replaced with 150 μL of *Sma*I restriction enzyme solution (New England Biolabs, Inc.) containing 40 U per plug. As a molecular weight standard, similarly prepared plugs of a *Salmonella* Branderup control strain were placed in *Xho*I enzyme solution. *Sma*1 and *Xho*I digests were allowed to incubate at 25ºC and 37ºC, respectively, for 3 h to overnight.

For electrophoresis, 1% SeaKem® gold agarose was mixed in 0.5X TBE, melted, and cooled to 55ºC. The gel solidified in a gel form and *Sma*I digested plugs were added to the gel wells along with the *Salmonella* Branderup marker. The wells were topped off with melted 1% Seakem gold agarose in 0.5 X TBE. PFGE was conducted in a CHEF Mapper™ system (Bio-Rad) containing 2.2 L of cooled (14ºC) 0.5 X TBE with 50 mM thiourea. The PFGE was run according to the following run conditions: 1.0 s initial time, 40.0 s final time, 200 V, 6 V / cm, 120º included angle, for 22 h.

Following the run, the gel bands were visualized by UV light after a 30 min stain with EtBr and 30 min destain with distilled H_2O.

Dendrograms were prepared using BioNumerics software version 5.10 (Applied Maths, Austin, TX) by the Unweighted Pair Group Method with Arithmetic mean [UPGMA]

clustering method using the similarity measurement of a Dice coefficient with position tolerance and optimization of 1.50%. PFGE clusters were compared to the Canadian Nosocomial Infections Surveillance Program [CNISP] database.

4.2.3 Ribotyping

Ribotyping of *C. difficile* isolates was conducted at the NML as described by Indra et al. (58).

4.3 RESULTS

4.3.1 Isolates from ground meats

The results obtained by typing ground meat isolates by toxigenic type characterization, ribotype and PFGE are depicted in Figure 8. Representative PFGE profiles are shown in Figure 9. Nearly all (85/93; 91.4%) of the ground meat isolates were toxigenic for both large clostridial toxins by multiplex-PCR (A^+B^+). All but three of these isolates contained the gene encoding for binary toxin, *cdtB* ($A^+B^+CdtB^+$). The three $A^+B^+CdtB^-$ strains had no deletion in *tcdC* and were found in lamb, chicken and turkey. Of the $A^+B^+CdtB^+$ strains, only one, originating from a beef sample, had no deletion in *tcdC*. The 81 other $A^+B^+CdtB^+$ isolates had a Δ39 bp in *tcdC* and were found amongst each ground meat type.

Isolates from eight of the 93 positive samples (8.6%) were non-toxigenic, lacking the three genes encoding for toxins as well as the negative toxin regulator, *tcdC*. All of the toxigenic type characteristics from the ground meat isolates are summarized in Table 10. There were 78 (83.9%) isolates belonging to the same ribotype, which was related to ribotype 078/126. (Ribotypes 078 and 126 could not be distinguished using the current CNISP database.) This most commonly isolated, ribotype (i.e., 078/126-like), differed from

Figure 8. Typing of *C. difficile* isolated from ground meats. Results of ribotyping, PFGE, and toxigenic type characterization are indicated in the dark rectangles. Percentages shown are the proportions of positive isolates by meat type. Pie slices are proportional to the total number of positive samples. Numbers within the pie slices represent the number of isolates per strain type and each slice is coloured by the strain's *tcdC* gene characteristics (bp deletion) by PCR. The 078/126-like ribotype lacked the 494 bp amplicon of 078/126. The 002-like and 020-like ribotypes both lacked two amplicons from the characterized 002 and 020 profiles. Ribotyping and PFGE profiles were compared to the CNISP database supplied by the National Microbiology Laboratory, Winnipeg, Canada.

Ground meat	# of Positives / Total samples (%)	# of Isolates (%)			
		Toxigenic			Non-toxigenic
		$A^+B^+CdtB^+$		$A^+B^+CdtB^-$	$A^-B^-CdtB^-$
		*Δ*39 bp*	*Δ0 bp*	*Δ0 bp*	*No tcdC*
Lamb	25 / 116 (21.6)	23 (92.0)	1 (4.0)	1 (4.0)	0
Veal	17 / 114 (14.9)	17 (100)	0	0	0
Chicken	18 / 147 (12.2)	11 (61.1)	1 (5.6)	1 (5.6)	5 (27.8)
Beef	18 / 152 (11.8)	17 (100)	1 (5.6)	0	0
Pork	10 / 101 (9.9)	10 (100)	0	0	0
Turkey	5 / 95 (5.3)	1 (20)	0	1 (20)	3 (60)

* Δ: deletion in *tcdC*.

Figure 9. Genetic relatedness, PFGE profiles, type characterization and origin of *C. difficile* isolates from foods. Scale bar indicates genetic relatedness. PFGE profiles are described by NAP and CNISP designations provided by the NML. Toxigenic type is described by the symbols + and –, which refer to the presence and absence of toxin genes by multiplex-PCR. The origin of the isolate is depicted in various colours, i.e., blue (cheese), red (ground meat) and green (milk).

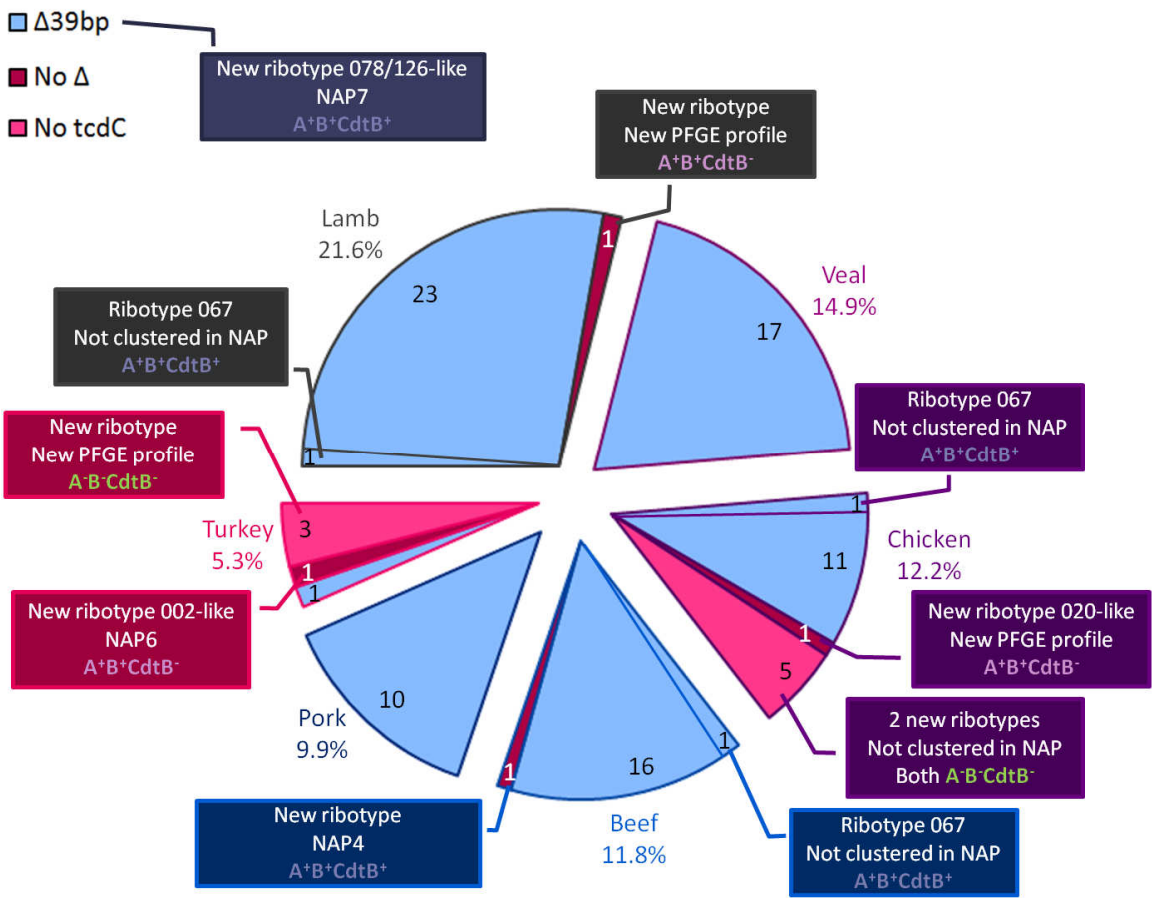
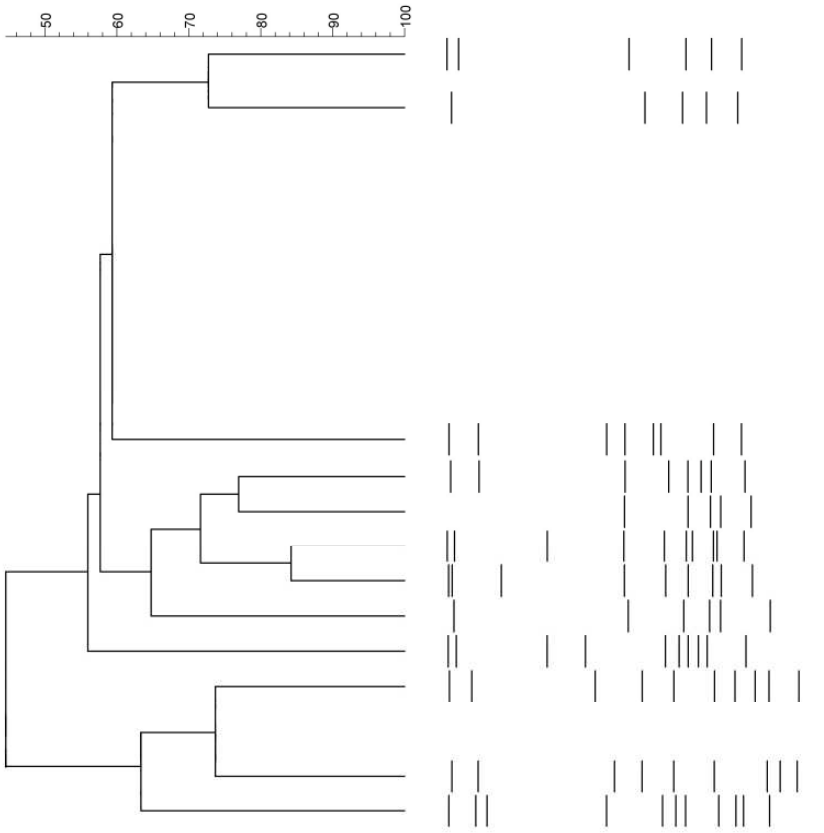

Δ39bp
No Δ
No tcdC
New ribotype 078/126-like
NAP7
A+B+CdtB+
New ribotype
New PFGE profile
A+B+CdtB-
Lamb
21.6%
23
1
1
Veal
14.9%
17
Ribotype 067
Not clustered in NAP
A+B+CdtB+
Ribotype 067
Not clustered in NAP
A+B+CdtB+
New ribotype
New PFGE profile
A-B-CdtB-
Turkey
5.3%
3
1
1
1
Chicken
12.2%
11
1
New ribotype 002-like
NAP6
A+B+CdtB-
New ribotype 020-like
New PFGE profile
A+B+CdtB-
1
5
2 new ribotypes
Not clustered in NAP
Both A-B-CdtB-
Pork
9.9%
10
1
16
1
New ribotype
NAP4
A+B+CdtB+
Beef
11.8%
Ribotype 067
Not clustered in NAP
A+B+CdtB+

Table 10. Toxigenic type characterization of *C. difficile* isolates by ground meat type.



NAP	CNISP Profile	Ribotype	Toxigenic Type			Δ*tcdC* (bp)	Origin	# of samples
			tcdA	*tcdB*	*cdtB*			
7	0536	033	+	-	+	39	Semi-soft	1
							Blue	1
7	0080	078/126-like	+	+	+	39	Lamb	23
							Veal	17
							Beef	16
							Chicken	11
							Pork	10
							Turkey	1
							Semi-soft	4
							Brie	2
							Blue	1
							Camembert	1
							Milk	2
		078/126	+	+	+	39	Blue	1
New[a]	New	New	+	+	-	0	Lamb	1
NN	0054	020-like	+	+	-	0	Chicken	1
New	New	New	-	-	-	0	Chicken	1
4	0131	New	+	+	+	0	Beef	1
New	New	New	-	-	-	0	Chicken	4
New	New	New	+	+	+	39	Camembert	1
NN[b]	0427	001	+	+	-	0	Camembert	1
NN	0106	067	+	+	+	39	Beef	1
							Lamb	1
			-	-	-	None	Chicken	1
New	New	New	-	-	-	None	Turkey	3
6	0339	002-like	+	+	-	0	Turkey	1
							Brie	1

[a] New: PFGE or ribotype profile was not previously described in the CNISP database.
[b] NN: PFGE profile did not cluster into a NAP.

ribotype 078/126 by only one amplicon at 494 bp. The PFGE profile of the 078/126-like isolates clustered in NAP7, the pulsotype to which 078 belongs. By meat type, the veal and pork isolates were all indistinguishable from this most prevalent strain. Only two lamb and two beef isolates were not this common strain. One of the unique lamb isolates, had no deletion in *tcdC* and corresponded to a new ribotype with a new PFGE profile. One of the unique beef isolates also had no deletion in *tcdC* and was a new ribotype that best clustered with NAP4. The other lamb and beef isolates had the same toxigenic characteristics as the most commonly isolated strain ($A^+B^+CdtB^+$); but they were ribotype 067 with a PFGE profile that has not been clustered into a NAP.

There was even more variety of *C. difficile* isolates from poultry (Table 10, Figure 8). Among the chicken isolates, 11 out of 18 were identified as the common 078/126-like/NAP7 strain. There was one other toxigenic isolate from chicken that was $A^+B^+CdtB^-$, with no deletion in *tcdC*. This strain was designated as a new ribotype that was 020-like, lacking two amplicons of ribotype 020. Its PFGE profile did not cluster with a known NAP. The remaining five isolates were non-toxigenic strains that spanned three different ribotypes that were each novel in ribotype and PFGE profile. Of the turkey isolates, only one was 078/126-like/NAP7 and three were a non-toxigenic strain of a novel ribotype and PFGE profile. The one remaining isolate had no deletion in *tcdC* and was designated as a new ribotype similar to 002, but missing two amplicons. By PFGE analysis, it belonged to the NAP6 cluster.

4.3.2 Isolates from cheese and milk

All of the 14 *C. difficile* isolates from cheese were positive by PCR for at least one toxin-producing gene. Only two of these isolates lacked the binary toxin gene, *cdtB*. By ribotyping, one of the $A^+B^+CdtB^-$ isolates was 002-like, lacking two amplicons of 002. By PFGE analysis, it belonged to the NAP6 cluster. This isolate was recovered from Brie and

was indistinguishable from a turkey isolate also found in this study. The other isolate was indistinguishable from ribotype 001. Its PFGE profile did not cluster into a NAP.

Nine of the toxigenic *C. difficile* isolates (64.3%) were the same strain as the one most commonly identified in meats in our study, i.e., $A^+B^+CdtB^+$ with a 39-bp deletion in *tcdC*, NAP7 by PFGE and 078/126-like, missing an amplicon at 494 bp. The remaining isolate found in blue cheese was indistinguishable from ribotype 078/126. Our typing results of the cheese isolates are depicted in Figure 10 and summarized in Table 11.

Both of the milk isolates were also the commonly identified 078/126-like/NAP7 strain.

The PFGE profiles of all of our isolates from ground meats, cheeses, and milk were analyzed using Bionumerics (Applied Maths, Austin, TX) to observe their genetic relatedness (Figure 9). We found that the strains isolated from dairy (blue, green) and ground meats (red) were distributed throughout the dendrogram, thus no association between the genetic relatedness and food origin was observed. Close relatedness by PFGE also did not correspond to a particular toxigenic type.

4.4 DISCUSSION

4.4. 1 A hypervirulent-related type was commonly isolated from ground meat

Interestingly, the majority of the *C. difficile* ground meat isolates contained the genes for all three toxins. This strongly suggests that the strains of *C. difficile* found in food may be able to cause disease in humans. One strain was very commonly isolated and represented 83.9% of all the ground meat isolates. With only one differing amplicon in its ribotype, a corresponding 39-bp deletion in *tcdC* and the same PFGE profile, this strain was highly

Figure 10. Typing of *C. difficile* isolated from cheeses. Results of ribotyping, PFGE, and toxigenic type characterization are indicated in the dark rectangles. Percentages shown are the proportions of positive isolates by cheese type. Pie slices are proportional to the total number of positive samples. Numbers within the pie slices represent the number of isolates per strain type and each slice is coloured by the strain's *tcdC* gene characteristics (bp deletion) by PCR. The 078/126-like ribotype lacked a 494 bp amplicon of 078/126. The 002-like ribotype lacked two amplicons from the characterized 002 profile. Ribotyping and PFGE profiles were compared to the CNISP database supplied by the National Microbiology Laboratory, Winnipeg, Canada.

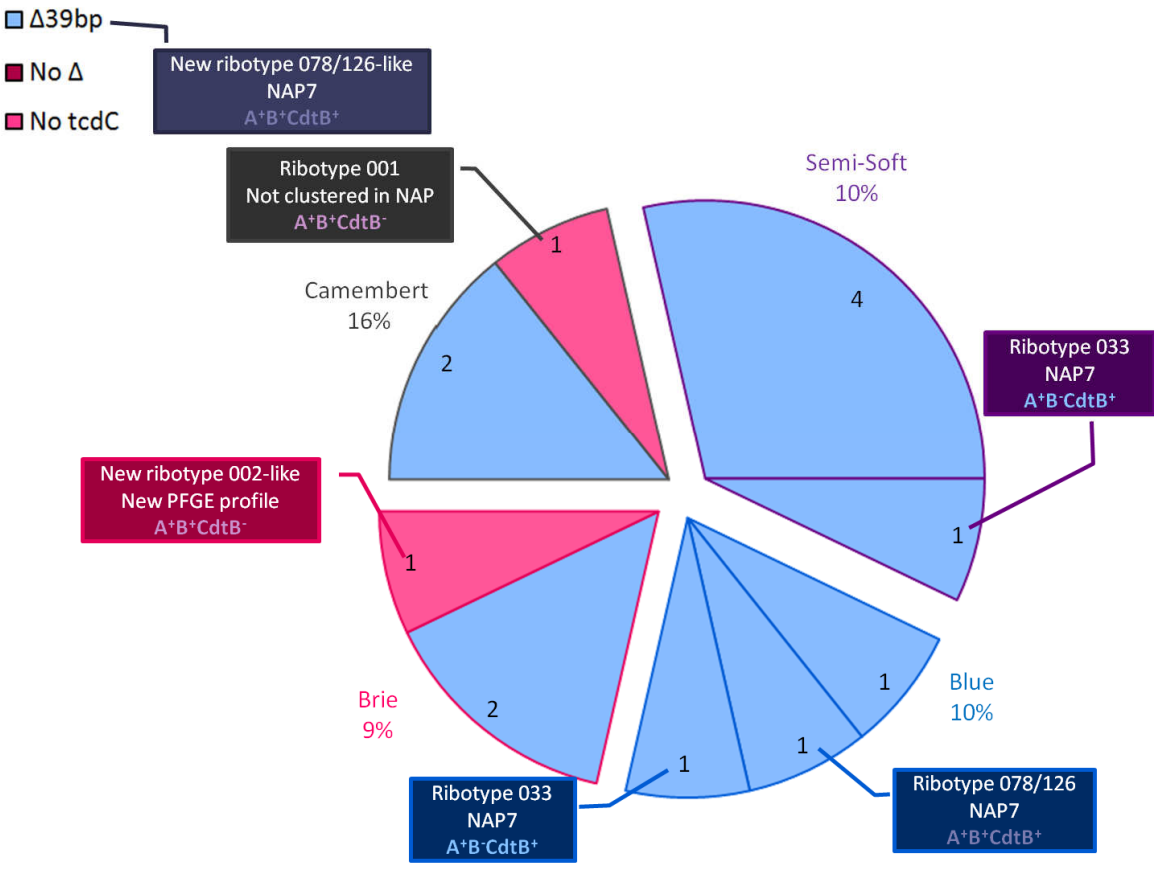

Δ39bp
No Δ
No tcdC
New ribotype 078/126-like
NAP7
A+B+CdtB+
Ribotype 001
Not clustered in NAP
A+B+CdtB-
Semi-Soft
10%
Camembert
16%
Ribotype 033
NAP7
A+B-CdtB+
New ribotype 002-like
New PFGE profile
A+B+CdtB-
Brie
9%
Blue
10%
Ribotype 033
NAP7
A+B-CdtB+
Ribotype 078/126
NAP7
A+B+CdtB+
1
2
4
1
1
2
1
1
1
1

Table 11. Characterization of *C. difficile* isolates from cheese by toxigenic type, ribotype and PFGE.

Cheese Type	# Samples tested	Total # (%) positive	Toxigenic type[a]	Δ*tcdC*, bp[b]	Ribotype[c]	PFGE profile[c]	# (%) of isolates
Camembert	19	3 (15.8)	$A^+B^+CdtB^+$	39	078/126-like[d]	NAP7	2 (10.5)
			$A^+B^+CdtB^-$	No Δ	001	Not clustered[e]	1 (5.3)
Semi-soft	50	5 (10.0)	$A^+B^+CdtB^+$	39	078/126-like	NAP7	4 (80.0)
			$A^+B^-CdtB^+$		033	NAP7	1 (2.0)
Blue	31	3 (9.7)	$A^+B^+CdtB^+$	39	078/126-like	NAP7	1 (3.2)
					078/126	NAP7	1 (3.2)
			$A^+B^-CdtB^+$		033	NAP7	1 (3.2)
Brie	34	3 (8.8)	$A^+B^+CdtB^+$	39	078/126-like	NAP7	2 (5.9)
			$A^+B^+CdtB^-$	No Δ	New ribotype 002-like[f]	New	1 (2.9)
Goat/Sheep's milk	12	0	-	-	-	-	-

[a] Toxigenic type was based on multiplex-PCR as described by Lemée et al (84).
[b] Deletion in the toxin negative regulator gene, *tcdC*.
[c] PFGE profiles were compared to the CNISP database provided by the National Microbiology Lab, Winnipeg, Canada. Ribotyping was performed and analyzed at the same site.
[d]Ribotype lacks the 494 bp amplicon of 078/126. (078 and 126 were indistinguishable with the CNISP database.)
[e] Did not cluster into a North American Pulsotype (NAP).
[f] Ribotype lacks two amplicons of 002.

characteristic of 078, the hypervirulent strain particularly attributed to infecting livestock and causing CA-CDI (28, 40, 133).

The missing ribotype amplicon, however, made this strain type distinguishable from 078. This does not agree with the findings of previous studies that had found primarily indistinguishable ribotype 078/NAP 7 or 8 strains from retail foods (Table 1). Our similar strain suggests that it could have been derived from a human or animal strain of 078. Our results suggest that there may be sources other than ground meat responsible for the transmission of CA-CDI.

4.4.2 Strain diversity in relation to meat type

In contrast to some earlier Canadian studies, we did not recover any 027/NAP1 strains from our retail meat samples (101, 123, 125, 141). We found a greater diversity of *C. difficile* strains in poultry meat (chicken and turkey) than in beef, veal or pork. This agrees with the finding that as compared to bovine or swine, poultry are colonized by more strain types of *C. difficile*. This finding of corresponding *C. difficile* strain diversity between the meat and source animal supports the notion that the *C. difficile* we isolated was shed from the animals. If the contamination had occurred in a common food processing area at the retailer, this common source would likely introduce the same strains as found in other meats prepared in the same environment. Only one report from France has examined *C. difficile* colonization in sheep. The authors found the bacteria in the ruminants of newborn lambs, but did not examine the strain types that colonized the animals (121). Because only one of our 25 positive lamb samples was not the most commonly isolated 078/126-like strain, it is possible that ovine *C. difficile* isolates would also exhibit the same low strain diversity found in bovine and swine.

4.4.3 Relevance of food isolates to characterized clinical and veterinary strains

From our ground meat samples, only one isolate, found in chicken, was indistinguishable from a previously characterized ribotype (067). Interestingly, this ribotype had been reported once in a case of community-onset CDI (CO-CDI) from the Netherlands. CO-CDI cases may include those of CA-CDI, whereby the disease onset occurs in the community or within 48 h of admission to a health care facility, with the proviso that the patient has not been discharged from a health care facility within the previous 3 months. For CO-CDI however, the patient's hospitalization history is not taken into account, so cases may also include those linked to acquisition within health care facilities. (17). The remainder of the *C. difficile* isolates from our study were novel, though some were closely related to previously characterized strains. An isolate similar to ribotype 002 was found in turkey; ribotype 002 is one of the five most isolated clinical types in the Netherlands and was also reported to have caused a case of CO-CDI in that country (12, 17, 51). Furthermore, it was previously isolated from a calf fecal sample (7). This ribotype has not been reported in poultry before, although data on *C. difficile* prevalence and type in poultry is very limited (138, 160, 169). Another novel strain was found in a chicken sample which was similar to ribotype 020, a ribotype commonly isolated from patients with CDI in Europe (12, 18, 28) that has also been isolated from equine and canine animals (68). One beef isolate had a novel ribotype, but its PFGE profile belonged to the NAP4 cluster. The NAP4 cluster includes ribotype 014, that has been previously isolated from animals and humans (51, 113, 123, 125).

The presence of at least one toxin gene in all of the *C. difficile* isolates from cheese, was indicative of the toxigenic types that may be pathogenic to humans. Among

the binary toxin negative isolates, ribotype 001 is a common clinical strain that has been primarily associated with HA-CDI (17, 68, 103, 147). Ribotype 002 has also been found to affect humans (28, 51). Ribotypes 001 and 002 are both among the five most isolated ribotypes from CDI patients in the Netherlands (51). Ribotype 033 was also found to be positive for the toxin A gene. Previous reports have shown that the ribotype 033 PaLoc has only a remnant portion of the *tcdA* gene and thus is unable to produce toxin A (7). This strain has been previously isolated from fecal samples of a calf and a horse, as well as from humans (7, 68, 113).

The majority of *C. difficile* isolates found in cheese, as well as the only two isolates from milk, were indistinguishable from the ribotype 078/126-like strain that we most commonly isolated from meat. It is possible that this major molecular type is a common contaminant on the farm or in the food preparation environment. Since *C. difficile* had never been isolated from cheese before, we wanted to check if there was any possibility that lab contamination had occurred. Among the *C. difficile* strains we isolated from food, three cheese samples contained strains (033 and 001) that had never before been cultured or isolated in our lab. Therefore, we believe that the positive samples from cheese do not reflect contamination during sample preparation in the lab.

Finding several previously uncharacterized strains from foods in this study may be a reflection of the small population of food isolates previously described in the CNISP database that we used for referencing strains. The fact, however, that these strains had almost identical ribotype patterns to previously characterized clinical and veterinary strains, suggests a high genetic-relatedness between these food isolates and clinical types.

All together, the molecular types of *C. difficile* that we found in various foods suggest that they are likely capable of causing disease in humans.

CHAPTER 5: OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

There were three major objectives of this research. Firstly, we wanted to determine the minimum thresholds for growth of *C. difficile* to examine if contaminated foods could support outgrowth of the bacteria and thus pose a risk for bacterial colonization upon ingestion. Since bacterial spores can persist in foods despite hurdle technology, we also wanted to examine the prevalence of *C. difficile* spores in foods. Finally, to examine the likelihood of food being a vector for CDI transmission, we characterized the food isolates and compared them to to known clinical and veterinary strains. While epidemiological studies will ultimately be required to establish if there is a link between contaminated food and community acquired cases of CDI, this research and future studies are needed to better understand the potential role, if any, of contaminated foods in CA-CDI.

We report for the first time the growth thresholds for vegetative *C. difficile* under suboptimal a_w, pH, and temperature. For the two toxinotype 0 strains assessed, in otherwise optimal media, the individual growth-limiting conditions of both strains were an a_w value of 0.966, a pH of 4.5, or a temperature of 10ºC. Modelling the G/NG of *C. difficile* under a combination of hurdles showed that further investigation of *C. difficile* growth, particularly near the thresholds would be warranted in order to obtain a more reliable fit. According to our model and observed data, we found that the growth of *C. difficile* was largely dependent on the intensity of the hurdles and could be prevented at

15ºC by a combination of low pH and a_w values. In the event of temperature abuse, however, some food environments, however, could support *C. difficile* growth.

Several genetic types of *C. difficile*, including the hypervirulent ribotype 027, seemed to have similar growth tolerance. The origin of the strain also did not seem to have a large effect. It would be interesting and relevant to continue this work to analyze more strains with diverse genetic backgrounds and origins.

Overall, the a_w and temperature tolerated for *C. difficile* growth was relatively narrow compared to other known foodborne pathogens. This suggests that proper storage conditions and a mild combination of hurdles could prevent *C. difficile* outgrowth. This data could be helpful for efficiently developing food processing standards to prohibit *C. difficile* growth in foods. For food microbial risk assessments, it will also be important to validate our results with food matrices and take into account the time to growth. Other hurdles should be assessed as well, including using other humectants and acidulents that may be more effective in inhibiting the growth of *C. difficile*.

Despite a combination of hurdles inhibiting vegetative growth, *C. difficile* spores may still persist in contaminated foods and pose a health risk. This study was the first and largest in Canada to test ground chicken, turkey, and lamb for *C. difficile*. Although we tested retail samples from a small region of Ottawa, Canada, our prevalence of positive ground meat samples was comparable to those obtained in other Canadian studies. In agreement with these reports as well, our prevalence was higher than that found in studies on meats from Europe. Standardized isolation and detection protocols will be required to enable better comparisons between reports of the prevalence of *C. difficile* in foods.

This was the second report of seasonality analysis of *C. difficile* in foods. Agreeing with the other Canadian report (123), we found a significantly higher rate of contamination in the winter than the other seasons. There may be an association between this increased prevalence of *C. difficile* in foods in the winter, and cases of CDI. Little has been done so far to examine the dynamics of CA-CDI. Further investigation will be required in order to link the disease and food intake epidemiologically.

We also report, for the first time, the isolation of *C. difficile* from cheese and milk. We found a total of 14 out of 146 (9.6%) packages of semi-soft cheeses to be contaminated with *C. difficile*. This was a similar prevalence to what we found in meat, which may reflect widespread dissemination of *C. difficile* spores in the farm environment. If this is the case, it is possible that we regularly ingest spores of *C. difficile* from foods. Continued food surveillance, as well as standardization of isolation and detection methods will be needed to examine this possibility. Analysis of stools from healthy human populations could also help in this regard. Interestingly, in examination of a limited number of samples, we found a low (2%) prevalence of *C. difficile* in milk. We speculate that contamination of milk may be from a different source than that from cheese, or may reflect different sterilization and/or processing procedures.

The majority of *C. difficile* strains isolated from raw ground meats and semi-soft cheeses in our study were toxigenic. Furthermore, the most commonly isolated type, which was found in both food types as well as two samples of milk, was highly related to the hypervirulent ribotype 078. Contrary to previous findings however, this 078/126-like/NAP7 strain was distinguishable from the hypervirulent clinical and veterinary type, 078/NAP7, by one ribotype amplicon. While this former strain may still be of concern to

human health, its genetic differences suggest that the samples we tested may not be direct vectors of *C. difficile* between humans and animals. Notably, the strain diversity of isolates suggests that the *C. difficile* isolates found in the meats we sampled originated from their respective animal hosts. Only four ribotypes from five food samples were isolated that were indistinguishable from previously characterized clinical human/animal types, namely, ribotypes 001, 033, 067, and one isolate of indistinguishable 078/126. Four of these contaminated samples were semi-soft cheeses, while the other was ground chicken. This could possibly implicate cheese as a more likely vector for clinically relevant *C. difficile* and warrants further study. Seven of the other ribotypes isolated were novel, one of which clustered with NAP4, a pulsotype previously associated with causing CDI in humans.

It would be important in the future to do molecular typing on a wider variety of food and water isolates and compare them with the clinical isolates in Canada. In addition, because of the high proportion of 078/126-like isolates we obtained in foods, we would be particularly interested in investigating its virulence and pathogenicity to examine its possible clinical significance in comparison to the hypervirulent strain 078. From this study, we conclude that *C. difficile* would likely be able to grow in some foods when stored above refrigeration temperatures. However, this could be prevented with various combinations of pH and a_w hurdles. While our isolates from ground meats, cheese and milk may have the ability to cause disease in humans and/or animals, the fact that they were distinguishable from clinical and veterinary strains suggests that these contaminated foods may not act as a vector for interspecies *C. difficile* transmission. However, the epidemiology behind the cases of CA-CDI is still unknown. Our report

provides the first real insight into what foods in Canada may be most at risk for containing the bacteria so that further research can be done on any role that food may play in transmitting *C. difficile* infections to humans, either in hospitals or in the community.

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CONTRIBUTIONS OF COLLABORATORS

Reference strains 11ACD0028, 11ACD0001and 11ACD0075 used in this project were supplied by Dr. Michael Mulvey of the National Microbiology Laboratory, Winnipeg, MB, Canada. Reference strain BI-7 was provided by Dr. Susan Logan of the National Research Council, Ottawa, ON, Canada.

Examination of ground meats for *C. difficile* spores was performed with the help of Jeff Bussey from the Bureau of Microbial Hazards of Health Canada, Ottawa, ON, Canada.

Statistical work and model fitting was performed by Dr. Loan Nguyen of the Microsimulation Modelling and Data Analysis Division of Health Canada, Ottawa, ON, Canada.

Ribotyping as well as some toxigenic typing for confirmation of our results was performed by members of the National Microbiology Laboratory under the direction of Dr. Michael Mulvey. All isolates were compared to the CNISP database supplied by the National Microbiology Laboratory.

APPENDIX A

Figure a1. Optical density of ATCC strain 9689 or 630 cultures over time under suboptimal water activity. Detection was recorded when the OD_{600} was greater than 0.2. Measurements are an average of three replicates. Standard error is shown.

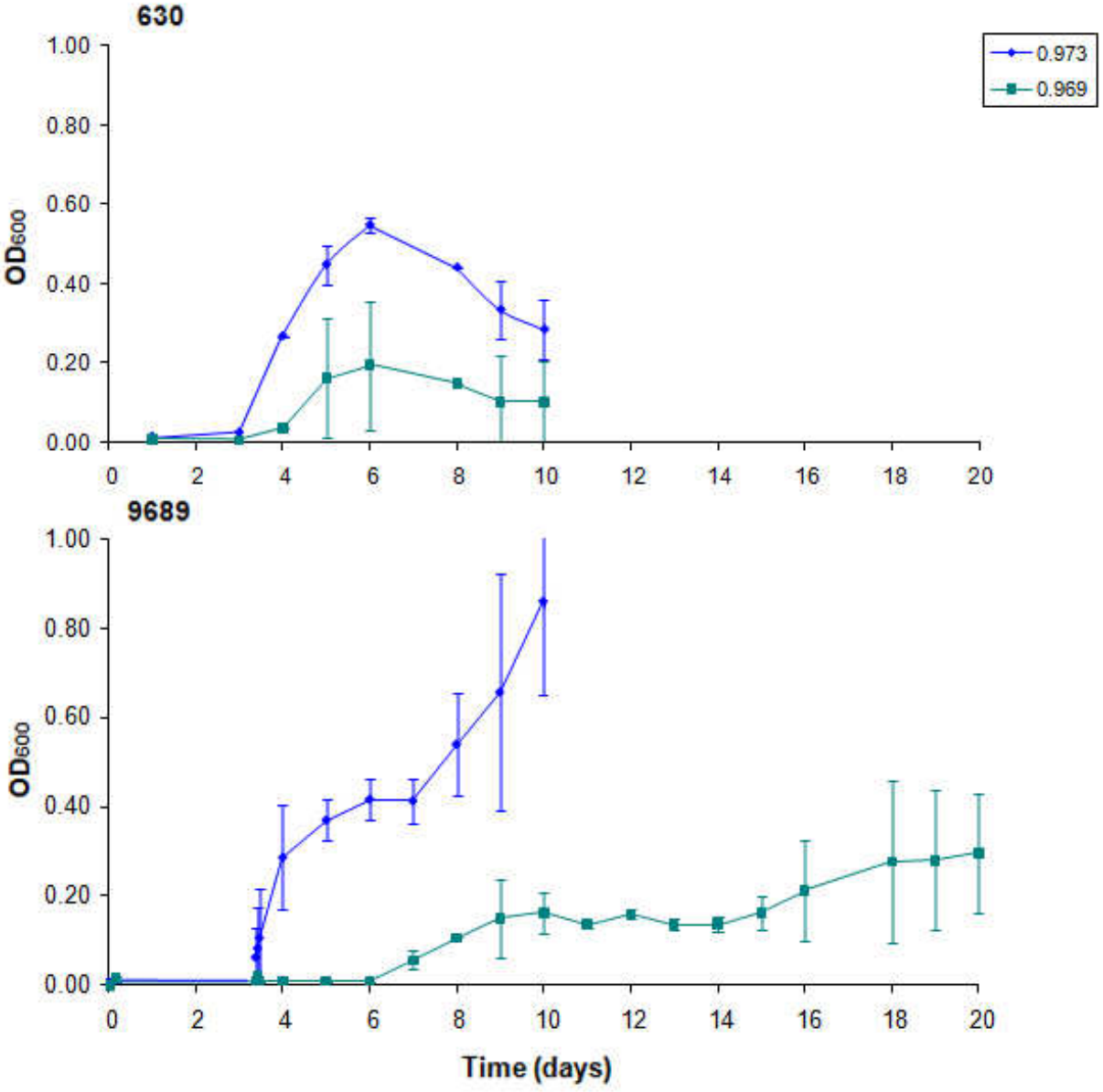

630
9689
0.973
0.969
OD600
Time (days)
1.00
0.80
0.60
0.40
0.20
0.00
0
2
4
6
8
10
12
14
16
18
20

Figure a2. Optical density of ATCC strain 9689 or 630 cultures over time under suboptimal pH. Detection was recorded when the OD_{600} was greater than 0.2. Measurements are an average of three replicates. Standard error is shown.

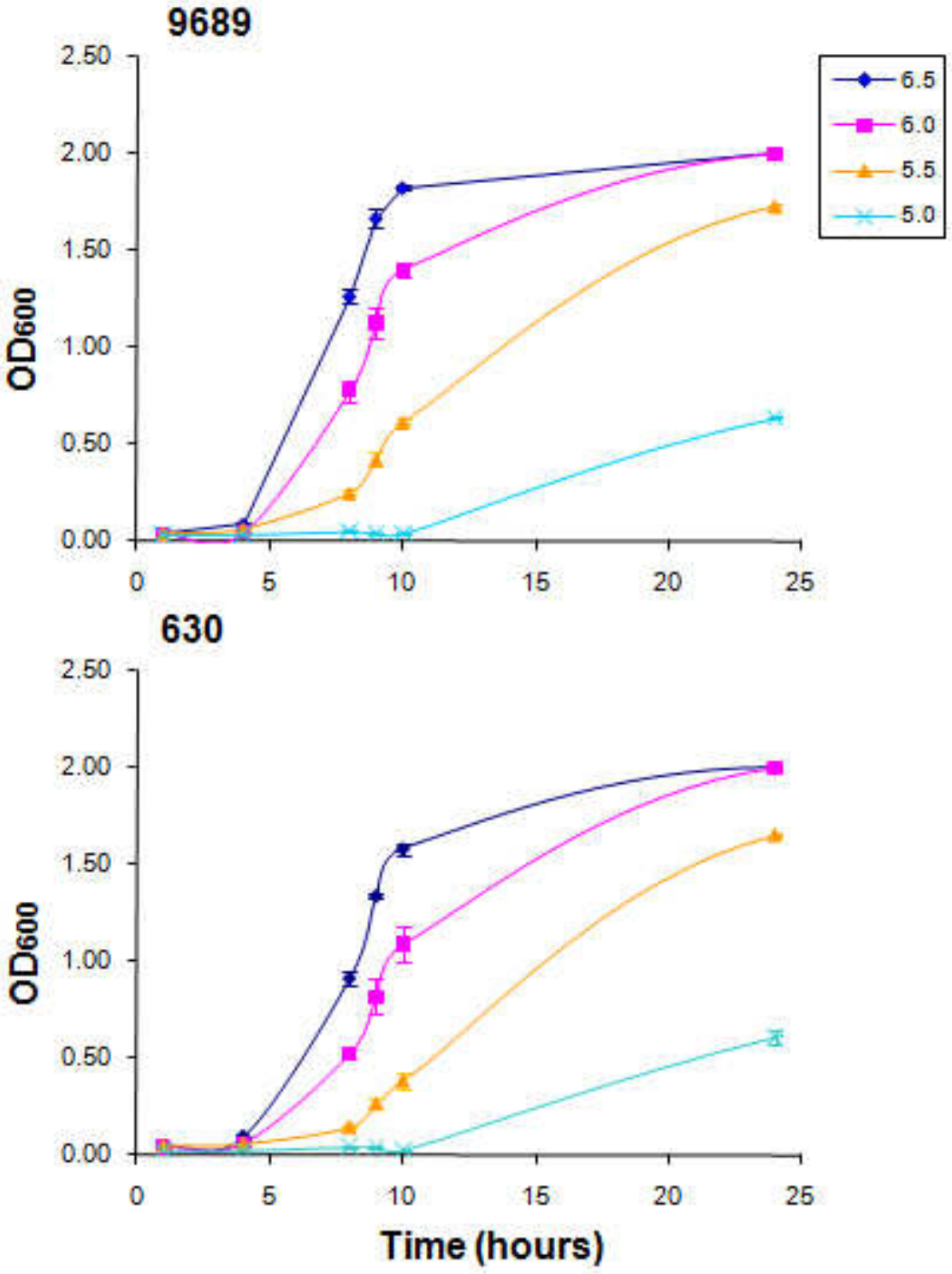
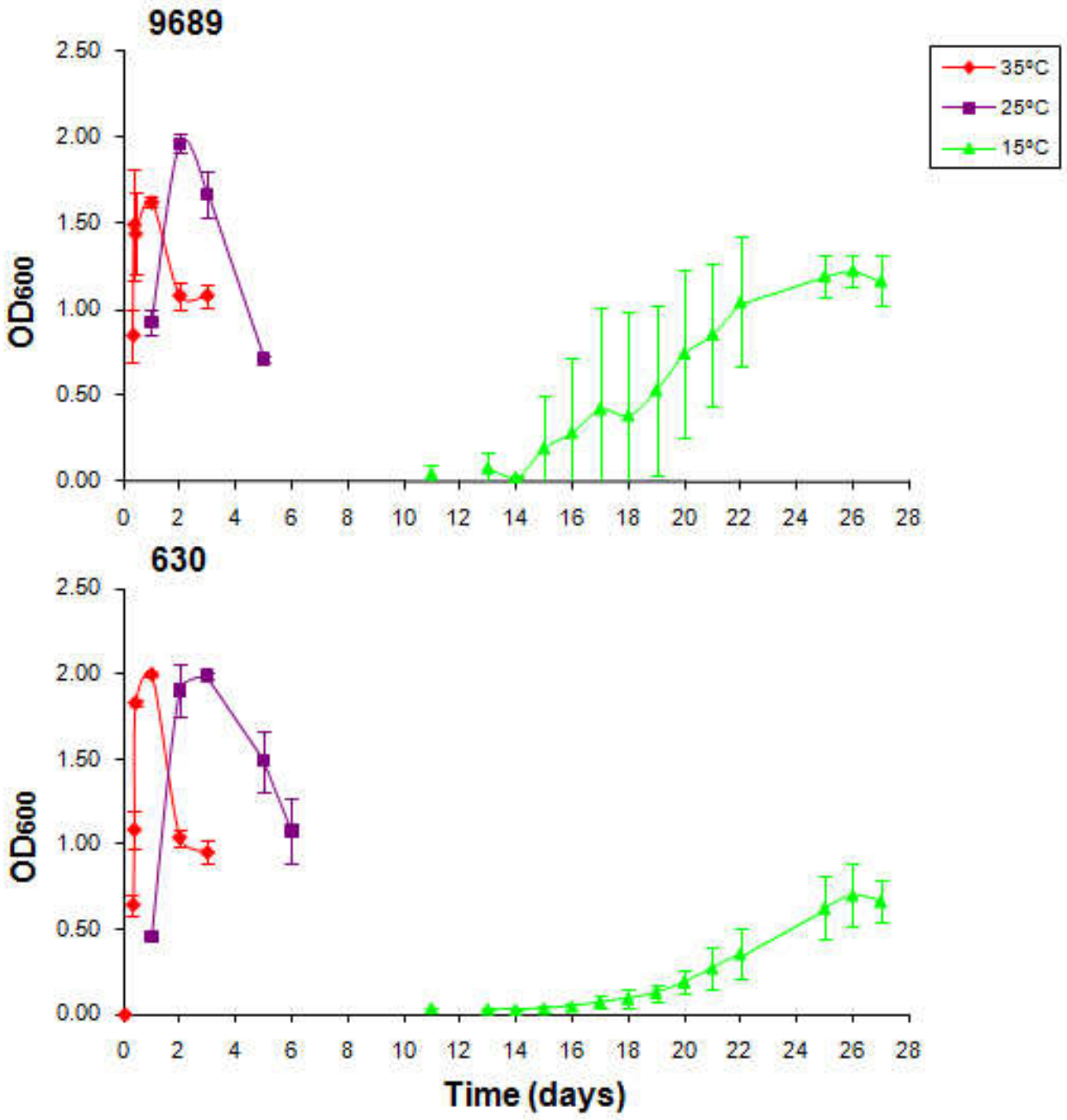
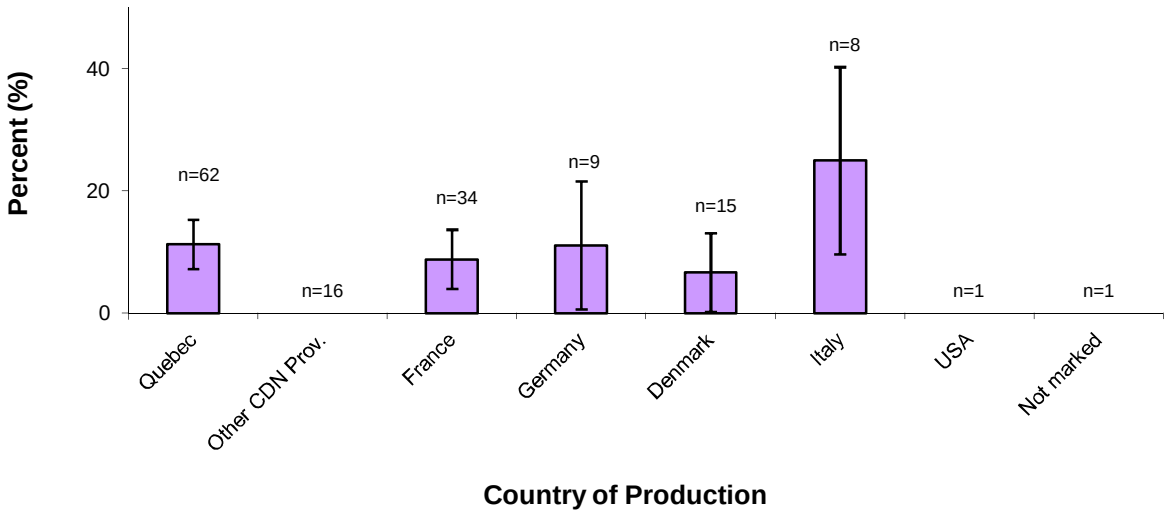

9689
2.50
2.00
1.50
1.00
0.50
0.00
OD600
0
5
10
15
20
25
6.5
6.0
5.5
5.0
630
2.50
2.00
1.50
1.00
0.50
0.00
OD600
0
5
10
15
20
25
Time (hours)

Figure a3. Optical density of ATCC strain 9689 or 630 cultures over time under suboptimal temperature. Detection was recorded when the OD_{600} was greater than 0.2. Measurements are an average of three replicates. Standard error is shown.


9689
630
35°C
25°C
15°C
OD600
Time (days)
0.00
0.50
1.00
1.50
2.00
2.50
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28

APPENDIX B

Figure b1. Proportion of positives cheese samples by country of production. Samples of cheese were enriched in selective *C. difficile* broth for 72 h. Aliquots of enrichment cultures were plated on to *C. difficile* agar for 24 – 72 h to observe colony growth. The cheese was designated positive after confirmation of *C. difficile* isolation from the enrichment culture. Standard error bars are shown. Numbers above the error bars indicate the sample size.


Percent (%)
40
20
0
n=62
n=16
n=34
n=9
n=15
n=8
n=1
n=1
Quebec
Other CDN Prov.
France
Germany
Denmark
Italy
USA
Not marked
Country of Production

CURRICULUM VITAE

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2010:	CIHR Frederick Banting and Charles Best CGS Master's Award
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2009 - 2011:	Graduate researcher, Bureau of Microbial Hazards, Health Canada, Ottawa, ON.
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PRESENTATION ABSTRACTS:

Sugeng, C., Austin, J. W., and Farber, J. M. "*Biological Characterization and Isolation of Clostridium difficile - A potential foodborne pathogen*". BMI Seminar Day, March, 2011, Ottawa, ON, Canada.

- 1st prize for M. Sc. in Microbiology

Austin, J. W., Bussey, J., **Sugeng, C.**, and Farber, J. M. "*Prevalence of Clostridium difficile in Various Types of Ground Meats*". Federal Food Safety and Nutrition Research Meeting, November 3-4, 2010, Ottawa, ON, Canada.

Bussey, J., **Sugeng C.**, Farber, J. M., and Austin, J. "Presence of Clostridium difficile in Various Types of Ground Meat and Poultry Products". International Association for Food Protection Annual Meeting, August 3, 2010, Anaheim, CA, USA.

CONFERENCE POSTERS:

Kongmanas, K., **Sugeng C.**, Souda, P., Whitelegge, J., Faull, K., and Tanphaichitr, N. "Roles of Sulfogalactosylglycerolipid (SGG) and Its Binding

Proteins in Fertilization". 43rd Annual Meeting of the Society for the Study of Reproduction, July 30-August 3, 2010, Milwaukee, WI, USA.

Sugeng, C., Austin, J. W., and Farber, J. M. "Characterization of growth boundaries for Clostridium difficile- a potential emerging foodborne pathogen". BMI Poster Day, May 20, 2010, University of Ottawa, Ottawa, ON, Canada.

Tanphaichitr, N., Kongmanas, K., **Sugeng, C.**, Faull, K.F., and Whitelegge, J. "Roles of Sulfogalactosylglycerolipid (SGG) in Fertilization: Studies Using a Proteomic Approach". 42nd Annual Meeting of the Society for the Study of Reproduction, July 18-22, 2009, Pittsburgh, PA, USA.

Kongmanas, K., **Sugeng, C.**, and Tanphaichitr, N. "Roles of Sulfogalactosylglycerolipid (SGG) and its Binding Proteins in Fertilization: Studies Using a Proteomic Approach". Gordon Research Conference: Fertilization & Activation of Development, July 12-17, 2009, Holderness, NH, USA.

Kongmanas, K., **Sugeng, C.**, and Tanphaichitr, N. "Roles of Sulfogalactosylglycerolipid (SGG) and its Binding Proteins in Fertilization: Studies Using a Proteomic Approach". 27th Annual Ottawa Reproductive Biology Workshop, May 5, 2009, Ottawa, ON, Canada.

Sugeng, C., Kongmanas, K., Faull, K.F., Whitelegge, J., and Tanphaichitr, N. "Proteomic analyses of pig sperm surface proteins associated with sulfogalactosylglycerolipid (SGG)". Biochemistry Undergraduate Research Poster Day, April 3, 2009, University of Ottawa, Ottawa, ON, Canada.