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CHARACTERIZATION, EXPOSURE, BURDEN, CONTROL AND DETECTION

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CONTRIBUTORS

EXPERTS

Declan Bolton, Teagasc (Agriculture and Food Development Authority), Ireland

Roger Cook, New Zealand Food Safety, New Zealand

Kathleen Glass, University of Wisconsin–Madison, United States of America

Tomasz Grenda, National Veterinary Research Institute, Poland

Caroline Le Maréchal, ANSES, Laboratory of Ploufragan-Plouzané-Niort, France

Chie Monma, Jumonji University, Japan

Thomas Riley, The University of Western Australia, Australia

Maja Rupnik, National Laboratory for Health, Environment and Food, Slovenia

Katja Selby, Faculty of Veterinary Medicine, University of Helsinki, Finland

Kristin Schill, University of Wisconsin–Madison, United States of America

Martin Webb, QIB Extra, Norwich, the United Kingdom of Great Britain and Northern Ireland

Xuefang Xu, National Institute for Communicable Diseases Control and Prevention, Chinese Center for Disease Control and Prevention, China

RESOURCE PERSONS

Sarah Cahill, Joint FAO/WHO Food Standards Programme

Akio Hasegawa, World Health Organization (WHO), Switzerland

Jason Brunt, the United Kingdom of Great Britain and Northern Ireland

SECRETARIAT

Jeffrey LeJeune, Food and Agriculture Organization of the United Nations (FAO), Italy

Kang Zhou, FAO, Italy

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DECLARATIONS OF INTERESTS

All participants completed a Declaration of Interests form in advance of the meeting. All experts were not considered by FAO to have declared any interest that may be perceived as a potential conflict with regard to the objectives of the meeting. All the declarations, together with any updates, were made known and available to all the participants at the beginning of the meeting.

All the experts participated in their individual capacities and not as representatives of their countries, governments or organizations.

ABBREVIATIONS

ACMSF	Advisory Committee on the Microbiological Safety of Food
BoNT	botulinum neurotoxin
CDI	*Clostridioides difficile* infection or *C. difficile*-associated disease
CPE	*Clostridium perfringens* enterotoxin
CPILE	*Clostridium perfringen*s iota-like enterotoxin
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EIA	enzyme immunoassay
EU	European Union
EUR	euro
EEA	European Economic Area.
FAO	Food and Agriculture Organization of the United Nations
FBO	food business operator
FDA/US FDA	United States Food and Drug Administration
FERG	Foodborne Disease Burden Epidemiology Reference Group
FSIS	United States Food Safety and Inspection Service
GAP	good agricultural practices
GDH	glutamate dehydrogenase
MBA	*in vivo* mouse lethality bioassay
MHLW	Japan Ministry of Health, Labour and Welfare
PCR	polymerase chain reaction
USD	United State dollars
WGS	whole-genome sequencing
WHO	World Health Organization

GLOSSRY

Anaerobic bacteria	Bacteria having the ability to grow in the absence of oxygen.
Antibiotic	Naturally occurring, semi-synthetic or synthetic substance used to treat or prevent infections, which kills or inhibits the replication of bacteria in or on the body, and which is typically administered orally, topically, or by injection.
Antimicrobial (n.)	Naturally occurring, semi-synthetic or synthetic substance that kills or inhibits the replication of microorganisms; includes inhibitory food ingredients (synthetic or natural preservatives) and antibiotics.
Antimicrobial (adj.)	Having the properties of an antimicrobial (n.), including substances used as medicines, preservatives (food grade antimicrobials) and, or sanitizers/disinfectants.
Botulinum cook	Heat treatment to deliver a 12-$\log_{10}$ reduction of Group I (proteolytic) *Clostridium botulinum* spores*;* 121 °C for 3 min or equivalent.
CPE	*Clostridium perfringens* enterotoxin which is responsible for causing foodborne gastroenteritis.
DALYs	Disability-adjusted life years. A measure used to assess the overall burden of disease and health conditions in a population. They combine the impact of premature mortality and the loss of healthy life due to disability into a single metric.
D-value	Decimal reduction time; time in which population of a microbe is reduced by 1-log.
EEA	European Economic Area. *Includes EU countries, Iceland, Liechtenstein and Norway. Switzerland is not an EU or EEA member but is part of the single market. The United Kingdom of Great Britain and Northern Ireland is not part of the European Union or the European Economic Area.
FSIS	United States Food Safety and Inspection Service, division of the US Department of Agriculture, responsible for developing and enforcing regulations related to meat and poultry processing and inspection.
GAP	Good agricultural practices. Knowledge and principles to address environmental, economic and social sustainability for on-farm production and post-production processes resulting in safe and healthy food and non-food agricultural products. May include general farm practices such as management, animal health management, veterinary medicines and biologicals, animal feeding and watering, environment and infrastructure, and animal and product handling.

GDH Glutamate dehydrogenase protein of *C. difficile* which can indicate presence of the bacteria in stool samples.

Microbial growth Increase in the number of microbial cells by binary fission; also referred to a proliferation; measured typically in $\log_{10}$ scale.

Nonprot Cbot cook Heat treatment to deliver a 6-$\log_{10}$ reduction of Group II (nonproteolytic) *Clostridium botulinum* for chilled foods; 90 °C for 10 min or equivalent.

PCR Polymerase chain reaction is a molecular laboratory technique that detects DNA sequences from microorganisms in a sample or enriched sample allowing timely determination of a pathogen's presence and its identification.

Spores Dormant (without metabolic activity) structures produced by clostridia, *Bacillus* and certain other bacterial genera in response to adverse environmental conditions. Bacterial spores are highly resistant to heat, dehydration, high hydrostatic processing, irradiation, and chemicals.

Vegetative bacteria Metabolically active, growing, and reproducing forms of bacterial cells. Killed by typical food processing such as cooking, pasteurization, high hydrostatic processing and sanitizers. Their proliferation is inhibited by variation in storage temperatures (chilled or frozen) increasing salt concentration, reduced water activity, acid and certain preservatives.

Water activity (a_w) Water activity refers to unbound water in a food product that is available to support the growth of microorganisms. Generally, a function of moisture and dissolved solutes, such as salt, sugar or other humectants.

WGS Whole-genome sequencing. A method to provide a high-resolution, base-by-base view of the genome. It allows for high-resolution analysis of bacterial pathogens, focusing on characteristics such as antibiotic resistance, molecular epidemiology, and virulence.

EXECUTIVE SUMMARY

Clostridia include several toxigenic bacterial species recognized as significant human pathogens, notably *Clostridium botulinum, Clostridium perfringens*, and *Clostridioides difficile*. Clostridia are commonly identified in various food and many environmental niches. Unlike other common bacterial foodborne pathogens, such as *Salmonella*, *Campylobacter* and *Listeria*, clostridia produce dormant structures known as spores that enable survival under harsh conditions. These spores persist for a long time, perhaps decades, in the farm and processing environments and from there, they may contaminate foods. Furthermore, spores are resistant to common food safety control measures such as cooking, pasteurization, and certain sanitizers. In the case of adult intestinal and infant botulism and infection by *C. difficile*, ingested spores must germinate and the vegetative bacteria multiply in the intestine and produce toxin to cause illness. With both foodborne botulism and *C. perfringens*, the spores must germinate and vegetative bacteria multiply in the food which, when eaten, results in foodborne illness. *Clostridium perfringens* causes gastrointestinal illness when high numbers of vegetative cells consumed in the food sporulate in the large intestine and enterotoxin is released. Food producers, processors, handlers and consumers should minimize contamination of foods with spores and/or prevent clostridial multiplication in foods to lessen the risk of foodborne disease.

This document has been prepared to provide a comprehensive review of the global status of foodborne clostridia. The focus is specifically on *C. botulinum* and other botulinum neurotoxin (BoNT)-producing species, *C. perfringens* and *C. difficile*, with the aim of providing a detailed analysis of their prevalence, associated risks and implications for food safety and public health. This review encompasses an extensive examination of peer-reviewed scientific literature, epidemiological data, and recent reports. It also includes an assessment of the epidemiology of outbreaks, and current detection and control methods for pathogenic clostridia in food systems. The document is structured to provide a clear and detailed overview of the current state of knowledge, highlighting critical areas for future research and policy development to address the challenges posed by foodborne clostridia.

Clostridia encompass two genera *Clostridium* and *Clostridioides* and includes several toxigenic species recognized as significant human pathogens, notably *C. botulinum, C. perfringens* and *C. difficile*. These bacteria produce resistant spores and are commonly detected in a broad range of food and environmental sources. *Clostridium botulinum* and *C. perfringens* are known foodborne pathogens. Although *C. difficile* spores have been isolated from certain foods, the evidence supporting its classification as a foodborne illness remains weak.

Clostridium botulinum strains are responsible for five forms of botulism in humans: foodborne, infant, adult intestinal, iatrogenic, and wound botulism. Although foodborne botulism is rare, it is a severe and potentially fatal neuroparalytic disease caused by ingesting preformed toxin in improperly preserved or insufficiently chilled foods. Consuming as little as 50 nanograms (ng) of botulinum neurotoxin can lead to severe and often fatal intoxication. Beyond spore elimination through proper canning procedures, the growth of toxigenic clostridia can be mitigated using established food safety practices. These include strict temperature control and additional preventive measures such as salt addition, pH reduction, lowered water activity, nitrite application, or the incorporation of inhibitory food ingredients (certain synthetic and natural preservatives).

Infant and adult intestinal botulism occur in individuals with immature or disrupted intestinal microbiota and result from the colonization of the intestine after ingesting spores of BoNT (botulinum neurotoxin) -producing clostridia, leading to *in situ* toxin production. In most cases, the source of spores remains unknown. However, when identified, the primary sources of spores associated with infant botulism have been honey and environmental sources such as dust and soil. Raising awareness among parents, caregivers, at-risk groups, and the medical community is essential for reducing the risk of infant and adult intestinal botulism.

Some strains of other *Clostridium* species such as *C. butyricum*, *C. baratii*, and some more recently identified *C. sporogenes*, produce BoNTs. However, their foodborne significance and associated public health burden remain poorly defined. The prioritization of food safety, as well as the collection and analysis of epidemiological data, varies substantially across countries.

Unlike foodborne botulism, *C. perfringens* is one of the most common – though often underreported – causes of foodborne gastrointestinal illness, frequently linked to large outbreaks in mass catering settings. This toxico-infection occurs when foods are consumed in which vegetative cells of *C. perfringens* have multiplied to high levels (exceeding 8-$\log_{10}$ per serving) due to temperature abuse. Once ingested, toxins are released in the gut as the cells sporulate. Symptoms are relatively mild and generally resolve within a few days without medical intervention. Preventing the proliferation of *C. perfringens* in high-risk foods depends on maintaining strict temperature control during cooling, hot-holding, reheating, and through inhibitory product formulations.

Clostridiodes difficile causes antibiotic-associated diarrhoea and colitis. Recent data indicate an increasing proportion of community-acquired *C. difficile* infections (with the environment and food as potential sources of spores) compared to hospital-acquired infections (human-to-human transmission). Although biologically plausible that both the environment and food can serve as vehicles for *C. difficile*, at this time there is minimal evidence for classifying the disease as a traditional foodborne illness, as seen with other clostridia species, which typically manifest symptoms within hours or days of consumption. Furthermore, unlike the association of infant botulism with foods such as honey, there is no known link with specific foods that a susceptible individual should avoid for *C. difficile*.

The estimates of incidence and burden for clostridia-associated diseases predominantly originate from data collected in high-income countries, leading to a notable global data gap in epidemiological understanding. Severe foodborne botulism ranks among the highest in disability weights (1 036 disability-adjusted life years [DALYs], due to its clinical impact, with an estimated cost of USD 1.68 million per case. *Clostridium perfringens* illness results in 6 963 DALYs in European and low-mortality countries and ranks as the second most common cause of bacterial-associated foodborne illness in the United States of America, imposing an annual economic burden estimated at USD 342.7 million. Costs for *C. difficile* infection are estimated at EUR 3.4 billion annually in Europe and USD 5.9 billion annually in the United States.

This review summarizes the recent literature on these topics, incorporating relevant reports such as the 2023 Advisory Committee on the Microbiological Safety of Food (ACMSF), the United Kingdom of Great Britain and Northern Ireland study on botulism, and the 2005 European Food Safety Authority (EFSA) report.


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CHAPTER 1
INTRODUCTION

1.1 CLOSTRIDIA

Clostridia encompass a diverse array of Gram-positive, rod-shaped, anaerobic, spore-forming bacteria. This group includes two genera and several toxigenic species that are significant human pathogens, notably *Clostridium botulinum*, *Clostridium perfringens*, and *Clostridioides difficile* (recently renamed from *Clostridium difficile*). In addition, whole genome sequencing (WGS) has identified toxigenic strains of *C. butyricum, C. baratii* and *C. sporogenes* that produce BoNTs that were likely previously classified as *C. botulinum* based on toxin production (ACMSF, 2023). While *C. butyricum* Type E is the leading cause of human botulism in Italy due to BoNT/E (Camareni, 2019) and in areas of China (Fu, 2008), the significance of *C. butyricum* and that of other BoNT-producing species, apart from *C. botulinum*, in other countries remains unclear.

Despite their classification as obligate anaerobes, the necessity for strict anaerobic conditions varies considerably among different species and strains. It has been observed that the mere presence of oxygen does not unequivocally ensure the safety of food with respect to toxigenic clostridia.

An important characteristic of clostridia compared to other common foodborne pathogens, such as *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes*, is that clostridia produce spores in response to unfavourable conditions. These spores exhibit resistance to extreme temperatures, desiccation, enzymatic degradation, and sanitizers that would typically inactivate non-spore-forming organisms. Spores will therefore survive for an indeterminate length of time, in some cases, many decades or longer, on the farm, in food ingredients, and in processing environments. The usual food safety control measures such as cooking, pasteurization and sanitation are ineffective. Although spores themselves cannot cause disease in healthy individuals, it is important for food producers and consumers to be cognizant of conditions in, and handling of, foods, required to prevent spore germination and subsequent growth of toxin-forming clostridia. Manufacturers and consumers should also be aware that spores have the potential to cause disease in individuals with immature or compromised intestinal microbiota, such as in the case of infant or adult intestinal botulism.

Human diseases caused by foodborne toxigenic clostridia can be differentiated by their pathogenesis into either intoxications or toxico-infections. Foodborne botulism is an intoxication in which *C. botulinum* multiplies in the food and produces botulinum neurotoxin, which is then ingested resulting in illness. In contrast, infant and adult intestinal botulism are toxico-infections characterized by the ingestion of toxin-producing clostridia spores in the food, followed by germination of the spores in the intestine, colonization by the resultant vegetative cells, proliferation, and subsequent toxin production. *Clostridium perfringens* enteritis is also a toxico-infection but it differs in that it requires proliferation to high numbers in foods, with toxin released when the ingested vegetative cells sporulate within the intestine. Less commonly, *C. perfringens* has been associated with antibiotic-associated diarrhoea and sporadic diarrhoea (Shrestha *et al.*, 2024). *Clostridium difficile* illness is also a toxico-infection. While there is some evidence that food may serve as a transmission vehicle for *C. difficile* spores from animals to humans, current data are insufficient to support *C. difficile* infections (CDI) as a "foodborne illness" in the same sense as those caused by other clostridial species.

1.2 *CLOSTRIDIUM BOTULINUM* AND OTHER BONT-PRODUCING CLOSTRIDIA SPECIES

Neurotoxigenic strains of clostridia are classified into six groups: Group I, II, and III *C. botulinum*, Group IV *C. argentinense*, plus groups that include *C. baratii* (Group V) and *C. butyricum* (Group VI), based on their proteolytic activity, growth characteristics, ecological niches, and the one or more antigenically distinct botulinum neurotoxin (BoNT) types (A-G) they produce (Smith *et al.*, 2018). These classifications help differentiate their pathogenic potential, environmental resilience, and implications for food safety.

Groups I (proteolytic; toxin types A and B) and II (nonproteolytic; type B, E, and F toxins) *C. botulinum* are predominantly implicated in foodborne botulism. In contrast, Group III (non-proteolytic; toxin type C and D) is primarily associated with animal botulism. However, a small number of cases of human foodborne botulism and one infant botulism case due to type C toxin have been reported as well as a few suspected cases due to type D (see Table 1). Strains of Group IV (proteolytic; type G toxins) have not been associated with human botulism. Additionally, certain strains of *C. baratii* (Group V; non-proteolytic; type F toxin) and *C. butyricum* (Group VI; non-proteolytic; type E toxin), and *C. sporogenes*, which is genetically closely related to Group I *C. botulinum*, have been identified as producing BoNT (type A), which contribute to instances of human botulism (Grenda *et al.*, 2024; Peck and van Vliet, 2016; Camerini *et al.,* 2019).

Clostridium botulinum Group I (also known as proteolytic *C. botulinum*) and *C. sporogenes* are closely related mesophilic bacteria that share genotypic and physiological characteristics, including a highly proteolytic nature and the ability to form spores with high thermal resistance (Brunt *et al.*, 2020a). Prior to the advent of genetic analysis, *C. sporogenes* was historically considered a non-toxigenic counterpart to *C. botulinum* Group I (Hatheway, 1990; Carter *et al.*, 2009; Peck *et al.,* 2011). More recently, the distinction between *C. sporogenes* and *C. botulinum* Group I has been based on core genome single-nucleotide polymorphism (SNP) analysis (Brunt *et al.*, 2020a). The study identified lineage-specific genes that serve as markers in Polymerase chain reaction (PCR)-based assays for differentiating the organisms; 20 percent of the 103 sequenced *C. sporogenes* strains contained BoNT/B genes (Brunt *et al.,* 2020a). Most strains of *C. botulinum* Group I (but not all) formed BoNT; for example, one study found 20 out of 452 (4.4 percent) strains sequenced lacked a BoNT gene (Brunt *et al.,* 2020a). Additionally, genetic analysis of historic outbreak strains implicates BoNT-producing *C. sporogenes* in cases of infant botulism and foodborne botulism linked to meat and fish (Brunt *et al.*, 2020a; Smith *et al.*, 2015; Mazuet *et al.,* 2016; Williamson *et al.*, 2016; Giordani *et al.,* 2015; McLauchlin *et al.*, 2006; Franciosa *et al.,* 2009).

Strains of *C. botulinum* Group I represent a major cause of the three most frequent types of botulism in humans, namely, foodborne, infant, and wound botulism and have also been implicated in botulism cases among horses (Peck and van Vliet, 2016). Foodborne botulism, characterized by severe neuroparalytic symptoms potentially leading to fatality if not promptly treated after onset of symptoms, can result from ingestion of as little as 50 ng of BoNT (Peck, 2009). The "botulinum cook" process (121 °C for 3 minutes) targets the heat-resistant *C. botulinum* Group I spores, aiming to ensure the safety of low acid canned foods (Stumbo *et al.*, 1975; Peck, 2009). Failure to apply this process to canned or bottled goods, as well as temperature abuse of products intended to be stored chilled, has been linked to foodborne botulism outbreaks (ACMSF, 2023). The commercial ramifications of such outbreaks can be profound, emphasizing the critical need for sustained vigilance to mitigate the incidence of foodborne botulism.

Strains of *C. botulinum* Group II are also ubiquitously distributed in the environment and can infiltrate the food chain posing a risk of foodborne botulism (Peck *et al.,* 2010; Barker *et al.,* 2016). Notably, *C. botulinum* Group II strains, particularly those producing type B, type E or rarely type F neurotoxin, are commonly implicated in foodborne botulism cases. They can also be involved in adult intestinal botulism cases. *Clostridium botulinum* Group II, also

referred to as non-proteolytic *C. botulinum* or psychrotrophic *C. botulinum*, is a saccharolytic bacterium with a minimum growth temperature of 3 °C that forms spores with moderate heat resistance (Eklund *et al.*, 1967; Brunt *et al.,* 2020b). Outbreaks of foodborne botulism have been associated with temperature abuse of mildly processed, vacuum/modified atmosphere-packed, and other chilled foods (Carter and Peck, 2015; Peck *et al.,* 2020) including fish, meat, soups, and low acid juices, accentuating the necessity for meticulous oversight to ensure safe production and maintenance of the integrity of the chill chain, especially as contamination is often unavoidable.

TABLE 1. HUMAN BOTULISM CASES LINKED TO BONT TYPE C AND D REPORTED WORLDWIDE

Country	Year	Cases	Form of botulism	BoNT Type	Source	Reference
United States	1950	4	Clinical suspicion	C	Food suspected	Meyer, 1953
France	1955	2	Clinical suspicion	C	Homemade "pâté"	Prevot *et al.*, 1955
Chad	1958	2	Clinical suspicion	D	Homemade ham	Demarchi *et al.,* 1958
Rhodesia (now Zimbabwe)	1960	4	Clinical suspicion	B or C	Homemade "pâté"	Fleming, 1960
USRR (now Russian Federation)	1965, 1966	2	Clinical suspicion	C	No data	Matveev *et al.,* 1967
France	1972	4	Foodborne botulism	C	Smoked chicken suspected	Maupas *et al.,* 1976
Japan	1990	1	Infant botulism	C	Environmental contamination suspected	Oguma *et al.,* 1990
French Guyana (now Guyana)	2006	1	Clinical suspicion	C or D	Chicken with clinical signs of botulism	Martrenchar *et al.,* 2019
Japan	2021	4	Foodborne botulism	C	Commercially prepared chicken dish was suspected	Maeda *et al.*, 2023
Japan	2025	1	Foodborne botulism	C	No confirmed source	Chie Monma personal communication, 2025

Sources: Adapted from Meurens *et al.* 2023 and Rasetti-Escargueil, Lemichez, & Popoff M.R. 2020. See Section 1.5 References section for full list of sources.

Infant botulism – a toxico-infection characterized by intestinal colonization and *in situ* neurotoxin production in infants under 12 months – has been primarily associated with *C. botulinum* Group I strains, and less frequently with Group II and neurotoxigenic *C. butyricum* or *C. baratii* (Harris et al., 2024). In cases of adult intestinal botulism, mostly Group I *C. botulinum* A and B and *C. baratii* F have been identified as causes (Guru et al., 2018)

Strains of Group III *C. botulinum* produce BoNT type C, D, as well as mosaic variants C/D and D/C, predominantly affecting animal species. However, some human botulism cases have been linked to Group III *C. botulinum*. Most of these cases were reported before 1990, although there was one in 2023 and one in 2025, both in Japan (**Table 1**).

1.3 *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens shares many characteristics with other clostridial species, including its ubiquitous presence in environmental and food matrices, and its consistent role as a resident of the gastrointestinal microbiota in both humans and animals (Mehdizadeh Gohari et al., 2021). This bacterium is implicated in a range of intestinal and non-intestinal diseases, such as gastroenteritis, gangrene, poultry necrotic enteritis, non-foodborne diarrhoea, pre-term necrotizing enterocolitis and nosocomial infection (Kiu and Hall, 2018; Wada et al., 1996; Kobayashi et al., 2009).

As with other clostridia, the formation of highly heat-resistant spores by *C. perfringens* is a key factor in its persistence and pathogenicity. These spores can survive typical cooking processes and subsequently germinate if foods are not cooled properly or if there is improper hot holding at temperatures that support spore germination and growth (Le Marc et al., 2008). At optimal growth temperatures and formulation conditions, the doubling time of *C. perfringens* can be under 10 minutes. These characteristics underscore the importance of stringent temperature-time food safety practices to prevent foodborne illness.

Clostridium perfringens is classified into seven types (A-G) based on the production of more than 20 distinct toxins (Rood et al., 2018; Grenda et al., 2023). All *C. perfringens* strains carry the *cpa* gene for α-toxin (responsible for gas gangrene) and may carry genes for other toxin types. The *C. perfringens* enterotoxin gene (*cpe*) encodes for enterotoxin (CPE), the key factor in food poisoning and antibiotic-associated diarrhoea (Camargo et al., 2024; Mehdizadeh Gohari et al., 2021; Rood et al., 2018). Prior to 2018, certain type A strains were classified as producing diarrhoeagenic toxin (Yonogi et al., 2014; Monma et al., 2015) but given they carry both the *cpa* and *cpe* gene have now been reclassified as type F (Rood et al., 2018). Type A are now defined as strains that produce α-toxin but do not produce other toxins. Type C carries the toxin genes *cpa, cpb, cpe* (Rood et al., 2018) and is associated with necrotizing enteritis, also known as pigbel (Camargo et al., 2024). Similarly, β (types B and C), ε (types B and D), iota (type E), and NetB (type G) toxins are associated with necrotizing enteritis and illness in animal species other than humans (Mehdizadeh Gohari et al., 2021).

After consumption of food in which *C. perfringens* has propagated to high numbers (e.g. 108 vegetative cells per serving; 106/g of food), the *C. perfringens* cells sporulate and release CPE toxin in the intestine, resulting in illness (FDA, 2012). *Clostridium perfringens* is a significant public health concern in the United States, ranking as the second most common cause of bacterial foodborne illness after non-typhoidal *Salmonella* (Scallan, 2011). The US Centers for Disease Control and Prevention (CDC) estimate that *C. perfringens* is responsible for approximately one million cases of foodborne illness annually (US CDC, 2024b). In Europe, the number of *C. perfringens* outbreaks and human cases between 2019 and 2023 ranged between 37 and 140 per year and 730 and 3 300 per year, respectively (EFSA FBO dashboard, 2025). In Japan, *C. perfringens* food poisoning has the highest number of patients per outbreak, and since 2021, it has ranked second in the number of bacterial food-poisoning incidents. In many countries, *C. perfringens* is not classified as a leading cause of foodborne illness, likely due to under-reporting. The illness often resolves quickly without medical intervention and thus frequently goes unnotified. Nevertheless, outbreaks are reported, usually with a high number of individual cases (New Zealand Food Safety, 2024). This highlights the critical need for effective monitoring and control measures in food production and handling to mitigate the risk posed by *C. perfringens*.

As noted above, the widespread presence of *C. perfringens* in the environment and food, coupled with its capacity to form heat resistant spores, rapid growth in susceptible foods, and release of toxin in the human intestine after ingestion, contribute to its significance as a major foodborne pathogen. The incidence of illnesses caused by this bacterium is best reduced by stringent implementation of known food safety practices by food handlers, specifically temperature control. The use of other formulation control measures is

encouraged to inhibit the growth of *C. perfringens* when temperature is not strictly controlled; further research on recooking to identify alternative time/temperature parameters may have applicability in certain food matrices (Xiao et al., 2024). When designing technological processes in food production, use of predictive models, as discussed later in this document, may be useful to identify critical product limits for foods and to design inoculated pack studies to validate the efficacy of formulation, cooling or inactivation of cells.

1.4 *CLOSTRIDIOIDES DIFFICILE*

Recent taxonomic revisions have led to the reclassification of *Clostridium difficile* as *Clostridioides difficile* (Lawson *et al.,* 2016). *Clostridioide difficile* is the predominant etiological agent of antibiotic-associated diarrhoea, which can lead to severe conditions such as pseudomembranous colitis, toxic megacolon, and fatal colitis (Di Bella *et al.*, 2024; *Smits et al.,* 2016). Extraintestinal infections are rare but have been reported (Mattila *et al.,* 2013). that CDI is an opportunistic, gastrointestinal disease that is a consequence of spore ingestion, not necessarily, but possibly, through food, and lack of protection provided by healthy gut microbiota; previous antibiotic treatment is an important predisposing factor for CDI.

The dose-response relationship between the number of *C. difficile* cells consumed, colonization and subsequent disease in susceptible individuals remains unknown. However, susceptible mice exposed to as few as seven environmental spores per cm^2 resulted in persistent infection (Lawley *et al.*, 2009). *Clostridioides difficile* synthesizes two potent exotoxins, TcdA and TcdB, both part of the large clostridial toxins family. These exotoxins glycosylate Rho family GTPases within host cells, thereby disrupting the actin cytoskeleton, inducing cell death, and eliciting a robust inflammatory response. TcdA and TcdB are considered the main virulence factors, but some strains can also produce a third toxin, binary toxin CDT, associated with increased virulence (Di Bella *et al.,* 2024). Non-toxigenic strains ($TcdA^-TcdB^-CDT^-$) do not cause disease. Standard treatment for primary CDI typically includes antibiotics such as vancomycin and fidaxomicin, with metronidazole occasionally used in combination with vancomycin in severe cases.

Despite established health guidelines aimed at the prevention, diagnosis, and treatment of CDI, incidence rates continue to rise. The main emerging issues in human CDI are increased mortality (since 2003), increased recurrent cases, and increasing rates of community-associated CDI (Di Bella *et al.,* 2024; Smits *et al.,* 2016).

Historically, CDI was viewed as a healthcare-associated infection. However, enhanced surveillance and the increased application of WGS have revealed that only 35 percent of CDI cases are attributable to transmissions in clinical settings with the majority of CDI considered community acquired (Eyre *et al.*, 2013). In the most recent data from Australia, 80 percent of CDI cases were reported as community-associated (ACSQHC, 2023).

The prevalence of *C. difficile* in live food animals, environmental samples, and various food types confirm that these are potential sources of community acquired CDI (Lund and Peck, 2015; Candel-Pérez *et al.,* 2019; Kong, 2020; Lim *et al.*, 2020b; Bolton and Marcos, 2023; Rui *et al.,* 2024; Rodriguez Diaz, 2024). Although foodborne *C. difficile* infections have to date not been proven, current data are consistent with food or the environment contaminated with soil or faecal material serving as a vehicle to transmit spores between locations and countries. Sequencing studies show that indistinguishable *C. difficile* clones colonize both humans and animals across various geographical regions (Knight and Riley 2019; Knetsch *et al.*, 2014). Highly related *C. difficile* strains were also shared between food (potatoes), humans, and animals in a recent pan-European study (Rupnik *et al.,* 2024). Examples for such mixed genomics clusters in the human/animal/food/environment were described based on analysis of deposited genomes (Cersosimo *et al.,* 2024). Additional evidence supporting foodborne transmission of *C. difficile* comes from a 2011–2012

outbreak in Australia and New Zealand involving the uncommon ribotype 244 strain. Comparative analysis with reference laboratory strains indicated that the outbreak clone shared a common ancestor with isolates from both the United States and the United Kingdom (Eyre *et al.*, 2015; De Almeida *et al.*, 2013). Ribotype 244 isolates from the United States were closely related to, but distinct from, the Australian ribotype 244 outbreak isolates. One UK isolate was found to be phylogenetically related to the outbreak strain, and follow-up revealed that the patient had recently travelled from Australia. Despite the geographic separation, the isolates were genetically highly similar and showed no evidence of geographic clustering – consistent with dissemination from a single point source.

Nonetheless, the pathogen's persistence along the food chain remains inadequately understood. A comparative analysis of the thermal resistance of *C. difficile* spores with those of *C. perfringens* showed that spores from both species withstand typical cooking temperatures used for meat and poultry, allowing them to remain as a source of contamination in cooked products (Lund and Peck, 2015).

The persistence of *C. difficile* as a major public health challenge underscores the need for continued research into its transmission dynamics, especially within the community setting. Identifying the precise sources of community-acquired CDI, the role of food in overall transmissions, and frequency and numbers of *C. difficile* vegetative cells and spores in foods will be crucial in developing more effective prevention strategies.

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CHAPTER 2
HAZARD IDENTIFICATION AND CHARACTERIZATION

2.1 HAZARD IDENTIFICATION – BONT-PRODUCING SPECIES OF CLOSTRIDIA

Foodborne botulism represents a severe intoxication resulting from the ingestion of food containing preformed botulinum neurotoxin (BoNT) produced by strains of *C. botulinum* Group I, Group II and rarely Group III, or less commonly, by neurotoxigenic strains of *C. sporogenes* (Group I), *C. baratii* (Group V) or *C. butyricum* (Group VI). There are seven traditionally accepted serotypes of BoNTs defined as A-G, although newly identified serotype and hybrid toxin types have been described (Peck *et al.,* 2017). Additionally, multiple subtype toxins have been recognized within each serotype.

Spores of toxigenic clostridia are ubiquitous in the environment. Consequently, preventing foodborne botulism necessitates the identification and implementation of control measures aimed at spore destruction or the inhibition of spore germination, cell multiplication, and neurotoxin formation (ACMSF, 2023).

Clostridium botulinum Group I, commonly referred to as proteolytic *C. botulinum*, are mesophilic bacteria characterized by their highly proteolytic nature and their ability to form spores with high thermal resistance. The spores of *C. botulinum* Group I are specifically targeted by the "botulinum cook" process (121 °C for 3 minutes) employed in the production of low acid canned foods. Outbreaks of foodborne botulism have been associated with failures to apply the botulinum cook to canned or bottled foods, particularly with artisanal foods, as well as with temperature abuse of products intended for refrigerated storage (Peck, 2009).

In contrast, *C. botulinum* Group II, also known as non-proteolytic or psychrotrophic *C. botulinum*, is a saccharolytic bacterium with a minimum growth temperature of 3 °C, forming spores of moderate heat resistance. Foodborne botulism outbreaks involving *C. botulinum* Group II have been linked to temperature abuse of products intended to be stored under refrigeration (not exceeding 8 °C), including vegetables, fish and meat, especially culturally prepared fermented marine products. Hence, ongoing vigilance is essential to ensure the safe production of mildly processed, vacuum/modified atmosphere packaged, and other chilled foods (Peck, 2009).

Clostridium botulinum Group III are non-proteolytic mesophilic bacteria that form spores with heat resistance typically higher than that of Group II strains, but lower than that of Group I strains (Segner *et al.,* 1971; Portinha *et al.*, 2022) and therefore may survive the mild thermal processes given to some foods. While Group III *C. botulinum* has been associated with a limited number of suspected and confirmed foodborne botulism cases, they are extremely rare (T**able 1** and Rasetti-Escargueil, 2020). Details of the food processing or handling failures that contributed to these human cases are unknown.

2.2 HAZARD CHARACTERIZATION – BONT-PRODUCING SPECIES OF CLOSTRIDIA

Foodborne botulism is a severe and potentially lethal neuroparalytic intoxication, which can result from the ingestion of as little as 50 ng of botulinum neurotoxin (Peck, 2009). Clinical manifestations may appear within hours of exposure but can also be delayed for several days depending on the toxin type and dose ingested. In contrast, infant botulism and intestinal (adult) botulism represent toxico-infections where the pathogen colonizes the gastrointestinal tract, leading to *in-situ* neurotoxin production (Harris and Dabritz, 2024; Harris *et al.*, 2020).

The World Health Organization (WHO) provides the following description of foodborne botulism symptoms (WHO, 2023; ACMSF, 2023):

> "Early symptoms include marked fatigue, weakness and vertigo, usually followed by blurred vision, dry mouth and difficulty in swallowing and speaking. Vomiting, diarrhoea, constipation, and abdominal swelling may also occur. The disease can progress to weakness in the neck and arms, after which the respiratory muscles and muscles of the lower body are affected. There is no fever and no loss of consciousness. Symptoms usually appear within 12 to 36 hours (within a minimum and maximum range of 4 hours to 8 days) after exposure. The disease can be fatal in 5 to 10% of cases".

Timely clinical diagnosis, immediate administration of the appropriate antitoxin (US CDC, 2010), and, in severe instances, respiratory support are critical interventions for treating the disease. The effects of foodborne botulism can endure for months to years, often resulting in significant long-term health impacts. The incidence of botulism is relatively low compared with other foodborne illnesses such as those caused by other enteric pathogens (ACMSF, 2023).

Botulinum neurotoxins, along with their associated complexes, are very stable within food matrices and can survive the acidic and highly proteolytic conditions encountered during passage through the gastrointestinal tract (Centurioni *et al.,* 2022; Pirazzini *et al.,* 2017; Rasetti-Escargueil and Palea, 2024). Unlike foodborne botulism, which affects anyone who consumes preformed toxin in contaminated food, infant and adult intestinal botulism occur in individuals with an immature or compromised intestinal microbiota. Illness results from oral exposure to spores of *C. botulinum*, BoNT-producing *C. butyricum*, or *C. baratii*, followed by germination in, and colonization of, the intestinal tract; the cells proliferate and produce botulinum neurotoxin *in situ*. The neurotoxin is then absorbed into the bloodstream, reaches neuromuscular junctions, and causes paralysis.

Infant botulism occurs in infants of an age of less than one year, although most cases present in younger patients under six months and have been associated with perturbations in the intestinal microbiota due to changes in diet and weaning (Arnon, 1986; Harris and Dabritz, 2024). Infant botulism usually presents initially with non-specific symptoms like constipation, poor feeding, and sleepiness, followed by symmetric progressive flaccid paralysis with loss of head control, and generalized hypotonia.

Like in the other forms of botulism, infant botulism can be fatal due to respiratory failure (Arnon and Chin, 1979; Thompson *et al.,* 1980). The symptoms can range from mild to severe and have been related to the sudden infant death syndrome (Arnon and Chin, 1979; Arnon *et al.,* 1979; Nevas *et al.,* 2005). During hospitalization, infants can suffer from secondary complications like pneumonia after aspiration of milk or food due to muscle weakness, or gastroenteric symptoms and colitis caused by *C. difficile* co-infections (Rosow and Strober, 2015; Fenicia and Anniballi, 2009). Initially, the non-specific clinical presentation often delays diagnosis and initiation of treatment. Supportive care and administration of antiserum preparations have proven effective in botulism treatment,

including use of a human-derived botulism immune globulin specifically formulated for infants (Arnon *et al.*, 2006).

Honey has been identified as a source of clostridial spores resulting in infant botulism (Arnon *et al.,* 1979; Brunt *et al.*, 2020a; Harris and Dabritz, 2024). Additionally, environmental exposure, such as dust and soil, has also been identified as a potential risk factor (Derman *et al.* 2014; Gladney *et al.*, 2021; Harris and Dabritz, 2024; Douillard *et al.,* 2022). As referenced above, botulism may coincide with other forms of clostridial disease. In the case of infant botulism, co-infection with *C. difficile* has been documented in several cases and significantly contributed to the severity of disease (Schechter *et al.,* 1999; Fenicia *et al.,* 2002; Douillard *et al.,* 2024). Local paralysis of the intestine leading to lowered intestinal motility has been proposed as a cause of increased susceptibility to *C. difficile* diarrhoea (Schechter *et al.,* 1999).

The true occurrence of infant botulism, especially in low- and middle-income countries, is likely to be largely underestimated due to its nonspecific clinical presentation and challenges associated with accurate diagnosis (Antonucci *et al.* 2021; Rosow *et al.*, 2015). Consequently, reported cases remain scarce. Nevertheless, infant botulism is becoming more frequently diagnosed. This may be attributed to greater awareness within the medical community coupled with access to advanced laboratory diagnostic tools, particularly in regions such as the United States (notably California), the European Union, China and Japan. For example, in the United States infant botulism was the most common form of botulism with 152 (71 percent) of the 201 laboratory-confirmed botulism cases in 2019 (Antonucci *et al.,* 2021; US CDC, 2019). In the European Union/European Economic Area, the incidence rate of infant botulism in 2022 was 0.2 cases per 100 000 infants per annum compared to an overall notification rate of 0.02 cases of foodborne botulism per 100 000 general population (ECDC, 2024). However, the relative incidence of infant botulism compared to foodborne botulism is lower in Europe than in the United States. In France, 17 infant botulism cases were reported out of 177 total botulism cases during the 2008–2018 reporting period, while Italy recorded 36 infant cases out of 466 total botulism cases (7.7 percent) from 1986 to 2015 (Le Bouquin *et al.,* 2022; Anniballi *et al.*, 2017; Moodley *et al.*, 2015). Between 2016 and 2021, China reported 20 cases of infant botulism (average 3.3 cases annually) (Wang Dai *et al.,* 2024) compared to 386 foodborne botulism cases recorded from 2004 to 2020 (average 22.7 cases annually) (Li *et al.*, 2022), resulting in a 12.7 percent relative incidence of infant botulism among total botulism cases.

Adult intestinal botulism has a similar aetiology as infant botulism and is also caused by *in situ* production of BoNT through intestinal colonization by BoNT-producing C. *botulinum, C. butyricum* or *C. baratii* after oral ingestion of clostridial spores. Its clinical presentation is identical to foodborne botulism, but the onset of symptoms may be slower. Adult intestinal botulism typically occurs in individuals with disrupted intestinal microbiota due to gastrointestinal surgeries, prolonged antibiotic use, or predisposing conditions such as diverticulitis or Crohn's disease (Harris *et al.,* 2020). However, cases have also been documented in patients lacking these identifiable risk factors (Guru *et al.,* 2018). Adult intestinal botulism is rare and, like infant botulism, is likely underreported due to diagnostic limitations and limited clinical awareness. (Harris *et al.,* 2020).

In contrast, wound botulism results from the proliferation of the bacterium within a deep wound, leading to localized production of botulinum neurotoxin. While historically associated with traumatic injuries, its incidence has increasingly been associated with injectable drug abuse in recent years (Peck, 2009; Chatham-Stephens *et al.,* 2018).

2.3 HAZARD IDENTIFICATION – *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens, like other clostridial species, is common in the environment, food, and gastrointestinal microbiota of humans and animals. Contamination of retail products varies depending on food matrix and country of origin. Although concentrations as high as 4-$\log_{10}$ CFU/g sulfite-reducing clostridia have been reported in raw meat samples, counts

were not confirmed as enterotoxigenic *C. perfringens* spores (Golden, 2009). One US survey of 494 retail processed meats showed that the number of *C. perfringens* spores, if present, were less than detectable level by direct plating (< 0.5 $\log_{10}$ CFU/g; no enrichments were conducted) in all but one sample (USDA-FSIS, 2023b). Comparable low numbers of total *C. perfringens* (2-$\log_{10}$ CFU/g or less of spores plus vegetative cells) were detected in raw and cooked meat and poultry products in US manufacturing (Taormina, 2003; Kalinowski, 2003). Spices may also be a source of *C. perfringens* (Powers *et al.*, 1975; Bhat *et al.*, 1987). In Japan, enterotoxigenic *C. perfringens* was detected in 2.5–13.3 percent of shellfish, and dried seafoods, curry powder and spices, whereas *C. perfringens* was not detected in retail beef and pork samples (Onishi *et al.,* 2024).

Compared to other clostridia, *C. perfringens* is relatively aerotolerant. Its optimal growth temperature range is 40–47 °C (min 12 °C, max 50 °C). Under optimal conditions of pH and water activity, the doubling time for vegetative cells is approximately 7.1 minutes at 41 °C. Additionally, the D-value for spores at 100 °C varies significantly, ranging from 0.01 to 123 minutes. A 2022 USDA survey found that cooked beef typically contains less than 1-$\log_{10}$ CFU/g of *C. perfringens* spores, confirming that normal cooking effectively eliminates vegetative cells. However, if spores survive the cooking process, beef, poultry and gravies held at growth temperatures between 27–50 °C for extended periods are the primary vehicles for *C. perfringens*-related foodborne illnesses (USDA-FSIS 2023a; US CDC, 2025).

2.4 HAZARD CHARACTERIZATION – *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens type F food poisoning is initiated by the consumption of food contaminated with a high number of vegetative cells, which can endure the acidic environment of the stomach (Grenda *et al.*, 2023). These vegetative cells subsequently undergo sporulation in the intestinal lumen, during which the lysis of mother cells releases the spores and concurrently, CPE.

Although the exact infective dose remains undetermined, the US Food and Drug Administration (FDA) reports that symptoms typically occur following the ingestion of large numbers of vegetative cells – specifically, more than 10^8 vegetative cells per 100-g serving of food (i.e. 10^6 CFU/g) (Johnson *et al.,* 2007; US FDA, 2012; Health Canada, 2025).

Primary clinical manifestations include stomach cramps, abdominal pain, and diarrhoea, typically emerging 8 to 12 hours after consumption of contaminated food. Diarrhoeal episodes are generally limited to 1 to 5, with some patients also reporting nausea and vomiting. In otherwise healthy individuals, the illness is usually mild and self-limiting, with symptoms resolving within 24 hours. Nevertheless, fatalities may occur in vulnerable populations, particularly the elderly or those with compromised health, such as hospitalized patients or residents of long-term care facilities (EFSA, 2005). Illness caused by CPILE (*C. perfringen*s iota-like enterotoxin) / CPE (enterotoxin) or BEC (binary enterotoxin of *C. perfringens*) strains exhibit similar symptoms (Irikura *et al.,* 2015; Yonogi *et al.,* 2014).

Clostridium perfringens type C is occasionally implicated in a severe and potentially fatal form of foodborne illness (Johnson, 2025). Pigbel disease, a condition marked by necrotizing enterocolitis, is notably associated with *C. perfringens* type C. The disease is observed in various low-income countries and, albeit rarely, in high-income countries where it is primarily confined to adults with chronic illnesses (Petrillo *et al.*, 2000). This disease exhibits a predilection for individuals suffering from severe protein malnutrition and diabetes, with a particularly high mortality rate in children (Wormald *et al.*, 2016). Clinically, the disease manifests as diarrhoea and abdominal pain. Histopathologically, *C. perfringens* type C infection is characterized by haemorrhagic necrosis of the intestinal wall, beginning in the mucosa and typically advancing to involve all intestinal layers (Uzal and McClane, 2011).

Management of clostridial necrotizing enteritis generally involves antibiotic therapy and surgical intervention (Wormald *et al.,* 2016).

2.5 HAZARD IDENTIFICATION – *CLOSTRIDIOIDES DIFFICILE*

Clostridioides difficile spores are widely present in soil and water, particularly following faecal contamination or the application of manure to fields. However, the primary site of multiplication is the intestinal tract of humans and animals, specifically when the gut microbiota is disrupted. This disruption occurs in young individuals whose microbiota and colonization resistance are not yet established, as well as in adults with dysbiotic microbiota due to factors such as antibiotic use, certain medications, poor diet, or long-distance travel.

Clostridioides difficile has been detected in raw and cooked foods, but quantitative studies of food contamination are limited. Meat, milk, seafood and vegetables are contaminated with *C. difficile* spores to varying degrees (Rodriguez-Palacios, 2020; Bolton and Marcos 2023; Rodriguez Diaz *et al.*, 2024). The average prevalence of food contamination with *C. difficile* is reported to be below 8 percent (Candel-Perez, 2019; Rodriguez-Palacios, 2020). The highest contamination rates are observed in seafood, poultry meat with skin intact, and soil-associated foods such as leafy greens, potatoes and onions (Rodriguez-Palacios, 2020). A German survey found 15.8 percent of retail chicken meat samples with skin on were positive for *C. difficile*, whereas none of the 42 turkey or chicken meat samples without skin were contaminated (Heise *et al.,* 2021). Notably, studies have found that up to 53 percent of potato skin samples contain *C. difficile* spores, likely due to soil contamination during cultivation (Tkalec *et al.,* 2020; Tkalec *et al.*, 2019; Lim *et al.,* 2018).

Quantitative assessments of contamination have yielded variable results. One study reported ground beef and ground pork samples containing between 20 and 240 *C. difficile* spores per gram (Weese, 2009) while another found calf carcasses (veal) with median counts of 7 CFU/cm² and maximum counts of 33 CFU/cm² in 16.7 percent (25/150) of samples exceeding the detection limit of 3 CFU/cm² (Knight *et al.*, 2016).

In recent years, the proportion of community-acquired *C. difficile* infections has increased, affecting individuals with no prior exposure to healthcare facilities (Fu *et al.*, 2021; Markovska, 2023). Despite evidence indicating the presence of *C. difficile* in water sources, livestock, slaughterhouses, and retail food products, the transmission dynamics of community-associated infections remain poorly understood. Foods contaminated with soil or faeces may serve as potential exposure vehicles (Bolton and Marcos, 2023; Candel-Pérez *et al.,* 2019, Lund and Peck 2015; Lim *et al.,* 2020a). However, a direct link between food consumption and CDI remains difficult to establish, as illness onset is often delayed and triggered by microbiota disruption – typically from antibiotic treatment or underlying conditions such as inflammatory bowel disease.

Over 900 *C. difficile* PCR-ribotypes have been identified, including highly virulent strains such as ribotypes 027 and 078 (Bolton and Marcos, 2023; Fu *et al.,* 2021). Many *C. difficile* ribotypes are shared between humans, animals and food (Lim *et al.,* 2020b). PCR ribotypes associated with community-acquired CDI have been detected in food products and livestock (Candel-Pérez *et al.,* 2019), highlighting the environmental role in transmission between food, animals, and humans (Lim *et al.,* 2020a).

Due to the heterogeneity of strains within the same ribotype, confirming these overlaps requires detailed molecular analysis. Genome sequencing frequently identifies clusters of closely related strains across animals, food and humans, substantiating the potential transmission pathways (Rupnik, Viprey *et al.,* 2024; Knight and Riley, 2019). Food, especially when covered with soil remnants, may therefore have a role as a vehicle introducing *C. difficile* spores into the domestic environment. These spores can persist in

household settings, where poor hygiene can lead to ingestion and pose a health risk for individuals with perturbed gut microbiota.

2.6 HAZARD CHARACTERIZATION – *CLOSTRIDIOIDES DIFFICILE*

As previously noted, *C. difficile* infection is an opportunistic disease, and to date, no confirmed cases of foodborne illness have been directly attributed to *C. difficile*. Nonetheless, indirect evidence implicates food as a potential source of human exposure. For example, while certain *C. difficile* ribotypes are geographically or institutionally confined, clustering within specific countries or hospitals, others are more widely distributed across multiple countries. This pattern raises the possibility of a shared source of contamination, such as food exposed to soil or faecal matter (Eyre et al., 2018).

It is not known if and to what extent *C. difficile* multiplies and/or persists in the intestines of healthy humans. However, given the extent of contamination identified in the environment and on food, transient colonization may frequently occur without adverse health effects. The dose-response for *C. difficile* is not known but is very likely to be host dependent.

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CHAPTER 3
SOURCE ATTRIBUTION AND EXPOSURE

3.1 DISTRIBUTION OF CLOSTRIDIUM BOTULINUM

Clostridium botulinum spores exhibit substantial resistance to various physical and chemical stressors, including desiccation, freezing temperatures, and most sanitizers. Consequently, these spores are ubiquitously present in the environment. Their presence has been documented in the gastrointestinal tracts of animals, including humans, as well as in soil and aquatic sediments, both saline and freshwater (Hauschild, 1989; Espelund and Klveness, 2014).

In the temperate zone of North America, soils in states west of the Mississippi River typically exhibit contamination with type A strains of *C. botulinum*, while eastern states commonly contain type B strains (Palmer *et al.*, 2019; Shapiro *et al.,* 1998); serotype E is most common in the areas of the Great Lakes and the US Pacific Northwest and Pacific coast of Canada. In Argentina, type A strains were the most prevalent (Luquez *et al.*, 2007). In Europe, type B strains are more prevalent in soils (Palmer *et al.* 2019; Le Maréchal, 2025) and type E is frequently found in aquatic sediments (Huss, 1980). In China, Type A and B are commonly found in soil (Ma *et al.,* 2022). Worldwide, Type E strains are notably abundant in both fresh water and marine sediments (Huss, 1980; Hielm *et al.*, 1998; Leclair *et al.*, 2013a).

Estimates of spore numbers in foods inherently entail uncertainties, underscored by surveillance revealing significant variability in both the prevalence of *C. botulinum* spores in different food materials and the number of spores. For example, Barker *et al.* (2016) conducted an extensive review of the literature examining the incidence of Group II *C. botulinum* in food commodities used in the manufacture of chilled foods in the United Kingdom and employed a Most Probable Number (MPN) approach to estimate the number of Group II *C. botulinum* spores present in the samples. Depending on the food category, the incidence of Group II *C. botulinum* positive samples ranged from 0 percent in fluid dairy to 22.0 percent in other dairy, whilst the estimated spore numbers ranged from 7.5 spores/kg in fluid dairy and 7.8 spores/kg in meat to 145.9 spores/kg in shellfish and 228.7 spores/kg in mushrooms and fungi (**Table 2**).

TABLE 2. PREVALENCE AND ESTIMATED NUMBERS OF CLOSTRIDIUM BOTULINUM SPORES IN FOOD MATERIALS (GROUP II) AND HONEY (GROUP I)

Food category	Number of samples tested	Number of positive samples	Percent positive	Estimated spore numbers/kg
Meat	4 642	98	2.1	7.8
Fish	30 285	3 343	11.0	51.4
Shellfish	3 879	375	9.7	145.9
Cereals	513	7	1.4	8.6
Plants	2 399	59	2.5	24.6
Dairy, fluid	222	0	0	7.5[a, b]
Dairy, nonfluid	1 563	344	22.0	34.0
Mushrooms & fungi	1 390	15	1.1	228.7
Dried herbs & spices	371	19	5.4	27.8
Honey	2 414	205	8.5[c]	20.3

Notes: [a] When all tests were negative, a single positive test result was added to achieve a conservative estimate of MPN. [b] University of Guelph, Canada reported contamination rate of 1 spore Group I *C. botulinum* per litre of milk (Collins-Thompson and Wood, 1993). [c] Depending on location of sampling, some surveys show positive rates for honey to be as high as 100 percent (Harris and Dabritz, 2024); one honey sample that was associated with infant botulism contained 15 000 spores/kg (Monetto *et al.,* 1999).

Sources: See 3.6 References section for full list of sources in Table 4.

Based on their literature review, and additional analyses of 483 food materials, Barker *et al.* (2016) concluded that ingredients used in chilled food production in the United Kingdom contain approximately 5 to 10 Group II *C. botulinum* spores per kilogram. These finding align with Group I spore levels found in ingredients used for refrigerated foods in France (Carlin *et al.*, 2004). More recent research examining 74 vegetable products from Finland and Germany (Pernu *et al.*, 2020) has further highlighted variability in both Group I and Group II spore numbers. Notably, spore counts in a vacuum-packaged vegetarian sausage product were elevated, reaching an estimated 1 200 spores/kg. Given these ranges, the complete absence of *C. botulinum* spores in raw food materials is considered unattainable (ACMSF, 2023).

Healthy livestock can carry *C. botulinum* spores in their intestines and serve as a reservoir for contamination of meat production environments and meat through faecal contamination of carcasses and thereby introduce *C. botulinum* spores into the food chain. Swedish studies reported 62 percent of faecal samples of pigs and 73 percent of faecal samples of cattle at slaughter being PCR-positive for *C. botulinum* type B, however, at low concentrations (Dahlenborg et al., 2001; Dahlenborg et al., 2003). In contrast, Schmid et al. (2013) were not able to detect *C. botulinum* in the faeces of 506 healthy cattle in Germany (Schmid et al., 2013); another German study reported a detection rate of 4.4 percent of *C. botulinum* in cattle faecal samples (Fohler et al., 2016), and a Finnish study reported a low prevalence of 3 percent in pig intestinal samples (Myllykoski, 2006).

3.2 FOODS ASSOCIATED WITH ILLNESS DUE TO BONT-PRODUCING CLOSTRIDIA

Foodborne botulism

The extensive body of literature on food-associated botulism has been comprehensively summarized (Peck, 2009; Peck and van Vliet, 2016; ACMSF, 2023). **Table 3** provides an overview of selected foodborne botulism outbreaks that have been documented globally since 1992. Notably, the largest outbreaks have been linked to commercial food production and multiple exposure events.

TABLE 3. FOODS IMPLICATED IN FOODBORNE BOTULISM

Food	Suspected or confirmed food	Toxin type	Cases	Country, year	Reference
Fish	Uneviscerated grey mullet fish (faseikh)[a]	E	97	Egypt,1991	Weber *et al.*,1993
	Hot smoked whitefish[a]	E	2	Germany, 1997	Korkeala *et al.,*1998
	Salted fish[b]	Unknown	2	Iran (Islamic Republic of), 2001	Vahdani *et al.*, 2002
	Vacuum-packed smoked salmon[a]	E	1	Germany, 2004	Dressler, 2005
	Smoked whitefish[a]	E	2	Finland, 2006	Lindström *et al.,* 2006
	Sardines – home canned[c]	B	15	France, 2023	Courtot-Melciolle *et al.*, 2023
	Atlantic wolffish[b]	E	1	Belgium, 2023	De Vet, 2023
Meat/poultry	Cooked boneless pork product[a]	A	1	Canada, 2001	Leclair *et al.,* 2013b
	Barbequed pork[c]	Unknown	5	Austria, 2006	Meusburger *et al.*, 2006
	Fermented raw goat meat (cinkrugan)[c]	B	5	Taiwan Province of China, 2006	Tseng *et al.,* 2009
	Sausage[a]	A	66	China, 2007	Zhang *et al.*, 2010
	Smoked ribs (restaurant)[a]	A	12	China, 2013	Feng *et al.,* 2015
	Vacuum-packaged RTE chili chicken feet[a]	E	4	China, 2019	Dong *et al.,* 2022
Dairy	Mascarpone cheese[a]	A	8	Italy, 1996	Aureli *et al.,* 1996
	Fermented milk drink (doogh)[c]	A	21	Iran (Islamic Republic of), 2023	Sadeghian *et al.*, 2023
Fruit	Canned tomatoes – home canned	B	4	Canada, 1999	Loutfy *et al.*, 2003
Egg-based	Potato omelette[a]	B	9	Spain, 2023	ECDC, 2023

TABLE 3. (CONT'D) FOODS IMPLICATED IN FOODBORNE BOTULISM

Food	Suspected or confirmed food	Toxin type	Cases	Country, year	Reference
Vegetables	Green beans – home canned	Unknown	2	Spain, 1996	Polo *et al.*, 1996
	Canned bamboo shoots – home canned	A	13	Thailand, 1998	Pantukosit, 2007
	Canned asparagus – home canned	B	9	France, 2000	Abgueguen *et al.*, 2003
	Canned mushrooms – home canned	Unknown	5	Türkiye, 2005	Cengiz *et al.*, 2006
	Canned bamboo shoots – home canned	A	209	Thailand, 2006	Kongsaengdao *et al.*, 2006
	Canned carrots and green beans – home canned	A	6	United States, 2008	Date *et al.*, 2011
	Canned green beans – home canned	A	3	United States, 2009	Date *et al.*, 2011
	Jar of olives[a]	B	2	Finland, 2011	Jalava *et al.*, 2011
	Canned red peppers – home canned	Unknown	4	Türkiye, 2011	Ahmet AĞAÇAYAK, 2011
	Canned potatoes – home canned	A	29	United States, 2015	McCarty *et al.*, 2015
	Canned peas – home canned	A	3	United States, 2018	Bergeron *et al.*, 2019
	Multipack roasted potatoes[a]	A	4	United States, 2019	Gayou *et al.*, 2022
	Canned vegetarian pâté[a]	B	12	Viet Nam, 2020	Ho *et al.*, 2022
Products	Pickled eggs[c]	A	5	China, 2024	Miao *et al.*, 2024
	Mayonnaise[a]	A & B	50	Saudi Arabia, 2024	Al Zuayr *et al.*, 2025
Soups	Fish soup[a]	A	1	France, 1999	Carlier JP *et al.*, 2001
	Potato soup[a]	A	1	United States, 2011	US CDC, 2011
	Broccoli soup[a]	Unknown	2	United States, 2012	Edmunds *et al.*, 2022
	Clam chowder[a]	A	1	United States, 2021	Edmunds *et al.*, 2022

Notes: [a] Commercial product. [b] Preparation type not confirmed. [c] Home preparation.

Sources: Adapted from Peck. & van Vliet, 2016; ACMSF. 2023. See 3.6 References section for full list of sources in Table 4.

The Advisory Committee on the Microbiological Safety of Food (ACMSF, 2023) report summarizes botulism associated foods globally. Between 2001 and 2017, the United States reported 326 laboratory confirmed foodborne botulism cases, with 277 linked to a specific food or beverage (Lúquez *et al*., 2021). Home-prepared foods, excluding canned foods, accounted for 47 percent of cases, followed by home-canned foods (29 percent), commercially canned foods (10 percent), and other commercially prepared foods (6 percent). A source was not identified in the remaining 8 percent of cases. Outbreaks from commercial foods implicated a chili meal, chili sauce, and nacho cheese sauce. Commercially prepared refrigerated foods (incorrectly stored) were associated with 13 foodborne botulism events in the United States between 1994 and 2021 (Edmunds *et al.,* 2022), with a total of 37 cases and four deaths. Eleven events were due to the consumer not storing the products refrigerated at home, and the other two events involved products stored unrefrigerated before purchase by the consumer. In three of the incidents, refrigerated storage instructions were inadequate or not readily accessible (Edmunds *et al*., 2022).

A review of botulism in China from 2004 to 2020 identified 80 foodborne outbreaks, resulting in 386 illnesses and 55 deaths (Li *et al.,* 2022). The foods most implicated were home-prepared, traditionally processed stinky tofu and dried beef, which accounted for 51.2 percent of outbreaks. Inadequate processing/fermentation/acidification and improper storage were contributory factors in 77.5 percent of outbreaks. An overview of type E botulism in China documented 11 outbreaks between 1965 and 2005, implicating soybean milk, fermented bean curd, raw dried beef, dried mackerel, and blood sausage. The bacteria involved were either Group II *C. botulinum* type E or *C. butyricum* type E (Fu and Wang, 2008).

From 2007 to 2017, Iran documented 252 confirmed cases of foodborne botulism, 743 suspected cases, and 48 fatalities (Khorasan *et al.,* 2020). Home-prepared, traditionally processed foods (including smoked fish, salted fish, ham, bacon, blood pudding, salami, and sausage) accounted for 34.1 percent of reported events. Commercially canned fish (28.6 percent), fish spawn (10.5 percent), dairy products (10.1 percent), vegetables and home prepared legumes (9.7 percent), cottage cheese (5.9 percent), and canned fruits (1.1 percent) were other implicated foods.

Canada reported 91 laboratory confirmed outbreaks of foodborne botulism between 1985 and 2005, involving 205 cases and 11 deaths. Group II *C. botulinum* type E outbreaks were linked to traditionally prepared marine mammal and fish products by native communities, mainly the Inuit of Nunavik in northern Quebec and the First Nations population of the Pacific coast of British Columbia. Two outbreaks were attributed to commercial ready-to-eat meat products (pâté and cooked boneless pork), and three were associated with foods served in restaurants (chopped garlic in oil, bottled chanterelle mushrooms, and baked potato). Additionally, eight outbreaks were linked to non-native home prepared foods (Leclair *et al*., 2013b). A review of foodborne outbreaks in British Columbia from 2009 to 2013 (Taylor *et al*., 2015) identified three botulism outbreaks and one death, with implicated foods including fruit and vegetables, seafood, and sauces/condiments. Between 2006 and 2021, 55 foodborne botulism outbreaks, involving 67 cases and seven deaths, were confirmed in Canada (Harris *et al.,* 2023). Most cases (52 percent) were caused by BoNT/E, whilst BoNT/A, BoNT/B and BoNT/F were responsible for 24 percent, 16 percent, and 3 percent, respectively, of the cases. Both type A and B botulinum neurotoxins were implicated in a single case (1 percent), and the serotype responsible could

not be determined in 3 percent of cases. Traditionally prepared Indigenous foods, that were stored under conditions where *C. botulinum* could grow and produce neurotoxin and were not heated before consumption, were the vehicle for 46 percent of the cases. Commercially-prepared foods (carrot juice, ground beef, salted fish, and Alfredo sauce) were responsible for four outbreaks involving eight cases. A further two outbreaks, involving three cases, were linked to home prepared foods (Harris *et al.,* 2023).

Italy recorded 1 173 suspected foodborne botulism cases from 1986 to 2015, with 421 laboratory confirmed cases, marking one of the highest incidence rates in Europe (Anniballi *et al.,* 2017). Homemade canned foods were implicated in 80.5 percent (95/118) of confirmed outbreaks, including an incident involving restaurant canned green olives. Among vegetable products, those canned in oil and brine/water accounted for 43.2 percent and 28.8 percent of laboratory-confirmed outbreaks, respectively. Other implicated foods included home bottled tuna (7.6 percent), ham (5.9 percent), home bottled meat (5.9 percent), salami/sausages (4.2 percent), cheese (2.5 percent), and tofu and seitan (1.7 percent). Among vegetables, mushrooms in oil, olives, and turnip tops were most frequently involved in botulism cases. Home canned tuna was the most common fish product linked to confirmed outbreaks. Despite infrequent association with cheese or dairy products, a notable outbreak was caused by mascarpone cheese (Aureli *et al*., 2000).

From 1987 to 2016, France reported 402 outbreaks of human botulism, comprising 731 cases and resulting in nine fatalities (Rasetti-Escargueil *et al.,* 2020). Where the food source was identified, 73.5 percent of these outbreaks were linked to cured ham, either homemade or from small scale producers, and mainly involved BoNT/B and Group II *C. botulinum*. Other implicated sources included home canned vegetables or fruits (e.g. beans, asparagus, aubergine, spinach, pumpkin, chestnut), homemade meat or fish preparation, and a limited number of commercially produced foods (e.g. fish soup, chicken/beef sausage, chicken/enchiladas, ground meat, olives/dried tomatoes, fresh pasta carbonara). A restaurant associated outbreak where ground meat was the implicated food vehicle was attributed to *C. baratii* and BoNT/F (Mazuet *et al.,* 2017).

A systematic review of botulism cases in Türkiye from 1983 to 2017 identified 91 foodborne cases (Karsen *et al.,* 2019). Among the 19 cases where laboratory confirmation for the presence of BoNTs was performed, 10 were positive for type A toxin (*C. botulinum* Group I). Canned green beans were the top food implicated (30 percent of cases) all of which were home canned, with other reported foods including strained yogurt, homemade local cheese, canned purslane, non-specified canned food, canned *Ferula communis* (giant fennel), canned peppers, scrambled eggs with garlic sausages and canned fried mushrooms.

Between 2014 and 2017, Poland reported 109 botulism cases, with 54 attributed to home-produced foods and 55 to commercial foods. Most cases (84) were linked to canned foods, with canned meat (excluding pork) being the most frequent source. Other implicated products included canned fish, sausages and cured meats, canned pork and vegetables, as well as canned mushrooms, fruits and vegetables (ACMSF, 2023). A further 54 cases of foodborne botulism were recorded in Poland during 2018 to 2021. The BoNT subtype was confirmed in 31 of the cases (57.4 percent): BoNT/B was responsible for 30 cases with BoNT/E implicated in the remaining case. The suspected food vehicle was identified in 46 cases and was mainly due to homemade preserved foods (Czerwiński and Czarkowski, 2023).

From 1955 to 2018, 8,614 botulism cases were recorded in Ukraine resulting in 659 deaths. Most cases were caused by BoNT/B (59.64 percent), followed by BoNT/E (25.47

percent), BoNT/A (7.97 percent) and suspected cases caused by BoNT/C (0.56 percent). The food products causing disease were identified in 85.3 percent of cases with the majority being canned meat products (49.5 percent), followed by canned fish including dried fish (32.3 percent), canned mushrooms (13.2 percent) and other canned vegetables (5.1 percent) during that observation period. Towards the end of this reporting period (2017–2018), the proportion of foodborne botulism cases due to commercially prepared food, in particular canned fish, increased to 32.6 percent of the recorded 258 cases. However, home canned meat and fish remained the main vehicle of botulism in Ukraine (Semenko *et al.*, 2020).

Type A foodborne botulism has been recorded in Ethiopia attributed to meal made with a traditional chili condiment called "Kochi-Kocha," cheese and clarified butter, which had been held in a tightly sealed container at room temperature for three days. This outbreak involved a family of 14, ten of whom died due to a delay in diagnosis, limited accessibility to ventilator support and a lack of regionally available antitoxin (Bacha *et al.,* 2021).

Earlier cases of two type A foodborne botulism outbreaks were reported in Kenya. The first occurred in 1978, involving nomadic people consuming traditionally prepared milk, in which there were six fatalities. A year later a second outbreak occurred in which contaminated termites were consumed leading to the death of five out of six cases (Smith *et al.,* 1979).

In April 1991, 97 patients were admitted to a hospital in Cairo, Egypt exhibiting symptoms of botulism. The implicated food product was a commercially produced uneviscerated grey mullet fish (faseikh) and 18 of the patients died. Serotype E toxin was detected in samples of leftover faseikh, and stools and gastric contents obtained from patients. It is thought that temperature abuse allowed *C. botulinum* spores in the viscera of the fish to germinate, multiply, and form toxin prior to salting (Weber *et al.,* 1993). Salted fish was responsible for another large outbreak of botulism recorded in Egypt in 2019 that affected 94 patients, two of whom died (Ghitani *et al.,* 2022).

In South Africa, in February of 2002, two siblings developed symptoms of botulism after consuming commercially processed canned fish in tomato sauce. Preformed type A neurotoxin was detected in both the stomach contents and blood of the patients, as well as in the food involved in the outbreak. *C. botulinum* type A, which was recovered from the implicated food, was believed to have contaminated the food from an environmental source via corrosion damage on the tin (Frean *et al.,* 2004).

In October 2008, an outbreak of botulism in a boarding school in Uganda sickened three students; all were hospitalized, and one died. All three had consumed a homemade oil-based condiment in which type A neurotoxin had formed. *Clostridium botulinum* type A was recovered from stool samples from one of the patients (Viray *et al.,* 2014).

A suspected botulism outbreak in Abuja, Nigeria affected three out of six family members following their consumption of pounded yam with fresh fish pepper soup. Two of the six family members died, while the third patient survived after administration of antitoxin. However, the BoNT serotype responsible for causing illness could not be determined by the mouse lethality bioassay in food remnants or in blood samples from the patients, and *C. botulinum* was not isolated from samples of food implicated in the outbreak (Okunromade *et al.,* 2020).

Thailand recorded separate botulism outbreaks that occurred in three different provinces in 2010. In April, a type F botulism outbreak affected seven individuals in Lampang Province, and in May seven cases involving a type A1(B) strain were identified in Saraburi Province and lastly, in December a type B8 strain was responsible for causing botulism in five individuals in Maehongson Province (Wangroongsarb *et al.*, 2014).

In 1998, a foodborne botulism outbreak involving 13 patients was reported in Thailand involving type A toxin in home-canned bamboo shoots (Pantukosit, 2007). However, North Thailand's largest botulism outbreak occurred on in March 2006, affecting 209 people, 42 of whom developed respiratory failure and 25 of those were referred to high facility hospitals for treatment of severe respiratory failure. The vehicle was found to be bamboo shoots, and BoNT/A was reported (Kongsaengdao *et al.,* 2006). This large outbreak prompted a concerted effort among four sources to obtain sufficient antitoxin to treat 90 patients, but with delays in treatment of 5–9 days from the time of exposure (Ungchusak *et al.,* 2007). This outbreak demonstrates the need for a global stockpile of botulinum antitoxin that can be rapidly deployed to any region throughout the world.

New Zealand has reported only three foodborne botulism events, all type A, each involving four or fewer cases. Two cases (1984, 2020) were linked to recreationally harvested mussels that were improperly fermented and stored, while one (2014) was associated with a commercially prepared, sealed-pack risotto meal that had been improperly stored at room temperature for six months, despite being labelled for refrigerated storage with a use-by date (Perchec and Cook, 2021; Smyth *et al.*, 2025).

While this report was being finalized, several botulism outbreaks were reported in different regions of Italy in 2025. These included an outbreak in Sardinia associated with street foods; an outbreak in Calabria linked to a street-food broccoli product and homemade mushrooms; and an outbreak in the Friuli-Venezia Giulia region associated with homemade eggplant (Beacon, 2025a). Two cases of botulism were reported in Brittany, France, linked to contaminated tortillas, and six individuals were hospitalized with foodborne botulism in Maine-et-Loire associated with home-canned carrots (Beacon, 2025b).

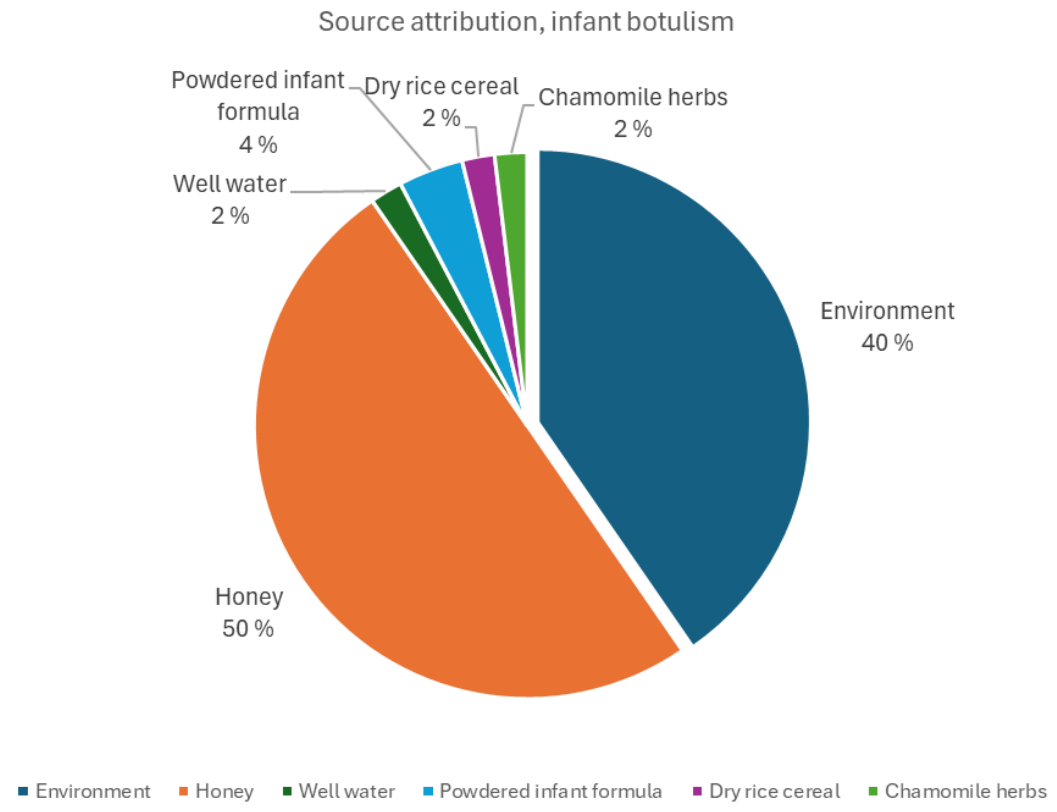
Infant botulism

A recent study has recorded 2 943 cases of infant botulism worldwide between 2007 and 2021, mostly type A and B and more rarely type E and F (Dabritz *et al.,* 2025). In most cases, the source of *C. botulinum* spores causing infant botulism remained unidentified. This is likely because infant botulism is a sporadic disease with a long incubation time of up to 30 days and an infectious dose of as few as 10–100 spores (Arnon *et al.*, 1979; Panditrao *et al.,* 2020; Harris and Dabritz, 2024). However, exposure to *C. botulinum* spores from the environment and through honey has been identified as a primary risk factor. An early study by Arnon *et al.*, (1979) reported that more than 30 percent of hospitalized infant botulism cases worldwide had ingested honey before falling sick and therefore identified honey as a significant source for *C. botulinum* spores. Harris and Dabritz (2024) reviewed available surveys and reported that *C. botulinum* was detected in 4 percent of all honey samples analysed worldwide. Based on their review of 52 cases investigations between 1976 and 2019 for which the source of contamination was identified, honey was the most common food source for infant botulism in 26 reports (Harris and Dabritz, 2024) (Figure 1). As a result, public health authorities in the United States and numerous other countries recommend against feeding honey to infants under one year of age – a measure that has reduced honey-associated cases of infant botulism.

More recently, the ingestion of spores from an infant's immediate environment has been recognized as a route of exposure (Panditrao *et al.,* 2020). Other food sources reported through 2019 were Chamomile herbs (1), dry rice cereal (1), and powdered infant formula (2) (Panditrao *et al.,* 2020; Harris and Dabritz, 2024). Two cases were related to untreated well water and terrapin tank water. As implied above, the remaining cases identified through 2019 were related to environmental sources like dust and soil (**Figure 1**). In Japan, homemade vegetable soup was implicated in one case of infant botulism (IASR, 2025). Although food items other than honey are seldom linked to infant botulism, the combination of a low infectious dose and the ubiquitous presence of clostridial spores suggests that food cannot be entirely ruled out as a potential source of exposure in this life-threatening disease.

FIGURE 1. DISTRIBUTION OF THE SOURCES IDENTIFIED IN INFANT BOTULISM 52 CASES REPORTED

(Argentina, Canada, China, Denmark, Finland, Ireland, Israel, Italy, Japan, Kuwait, Norway, Portugal, Spain, the United Kingdom, and the United States between 1976 and 2019.)



Sources: Based on Harris & Dabritz, 2024.

Harris, R.A. & Dabritz, H.A. 2024. Infant botulism: in search of *Clostridium botulinum* spores. *Current Microbiology*, 81: 306. https://doi.org/10.1007/s00284-024-03828-0

Although infant botulism is typically regarded as a sporadic illness, while finalizing this report, an unprecedented outbreak in 2025 was epidemiologically linked to powdered infant formula manufactured in the United States. Between December 2023 and November r 2025, 48 cases of infant botulism (28 confirmed cases; 20 probable cases) were reported across 187states, where the infant consumed a single brand of organic whole milk powdered infant formula. A pronounced temporal clustering was observed, with 38 cases identified between August and November 2025. A retrospective review of epidemiologic interview forms for infant botulism identified an additional 10 cases with illness onset between December 2023 and July 2025 who had also consumed the same powdered infant formula (PIF) brand. All 48 infants were hospitalized and treated with BabyBIG®, a medication used to treat infant botulism. No deaths were reported. Affected infants ranged in age from 16 to 264 days old, and 22 (46 percent) are female (US CDC, 2026).

Microbiological investigation demonstrated the presence of Type A BoNt-producing *C. botulinum* spores in unopened and opened cans from two production lots of the implicated formula and from organic whole milk powder used as an ingredient in the formula (US FDA, 2026). These isolates matched several clinical isolates obtained from the August–November 2025 cases. Whole-genome sequencing revealed genetic heterogeneity, with at least two genetic clusters and 17 different strains isolated from among individual ingredients, finished products and clinical samples. At the time of the writing of this report, CDC's outbreak summaries and investigation updates did not mention detail sequencing of clinical isolates or identification of genetic clusters among infant-derived samples (USCDC, 2026).

Adult intestinal botulism

It is commonly accepted that occasional ingestion of low numbers of clostridial spores in the absence of neurotoxin does not lead to disease in healthy adult individuals, as the intestinal tract of healthy adults does not support germination and subsequent toxin production. However, in the presence of predisposing factors (e.g. disrupted intestinal microbiota), the ingestion of clostridial spores in food has been associated with cases of adult intestinal botulism (Harris *et al.* 2020; McCroksey and Hatheway, 1988). Potential food sources have been identified in some of the cases: coconut cream and home-canned blackberries in the United States (McCroksey & Hatheway, 1988) and peanut butter in Canada (Sheppard *et al.,* 2012). In addition, it has been suggested that in some cases foodborne botulism might result in persistent intestinal colonization and neurotoxin production by *C. botulinum* (Kobayashi *et al.,* 2003). Identifying implicated food items is challenging, as even low spore counts – often below the detection limits of standard analytical methods – could be sufficient to cause disease.

3.3 DISTRIBUTION OF *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens is frequently detected in many foods, albeit typically at low concentrations (Wen and McClane, 2004). Recognized as a significant foodborne pathogen in Japan, Europe, Canada and the United States (Thomas *et al.,* 2013, 2014; Wen and McClane, 2004), *C. perfringens* is not routinely isolated and enumerated from commercial food products beyond baseline surveys and in outbreak investigations.

Despite the common isolation of *C. perfringens* from food products, only a small fraction (approximately 1 to 5 percent) of these isolates, primarily type F (previously Toxinotype A with the ability to produce CPE), carry the *cpe* gene responsible for food poisoning (Wen and McClane, 2004). **Table 4** presents a summary of meats from which type F strains carrying the *cpe* gene have been isolated. For instance, in a survey of retail foods in the United States, about 1.4 percent of the samples tested were contaminated with enterotoxigenic *C. perfringens* (Wen and McClane, 2004). Notably, all these isolates harboured the *cpe* gene on their chromosome and exhibited extremely high spore heat resistance.

TABLE 4. *CLOSTRIDIUM PERFRINGENS* TYPE F STRAINS, CARRYING THE *CPE* GENE, ISOLATED FROM BEEF, PORK, AND POULTRY MEAT

Food	Product information	Number of type F positive samples/total number of samples (percentage)	Country	Year	Reference
Beef	Whole meat	5/133 (3.7%)	Iran	2022	Hassani *et al.,* 2022
	Beef	2/35 (5.7%)	Japan	2006	Miki *et al.,* 2008
	Beef	0/95 (0%)	Japan	2025	Ohnishi *et al.,* 2025
	Beef	4/155 (2.6%)	Egypt	2017	Ghoneim and Hamza, 2017
Pork	Pork	3/144 (2.1%)	United States	2004	Wen and McClane, 2004
	Ground pork	1/99 (1%)	United States	2004	Wen and McClane, 2004
	Pork	0/110 (0%)	Japan	2025	Ohnishi *et al.,* 2025
Poultry	Turkey	2/68 (2.9%)	United States	2004	Wen and McClane, 2004
	Chicken	1/147 (0.7%)	United States	2004	Wen and McClane, 2004
	Chicken	1/33 (3%)	Japan	2006	Miki *et al.,* 2008
	Chicken	1/150 (0.7%)	Turkïye	2011	Guran and Oksuztepe, 2013
	Chicken	2/252 (0.8%)	China	2018	Zhang *et al.,* 2018

Sources: See 3.6 References section for full list of sources in Table 4.

3.4 FOODS ASSOCIATED WITH ILLNESS DUE TO *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens is frequently implicated in foodborne illnesses linked to cooked, but inadequately cooled or improperly stored foods, especially meat, poultry, other high protein foods, gravies and foods thickened with starch or flour (**Table 5** summarizes implicated foods in Australian outbreaks). Bulk meat preparations, cooked in large quantities, present a particular risk (Le Marc *et al.,* 2008).

TABLE 5. FOOD CATEGORIES IMPLICATED IN *CLOSTRIDIUM PERFRINGENS* TOXIN-MEDIATED OUTBREAKS, AUSTRALIA 2001 TO 2013

Food categories	n	%
Solid masses of potentially hazardous foods*	19	24
Liquid or semi-solid mixtures of potentially hazardous foods	13	16
Roasted meat/poultry/fish	6	7
Cook/serve foods	3	4
Salads prepared with one or more cooked ingredients	0	0
Salads with raw ingredients	0	0
Multiple foods	0	0
Baked goods	2	3
Sandwiches	0	0
Beverages	0	0
Unknown food vehicle	38	47

Note: *Potentially hazardous foods are also referred to as time-temperature control for safety.

Source: May, Polkinghorne and Fearnley. 2016.

May, F.J., Polkinghorne, B.G. & Fearnley, E.J. 2016. Epidemiology of bacterial toxin-mediated foodborne gastroenteritis outbreaks in Australia, 2001 to 2013. Communicable Diseases Intelligence, 40(4): E460–E469. https://doi.org/10.33321/cdi.2016.40.51

A retrospective study of foodborne outbreaks in the United States from 1998 to 2010 identified that the most common contributing factor (reported in 114 outbreaks, or 39 percent) was the practice of leaving foods at ambient or warm outdoor temperatures for extended periods. Furthermore, over 90 percent of *C. perfringens* outbreaks linked to a single food commodity were associated with meat and poultry products (Grass *et al.,* 2013). Similarly, a report by the Department for Environment, Food and Rural Affairs (DEFRA, 2021) identified beef products as the most frequently implicated vehicle in *C. perfringens* outbreaks in the United Kingdom. Specifically, beef was associated with 31 percent of *C. perfringens* outbreaks, accounting for 267 outbreak cases. A 2009 risk assessment model estimates that deviations for cooling of commercially processed meats accounted for less than 1 percent of illnesses (Golden *et al.,* 2009).

In Japan, about 20–30 outbreaks are reported annually (MHLW, 2025). In Japan, foods thickened with corn starch and flour (*TOROMI*) including curry, nimono and ankake have been associated with multiple outbreaks. These foods pose a heightened risk of microbial growth and subsequent illness due to two key factors: Their viscous nature prolongs cooling time, and the ingredients are commonly contaminated with *C. perfringens* spores.

The European Centre for Disease Prevention and Control (ECDC) and EFSA annually collect data on zoonotic diseases from European Union (EU) member states and other European countries. In 2022, it was reported that *C. perfringens* toxin-producing strains in "other or mixed red meat and products thereof" constituted the agent/food pair responsible for the highest number of cases in outbreaks reported by EU member states (EFSA, 2023).

Increased concern regarding the safety of spices and dried aromatic herbs led the Codex Committee on Food Hygiene to request that the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) conduct a risk assessment of microbiological hazards in these food commodities. The committee concluded that spore forming *C. perfringens* should be regarded as a foodborne pathogen of particular concern in relation to spices and dried aromatic herbs (FAO and WHO, 2022).

While finalizing this report, two separate *C. perfringens* outbreaks were identified, both linked to food served in a restaurant in Wales in October and November 2025 (BBC, 2025a; 2025b). The specific food responsible was not confirmed.

3.5 DISTRIBUTION OF *CLOSTRIDIOIDES DIFFICILE* IN FOODS

Clostridioides difficile can potentially be found in all foods as its spores are ubiquitous in the environment (soil, water, wastewater effluents, compost, manure, and so forth) and are often found in the intestinal content of animals.

Rodriguez-Palacios *et al.* (2020) conducted a systematic review and meta-analysis to assess the prevalence of *C. difficile* in food samples, drawing on 79 studies from 25 countries published between January 1981 and November 2019. They reported a 4.1 percent overall prevalence of *C. difficile* across 21 886 food samples. Risk ratio rankings and meta-regression analysis identified seafood (shellfish and finfish), leafy green vegetables, pork, and poultry as higher-risk sources, whereas milk was the least likely to harbour *C. difficile* contamination.

The prevalence in meat, seafood and vegetables has a wide range (**Table 6**) (Candal-Perez *et al.* 2019; Lim *et al.,* 2020a; Bolton and Marcos, 2023; Rodriguez-Diaz *et al.,* 2024; Rodriguez-Palacios 2020), and there is also a difference within individual food type categories. For example, the level of contamination on retail poultry without skin (0 out of 42 samples) was low compared to that on poultry with skin (54 of 322 samples, 15.8 percent) (Heise *et al.*, 2021).

Although Rodriguez-Palacios *et al.* (2020) did not identify root vegetables as higher-risk sources in their meta-analysis, other studies have shown that potatoes – one of the most widely consumed staple foods worldwide – exhibit high rates of *C. difficile* contamination. A European study by Tkalec *et al.* (2022) found that 22.4 percent of potato samples tested positive for *C. difficile*. However, country-level variation was substantial: None of the samples from Slovakia were positive, while all samples from Romania tested positive. Notably, at least 10 percent of samples were positive in 9 out of the 12 countries examined. An Australian study reported 25.7 percent (18/70) positivity (Lim *et al.,* 2018), 5 percent in Vietnam (1/20) (Khun *et al.,* 2023), 25.7 percent (18/70) in Slovenia (Tkalec *et al.,* 2022) and 20 percent in Germany (6/30) (Scholtzek *et al.,* 2022). The overall positivity rate for potatoes from five studies totalling 312 samples was 23.6 percent (82 positive samples). Cleaning methods such as brushing, washing and peeling can substantially reduce the microbial load. However, *C. difficile* spores introduced into the kitchen may still contaminate surfaces and the surrounding environment.

Multiple PCR-ribotypes have been isolated from foods (some most common in human CDI also shown in **Table 6**), and the substantial overlap in ribotypes suggests extensive transmission. A meta-analysis of food prevalence and the ribotypes associated with community acquired CDI identified a higher risk of exposure when consuming shellfish or pork, with the latter being the main foodborne route for ribotypes 027 and 078 (Bolton

and Marcos, 2023). European Centre for Disease Prevention and Control 2018–2020 top 15 most prevalent ribotypes in EU surveillance in human CDI are 014/020, 002, 078, 005, 015, 001, 023, 106, 012, 017, 027, 081, 126, 056, 011 (ECDC, 2024). Most common RTs in humans are 014/020, 106, 078 and 027, and 017 (in Asia only) (Hong *et al.,* 2020; Collins *et al.,* 2013).

TABLE 6 THE REPORTED PREVALENCE OF *CLOSTRIDIOIDES DIFFICILE* IN MEAT, SEAFOOD, VEGETABLES, AND FOR POTATO ONLY RIBOTYPES MOST COMMONLY FOUND IN HUMAN CDI ARE GIVEN IN THE TABLE BELOW.

Food	Prevalence	Ribotypes	Remarks
Meat	0–62.5%	078, 027, 001, 002, 005, 012, 014/020	Pork meat and poultry with skin have highest positivity rates
Milk	0–10%	078, 011/018, 060	One study has reported detection of *C. difficile* but not the prevalence
Seafood	3.9–75%	126, 078, 002, 001, 014/020, 017, 081, 106, 012	Small number of studies
Vegetables (various, excluding potato)	1.9–20%	001, 002, 005,011,012,015, 014/020, 023, 027, 056,078, 081, 106, 126	Leafy green vegetables have the highest positivity rates; 20% was reported for spinach
Potato	0–53, 4% (100%) *	014/020, 078, 010, 023, 106, 126,	Not included in meta-analyses *Up to 100% positive potato samples were reported based on small (n = 7) sample number

Sources: Tkalec *et al*, 2022; Scholtzek 2022; Khun *et al.*, 2023; Candal-Perez *et al.*, 2019; Lim *et al.*, 2018; Bolton and Marcos, 2023; Rodriguez-Diaz *et al.*, 2024

See 3.6 References section for full list of sources in Table 6.

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CHAPTER 4
THE GLOBAL BURDEN OF FOODBORNE DISEASE

4.1 INTRODUCTION

Microbiological foodborne diseases pose significant challenges to public health and incur substantial economic costs globally. Annually, across the globe, the consumption of contaminated and mishandled food leads to an estimated 600 million incidents of foodborne illnesses and results in approximately 420 000 fatalities (WHO, 2015). However, the prioritization of food safety, as well as the reporting and analysis of data, vary considerably between countries. Regarding clostridia, incidence data and burden estimates are predominantly available from a limited number of countries with effective human health notification systems, creating a significant data gap in global understanding.

The 2015 World Health Organization (WHO) report (Kirk *et al.,* 2015; WHO, 2015) highlighted the shortage of comprehensive global data on foodborne diseases. This limitation underscores that the existing data from high-income countries cannot be reliably extrapolated to provide accurate global estimates (WHO, 2015). The dearth of data from low- and middle-income countries severely hampers efforts to understand the true global burden of foodborne diseases caused by clostridia. It is important to note that the methods utilized for calculating and reporting these data have not been evaluated within the scope of this review. The focus remains on the availability of data, rather than on the methodological rigor or merit of the data collection and analysis techniques employed by various countries. This delineation is intended to highlight the pressing need for enhanced global data collection and reporting mechanisms to assess and address the burden of foodborne infectious diseases worldwide more accurately.

4.2 WORLD HEALTH ORGANIZATION REPORTS ON THE GLOBAL BURDEN OF BOTULISM AND *CLOSTRIDIUM PERFRINGENS* FOODBORNE DISEASE

In 2015, the Foodborne Disease Burden Epidemiology Reference Group (FERG) 2007–2015 of the WHO presented comprehensive estimates on the global and regional burden of foodborne botulism and *C. perfringens* foodborne illness (Kirk *et al.*, 2015; WHO, 2015). For botulism, clinical outcomes were categorized into mild to moderate botulism, with a duration of 10 days (ranging from 5 to 20 days), and severe botulism, lasting 30 days (ranging from 15 to 180 days). It was assumed that 35 percent of botulism cases progressed to severe botulism. In the case of *C. perfringens* foodborne illness, the clinical outcomes included acute gastroenteritis, lasting 1 day (ranging from 0.25 to 2.5 days), and mortality due to the foodborne disease.

The incidence estimates for botulism and *C. perfringens* foodborne disease were confined to the 61 countries in the European region and other subregions with very low child and adult mortality (Subregion A in Europe, the Americas and western Pacific area). The WHO report identified national estimates for botulism incidence from five countries: Canada, France, Georgia, Poland, and the United States. For *C. perfringens* foodborne illness, the report identified national incidence estimates from seven countries: Australia, Canada, France, the Kingdom of the Netherlands, New Zealand, the United Kingdom, and the United States (WHO, 2015).

In 2010, the estimated incidence of foodborne botulism was 475 cases, with a 95 percent uncertainty interval (UI) ranging from 183 to 990 cases in European and other low-mortality countries alone (WHO, 2015). These cases were further estimated to result in 24 deaths (95 percent UI: 7–65) and 1 036 disability adjusted life years (DALYs) (95 percent UI: 299–2 805) in the region and countries. According to the WHO report, as well as independent reports from Taiwan Province of China (Lai *et al.,* 2020) and Greece (Gkogka, 2011), botulism exhibits one of the lowest overall DALYs of all foodborne illnesses due to its low incidence rate. However, when evaluated based solely on clinical outcomes, severe botulism ranks among the highest in disability weights.

For *C. perfringens*, the estimated incidence was 3 998 164 cases, with a 95 percent UI ranging from 837 262 to 11 529 642 cases in 61 European and other low-mortality countries alone. These illnesses were further estimated to result in 120 deaths (95 percent UI: 25–351) and 6 963 DALYs (1 423–20 493) in the region and countries. In the United States, it is estimated that approximately one million individuals experience foodborne illness attributable to *C. perfringens* annually, leading to economic losses estimated at around USD 400 million per year (Grenda *et al.,* 2023). The assessment of the disease burden caused by *C. perfringens* is challenging due to the typically mild and self-limiting nature of the illness, resulting in significant underreporting (FSA, 2011).

The World Health Organization (WHO) is currently updating its estimates of the burden of foodborne diseases based on 42 hazards, including the incidence, death and DALYs due to botulism. Currently, estimates on the global burden of infant and adult intestinal botulism are not available.

4.3 GLOBAL BURDEN OF *CLOSTRIDIOIDES DIFFICILE* INFECTIONS

To date, there have been no confirmed instances of CDI definitively attributed to consumption of contaminated food. Consequently, most prevalence data pertains to hospital acquired infections.

Despite the establishment of comprehensive health practice guidelines aimed at preventing, diagnosing, and treating CDI, the incidence continues to escalate (GBD 2021 Diarrhoeal Diseases Collaborators, 2025). The burden of CDI is multifactorial and includes an economic burden for health care systems, a clinical burden in term of disease severity, and significant effects on patient quality of life, especially in the case of recurrent CDI (Feuerstadt *et al.,* 2023). Although not a leading pathogen globally, *C. difficile* was the main cause of diarrhoea-related deaths in high-income countries in 2021 (GBD 2021 Diarrhoeal Diseases Collaborators, 2025; Chen *et al.* (2024) analysed the global burden and trends of *C. difficile*-associated deaths and DALYs from 1990 to 2019 and reported that globally CDI increased at a higher rate in terms of diarrhoeal deaths and disability-adjusted life years (DALYs) compared to other bacterial pathogens over that time. The age-standardized death rate (ASDR) increased from 0.19 in 1990 to 0.43 in 2019, with the elderly and females at higher risk. The authors highlighted the rapid increase in ASDR in high to middle sociodemographic index (SDI) regions such as North America (average annual percentage change (AAPC) = 7.71 percent), Andean region (AAPC = 7.82 percent), and Southern Latin America (AAPC = 11.08 percent), and antibiotic consumption had a significant positive correlation with *C. difficile*-associated illness.

In the United States CDI incurs more than USD 5.9 billion in excess medical costs (Desai *et al.*, 2016). More contemporary cost calculations reported per patient were USD 71 980 per case with no recurrence and USD 207 733 for recurrent CDI (Feuerstadt, 2020). The estimated costs for *C. difficile* infection are up to EUR 3.4 billion annually in Europe, with reported costs per case ranging from EUR 5 798 to EUR 15 242 per primary episode (Reigadas *et al.,* 2024).

In 2022, the European Centre for Disease Prevention and Control (ECDC) performed a Europe wide survey on the incidence of CDI in 559 hospitals. The mean incidence of CDI was 3.48 cases per 10 000 patient days. Most cases (60.9 percent) were healthcare-associated CDI, with 32.7 percent being community-associated (mostly presenting with severe symptoms to emergency departments) or of unknown origin, and the remaining 6.4 percent reported as recurrent cases (ECDC, 2022). These cases substantially contributed to morbidity and fatality. In the United States, the CDC report that CDI is associated with half a billion infections and over 29 000 deaths annually (US CDC, 2024a).

In a systematic review conducted by Balsells *et al.* (2019) on the global burden of CDI, 229 publications from 41 countries were included. An extensive internet search was conducted to identify the most recent data from national surveillance reports, encompassing studies and reports published between 2005 and 2015. Most of the reports (195/229) originated from Europe and North America, with fewer studies from the Western Pacific, Latin America, Eastern Mediterranean, and Africa. The overall incidence rate of health care-associated CDI was reported as 2.24 (95 percent confidence interval [CI]: 1.66–3.03) per 1 000 admissions per year and 3.54 (95 percent CI: 3.19–3.92) per 10 000 patient days per year. For CDI occurring in intensive care units or internal medicine wards, the incidence rates were 11.08 (95 percent CI: 7.19–17.08) and 10.80 (95 percent CI: 3.15–37.06) per 1 000 admissions per year, respectively. Community-associated CDI rates were lower, at 0.55 (95 percent CI: 0.13–2.37) per 1 000 admissions per year. CDI incidence was higher in North America and in elderly populations, although similar rates were observed in other regions and age groups (Balsells et al., 2019). The extent to which any of these data pertain to foodborne disease in the community remains unknown.

4.4 REFERENCES

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CHAPTER 5
CONTROL MEASURES AND PROCESSES

The initial contamination of food products by clostridia presents a significant challenge to control measures. Clostridia are ubiquitous in the environment, being isolated from soil, raw vegetables, animal skin or hide contaminated with faecal material or soil, and from the environment of processing facilities, which ultimately leads to contamination of food (**Tables 2, 4,** and **6**). Physical removal by washing, peeling, careful removal of skin, hide, feathers and viscera will reduce the prevalence but not eliminate spores. Many food grade sanitizers (e.g. those used for fresh produce washes and carcass washes) are effective against vegetative microbes but not against spores. Low level ethylene oxide and irradiation treatments (used for meat, poultry, and produce) are not sporicidal whereas high doses used to sterilize some spices and medical foods are effective against spores. Cleaning to physically remove most filth and microorganisms and disinfection of the processing environment including equipment is essential to prevent cross-contamination of food. Eliminating spores requires the application of sporicidal agents that include compounds such as glutaraldehyde, formaldehyde, hydrogen peroxide, peroxy acids, as well as iodine and/or chlorine-based compounds, individually or in combination (Broda, 2007; Russell, 1990). However, disinfectants can be corrosive to food-processing equipment with regular use. In domestic and institutional kitchens that serve individuals that have disrupted gut microbiota, regular cleaning and disinfecting of work surfaces, equipment, and utensils with disinfectants that are effective against *C. difficile* spores is essential to reduce exposure.

Drying, low water activity, and freezing will prevent growth, but spores can remain viable for decades (Gao *et al.,* 2023). Empirical evidence has shown that the proper storage temperature of food products throughout the entire distribution chain, including retail and consumer phases, along with inhibitory formulation strategies (such as pH and water activity [a_w]), significantly reduces the risk of clostridial proliferation in foods (**Table 7**).

TABLE 7. EFFECT OF TEMPERATURE (° C), PH AND WATER ACTIVITY (AW) ON GROWTH OF *C. BOTULINUM, C. PERFRINGENS AND CLOSTRIDIOIDES DIFFICILE* (AT OTHERWISE OPTIMAL GROWTH CONDITIONS)

Organism	Min (° C)	Optimum (° C)	Max (° C)	No growth at pH values	No growth at a_w values*
***C. botulinum* GI**	10–12	35–45	48	<4.6	<0.93–0.94
***C. botulinum* GII**	3–4	28–30	37	<5.0	<0.97
***C. botulinum* GIII**	15	37–40	–	<5.1	<0.97
***C. baratii* GV**	10–15	30–45	–	<3.7	–
C. butyricum	10–11	30–37	–	<4.2	<0.96
C. perfringens	10–12	43–47	50	<5.5–5.8	<0.95–0.97
C. difficile	22**	30–37	45**	<6.5**	–

Notes: *Limits for growth are based on salt or sugar as a humectant (ref ICMSF 5); water activity of 0.94, 0.95 and 0.97 equivalent to 10, 7 and 5 percent salt, respectively; the minimum water activity limit for growth is lower when using glycerol as a humectant. **Limits of growth for *C. difficile* ribotype 078 and 027 only. Lack of information on minimum and maximum growth temperatures for *C. difficile*.

Sources: EFSA 2005; Warriner *et al.* 2017; Candel-Pérez *et al.,* 2019; ACMSF,2023; ICMSF, 1996. Ghoddusi, Sherburn and Aboaba, 2013.

Based on these growth parameters, **Table 8** outlines limits for thermal processing to destroy spores and vegetative cells, and storage temperature, pH, water activity, and salt-in-moisture phase used to inhibit growth of Groups I and II *C. botulinum* and *C. perfringens*.

TABLE 8. CONTROLLING FACTORS AND GENERALLY ADOPTED VALUES TO ELIMINATE OR INHIBIT GROWTH FOR *CLOSTRIDIUM BOTULINUM* GROUP I AND GROUP II AND *CLOSTRIDIUM PERFRINGENS* IN MANUFACTURING OR FOOD SERVICE, RESTAURANTS, OR HOME

Characteristic	Group I *C. botulinum*	Group II *C. botulinum*	*C. perfringens*
Thermal destruction of spores – Value	$D_{121.1°C}$ = 0.19–0.21 minutes	$D_{90°C}$ = 1.1 minutes	Spores: D100 °C = 0.31–13 minutes Vegetative cells: D65 °C = 0.9 minutes
Thermal destruction of spores – Generally Adopted Control	Heat process low acid ambient foods pH > 4.6 at 121.1 °C for 3 minutes or equivalent using z = 10 centigrade degrees to achieve a 12-log_{10} reduction in spore number	Heat process foods intended to be chilled (8 °C or less) with extended life (greater than 10 days) at 90 °C for 10 minutes or equivalent[a]	Processing used for Group I *C. botulinum* control sufficient for *C. perfringens*. Recook foods to 72 °C or higher to inactivate vegetative cells
Minimum growth temperature	Store foods at less than 10 °C	Store foods at less than 3 °C	Store foods at less than 12 °C
Inhibitory pH	Formulate to pH = 4.6 or less throughout the food[b]	Formulate to pH = 5.0 or less throughout the food[b]	Formulate to pH = 5.0 or less throughout the food[b]
Inhibitory a_w (with salt)	Formulate to a_w = 0.94 or less throughout the food[c]	Formulate to a_w = 0.97 or less throughout the chilled food[c]	Formulate to a_w = 0.97 or less throughout the food[c]
Inhibitory a_w (with glycerol)	Formulate to a_w = 0.93 or less throughout the food	Formulate to a_w = 0.97 or less throughout the chilled food	Formulate to a_w = 0.93 or less throughout the food
Inhibitory salt concentration (in aqueous phase)	Formulate to 10% or greater aqueous salt throughout the food	Formulate chilled food to 3.5% or greater aqueous salt throughout	Formulate chilled food to 3.5% or greater aqueous salt throughout

Notes: [a] Calculate equivalent thermal processes using z = 7 for process temperatures below 90 °C and z = 10 for process temperatures above 90 °C. [b] Ensure that precautions are taken to prevent contamination and growth of microorganisms that could elevate pH (e.g. moulds, butyric anaerobes, bacilli).[c] If using humectants other than sodium chloride or glycerol, lower values of a_w may be necessary.

Source: adapted from ACMSF, 2023

Toxigenic *C. baratii*, *C. butyricum* and Group III *C. botulinum* are emerging hazards for human foodborne botulism. These toxigenic strains have been identified as mesophilic, like Group I *C. botulinum*. While thermal inactivation parameters have been characterized, other control strategies for these hazards in food remain underdeveloped. This presents opportunities for research to identify growth-limiting factors – such as minimum pH, water activity, and storage temperature thresholds – to inform effective control mechanisms. Low numbers of confirmed illnesses indicate the risks presented by these neurotoxigenic clostridial species are not rapidly changing (ACMSF, 2023).

5.1 EFFECT OF TEMPERATURE ON TOXIGENIC CLOSTRIDIA SPORE INACTIVATION AND GROWTH INHIBITION

Thermal treatment of food at 68–70 °C effectively eliminates vegetative cells but does not deactivate spores (EFSA, 2005). Deviation from appropriate processing protocols, including cooling, at any stage, commercially or during consumer handling, could facilitate the growth of clostridia and toxin production.

The storage temperature is also a critical determinant for preventing the germination of spores and subsequent vegetative cell growth of clostridia. The interactions between temperature, pH, water activity, inhibitory food ingredients, and competitive microbiota significantly influence the growth of clostridia. Therefore, a comprehensive understanding of these parameters and their synergistic effects is imperative for ensuring food safety of food products susceptible to clostridia.

5.1.1. THERMAL INACTIVATION OF SPORES

Thermal treatment is the most effective method for inactivating bacterial spores, with their destruction directly influenced by temperature and pressure – higher levels accelerate inactivation. Additionally, the thermal resistance of bacterial spores is modulated by various food matrix properties, including pH, water activity (a_w), and fat content.

Due to their thermal resistance, most clostridial spores can survive standard cooking processes used in commercial food production, restaurants, and home kitchens, including heating to an internal temperature of 70 °C for up to 2 minutes or a core temperature of 74 °C (Lund and Peck, 2015). Mild cooking methods, such as *sous vide* processing at 60 °C for 1 hour, are insufficient to inactivate *C. difficile* spores (Marcos *et al.*, 2023).

For shelf-stable low acid canned foods, a standard thermal process involves heating at 121 °C for at least 3 minutes (or equivalent) to achieve a 12-$\log_{10}$ reduction (12D) in *C. botulinum* Group I spores (**Table 8)**. This treatment regime is also effective against *C. perfringens* spores (Talukdar *et al.,* 2017) as well as other BoNT-producing species of clostridia and *C. difficile*.

Clostridium botulinum Group II spores are more heat sensitive than Group I *C. botulinum* spores. Heating of chilled foods – intended for storage of no greater than 42 days – at 90 °C for 10 minutes or equivalent treatment is sufficient to achieve a 6-$\log_{10}$ reduction (referred to as a "nonprot Cbot cook"; FSA, 2020).

Reported results for spore heat resistance vary based on recovery methods for injured cells, heating matrices, and strain selection (Lund and Peck, 2015). D-values at 100 °C for *C. perfringens* spores in buffer ranged from 15.5 to 21.4 min (Juneja *et al.,* 2003). Other researchers reported *C. perfringens* D-values ranging from 2.2 minutes at 100 °C to 34.2 minutes at 90 °C in pork luncheon roll (Byrne *et al.,* 2006). Studies on the thermal inactivation of 108 *C. difficile* strains in phosphate buffer revealed $D_{100°C}$ values ranging from 2.5–33 minutes (Nakamura *et al.,* 1985). Cooking at 80 °C for 40 minutes achieves a 1.0–1.5 $\log_{10}$ spore/g reduction of *C. difficile* (Marcos *et al.,* 2023). Other research indicates that *C. difficile* spore populations require temperatures exceeding 85 °C for more than 10 minutes for significant reduction, a treatment approach similar to that used for Group II non-proteolytic *C. botulinum* (Candel-Pérez *et al.,* 2019; FSA, 2020). Additionally, thermal

inactivation studies of *C. difficile* PCR ribotypes 014, 027, 033, 077, 078, and ATCC6969 spores in phosphate-buffered saline estimate D-values at 85 °C ranging from 6.0 to 8.5 minutes (Rodriquez-Palacios and LeJeune, 2011)

Sublethal thermal treatments can trigger clostridial spore germination, potentially resulting in vegetative cell proliferation if the food is subsequently stored under conditions conducive to bacterial growth (Lund and Peck, 2015). However, germinating spores are more susceptible to environmental stresses, a synergistic effect that can be exploited to enhance their inactivation through subsequent control measures such as secondary heating (Wen *et al.*, 2022; Nerandzic and Donskey, 2013).

5.1.2. EFFECT OF COOLING AND STORAGE TEMPERATURE ON GROWTH INHIBITION OF CLOSTRIDIA

5.1.2.1 *BoNT-producing clostridia*

Established controls aimed at preventing and monitoring foodborne botulism are based on rigorous scientific principles. These controls are designed to mitigate the risk of C. botulinum growth and neurotoxin production and to ensure the production of safe food. A summary of controls (**Table 8**) was produced by the Advisory Committee on the Microbiological Safety of Food (ACMSF), which outlined various controlling factors and recommended processes for C. botulinum in foods (ACMSF, 2023).

While specific cooling regimes for *C. botulinum* are not detailed in this section, cooling regimes described for *C. perfringens* (section 5.1.2. [b]) will prevent growth and toxin production by *C. botulinum*.

Spores of *C. botulinum* Group I exhibit prolonged viability under standard frozen storage conditions applied to foods (-18 °C) (ICMSF, 1996). The growth range for Group I *C. botulinum* is between 12 °C and 48 °C (Hinderink *et al.*, 2009) with optimal growth between 35 °C and 45 °C. Growth has not been observed at or below 10 °C (Peck, 2009).

Spores of *C. botulinum* Group II also survive prolonged frozen storage in food at -18 °C (ICMSF, 1996). The temperature range for growth and toxin production by Group II is between 3 °C and 37 °C, with some evidence suggesting growth at higher temperatures (Jensen, 1986; Derman *et al.,* 2011). Optimal growth occurs between 28 °C and 30 °C (Peck, 2009), while growth at 8 °C under otherwise favourable conditions is considered slow (ACMSF, 2023). Guidance in the United Kingdom for chilled foods intended to be stored for more than 10 days recommends that in addition to storage at 3 to 8 °C throughout the chill chain, one or more control factors, such as a heat-treatment of 90 °C for 10 minutes, a pH < 5.0, water activity < 0.97, a salt concentration > 3.5 percent, should be used in combination to prevent growth and toxin production by Group II *C. botulinum*.

To date, all strains of toxigenic *C. baratii*, *C. butyricum* and Group III *C. botulinum* have been identified as mesophilic, like Group I *C. botulinum*. Toxigenic *C. baratii* strains are capable of growth at a minimum temperature of 10 °C with an optimum growth temperature between 30 and 45 °C. Toxigenic *C. butyricum* strains have a minimum growth temperature of 10–11 °C and an optimum growth temperature between 30 and 37 °C (Ghoddusi, 2013). The minimum growth temperature for *C. botulinum* Group III strains is 15 °C, with optimal growth occurring at 37–40 °C (Peck, 2009).

5.1.2.2 *Clostridium perfringens*

Clostridium perfringens illness is attributed to consumption of high levels (typically 8-$\log_{10}$ per serving based on a 100-g serving) of the vegetative cells, which then sporulate in the human gut, releasing enterotoxin and causing illness. Based on surveys of food ingredients, these high numbers can be achieved only if there is microbial growth in the food.

Commercial and regulatory thermal guidelines for processing to prevent *C. perfringens*-related foodborne illness are more detailed than those for other clostridia due to its rapid growth characteristics during cooling and hot-holding. These guidelines outline several critical control measures across the production chain, from cooking to reheating:

- cooking process to kill any existing vegetative cells;
- rapid cooling through the temperature range of 54 to 15 °C;
- maintaining food storage temperatures below 10 °C or greater than 60 °C; and,
- reheating the product to an internal temperature of 72 °C prior to consumption to destroy any vegetative cells that may have multiplied during cooling.

Studies demonstrate optimal growth of *C. perfringens* at temperatures ranging from 43–47 °C, with significant inhibition of growth occurring below 10–12 °C and above 54 °C (**Table 7**). Within the optimal temperature range, the generation time for *C. perfringens* can be as short as 7 minutes. When the post-cooking cooling process is delayed or hot holding of the food is insufficient (such as in a mass catering situation), spores can germinate and the vegetative cells rapidly multiply.

Consistent with the growth parameters of *C. perfringens*, United States control guidelines recommend that cooked meat and poultry products should be cooled from 54 °C to 27 °C within one hour, followed by continued cooling to 12 °C within the next five hours (or variations depending on formulation) (US FSIS, 2021). An additional five-hour limit is recommended for further cooling to 4 °C to inhibit the growth of other pathogens. Other foods under FDA jurisdiction must follow a two-stage rule cooling from 57 °C to 21 °C in two hours and then to 5 °C within an additional 4 hours (US FDA Food Code, 2022). Although the first stage of cooling is designed specifically to control growth of *C. perfringens* in foods with high pH and high-water activity, other spore-forming pathogens should be considered. For example, if products take longer than 3 hours to cool between 54 to 27 °C, not only can there be excessive growth of *C. perfringens,* but proliferation and production of heat-stable toxins of *C. botulinum* may also occur. The Pathogen Modelling Program (PMP) can be consulted to determine if either pathogen will grow to levels associated with toxin production (Juneja *et al.,* 2018, 2019a, 2019b; USDA, 2023; ComBase, 2025).

Thermal inactivation studies in a pork luncheon roll determined that vegetative cells of *C. perfringens* exhibited D-values of 0.9 min (65 °C) and 16.3 minutes (55 °C) (Byrne *et al.,* 2006). Therefore, reheating to greater than 72 °C serves as an effective control measure to reduce the populations of *C. perfringens* vegetative cells, particularly when the prior thermal history of the product is uncertain (EFSA, 2005). After reheating the product to 72 °C, product should be again maintained above 60 °C or discarded.

5.1.2.3 *Clostridioides difficile*

Clostridioides difficile is a mesophilic bacterium, with a reported minimum growth temperature of 22 °C (Warriner *et al.,* 2017). As with other clostridial spores, populations of *C. difficile* remain unchanged under refrigeration (4 °C) and freezing temperatures (−18 °C and −80 °C) (Bolton and Marcos, 2023). To date, there is no published evidence that spores of *C. difficile* would germinate and multiply in foods during typical storage conditions; however, considering that 22 °C is the minimum temperature required for growth (**Table 7**), other pathogens and spoilage microbes are more likely to grow to dangerous levels if a food were temperature abused. In the domestic or catering kitchen, *C. difficile* spores survive for prolonged periods of chilled and frozen storage in different foods. Furthermore, *C. difficile* spores remain viable after desiccation, chilled and frozen storage, hydrostatic pressure pasteurization, and are not inhibited at room temperature by several food preservatives when used at levels within regulatory limits (Lim *et al.*, 2016; Marcos *et al.,* 2023).

5.2 IMPACT OF PH ON GROWTH OF CLOSTRIDIA

Acidic conditions exert an inhibitory effect on the germination and subsequent growth from clostridial spores. The minimum pH for the growth of *C. perfringens* is 5.5, whereas *C. botulinum* Group I is within the pH range of 4.6 to 4.8 (ICMSF, 1996). It is widely accepted that at or below a pH of 4.6, the growth of *C. botulinum* Group I and its toxin production in food are effectively inhibited under otherwise optimal conditions (Peck, 2009). The pH 4.6 threshold has been acknowledged as the point of demarcation for acid or acidified food products, below which *C. botulinum* Group I is unable to grow (ACMSF, 2023). The critical inhibitory pH for *C. botulinum* Group II is established at pH 5.0, serving as a well-accepted control parameter in food safety protocols (ICMSF, 1996).

Acid type also impacts the minimum pH required for growth. Toxigenic *C. butyricum* strains have been shown to be capable of growth in laboratory medium at a pH as low as pH 4.2 with HCl as the acidulant whereas inhibition occurred at higher pH values in substrates prepared with citric (pH 4.7), lactic (pH 4.8), and acetic (pH 4.8) acids (Ghoddusi, 2013). Another study observed growth and toxin production of *C. butyricum* strains after 43 days storage in laboratory media adjusted to pH 4.8 with HCl, but not at pH 4.6 (Anniballi *et al.,* 2002). The minimum pH for growth of *C. botulinum* Group III is reported to be pH 5.1 (Peck, 2009).

The effect of pH on *C. difficile* has been evaluated in a limited number of strains, primarily in the context of the intestinal environment (Wetzel and McBride, 2020; Yuille *et al.,* 2020). These studies did not focus on pH as a control parameter for growth and survival in food matrices. As with other clostridia, low pH decreased growth and sporulation. The effect on toxin production showed strain dependent pattern. Vegetative cell growth is inhibited at pH levels below 5.5, while growth from spore suspensions does not occur below pH 6.19. Optimal germination occurs between pH 6.5 and 8.5 (Kochan *et al.,* 2018). In an earlier study, Scaria *et al.* (2015) determined optimal growth at pH 6.0, while at pH 4.0 growth was reduced to a quarter of the optimal growth.

5.3 IMPACT OF WATER ACTIVITY ON GROWTH OF CLOSTRIDIA

The growth of clostridia is progressively inhibited as the water activity (a_w) decreases due to drying or the addition of solutes such as sodium chloride or sugars (humectants). According to ICMSF, growth of the majority of *C. botulinum* Group I strains is inhibited when the salt concentration reaches or exceeds 10 percent ($a_w \leq 0.94$) (ICMSF, 1996). For *C. botulinum* Group II strains, most strains are incapable of growth under otherwise favourable conditions if the salt concentration is 5 percent or above ($a_w \leq 0.97$) (ICMSF, 1996). Growth of *C. perfringens* is likewise inhibited at $a_w \leq 0.97$ when salt is the humectant but at 0.94–0.95 when glycerol is the humectant.

5.4 IMPACT OF ATMOSPHERE (OXYGEN) ON GROWTH OF CLOSTRIDIA

There is a varying degree of aerotolerance among clostridial strains. Despite its classification as an obligate anaerobic bacterium, *C. botulinum* demonstrates the capacity to proliferate in substrates with positive redox potential, although growth rate is faster when strict anaerobiosis is present.

High levels of oxygen within a food package do not prevent clostridia from growing or producing toxin within microenvironments where cells may be protected from the effects

of oxidative stress (ACMSF, 2023). Therefore, head space oxygen content, gaseous atmosphere or redox potential of a food product is not reliable to control the growth and toxin production of botulinum neurotoxin-producing species of clostridia. Spoilage microbes may contribute to oxygen depletion during storage, creating a permissive anaerobic environment.

Reports indicate that *C. botulinum* Group I strains can grow and produce toxin in various aerobic conditions, including crumpets initially packaged in air (Daifas *et al.*, 1999), laboratory media with 15 percent headspace oxygen (Whiting and Naftulin, 1992), and mushrooms packed within semi-permeable plastic films (Sugiyama and Yang, 1975).

Under certain conditions, toxin formation by *C. botulinum* strains in foods packaged in air can approach that observed under modified atmosphere packaging with reduced oxygen or vacuum packaging (Peck *et al.,* 2008). At a redox potential of +217 mV, the probability of growth from single spores of Group II *C. botulinum* type E within five days at 20 °C equals that observed under strict anaerobic conditions at a redox potential of -400 mV (Lund *et al.,* 1984). Others reported that Group I *C. botulinum* type A spores growing within an Eh range of -436 to -18 mV (Montville, 1982). The growth of Group II non-proteolytic type B and E strains in culture medium occurs at a slower rate when exposed to > 70 percent CO_2 (Gibson *et al.,* 2000), yet toxin expression has been shown to be significantly increased (Artin *et al.,* 2008). In contrast, levels of CO_2 had little effect on the growth and toxin production of Group I *C. botulinum* (Artin *et al.,* 2010).

Compared with *C. botulinum*, *C. perfringens* exhibits greater aerotolerance, demonstrating 1-$\log_{10}$ growth in beef under microaerophilic conditions (6–8 percent oxygen) at 22 °C (FSANZ, 2015). *Clostridium perfringens* has been reported to grow at oxygen levels up to 20 percent. Its aerotolerance, combined with reduced redox potential below the food surface, contributes to its ability to proliferate in catered foods and food service buffets where temperature control is insufficient (Briukhanov and Netrusov 2007).

Nonetheless, oxygen in the form of aqueous ozone (O_3) can be used to reduce the number of microbes contaminating meat surfaces and has been shown to have an inhibiting effect on clostridia (Xu *et al.* 2025; Novak and Yuan, 2003). Further, a decrease in pH tolerance after ozone treatment has been shown for *C. perfringens* (Novak and Yuan, 2003).

5.5 EFFECT OF NITRITE/NITRATE ON INHIBITION OF CLOSTRIDIA

Nitrite inhibits both the germination and outgrowth of spores of *C. botulinum* and *C. perfringens*, but it is not sporicidal. This inhibitory effect is intricately dependent on several parameters, including ingoing nitrite concentration and pH levels (ACMSF, 2023).

Nitrate chemistry is complex and relies on conversion to nitrite and further to nitric oxide which imparts the antimicrobial activity through interference with conserved bacterial metabolic targets, resulting in disruption of cellular metabolism. Cure accelerators such as sodium erythorbate or ascorbate hasten the conversion from nitrite to nitric oxide. Depending on the exposure of the pathogen to optimal growth conditions vs. to the nitrite source, the use of the cure accelerator can either potentiate the growth inhibition effect of nitrite or have no enhancing effect (King, 2015, 2016; Bowen 1974). Cure accelerators also reduce the formation of nitrosamines during high-temperature cooking, such as frying.

In the context of food preservation, nitrite is utilized extensively to control *C. botulinum* in various meat products. It is particularly effective in chilled meats, such as bacon, as well as in ambient stored products, including canned cured ham. Notably, nitrite demonstrates efficacy against both *C. botulinum* Group I and Group II strains (Hilliard *et al.,* 1969; Lebrun *et al.,* 2020). For most cured meat products, an addition of sodium nitrite ranging from 50 to 150 mg per kg of meat is considered adequate to inhibit the growth of *C. botulinum* (ACMSF, 2023). The ACMSF 2023 reported that there was no data on the inhibitory effect

of nitrite on other botulinum neurotoxin-producing clostridia, and this potentially represents a knowledge gap prompting further research.

The application of nitrite and nitrate in food preservation is subject to stringent regulatory oversight to ensure food safety. In Australia and New Zealand, Food Standards Australia New Zealand (FSANZ) mandates a maximum allowable concentration of 125 mg/kg nitrite in meat products (New Zealand Gazette, 2015). Effective October 2025, the European Union's regulatory framework stipulates a maximum addition of 120 mg/kg nitrite ion for heat-treated (not sterilized) meat products with maximum residual nitrite ion not to exceed 67 mg/kg throughout shelf-life, and a maximum of 250 mg/kg nitrate in long-cured products when no nitrite is added; other limits apply to unheated, sterilized and traditionally cured products (Commission Regulation EU, 2023). In the United States, the regulatory limit for ingoing nitrite in comminuted products is 156 mg/kg (USDA FSIS, 2024a, 2024b) whereas the minimum ingoing nitrite is 100 mg/kg to qualify for extended cooling of cured meat products (USDA 2021, 2024).

Conventional curing processes involving nitrite supplementation have similarly been observed to inhibit the proliferation of *C. perfringens* (Redondo-Solano *et al.*, 2013; Myers *et al.*, 2016). As with *C. botulinum*, the antimicrobial activity of nitrite against *C. perfringens* has been demonstrated to be dependent on its concentration (Gibson and Roberts, 1986; King *et al.*, 2015). This bacterium is notably absent in cases of diarrhoea associated with cured meats owing to its susceptibility to nitrite (EFSA, 2005).

Certain vegetables, including celery, beets and spinach, are naturally rich in nitrates. In some applications, synthetic sodium nitrite has been replaced with cultured vegetable juice, where naturally occurring nitrate is converted to nitrite by specific starter cultures (e.g., *Staphylococcus carnosus*). This process yields a "natural" source of nitrite. The appropriate use of vegetable juice must account for both the maximum regulatory limits set by each country and food product (EU, 2023; New Zealand Gazette, 2015) as well as the minimum concentration necessary to achieve inhibition comparable to conventionally cured meat products (EFSA, 2004; Canada, 2025; USDA, 2021). Specifically, the formulation must effectively suppress *C. perfringens* growth during extended cooling and prevent *C. botulinum* outgrowth in cases of temperature abuse.

Data on the impact of nitrite on *C. difficile* remains limited. As observed with *C. botulinum* and *C. perfringens*, nitrite and nitrate exhibit sporostatic, rather than sporicidal, effects. Consequently, spores present in cured meat products will survive and remain viable. Lim *et al.* (2016) reported that the minimum inhibitory concentrations (MIC) for sodium nitrite, sodium nitrate and sodium metabisulphite against 11 strains of *C. difficile* were 250 µg/ml, > 4 000 µg/ml and 1 000 µg/ml, respectively, when the substrate is stored at 37 °C. These levels "exceed established permissible thresholds" in ready-to-eat meats. No data was provided for inhibition at lower storage temperatures.

5.6. OTHER FORMULATION STRATEGIES TO INHIBIT GROWTH OF CLOSTRIDIA

The interplay among water activity, pH, and temperature offers strategic avenues to prevent growth by *C. botulinum* and *C. perfringens* to sufficient numbers to cause illness (Leistner, 2000). Specifically, the interaction of pH < 5.6 and water activity < 0.95 will inhibit Group I botulinum spore outgrowth in foods when chilled storage temperatures are not maintained (Busta, 2003). Combinations for *C. perfringens* and Group II *C. botulinum* are likely to include higher pH and water activity than that needed to control Group I *C. botulinum* because the individual minimum values for pH (5.0), salt in aqueous phase (3.5–5.0 percent), and water activities (0.97) for growth are higher than Group I C. *botulinum*. Nonetheless, specific combinations of pH and water activity have not been reported. In general, as the storage temperature decreases to the minimum requirements for growth,

higher water activity and pH may still be inhibitory. Other measures to control growth of toxigenic *C. baratii*, *C. butyricum,* Group III *C. botulinum* and *C. difficile* in foods have not been well established and present opportunities for research to identify growth limiting factors (minimum pH, water activity, and maximum temperature, and so on) aimed at establishing control mechanisms.

5.7. OTHER FOOD INGREDIENTS WITH INHIBITORY PROPERTIES AGAINST *CLOSTRIDIUM BOTULINUM* AND *CLOSTRIDIUM PERFRINGENS*

In addition to temperature, a_w, pH and nitrite as major drivers for growth inhibition, other food ingredients with growth inhibitory properties (preservatives) can be used as additional hurdles to ensure safety of foods subject to temperature abuse.

Sodium and potassium lactate (salts of lactic acid) have been used as a substitute for nitrite in uncured RTE meats and *sous vide* products to inhibit growth and toxin production by *C. botulinum* (Maas *et al.,* 1989; Meng and Genigeorgis 1993, 1994). Sodium diacetate (acetic acid buffered with sodium hydroxide), and its clean label counterpart, buffered vinegar, also inhibits *C. botulinum* (Miller *et al.*, 1993). Similar inhibition has been noted against *C. perfringens* during extended cooling of uncured meat products (Kennedy *et al.*, 2013).

In response to demand from consumers for foods free of synthetic preservatives, "clean label" alternatives (ingredients with names that are recognized by the consumer) have been gaining popularity, particularly in the United States. Ingredients such as cultured sugars are single or blends of organic acids, such as lactic or propionic, and potentially antimicrobial peptides, such as bacteriocins.

Although the bacteriocin, nisin, has been reported to be inhibitory to *C. botulinum* in refrigerated meals and shelf-stable pasteurized processed cheese, the efficacy is decreased when product pH exceeds 6.0 or in the presence of lipids or hydrophobic proteins (Scott and Taylor, 1981). Levels that are needed for the efficacy of *C. botulinum* control typically are higher than for other spore formers and may approach regulatory limits for addition (Glass *et al.,* 2024).

Recently, an antimicrobial phage lysin has been reported to selectively lyse vegetative Group I *C. botulinum* cells and might have potential as a novel food preservative (Zhang *et al.*, 2020).

5.8 EFFECT OF COMPETITIVE MICROBIOTA IN FOODS, INCLUDING TRADITIONAL FERMENTATIONS

The presence of competitive microbes is known to be inhibitory to pathogenic clostridia in foods, as it is for other foodborne pathogens. The recommendation to use oxygen permeable film (oxygen transmission rate of at least 10 000 cc/m2/24 hours at 24 °C) for fishery products is to promote the growth of aerobic spoilage microorganisms that will produce acid and compete with clostridia to inhibit growth (USFDA, 2022). Similarly, indigenous spoilage microbes can reduce pH in prepared meals to inhibit *C. botulinum* (Golden et al., 2017). Added lactic acid producing starter cultures and protective cultures, in combination with a fermentable carbohydrate source, have a long history for the preservation of fermented meats, cultured dairy products, and other foods to inhibit growth of pathogenic bacteria, including clostridia (Smith and Palumbo, 1983; Rodgers et al., 2003a, b). Failed or inadequate fermentations have contributed to outbreaks of foodborne and animal botulism (Akdeniz et al., 2007; Horowitz, 2010; Lindstrom et al., 2004; Smith and Palumbo, 1983; Rodgers et al., 2003a, b)

Whilst not in food, competitive microbiota influences the colonization and proliferation of *C. difficile* and BoNT-producing clostridia linked to infant and adult intestinal botulism. Subsequent illness occurs when the human intestinal microbiota is disrupted. Understanding this process is crucial for recognizing the protective role of competitive microbiota in preventing colonization.

5.9 PREDICTIVE MODELS

Predictive growth models are useful tools for determining whether *C. botulinum or C. perfringens* will grow in a food based on the intrinsic properties of the food (namely, pH, water activity and salt content) and temperature at which the food is intended to be stored. These models are often based on data collected in a microbiological growth medium, but some models are based on data collected in food matrices. It is important to understand the limitations when using any given model.

Several online user-friendly models that include *C. botulinum* and/or *C. perfringens* are ComBase (Combase, 2024), Agricultural Research Service Pathogen Modeling Program (PMP) (USDA ARS, 2023), and the Danish Meat Research Institute (DMRI) (DMRI, 2025) predictive models.

The ComBase Perfringens predictor is particularly accurate for cooling of uncured meats, but overpredicts growth for cured meat products because the studies did not include erythorbate, which accelerates the conversion of nitrite to nitric oxide as the primary antimicrobial. ComBase models for Group I and Group II *C. botulinum* were developed in laboratory media, and include factors such as temperature, pH and salt or a_w.

The PMP has several models for *C. perfringens* during cooling of cured and uncured beef, chicken and pork, as well as *C. botulinum* during cooling of cooked uncured ground chicken, pork and beef (without any salt or food grade antimicrobials).

The DMRI growth/no-growth model for *C. botulinum* in meat products includes variables for sodium and potassium lactate, sodium nitrite, pH, salt, moisture and storage temperature.

Other published models in specific foods are available, but they require modest statistical skills to convert them to an easier to use Excel format.

5.10 CONDUCTING CHALLENGE STUDIES TO VALIDATE THE EFFECTIVENESS OF CONTROL MEASURES

Microbiological food challenge testing involves the deliberate contamination of food products with pathogenic or spoilage microorganisms to evaluate the effect of control measures applied to the food (e.g. thermal and other treatments), or defined storage conditions, on the microorganism of concern. They are an invaluable tool for the food industry to investigate survival, multiplication or reduction of the added microorganism in food products and are used to validate how processing procedures (such as heat treatment), product reformulation or changes in product storage conditions affect food safety. In addition, they enable validation of date marking, especially use-by dates, that are important for providing food safety assurance to the consumer.

There are several established control measures (known as "safe harbours") to prevent outgrowth of *C. botulinum* spores and cell proliferation in food products and hence prevent toxin formation. Maintaining a pH of 4.6 or less, a water activity of 0.92 or less, or a salt concentration of 10 percent or more throughout the food will allow spore survival but will prevent toxin formation under ambient storage. For chilled vacuum or modified

atmosphere packaged foods in the United Kingdom and European Union, shelf-life is limited to 10 days stored at 3–8 °C (Callaghan, 2008). If shelf-life exceeds 10 days, additional controls must be in place to prevent non-proteolytic *C. botulinum* growth. Heat treatment at 121 °C for 3 minutes for low acid canned foods is designed to eliminate 12-$\log_{10}$ Group I *C. botulinum*. Heating products to 90 °C for 10 minutes (or equivalent) will reduce populations of Group II psychrotrophic *C. botulinum* by 6-$\log_{10}$ to qualify for extended shelf-life of up to 42 days. Other validated controls for Group II *C.* botulinum such as pH reduction (pH $\leq$5.0 throughout the food), water activity control ($\leq$ 0.97 throughout food), salt (NaCl $\geq$ 3.5 percent throughout food) can similarly extend storage time.

However, when it is not possible to process or reformulate a food product to meet one of these accepted control measures, or when a combination of factors is being used to control growth of the pathogen, food challenge tests provide an essential tool for the food industry to use to assess product safety (Bowe *et al.*, 2022; ACMSF, 2023).

Since foodborne botulism is an intoxication caused by ingestion of preformed neurotoxin in food, guidance documents highlight the importance of confirming the absence of neurotoxin production during *C. botulinum* food challenge tests (US Food and Drug Administration, 2003; National Advisory Committee on Microbiological Criteria for Foods, 2010; Health Canada Food Directorate, 2010; International Organization for Standardization, 2019, Chilled Food Association, 2018; ACMSF, 2023). Neurotoxin formation has been observed even when no measurable increase in viable cell numbers is detected. (National Advisory Committee on Microbiological Criteria for Foods, 2010; Carlin and Peck, 1996; Hyytiä et al., 1999; ACMSF, 2023). This may reflect limitations in sampling or enumeration methodology that miss earlier growth followed by cell death, or the possibility that the cells remain metabolically active and capable of producing neurotoxin, without an accompanying increase in viable counts (Chilled Food Association, 2018). Nevertheless, an increase in the *C. botulinum* viable count should be considered as a potentially hazardous situation, even if toxin is not detected, because cell proliferation may precede toxin formation (ACMSF, 2023).

Unlike foodborne botulism, which results from consuming preformed BoNT in food, *C. perfringens* illness requires ingestion of large numbers (likely 10^8) of vegetative cells that sporulate in the intestine, releasing enterotoxin. Food challenge studies typically involve inoculation with *C. perfringens* spores, followed by cooking, cooling, or extended holding at target temperatures, with subsequent enumeration of total cell counts on selective media under anaerobic conditions.

The United States Food and Drug Administration (USFDA) does not establish specific allowable growth limits for *C. perfringens* in food but emphasizes proper handling, cooling, and reheating to limit its proliferation, so the food does not exceed the 10^6 CFU per gram threshold. In contrast, the USDA Food Safety and Inspection Service assumes an initial contamination level of 4-$\log_{10}$ CFU per gram and conservatively restricts growth to a maximum 1-$\log_{10}$ increase as pass/fail criteria (*Golden et al.,* 2009; USDA FSIS, 2021).

5.11 NEW AND EMERGING TECHNOLOGIES

There has been a sustained effort to develop advanced preservative technologies that ensure food safety, particularly concerning botulism, while also preserving or enhancing the organoleptic properties of food products. Traditional food safety methods, such as stringent heat treatments and freezing, often compromise product quality. In response, both novel thermal and non-thermal technologies have been explored and implemented to address these challenges. **Table 9** lists some of these emerging technologies and advantages and disadvantages associated with them.

TABLE 9 NEW AND EMERGING TECHNOLOGIES

Technology	Description	Advantages	Disadvantages	References
Non-thermal technologies				
High pressure processing (HPP)	Involves pressures between 100 and 10 00MPa, used to inactivate vegetative spoilage organisms and pathogens	Often combined with other controls like refrigeration and low pH for better bacterial spore control	Ineffective for substantial reduction of bacterial spores when used alone	Huang *et al.,* 2017; ACMSF, 2023
Pulsed light	Uses high-frequency light pulses (200–1 100 nm) to inactivate vegetative bacteria	Effective against vegetative bacteria	Ineffective for inactivating spores	Skowron *et al.,* 2014
Cold plasma	Generates reactive oxygen and nitrogen species, and UV radiation to inactivate bacteria, fungi and viruses	Wide range of activity against major foodborne pathogens	Mechanisms for inactivating bacterial spores not fully elucidated	Singh *et al*., 2020
Packaging technologies	Packaging materials include antimicrobials, antioxidants, light blockers, oxygen scavengers, CO_2 emitters, and moisture regulators	Rapid monitoring intervention for food safety	Concerns and uncertainties about safety and effectiveness of some packaging materials	Singh *et al.,* 2020; ACMSF, 2023
Thermal technologies				
Ohmic heating	Alternating electrical current heats food and rapidly destroys bacterial cell membranes, causing lethal leakage	Faster than steam heating	Ineffective for fat-rich foods due to poor electrical conductivity	Knirsch *et al*., 2010; Singh *et al.,* 2020
High-frequency heating	Radio frequency heating system induces molecular friction and heat to inactivate pathogens	Rapid and uniform heat distribution, minimizing product quality reduction	Ineffective against clostridial spores	ACMSF, 2023
Power ultrasound/ thermo-sonication	Uses pressure waves (20–100kHz) to cause cavitation, generating hydroxyl radicals and bacterial death	Efficiently removes microorganisms while maintaining food's nutritional and sensory properties	Not tested on bacterial spores	ACMSF, 2023
Microwave thermal sterilization	Combines microwave heating with thermal sterilization using water as an initial heating medium	Destroys clostridial spores and cells while preserving desirable food attributes	Complex handling of process deviations compared to conventional thermal processes	Singh *et al.,* 2020; ACMSF, 2023

Sources: See 5.18 References section for full list of sources cited in Table 9.

5.12 PREVENTION OF INFANT AND ADULT INTESTINAL BOTULISM

The toxico-infections of infant and adult intestinal botulism are caused by ingestion of the clostridial spores which can be found ubiquitously in the environment. As discussed earlier, prevention of food contamination by *C. botulinum* spores is not an achievable objective and the measures to inhibit growth and neurotoxin production by *C. botulinum* in food presented above will not prevent infection of infants or susceptible adults with spores.

Furthermore, a definitive dose-response has not been established for infant or adult intestinal botulism. However, the infection can result following exposure to as few as 10–100 spores, based on recovered cells from foods implicated in human illness (Arnon et al., 1979). Reducing the risk of spore ingestion with food therefore relies solely on the avoidance of food items in which substantial risk of spore contamination can be anticipated, or by consuming only sterilized food.

With honey being one of the main food items associated with infant botulism because of its relatively high rate of contamination (Harris and Dabritz, 2024) and likelihood for consumption by young infants compared to other foods, it is common practice in some countries to warn parents and caregivers to avoid giving honey to infants under the age of one year (US CDC, 2025). There is no other control measures applied to honey to reduce the risk of infant botulism.

Infants younger than 12 months, who represent a particularly vulnerable age group, are frequently fed infant formula. With the exception of the 2025 U.S. outbreak (US CDC, 2026; US FDA, 2026), the incidence of infant botulism associated with powdered infant formula (PIF) has historically been very low (Figure 1) (FAO/WHO, 2004, 2006; ACMSF, 2006; Harris and Dabritz, 2024). However, the extensive 2025 US outbreak associated with one manufacturer indicates that a risk still exists (USFDA, 2026; USCDC2026b). When detected in PIF, *C. botulinum* numbers are low (Carlin et al., 2004; Barash et al. (2010) and routine testing for *C. botulinum* spores has previously not been recommended (ICSMF, 2014). Currently, there are no standards or regulations targeting clostridia for powdered infant formula in the United States or Canada (Harris and Dabritz, 2024). FAO and WHO classified *C. botulinum* as a "Category C" organism, indicating causality is less plausible or not yet demonstrated (FAO and WHO, 2004, 2006). Although the likelihood of infant botulism associated with powdered infant formula (PIF) is very low, the potential severity of the illness is extremely high. Therefore, conducting a risk assessment for infant botulism in relation to PIF, and correlation with indicator organisms, may be warranted to improve current practices and identify effective approaches to minimize this risk. Some countries have microbiological specifications for clostridia (e.g. a maximum of 10 CFU/g of *C. perfringens*) (GSO, 2025) or process control recommendations (e.g. 100 CFU/g of sulphite-reducing clostridia) (ICSMF, 2014). In practice, particularly in countries like the United States and Canada, Gram-negative bacteria – such as *Salmonella*, *Cronobacter*, Enterobacteriaceae, coliforms, and generic *E. coli* – are commonly used as sanitation and process control indicators (Fusi et al., 2025). Nevertheless, end-product testing is useful in infant botulism outbreak investigation and control.

The United Kingdom ACMSF performed a risk assessment for *C. botulinum* in infant foods regarding chilled and frozen minimally processed infant weaning foods and concluded that they do not pose a greater risk of containing Group I *C. botulinum* spores than current commercially available and home-produced food. Therefore, a 12D botulinum cook was not regarded as necessary, whereas a heating step aiming at a 6D reduction of Group II *C. botulinum* spores or a formulation preventing any growth during shelf-life were strongly recommended (ACMSF, 2006).

Improving the awareness of healthcare professionals about infant and adult intestinal botulism may be the primary control measure for infant botulism as it will help in improving rapid diagnosis, setting up treatments earlier and advising people at risk and their caretakers (for example patients with intestinal diseases) to avoid some specific food or practices.

There are no specific control measures for adult botulism. However, some bacteria such as *L. plantarum, L. lactis,* and *Pediococcus pentosaceus*, are able to inhibit the growth of *C. botulinum* (Okereke, 1991a, 1991b; Rodgers et al., 2003a, b, Scott and Taylor, 1981) so theoretically their use as probiotics might be beneficial as prophylactic to reduce the severity of intestinal botulism (Harris and Dabritz, 2024). Additional research is needed to confirm efficacy.

5.13 RANGE OF REGULATORY REQUIREMENTS

Regulations and guidelines regarding control of clostridia vary among countries. Standards for cooling and hot-holding of cooked foods under which *C. perfringens* and *C. botulinum* will not grow to dangerous levels are justifiable. For example, in meat and poultry products under USDA FSIS jurisdiction, increase in populations of *C. perfringens* is limited to no more than 1-$\log_{10}$, and *C. botulinum* increase is limited to no more than 0.3 $\log_{10}$ (USDA, 2021). Standards based on the ComBase Perfringens Predictor, USDA predictive models (Juneja et al., 1999) or other challenge studies describe alternate regimes for cooling of foods such that temperature of the food is chilled from:

- 57 °C to 21 °C within no longer than 2 hours followed by cooling to 5 °C in no longer than 4 hours (USFDA, 2022b); or
- 52 °C to 12 °C within 6 hours (uncured meats) or 7.5 hours (cured meats) or to 5 °C within 24 hours (FSANZ, 2015); or
- 60 °C to 5 °C (or below) in less than 6 hours or it must be thrown out (New Zealand Food Safety, 2025); or
- 60 °C to room temperature or 21 °C (whichever is colder) in less than 2 hours, then room temperature or 21 °C (whichever is colder) to 5 °C (or below) in less than 4 hours (New Zealand Food Safety, 2025).

For foods prepared and cooled at retail, the US FDA Food Code recommends that foods be reheated to reach a temperature of 74 °C for 15 seconds (USFDA, 2022b) or equivalent prior to consumption to eliminate any *C. perfringens* vegetative cells should they have growth during cooling and storage.

Similarly, regulations and guidelines for control of clostridia in shelf-stable low acid canned foods generally require them to either undergo thermal processing sufficient to kill the equivalent of 12-$\log_{10}$ *C. botulinum* spores or demonstrate there is no growth of the pathogen via acidification (equilibrated pH < 4.6), reduced water activity, or other formulation safety parameters validated by a microbial challenge study with the pathogen.

Except for refrigerated juices (USFDA, 2007) and fish/foods containing seafood (USFDA, 2022a), the FDA does not mandate control Group II *C. botulinum* in refrigerated foods. However, individual states require manufacturers to validate the inhibition of *C. botulinum* in products with reduced-oxygen packaging.

Conversely, in the United Kingdom the shelf-life of vacuum/modified atmosphere packed (VP/MAP) chilled foods held between 3 and 8 °C is limited to 10 days unless additional controlling factors are used to prevent growth and toxin production by Group II *C. botulinum* (FSA, 2020).

The following controlling factors can be used either individually or in combination to extend the shelf-life of chilled foods to greater than 10 days (ACMSF, 2023):

- a heat treatment of 90 °C for 10 minutes or equivalent lethality at the slowest heating point in the food; or
- a pH of 5.0 or less throughout the food and throughout all components of complex foods; or
- a minimum salt level of 3.5 percent in the aqueous phase throughout the food and throughout all components of complex foods; or
- a water activity (Aw) of 0.97 or less throughout the food and throughout all components of complex foods; or
- a combination of heat and preservative factors which can be shown consistently to prevent growth and neurotoxin production by Group II *C. botulinum*.

This guidance does not apply to VP/MAP chilled fresh meat (beef, lamb and pork) which has not been processed beyond cutting, packing, chilling, freezing and quick-freezing (FSA, 2020). It is the responsibility of the food business operator (FBO) producing VP/MAP chilled fresh meat to identify and apply a safe shelf-life in line with the company's existing food safety management systems. Smaller FBOs, lacking resources or expertise to identify an appropriate shelf-life for their VP/MAP chilled fresh meat products, can apply a maximum shelf-life of 13 days (FSA, 2020).

While some countries (e.g. the United States) recommend that producers include the statement on bottles of honey "WARNING: Do Not Feed Honey to Infants Under One Year Old," there is generally no mandate for such labelling (e.g. United States, European Union, Australia and New Zealand).

5.14 OVERARCHING STATEMENT ON CONTROL OF BONT-PRODUCING CLOSTRIDIA

The prevention of foodborne botulism relies on inactivating spores through validated thermal processing, or inhibiting their growth via strict temperature control, formulation adjustments such as acidification or reduced water activity, rapid and adequate fermentation, or the use of food-grade antimicrobial ingredients in combination with other hurdles. Validation of growth inhibition and assessment of shelf-life of low acid foods (especially in food kept in room temperature) is needed; labelling should be prominent on food package to show critical storage temperature requirements and use by dates for safety.

Government and schools should educate the consumers about proper storage of foods in their homes and risks associated with home-prepared and home-canned foods or temperature abuse of chilled foods; inspections of manufacturers, restaurants, institutions and caterers should reinforce the strict adherence to temperature control. For foods and communities where temperature control is unreliable, governments should encourage manufacturers to make formulation changes to inhibit botulinum growth and toxin production if the product were temperature abused.

Government, medical professionals, and organizations, and science-based information websites and blogs that support parent and caregivers should provide education to remind them of risks on honey and other environmental exposures that lead to infant botulism.

Clinicians should be trained in rapid diagnosis and treatment of foodborne, infant and adult intestinal botulism to reduce severity and duration of illness.

5.15 OVERARCHING STATEMENT ON CONTROL OF *CLOSTRIDIUM PERFRINGENS*

Effective prevention of foodborne illness caused by *C. perfringens* requires strict adherence to validated thermal control measures. Current recommendations from EFSA (2005) and the USDA's FSIS Stabilization Guidelines for Meat Products (USDA, 2021) emphasize key interventions: thorough cooking, rapid cooling of food through the critical temperature range of 55 °C to 15 °C, maintaining holding temperatures above 60 °C or below 12 °C, and reheating to an internal temperature of at least 72 °C prior to consumption. Reheating is especially important when the product's prior thermal history is unknown, serving as an additional safeguard against potential vegetative cell survival (EFSA, 2005). Each step in this temperature-control strategy plays a vital role in mitigating the risk of *C. perfringens* proliferation and should be explicitly integrated into a food business operator's (FBO's) control programme. For high-mass or thick foods – such as Toromi-style products or stuffed poultry – cooling must be managed with consideration for extended heat retention characteristics, which can delay temperature reduction and elevate risk if not properly addressed. When rapid cooling or consistent temperature control is impeded due to product characteristics or limitations in food establishment infrastructure, product reformulation may be employed to enhance safety margins.

5.16 OVERARCHING STATEMENT ON CONTROL OF CLOSTRIDIOIDES DIFFICILE

Although evidence supporting *C. difficile* as a traditionally defined foodborne pathogen remains limited, implementing control measures may reduce the risk of transmission across regions, between countries, and via food. These interventions may also contribute to minimizing community-acquired infections attributed to environmental and food-related exposure pathways.

The scale, complexity and international dimension of modern food production, processing and trade and the relatively widespread use of antibiotics in some parts of the world that enable *C. difficile* to proliferate means that *C. difficile* control along the food chain and prevention of community acquired CDIs is not as easily achieved and maintained in agriculture as in a healthcare setting (Lim et al., 2020b). Achieving some measure of risk management is therefore reliant on applying multiple actions or hurdles along the food chain. Because CDI is thought to be mediated by relatively low levels of spores present in the environment, including foods, and that growth of *C. difficile* is not likely under storage conditions that control other clostridia, control will be more likely to be effective if focused on minimizing antibiotic use that selects for *C. difficile*.

Mitigating the risk of amplifying *C. difficile* in food-producing animals therefore requires observation by both farmers and veterinarians of good agricultural practices (GAP), prudent use of antibiotics, and judicious use of animal manure.

Pathogen carriage in farm animals is affected by multiple factors (e.g. biosecurity, animal husbandry practices, use of antibiotics, feed) while shedding is influenced by the environment, gut microbiota, the immune status of the animal and the pathogen characteristics (need citations). Pathogen prevalence and cross-contamination of food may be minimized at the production stage through the application of good agricultural practices (GAPs) for food safety which cover general farm management, animal health management, veterinary medicines and biologicals, animal feeding and watering, environment and infrastructure, and animal and product handling, irrigation and soil amendment practices (Poisot et al., 2007). These should be defined at national or local level, for individual sectors (e.g. beef, dairy, sheep, poultry, cereals, fruit and vegetables) to maximize control

of zoonotic agents and ensure compliance with the requirements of national or international legislation and trade.

Although *C. difficile* is ubiquitous in the environment, the application of specific actions under GAP may be applied to minimize animal carriage and shedding as well as environmental and ultimately food contamination. Clostridioides difficile in farm animals, for example, is affected using select antibiotics, especially cephalosporins, and it has been hypothesized that these eliminate susceptible microflora in the intestines providing niches, reducing competition and facilitating the outgrowth of *C. difficile* (Arruda et al., 2013). Good stewardship of antibiotics, informed by research, under the GAP programme (Poisot et al., 2007) is therefore essential to minimize the opportunity for this pathogen to establish, multiply and be shed in faeces thereby cross contaminating the environment, infecting other livestock and increasing the probability of transfer to humans.

Clinically important *C. difficile* strains have been isolated from animal wastes and biosolids (Romano et al., 2012; Steyer et al., 2015; Moradigaravand et al., 2018; Rivas et al., 2020, Subirats et al., 2022) and associated composted products (Lim et al., 2020) which therefore should be effectively managed to minimize environmental and public health risks. As with composting, anaerobic digestion is also ineffective at reducing the concentration of *C. difficile* spores (Le Maréchal et al., 2020) which can survive in the environment for several months (Vonberg et al., 2008). The use of animal slurries, manure, biosolids, mulches and digestate as organic fertilizers and soil improvers therefore facilitates cross-contamination of the environment, cross infection of livestock and ultimately contamination of retail foods. The use of animal wastes and biosolids as organic fertilizers, along with related horticultural practices, should adhere to GAP. These guidelines provide recommendations on seasonality, climatic conditions, application rates, pre-grazing waiting periods, buffer zones, and other measures to minimize human health risks from environmental contamination.

The use of probiotics to manage the *C. difficile* risk associated with food is inconclusive (Valdés-Varela et al., 2024; Kelly et al., 2021)

5.17. ROLE OF CONSUMER EDUCATION IN REDUCING ILLNESS

As noted above, consumer education is an important activity in the strategies to control illnesses attributed to clostridia. If risk communication strategies are to be effective and change consumer behaviour, the risk perceptions and factors that have influenced consumer perceptions must be elucidated and understood (Burnett and Corlett, 2017).

Low acid foods subjected to insufficient thermal treatment for home canning, or home-fermented foods where a starter culture was either not used or ineffective, are a particular risk for botulism. Consumers should be educated on the importance of using clean ingredients and hygienic production as well as risks associated with these activities and how these can be minimized by rapid chilling, chilled storage at temperatures below 4 °C, rapid fermentation, or rapid acidification throughout the food matrix using acids such as vinegar. Government, medical professionals, and organizations that support parents and caregivers should provide warnings on the risks related to exposure of young infants to honey and other environmental exposures that lead to infant botulism.

The key message for *C. perfringens* control is to ensure thorough cooking, followed by rapid cooling of cooked foods – particularly meat, poultry, and gravies – not intended for immediate consumption. Cooling should occur through the temperature danger zone, from approximately 55 °C to below 10–12 °C, within four hours to inhibit spore germination and outgrowth. Prior to consumption, such foods should be reheated to a core temperature of at least 72 °C to ensure microbial safety.

While many organizations provide information leaflets for patients focusing on nosocomial *C. difficile* infection, the equivalent is not currently available for risks associated

with foods known to carry spores. The development of a key message(s) is complicated by the potential of multiple foods as sources of spores (Rodriguez-Diaz *et al.,* 2024) and the absence of specific interventions to reduce exposure to spores, other than physical removal of spore-containing soils and severe thermal processing.

Consumers should be made aware that all foods are potentially contaminated with bacterial spores and that the spores survive routine cleaning with detergents, and that hand hygiene using alcohol-based gels is not effective. They should be informed that in areas where food is prepared, enhanced cleaning is required of all potentially contaminated surfaces with sporicidal agents such as sodium hypochlorite (bleach) while handwashing with chlorhexidine or with soap and water is effective in physically removing spores (Gerding *et al.*, 2008). Cooking is a common household practice, and consumers should also be advised that heating their food to at least 85 °C for a minimum of 10 minutes will generally reduce the risk of foodborne transmission by vegetative cells (Rodriguez-Diaz *et al.*, 2004).

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CHAPTER 6
LABORATORY METHODS FOR DETECTION AND CHARACTERIZATION

6.1 INTRODUCTION

In the context of clostridia in food, the detection process is intricate and multilayered. It encompasses the identification and quantification of viable organisms (spores and/or vegetative cells), the detection and typing of toxins, and the identification of toxin-encoding genes. Detecting botulinum neurotoxin (BoNT) in a laboratory setting, following a clinical diagnosis and treatment of the illness, is of utmost importance. Additionally, identifying toxins or spores of clostridia in food or environmental samples is essential for incident investigations and plays a critical role in scientific research programmes and risk assessments. However, routine monitoring for the presence of spores, cells, or toxins in food is not a practical aspect of food safety protocols (ACMSF, 2023). While sample enrichment methods such as heat or alcohol treatment can enhance spore detection by eliminating competitive vegetative microbes, the microbiological cultivation of clostridia requires specialized laboratory equipment, personnel training, and infrastructure, including Biosafety Level 2 (BSL-2) facilities to manage the associated risks effectively. Enhanced biosecurity is required in the United States for handling *C. botulinum* and its toxins; Biosafety Level 3 may be necessary depending on the quantities of BoNT handled or specific country regulations.

6.2 METHODS FOR THE DETECTION AND CHARACTERIZATION OF CLOSTRIDIA IN FOODS AND ENVIRONMENTAL SAMPLES

An international standard for the enumeration of sulfite-reducing clostridia, which includes both non-pathogenic and pathogenic strains, in food and animal feed is provided by International Organization for Standardization (ISO) 15213-1 (ISO, 2023a). Cultural and genomic-based detection and enumeration methods for *C. botulinum*, *C. perfringens* and *C. difficile* are detailed in the sections below, along with recognized limitations. Since foodborne botulism is caused by BoNT rather than the bacterial cells, the toxin is the target for analysis rather than the simple presence of cells.

Most studies evaluating the impact of storage temperature on the detection of the bacteria or the toxins have been conducted in a diagnosis context (faecal, serum samples). For detection of BoNT or *C. botulinum*, freezing the samples appears to be a reliable method to maintain sensitivity of the method (Le Maréchal *et al.,* 2017; Piazza *et al.,* 2011; Smart *et al.,* 1987). For *C. difficile*, storage at 4 °C is appropriate for both the toxins and bacteria detection, but storage at – 20 °C can also be used (Nho *et al.*, 2021; Freeman and Wilcox, 2003; Scottish Microbiology & Virology Network, Scottish *C. difficile* Reference Service and Health Protection Scotland, 2018). In all cases, storage at room temperature, and freeze–thaw cycles, should be avoided.

6.3 *CLOSTRIDIUM BOTULINUM*

Clostridium botulinum represents a significant threat to human health, necessitating stringent biosecurity measures during research and the detection process due to the involvement of potentially dangerous materials. Consequently, research and advanced diagnostic work on *C. botulinum* is restricted to specialized laboratories with appropriate biohazard containment facilities and procedures.

In the United States, any state public health lab can conduct the detection assays for botulinum organism and toxin in a food that is not known to carry toxin. Once the organism or toxin is detected, the lab must notify the CDC and either destroy the sample or transfer the sample to a Select Agent Tier I registered laboratory within 7 days (CFR, 2025).

An International Organization for Standardization (ISO) standard is available (ISO/TS 17919:2013 (ISO, 2023b) which utilizes the polymerase chain reaction (PCR) for detection of botulinum type A, B, E and F neurotoxin-producing clostridia. However, this method detects the genes rather than the toxins themselves, so a positive result does not necessarily indicate the presence of these toxins in the sample being analysed.

6.3.1 CULTURAL METHODS FOR *CLOSTRIDIUM BOTULINUM* DETECTION

Detection and enumeration of *C. botulinum* spores or vegetative cells present substantial challenges due to the limited sensitivity and specificity of available selective culture media. A key limitation is the inability to reliably distinguish between toxigenic and non-toxigenic colonies, complicating interpretation and risk assessment. Confirmation typically necessitates specialized microbiological techniques, beginning with anaerobic incubation of food samples in enrichment medium, followed by neurotoxin detection or identification of the neurotoxin-encoding gene from the enrichment broth or from a subculture. If the assay is designed exclusively to recover spores, food samples may be heat-treated or exposed to ethanol before enrichment to reduce competitive microbiota. However, care must be taken to avoid inadvertently destroying *C. botulinum* vegetative cells, as this could affect total cell enumeration.

Commonly, enrichment will be performed at 35 °C (for proteolytic strains) and at 28 °C for non-proteolytic strains). However, in cases of infant or adult intestinal botulism an enrichment temperature of 37 °C should be considered. Incubation for 7 days or longer at 12 °C can help to reduce the detection thresholds for non-proteolytic Group II *C. botulinum* (Peck *et al.,* 2010). Also, an increase of the sample volume from 25 g (as recommend in the ISO standard) to 200 g can significantly reduce the detection limit for both groups of human-relevant *C. botulinum* (Peck *et al.,* 2010).

Enumeration of cells may be achieved through statistical analysis of positive detections in serially diluted samples prior to enrichment. Research methodologies such as the most probable number (MPN) technique can estimate viable counts as low as 5 to 100 spores per kilogram, depending on the specific method applied and the characteristics of the food matrix (Peck *et al.,* 2010).

Although plating on solid nutrient laboratory media (such as Reinforced Clostridial Agar) and incubation under anaerobic conditions is appropriate for Group I *C. botulinum*, enumeration of Group II non-proteolytic strains is less reliable and may result in under estimating cell numbers. Culturing using the MPN method in liquid media provides more accurate enumeration; methods for the enumeration of Group II *C. botulinum* spores from a variety of foods have been described combining heat treatment at 65 °C (to kill vegetative cells) and low-temperature selective enrichment in anaerobic medium at 12 °C with detection of BoNT genes by multiplex PCR (Peck *et al.,* 2010). The method facilitated selective detection of Group II *C. botulinum* but was not effective for Group I strains. Detection limits for each tested food matrix were established using control samples

inoculated with known concentrations of Group II spores. Additionally, the use of selective enrichment and large sample volumes (200 g) enabled achievement of low detection thresholds (Peck *et al.,* 2010).

Confirmation of BoNT-producing cells relies on toxin detection described below (**Table 10**) or using multiplex or singleplex PCR techniques with specialized primers to detect the presence of neurotoxin-encoding genes indicating the presence of bacterial cells (**Table 10**).

The detection of *C. botulinum* in foods and ingredients is inherently limited in its ability to guarantee the complete absence of such cells. Consequently, routine pre-emptive testing of food materials for *C. botulinum* offers limited value for food safety (FSA, 2020). However, accurate enumeration of toxigenic cells is needed to conduct challenge tests that validate the ability of new food formulations and processes to prevent growth and toxin production.

6.3.2 DETECTION OF BOTULINUM NEUROTOXIN: BIOASSAY

In clinical settings, the detection of botulinum neurotoxin in a specimen, whether serum or faeces, immediately follows any clinical diagnosis of human botulism. However, the identification of the toxin is not a prerequisite for initiating treatment. Prompt administration of antitoxin significantly mitigates the severity of botulism outcomes (WHO, 2023).

The *in vivo* mouse bioassay (MBA) remains the gold standard for botulinum neurotoxin detection in clinical and epidemiological contexts. This assay concurrently verifies multiple neurotoxin functionalities including binding, internalization, and intracellular activity, collectively confirming the ability of the sampled material to cause botulism (ACMSF, 2023). The MBA is universally effective across all toxin types, boasting a typical sensitivity of approximately 5–10 pg per ml. With regards to neurotoxins produced by non-proteolytic *C. botulinum*, the assay's sensitivity can be enhanced by trypsin treatment of the sample before injection (Duff *et al.,* 1956). Typically, the assay involves a 0.5 ml intraperitoneal injection of patient serum or an extract from the implicated food or faecal sample; with the mouse LD50 (median lethal dose) in the range of 0.1 to 1ng per kg (Rosseto and Montecucco, 2019); the human oral lethal dose of botulinum neurotoxin is approximated at 70 µg per kg body weight (Arnon *et al.*, 2001).

In clinical practice, MBA is often paired with an array of antitoxins, when available, in cross neutralization reactions, facilitating toxin type identification, even in complex scenarios with multiple toxin types, thereby bolstering outbreak tracking efforts (Thirunavukkarasu *et al.,* 2018; ACMSF, 2023). The benefit of the MBA is its low detection limit, detection of biological activity of the neurotoxin, and its non-reliance on antibodies, as these would limit the detection to serotypes for which antibodies are readily available. Especially in the light of the recent discovery of new sero- and subtypes of botulinum neurotoxin and neurotoxin-like toxins (Zhang *et al.,* 2017; Zhang *et al.,* 2018). This limitation by antibody-based methods in general needs to be considered when selecting botulinum neurotoxin detection methods.

Despite its sensitivity and robustness, particularly from a public health standpoint, MBA has several significant drawbacks. The assay necessitates costly specialized animal facilities, has a turnaround time of approximately 4-5 days, and presents challenges in interpreting negative results. Additionally, animal welfare concerns and logistical limitations have catalysed the pursuit of improved, rapid, and validated technologies for botulinum neurotoxin detection (EFSA, 2005; Thirunavukkarasu *et al.,* 2018; ACMSF, 2023).

A modified form of the MBA, the local paralysis assay, has a sensitivity at a similar level to MBA. In contrast to MBA, this assay does not employ death as endpoint and reduces animal distress and therefore might be more acceptable from an animal welfare perspective. It utilizes local injection into the gastrocnemius muscle of mice followed by evaluation of

the neurotoxin-induced local paralysis using the mouse digit abduction score (Sugiyama *et al.*,1975; Akoi, 1999). While this assay requires a significant level of training of the assay performers in test substance application and assessment of paralysis, it is sensitive and reduces the turnover time for example in BoNT potency testing (Jones *et al.,* 2006).

6.3.3 DETECTION OF BOTULINUM NEUROTOXIN: TECHNOLOGICAL SOLUTIONS

Various alternative methods have been developed to detect a range of toxin types, with some but not all, having sensitivity comparable to that achieved by the MBA (**Table 10**). However, limitations include assay inaccuracies caused by interference from complex food samples, the unavailability of reliable antibodies for all toxin types, restricted access to costly equipment, and the absence of regulatory approval.

Innovations for alternative methods include:

Modified *in vivo* and *ex vivo* methods: These techniques utilize animal tissues, stem cells or differentiated neurogenic cells to detect toxin activity directly.

The *ex vivo* mouse phrenic nerve hemidiaphragm assay (Bigalke and Rummel, 2015) utilizes isolated *Nervus phrenicus* (*N. phrenicus*) hemidiaphragm tissue instead of living animals.

Exposure to botulinum neurotoxin will lead to reduced contraction upon muscle stimulation in a dose-dependent manner. This test requires 2-3 days and can reduce the number of animals used for testing, as two tissue preparations can be done from one mouse. The detection thresholds can be as low as 50 pg BoNT/ml sample. The inhibiting effect of the sample matrix can be reduced by dilution and dialysis of the sample, possibly increasing the detection threshold for this method. Specialized laboratory equipment and training are required to perform this assay.

Cell culture-based assays have been primarily developed for potency testing of botulinum neurotoxin-based pharmaceuticals and commonly employ cell lines such as neuroblastoma SiMa cells in combination with immunodetection of cleaved target SNARE proteins (Fernández-Salas *et al.*, 2012; Bak *et al.* 2017). These assays are highly sensitive but need a significant level of technical skills and experience to be performed. Additionally, their pronounced sensitivity to the composition of the delivery matrix may limit their applicability for detecting neurotoxins in food or clinical samples. Although primarily employed in pharmaceutical research environments for potency testing, cell-based methods offer minimal improvement over the mouse bioassay (MBA) in terms of turnaround time and exhibit variability levels that do not meet the stringent requirements of clinical diagnostics (Kiris *et al.,* 2014; Thirunavukkarasu *et al.,* 2018; ACMSF, 2023).

Immunological detection methods: ELISA, which employ antibodies specific to botulinum neurotoxin antigens, offer rapid turnaround times (typically a few hours). When combined with other technologies like mass spectrometry, these methods achieve high specificity and can be automated. However, they may also detect biologically inactive toxin (Thirunavukkarasu et al., 2018), and the assays are, as mentioned above, limited to neurotoxin serotypes and possibly subtypes to which the antibodies reliably bind.

Enzymatic activity assays: These systems exploit the enzymatic properties of botulinum neurotoxins, such as their ability to cleave the SNAP-25 protein. By using combinations of specific antibodies, flow cytometry, fluorescent dyes, or mass spectrometry, these methods provide automated in vitro detection systems. While highly specific to each toxin type, extending these assays to detect a wide array of known and potential new toxins remains challenging (Rhéaume et al., 2015; ACMSF, 2023).

Nucleic acid-based methods: Techniques such as PCR, real-time PCR, Multi-Locus Sequence Typing (MLST), and Whole genome sequencing (WGS) facilitate the detection and characterization of *C. botulinum* and its toxin encoding genes, though they do not directly detect the toxin itself. The extensive gene sequence data available in accessible

databases enhance their utility in epidemiological, surveillance, and research applications. However, WGS and other nucleic acid-based methodologies are fundamentally dependent on culture enrichment processes, which inherently extend the time required to obtain results by several days (EFSA, 2005; Thirunavukkarasu et al., 2018; ACMSF, 2023).

Although some of these methods have gained regulatory approval, none currently meet the rigorous standards required to replace the MBA for detecting botulinum neurotoxins in clinical settings (ACMSF, 2023).

TABLE 10. BOTULINUM NEUROTOXIN DIAGNOSTIC ASSAYS AND DESCRIPTION

Method	Description	Benefits/limitations
Cell-based assays (SNAP-25 cleavage)	Detects BoNT/A-E, analysis time 3–5 days, detection limits 0.55–1 500 pg/mL	Sensitive, detects functional toxin/technically demanding
Cell-based assays using differentiated cell lines	Detects BoNT/A-E, analysis time 3–5 days, detection limits 825–150 000 pg/mL	Detects functional toxin/technically demanding
Electrical conductance	Detects BoNT/A, analysis time 1–3 days, detection limits 25 000 pg/mL	Detects functional toxin/technically demanding
Endopeptidase ELISA-based or MS based	Detects BoNT/A-G, analysis time 7–8 hours, detection limits 0.1–1 000 pg/mL	Rapid detection/only detects cleavage, not full toxin functionality
Endopeptidase-suspension immuno-assay	Detects BoNT/A-F, analysis time 2 days, detection limits 0.3–80 pg/mL (von Berg *et al.,* 2019)	Sensitive, detects functional toxin, potential to replace mouse lethality bioassay
Immunosensors and FRET assays	Detects BoNT/A, analysis time 2–5 hours, detection limits 0.1–20 pg/mL	Rapid detection/detects both active and inactive BoNTs
Lateral flow assay	Detects BoNT/A & B, analysis time 30 minutes, detection limits 10–50 ng/mL	Rapid detection/ detects both active and inactive toxins, hindered by neurotoxin associated proteins
Mass spectrometry	Detects BoNT/A-F, analysis time 5–8 hours, detection limits 0.1–1 pg/mL	Rapid detection/detects both BoNT serotypes and subtypes
Mouse lethality bioassay	Detects BoNT/A-F, analysis time 4 days, detection limits 1–10 pg/mL	Sensitive, detects functional toxin/ethical concerns, assay variability and duration
Mouse phrenic nerve hemidiaphragm assay	Detects BoNT/A, B, E, analysis time 2–3 days, detection limits 30–50 pg/mL (Bigalke and Rummel, 2015)	Sensitive, detects functional toxin/ethical concern, technically demanding
Sandwich ELISA, electro-chemiluminescent assay	Detects BoNT/A-F, analysis time 6–7 hours, detection limits 2–176 pg/mL	Rapid detection/detects both active and inactive toxins, hindered by neurotoxin associated proteins

Sources: Adapted from Rasetti-Escargueil and Popoff, 2022

Rasetti-Escargueil, C. & Popoff, M.R. 2022. Recent developments in botulinum neurotoxins detection. *Microorganisms*, 10(5): 1001. https://doi.org/10.3390/microorganisms10051001

6.4 *CLOSTRIDIUM PERFRINGENS*

As with *C. botulinum*, microbiological testing for *C. perfringens* in foods and food ingredients offers limited utility in ensuring food safety, given the widespread presence of this organism in various foods and the environment. A positive result is typically inconsequential unless it reveals significantly elevated counts (EFSA, 2005). An international standard for the enumeration of *C. perfringens* in food and animal feed is provided by ISO 15213-2 (ISO, 2023a, 2023c).

6.4.1 CULTURAL METHODS FOR *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens is readily detected through traditional culture methods, with isolates evaluated based on characteristic species traits. The ISO 15213-2 standard and the AOAC International, FDA, recommend detection and enumeration of *C. perfringens* on Tryptose Sulfite Cycloserine (TSC) agar (Rhodehamel and Harmon, 2001; ISO, 2023a, 2023c). *Clostridium perfringens* exhibits less fastidious growth requirements compared to other clostridial species and hence can proliferate in conditions that are not strictly anaerobic (Pearson and Walker, 1976). On TSC agar supplemented with egg yolk, *C. perfringens* typically forms black colonies characterized by a 2–4 mm opaque halo indicative of lecithinolysis.

Species confirmation of *C. perfringens* is achieved through Gram staining and further verification on lactose gelatine medium, where assessment of gas production, lactose fermentation, and motility is conducted (Grenda et al., 2023). Typically, the results obtained through culture and biochemical methods are validated via PCR testing (Selim et al., 2017).

For purposes of validating the efficacy of strategies, such as formulation adjustments and cooling rate, to inhibit growth in foods, manufacturers rely on inoculated pack/challenge studies with known *C. perfringens* strains; counts of presumptive positives on TSC agar are sufficient to determine pass–fail of the product.

6.4.2 OTHER METHODS FOR *CLOSTRIDIUM PERFRINGENS*

In cases of suspected *C. perfringens* infection, diagnostic evaluation should include stool and suspected food analysis, encompassing both culture and instrumental methods (e.g. based on ELISA, Immunological methods, PCR or other reliable techniques) for the determination of *C. perfringens* toxin type.

In the context of outbreak investigations, appropriate testing protocols are crucial for confirming etiological agents (**Table 11**). Confirmation of an outbreak can be established through the following criteria: (1) the isolation of 10^6 organisms per gram from the stools of two or more symptomatic individuals, contingent upon proper specimen handling; (2) the detection of enterotoxins in the stools of two or more symptomatic individuals; or (3) the isolation of 10^6 to 10^7 organisms per gram from food that is epidemiologically implicated, provided the specimen is adequately handled. Given likely uneven distribution of microbial contamination in implicated food, the detection of more than 10^4 organisms per gram in unused food (Golden *et al.,* 2009), when associated with temperature abuse during preparation of the food, also serves as supporting evidence of an outbreak (UK Health Security Agency, 2024). These measures are essential for accurate identification and control of the source of infection (EFSA, 2005).

TABLE 11. OVERVIEW OF IDENTIFICATION METHODS FOR *CLOSTRIDIUM PERFRINGENS*

Method	Description	References
16S ribosomal RNA PCR	Primers specific to the 16S rRNA gene are utilized in PCR to amplify the 16S rRNA gene. Post amplification, the PCR products undergo sequencing. The resultant sequences are subsequently analysed with bioinformatics tools. Taxonomic assignment is determined based on sequence similarity exceeding 97%.	Browne *et al.,* 2016
ELISA	ELISA characterized by their affordability, rapidity, and operational simplicity. Assay may have low diagnostic sensitivity.	Wimsatt *et al.,* 1986
LAMP-LFB	The Loop-Mediated Isothermal Amplification coupled with a Lateral Flow Biosensor (LAMP-LFB) assay is a cost effective and readily accessible qualitative method for the routine detection of *C. perfringens* in food samples.	Sridapan *et al.,* 2021
Lecithinase test (Nagler's reaction)	*Clostridium perfringens* is cultured on agar containing egg yolk; an opalescent halo is formed around colonies that produce lecithinase (alpha-toxin). However, the test is not specific to *C. perfringens*.	Nagler FPO, 1939
MALDI-TOF mass spectrometry	Characterized by straightforward procedural steps, affordability, rapid detection, and low operational expenses, coupled with high specificity. However, its effectiveness hinges on the availability of precise and dependable microbial spectral databases for comparative analysis.	Brodzik *et al.,* 2016
Multiplex PCR	The multiplex PCR approach is routinely employed to amplify key toxin genes, enabling the classification of *C. perfringens* into seven toxin types (A to G) based on the combination of toxin genes present.	Rood *et al.,* 2018
RAA-CRISPR/Cas12a-based	Rapid, sensitive, and instrument free. The whole process can be completed in 1 h.	Xiao *et al.,* 2023
Reverse CAMP test	A streak of *Streptococcus agalactiae* is applied onto a blood agar plate, followed by a perpendicular streak of *C. perfringens*. After anaerobic incubation, the presence of a "bow-tie" zone of synergistic haemolysis confirms the presence of *C. perfringens*. This method is highly sensitive, although it may produce false-positive reactions.	Hansen and Elliott, 1980
TSC+EYG	Plating on Tryptose Sulfite Cycloserine (TSC)+ Egg Yolk Agar (EYG). *C. perfringens* colonies exhibit distinct black pigmentation accompanied by opaque halos. This medium facilitates a sensitive, rapid, and accurate presumptive identification of *C. perfringens*.	Kotsanas *et al.,* 2010
WGS	A genome wide search for relevant toxin genes in *C. perfringens* is conducted utilizing sequence similarity search programs such as BLAST. This approach facilitates toxinotyping by identifying and characterizing toxin genes within *C. perfringens* genomes.	Kiu *et al.,* 2017

Sources: Adapted from Kiu and Hall, 2018; Rood, *et al.*, 2018. See 6.8 References section for all other sources in Table 11.

6.5 *CLOSTRIDIOIDES DIFFICILE*

Detection methods are primarily tailored for clinical samples. As the disease is caused by toxins, diagnostics must include confirmation of either free toxin, presence of toxin genes or toxigenic status of the strain. *Clostridioides difficile* detection methods include cultivation, detection of free toxins TcdA and TcdB or detection of toxin genes (*tcdA* and/or *tcdB*) (Guery *et al.,* 2019, Carroll and Mizusawa, 2020). Detection of one of the two genes for binary toxin CDT is included in some commercially available diagnostic tests. Several primers for *tcdA* and *tcdB* genes are also described and are used in epidemiological studies. In this case it is important to note that not all published primers will detect all forms of variant toxin genes called *C. difficile* toxin types. The presence of binary toxin and a specific deletion in the accessory gene *tcdC* are molecular markers for some ribotypes with increased virulence potential (called also hypervirulent strains); however, both markers are present also in related but non-hypervirulent types.

For human clinical samples a two-step algorithm is suggested in several guidelines, and detection of common *C. difficile* antigen (GDH – glutamate dehydrogenase) or toxin genes (A/B) detection by molecular approaches can be used as the first screening stage followed by free toxin detection by enzyme immunoassay (EIA) (Guery *et al.,* 2019). Cultural methods for detection are very rarely used in routine human diagnostic laboratories.

From the available methodological approaches, culture or sometimes molecular detection of toxin genes is used for animal faecal and food samples. Free toxin EIAs are not validated for animal faecal specimens and are used only occasionally. Testing free toxin in food samples is not relevant, as spores are the infectious agent. The GDH test is usually not included in testing algorithms for animal faecal and food samples.

Methods for detecting *C. difficile* in food samples are not standardized and differ widely in sample quantity, enrichment media and incubation times, and choice of solid selective media. Molecular approaches also vary, particularly in the primers used for *tcdA*/*tcdB* detection (Candel-Pérez *et al*., 2019; Rodriguez-Palacios *et al.,* 2020).

Another issue in *C. difficile* detection is the emergence of strains from divergent or cryptic clades (C-I to C-V). They are common in soil and therefore often found in soil-covered vegetables (e.g. root vegetables). These strains are potentially incorrectly recognized as non-toxigenic. Occasionally, divergent strains can have toxin genes for TcdA or TcdB; however, the genes show a lower level of homology to ordinary *tcdA*/*tcdB* genes, and commercial molecular tests will not detect them. The free toxins of divergent clades will be detected with EIAs.

6.5.1 CULTURAL METHODS FOR *CLOSTRIDIOIDES DIFFICILE*

Cultural methods are used to detect *C. difficile* in food samples regardless of food type. One of the main differences to human diagnostic methods is the introduction of enrichment as the first step in the cultivation protocol. Temperature or ethanol shock are included in most protocols to destroy vegetative cells of remaining microbes and select for spores, where appropriate. Selective media, like those used in human diagnostics, are then used for selection of *C. difficile.* A variety of culture media have been employed to ascertain the prevalence of *C. difficile* in food samples (Candel-Pérez *et al.,* 2019). One of the meta-analyses of *C. difficile* in food samples (Rodriguez-Palacios *et al.,* 2020) listed six different cultivation approaches used in the studies and showed that cultivation methods were not significantly different for *C. difficile* recovery. The main differences in the methods were direct plating vs. an enrichment step, and where selective ethanol shock was positioned within the overall protocol (before or after enrichment).

Clostridioides difficile must be incubated in an anaerobic atmosphere and at a temperature of 37 °C. There is a notable paucity of data regarding the precise minimum and maximum thermal thresholds that permit growth, as well as the specific rates of growth under varying temperature conditions (Lund and Peck, 2015). In newer studies, strains isolated from food are commonly further characterized with ribotyping and/or WGS.

6.5.2 TECHNOLOGICAL SOLUTIONS FOR CLOSTRIDIOIDES DIFFICILE

Innovations in the field of *C. difficile* detection include semi-quantitative highly sensitive methods for free toxin detection (Sandlund, Davies and Wilcox, 2020). However, this approach has not yet been adopted in routine diagnostics. Also *C. difficile* toxin gene targets are included in syndromic panels for intestinal infections.

Antibiotic resistance testing is currently not part of routine diagnostics of *C. difficile* in humans. Resistance to three antibiotics used for the treatment (metronidazole, vancomycin, fidaxomicin) is low. Metronidazole resistance is increasing; however, phenotypic detection is greatly influenced by media and conditions used.

Resistance to other antimicrobial classes is important for *C. difficile* in terms of selective advantage and, in some cases, also increased virulence. Resistance testing is therefore often part of the characterization of stains isolated from foods.

Traditional culture serves as the basis for the exploration of additional typing methodologies, a necessity in the conduct of epidemiological investigations. Recently, non-culture methodologies have emerged as pivotal in the detection and quantification of anaerobic faecal bacteria (**Table 12**). Polymerase chain reaction (PCR) ribotyping has achieved widespread recognition in Europe as the predominant typing technique, characterized by the adoption of a uniform nomenclature across most nations, albeit with the emergence of some localized systems. Capillary gel-based electrophoresis PCR ribotyping has undergone standardization and validation through a collaborative initiative involving the European Centre for Disease Control and Prevention, the US CDC, and the Public Health Agency of Canada (Fawley *et al.*, 2015).

While PCR ribotyping remains the conventional typing method for *C. difficile*, the adoption of novel molecular methodologies leveraging WGS data warrants further exploration, akin to endeavours undertaken for various bacterial taxa. Preliminary endeavours employing a core genome multilocus sequence typing (cgMLST) framework show promise and are anticipated to enhance the standardization of *C. difficile* typing methodologies in the foreseeable future (Bletz *et al.,* 2018; ECDC, 2018).

TABLE 12. *CLOSTRIDIOIDES DIFFICILE* DETECTION METHODS

Method/test	Description
Cell cytotoxicity assay	Historically considered the gold standard. Not used routinely.
Enzyme immunoassay	Detect free toxin; usually both toxins are included TcdA and TcdB; considerable variability in sensitivity among products from different manufacturers.
Glutamate dehydrogenase	Glutamate dehydrogenase assays exhibit high sensitivity, yielding rapid negative results with minimal manual intervention. Because of low specificity, presence of toxigenic *C. difficile* must be confirmed with additional test.
Nucleic acid amplification methods	Feature rapid detection, exceptional specificity, and high sensitivity. Targeted at toxin genes *tcdA* and *tcdB*. Commercial tests detect all strains with variant toxin genes, in-house methods can miss some *C. difficile* ribotypes with variant toxin genes.
Toxigenic culture	While toxigenic culture can be time consuming, it offers high sensitivity, and strains are available for further characterizations.

Sources: Carroll, 2020; Guerry, 2019; Scottish Microbiology & Virology Network, 2016; Rui *et al.*, 2024; Sandlund *et al.*, 2020

6.6 CONCLUSIONS ON TESTING OF FOOD PRODUCTS

Due to low levels and sporadic nature of the pathogens, routine testing of food products for *C. botulinum*, *C. perfringens* or *C. difficile* is not considered cost effective and is therefore not recommended (EFSA, 2005). Special laboratory techniques can effectively identify spores and viable cells of clostridia in food matrices, thereby facilitating research endeavours. However, these methods do not provide a feasible basis for implementing surveillance systems at the food business operator level.

The resources required to implement robust monitoring programmes and laboratory facilities for clostridial analyses, especially if MBA and high-level containment facilities are needed, will likely restrict implementation to competent authorities in individual countries, or potentially on a regional basis. Additionally, monitoring efforts may generate critical data on causative foods in foodborne illness cases, informing decisions that contribute to a reduction in foodborne diseases.

6.7 ADDITIONAL INFORMATION REGARDING DETECTION

The principal entities engaged in various aspects of method recognition, surveillance and/or validation include ISO, AOAC, FDA/USDA, Health Canada, AFFNOR, EFSA, and FSA/ACMSF.

Enrichment and cultivation requirements: While sample enrichment methods such as heat or alcohol treatment can enhance spore detection, the microbiological cultivation of clostridia requires specialized laboratory equipment, personnel training, and infrastructure, including BSL-2 and BSL-3 containment facilities to manage the associated risks effectively. However, pretreatment of samples may eliminate vegetative clostridia that may be of concern or interest in an inoculated pack/challenge study, research, or clinical diagnosis.

Interference with immunological tests: The presence of various food components can significantly interfere with immunologically based assays, leading to a reduction in test sensitivity.

Need for rapid detection methods: The development of rapid, sensitive, and specific detection methods for pathogenic bacteria and their toxins is crucial, particularly for field applications. The physical and chemical complexities of food matrices and the typically low concentrations of pathogens and/or toxins in contaminated foods present ongoing challenges to rapid detection.

Emerging test methodologies: Advances in technology have led to new test methodologies that retain high sensitivity and potentially reduce reliance on experimental animals. Such developments merit broad support to enhance pathogen detection capabilities.

Metagenomic approaches: Metagenomic techniques have been introduced to detect pathogenic clostridia in food and samples with complex microbial communities.

Requirements for genomic surveillance: Effective genomic-based surveillance requires next-generation sequencing technologies, high throughput computing systems, bioinformatics pipelines, computational expertise, and robust data management practices to process and analyse large datasets efficiently.

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CHAPTER 7

FUTURE CONSIDERATIONS

7.1 STRAINS PRODUCING BOTULINUM TOXINS AND THEIR TAXONOMIES

Botulinum neurotoxin production is not exclusive to *C. botulinum*. Notably, *C. sporogenes* has occasionally been implicated in cases of foodborne botulism. A comprehensive genetic study on the diversity and dissemination of *C. botulinum* Group I and *C. sporogenes* strains, alongside their neurotoxin genes, has identified two major lineages: C. *botulinum* Group I (predominantly harbouring neurotoxin genes of types A, B, and/or F) and *C. sporogenes* (with some strains containing a type B neurotoxin gene). Both lineages encompass strains that are responsible for foodborne, infant, and wound botulism, and include both toxigenic and non-toxigenic variants (Brunt *et al.,* 2020a).

Additionally, certain strains of *C. baratii* and C. *butyricum* have demonstrated the ability to produce botulinum neurotoxins. Specifically, *C. butyricum* strains that produce neurotoxin type E and *C. baratii* strains that produce neurotoxin type F have occasionally been linked to foodborne botulism cases (Anniballi et al., 2002; Mazuet *et al.,* 2016). Recently, advances have been made identifying novel botulinum and botulinum-like toxins using data mining of publicly available metagenomic data (Doxey *et al.,* 2018). Zhang *et al.* (2017) reported a novel BoNT type in *C. botulinum* strain 111, named BoNT/X, which can cleave the SNARE protein VAMP *in vitro*. However, the strain showed very limited toxicity in the mouse model (Gregg *et al.,* 2024). Other newly identified botulinum neurotoxin-like toxins were also identified in species outside the genus *Clostridium* like *Weissella oryzae* (BoNT/Wo) (Zornetta *et al.,* 2016), *Enterococcus faecium* (BoNT/En) (Zhang *et al.,* 2018), *Paraclostridium bifermentans* (PMP1) (Contreras *et al.,* 2019) and *Paeniclostridium ghonii* (PGT1 & 2) (Wei *et al.,* 2022). Whilst the *in vivo* activity and target species of most of the novel toxins remain to be characterized, it has been shown that PMP1 targets insects (Contreras *et al.,* 2019). With increasing availability of WGS data it is likely that more potential botulinum neurotoxin-encoding genes will be identified in the future.

This review has predominantly focused on *C. botulinum*. Consequently, future reviews should incorporate an assessment of non-*C. botulinum* strains as potential botulinum neurotoxin producers.

7.2 GENOMICS AND RISK ASSESSMENTS

Bacterial genomics, through the identification of distinct lineages and clusters as well as the characterization of strains within these groupings, play a crucial role in enhancing risk assessments and preventing clostridial contamination in foods. Although this review does not delve deeply into this topic, the increasing accessibility of WGS data will facilitate further comparative genomic studies. Such studies and reviews should be incorporated to comprehensively assess population structure, genetic diversity, whole genome lineages, variants, epidemiology, and geographical distribution (Peck and van Vliet, 2016).

Further, epidemiological tools based on molecular biological methods like real-time PCR, rely on increasing availability of WGS data to enhance their utility. Expanding the knowledge of the diversity of neurotoxin-coding genes and toxin subtypes in *C. botulinum* has led to the recent development of new primer sets detecting all to-date known human relevant BoNTs (Pillai *et al.*, 2024).

7.3 OTHER CONSIDERATIONS

7.3.1 CLIMATE CHANGE

Climate change is a major challenge, especially in the agricultural sector that is already impacted by drought, flooding and temperature change which impacts production, quality and yields. Climate change will also have consequences for food safety, potentially affecting the occurrence and intensity of some foodborne diseases (FAO, 2020). For example, higher temperature can result in heat stress in animals that may result in greater excretion of zoonotic pathogens and affect the persistence of foodborne pathogens in the environment. A rise in air temperature might also result in new issues such as consistently achieving appropriate storage temperatures.

Avian botulism in wildlife is a seasonal disease, currently reported during summer when the temperature is warm. A rise in the number of outbreaks is anticipated in the future due to the increase of temperatures throughout the year (Espelund *et al.,* 2014; Gutiérrez-Arnal and Marin, 2024). Such outbreaks can have a huge impact on bird populations, with reported outbreaks exceeding several tens of thousands of dead birds, impacting endangered species, biodiversity, and ecosystem balances. Importantly, because BoNT-E is associated with avian botulism, any potential increased risk to humans from consuming products (e.g. fish, waterfowl) harvested in areas experiencing active outbreaks of avian botulism or in regions with recurrent history of botulism outbreaks should be carefully evaluated.

New agricultural practices need to be developed to cope with the challenges of climate change, but their impact on food safety should be considered, and risks need to be evaluated to avoid new sources of contamination of the food chain. For example, European regulations were changed in 2023 to allow the reuse of wastewater for crop irrigation, even for production of vegetables, to reduce the competition for water use. However, the impact of water reuse on dissemination of clostridia in the environment and how this may impact food safety has not been evaluated.

7.3.2 MANURE AND BIOSOLIDS TRANSFORMATION AND USE ON FARMS

Animals can be asymptomatic carriers of clostridia (Dalhenborg *et al.,* 2001, 2003; Rodriguez-Diaz *et al.,* 2024; Camargo *et al.*, 2024) which results in the deposition of contaminated manure (Pourcher *et al.,* 2023). Manure may be either directly spread on fields or transformed through anaerobic digestion or composting, which are then spread on soils as a fertilizer and amendment. Biosolids from wastewater treatment plants, which are known to be contaminated by clostridia (Chisholm *et al.*, 2023; Flemming *et al.,* 2017), are also commonly spread on agricultural soils either directly or after anaerobic digestion or composting. Use of anaerobic digestion of biological material has grown considerably over the last decade, especially on farms in Europe. Published studies show that wet anaerobic digestion, either mesophilic or thermophilic, does not achieve a significant reduction in clostridial populations (Pourcher *et al.,* 2023; Subirats *et al.,* 2022), specifically those of *C. difficile* (Fröschle *et al.,* 2015; Le Maréchal *et al.,* 2019; Le Maréchal *et al.,* 2020; Pourcher *et al.,* 2023; Derongs *et al.,* 2021). Dry anaerobic digestion has a higher impact on spore-forming bacteria, achieving a $\log_{10}$ reduction ranging from 1.8 to 3.1 (Subirats *et al.,* 2022). Unfortunately, dry anaerobic digestion can only be applied to organic materials with high solid content, which is not always the case at the farm level, especially with slurry. Aerobic composting, when properly undertaken, seems to be more effective at reducing clostridial populations achieving 2.8 to 3.4 $\log_{10}$ after reaching thermophilic temperatures of > 70 °C for 27 days (Subirats *et al.,* 2022) and could be combined with anaerobic digestion to handle the solid phase of digestates.

Spread of animal manure disseminates spores (Frentrup *et al.,* 2021) and enables persistent soil contamination (Xu *et al.*, 2016). Agricultural soils are one of the primary reservoirs of

human pathogens, which is amplified when biosolids and manure are spread for soil amendments or stored on soils (Iwu and Okoh, 2019). This practice plays an important role in the perpetuation of the contamination of the food chain either directly through contamination of fresh products or indirectly through contamination of crops for animal feed.

7.3.3 ROLE OF INSECTS

Maggots, known to be involved in the "carcass-maggot" cycle in avian botulism outbreaks in wildlife, are not sensitive to BoNT and concentrate both BoNT and clostridia. Flies can also carry *C. botulinum* (Anza *et al.,* 2014) and *C. difficile* (Davies *et al.,* 2016) and be mechanical vectors of these spores. A recent study showed that genetic signatures for BoNT-producing clostridia can be detected in the metagenomes of microbiomes of major insect orders, including Diptera, Coleoptera, Lepidoptera, Orthoptera, Hymenoptera, Hemiptera and Blattodea (Douillard *et al.,* 2025; Davies *et al.,* 2016).

The increasing introduction of insects as food into the human diet as alternative protein sources is expected to have significant ecological and economic benefits as compared to animal-derived proteins and could improve food security (Colamatteo *et al.,* 2024). Traditionally consumed in many parts of the world, a diversity of commercial insect-based food items is now available on the markets for example in Europe and the United States (Colamatteo *et al.*, 2024). Nevertheless, potential risks associated with these foods have not been fully evaluated. Interestingly, metagenomic analysis of edible insects intended for food production frequently showed the presence of clostridial species (Vandeweyer *et al.,* 2017; Garofalo *et al.,* 2017). Similar to the mechanism of transmission with avian botulism, insect protein-based animal feed might pose a hazard for entry of pathogenic clostridia into the human food chain (Grenda *et al.,* 2021).

Based on this context and on the limited data currently available on this topic, specific attention should be paid to clostridia in insects and clostridial contamination in products manufactured in this sector to prevent or manage a potential new source of clostridial contamination in the food chain.

7.3.4 ANTIMICROBIAL (ANTIBIOTIC) RESISTANCE

Clostridia exhibit resistance to various antibiotics. In 2013, the CDC identified *C. difficile* as an antibiotic resistance threat (US CDC, 2019). Antibiotic resistance plays a critical role in promoting *C. difficile* infection (CDI) during treatment for unrelated infections. By disrupting the normal intestinal microbiota, antibiotic use creates favourable conditions for *C. difficile* colonization.

While clostridia are not often considered as priority pathogens for which strategies to control and prevent antibiotic resistance need to be developed, they should be considered as a reservoir of antibiotic resistance genes. For instance, it has been shown *in vitro* that *C. difficile* is able to transfer *erm*B to *S. aureus*, *B. subtilis* (Hachler *et al.,* 1987; Mullany *et al.,* 1995, Farrow *et al.,* 2001), *E. faecalis* (Wasels *et al.,* 2014) or other *C. difficile* strains (Goh *et al.,* 2013; Wasels *et al.,* 2015, Imwattana *et al.,* 2020) and *tetM* to *B. subtilis* and *E. faecalis* (Mullany *et al.,* 1990; Jasni *et al.,* 2010).

Regarding intestinal forms of botulism (toxico-infections), the use of antibiotics is highly controversial as it may lead to increased release of neurotoxin when the bacterial cells are lysed and therefore needs to be accompanied by specific treatment with antitoxin. A United States study to identify antibiotic resistance in 260 infant botulism isolates reported an overall low level of antibiotic resistance to 12 clinically relevant antimicrobial agents (Barash *et al.,* 2018). Nevertheless, antibiotic resistance to both penicillin and metronidazolehas been reported in a strain implicated in infant botulism in France (Mazuet *et al.,* 2016).

7.3.5 ATYPICAL FORMS OF DISEASE

The diagnosis of clinical botulism relies, in addition to the clinical presentation, on the detection of botulinum neurotoxin from human specimens (blood and faeces) or implicated food items. As discussed above, detection of botulinum neurotoxin is challenging, and detection thresholds are dependent on detection tools, sampling methods, and timing. Thus, potential forms of disease caused by low concentrations of this potent toxin may not be identified.

Recent research using murine models has indicated that low, non-paralytic concentrations of botulinum neurotoxin might act locally on the enteric nervous system and be of medical significance. It has been reported that low BoNT/A and B doses administered orally to mice acted on cholinergic intestinal neurons, potentially altering intestinal function, motility and defence mechanisms, and facilitated invasion by the intestinal pathogens *Salmonella typhimurium* and *Shigella flexneri* (Fabris *et al.,* 2024). The fact that cases of infant botulism frequently show *C. difficile* co-infections (Schechter *et al.,*1999; Fenicia *et al.,* 2002, Douillard *et al.,* 2024) further supports the suggested botulinum neurotoxin impact on intestinal immunity. Therefore, to date unknown local forms of intestinal botulism may exist in humans and present with atypical intestinal symptoms and neurotoxin levels in the blood which are below current detection levels. The development of more sensitive analytical methods will enhance the awareness, understanding, and treatment of previously unknown disease manifestations.

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CHAPTER 8
DISCUSSION AND CONCLUSIONS

8.1 *CLOSTRIDIUM BOTULINUM*

Clostridium botulinum Groups I and II predominantly cause foodborne botulism, a severe form of intoxication resulting from the ingestion of botulinum neurotoxin preformed in contaminated food. A wide range of food products have been associated with outbreaks of botulism. Foodborne botulism results from the ingestion of preformed toxin in foods that are improperly preserved and/or time-temperature abused and in which clostridia have grown. Although uncommon, foodborne botulism has a rapid onset, is very severe and potentially fatal, requires prompt treatment and frequently results in prolonged hospital stays with a high economic burden.

The clinical impact of severe botulism is profound, reflected in its high disability and an estimated economic burden of USD 1.68 million per case. The disease carries a fatality rate of 5–10 percent.

The "botulinum cook" process (121 °C for 3 minutes) specifically targets *C. botulinum* Group I spores, ensuring the safety of low acid canned foods. The inactivation of *C. botulinum* Group II spores for refrigerated foods is typically achieved by heating at 90 °C for 10 minutes. Failures in the application of these processes, as well as temperature abuse of low-acid chilled products, have been linked to outbreaks of foodborne botulism.

There has been a sustained effort to develop advanced preservative technologies to enhance food safety. However, there remains a lack of universal consensus on the categorization of foods and food processes regarding the control of C. *botulinum*. Although Group II *C. botulinum* grows at refrigeration temperatures, most foodborne botulism cases have been associated with foods stored at 10 °C to 40 °C.

Infant and adult intestinal botulism are severe illnesses resulting from colonization of the intestine and toxin production following ingestion of the spores of BoNT-producing clostridia. Intestinal infection likely requires predisposing factors like an immature or disrupted intestinal microbiota. Confirmed cases of intestinal botulism are rare, but the diseases are likely underdiagnosed and underreported. Confirmed cases of infant botulism are increasing in number in regions of the world where there is a high level of awareness and well-established diagnostic tools are used. The source of spores is unknown in most cases of adult intestinal and infant botulism. However, when identified, the predominant sources in infant botulism have been honey and the environment (e.g. from dust and soil). Education and raising awareness among parents, caregivers, risk groups and the medical community is crucial for reducing the risk of infant and adult intestinal botulism.

The *in vivo* MBA remains the gold standard for BoNT detection in clinical and epidemiological contexts. However, this assay requires costly specialized animal facilities, has a turnaround time of approximately 4–5 days, and presents challenges in interpreting negative results. These, as well as animal welfare and logistical concerns, necessitate the development of alternative detection methods. Molecular analytical tools are available but may have limited application for clinical or food testing, and their sensitivity may vary. Development and validation of new tests is ongoing.

8.2 CLOSTRIDIUM PERFRINGENS

Clostridium perfringens illness is a toxico-infection caused by consuming foods where vegetative cells of *C. perfringens* multiply to high levels (exceeding 8-$\log_{10}$ per serving) due

to temperature abuse. Upon ingestion, toxin is released in the gut as the vegetative cells undergo sporulation.

Clostridium perfringens is classified into seven distinct types (A–G), with Type F strains being the primary gastrointestinal pathogens affecting humans. It is among the most common causes of foodborne illness, often responsible for large outbreaks linked to mass catering. Globally, foodborne outbreaks of *C. perfringens* are most frequently associated with meat and poultry dishes, as well as gravies and other thickened foods that facilitate bacterial growth when subjected to improper temperature control.

Although *C. perfringens* spores are commonly detected at low concentrations (10^2 spores per gram) in various food products – including beef, poultry, and thickened dishes – these spores can proliferate rapidly if food is cooled slowly or held at temperatures between 15–50 °C for extended periods. As a result, such conditions are a major contributor to foodborne illness.

In the United States, *C. perfringens* ranks among the leading causes of foodborne illness, with approximately one million cases reported annually. The associated public health burden has an estimated economic impact of USD 400 million per year. While the illness is typically self-limiting, with symptoms resolving within 24 hours, fatalities can occur particularly among elderly or immunocompromised individuals. The incidence of *C. perfringens*-associated illnesses in other countries is less documented.

Effective prevention of foodborne diseases caused by *C. perfringens* involves critical control measures: thorough cooking, proper hot holding, rapid chilling, and addition of food-grade growth inhibitors such as nitrite or organic acid salts.

8.3 CLOSTRIDIOIDES DIFFICILE

Clostridioides difficile is a leading cause of antibiotic-associated diarrhoea. Whole genome sequencing data reveals that a decreasing proportion of *C. difficile* infections are attributable to healthcare-associated transmission, with most cases now deemed to be community-acquired. The exact source of community-acquired CDI remains elusive, although there is speculation regarding food as a source of exposure.

Clostridioides difficile has been isolated from a diverse array of retail food products and agricultural commodities. Some foods and soil can introduce spores into the human domestic environment and provide a route for ingestion.

There is no doubt that *C. difficile* can cause human illness and is present in various foods, making it a potential foodborne hazard. However, current evidence does not confirm that *C. difficile* poses a significant foodborne risk, as no documented cases of illness have been directly linked to its transmission through food. In the United States, the financial burden of *C. difficile* infections surpasses USD 5.9 billion annually, with over 29 000 associated fatalities each year.

Spores of *C. difficile* are likely to withstand common food-cooking processes, which typically involve heating to an internal temperature of 70 °C for up to 2 minutes or up to 74 °C. Specific strategies to eliminate its presence in foods other than retort thermal processing (canning) remain undetermined. Although research on controlling *C. difficile* in food is limited, other clostridia tend to exhibit faster growth rates due to their optimal growth temperatures. Consequently, strategies used to control these pathogens are likely to also inhibit the proliferation of *C. difficile*.

CHAPTER 9
NEEDS ASSESSMENT AND KNOWLEDGE GAPS

- *Clostridium*-mediated illnesses are likely underreported. Improved worldwide surveillance of *Clostridium*-mediated illness is needed to determine the true global burden.
- Improved investigation of foodborne illness and outbreaks will enhance the understanding of contributing causes and inform the development of intervention strategies to prevent further illness.
- An improved understanding of the role and extent to which antibiotic use in livestock production contributes to the selection and amplification of *C. difficile* in food animals, and subsequent contamination of foods with *C. difficile,* would provide the scientific basis for the development of on-farm control measures for this pathogen.
- An investigation of the impact of scalding pig and poultry carcasses on the level of clostridial spore contamination on the carcasses would inform better control measures.
- Simple, sensitive, specific, standardized, animal-free, rapid botulinum toxin detection assays that recognize all serotypes and subtypes and are field deployable are needed.
- Intestinal botulism (infant and adult) may present atypical symptoms and at neurotoxin levels below current detection levels. The development of more sensitive analytical methods will enhance the awareness, understanding, and treatment of previously unknown disease manifestations.
- The use of probiotics may prove helpful to minimize the duration or severity of infant or adult intestinal botulism.
- Development and validation of hurdle approaches for novel foods and changed product formulations can contribute to reduce microbial proliferation and enhance food safety.

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ANNEX
REVIEW OF THE CODEX DOCUMENTS

There are several Codex documents related to clostridia. The experts reviewed and provided comments based on the most recent science.

- CXC23-1979(1993) Code of Hygienic Practice for Low and Acidified Low Acid Canned Foods (mentioned **botulinum)**
- CXC39-1993 Code of Hygienic Practice for Precooked and Cooked Foods in Mass Catering (mentioned **botulinum, perfringens)**
- CXC40-1993 Code of Hygienic Practice for Aseptically Processed and Packaged Low-Acid Foods **(**mentioned **botulinum)**
- CXC46-1999 Code of Hygienic Practice for Refrigerated Packaged Foods with Extended Shelf Life **(**mentioned **botulinum)**
- CXC53-2003(2017) Code of Hygienic Practice for Fresh Fruits and Vegetables **(**mentioned **botulinum)**
- CXC57-2004 Code of Hygienic Practice for Milk and Milk Products (mentioned **botulinum)**
- CXC 58-2005 Code of Hygienic Practice for Meat
- CXC66-2008 Code of Hygienic Practice for Powdered Formulae for Infants and Young Children (mentioned **botulinum, difficile, perfringens)**
- CXC75-2015(2018) Code of Hygienic Practice for Low-Moisture Foods (mentioned **botulinum, perfringens)**

The **bold** words are from the Codex document; the texts without bold are comments and recommendations after the experts' review.

A1. CXC23-1979(1993) CODE OF HYGIENIC PRACTICE FOR LOW AND ACIDIFIED LOW ACID CANNED FOODS

- **Flora** needs to be changed to microbiota.
- **7.5.2.1 Product composition or formulation.** Does this include size of inclusions? Dimension of pieces?
- **7.5.2.2 The second step is to determine the scheduled process taking into account the sterilizing facilities available and the desired product quality by carrying out heat penetration tests. The heat penetration into the product must be determined under the most adverse conditions that are likely to be met in production. For this purpose the temperature in the slowest heating point in the container contents should be monitored during a heat process. It is essential to carry out an adequate number of heat penetration (**How many are adequate? Is this defined somewhere?**) tests to determine the variations, which should be taken into account in the scheduled process. The scheduled process can be determined from the time temperature graph obtained.**
- **7.5.3.3 Heat processing should be commenced as soon as possible after closing to avoid microbial growth or changes in heat transfer characteristics of the products. If during breakdowns the production rate is low, the product should be processed in partly filled retorts. Where necessary, a separate scheduled process should be established for partly filled retorts.** During a breakdown – and if product cannot be retorted – should there be storage recommendations?
- **7.5.3.2 Acidified, fermented and pickled foods shall be so manufactured, processed and packaged that an equilibrium pH value of 4.6 or lower is achieved within the time (**For fermented foods this should be within 24 hours.**) designated in the scheduled process and maintained.**
- The flow chart at the end of the document suggests conducting the mouse bioassay. That should be changed perhaps to contact an appropriate testing facility for confirmation of the presence of botulinum toxin

A2. CXC39-1993 CODE OF HYGIENIC PRACTICE FOR PRECOOKED AND COOKED FOODS IN MASS CATERING

- The **definition** of mass catering does not specifically cover food service/restaurants or food trucks. Is this covered elsewhere, or can this be added to the definition?
- **2.1 Catering definition** does not clearly state restaurants. Restaurants could potentially consider themselves exempt from this definition. This should be clarified.
- **4.3.7. Windows** – Food trucks often do not have screened windows.
- **4.3.16 Hand washing facilities in processing areas** – Add notices to wash hands after using the toilet, handling raw foods or dropped foods, or coughing/sneezing that may have contaminated hands.
- **4.3.19.1** – Change term "plant" to premises.

- **4.4.1 Materials.** *CCP Note: Equipment and utensils constitute a source of potential cross-contamination. In addition to regular routine cleaning, it is essential that all equipment and utensils used for raw foods be thoroughly (insert "cleaned and") disinfected before they are used for cooked and precooked foods. If at all possible, separate utensils should be used for raw and cooked products. If this is not possible, thorough cleaning and disinfection is necessary.*

- **7.2.1 Effective measures should be taken to prevent contamination of cooked and precooked foods by direct or indirect contact with material at an earlier stage of the process. Raw food (**Should this be raw animal products?**) should be effectively separated from cooked and precooked foods.** Clean RTE vegetables are raw; how should this be handled?

- **7.4.2 b) running potable water maintained at a temperature not above 21 °C for a period not exceeding 4 hours.** Do we need to add directions about handling to not allow cross-contamination if using running water thawing method?

- **7.5.1** *Note: Boned rolled joints of meat are convenient for cooking, but the operation of removing the bone and rolling the meat will transfer microbes from the surface to the centre, where they are better protected from the heat of cooking. For the safe production of rare-cooked beef, the centre of joints must reach a minimum of 63* °C *(*for how long? 2 hours?*) in order to eliminate contaminating* **salmonellae.** *The proper use of other time/temperature combinations which would ensure safety is acceptable.*

- **7.5.1 Stuffed birds cool very slowly, and *C. perfringens* will germinate and multiply during this time. The effectiveness of the cooking process should be checked regularly by measuring the temperature in the relevant parts of the foods.** Cooling is applicable to more than just poultry. How should other large diameter meats be included?

- **7.5.2 When grilled, roasted, braised, fried, blanched, poached, boiled, or cooked products are not intended for consumption on the day they are prepared** (within two hours of preparation)**, the cooking process should be followed by cooling as quickly as possible.** This does not mention hot-holding.

- **7.7.2** *Note: Epidemiological information indicates that the most important factors contributing to the occurrence of foodborne disease outbreaks are related to operations that follow cooking; for instance, if cooling is far too slow,* **(**for instance, if cooling is far too slow and continuous cooling to get 10 °C as quick as possible.) *so that any part of the food stays for a dangerously long time in the temperature range between 60 °C and 10 °C where harmful microorganisms may grow; therefore, the product should not be maintained in this temperature range for more than 4 hours* (Do we need to be more prescriptive, such as cooling to 27 °C in one hour and to 10°C in the additional 3 hours?). *Hazard analysis must assess conditions of chilling.*

- **7.11.3 Where appropriate for safety a sample of at least 150 g of each item of food taken from each lot should be kept in a sterile contained at 4 °C or below until at least three days after that whole lot has been consumed.**

Some organisms do not tolerate freezing and thus refrigeration of samples is recommended in lieu of freezing. The sample should be obtained from the lot at the end of the portioning period. These samples should be available for investigation in the event of any suspected foodborne disease. How would an operator know which organisms are freeze sensitive? High salt foods do not freeze at 0 °C.

A3. CXC40-1993 CODE OF HYGIENIC PRACTICE FOR ASEPTICALLY PROCESSED AND PACKAGED LOW-ACID FOODS

No comment.

A4. CXC46-1999 CODE OF HYGIENIC PRACTICE FOR REFRIGERATED PACKAGED FOODS WITH EXTENDED SHELF LIFE

- **4.4.5.2 Cooling facilities – Refrigeration without delay** (refrigeration rapid and continuous) **after the heat treatment, as soon as the internal temperature reaches 60 °C and an even temperature distribution in the batch when it is cooled.**
- **5.1.2.3 Development of cooling method – Products should be cooled so that their temperature remains for a minimum of time between 60 °C and 10 °C, the temperature range most favourable for microbiological proliferation. When feasible, it is recommended to bring the temperature at the centre of the product to under 10 °C in two hours or less.** Use this to replace the Mass Catering document or the others. Add rapid and continuous.
- **5.2.1 Time and temperature control – In all steps of processing, critical temperatures (**core temperature**) for multiplication of microorganisms (10 °C to 60 °C) should be avoided or in any case passed through rapidly (**rapidly and continuously**).**
- **APPENDIX – HURDLES, b) pH - To illustrate these concepts, if a refrigerated food is to be packed in a reduced oxygen atmosphere and has a shelf-life longer than 10 days, it is necessary to assess the potential risk from psychrotrophic strains of *C. botulinum* and, if necessary, to control these strains through the appropriate use of hurdles in combination with a heat process, as long as the heat process is not equivalent to 90 °C for 10 minutes. Examples of hurdles are (**Examples of hurdles to be achieved throughout the product and each component of the product:**):**
- **add sodium chloride to 5 percent in brine;** (add sodium chloride in product to achieve the concentration no less than 3.5 percent)

A5. CXC53-2003(2017) CODE OF HYGIENIC PRACTICE FOR FRESH FRUITS AND VEGETABLES

- **INTRODUCTION. The microbiological pathogens associated with fresh fruits and vegetables include *Salmonella* spp., *Shigella* spp., *Campylobacter*, pathogenic strains of *Escherichia coli*, *Listeria monocytogenes*, *Yersinia pseudotuberculosis*, norovirus, hepatitis A virus and parasites such as *Cyclospora cayetanensis, Giardia lamblia* and *Cryptosporidium parvum*.** Add "There is emerging evidence that *C. perfringens* and *C. difficile* and their spores are found on some vegetables and potentially associated with foodborne illness."
- **3.2.1.2 *Manure, biosolids and other natural fertilizers*** – *Manure, biosolids, digestates and other natural fertilizers*
- **The use of manure, biosolids**, *digestates* **and other natural fertilizers in the production of fresh fruits and vegetables should be managed to limit the potential for microbial, chemical and physical contamination.** Add "Management should consider pathogenic and spoilage spore-formers will not be activated by composting."
- **Composting, if done properly, can be a practical and efficient method to inactivate foodborne pathogens in manure (**manure but not spores**). In general, only fully composted animal waste or plant material should be applied to production fields.**
- **ANNEX I, INTRODUCTION. Some of the microbiological pathogens associated with fresh fruits and vegetables include *Salmonella* spp., *Shigella* spp., pathogenic strains of *Escherichia coli*, *Listeria monocytogenes*, norovirus and hepatitis A virus and parasites such as *Cyclospora cayetanensis.*** *C. perfringens and C. difficile and their spores may also be included.*
- **ANNEX II, INTRODUCTION. Microbial pathogens associated with sprouted seeds include *Salmonella* spp, pathogenic *Escherichia coli*, *Listeria monocytogenes* and *Shigella* spp** and *C. perfringens*.
- **5.2.3 *Microbiological and other specifications.* It is recommended that seed and sprouts or spent irrigation water be tested for the presence of pathogens (**aerobic and anaerobic pathogens**).**
- **ANNEX III, INTRODUCTION.** *C. perfringens* and *C. difficile* and their spores may also be included *C. perfringens* and *C. difficile* and their spores.

A6. CXC57-2004 CODE OF HYGIENIC PRACTICE FOR MILK AND MILK PRODUCTS

- OIE should be replaced with WOAH.

A7. CXC 58-2005 CODE OF HYGIENIC PRACTICE FOR MEAT

- **INTRODUCTION. 1. Meat has traditionally been viewed as a vehicle for a significant proportion of human foodborne disease. Although the spectrum of meat-borne diseases of public health importance has changed with changing production and processing systems, continuation of the problem has been well illustrated in recent years by human surveillance studies of specific meat-borne pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter* spp. and *Yersinia enterocolitica*.** Add "and the emergence of food associated *C. difficile"*

- **INTRODUCTION. 5. The principles of food safety risk management should be incorporated wherever appropriate in the design and implementation of meat hygiene programmes. Specifically, work conducted by JEMRA, JECFA and FAO/WHO Expert Consultations and resulting risk management recommendations should be considered. Further, newly-recognized meat-borne risks to human health may require measures additional to those usually applied in meat hygiene, e.g. the potential for zoonotic transmission of central nervous system disorders of slaughtered livestock means that additional animal health surveillance programmes may need to be undertaken.** Add "and where necessary consideration be given to measures for specific control of clostridial spores."

- **14. Primary production is a significant source of hazards associated with meat. A number of hazards are present in animal populations intended for slaughter, and their control during primary production often presents considerable challenges, e.g. *E. coli* O157:H7, *Salmonella* spp. *Campylobacter* spp. and** (emerging clostridia)**, and various chemical and physical hazards.**

- **40. Facilities are operated in a way that soiling and cross-contamination of animals with foodborne pathogens** (add [e.g. *C. difficile*]) **are minimized to the greatest extent practicable;**

- **68. effective cleaning, sanitation and maintenance can be carried out during and between periods of operation** (Add "including the reduction or elimination of clostridial spores".)

- **73. All equipment used in areas where bodies of animals are dressed or meat may be present should facilitate good hygienic practices (GHP).** The clostridial spore concentrations in scald tank water in the broiler and pig processing plants should be monitored and measures taken to prevent the accumulation of spores to ensure post-scalded carcasses do not have a higher prevalence of spores. *Note:* not suggesting the carcasses will not be cross contaminated but rather that the spore concentration will be lower post scalding. Risk assessment is required to set a limit for spores on carcases post scalding as a general recommendation in the whole report.

- **77. Airborne contamination (**add "including clostridial spores"**) from aerosols and condensation droplets is minimized.**

- **79. Sanitizing solution (**add "including where necessary sporicidal agents") **is used according to manufacturers' specifications supplied as and where necessary**

- **95.**
  Microbiological verification of SSOPs can utilize a range of direct or indirect methods (Add "to evaluate the effectiveness of the Sanitation Standard Operating Procedures (SSOPs) for controlling foodborne pathogens including spore formers."). **Establishment operators should use statistical process control or other methods to monitor sanitation trends.**

- **101. When developing HACCP plans for heat-treated meat preparations and manufactured meat, the establishment operator should fully document, as appropriate to the process, all thermal process parameters, post-heat treatment handling, and additional preservation treatments appropriate to the intended process outcome (e.g. a pasteurized product). Process parameters for cooling of heat-treated products may incorporate as appropriate to the product, rapid cooling, slow cooling, or interrupted cooling. Previously heated products should not be packaged above a minimum temperature (e.g. 4 °C), unless it can be demonstrated that cooling after packaging does not compromise product safety** (Replace with "unless it can be demonstrated that cooling after packaging does not compromise product safety for all relevant pathogens.").

- **174. Appropriate product information and adequate knowledge of food hygiene is necessary to prevent mishandling at later stages in the food chain. Pre-packaged foods should be labelled with clear instructions to enable the next person in the food chain to handle, display, store and use the product safely. Principles and guidelines for product information and consumer awareness in the context of safety and suitability of meat are described in general terms in Section IX of the General Principles of Food Hygiene (CXC 1-1969).** These need to be checked to make sure they include, where relevant, instructions about correct chilled storage, and so on (mentioned below in 176).

A8. CXC66-2008 CODE OF HYGIENIC PRACTICE FOR POWDERED FORMULAE FOR INFANTS AND YOUNG CHILDREN

- **SECTION IV – ESTABLISHMENT: DESIGN AND FACILITIES. Facilities and equipment should be designed, constructed and laid out to prevent entry of *Salmonella* and *E. sakazakii* (*Cronobacter* species) into high hygiene areas and to minimize their establishment or growth in harbourage sites** (replace site with "sites and build-up of bacteria spores.").

A9. CXC75-2015(2018) CODE OF HYGIENIC PRACTICE FOR LOW-MOISTURE FOODS

- No comment.

FOOD SAFETY AND QUALITY SERIES

1. FAO. 2016. Risk based imported food control, manual.

2. FAO and WHO. 2016. Risk communication applied to food safety, handbook.

3. FAO. 2016. Enhancing early warning capabilities and capacities for food safety.

4. FAO. 2017. Food safety risk management: evidence-informed policies and decisions, considering multiple factors.

5. FAO and WHO. 2018. Technical guidance for the development of the growing area aspects of bivalve mollusc sanitation programmes.

5a.FAO and WHO. 2021. Technical guidance for the development of the growing area aspects of bivalve mollusc sanitation programmes, second edition.

6. FAO. 2019. Technical guidance principles of risk-based meat inspection and their application.

7. FAO and WHO. 2019. Food control system assessment tool
   - Introduction and glossary
   - Dimension A – inputs and resources
   - Dimension B - control functions
   - Dimension C - interaction with stakeholders
   - Dimension D - science/knowledge base and continuous improvement

8. FAO.2020. Climate change: unpacking the burden on food safety.

9. FAO and WHO. 2020. Report of the expert meeting on ciguatera poisoning.

10. FAO. 2020. FAO guide to ranking food safety risks at the national level.

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TOXIGENIC CLOSTRIDIA IN FOODBORNE DISEASE

CHARACTERIZATION, EXPOSURE, BURDEN, CONTROL AND DETECTION

Clostridia include several toxigenic bacterial species recognized as significant human pathogens, notably *Clostridium botulinum, Clostridium perfringens*, and *Clostridioides difficile*. Clostridia are commonly identified in various food and many environmental niches. Unlike other common bacterial foodborne pathogens, such as *Salmonella*, *Campylobacter* and *Listeria*, clostridia produce dormant structures known as spores that enable survival under harsh conditions. These spores persist for a long time, perhaps decades, in the farm and processing environments and from there, they may contaminate foods. Furthermore, spores are resistant to common food safety control measures such as cooking, pasteurization, and certain sanitizers.

This document has been prepared to provide a comprehensive review of the global status of foodborne clostridia. The focus is specifically on *C. botulinum* and other botulinum neurotoxin (BoNT)-producing species, *C. perfringens* and *C. difficile*, with the aim of providing a detailed analysis of their prevalence, associated risks and implications for food safety and public health. This review encompasses an extensive examination of peer-reviewed scientific literature, epidemiological data, and recent reports. It also includes an assessment of the epidemiology of outbreaks, and current detection and control methods for pathogenic clostridia in food systems. The document is structured to provide a clear and detailed overview of the current state of knowledge, highlighting critical areas for future research and policy development to address the challenges posed by foodborne clostridia.

AGRIFOOD SYSTEMS AND FOOD SAFETY DIVISION – ECONOMIC AND SOCIAL DEVELOPMENT

WEBSITE: WWW.FAO.ORG/FOOD-SAFETY

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

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