Opinion of the Scientific Panel on Biological Hazards on the request from the Commission related to *Clostridium* spp in foodstuffs.[1]

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SUMMARY

Anaerobic spore-forming bacteria spoil a wide range of foods including dairy products, meat and poultry products, fresh and canned fruits and vegetables, typically producing gas and/or putrid odours. A few of those species can cause illness.

The species of the genus *Clostridium* most commonly involved in food-borne illness are *Clostridium perfringens* and *C. botulinum*. Intoxication due to *C. perfringens* is usually brief, self-limiting, and is rarely fatal. However, the neurotoxins of *C. botulinum* are among the most toxic naturally-occurring substances and cause severe food-borne illness, sometimes fatal, with symptoms continuing for several months.

Clostridia occur commonly in soil, dust, the aquatic environment and in the intestines of animals. Consequently, *C. perfringens* and *C. botulinum* can be present in a wide range of foods. Good Agricultural Practices and Good Hygienic Practices contribute to reducing numbers of clostridia by minimising contamination with soil and animal faeces.

C. perfringens is commonly present in foods and ingredients, occasionally at hundreds per gram. *C. botulinum* is present less frequently, normally at a few spores per kg. Spores of both *C. perfringens* and *C. botulinum* can be eliminated from foods by heating.

C. perfringens

Illness due to *C. perfringens* occurs after ingestion of large numbers of enterotoxin-producing vegetative cells after food has been temperature-abused, either during cooling after a heat process, or stored unrefrigerated, both of which allow spores to germinate and multiply. Illnesses due to *C. perfringens* are commonly associated with cooked meat, cooked uncured meat products, the associated gravy, casseroles, or pea soup produced on a large scale, at mass catering in food service establishments, care homes and similar establishments. Almost all outbreaks are the result of cooling slowly, or holding without refrigeration, allowing multiplication of *C. perfringens*, numbers reaching 10^6-10^7 /g, and implying an infective dose of the order 10^8 of vegetative cells of enterotoxin-producing *C. perfringens*. Some vegetative cells survive the acid conditions of the stomach and subsequently form spores in the large intestine, at the same time producing enterotoxin. Because the illness lasts only 12-24 h, and the symptoms are usually not serious enough to consult a physician, recorded cases and outbreaks are probably under-reported. Occasionally death occurs, usually in elderly patients. The enterotoxin of *C. perfringens* is heat labile, heating at 60°C for 5 min destroying the biological activity.

The most essential measures to prevent foodborne diseases caused by *C. perfringens* are: appropriate cooking, cooling rapidly through the temperature range 55°C to 15°C, holding foods at temperatures <10°-12°C, and re-heating the product to an internal temperature of 72°C before consumption.

Microbiological testing for *C. perfringens* has limited value in ensuring food safety, because the organism is so common in or on foods that a positive result means little, unless very high numbers are present. Moreover, cultural methods detect all *C. perfringens*, while enterotoxin is produced by only a fraction of strains.

C. botulinum

Botulism occurs after ingestion of a neurotoxin formed when spores of *C. botulinum* type A, B, E or F germinate and multiply in a food. It has even resulted after a person, suspecting a food might be spoiled, merely tasted the food after dipping one finger into it. Hence, any multiplication of *C. botulinum* in foods must be prevented. The toxins of *C. botulinum* are relatively sensitive to heat and are inactivated by heating at 80°C for 10 min or an equivalent process.

Consumption of a raw product that is contaminated with spores of *C. botulinum* does not cause botulism. Germination and multiplication of *C. botulinum*, accompanied by neurotoxin production, must occur before the food is consumed. Outbreaks occur after failures in formulation (recipe) or the process applied, or lack of temperature control in one of the many steps before consumption. Botulism from commercially prepared food is usually associated with a failure of the process, or of container/pack integrity. Historically a high percentage of botulism outbreaks have been associated with home-preserved foods. Those who practise home preservation should be advised of the risks from *C. botulinum*, should follow well-established practices and procedures, and should not deviate from those procedures e.g. pack size and heating process. Thorough reheating before consumption would inactivate preformed botulinal neurotoxin (BoNT).

Outbreaks have also been traced to small-scale manufacture of "home-made" style foods, where technological knowledge and support may be weak.

Botulism is also associated with traditional fermented fish products that are eaten without heating.

C. botulinum is able to multiply in a very wide range of foods if the temperature is suitable. It is unable to multiply in some fruits because of the low pH value (< 4.5).

Spores of *C. botulinum* Group I (proteolytic) survive all but the most severe heat process e.g. that applied to low acid canned foods (121°C for 3 min). Spores of *C. botulinum* Group II (non-proteolytic) are less heat resistant and are inactivated by heating at 90°C for 10 min, or by equivalent processes. Spores that survive heating, or those present on foods that are not heated, are prevented from growing by the pH and/or water activity of the food, preservatives, and the temperature and time of storage.

Laboratory detection methods for *C. botulinum* are not suited to routine food microbiology laboratories because it is necessary to test for the neurotoxin, and special safety precautions are necessary. Hence, testing for *C. botulinum* and its toxins is not recommended. Good Hygienic Practices and Good Manufacturing Practices should be built into control of the process, with particular attention paid to the formulation (recipe), the heating process, the storage temperature and the intended duration of storage.

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BACKGROUND

Clostridium botulinum is a well-known cause of severe food-borne illnesses in humans. *Clostridium perfringens* is another species of the *Clostridium* family, which is often reported as the cause of food-borne diseases. Clostridia are commonly found in soil, the aquatic environment and in the intestines of animals. Consequently, these bacteria are also often present in different types of foodstuffs.
The current Community legislation does not include any specific provisions for *Clostridium spp*. in foodstuffs, apart from the special conditions laid down for pasteurised or sterilised meat and fishery products in hermetically sealed containers by Directives 77/99/EEC[2] and 91/493/EEC[3]. However, several Member States have in their national legislation or guidelines criteria for *Clostridium perfringens* or sulphite-reducing clostridia in various foodstuffs.
The Scientific Committee on Veterinary Matters related to Public Health of the European Commission issued an opinion on honey and microbiological hazards on 19-20 June 2002. In this opinion, the infant botulism is specifically addressed.
Community legislation on food hygiene is currently under revision. In this framework a revision of the microbiological criteria in Community legislation is taking place. The revised hygiene legislation provides also a legal basis to set specific targets and temperature control requirements for foodstuffs, when appropriate.

Question

The European Food Safety Authority is asked to:
- identify the categories of foodstuffs and the food manufacturing and preparation processes where *Clostridium* spp. may pose a risk for human health;
- establish, for the different categories of foodstuffs identified, the relation between the number of *Clostridium* spp. bacteria in the foodstuffs and the ability of these foodstuffs to cause food-borne illness;
- list and evaluate specific control measures, including microbiological or other types of testing and time-temperature requirements, to manage the risk for human health caused by the presence of *Clostridium spp*. or their toxins in foodstuffs. In doing so attention should be paid to different species of *Clostridia*.

ASSESSMENT

1 INTRODUCTION

Anaerobic spore-forming bacteria have been reported to spoil food products and to cause foodborne disease. Spoilage of dairy products, meat products, poultry products, fresh and canned fruits and vegetables, is caused by *Clostridium barati*,

[2] Council Directive 77/99/EEC on health problems affecting the production and marketing of meat products and certain other products of animal origin. OJ L 26, 31.1.1977, s.25

[3] Council Directive 91/493/EEC laying down the health conditions for the production and the placing on the market of fishery products. OJ L 1, 3.1.1994, p.220

C. beijerinckii, *C. bifermentans*, *C. butyricum*, *C. pasteurianum*, *C. puniceum*, *C. putrefaciens*, *C. sporogenes* and *C. tyrobutyricum* (de Jong, 1989; Chapman, 2001). They typically produce gas and/or putrid odours.

For many years the only recognized psychrophilic species of the genus *Clostridium* was *C. putrefaciens*, first described by McBryde (1911) who isolated it from sour ham. Ross (1965) described the properties of a strain isolated in the UK (NCTC 9836), noting that it has an optimum temperature of 20-25°C and does not grow at 37°C. Roberts and Derrick (1975) reported that it grew at 1°C.

Various workers have failed to isolate strictly anaerobic psychrophilic spore-forming bacteria from a range of materials including soil, sewage, river mud, raw and pasteurised milk, ice cream, cheese meat and fish, although Sinclair and Stokes (1964) isolated 11 strains from river mud, soil and sewage. At that time it was recognised that the apparent scarcity of psychrophilic spoilage clostridia might be an illusion due to inappropriate isolation methods.

Dairy products: Semi-hard cheeses such as Gouda, Emmental, Provolone are susceptible to a defect known as "late-blowing", gas production by *C. tyrobutyricum*, resulting in holes, fissures, and even bursting of the cheese. Bhadsavle *et al*. (1972) isolated four psychrophilic clostridia from milk: two with a minimum temperature for growth of ca 4°C resembling *C. hastiforme*, and two with a minimum growth temperature of 0°C and resembling *C. carnis*.

Fruit and vegetables: *C. puniceum* causes soft rot of potatoes and carrots. *C. beijerinckii*, and *C. butyricum* grow in olives prior to oil extraction resulting in oil of poor quality. Canned fruits and fruit juices are spoiled by *C. pasteurianum* and *C. butyricum* (Chapman, 2001).

Meat products: Vacuum-packed beef primals, stored below 3°C but for longer than the normal time, showed extreme gas production and pack distension due to growth of a psychrophilic *Clostridium* spp., (psychrophilic because it did not grow above ca 20°C). The gas comprised mainly hydrogen and carbon dioxide with some butanol, butanoic acid, ethanol, acetic acid and a range of sulphur containing compounds (Dainty *et al*., 1989). That isolate was characterised by 16S rRNA sequencing and named *C. estertheticum* (Collins *et al*., 1992). US workers reported a similar problem and named the causative organism *C. laramie* (Kalchayanand *et al*., 1989; 1993). In Norway, vacuum-packed cooked pork was spoiled with much less gas production and with offensive ("sickly") odours by *C. algidicarnis* (Lawson *et al*., 1994). In New Zealand numerous cold-tolerant spoilage clostridia have been isolated from a range of meat products (Broda *et al*., 1996a,b, 1998, 2000, 2003a,b), believed to originate from the soil (Boerema *et al*., 2003). In the USA, cold-tolerant clostridia have spoiled non-cured turkey breast and roast beef (Kalinowski and Tompkin, 1999).

None of the above examples of spoilage was associated with illness and none was suspected of causing illness.

Other clostridia: *Clostridium difficile* can cause diarrhoea and more serious intestinal conditions such as colitis in humans. It can be part of the normal bacterial flora occupying the large intestine. Taking certain antibiotics can change the balance of bacteria in the large intestine making it easier for *C. difficile* to grow and cause an infection. Those in good health rarely get *C. difficile* disease. People having other illnesses, conditions requiring prolonged use of antibiotics, and the elderly, are at greater risk of acquiring this disease.

Because *C. difficile* is found in faeces, healthcare workers can spread the bacteria to other patients or contaminate surfaces through hand contact (Walter et al. 1982). People can become infected if they touch items or surfaces that are contaminated with faeces and then touch their mouth or mucous membranes. In principle, foodborne transmission is possible (Borriello *et al*., 1983) but has, so far, not been described.

The species of the genus *Clostridium* most commonly involved in food-borne illness are *C. perfringens* and *C. botulinum*.

2 *CLOSTRIDIUM PERFRINGENS*

2.1 HAZARD IDENTIFICATION

2.1.1 Foodborne illness due to C. perfringens *and taxonomy*

C. perfringens was first recognized as being responsible for food-poisoning in the 1940's, since when outbreaks have been reported (Knox & MacDonald, 1943; McClung, 1945; Hobbs *et al*., 1953; Fruin, 1977). Brynestad and Granum (2002) reviewed the role of *C. perfringens* in foodborne illness.

The first food vehicle suspected of causing *C. perfringens*-associated diarrhoea was rice pudding. Because the organism is commonly found in stools of man and animals, in food, and is widespread in the environment in soil, dust, flies and vegetation, its role as a common agent of food poisoning was slow to be accepted.

C. perfringens is a Gram-positive square-ended anaerobic (microaerophilic) bacillus classified in Group III of the Family Bacillaceae. This non-motile member of the clostridia forms oval, central spores rarely seen in culture unless grown in specially formulated media, although the spores are produced readily in the intestine. Capsules may be seen in smears from tissue. Nitrate is reduced and lecithinase (alpha-toxin activity) can be demonstrated in egg yolk medium (Nagler reaction). Sugar reactions (acid and gas) may be irregular. *C. perfringens* is antigenically related to *C. bifermentans* and *C. sordellii*, which also produce pearly opalescence on egg yolk agar partially inhibited by *C. perfringens* type A antitoxin. Unlike *C. perfringens*, these organisms are motile and sporulate freely and abundantly, particularly *C. bifermentan*s and are proteolytic. Lactose added to media helps to differentiate *C. perfringens*, which ferments lactose, from *C. sordellii* and also *C. novyi*, which *C. perfringens* resembles in young cultures. The sera available for typing *C. perfringens* type A are also helpful for differentiation.

2.2 HAZARD CHARACTERIZATION

2.2.1 Type of illness

Food poisoning from *C. perfringens* gives rise to abdominal pain, nausea and acute diarrhoea 8-24 h after the ingestion of large numbers of the organism, a proportion of which survive the acid conditions of the stomach (Sutton & Hobbs, 1971). The illness is usually brief and full recovery within 24-48 h is normal. However, death occasionally occurs in the elderly or otherwise debilitated patients, e.g. in hospitals or institutions (Smith, 1998). The symptoms of the disease are caused by an enterotoxin.

C. perfringens diarrhoea has not been associated with cured meats because it is relatively sensitive to sodium chloride and nitrite and therefore does not grow to high numbers (Riha and Solberg, 1975; Sauter *et al*., 1977; Brett and Gilbert, 1997; Roberts and Derrick, 1978; Gibson and Roberts, 1986).

2.2.2 Toxins of C. perfringens

C. perfringens is grouped into 5 types A-E according to the exotoxins (soluble antigens) produced (Oakley & Warrack, 1953). Types A, C and D are pathogens for humans, types B, C, D and E, and possibly A also, affect animals. The enterotoxin produced by types A and C is distinct from the exotoxins and is responsible for the acute diarrhoea that is the predominant symptom of *C. perfringens* food poisoning. The beta-toxin of type C appears to be the necrotic factor in the disease enteritis necroticans jejunitis ("pig-bel"). Type A strains are responsible for gas gangrene (myonecrosis), necrotizing colitis, peripheral pyrexia, septicaemia as well as food poisoning. The enterotoxin involved in food-poisoning have been reviewed (Granum, 1990; McClane, 1992; Labbe, 2000; Sarker *et al*., 2000).

The disease-causing enterotoxin of *C. perfringens* (CPE) is produced during sporulation. To cause disease vegetative cells of *C. perfringenes* have to be ingested with the food. The vegetative cells that survive the acidity of the stomach sporulate in the intestinal lumen. During lysis of the mother-cells to release the spores, CPE is also released. Subsequently, the CPE is converted to a more active toxin by trypsin and chymotrypsin, after which it binds to receptors present on the brush-border membrane of the intestinal epithelial cells (Wnek and McClane, 1983). The bound CPE inserts into the cell membrane and pores are produced that makes the cells permeable for ions and small molecules. As a consequence a reversal of ileal transport of water is induced (McDonell, 1974)

It is important to recognise that not all *C. perfringens* are able to produce the enterotoxin that causes foodborne disease. Tschirdewahn *et al.* (1991) tested for the presence of gene encoding the enterotoxin of random isolates of *C. perfringens* from faeces of horses, cattle, poultry and pigs. Faecal samples containing enterotoxigenic strains, assessed by colony hybridization, amounted to 4%, 22%, 10% and 0% respectively. .

Skjelkvale *et al*. (1979) reported that a high proportion of serotypes of *C. perfringens* isolated from food-poisoning outbreaks produced enterotoxin in broth culture, while the same serotypes isolated from beef, lamb and pig carcasses did not.

Damme Jongsten *et al*. (1990a) tested enterotoxin production of *C. perfringens* strains from diarrhoeic syndrome in piglets. None contained the gene encoding for the enterotoxin.

Damme Jongsten *et al*. (1990b), used four DNA probes encoding different parts of the gene of *C. perfringens* enterotoxin to test the enterotoxigenicity of *C. perfringens* strains isolated from confirmed outbreaks of food poisoning. Of the 245 strains isolated from food and faeces originating from 186 separate outbreaks, 145 (59%) gave hybridization reactions with each of the four DNA probes used, while 104 strains did not hybridize with any of the probes.

2.2.3 Heat sensitivity of the enterotoxin

The enterotoxin is heat labile, heating in saline at 60°C for 5 min destroying the biological activity (Granum and Skjelkvale, 1977; Bradshaw *et al*., 1982). Others have reported a 90% loss in biological activity within 1 min at 60°C in buffer (Naik and Duncan, 1978).

2.3 EXPOSURE ASSESSMENT

2.3.1 Distribution of C. perfringens

C. perfringens is ubiquitous and widely distributed in soil, dust, vegetation and raw foods. It is part of the normal flora of the intestinal tract of man and animals. Although the clostridia are anaerobes, *C. perfringens* is one of the less fastidious species and is able to grow under conditions that are not strictly anaerobic (Pierson and Walker, 1976). The spores exhibit a range of resistance to heating, and spores of some strains readily survive cooking.

C. perfringens can be detected in a wide range of foods as a result of contamination by soil or with faecal matter e.g. meat (Smart *et al*., 1979), poultry, fish, vegetables, dairy products, dehydrated foods such as soups and gravies, spices, milk, gelatin, pasta, flour, soy protein and animal feeds (Keoseyan, 1971; Bauer *et al*., 1981; ICMSF, 1998, 2005).

2.3.2 Numbers of C. perfringens *causing illness*

Illness occurs after ingestion of large numbers of enterotoxin-producing vegetative cells of *C. perfringens*, some of which survive the acid conditions of the stomach and subsequently form spores in the large intestine, at the same time producing enterotoxin. From reported and investigated outbreaks, approximately 10^8 vegetative cells per serving are necessary to cause diarrhoea This occurs a few hours after consuming food, usually cooked meat or gravy, in which multiplication of *C. perfringens* has occurred and vegetative cells are present. The illness commonly lasts only ca 12-24 h, and the symptoms are usually not serious enough to consult a

physician – hence the recorded number of outbreaks may be an underestimate (Brett, 1998). Occasionally death occurs, usually in elderly patients (Smith, 1998).

2.3.3 Foods associated with illness due to C. perfringens

C. perfringens has the capacity to grow in protein-rich foods held at temperatures above 12°C. Not all strains of *C. perfringens* have the capacity to produce enterotoxin. Cooked meat and poultry are the foods most commonly involved in *C. perfringens* food poisoning outbreaks. Fish and fish products are rarely implicated.

Intoxications with *C. perfringens* are commonly associated with cooked uncured meat and the associated gravy, at mass catering in food service establishments, care homes, schools, and similar establishments (Tallis *et al*., 1999). Almost all outbreaks are the result of cooling slowly, or holding without refrigeration, allowing multiplication of *C. perfringens*, numbers reaching 10^6-10^7 /g (Pace *et al*., 1976), implying an infective dose of the order of 10^8 vegetative cells of enterotoxin-producing *C. perfringens*

Occasionally illness has been caused by pea soup prepared on a catering (food service) scale (Duynhoven and de Wit, 1998; Anon, 2002; de Jong, 2003; de Jong *et al*., 2004). Even after slow cooling and multiplication of *C. perfringens* that survives cooking; thorough re-heating will inactivate the vegetative cells and prevent illness. In the Netherlands, pea soup is traditionally composed of cut vegetables (peas, celery, leek, and carrots) and pork meat. It has a neutral pH. It is usually produced in winter and often in large quantities e.g. at sporting events.

The inevitable presence of *C. perfringens* in raw materials is taken into account by companies producing dried soups and gravy mixes, and there is no history of illness associated with those foods. Foods should be prepared for consumption as described in Control Measures (see chapter 4).

2.3.4 Risk factors

C. perfringens is ubiquitous and present in most raw food product materials, including vegetables and meat products. Therefore, its presence in many food products must be accepted. The extent of multiplication of *C. perfringens* in food is determined mainly by the storage temperature. In addition, *C. perfringens* requires a protein-rich substrate for growth, explaining why foods associated with *C. perfringens* outbreaks are limited to meat and poultry products, gravy, casseroles and pea soup, usually after temperature-abuse after cooking. Low numbers of *C. perfringens* spores often survive cooking and multiply to food- poisoning levels during slow cooling and unrefrigerated storage of prepared foods. Larger quantities of food, large joints of meat, and large containers of pea soup are difficult to cool quickly. Hence cooling rate and temperature of storage are risk factors.

Herbs, spices and seasonings may contain *C. perfringens* in numbers varying from <100 - 500/g (ICMSF, 1998 chapter 7, Table 7.2, p.279; Rodriguez-Romo *et al*., 1998). Use of herbs and spices is considered by some to be a risk factor. However, there is no evidence that adding even substantial amounts of herbs and spices to cooked foods is a real risk factor for *C. perfringens*-induced disease. Herbs and spices

containing *C. perfringens* will only cause disease if the cooked food in question is not cooled adequately or left-overs are not reheated sufficiently (70-72°C throughout) and is therefore only a temperature-related risk factor.

3 CLOSTRIDIUM BOTULINUM

3.1 HAZARD IDENTIFICATION

3.1.1 Foodborne illness due to C. botulinum

Van Ermengem (1897) isolated an anaerobic, spore-bearing, toxin-producing bacillus from the remains of a salted ham, which had caused neuroparalytic illnesses and 3 fatalities among 34 musicians in Belgium in 1895. He named the organism *Bacillus botulinus*. In 1917, the generic term *Clostridium* was adopted for anaerobic bacilli showing a spindle-shaped enlargement at the site of spore formation.

Clostridium botulinum was defined by Prévot (1953) as the species name for all organisms producing *botulinum* neurotoxin.

Four different forms of botulism are recognized. If *C. botulinum* multiplies in a food, and that food is subsequently ingested without heating, the person consuming it suffers botulism via **intoxication** from pre-formed toxin. There are reports that another form of botulism occurs when *C. botulinum* multiplies in the gut, producing neurotoxin and causing a "**toxico-infection**" (Matveev *et al*., 1967; Chia *et al*., 1986; McCroskey and Hatheway, 1988). In **wound botulism**, *C. botulinum* establishes itself and multiplies in damaged tissues, and in doing so produces neurotoxin, which is absorbed, causing botulism (Merson and Dowell, 1973), which has been associated with traumatic wounds, tooth abscesses (Weber *et al*., 1993a) and in drug users such as those injecting heroin or sniffing cocaine (Kudrow *et al*., 1988; Passaro *et al*., 1998; Mulleague *et al*., 2001; Brett *et al*., 2004). The fourth form is **infant botulism** when multiplication and toxin production occurs in the infant gut (Chin *et al*., 1979; Arnon, 1998; SCVPH (2002) Opinion on Honey and Microbiological Hazards).

3.1.2 Taxonomy of the C. botulinum group

C. botulinum is a Gram-positive spore-forming anaerobic (microaerophilic) bacillus classified in the Family Bacillaceae. It may be saccharolytic, proteolytic, or both.

Table 1: Main distinguishing features of metabolic groups of *C. botulinum* (modified from Hatheway, 1993)

	Group			
Feature	**I**	**II**	**III**	**IV**
Toxin produced	A, B, F	B, E, F	C1, C2, D	G
Proteolysis	+	-	+	+
Lipolysis	+	+	+	-
Fermentation of glucose	+	+	+	-
Fermentation of	-	+	+	-

mannose				
Minimum growth temperature	10°-12°C	3.3°C.	15°C	*
Inhibited by NaCl (%)	10	5	3	>3
Volatile fatty acids**	A, iB, B, iV	A, B	A, P, B	A, iB, B, iV, PP, PA

*minimum growth temperature not determined;**Key to fatty acids: A = acetic; P = propionic; iB = iso-butyric; B = butyric; iV = iso-valeric; V = valeric; PA = phenylacetic; PP = phenylpropionic (hydrocinnamic).

Strains of *C. botulinum* are divided into four groups, the most important in food-borne botulism in man being Group I (proteolytic, mesophilic), and Group II (non-proteolytic / saccharolytic, psychrotrophic). One phenotypic group of organisms may produce more than one type of toxin. The main distinguishing biochemical features differentiating Groups I and II, and those of other clostridia producing *botulinum* neurotoxin, are shown in Table 1. Hatheway (1993) reviewed the taxonomic status of *C. botulinum*, based on phenotype. The relationship of the different types of *C. botulinum* and of other clostridia capable of producing *botulinum* neurotoxin, based on 16S rRNA sequencing, was investigated by Lawson *et al*. (1993), and the phylogeny and taxonomy of *C. botulinum* and its neurotoxins by Collins and East (1998).

3.2 HAZARD CHARACTERIZATION

3.2.1 Type of illness

Botulinum neurotoxin (BoNT) has the capacity to cause disease in essentially all vertebrates (Table 2).

Symptoms may appear in a few hours or take several days to appear. Initial symptoms such as weakness, fatigue and vertigo, are usually followed by blurred vision and progressive difficulty in speaking and swallowing. In type E botulism nausea and vomiting often occur early in the illness and probably contribute to its lower mortality than types A and B. Disturbed vision and difficulty in speaking and swallowing are due to neurological implications involving extra-ocular and pharynx muscles. Weakening of diaphragm and respiratory muscles also occurs and death is usually due to respiratory failure. Specifically neurotransmission of the peripheral nerve system is blocked. The mortality rate has fallen due to early diagnosis, prompt administration of antitoxin, and artificial maintenance of respiration. The illness is serious and full recovery usually takes many months.

3.2.2 Toxins of C. botulinum

The toxins of *C. botulinum* types A, B and E primarily affect humans, with a few reports of type F. There are reports in the scientific literature that types C and D havecaused human botulism, but, by the criteria currently applied, types C and D botulism have not been confirmed in man (discussed in Roberts and Gibson, 1979; Hatheway, 1993).

While surveying soil in Argentina, Gimenez and Ciccarelli (1970) isolated an organism that produced a toxin that killed mice with symptoms of botulism, but was not neutralised by any monovalent or polyvalent *C. botulinum* antitoxins. This was named "*C. botulinum* type G" but has since been reclassified as *C. argentinense* (Suen *et al*., 1988). *C. argentinense* (type G) was isolated retrospectively after sudden death in adults (Sonnabend *et al*., 1981) and infants (Sonnabend *et al*., 1985), but it is doubtful whether it was the cause of the deaths (Hatheway, 1993). *C. argentinense* (type G) has never been demonstrated in, or isolated from, foods.

Definitive molecular techniques demonstrated that the four phenotypic groups (Groups I to IV) of *C. botulinum* (Hatheway, 1993) correspond to four distinct genospecies (Hutson *et al*., 1993a,b; Campbell *et al*., 1993, East *et al*., 1992). For Group I, proteolytic clostridia that produce usually one, but occasionally two, toxin(s) of type A, B or F, the most closely related organism is *C. sporogenes.* Group II, comprising non-proteolytic strains producing BoNT/B, BoNT/E or BoNT/F have no named non-toxigenic equivalent species. However, non-toxic variants of Group II *C. botulinum* have been reported (Ghanem *et al*. 1991) and sequencing of 23S rRNA showed that these non-toxic strains are part of the Group II *C. botulinum* genospecies (Campbell *et al*. 1993). In the case of group III, producing BoNT/C and BoNT/D and causing botulism in animals and birds, the most related species is *C. novyi*. Group IV (*C. argentinense*) includes both toxin-producing and non-toxigenic strains, including some strains previous known as *C. subterminale* (Suen *et al*., 1988).

Table 2. Types of *Clostridium botulinum*, species affected and vehicles

Type	Species affected	Vehicle
A	Humans (neuroparalytic, wound & infant)	Home - canned vegetables, fruit, meat, fish
A	Chicken ("limberneck")	Decomposing kitchen waste
B	Humans (neuroparalytic, wound & infant)	Prepared meats (esp. pork), cattle, horses
Cα	Aquatic wild birds ("Western duck sickness")	Toxin-bearing invertebrates Carrion, forage
Cβ	Cattle ("Midland cattle disease"), Horses, ("forage poisoning")	Feed or grazing contaminated with decomposing poultry waste; accidental ingestion of parts of putrid mice or cats
	Mink	Feed is often raw minced (ground) offal and abattoir waste; unless temperature is reduced rapidly and controlled below 10°C, type C is able to grow and produce toxin. Feed sometimes acidified and/or heated.
D	Cattle ("lamziekte")	Carrion
E	Humans (neuroparalytic,	Uncooked products of fish and

	wound and infant)	marine mammals
F	Humans (neuroparalytic, wound and infant)	Home-made liver paste "deer-jerky"
G*	Unknown	Unknown

* *"C. botulinum* type G" = *C. argentinense* (see text)

BoNTs occur in complexes of four molecular sizes, three of which are associated with non-toxic proteins (Lund and Peck, 2000). The toxic component of the complexes is a single-chain polypeptide (protoxin) (ca 150 kDa) of relatively low toxicity, which are converted enzymatically into a light chain (ca 50 kDa) and a heavy (ca 100 kDa) linked by a disulphide bond. The BoNTs act by blocking the release of acetylcholine at cholinergic synapses of the peripheral nerve system.

Epidemiological information worldwide on infant botulism indicates that *C. botulinum* types A and B are most frequently involved, while types E and F have also caused the disease (Arnon, 1998). *C. botulinum* producing 2 neurotoxins (types B and F) at the same time has been isolated from infant botulism (Hatheway and McCroskey, 1987). Unusual strains of *C. butyricum* and *C. baratii* that produce BoNT type E and F, respectively, have also caused infant botulism (Aureli *et al.*, 1986; Hall *et al.*, 1985; Trethon *et al.*, 1995).

3.3 EXPOSURE ASSESSMENT

3.3.1 Distribution of C. botulinum

C. botulinum is ubiquitous and may occur in or on almost all foods, whether of vegetable or animal origin. Comprehensive listings of the breadth and frequency of its occurrence have been published (Tompkin, 1980; Rhodehamel *et al*., 1992; Hauschild and Dodds, 1993; Lund and Peck, 2000). Spores of *C. botulinum* are widely distributed in the soil, on shores and bottom deposits, lakes and coastal waters and occur in the intestinal tracts of fish and animals. In the USA, soil in western states is commonly contaminated with type A and soils in the east with type B. Soils in Europe carry type B more commonly. Type E is especially common in fresh water and marine sediments. Searches for type E after an outbreak of type E botulism due to smoked whitefish chubs from the Great Lakes in N. America resulted in the discovery of psychrotrophic saccharolytic strains of types B and F (Group II).

Rhodehamel *et al*. (1992) and Dodds (1993) cite examples of contamination of spinach, onion skins, mushrooms, garlic cloves, cabbage, honey, fresh and processed meats. *C. botulinum* is also common in some fish and fishery products e.g. farmed trout (Hielm *et al*., 1998), and especially trout farmed in ponds with mud bottoms (Huss *et al*., 1974 a,b) in Denmark where *C. botulinum* type E was predominant, at levels up to 5.3 spores/g (Huss *et al*. 1974a). The incidence of *C. botulinum* in meats appears from the literature to be low (Hauschild, 1989; Tompkin, 1980), but if sufficient large samples were examined its presence could be demonstrated readily in pork (Abrahamsson and Riemann, 1971; Roberts and Smart, 1976).

Although spores of anaerobes are present in herbs and spices (ICMSF, 1998, 2005) a search of the literature failed to find a single report of the presence of spores of *C. botulinum*. Spores of *C. perfringens* are commonly present (ICMSF, 1998, 2005).

Concentrations of *C. botulinum* spores in raw materials intended for use in "refrigerated, processed foods of extended durability" (REPFEDs) were generally low: fish and shellfish 2-3/kg (102 samples); meat 2-4/kg (143 samples); dairy products 2-5/kg (26 samples); thickening agents 3-7/kg (25 samples), or very low: flavourings and sauces 0.3-0.6/kg (25 samples); spices and herbs <0.6/kg (65 samples). Only types A and B were detected (Carlin *et al*., 2004).

3.3.2 Numbers of C. botulinum causing illness

The neuroparalytic toxin of *C. botulinum* is one of the most toxic naturally occurring substances (Gill, 1982). The amount of BoNT type A needed to cause death in man has been estimated to be between 0.1 and 1µg, corresponding to 3,000 to 30,000 intraperitoneal mice LD_{50} doses (Schantz and Sugiyama, 1974). Botulism has resulted after a person suspected that a food might be spoiled, and merely tasted the food after dipping one finger into it. Even modest multiplication of *C. botulinum* must be prevented.

C. botulinum has the capacity to grow in almost every food excepting those with a low pH (Table 6) or low a_w (Table 7).Tables 3 and 4 illustrate the wide range of foods that have been associated with botulism.

A raw product that is already contaminated with *C. botulinum* does not necessarily cause botulism. Foodborne botulism occurs after ingestion of BoNT pre-formed in a food. Outbreaks occur due to failures in formulation (recipe) or the process applied, or lack of control in one of the many steps before consumption, usually inadequate refrigeration.

Any multiplication of *C. botulinum* must be avoided because of the extreme toxicity of BoNT

3.3.3 Foods associated with botulism

The literature on food-associated botulism is vast, but has been comprehensively summarized by Hauschild and Dodds (1993) and more recently by Lund and Peck (2000).

Examples of the wide range of foods that have been responsible for botulism are shown in Table 3.

Table 3. Examples of foods associated with botulism.

Commodity	Reported cases/outbreaks of botulism from (reference)
Meat*	Raw cured (1); Cooked cured; Fermented
Poultry*	None recorded
Fish*	Lightly preserved; Smoked, vacuum-packed salmon (2, 3); Home-smoked

	vacuum-packed fish (4)
	Fermented** (5, 6, 7, 8)
Vegetables	Asparagus, home bottled (9); Home-canned bamboo shoots (31)
	Bottled mushrooms (10, 11)
	Home-preserved mushrooms (12); Green Beans / artichokes (13);
	Garlic (14); Potatoes (15, 16, 17)
	Olives, black (19)
	Canned peppers (20)
Fruits	Tomatoes
Spices, soups and oriental flavourings	Fish sauce
	Shrimp sauce
Cereals*	None reported
Nuts, oilseeds, dried legumes	Hazelnut purée (in yoghurt) (26)
	Unsalted peanuts in water (28)
Cocoa, chocolate, confectionery	None reported
Oil- and fat-based foods	Potato salad (15, 16, 17)
	Garlic in oil (14)
	Egg plant in oil (18)
Sugar, syrups, honey	Honey associated with infant botulism (21)
Soft drinks, fruit juices, concentrates and fruit preserves	None
Water	None
Eggs*	Pickled eggs (29)
Milk*	Cheese with onion (22); Brie cheese (23); Mascarpone cheese (24)
	Cheese preserved in oil (25); Yoghurt with hazelnut purée (26)
	Canned cheese sauce (27)
Fermented beverages	None
Recipe dishes	Kosher airline meal, veal and rice (30)

*and products thereof; ** especially traditional fermented products

(1) Troillet and Praz, 1995, (2) Jahkola and Korkeala, 1997, (3) Korkeala *et al*., 1998, (4) Anon. 1998a, (5) Dawar *et al*., 2002 (6) Proulx *et al*., 1997, (7) Slater *et al*., 1989, (8) Weber *et al*., 1993b, (9) Paterson *et al*., 1992, (10) CDC 1987, (11) McLean *et al*., 1987, (12) Roberts *et al*., 1998, (13) Mangas-Gallardo and Hernandez-Pezzi, 1995, (14) St Louis *et al*., 1988, (15) Seals *et al*., 1981, (16) Sugiyama *et al*., 1981, (7) MacDonald *et al*., 1986, (18) CDC 1995, (19) Fenicia et al. 1992, (20)Terranova *et al*., 1978, (21) SCVPH Opinion 2002, (22) de Lagarde 1974, (23) Billon *et al*., 1980, (24) Aureli *et al*., 2000 (25) Pourshafie *et al*., 1998, (26) O'Mahony *et al*., 1990, (27)Townes *et al*., 1996, (28) Chou *et al*., 1988, (29) Anon., 2000, (30) Gilbert *et al*., 1990, (31) CDC, 1999b.

The types of *C. botulinum* involved in outbreaks differ in different countries, reflecting the distribution of the organism in the soil, and also different patterns of food production, processing and preservation. Foods commodities involved in outbreaks of botulism in different countries are summarised in Table 4.

Table 4. Foods involved in outbreaks of botulism (adapted from Hauschild, 1993, his Table 3).

	Number of outbreaks with food identified	Meats (%)	Fish (%)	Fruit and vegetables (%)	Other (%)[a]	Home prepared (%)
USA	222	16	17	59	9	92
Canada	75	72	20	8	0	96

Argentina	14	29	21	36	14	79
Poland	1500	83	12	5	0	75
Czechoslovakia	14	72	7	14	7	100
Germany						
East	31	52	26	19	3	73
West	55	78	13	9	0	100
Belgium	8	75	12	0	62	38

[a] includes mixed vehicles

Type E botulism is most common in the north (Baltic countries, Japan (Hokkaido), Alaska and British Columbia, partly because it is commonly found in the marine environment, and partly because of preparation of traditional fermented fish and fermented marine mammals consumed without heating (Dolman and Iida, 1963; Wainwright, 1993). Outbreaks are also common in Iran where similar fermented fish are eaten. Outbreaks in European countries are most often type B while those in the USA, Argentina and China are most often type A.

Although *C. botulinum* and botulism are often associated particularly with meat and fish products, it has the capacity to grow in a wide range of foods including fresh-cut packaged vegetables (Austin *et al*., 1998), cooked vegetables (Carlin and Peck, 1995), fresh pasta (Glass and Doyle, 1994), melons (Larsen and Johnson, 1999), sautéed onions (Macdonald *et al*., 1985; Solomon and Kautter, 1986), shredded cabbage (Solomon *et al*., 1990; Petran *et al*., 1995), uneviscerated fish (Telzak *et al*., 1990), home preserved asparagus (Paterson *et al*., 1992), bottled chopped garlic (Solomon and Kautter, 1988; Morse *et al*., 1990), peyote (Hashimoto *et al*., 1998), garlic in chili oil (Lohse *et al*., 2003), baked potato (Angulo *et al*., 1998), fried lotus rhizome in mustard (Otofuji *et al*., 1987) and home canned bamboo shoots (CDC 1999b; Swaddiwudhipong and Wongwatcharapaiboo, 2000).

C. botulinum is widely distributed in the soil and on raw agricultural products, but the level of contamination is generally low, or very low. Nevertheless, because huge quantities of agricultural produce are stored, processed in many different ways, and consumed by animals and man, the possible survival of *C. botulinum* spores and potential for growth and toxin production must be taken into account. In particular foods the incidence is much higher e.g. Baltic herring (Johannsen, 1963), trout farmed in ponds with mud bottoms (Huss *et al*., 1974a,b).

3.3.4 Risk factors

Since *C. botulinum* is a ubiquitous organism, small numbers of spores of that organism may be present in all types of raw food materials. Growth of the organisms, accompanied by toxin production in the food, is the main risk factor. A wide range of food products has the capacity to support growth of *C. botulinum*. Even when food is stored in air, the interior is often sufficiently anaerobic to permit growth of *C. botulinum* and BoNT production. The main risk factors are:

- **Under-processing in heat preservation of food products.** For heat-preserved low acid foods it is important that spores of *C. botulinum* are

completely inactivated. To achieve this, industrial processing equipment is essential, as well as knowledge of the necessary processing conditions. Home preservation, which is practised in many EU Member States, does not kill spores of proteolytic strains of *C. botulinum*.

- **Incomplete curing of pork products.** Certain pork products cannot be heat treated to kill the spores of *C. botulinum* without damaging the quality of the product. This applies to a wide range of meat products preserved in different ways.

- **Slow fermentation /traditionally fermented fish and marine mammals/some cheeses.** Botulism has been caused by slow fermentation processes, used for the production of traditional products such as rakefisk (Norway), izushi (Japan) and muk tuk (Alaska).

- **Storage/time/temperature, not under control.** Growth of *C. botulinum* has been observed in over-wrapped fresh mushrooms, in garlic in oil, and in baked potatoes wrapped in aluminium foil when stored at ambient temperatures.

- **Water activity not under control.** Cases of botulism have been caused by home drying of fish products and adding too little salt.

- **pH not under control e.g. marinated mushrooms.** For marinated vegetable products such as mushrooms, failure of rapid pH-decrease of the internal contents has been the cause of several outbreaks of botulism and should be considered as an important risk factor.

- **Process failure.** Cases of botulism have been caused by process failures. Examples include under-heating of canned food products, cooling of sterilised cans in un-chlorinated water, and recontamination of commercially sterile canned salmon via damaged cans.

4 CONTROL MEASURES

4.1 Initial contamination of food

The initial contamination of foods with *C. botulinum* and *C. perfringens* is difficult to control. While Good Agricultural Practices (GAP) can help to reduce numbers of infectious pathogens such as salmonellae, the only means of reducing the initial load of bacterial spores is to minimise contamination of raw foods by soil and by animal faeces. Good Hygienic Practices (GHP) in the manufacturing environment minimise chances of recontamination. There is some evidence that *C. perfringens* can reside in pipelines and contaminate product, resulting in spoilage of the product (Ernst, 1974).

Because *C. botulinum* and *C. perfringens* occur so commonly in the environment, many foods will be contaminated. Experience has demonstrated that if foods are handled properly during all stages of distribution, retailing and consumer use, the likelihood of multiplication of clostridia is very low. Heating food to ca 68-70°C kills vegetative cells but not spores. Inappropriate treatment of the food at any stage during

the commercial processing or consumer use could result in an increase in the numbers of cells of *C. botulinum* and toxin production.

Table 5. Effect of temperature (°C) on growth of *C. botulinum* and *C. perfringens*

Strains	Minimum	Optimum	Maximum
C. botulinum Group I (proteolytic)	10-12	35-40	40 – 42
C. botulinum Group II (non-proteolytic)	3-4	28-30	34-35
C. perfringens	10 –12	43 – 47	50

Table 6: Effect of pH on growth of *C. botulinum* and *C. perfringens*

Strains	No growth at pH values
C. botulinum Group I	< 4.6
C. botulinum Group II	< 5.0
C. perfringens	< 5.5-5.8

Table 7: Effect of water activity (a_w) on growth of *C. botulinum* and *C. perfringens*

Strains	No growth at a_w values
C. botulinum Group I	< 0.94
C. botulinum Group II	< 0.97
C. perfringens	< 0.95-0.94

4.2 Growth limitation of *C. perfringens* in the food chain

4.2.1 Effect of temperature on growth

There are numerous publications illustrating the importance of rate of chilling and holding temperatures on growth of *C. perfringens* (Bryan and Kilpatrick, 1971; Blankenship *et al*., 1988; Juneja & Marmur, 1996; Steele and Wright, 2001; Danler *et al*., 2003; Zaika, 2003; Andersen *et al*., 2004; de Jong *et al*., 2004). The optimum temperature for growth of *C. perfringens* is 43-47°C. Growth does not occur below 10-12°C.

Most meat and poultry products receive a cook much lower than the sterilizing process for low-acid canned foods, and spores of *C. perfringens* survive. If the product then cools slowly, surviving spores germinate and multiply rapidly. After heating, meat and poultry products should be cooled from 55°C to below 15°C as quickly as is reasonably possible. US regulations serve as a guide, requiring the product's internal temperature not to remain between 54.4°C (130°F) and 26.7°C (80°F) for more than 1.5 h or between 26.7°C and 4.4°C (40°F) for more than 5 h (FSIS, 1999). These guidelines are presented in Annex I. Similarly, if intact muscle e.g. roast beef, is cooked, chilling must be initiated within 90 min of the end of the cooking cycle. The product must also be chilled from 48°C (120°F) to 12.7°C (55°F) in not more than 6 h, and chilling must continue and the product not packed for transportation until it has reached 4.4°C (40°F). However, study of *C. perfringens* in commercially cooked products during chilling and refrigerated storage concluded that the above chilling regimes are more severe than necessary to maintain safe product (Kalinowski *et al*., 2003; Taormina *et al*., 2003).

Even if rapid cooling is not achieved, vegetative cells of *C. perfringens* can be killed and pre-formed toxins inactivated by thorough re-heating to above 70°C (centre or core temperature of product) immediately before consumption of the product. This is an effective control measure if the prior cooking and cooling temperature history of the product is not known.

A user-friendly software tool, "Perfringens Predictor", has been developed from research funded by the UK Food Standards Agency at the Institute of Food Research (IFR), Norwich, UK, complementing "Growth Predictor". After cooling profiles are entered, the software calculates the extent of multiplication of *C. perfringens*. In the case of abused cooling and holding conditions, reheating of the product before consumption is advised. Heating to an internal temperature above 72°C will destroy both vegetative cells and CPE present and is an additional preventive measure.

4.3 Growth limitation of *C. botulinum* in the food chain

Growth of C. botulinum in foods is presented by a variety of measures.

4.3.1 Effect of temperature on growth

Growth of Group I *C. botulinum* (proteolytic strains) and *C. perfringens* is prevented by storage at 10-12°C, and growth of both types is slow below 15°C. Growth of Group I *C. botulinum* often produces offensive odours that render the food unacceptable.

However, Group II *C. botulinum*, the so-called psychrotrophic strains, are able to multiply and form toxin at temperatures found in food distribution chains, typically below 7°C, and in domestic refrigerators where temperatures may reach 10-12°C. Organisms of this group produce spores which are much less resistant to heat and a 6 $\log_{10}$ (6-D) reduction is obtained by heating at 90°C for 10 min (Lund and Notermans, 1993). A heat treatment of 90°C for 10 min has also been included in the European Code of Hygienic Practice for REPFEDs (ECFF, 1996).

Growth of Group II (non-proteolytic) strains produces no such foul odours and the foods appear to be normal, but contain lethal amounts of BoNT. Growth and toxin production have been reported at temperatures as low as 3°C (Graham *et al*., 1997).

Exceptions to the above consideration of spoilage odours are particular traditional fermented fish products where proteolysis and strong odours and flavours are an important part of the products' appeal, e.g. izushi in Japan, rakefisk in Norway, muk tuk among the native Inuit population in Alaska (Dolman and Iida, 1963; Wainwright, 1993; Wainwright *et al*., 1998; Chiou *et al*., 2002). All have been responsible for botulism, usually type E (Group II), when the fermentation does not reduce the pH value quickly enough to prevent multiplication of *C. botulinum* type E.

4.3.2 Effect of pH on growth

Growth of all microbes slows as pH falls from an optimum pH value (for foodborne microorganisms near neutrality, pH 7.0) to acid pH values, to reach a minimum pH for growth.

Many years ago tomatoes were a product with relatively low pH and could be preserved by canning processes much lower then F_0=3. However, selection for faster rate of growth and increased yield has resulted in many modern varieties grown for canning having a pH value nearer neutrality. Consequently, prior to canning, tomatoes are commonly acidified with citric acid.

Fermented foods, including meat, fish and vegetables, have historically prevented the growth of a wide range of pathogenic bacteria. This is a consequence of rapid development of a "low" pH, below ca pH 4.5. Traditional fermentations are not always under control and slow development of the low pH value has led to botulism. Reaching the "low" pH value as quickly as possible is facilitated by using starter cultures and a fermentable carbohydrate e.g. glucono-delta-lactone (GDL).

A rapid equilibration of the pH, for example in onions, is important to prevent growth of clostridia. Odlaug and Pflug (1978) reviewed the microbiology of acid foods.

4.3.3 Effect of water activity on growth

As water activity is reduced by drying, or by adding, for example, sodium chloride, sugar or other solutes, growth of clostridia slows, and eventually stops (Leistner and Russell, 1991; Brown, 2000).

4.3.4 Effect of the atmosphere above the food

Although *C. botulinum* is an anaerobic bacterium, it is able to multiply at the surface of many foods, especially meat and fish. Consequently vacuum-packing, or packing under modified atmospheres, has a smaller influence on toxin production than might be expected, safety being determined mainly by the temperature and duration of storage (Reddy *et al*., 1992; Larsen *et al*., 1997; Smith *et al*., 2004). Even if the atmosphere above the food contains oxygen, clostridia may still multiply because conditions at the food surface are sufficiently anaerobic.

4.3.5 Combination of factors

Extensive research has confirmed that microbial growth in many meat products is controlled by inhibitory factors acting in combination e.g. pH, NaCl, $NaNO_2$, storage time and temperature. This applies to growth of both *C. botulinum* (Braithwaite and Perigo, 1971; Riemann *et al*., 1972; Lücke and Roberts, 1993; Graham *et al*., 1996, 1997; EFSA (2003) Opinion, on Effects of Nitrites/Nitrates on Microbiological Safety of Meat Products) and *C. perfringens* (Gibson and Roberts, 1986). In addition, the severity of any heat process applied helps to prevent growth and toxin production (Jarvis *et al*., 1976; Bean, 1983; Stringer *et al*., 1997). Detailed laboratory investigations also identified the concentration of spores present to be important, with growth and toxin production occurring much more readily at high concentrations than low (Roberts *et al*., 1976).

Predictive models of growth have becoming generally available for predicting the effect of some conditions and can give a rapid estimate of whether or not growth will occur, sometimes including "time to toxin production" (e.g. Graham *et al*., 1996).

Some combinations of factors that inhibit growth and toxin production by *C. botulinum* have evolved from years of experience of manufacture of particular products (e.g. Lücke and Roberts, 1993).

An alternative to assuring the safety of a product against growth of clostridia is to perform challenge testing (Notermans *et al*., 1993). Relatively high numbers of spores are generally inoculated into one or more product formulations stored at temperatures representing appropriate and abuse temperatures. The use of large numbers of the challenge organism in an attempt to find a margin of safety is difficult to extrapolate to the low and sporadic numbers of *C. botulinum* normally present.

4.4 Inactivation of clostridia in the food chain

4.4.1 Effect of heating

Heating is the most reliable method of inactivating bacterial spores. The rate of inactivation of spores is dependent on the temperature of heating, spores dying more rapidly as temperature rises. The heat resistance of spores is also affected by properties of food (pH, water activity, fat content).

Spores are more heat resistant at low aw and neutral pH values. Spores are more heat sensitive at low pH values (below ca pH 4.5); hence canned fruits are made safe and shelf-stable without refrigeration by much lower heat processes than those applied to low acid canned foods. (Stumbo, 1973; ICMSF, 1998).

Low acid canned foods are heated at 121°C for ca 3 min to eliminate spores of *C. botulinum* (12D reduction of Group I spores). That process would also eliminate spores of *C. perfringens*. Elimination of Group II spores is achieved by heating at 90°C for 10 min (6D reduction) (ACMSF, 1992).

Foods subjected to less severe heat treatments will occasionally carry spores of *C. botulinum* and often spores of *C. perfringens*, reflecting levels of contamination on the commodity and ingredients.

4.4.2 Effect of lysozyme on heated spores

Lysozyme and some other proteolytic enzymes can initiate germination of spores of the non-proteolytic *C. botulinum* types after apparently lethal damage by heat treatment. The role of lysozyme in the recovery of heat-treated spores has been attributed to the replacement of thermally inactivated enzymes (Alderton *et al.*, 1974; Scott and Bernard, 1985).

It has also observed that adding 5% lysozyme to the recovery medium for heat-treated spores of non-proteolytic C. *botulinum* considerably increases the recovery (Smelt, 1980). While this might have consequences for thermal inactivation of spores of non-proteolytic C. *botulinum* in foods containing lysozyme, this phenomenon has never been identified as a risk factor in foodborne botulism.

4.4.3 Other processes

Until *ca* 20-25 years ago inactivation of microbes in food processing used heating. Today many novel processes are being explored e.g. high hydrostatic pressure, pulsed electrical fields, irradiation used alone, or in combination with heat (Gould, 1995; Ross *et al.*, 2003).

Several chemical and physical stresses can inactivate vegetative cells of *Clostridium* spp. and a proportion of their spores. Conditions in the food (pH, a_W, fat content) may influence their effectiveness.

Spores of all types of *C. botulinum* are relatively resistant to ionizing radiation. Radiation resistance is greater at ca -80°C than at ambient temperatures, and increases as water activity is reduced. A 12D reduction of spores of Group I *C. botulinum* by irradiation at -30°C ± 10°C requires a dose of *ca* 25-45 kGy (WHO, 1999).

Spores of *C. perfringens* are similarly resistant to irradiation (Clifford and Anellis, 1975).

High hydrostatic pressure inactivates vegetative bacteria and yeasts (Knorr, 1995; 1999). It is used commercially, but sometimes relies on the low pH of the products to prevent multiplication of surviving spores. Currently, high hydrostatic pressure is not used commercially to inactivate spores. Applying high hydrostatic pressure in the presence of chemicals known to promote germination increases the proportion of spores inactivated, but there are concerns that a small fraction of "super-dormant" spores remains and presents a risk (Gould *et al.*, 1968)

Table 8: Effect of temperature on the heat resistance of spores of *C. botulinum* Group I (based on ICMSF, 1996).

C. botulinum (spores) Group I proteolytic			
Temperature	Type A	Type B	Type F
110°C	0.61-2.48*-	0.49-3.33-	1.45-1.82
115.6°C	0.23-0.83-	0.26-1.18-	-
121°C	0.05-0.23	0.3-0.32	0.14-0.22-

* D value (min). N.B. D values vary with the medium in which the spores are heated (e.g. buffer or food), the pH and aw, and with the manner in which spores were produced, and depending on strain.

Table 9: Effect of temperature on the heat resistance of spores of *C. botulinum* Group II (based on ICMSF, 1996)

C. botulinum (spores) Group II non- proteolytic			
Temperature	Type B	Type E	Type F
77°C	-	2.4-4.1	0.2-9.5
80°C	-	0.55-2.15	-
82.2°C	1.5-32* (100**)	0.49-1.2	0.25-42.4

*D value (min). N.B. D values vary with the medium in which the spores are heated (e.g. buffer or food), the pH and aw, and with the manner in which spores were produced, and depending on strain.
** 10 µg/ml lysozyme in recovery medium

Table 10: Effect of temperature on the heat resistance of spores of *C. perfringens* (based on ICMSF, 1996)

Temperature (°C)	D value (min)
90	0.015-8.7
100	0.31-13
104.4	3.15-6.6
110	0.5-1.29
115.6	0.2-0.6

The toxins of *C. botulinum* are relatively sensitive to heat and are inactivated by heating for 10 min at 80°C or equivalent time/temperature conditions (Losikoff, 1978; Smart and Rush, 1987; Siegel, 1993).

4.4.4 Effect of food additives

For many centuries the curing of meat and fish has relied upon curing salts to control the growth of *C. botulinum* and *C. perfringens*. Concerns that sodium nitrite in meat products might result in the formation of carcinogenic N-nitrosamines and other nitrosated products led to extensive research, mainly in N. America and Europe, to try to find alternatives to sodium nitrite and sodium nitrate. Some anti-clostridial activity was identified in potassium sorbate and certain polyphosphates (Ivey *et al*., 1978; Sofos *et al*., 1979; Nelson *et al*., 1983), parabens (Robach and Pierson, 1978; Dymicky and Huhtanen, 1979; Reddy *et al*., 1982), antioxidants (Robach and Pierson, 1979; Reddy *et al*., 1982; Pierson and Reddy, 1982), nisin (Scott and Taylor, 1981) and sodium lactate (Maas *et al*., 1989; Meng and Genigeorgis, 1993; Houtsma *et al*., 1994) when used in combination with other conditions.

Cured meat producers have minimised the risk of formation of nitrosamines by reducing the amount of nitrite used, and eliminating nitrate from most cured products.

A few foods have a single controlling factor that prevents multiplication of *C. botulinum* e.g. fruits (low pH), dried foods (low a_W). Most foods rely on combinations of at least 2 factors to prevent multiplication (Riemann *et al*., 1972; Roberts and Ingram, 1973; Roberts and Derrick, 1978; Graham *et al*., 1996, 1997; EFSA, 2003).

Examples of products that are rendered microbiologically safe and shelf-stable without refrigerated storage are the various "shelf-stable canned cured meats" (SSCCM) such as luncheon meat. In such products a combination of sodium nitrite, sodium chloride and a modest heat process, sometimes less than one tenth of the process applied to low acid canned foods, has effectively controlled *C. botulinum* over many years of large scale production (Hauschild and Simonsen, 1985; Lücke and Roberts, 1993). After collecting production data including the product formulation (recipe) and details of the process from many companies Simonsen and Hauschild (1986) estimated product safety by totalling the weight and number of units sold without spoilage or safety problems.

4.5 Controlling *C. perfringens* in foods

The most essential measures to prevent foodborne diseases caused by *C. perfringens* are: appropriate cooking, cooling rapidly through the temperature range 55°C to 15°C, holding foods at temperatures <12°C, and re-heating the product to an internal temperature of 72°C before consumption.

To prevent foodborne diseases caused by *C. perfringens* it is essential to cool prepared cooked foods adequately to temperatures that do not allow growth of the organism (<12°C). Also stored products should be kept at temperature where no growth will occur (<12°C).

Since cooling after cooking is important to control diseases caused by *C. perfringens* and other foodborne pathogens several countries have set guidelines for cooling conditions. An example is given in Annex I.

4.6 Controlling *C. botulinum* in foods

Because *C. botulinum* includes proteolytic and saccharolytic strains, and both mesophilic and psychrotrophic strains, many different foods can be implicated in botulism. However, there are many control options to prevent growth of *C. botulinum*. They include:

4.6.1 Heat treatment

Numbers of spores of *C. botulinum* can be dramatically reduced by heat treatment

The application of the '*botulinum* cook'[4] is defined as equivalent to 3 minutes heating at 121°C. This value is also the F_o value or the process value. This heating regime is used for low acid canned food products and results in a 12 $\log_{10}$ units reduction in numbers of spores.

10 min heating at 90°C to kill spores of non-proteolytic *C. botulinum* (group II) species by at least 6 $\log_{10}$ units. This heating regime is included in the production code for refrigerated, processed foods of extended durability (REPFEDS).

4.6.2 Product composition

Growth can be influenced depending on the composition of the product. The main parameters are:

- **pH:** no growth at pH <4.6 for *C. botulinum* Group I and <5.0 for *C. botulinum* Group II.
- **a_w**: no growth at a_w <0.94 for *C. botulinum* Group I and <0.97 for *C. botulinum* Group II.
- **Preservative agents:** curing agents for meat products, grow limiting additives such as lactate, etc.
- **Combinations** of pH, a_w and curing (preservative) agents.

These must be established throughout the product.

4.6.3 Storage conditions

Growth of *C. botulinum* Group I (proteolytic strains) cannot occur at temperatures lower than 10°C or higher than 42°C.

[4] The Fo value required for canned food products is equivalent to 12-decimal reductions of proteolytic *C. botulinum* spores. Using the highest known D-values (0.25 min at 121 °C) the Fo is therefore equal to 12 x 0.25 = 3. This is the so-called 12 D-concept designed to reduce the bacterial load of one billion spores in each of 1000 cans to 1 spore in a thousands cans.

Growth of *C. botulinum* Group II (non-proteolytic or saccharolytic strains) cannot occur at temperatures lower than 3°C or higher than 35–43°C, depending on the strain.

There is no universally agreed categorization of foods and food processes with respect to controlling *C. botulinum*. Two examples are provided to illustrate the wide range and combinations of controlling factors that have led to botulism being a rare intoxication. Botulism is most often associated with home preservation, traditional preservation, or failure of a commercial process (see Annex 5).

4.7 Microbiological Testing

C. perfringens

Microbiological testing has limited value in ensuring food safety, because *C. perfringens* is so common on foods that a positive result means little, unless very high numbers are present. An international standard for the enumeration in food and animal feeding stuffs is available: ISO 7937. Testing for the capacity to produce enterotoxin is possible by PCR methods although validated commercially available methods are not yet available.

Testing is appropriate when investigating outbreaks of illness. Outbreaks can be confirmed by: isolation of 10^6 organisms/g from the stools of two or more ill persons, if the provided specimen is properly handled, or demonstration of enterotoxin in the stools of two or more ill persons or isolation of 10^6 to 10^7 organisms/g from epidemiologically implicated food, provided the specimen is properly handled. Testing on behalf of the manufacturer is sometimes appropriate when designing a new process, or developing a new formulation (recipe) for a food. An alternative approach is a round table Risk Profile with appropriately experienced persons and a protocol for GMP.

C. botulinum

Detection of *C. botulinum* is still based on the presence of *C. botulinum* toxin in the "enrichment"-fluid of a test sample. The mouse-bio assay is still the method of choice for detecting the *C. botulinum* neurotoxin in enrichment cultures.

Cultural methods for the clostridia are generally poor and there is no cultural method specific for *C. botulinum*. Media containing sulphite are often recommended for clostridia but sulphite reduction to sulphide is inconsistent. Some strains of *C. botulinum*, particularly non-proteolytic (saccharolytic) strains in Group II, do not consistently form black colonies on such media. It is usually sufficient to demonstrate the presence of neurotoxin after enrichment culture and toxin tests (see below). In laboratory investigations using pure cultures, plate counts incubated anaerobically are adequate.

Microbiological testing for *C. botulinum* has limited value in ensuring food safety. Methods are not suited to routine food microbiology laboratories because it is necessary to test for the neurotoxin, and special safety precautions are necessary

(CDC, 1999a, 2000a). Furthermore the traditional test for toxin uses mice (Schantz and Kautter, 1978) and although other methods have been developed (see Table 10 in SCVPH (2002) Opinion on Honey and Microbiological Hazards", reproduced in Annex II)), no validated alternative method is yet available commercially.

Microbiological testing is appropriate in investigating outbreaks of illness. Outbreaks can be confirmed by the detection and neutralization of neurotoxin in patient's stool and serum samples or in the suspected food (for laboratory confirmation see CDC, 1998) Testing for growth and toxin production by *C. botulinum* is occasionally performed for a manufacturer designing a new process, developing a new formulation (recipe), or changing conditions of storage. Such work would be performed in a specialist laboratory with extreme biosecurity.

5 CONCLUSIONS

5.1 General

- The species of the genus *Clostridium* most commonly involved in food-borne intoxication are *C. perfringens* and *C. botulinum* and cause disease only after spores germinate and multiply in the food.

- In foods not subjected to severe heating processes, spores will be present from time to time; *C. botulinum* rarely and in low numbers, *C. perfringens* frequently and sometimes at numbers high enough to represent a hazard.

- Other species of the genus *Clostridium* that cause food spoilage have not been associated with illness.

- Good Agricultural Practices and Good Hygienic Practices minimise contamination with soil and animal faeces, but there is currently no technological solution to reducing the initial load of spores on raw agricultural products.

- Although herbs and spices may contain spores of *C. perfringens* and *C. botulinum*, levels are of the same order of magnitude as other raw food materials and it is very unlikely that using these ingredients will increase the risk of the associated foodborne illnesses.

ToR1: - identify the categories of foodstuffs and the food manufacturing and preparation processes where *Clostridium* spp. may pose a risk for human health.

- *C. perfringens* causes a short self-limiting illness that is rarely fatal except in the elderly, involving protein-rich foods such as cooked uncured meat and poultry and the associated gravy, casseroles, and pea soup, most often at food service establishments, care homes, or where there is communal cooking and feeding.

- *C. botulinum* is capable of growth and production of botulinal neurotoxin (BoNT) in essentially all foods except those with a natural pH value < 4.5 (e.g. some fruits) or a_w <0.94 for *C. botulinum* Group I and <0.97 for *C. botulinum* Group II.

- In commercially produced foods, botulism is usually the result of failure of part of the process or failure of container / pack integrity, and storage at temperatures allowing spore germination, multiplication and neurotoxin production.

- A high proportion of outbreaks of botulism are associated with home preservation.

- Several outbreaks of botulism have been traced to small food companies, where technological support may be weak.

- Outbreaks of botulism are also the result of preparation of traditional fermented foods that are eaten without heating, usually fish or marine mammals, where the fermentation is not under control, e.g. in Japan, the Nordic countries, N. America (especially British Columbia and Alaska) and elsewhere (e.g. Poland, Rep. Georgia, Iran, Israel).

ToR2: - establish, for the different categories of foodstuffs identified, the relation between the number of *Clostridium* spp. bacteria in the foodstuffs and the ability of these foodstuffs to cause food-borne illness.

- To cause diarrhoea, high numbers of enterotoxigenic vegetative cells of *C. perfringens* (10^8 cells) must be consumed. This can be avoided if the numbers of *C. perfringens* are kept below ca 10^5 /g through proper heating, rapid cooling and storage below 10°-12°C.

- *C. botulinum* causes a serious intoxication, sometimes fatal, with symptoms lasting for several months

- *C. botulinum*: due to the extreme toxicity of botulinal neurotoxin (BoNT), multiplication of *C. botulinum* in foods must be prevented.

ToR3: - list and evaluate specific control measures, including microbiological or other types of testing and time-temperature requirements, to manage the risk for human health caused by the presence of *Clostridium* spp. or their toxins in foodstuffs. In doing so attention should be paid to different species of clostridia.

C. perfringens:

- After cooking, foods should be cooled as quickly as possible through the temperature range over which *C. perfringens* multiplies (55°C to 15°C), and then stored below 10°-12°C.

- Rapid cooling is only possible if the portions / packs are relatively small and well separated. Alternatively, heating the food to >70°C throughout before consumption would inactivate enterotoxigenic vegetative cells and enterotoxin.

- If food is stored to be consumed on subsequent days, if stored below 10°-12°C, it may be consumed safely when cold (e.g. cooked uncured meats) or at room temperature. If storage conditions are not known and storage below 10°-12°C cannot be guaranteed, re-heat to 70-72°C (72°C in the centre of the product or throughout a liquid product).

- Codes of Good Manufacturing Practices (GMP) should always take into account the presence of *C. perfringens* in raw food ingredients and in final products.

- Food processors must ensure that the intended storage conditions (temperature and time) do not result in a risk to the consumer.

- Microbiological testing for *C. perfringens* may be used to verify that a HACCP-based system is in place and functioning effectively.

- Routine microbiological testing for *C. perfringens* is not recommended because it is present in almost all foods.

C. botulinum:

- *C. botulinum* is ubiquitous and its spores survive most food processing treatments. Presence of spores of *C. botulinum* in low numbers in foods is almost inevitable, but does not represent a hazard for consumers.

- The most reliable and safest option is to inactivate the spores of C. botulinum by heating (e.g. F_0 3.0 for Group I *C. botulinum* spores; 90°C for 10 min for Group II *C. botulinum* spores).

- The toxins of *C. botulinum* are relatively sensitive to heat and are inactivated by heating at 80°C for 10 min, or equivalent time/temperature conditions. If it is suspected that multiplication and neurotoxin production may have occurred and the food is not "spoiled", thorough heating would render the food safe to eat

- Codes of Good Manufacturing Practices (GMP) should always take into account the possible presence of *C. botulinum* in raw food ingredients and in final products (e.g. Huss *et al*., 1995).

- Food processor should re-evaluate the safety of foods when modifying a formulation (recipe), a process, or storage conditions.

- Food processors should use well-established Codes of Good Manufacturing Practice (e.g. see www.ifst.org; FDA, 1979; Codex, 1983)

and ensure that the intended storage conditions (temperature and time) do not result in a risk to the consumer. For new products where no experience exists, the use of predictive models and/or challenge testing can be considered.

- Historically a high percentage of botulism outbreaks have been associated with home-preserved foods (Sobel *et al*., 2004; Varma *et al*., 2004). Those practising home preservation should be advised of the risks from C. botulinum, should follow well-established practices and procedures, and should not deviate from those procedures.

- Growth of other *Clostridium* spp. that cause spoilage is also controlled by the same measures (pH, water activity and temperature). Although definitive data are lacking, spoilage by proteolytic clostridia is largely prevented by storage below 10°C (i.e. the same temperature as Group I (proteolytic) *C. botulinum*). Similarly, spoilage by non-proteolytic (saccharolytic) clostridia is largely prevented by storage below 3°-5°C.

- The analytical methods for *C. botulinum* mainly rely on production and detection of the neurotoxin and are not suited to routine food microbiology laboratories. Hence, routine testing of foods for *C. botulinum* does not seem appropriate

6 .RECOMMENDATIONS

- Routine testing of foods for *C. perfringens* and *C. botulinum*, or for "sulphite reducing clostridia", is not regarded as a cost-effective control measure and is not recommended.

- Similarly, the setting of microbiological criteria is not regarded as a cost-effective control measure and efforts should concentrate on establish HACCP-based systems with effective process controls and assured refrigerated storage for a defined time.

- Because domestic cooking does not destroy spores, cooked foods should be eaten soon after cooking, or kept hot (above 63°C), or cooled rapidly and kept below 7-8°C (ideally below 4°C to control microorganisms other than clostridia), preferably for a short time (a few days) before consumption.

- When new or modified products are developed, that might support the growth of *C. perfringens* and/or enterotoxin production, processors should ensure that target levels of 10^5/g are not exceeded under the anticipated conditions of storage and handling. These levels are particularly important in dehydrated foods to be reconstituted with hot water.

- Codes of Good Manufacturing Practices (GMP) should include recommendations not to overlook *C. perfringens* and *C. botulinum*, and, if relevant, provide information how to control these organisms.

- Data in the scientific literature on the limiting conditions for growth of clostridia other than *C. perfringens* and *C. botulinum* are inadequate and additional research effort to determine growth rates at pH values, water activities and temperatures representative of food formulations and storage conditions is desirable.

- It is recommended that testing for "surrogate" organisms e.g. "sulphite reducing clostridia" not be relied upon to reflect the behaviour of *C. botulinum* in foods

7 REFERENCES

Abrahamsson, K. and Riemann, H. (1971) Prevalence of *Clostridium botulinum* in semi-preserved meat products. Appl. Microbiol., 21, 543-544.

ACMSF (Advisory Committee on the Microbiological Safety of Food, UK). (1992) Report on vacuum packaging and associated processes. HMSO. London (UK).

Alderton, G., Chen, J.D. & Ito, K.A. (1974) Effect of lysozyme on the recovery of heated *Clostridium botulinum* spores. Appl. Microbiol., 27(3), 613-615.

Andersen, K.G., Hansen, T.B. and Knochel, S. (2004) Growth of heat-treated enterotoxin-positive Clostridium perfringens and the implications for safe cooling rates. J. Food Protect., 67(1):83-9.

Angulo, F.J., Getz, J., Taylor, J.P., Hendricks, K.A., Hatheway, C.L., Barth, S.S., Solomon, H.M., Larson, A.E., Johnson, E.A., Nickey, L.N. and Ries, A.A. (1998) A large outbreak of botulism: the hazardous baked potato. J. Infect. Dis., 178(1), 172-7.

Anon. (1998a) Fallericht: Botulismus nach dem Verzehr von geräucherten Lachsforellen. Epidemiol. Bull., 4/98, 20.

Anon. (2000) Foodborne botulism from eating home-pickled eggs--Illinois, 1997. MMWR Morb Mortal Wkly Rep., 49(34), 778-80.

Anon. (2002) Matförgiftning I Tierp. *EPI-aktuellt,* 1, 2-3.

Arnon, S.S. (1998) Infant botulism. In: "Textbook of Pediatric Infectious Diseases", 4th ed., (eds R.D. Feigen and J.D. Cherry), Saunders, W.B., Philadelphia, pp. 1570-1577.

Aureli, P., Fenicia, L., Pasolini, B., Ginafranceschi, M., McCroskey, L.M., Hatheway, C.L., (1986) Two cases of type E infant botulism in Italy caused by neurotoxigenic *Clostridium butyricum*. J. Infect. Dis., 54: 207-211.

Aureli, P., Di Cunto, M., Maffei, A., De Chiara, G., Franciosa, G., Accorinti, L., Gambardella, A.M. and Greco, D. (2000) An outbreak in Italy of botulism associated with a dessert made with mascarpone cream cheese. Eur. J. Epidemiol., 16(10), 913-8.

Austin, J.W., Dodds K. L., Blanchfield, B. and Farber J. M. (1998) Growth and toxin production by *Clostridium botulinum* on inoculated fresh-cut packaged vegetables. J. Food Protect., 61, 324-8.

Bauer, F.T., Carpenter, J.A. and Reagan, J.O. (1981) Prevalence of *Clostridium perfringens* in pork during processing. J. Food Protect., 44, 279-283.

Bean, P.G. (1983) Developments in heat treatment processes for shelf-stable products, pp. 97-112 in "Food Microbiology: Advances and Prospects" (eds T.A. Roberts and F.A. Skinner), Society for Applied Bacteriology Symposium Series No. 11, Academic Press, London.

Bhadsavle, C.H., Shehata, T.E. and Collins, E.B. (1972) Isolation and identification of psychrophilic species of *Clostridium* from milk. Appl. Microbiol. 24(5): 699-702.

Billon, J., Guérin, J. and Sebald, M. (1980) Etude de la toxinogénèse de *Clostridium botulinum* au cours de la maturation de fromages à pâte molle. Le Lait, 60, 392-396.

Blankenship, L.C., Craven, S.E., Leffler, R.G. and Custer, C. (1988) Growth of *Clostridium perfringens* in cooked chilli during cooling. Appl. Environ. Microbiol., 54(5), 1104-1108.

Boerema, J.A., Broda, D.M. and Bell, R.G. (2003) Abattoir sources of psychrophilic clostridia causing blown pack spoilage of vacuum-packed chilled meats determined by culture-based and molecular detection procedures. Lett. Appl. Microbiol., 36(6), 406-11.

Borriello, S.P., Honour, P. and Barclay, F. (1983) Foodborne transmission of *Clostridium difficile*. Gastroenterology, 84(1), 201.

Bradshaw, J.G., Stelma, G.N., Jones, V.I., Peeler, J.T., Wimsatt, J.C., Corwin, J.J. and Twedt, R.M. (1982) Thermal inactivation of *Clostridium perfringens* enterotoxin in buffer and in chicken gravy. J. Food Sci., 47(3), 914-916.

Braithwaite, P.J. and Perigo, J.A. (1971) The influence of pH, water activity and recovery temperature on the heat resistance and outgrowth of Bacillus spores, pp. 289-302 in "Spore Research 1971" (eds A.M. Barker, G.W. Gould and J. Wolf), Academic Press, London.

Brett, M. M. (1998) 1566 outbreaks of *Clostridium perfringens* food poisoning, 1970-1996. Proceedings 4th World Congress on Foodborne Infections and Intoxications. Berlin. 1, 243-244.

Brett, M.M. and Gilbert, R.J. (1997) 1525 outbreaks of *Clostridium perfringens* food poisoning, 1970-1996. Review of Medical Microbiology 8 Supplement 1 S64 - S65.

Brett, M.M., Hallas, G. and Mpamugo, O. (2004) Wound botulism in the UK and Ireland. J. Med. Microbiol., 53(6), 555-61.

Broda, D.M., DeLacy, K.M., Bell, R.G. and Penney, N. (1996a) Association of psychrotrophic *Clostridium* spp. with deep tissue spoilage of chilled vacuum-packed lamb. Int. J. Food Microbiol., 29(2-3), 371-8.

Broda, D.M., DeLacy, K.M., Bell, R.G., Braggins, T.J. and Cook, R.L. (1996b) Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int. J. Food Microbiol., 29(2-3),335-52.

Broda, D.M., De Lacy, K.M. and Bell, R.G. (1998) Influence of culture media on the recovery of psychrotrophic *Clostridium* spp. associated with the spoilage of vacuum-packed chilled meats. Int. J. Food Microbiol., 39(1-2), 69-78.

Broda, D.M., Musgrave, D.R. and Bell, R.G. (2000) Use of restriction fragment length polymorphism analysis to differentiate strains of psychrophilic and psychrotrophic clostridia associated with 'blown pack' spoilage of vacuum-packed meats. J. Appl. Microbiol., 88(1), 107-16.

Broda, D.M., Musgrave, D.R. and Bell, R.G. (2003a) Molecular differentiation of clostridia associated with "blown pack" spoilage of vacuum-packed meats using internal transcribed spacer polymorphism analysis. Int. J. Food Microbiol., 84(1), 71-7.

Broda, D.M., Boerema, J.A. and Bell, R.G. (2003b) PCR detection of psychrophilic *Clostridium* spp. causing 'blown pack' spoilage of vacuum-packed chilled meats. J. Appl. Microbiol., 94(3), 515-22.

Brown, K.L. (2000) Control of bacterial spores, pp. 158-171 in "Health and the Food Chain", (eds D.I. Thurman and T.A. Roberts), British Medical Bulletin, 56 (1).

Bryan, F.L. and Kilpatrick, E.G. (1971) *Clostridium perfringens* related to roast beef cooking, storage, and contamination in a fast food service restaurant. American Journal of Public Health, 61:1869-1885.

Brynestad, S. and Granum, P.E. (2002) *Clostridium perfringens* and foodborne infections. Int. J. Food Microbiol., 74(3), 195-202. Review. PMID: 11981970

Campbell, K.D., East, A.K., Thompson D.E. & Collins, M.D. (1993) Studies on the large subunit ribosomal RNA genes and intergenic spacer regions of non-proteolytic *Clostridium botulinum* types B, E, and F. Research in Microbiology 144 171-180.

Carlin, F. and Peck, M.W. (1995) Growth and toxin production by non-proteolytic and proteolytic *Clostridium botulinum* in cooked vegetables. Letters in Appl. Microbiol., 20, 152-6.

Carlin, F., Broussolle, V., Perelle, S., Litman, S. and Fach, P. (2004) Prevalence of *Clostridium botulinum* in food raw materials used in REPFEDs manufactured in France. Int. J. Food Microbiol., 91(2), 141-5.

CDC (1973) Botulism traced to commercially canned mushrooms. Morbid. Mortal. Week. Rep., 22, 241-2.

CDC (1987) Restaurant associated botulism from mushrooms bottled in-house – Vancouver, British Columbia, Canada. Morbid. Mortal. Week. Rep., 36, 103.

CDC (1995) Type B botulism associated with roasted egg-plant in oil – Italy, 1993. MMWR, 44(2), 33-36.

CDC, (1973). Botulinal toxin in a commercial food product. MMWR. 22, 57-58

CDC, (1984). Foodborne Botulism - Illinois 1984. MMWR 33(2), 22-3

CDC (1998) Botulism in the United States, 1899-1996. Handbook for epidemiologists, clinicians, and laboratory workers. CDC, Division of Bacterial and Mycotic Diseases, Atlanta, USA).

CDC (2000) Guide to Confirm the Diagnosis of Foodborne Diseases, Foodborne Outbreak Response and Surveillance Unit, CDC Atlanta, USA.

CDC (1999a) Biosafety in Microbiological and Biomedical Laboratories 4th Edition, Office of Health and Safety, CDC Atlanta, USA.

CDC, (1999b). Foodborne Botulism Associated With Home-Canned Bamboo Shoots - Thailand, 1998. MMWR, 48(21), 437-439

CDC, (2000). Foodborne botulism from eating home-pickled eggs -Illinois, 1997. MMWR 49(34), 778-780.

CDC, (2001). Botulism outbreak associated with eating fermented food – 2001. MMWR 50(32), 680-2

CDC, (2002). Outbreak of Botulism Type E associated with eating a beached whale - Western Alaska, July 2002. MMWR, January 17, 2003 / 52(02);24-26

Chapman, B. (2001) Anaerobic spore-forming rods, Chapter 5.2 pp. 295-306 in "Spoilage of Processed Foods: Causes and Diagnosis", Food Microbiology Group, Australian Institute of Food Science and Technology (NSW Branch). ISBN 0-9578907-0-2, www.aifst.asn.au

Chia, J.K., Clark, J.B., Ryan, C.A., Pollack, M. (1986) Botulism in an adult associated with food-borne intestinal infection with *Clostridium botulinum*. New Engl. J. Med., 315: 239-241.

Chin, J., Arnon, S.S., Midura, T.F. (1979) Food and environmental aspects of infant botulism in California. Rev. Infect. Dis., 1: 693-696.

Chiou, L.A., Hennessy, T.W., Horn, A., Carter, G. and Butler, J.C. (2002) Botulism among Alaska natives in the Bristol Bay area of southwest Alaska: a survey of knowledge, attitudes, and practices related to fermented foods known to cause botulism. Int. J. Circumpolar Health, 61(1), 50-60. PMID: 12002947

Chou, J.H., Hwang, P.H. and Malison, M.D. (1988) An outbreak of type A foodborne botulism in Taiwan due to commercially preserved peanuts. Int. J. Epidemiol., 17, 899-902.

Clifford, W.J. and Anellis, A. (1975) Radiation resistance of spores of some *Clostridium perfringens* strains. Appl. Microbiol., 29(6), 861-863.

Codex (Codex Alimentarius Commission) (1983) Recommended International Code of Hygienic Practice for Low-Acid and Acidified Canned Foods. 1st edn. CAC/RCP 23-1979, FAO, Rome.

Collins, M.D. and East, A.K. (1998) Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J Appl Microbiol., 84(1), 5-17.

Collins, M.D., Rodriguez, U.M., Dainty, R.H., Edwards, R.A. and Roberts, T.A. (1992) Taxonomic studies on a psychrotrophic *Clostridium* from vacuum-packed beef: Description of *Clostridium* estertheticum sp. nov. FEMS Microbiology Letters, 96: 235-240.

Dainty, R.H., Edwards, R.A. and Hibbard, C.M. (1989) Spoilage of vacuum-packed beef by a *Clostridium* sp. Journal of the Science of Food and Agriculture, 49:473-486.

Danler, R.J., Boyle, E.A., Kastner, C.L., Thippareddi, H., Fung, D.Y. and Phebus, R.K. (2003) Effects of chilling rate on outgrowth of *Clostridium perfringens* spores in vacuum-packaged cooked beef and pork. J. Food Protect., 66(3):501-3.

Dawar, M., Moody, L., Martin, J.D., Fung, C., Isaac-Renton, J. and Patrick, D.M. (2002) Two outbreaks of botulism associated with fermented salmon roe – British Columbia, August 2001. Can. Commun. Dis. Rep., 28(6), 45-49.

Damme-Jongsten, M. v., Haagsma, J. and Notermans, S.H.W. (1990a) Testing strains of *Clostridium perfringens* type A isolated from diarrhoeic piglets for the presence of the enterotoxin gene. Vet. Rec., 126, 191-192.

Damme-Jongsten, M. v., Rodhouse, J., Gilbert, R.J. and Notermans, S.H.W. (1990b) Synthetic DNA probes for detection of enterotoxigenic *Clostridium perfringens* strains isolated from outbreaks of food poisoning. J. Clin. Microbiol., 28, 131-133.

de Jong, A.E.I. (2003) *Clostridium perfringens*: Spores & cells, media & modelling. Thesis Wageningen University, The Netherlands.

de Jong, A.E., Rombouts, F.M. and Beumer, R.R. (2004) Effect of cooling on *Clostridium perfringens* in pea soup. J Food Protect., 67(2), 352-6.

de Lagarde, E.A. (1974) Boletin Informativo del Centro Panamericano de Zoonosis, Vol. 1. Centro Panamericano de Zoonosis, Buenos Aires, Argentina.

Dodds, K.L. (1993) *Clostridium botulinum* in foods. pp. 53-68 in "*Clostridium botulinum*: Ecology and Control in Foods", (eds A.H.W. Hauschild and K.L. Dodds), Marcel Dekker Inc., New York.

Dolman, C.E. and Iida, H. (1963) Type E botulism: Its epidemiology, prevention and specific treatment. Can. J. Publ. Hlth., 54, 293-308.

Dolzhkevich, N.Ia, Lobarets, I.M, Tkachenko, V.P. (1978) Botulism caused by home-preserved stewed pork. Gig Sanit. 6:93-4.

Duynhoven, Y.T.H.P.v. and de Wit, S.M.A.S. (1998). Registratie van voedselinfecties en –vergiftigingen onderzocht door de GGD's en Regionale Inspecties Gezondheidsbescherming/Keuringsdientsen van Waren, !997, RIVM, Bilthoven, The Netherlands

Dymicky, M. and Huhtanen, C.N. (1979) Inhibition of *Clostridium botulinum* by p-hydroxybenzoic acid esters and n-alkyl esters. Antimicrobial Agents and Chemotherapy, 15(6), 798-801.

East, A.K., Thompson, D.E. & Collins, M.D. (1992) Analysis of operons encoding 23S rRNA of *Clostridium botulinum* type A. J. Bact., 174, 8158-8162.

ECFF (European Chilled Food Federation) (1996) Guidelines for the hygienic manufacture of chilled foods. European Chilled Food Federation, London, United Kingdom.

Ecklund, M.W. (1993) Control in fishery products. pp. 209-232 in "*Clostridium botulinum*: Ecology and Control in Foods", (eds A.H.W. Hauschild and K.L. Dodds), Marcel Dekker Inc., New York.

EFSA, (2003). Opinion of the Scientific Panel on Biological Hazards on a request from the Commission related to the Effects of Nitrites/Nitrates on the Microbiological Safety of Meat products. The EFSA Journal, 14, 1-31.

Ernst, H. (1974) *Clostridium perfringens* in a pipeline, and its implication in spoilage of sausages. *Clostridium perfringens* in einer Rohrleitung - Ursache fuer ungeklaerten Wurstverderb. Fleisch., 28 (11) 214-215.

Eurosurveillance Weekly archives (2001),Volume 5 / Issue 15

Fenicia, L., Ferrini, A.M., Aureli, P. & Padovan, M.T. (1992) Botulism outbreak from black olives (Italian). Ind. Aliment., 31, 307-308.

FDA (Food and Drug Administration) (1979) Rules and Regulations 21CFR Part 113 – Thermally Processed Low-Acid Foods Packaged in Hermetically Sealed Containers: Current Good Manufacturing Practice. Federal Register, 44, No. 53, 16209-16238.

Fruin, J.T. (1977) Significance of *Clostridium perfringens* in processed foods. J. Food Protect., 40(5), 330-332 (review).

FSIS (Food Safety and Inspection Service) (1999) Annex B: Compliance Guidelines for Cooling Heat-Treated Meat and Poultry Products (Stabilization). FSIS/USDA, Washington, DC.

Ghanem, F.M., Ridpath, A.C., Moore, W.E.C. & Moore, L.V.H. (1991) Identification of *Clostridium botulinum*, *Clostridium argentinense*, and related organisms by cellular fatty acid analysis. J. Clin. Microbiol., 29, 1114-1124.

Gibson, A.M. & Roberts, T.A. (1986) The effect of pH, sodium chloride, sodium nitrite and storage temperature on the growth of *Clostridium perfringens* and faecal streptococci in laboratory media. Int. J. Food Microbiol., 3(4), 195-210.

Gilbert, R.J., Rodhouse, J.C. and Haugh, C.A. (1990) Anaerobes in food poisoning, in "Clinical and Medical Aspects of Anaerobes: VI Biennial Anaerobe Discussion Group", International Symposium, University of Cambridge, 20-22 July 1989, Wrighton Biomedical Publications Ltd., Petersfield, UK.

Gill, D.M. (1982) Bacterial toxins: A table of lethal amounts. Microbiol. Rev., 46(1), 86-94.

Glass, K.H. and Doyle, M.P. (1991) Relationship between water activity of fresh pasta and toxin production by proteolytic *Clostridium botulinum*. J. Food Protect., 54, 162-5.

Gould, G.W. (ed) (1995) New Methods of Food Preservation. Blackie Academic & Professional, London.

Gould, G.W., Jones, A. & Wrighton, C. (1968) Limitations of the initiation of germination of bacterial spores as a spore control procedure. J. Appl. Bact., 31, 357-366.

Graham, A.F., Mason, D.R. and Peck, M.W. (1996) Inhibitory effect of combinations of heat treatment, pH, and sodium chloride on a growth from spores of non-proteolytic *Clostridium botulinum* at refrigeration temperature. Appl. Environ. Microbiol., 62(7), 2664-8.

Graham, A.F., Mason, D.R., Maxwell, F.J., and Peck, M.W. (1997) Effect of pH and NaCl on growth from spores of non-proteolytic *Clostridium botulinum* at chill temperature. Lett. Appl. Microbiol., 24, 95-100.

Granum, P.E. (1990) *Clostridium perfringens* toxins involved in food poisoning. Int. J. Food Microbiol., 10(2), 101-112 (review).

Granum, P.E. and Skjelkvale, R. (1977) Chemical modification and characterization of enterotoxin from *Clostridium perfringens* type A. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 85, 89-94.

Hall, J.D., McCroskey, L.M., Pincomb, B.J., Hatheway, C.L. (1985) Isolation of an organism resembling *Clostridium* baratii which produces type F botulinal toxin from an infant with botulism. J. Clin. Microbiol., 21: 654-655.

Hashimoto, H., Clyde, V.J. and Parko, K.L. (1998) Botulism from peyote. N. Engl. J. Med., 339(3), 203-4.

Hatheway, C.L. (1993) *Clostridium botulinum* and other clostridia that produce *botulinum* neurotoxin. pp. 3-20 in "*Clostridium botulinum*: Ecology and Control in Food", (eds A.H.W. Hauschild and K.L. Dodds), Marcel Dekker Inc., New York.

Hatheway, C.L., McCroskey, L.M. (1987) Examination of feces and serum for diagnosis of infant botulism in 336 patients. J. Clin. Microbiol., 25, 2334-2338.

Hauschild, A.H.W. (1989) *Clostridium botulinum*. in "Foodborne Bacterial Pathogens" (ed. M.P. Doyle), Marcel Dekker, New York, pp.111-89.

Hauschild, A.H.W. (1993) Epidemiology of human botulism, pp. 69-104 in "*Clostridium botulinum*: Ecology and Control in Foods" (eds A.H.W. Hauschild and K.L. Dodds), Marcel Dekker Inc., New York.

Hauschild, A.H.W. & Dodds, K.L. (eds) (1993) *Clostridium botulinum*: Ecology and control in foods. (1993) Marcel Dekker, New York.

Hauschild, A.H.W. & Simonsen, B. (1985) Safety of shelf-stable canned cured meats. J. Food Protect., 48 (11), 997-1009.

Hielm,S., Bjorkroth, J., Hyytia, E. and Korkeala, H. (1998). Prevalence of *Clostridium botulinum* in Finnish trout farms: pulsed-field gel electrophoresis typing reveals extensive genetic diversity among type E isolates. Appl. Environ. Microbiol., 64(11), 4161-4167.

Hobbs, B.C., Smith, M.E., Oakley, C.L., Warrack, G.H. and Cruickshank, J.C. (1953). *Clostridium welchii* food poisoning. J. Hyg., (Cambridge), 51, 75-101.

Houtsma, P.C., Heuvelink, A., Dufrenne, J. and Notermans, S. (1994). Effect of sodium lactate on toxin production, spore germination and heat resistance of proteolytic *Clostridium botulinum* strains. Journal of Food Protection, 57, 327-330.

Huss, H.H., Pedersen, A. and Cann, D.C. (1974a). The incidence of Cl. *botulinum* in Danish trout farms. II: measures to reduce the contamination of the fish. Journal of Food Technology, 9:451-458.

Huss, H.H., Dalsgård, D., Hansen, L., Ladefoged, H., Pedersen, A. and Zittan, L. (1974b). The influence of hygiene in catch handling on the storage of cod and plaice. Journal of Food Technology, 9:213-221.

Huss, H.H., Ben Embarek, P.K. and From Jeppesen, V. (1995). Control of biological hazards in cold-smoked salmon production. Food Control, 6:335-340.

Hutson, R.A., Thompson, D.E. & Collins, M.D. (1993a). Genetic interrelationships of saccharolytic *Clostridium botulinum* types B, E and F and related clostridia as revealed by small-subunit rRNA sequences. FEMS Microbiology Letters, 108, 103-110.

Hutson, R.A., Lawson, P.A., Schocken-Itturino, R.P., Böttger, E.C. & Collins, M.D. (1993b). Genetic interrelationships of proteolytic *Clostridium botulinum* types A, B, and F and other members of the *Clostridium botulinum* complex as revealed by small-subunit rRNA gene sequences. Antonie van Leewenhoek, 64, 273-283.

ICMSF (International Commission on Microbiological Specifications for Foods) (1996). Microorganisms in Foods 5: Characteristics of Microbial Pathogens. Blackie Academic & Professional, London.

ICMSF (International Commission on Microbiological Specifications for Foods) (1998). Microorganisms in Foods 6: Microbial Ecology of Food Commodities. Blackie Academic & Professional, London.

ICMSF (International Commission on Microbiological Specifications for Foods) (2005). Microorganisms in Foods 6: Microbial Ecology of Food Commodities, 2nd edn. Kluwer Academic/Plenum Publishers, New York.

IFST (Institute of Food Science and technology ,UK) (1993) Listing of Codes of Practice Applicable to Foods, IFST, London.

Ivey, F.J., Shaver, K.J., Christiansen, L.N. and Tompkin, R.B. (1978). Effect of potassium sorbate on toxinogenesis by *Clostridium botulinum* in bacon. Journal of Food Protection, 41(8), 621-625.

Jahkola, M. and Korkeala, H. (1997). Botulismi saksassa suomessa pakatusta savusiiasta. Kansanterveys, 3: 8-9.

Jarvis, B., Rhodes, A.C., King, S.E. & Patel, M. (1976). Sensitization of heat-damaged spores of *Clostridium botulinum* type B to sodium chloride and sodium nitrite. J. Food Technol., 11(1), 41-50.

Johannsen, A. (1963). *Clostridium botulinum* in Sweden and the adjacent waters. J. Appl. Bact., 26, 43-47.

Jong, J. de (1989) Spoilage of an acid food product by *Clostridium perfringens*, *C. barati* and *C. butyricum*. International Journal of Food Microbiology, 8 (2) 121-132.

Juneja, V. K. and Marmer, B. S. (1996). Growth *Clostridium perfringens* from spore inocula in sous-vide turkey products. International Journal of Food Protection 32:115-123.

Kalchayanand, N., Ray, B., Field, R.A. and Johnson, M.C. (1989). Spoilage of vacuum-packed beef by a *Clostridium*. Journal of Food Protection, 52, 424-426.

Kalchayanand, N., Ray, B. and Field, R.A. (1993). Characteristics of psychrotrophic *Clostridium* laramie causing spoilage of vacuum-packaged refrigerated fresh and roasted beef. Journal of Food Protection, 56:13-17.

Kalinowski, R. and Tompkin, R.B. (1999). Psychrotrophic clostridia causing spoilage in cooked meat and poultry products. J. Food Protect., 62(7): 766-772.

Kalinowski, R.M., Tompkin, R.B., Bodnaruk, P.W. and Pruett, W.P. Jr. (2003). Impact of cooking, cooling, and subsequent refrigeration on the growth or survival of *Clostridium perfringens* in cooked meat and poultry products. J. Food Prot., 66(7):1227-32.

Keoseyan, S.A. (1971). Incidence of *Clostridium perfringens* in dehydrated soup, gravy, and spaghetti mixes. J.A.O.A.C., 54, 106-8.

Knorr, D. (1995). Hydrostatic pressure treatment of food: microbiology, pp. 159-175 in "New Methods of Food Preservation", (ed. G.W. Gould), Blackie Academic & Professional, London.

Knorr, D. (1999). Novel approaches in food-processing technology: new technologies for preserving foods and modifying function. Curr. Opin. Biotechnol., 10(5), 485-491.

Knox, R. and MacDonald, E.K. (1943). Outbreaks of food poisoning in certain Leicester institutions. Med. Offr., 69, 21-22.

Korkeala, H., Stengel, G., Hyytia, E., Vogelsang, B., Bohl, A., Wihlman, H., Pakkala, P. and Hielm, S. (1998). Type E botulism associated with vacuum-packaged hot-smoked whitefish. Int. J. Food Microbiol., 43(1-2), 1-5.

Kudrow, D.B., Henry, D.A., Haake, D.A., Marshall, G. and Mathisen, G.E. (1988). Botulism associated with *Clostridium botulinum* sinusitis after intranasal cocaine abuse. Ann. Intern. Med., 109(12), 984-5.

Labbe, R.L. (2000). *Clostridium perfringens*, Chapter 42, pp. 1110-1135 in "The Microbiological Safety and Quality of Food", Volume 2, (eds. B.M. Lund, T.C. Baird-Parker and G.W. Gould); Aspen Publishers, Maryland.

Larsen, A. E. and Johnson, E. A. (1999). Evaluation of botulinal toxin production in packaged fresh-cut cantaloupe and honeydew melons. J. Food Protect. 62, 948-952.

Larsen, A.E., Johnson, E.A., Barmore, C.R., and Hughes, M.D. (1997). Evaluation of the botulism hazard from vegetables in modified atmosphere packaging. J. Food Prot., 60, 1208-14.

Lawson, P.A., Llop-Perez, P., Hutson, R.A., Hippe, H., Collins, M.D., (1993) Towards a phylogeny of the clostridia based on 16S rRNA sequences. FEMS Microbiol. Lett., 113, 87-92.

Lawson, P., Dainty, R.H., Kristiansen, N., Berg, J. and Collins, M.D. (1994). Characterization of a psychrotrophic *Clostridium* causing spoilage in vacuum-packed cooked pork: description of *Clostridium* algidicarnis sp. nov. Lett. Appl. Microbiol., 19:153-157.

Leistner, L. and Russell, N.J. (1991) Solutes and low water activity, pp.111-134 in "Food Preservatives" (eds N.J. Russell and G.W. Gould), Blackie, London.

Lohse N, Kraghede PG, Molbak K. (2003). Botulism an a 38-year-old man after ingestion of garlic in chilli oil. Ugeskr Laeger., 165(30), 2962-3.

Losikoff, M.E. (1978). Establishment of a heat inactivation curve for *Clostridium botulinum* 62A toxin in beef broth. Appl. Environ. Microbiol., 36(2), 386–388.

Loutfy,M. R., Austin, J.W., Blanchfield, B. and Fong, I.W., 2003. An outbreak of foodborne botulism in Ontario. Infectious Diseases & Medical Microbiology, 14, 206-209

Lücke, F.-K. and Roberts, T.A. (1993). Control in meat and meat products, pp. 177-207 in "*Clostridium botulinum*: Ecology and Control in Foods", (eds A.H.W. Hauschild and K.L. Dodds), Marcel Dekker Inc., New York.

Lund, B.M. and Peck, M.W. (2000). *Clostridium botulinum*. Chapter 41, pp. 1057-1109 in "The Microbiological Safety and Quality of Food", Volume 2, (eds. B.M. Lund, T.C. Baird-Parker and G.W. Gould); Aspen Publishers, Maryland.

Lund, B.M. and Notermans, S.H.W. (1993). Potential hazards associated with REPFEDs, chapter 11, pp. 279-303 in "*Clostridium botulinum*: Ecology and

Control in Foods", (eds A.H.W. Hauschild and K.L. Dodds), Marcel Dekker Inc., New York..

Maas, M.R., Glass, K.A. and Doyle, M.P. (1989). Sodium lactate delays toxin production by *Clostridium botulinum* in cook-in-bag turkey products. Appl. Environ. Microbiol., 55(9), 2226-2229.

MacDonald, K.L., Spengler, R.F., Hatheway, C.L., Hargrett, N.T. and Cohen, M.L. (1985). Type A botulism from sautéed onions: clinical and epidemiologic observations. J. Am. Med. Assoc., 253, 1275-8.

MacDonald. K.L., Cohen, M.L. and Blake, P.A. (1986) The changing epidemiology of adult botulism in the United States. Am. J. Epidemiol., 124, 794-9.

Mangas-Gallardo, I. And Hernandez-Pezzi, G. (1995) Vigilancia del botulismo en España, Años 1992-1994. Bol. Epidemiol. Sem., 3, 209-212.

Matveev, K.I., Nefedjeva, N.P., Bulatova, T.I., Sokolov, I.S., (1967). Epidemiology of botulism in the USSR. pp. 1-10 in "Botulism 1966", Proceedings of the Fifth International Symposium on Food Microbiology, Moscow, July 1966 (eds M. Ingram and T.A. Roberts), Chapman and Hall, London.

McBryde, C.N. (1911). A bacteriological study of ham souring. U.S. Bureau of Animal Industry, Bulletin No. 132.

McClane, B.A. (1992). *Clostridium perfringens* enterotoxin: structure, action and detection. J. Food Safety, 12(3), 237-252 (review).

McClung, L.S. (1945). Human food poisoning due to the growth of *Clostridium perfringens* (C. welchii) in freshly cooked chicken. Preliminary note. J. Bact., 50, 229-231.

McCroskey, L.M., Hatheway, C.L., (1988). Laboratory findings in four cases of adult botulism suggest colonisation of the intestinal tract. J. Clin. Microbiol., 26: 1052-1054.

McDonell, J.L. (1974) In: McDonell, J.L. (1980). Mechanism of action of *Clostridium perfringens* enterotoxin. Food Technol., 34, 91-95.

McLean, H.E., Peck, S., Blatherwick, F.J. *et al.* (1987). Restaurant-associated botulism from in-house bottled mushrooms – British Columbia. Can. Commun. Dis. Rpt., 13-8, 35-36.

Meng, J. and Genigeorgis, C.A. (1993). Modeling lag phase of non-proteolytic *Clostridium botulinum* toxinogenesis in cooked turkey and chicken breast as affected by temperature, sodium lactate, sodium chloride and spore inoculum. Int. J. Food Microbiol. 19, 109-122.

Merson, M.H., Dowell, V.R. Jr. (1973). Epidemiological, clinical and laboratory aspects of wound botulism. New Engl. J. Med., 289, 1005-1010.

Morse, D.L., Pickard, L.K., Guzewich, J.J., Devine, B.D., Shayegani, M. (1990) Garlic-in-oil associated botulism: episode leads to product modification. Amer. J. Publ. Hlth., 80(11), 1372-3.

Mulleague, L., Bonner, S.M., Samuel, A., Nichols, P., Khan, M., Shaw, S. and Gruning, T. (2001) Wound botulism in drug addicts in the United Kingdom. Anaesthesia, 56(2), 120-3. (Comment in: Anaesthesia, 2002, 57(3), 301-2.

Naik, H.S. and Duncan, C.L. (1978) Thermal inactivation of *Clostridium perfringens* enterotoxin. J. Food Protect., 41(2), 100-103.

Nelson, K.A., Busta, F.F., Sofos, J.N. and Wagner, M.K. (1983) Effect of polyphosphates in combination with nitrite or sorbate on *Clostridium botulinum* growth and toxin production in chicken frankfurter emulsions. J. Food Protect., 46(10), 846-850.

Notermans, S., Veld, P., Wijtzes, T. and Mead, G.C. (1993) A user's guide to microbiological challenge testing for ensuring the safety and stability of food products. Food Microbiol., 10(2), 145-157.

Oakley, C.L. and Warrack, G.H. (1953) Routine typing of *Clostridium* welchii. J. Hyg., (Cambridge), 51, 102-107.

Odlaug, T.E. and Pflug, I.J. (1978) *Clostridium botulinum* and acid foods. J. Food Protect., 41: 566-573.

O'Mahony, M., Mitchell, E., Gilbert, R.J., Hutchinson, D.N., Begg, N.T., Rodhouse, J.C. and Morris, J.E. (1990) An outbreak of foodborne botulism associated with contaminated hazelnut yoghurt. Epidemiol. Inf., 104, 389-395.

Otofuji, T., Tokiwa, H. and Takahashi K (1987) A food-poisoning incident caused by *Clostridium botulinum* toxin A in Japan. Epidemiol. Inf., 99, 167-72. (mustard prepared with fried lotus rhizomes)

Pace, P.J., Duncan, C.L., Dowell, V.R., Antonmattei, J.C. and Wisniewski, H.J. (1976) *Perfringens* illness associated with beef prime rib served in a restaurant. J. Milk Food Technol., 39(4), 305 (letter).

Passaro, D.J., Werner, S.B., McGee, J., MacKenzie, W.R. and Vugia, D.J. (1998) Wound botulism associated with black tar heroin among injecting drug users. JAMA., 279(11), 859-63.

Paterson, D.L., King, M.A., Boyle, R.S., Pond, S.M., Whitby, M., Wright, M. and Henderson, A. (1992) Severe botulism after eating home-preserved asparagus. Med. J. Aust., 157(4), 269-70.

Petran, R.L., Sperber, W.H. and Davis, A.B. (1995) *Clostridium botulinum* toxin formation in Romaine lettuce and shredded cabbage: effect of storage and packaging conditions. J. Food Prot., 58, 624-7.

Pierson, C.B. and Walker, H.W. (1976) Effect of oxidation-reduction potential on growth and sporulation of *Clostridium perfringens*. J. Milk Food Technol., 39, 421-425.

Pierson, M.D. and Reddy, N.R. (1982) Inhibition of *Clostridium botulinum* by antioxidants and related phenolic compounds in comminuted pork. J. Food Sci., 47, 1926-1929 and 1935.

Pourshafie, M.R., Saifie, M., Shafiee, A., Vahdani, P., Aslani, M. and Salemian, J. (1998) An outbreak of food-borne botulism associated with contaminated locally made cheese in Iran. Scand. J. Infect. Dis., 30(1), 92-4.

Prévot, A.R. (1953) Rapport d'introduction du Président du Sous-Comité *Clostridium* pour l'unification de la nomenclature des types toxigeniques de C. *botulinum*. International Bulletin of Bacteriological Nomenclature and Taxonomy, 3: 120-123.

Proulx, J.F., Milot-Roy, V. and Austin, J. (1997) Four outbreaks of botulism in Ungava Bay, Nunavik, Quebec. Can. Commun. Dis. Rep., 23-4: 30-32.

Przybylska, A. (2004) Botulism in Poland in 2002. Przegl. Epidemiol., 58,103-10.

Reddy, N.R., Pierson, M.D. and Lechowich, R.V. (1982) Inhibition of *Clostridium botulinum* by antioxidants, phenols and related compounds. Appl. Environ. Microbiol., 43(4), 835-839.

Reddy, N.R., Armstrong, D.J., Rhodehamel, E.J. and Kautter, D.A. (1992) Shelf-life extension and safety concerns about fresh fishery products packaged under modified atmospheres: A review. Journal of Food Safety, 12:87-118.

Rhodehamel, E.J., Reddy, N. and Pierson, M.D. (1992) Botulism: the causative agent and its control in foods. Food Control, 3, 125-143.

Riemann, H., Lee, W.H. and Genigeorgis, C.A. (1972) Control of *Clostridium botulinum* and Staphylococcus aureus in semi-preserved meat products. J. Milk Food Technol., 35(9), 514-523.

Riha,W.E. Jr and Solberg, M. (1975) *Clostridium perfringens* inhibition by sodium nitrite as a function of pH, inoculum size and heat. J. Food Sci., 40 (3) 439-442.

Robach, M.C. and Pierson, M.D. (1978) Influence of parahydroxybenzoic acid esters on the growth and toxin production of *Clostridium botulinum* 10755A. J. Food Sci., 43(3), 787-789 and 792.

Robach, M.C. and Pierson, M.D. (1979) Inhibition of *Clostridium botulinum* types A and B by phenolic antioxidants. J. Food Protect., 42(11), 858-861.

Roberts, T.A. & Derrick, C.M. (1975) Sporulation of *Clostridium* putrefaciens NCTC 9836, and the resistance of the spores to heat, gamma radiation and curing salts. J. Appl. Bact., 38: 33-37.

Roberts, T.A. and Derrick, C.M. (1978) The effect of curing salts on the growth of *Clostridium perfringens* (welchii) in a laboratory medium. J. Food Technol., 13(4). 349-353.

Roberts, T.A. & Ingram, M. (1973) Inhibition of growth of *Clostridium botulinum* at different pH values by sodium chloride and sodium nitrite. J. Food Technol., 8, 467-475.

Roberts, T.A. and Gibson, A.M. (1979) The relevance of *Clostridium botulinum* type C in public health and food processing. J. Food Technol., 14, 211-226.

Roberts, T.A. and Smart, J.L. (1976) The occurrence and growth of *Clostridium* spp. in vacuum-packed bacon with particular reference to Cl. *perfringens* (welchii) and Cl. *botulinum*. J. Food Technol., 11(3), 229-244.

Roberts, T.A., Jarvis, B. & Rhodes, A.C. (1976) Inhibition of *Clostridium botulinum* by curing salts in pasteurized pork slurry. J. Food Technol., 11(1), 25-40.

Roberts, D., Watson, G.N. & Gilbert, R.J. (1982) Contamination of food plants and plant products with bacteria of public health concern, in *Bacteria and Plants* (eds M.E. Rhodes-Roberts and F.A. Skinner). Academic Press, London, pp.169-95.

Roberts, E., Wales, J.M., Brett, M.M. and Bradding, P. (1998) Cranial-nerve palsies and vomiting. Lancet, 352(9141), 1674.

Rodriguez-Romo, L.A., Heredia, N.L., Labbe, R.G. and Garcia-Alvarado, J.S. (1998) Detection of enterotoxigenic *Clostridium perfringens* in spices used in Mexico by dot blotting using a DNA probe. J. Food Prot., 61(2), 201-204.

Ross, H.E. (1965) *Clostridium* putrefaciens: a neglected anaerobe. J. Appl. Bact., 28, 49-51.

Ross, A.I., Griffiths, M.W., Mittal, G.S and Deeth, H.C. (2003) Combining nonthermal technologies to control foodborne microorganisms. Int. J. Food Microbiol., 89(2-3), 125-138.

Sarker, M.R., Singh, U. and McClane, B.A. (2000) An update on *Clostridium perfringens* enterotoxin. J. Nat. Toxins, 9(3), 251-66.

Sauter, E.A., Kemp, J.D. and Langlois, B.E. (1977) Effect of nitrite and erythorbate on recovery of *Clostridium perfringens* spores in cured pork. J. Food Sci., 42(6), 1678-1679.

Schantz, E.J. & Sugiyama, H. (1974) The toxins of *Clostridium botulinum*. Essays Toxicol., 5, 99-199.

Schantz, E.J., Kautter, D.A. (1978) Standardized assay for *Clostridium botulinum* toxins. J. AOAC Int., 61, 96-99.

Scott, V.N. and Taylor, S.L. (1981) Temperature, pH, and spore load effects on the ability of nisin to prevent the outgrowth of *Clostridium botulinum* spores. J. Food Sci., 46, 121-126.

Scott, V.N. and Bernard, D.T. (1985) The effect of lysozyme on the apparent heat resistance of non-proteolytic type B *Clostridium botulinum*. J. Food Safety 7: 145.

SCVPH (2002) Opinion of the Scientific Committee on measures related to Public Health on Honey and Microbiological Hazards, adopted June 19-20 2002.

Seals, J.E., Snyder, J.D., Edell, T.A., Hatheway, C.L., Johnson, C.J., Swanson, R.C. and Hughes, J.M. (1981) Restaurant-associated type A botulism: transmission by potato salad. Amer. J. Epidemiol., 113(4), 436-44.

Siegel, L.S. (1993) Destruction of *botulinum* toxins in food and water, pp. 323-341 in "*Clostridium botulinum*: Ecology and Control in Foods", (eds A.H.W. Hauschild and K.L. Dodds), Marcel Dekker Inc., New York.

Silset, T.O. & Hole, S. (1975) A new case of human botulism type E from fermented fish 'rakefisk'. Norsk.Vet.Tidsskr., 87(7/8), 480-483.

Simonsen, B. & Hauschild, A.H.W. (1986) Safety assessment for shelf-stable canned cured meats - an unconventional approach. Fd Technol., 40(4), 155-158.

Sinclair, N.A. and Stokes, J.A. (1964) Isolation of obligately anaerobic psychrophilic clostridia. J. Bact., 87, 562-4.

Skjelkvale, R., Stringer, M.F. and Smart, J.L. (1979) Enterotoxin production by lecithinase-positive and lecithinase-negative *Clostridium perfringens* isolated from food poisoning outbreaks and other sources. J. Appl. Bacteriol., 47(2), 329-339.

Slater, P.E., Addiss, D.C., Cohen, A., Leventhal, A., Chassis, G., Zehavi, H., Bashari, A. and Costin, C. (1989) Foodborne botulism: an international outbreak. Int. J. Epidemiol. 18, 693-696.

Smart, J.L. and Rush, P.A.J. (1987) In-vitro heat denaturation of *Clostridium botulinum* toxins types A, B and C. Int. J. Food Sci. Technol., 22, 293-298.

Smart, J.L., Roberts, T.A., Stringer, M.F. and Shah, N. (1979) The incidence and serotypes of *Clostridium perfringens* on beef, pork and lamb carcasses. J. Appl. Bacteriol., 46, 377-383.

Smelt, J.P.P.M. (1980). Heat resistance of *Clostridium botulinum* in acid ingredients and its significance for the safety of chilled foods. PhD-thesis, University of Utrecht, Utrecht, The Netherlands

Smith, J.L. (1998) Foodborne illness in the elderly. J. Food Prot., 61(9), 1229-39.

Smith, J.P, Daifas, D.P., El-Khoury, W., Koukoutsis, J. and El-Khoury, A. (2004) Shelf life and safety concerns of bakery products--a review. Crit. Rev. Food Sci. Nutr., 44(1), 19-55.

Sobel, J., Tucker, N., Sulka, A., McLaughlin, J. and Maslanka, S. (2004) Foodborne botulism in the United States, 1990-2000. Emerg. Infect. Dis., 10(9), 1606-1611.

Sofos, J,N., Busta, F.F, Bhothipaksa, K. and Allen, C.E. (1979) Sodium nitrite and sorbic acid effects on *Clostridium botulinum* toxin formation in chicken frankfurter-type emulsions. J. Food Sci., 44, 668-672 and 675.

Solomon, H.M. and Kautter, D.A. (1986) Growth and toxin production by *Clostridium botulinum* in sautéed onions. J. Food Protect., 49, 618-20.

Solomon, H.M. and Kautter, D.A. (1988) Outgrowth and toxin production by *Clostridium botulinum* in bottled chopped garlic. J. Food Protect., 51, 862-5.

Solomon, H.M., Kautter, D.A., Lilly, T. and Rhodehamel, E.J. (1990) Outgrowth of *Clostridium botulinum* in shredded cabbage at room temperature under a modified atmosphere. J. Food Prot., 53, 831-3, 845.

Sonnabend, O., Sonnabend, W., Heinzle, R., Sigrist, T., Dirnhofer, R. and Krech, U. (1981) Isolation of *Clostridium botulinum* type G and identification of type G botulinal toxin in humans: report of five sudden unexpected deaths. J. Infect. Dis., 143, 22-27.

Sonnabend, O.A. R., Sonnabend, W. F. F., Krech, V., Molz, G. and Sigrist, T. (1985) Continuous microbiological and pathological study of 70 sudden and unexpected infant deaths. Toxigenic intestinal *Clostridium botulinum* infection in 9 cases of sudden infant death syndrome. Lancet, i, 237-241.

St. Louis, M.E., Peck, S.H.S., Bowering, D., Morgan, G.B., Blatherwick, J., Banerjee, S., Kettyls, G.D.M., Black, W.A., Milling, M.E., Hauschild, A.H.W., Tauxe, R.V., and Blake, P.A. (1988). Botulism from chopped garlic: Delayed recognition of a major outbreak. Annals of Internal Medicine, 108, 363-368.

Steele, F.M. and Wright, K.H. (2001) Cooling rate effect on outgrowth of *Clostridium perfringens* in cooked, ready-to-eat turkey breast roasts. Poult Sci., 80(6), 813-6.

Stringer, S.C., Fairbairn, D.A. and Peck, M.W. (1997) Combining heat treatment and subsequent incubation temperature to prevent growth from spores of non-proteolytic *Clostridium botulinum*. J. Appl. Microbiol., 82(1), 128-36.

Stumbo, C.R. (1973) Thermobacteriology in Food Processing, 2nd edition, Academic Press, New York.

Suen, J.C., Hatheway, C.L., Steigerwalt, A.G. & Brenner, D.J. (1988) *Clostridium* argentinense sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* type G and some non-toxigenic strains previously identified as *Clostridium* subterminale or *Clostridium* hastiforme. Int. J. Sys. Bacteriol., 38, 374-381.

Sugiyama H, Yang KH. (1975) Growth potential of *Clostridium botulinum* in fresh mushrooms packaged in semipermeable plastic film. Appl Microbiol 30, 964-9.

Sugiyama, H., Woodburn, M., Yang, K.H. and Movroydis, C. (1981) Production of *botulinum* toxin in inoculated pack studies of foil-wrapped baked potatoes. Journal of Food Protection, 44(12), 896-898, 902.

Sutton, R.G.A. and Hobbs, B.C. (1971) Resistance of vegetative cells of *Clostridium* welchii to low pH. J. Med. Microbiol., 4, 539-543.

Swaddiwudhipong W, Wongwatcharapaiboon P. (2000) Foodborne botulism outbreaks following consumption of home-canned bamboo shoots in Northern Thailand. J. Med. Assoc. Thai., 83(9), 1021-5.

Tallis, G., Ng, S., Ferreira, C., Tan, A. and Griffith, J. (1999) A nursing home outbreak of *Clostridium perfringens* associated with pureed food. Aust. N. Z. J. Public Health, 23(4), 421-3.

Taormina, P.J., Bartholomew, G.W. and Dorsa, W.J. (2003) Incidence of *Clostridium perfringens* in commercially produced cured raw meat product mixtures and behavior in cooked products during chilling and refrigerated storage. J Food Protect., 66(1), 72-81.

Telzak, E.E., Bell, E.P., Kautter, D.A., Crowell, L., Budnick, L.D., Morse, D.L. and Schultz, S. (1990) An international outbreak of type E botulism due to uneviscerated fish. J. Inf. Dis., 161, 340-342.

Terranova, W., Breman, J.G., Locey, R.P. and Speck, S. (1978) Botulism type B: epidemiologic aspects of an extensive outbreak. Amer. J. Epidemiol., 108(2):

Tompkin, R.B. (1980) Botulism from meat and poultry products - a historical perspective. Food Technol., 34: 229-236, 257.

Townes, J.M., Cieslak, P.R., Hatheway, C.L., Solomon, H.M., Holloway, J.T., Baker, M.P., Keller, C.F., McCroskey, L.M. and Griffin, P.M.. (1996) An outbreak of type A botulism associated with a commercial cheese sauce. Ann. Intern. Med., 125(7), 558-63.

Trethon, A., Budai, J., Herendi, A., Szabo, V., Geczy, M., (1995) Botulism in infancy. Orv. Hetil., 136, 1497-1499.

Troillet, N. and Praz, G. (1995) [Epidemic of type B botulism: Sion, December 1993-January 1994][in French] Schweiz Med. Wochenschr., 125(39): 1805-12.

Tschirdewahn, B., Notermans, S., Wernars, K. and Untermann, F. (1991) The presence of enterotoxigenic *Clostridium perfringens* strains in feces of various animals. Internat. J. Food Microbiol. 14, 175–178.

Van Ermengem, E. (1897) Ueber einen neuen anaeroben Bacillus und seine Beiziehungen zum Botulismus. Z. Hyg. Infektionskr., 26, 1-56

Varma, J.K., Katsitadze, G., Moiscrayfishvili, M. *et al*. (2004) Foodborne botulism in the Republic of Georgia. Emerg. Infect. Dis., 10(9), 1601-1605.

Wainwright, R.B. (1993) Hazards from northern native foods. pp. 305-322 in "*Clostridium botulinum*: Ecology and Control in Foods". (eds A.H.W. Hauschild and K.L. Dodds) Marcel Dekker Inc., New York.

Wainwright, R.B., Heyward, W.L., Middaugh, J.P., Hatheway, C.L., Harpster, A.P. and Bender, T.R. (1988) Food-borne botulism in Alaska, 1947-1985: epidemiology and clinical findings. J. Infect. Dis., 157, 1158-1162.

Weber, J.T., Goodpasture, H.C., Alexander, H., Werner, S.B., Hatheway, C.L. and Tauxe, R.V. (1993a) Wound botulism in a patient with a tooth abscess: case report and review. Clin. Infect. Dis., 16(5), 635-9.

Weber, J.T., Hibbs, R.G. Jr., Darwish, A., Mishu, B., Corwin, A.L., Rakha, M., Hatheway, C.L., El-Sharkawy, S., El-Rahim, S.A., al-Hamd, M.F. *et al*. (1993b) A massive outbreak of type E botulism associated with traditional salted fish in Cairo. J. Infect. Dis., 167, 451-454.

WHO (World Health Organization) (1999) "High-Dose Irradiation: Wholesomeness of Food Irradiated with Doses Above 10 kGy". Report of a Joint FAO/IAEA/WHO Study Group, WHO Technical Report Series 890, WHO, Geneva.

Wnek, A. P. and McClane, B.A. (1983) Identification of a 50,000 Mr Protein from rabbit brush border membranes that binds *Clostridium perfringens* enterotoxin. Biochem. Biophys. Res. Commun., 112, 1099-1105.

Yndestad, M. (1970) Outbreak of type E botulism in humans after consumption of home-prepared salted fish (rakefisk). Norsk Veterinaertidsskrift, 82(6), 321-323.

Zaika, L.L. (2003) Influence of NaCl content and cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ham and beef. J Food Protect., 66(9), 1599-603.

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8 ANNEX

8.1 Annex I Compliance Guidelines for Cooling Heat-Treated Meat and Poultry Products (Stabilization).

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Food Safety and Inspection Service
United States Department of Agriculture
Washington, D.C. 20250-3700

Introduction

Establishments producing ready-to-eat roast beef, cooked beef and corned beef products, fully cooked, partially cooked, and char-marked meat patties, and certain partially cooked and ready-to-eat poultry products are required by FSIS to meet the stabilization performance standards for preventing the growth of spore-forming bacteria (9 CFR §§ 318.17(a)(2), 318.23(d)(1), and 381.150(a)(2), respectively). Further, FSIS requires meat and poultry establishments, if they are not operating under a HACCP plan, to demonstrate how their processes meet these stabilization performance standards within a written process schedule validated for efficacy by a process authority (§ 318.17(b) and (c); 318.23(d)(2) and (3); and 381.150(c) and (d)).

To assist establishments in meeting the stabilization requirements, FSIS is issuing these compliance guidelines, which are based upon FSIS Directives and the product cooling requirements contained in previous regulations. Establishments may choose to employ these guidelines as their process schedules. FSIS considers these guidelines, if followed precisely, to be validated process schedules, since they contain processing methods already accepted by the Agency as effective.

Also within these guidelines, FSIS has provided discussion regarding disposition of product following cooling deviations and advice for the development of customized procedures for meeting the stabilization performance standards.

Stabilization Guidelines

It is very important that cooling be continuous through the given time/temperature control points. Excessive dwell time in the range of 130° to 80°F is especially hazardous, as this is the range of most rapid growth for the clostridia. Therefore cooling between these temperature control points should be as rapid as possible.

1. During cooling, the product's maximum internal temperature should not remain between 130°F (54.4°C) and 80°F (26.6°C) for more than 1.5 hours nor between 80°F (26.6°C) and 40°F (4.4°C) for more than 5 hours. This cooling rate can be applied universally to cooked products (e.g., partially cooked or fully cooked, intact or non-intact, meat or poultry) and is preferable to (2) below.

2. Over the past several years, FSIS has allowed product to be cooled according to the following procedures, which are based upon older, less precise data: chilling should begin within 90 minutes after the cooking cycle is completed. All product should be chilled from 120°F (48°C) to 55°F (12.7°C) in no more than 6 hours. Chilling should then continue until the product reaches 40°F (4.4°C); the product should not be shipped until it reaches 40°F (4.4°C).

This second cooling guideline is taken from the former ("Requirements for the production of cooked beef, roast beef, and cooked corned beef", 9 CFR 318.17(h)(10)). It yields a significantly smaller margin of safety than the first cooling guideline above, especially if the product cooled is non-intact product. If an establishment uses this older cooling guideline, it should ensure that cooling is as rapid as possible, especially between 120°F (48°C) and 80°F (26.6°C), and monitor the cooling closely to prevent deviation. If product remains between 120 °F (48°C) and 80°F (26.6°C), more than one hour, compliance with the performance standard is less certain.

3. The following process may be used for the slow cooling of ready-to-eat meat and poultry cured with nitrite. Products cured with a minimum of 100 ppm ingoing sodium nitrite may be cooled so that the maximum internal temperature is reduced from 130°F (54.4°C), to 80 °F (26.6°C), in 5 hours and from 80 (26.6°C), to 45 °F (7.2°C), in 10 hours (15 hours total cooling time).

This cooling process provides a narrow margin of safety. If a cooling deviation occurs, an establishment should assume that their process has exceeded the performance standard for controlling the growth of *Clostridium perfringens* and take corrective action. The presence of the nitrite, however, should ensure compliance with the performance standard for *Clostridium botulinum*.

Establishments that incorporate a "pasteurization" treatment after lethality and stabilization treatments (e.g., applying heat to the surface of a cooled ready-to-eat product after slicing) and then re-stabilize (cool) the product should assess the cumulative growth of *C. perfringens* in their HACCP plans. That is, the entire process should allow no more than 1-$\log_{10}$ total growth of *C. perfringens* in the finished product. When employing a post-processing "pasteurization," establishments may want to keep in mind that at temperatures of 130 °F (54.4°C), or greater, *C. perfringens* will not grow.

Support documentation for this process was filed by the National Food Processors Association on April 14, 1999. It is available for review in the FSIS Docket Room, Room 102, Cotton Annex, 300 12th St., SW, Washington, DC 20250-3700.

Discussion

Cooling Deviations

In spite of the best efforts of an establishment to maintain process control, cooling deviations will occasionally occur. Power failures or breakdowns of refrigeration

equipment cause situations that cannot always be anticipated. However, it is important that the establishment plan how to cope with such eventualities before they occur.

The recommended time/temperature combinations in these guidelines incorporate a small safety margin. Therefore, an occasional small lapse in and of itself may not cause a problem in every instance. If the cause of a small cooling deviation is not traced and corrected when first noticed, however, the problem will likely recur and possibly become more frequent and more severe. The processor should consider an occasional small deviation an opportunity to find and correct a control problem. Of course, a large deviation or continual small ones will always constitute unacceptable risk.

After it is determined that a cooling deviation has occurred, the processor should:

1. Notify the inspector, the QC unit, and other concerned units, such as refrigeration maintenance and production.

2. Hold the involved product and determine the potential adulteration by bacteria, particularly clostridial pathogens. If adulteration is confirmed or appears to be likely, inform the inspector.

3. Postpone further product manufacturing using that chill facility until the processor has:

a. determined the cause of the deviation;

b. completed adjustments to assure that the deviation will not recur; and

c. informed the inspector and the production units of the determinations and adjustments and make any needed amendments in the written processing procedures.

Computer modeling and sampling

In the event that a cooling deviation does occur, the product may often be salvaged if the results of computer modeling and/or sampling can ensure product safety. Because of a lack of information concerning the distribution of *C. perfringens* in product, sampling may not be the best recourse for determining the disposition of product following cooling deviations. However, computer modeling can be a useful tool in assessing the severity of a cooling deviation. While computer modeling cannot provide an exact determination of the possible amount clostridial growth, it can provide a useful estimate.

A technical document (available from the FSIS Docket Room) provides description of the calculations that are used to estimate relative growth.

With careful continuous monitoring of the heating and cooling time/temperature profile of each lot, there will always be many available data points, enhancing the accuracy of computer modeling. Conversely, when there are few documented

time/temperature data points, the accuracy of the modeling decreases markedly. If time/temperature monitoring has not been conducted through the end point internal product temperatures of 40° F or less, sampling is not an option and the product should be destroyed.

Options after computer determination of cooling deviation severity.

If computer modeling suggests that the cooling deviation would likely result in more than one log increase in *C. perfringens*, without any multiplication (remains in lag phase) of *C. botulinum*, then the establishment can choose to recook or sample the product.

Recook only when:

- All product was either immediately refrigerated after the deviation or can be immediately recooked after the deviation; and

- The recooking procedure can achieve a final internal product temperature of at least 149°F (65°C) for two minutes. Subsequent to recooking, the product must be cooled in strict conformance to existing guidelines. When the product is to be reworked with another raw product, the recooking procedure for the combined product must achieve a minimum internal temperature of 149°F, to address the cooling deviation, and further to an increased time\temperature if necessary to be in accord with any other requirement relative to microbiological safety for the intended final product. Subsequent to recooking, the product must be cooled in strict conformance to existing guidelines.

Custom Stabilization Processes

While compliance with the guidelines above will yield product that meets the cooling performance standards, some establishments may want to develop customized stabilization procedures. Because customized process schedules must be validated by process authorities for efficacy, most establishments will probably rely upon processing authorities to develop such procedures, demonstrate their efficacy, and attest to their safety. Process authorities may obtain information from the literature, or likely compare peer reviewed methods in determining safe procedures that meet the performance standards.

Probably one of the most definitive tools at the disposal of the processing authority is the inoculated pack study. Such studies should, of course, be conducted only in the laboratory, not in the plant.

Further, such studies should be undertaken by individuals who have a thorough knowledge of laboratory methods used in clostridial research. *C. perfringens* can be used alone in an inoculated pack study to demonstrate that the cooling performance standard is met for both microorganisms, *C. perfringens*, and *C. botulinum*. This is because conditions of time/temperature that would limit the growth of *C. perfringens* to one log or less would also prevent multiplication of *C. botulinum*, which is much slower. A cocktail of various strains of *C. perfringens* spores is often used for this purpose. Relatively "fast" toxigenic strains should be used to develop a worst case. However, the strains selected should be among those that have been historically implicated in an appreciable number of outbreaks, especially in products similar to those being prepared in the establishment.

8.2 Annex II. Methods of detection for *C. botulinum*

(from SCVPH Opinion on Honey and Microbiological Hazard, 2002)

Microbiological monitoring for *C. botulinum* currently requires culturing for 3-5 days, and testing for toxin in a mouse bio-assay (slow, expensive, requires dedicated facilities, and in many countries a licence for animal experimentation).

Alternative molecular / genetic assays are being developed, but none available commercially have been validated against the mouse bio-assay.

The reference method for detection of botulinum neurotoxin remains the mouse bioassay (Schantz and Kautter, 1978) coupled with neutralisation with monovalent antisera. Production of those antisera has offered poor commercial returns, and, as a consequence, antisera are difficult to obtain.

For many years attempts to find alternative methods suffered from lack of sensitivity and specificity. In recent years many alternative methods of detection have been developed (see Table 10 belowfor examples), but none are being produced commercially.

The (UK) Centre for Applied Microbiology and Research (CAMR) has developed a panel of sensitive assays for the botulinum toxin serotypes associated with human disease (types A, B, E & F). These novel *in vitro* assay systems are a modified ELISA (Hallis *et al*., 1996) in which the biological (endopeptidase) activities contained within the toxins are used to amplify the assay signal to provide tests that are of equivalent sensitivity to the mouse bioassay. The *in vitro* assay for botulinum type B neurotoxin has been assessed in broad range of foodstuffs and shown to provide a robust method of detection with a sensitivity that exceeds that of the mouse assay (Wictome *et al*., 1999b). A major advantage of the assay format is the extremely low incidence of false-positive results, because the assay signal depends on both the recognition of the neurotoxins by specific antibodies and also the unique endopeptidase activities contained within the toxin light subunits.

Continuing research at CAMR is aimed at generating validated assays systems and reagents for all for botulinum serotypes associated with human disease, and also improving and simplifying the assay protocols to provide a reliable alternative to the mouse bioassay.

Table 10 Detection of *Clostridium botulinum* and botulinal neurotoxins – examples

Procedure	Type(s)	References
Mouse lethality	A, B, C, D, E, F, G	Schantz and Kautter, 1978
ELISA	A, B	Shone *et al*., 1985
ELISA	G	Lewis *et al* ., 1981
endopeptidase	A, B	Hallis *et al*., 1996; Wictome *et al*., 1999a
PCR + 5 gene probes	A, B, E, F G	Fach *et al*., 1995
electroimmunodiffusion	A, E	Jay, 1996
ELISA-ELCA	E	Roman *et al*., 1994
PCR + capillary electrophoresis	E	Sciacchitano and Hirschfield, 1996
colorimetric capture ELISAS	A, B	Szilagyi *et al*., 2000
PCR	E	Kimura *et al*., 2001
fluoroimmunoassays using ganglioside-earing liposomes	All*	Singh *et al*., 2000
PCR and molecular probes	A, B, E	Braconnier *et al*., 2001
immuno-PCR	A	Wu *et al*., 2001
enrichment PCR	BEF	Dahlenborg, *et al*., 2001
Multiplex PCR	ABEF	Lindstrom *et al*., 2001
PCR	AB	Nevas *et al*., 2002
Fibre optic-based biosensor	A	Ogert *et al*., 1992
enzyme-linked immunosorbent assay and enzyme-linked coagulation	ABE	Doellgast *et al*., 1994
evanescent wave immunosensor	All*	Kumar *et al*., 1994
PCR	A	Fach *et al*., 1993
PCR Gene probes	BEF	Campbell *et al*., 1993
enzyme-linked immunosorbent assay and signal amplification via enzyme-linked coagulation assay	ABE	Doellgast *et al*., 1993
PCR and DNA probes	ABEF	Aranda *et al*., 1997

All * = in principle

For references, see SCVPH Opinion on "Honey and Microbiological Hazards" 19-20 June, 2002

8.3 Annex III. Effect of number of spores on toxin production by *C. botulinum* (in challenge tests)

(EFSA (2003) Opinion on the Effects of Nitrites/Nitrates on the Microbiological Safety of Meat Products)

Table 7. The effect of inoculum size of proteolytic *C. botulinum* type A and B in pork slurry system (pH 6.5, 2.5% NaCl) (data from Robinson et al., 1982)

Treatment	Inoculum (per 28 g)			
	1000		10	
	Incubation temp.		Incubation temp.	
	15 °C	20 °C	15°C	20 °C
$NaNO_2$ 100 mg/kg*	71**	91	27	59
$NaNO_2$ 200 mg/kg	20	50	4	13
$NaNO_2$ 300 mg/kg	2	9	0	1

* nitrite input
**probability (%) of toxin production

The results demonstrate that the number of spores present initially strongly influences the probability of growth and toxin production, confirming indications in the 1960's . Even the lower inoculum was appreciably higher than levels of *C. botulinum* spores occurring naturally – 10 spores per 28 g is equivalent to 350 spores per kg, compared with not more than 2 per kg detected in a range of meat products (Table 1).

It is also clear that the protective effect increases with increasing concentrations of added nitrite.

8.4 Annex IV: Examples of foods historically associated with botulism

- **Fish and marine mammals caught in the Baltic Sea and N. Pacific Ocean (British Columbia, Alaska, Japan, Scandinavia**). Products involved include eating a beached whale, parts of which had been stored in plastic bags (CDC, 2002), home-made 'rakefisk' (Yndestad, 1970; Silset and Hole, 1975) "izushi", a similar traditional fermented fish product in Japan, vacuum-packed white-fish chubs from the US Great Lakes that were under-processed, contained very little salt, and were grossly temperature abused during transportation (Ecklund, 1993) and vacuum-packaged hot-smoked whitefish (Korkeala *et al*, 1998). Other fish-related cases of botulism have been caused by faseikh, a whole, uncut (uneviscerated) salt-cured fish *(http://www.news-medical.net/?id=3344)* and ribbetz or kapchunka, a salt-cured, air-dried whitefish that is processed and sold uneviscerated (Slater *et al.*, 1989; Telzak *et al*., 1990).

- **Meat (especially pork) - Europe and N. America.** Products involved include home prepared stewed pork (Dolzhkevich *et al*.,1978), home cured ham (van Ermengem, 1897) and canned pork (Przybylska, 2004).

  A total of 85 foodborne botulism were reported in Poland in 2002, corresponding to an incidence 0.22 per 100,000 population. In rural areas 67% of cases occurred and 33% in urban areas (incidence 0.39 and 0.12 per 100,000). There were 53 outbreaks involving one person, 11 outbreaks of two people, 2 outbreaks of three, and 1 outbreak of four people. Meat dishes were the main vehicle of botulinum toxin (58 cases, 68.2%). Home-made conserves (bottling jars) prepared from pork meat (23.5% of cases) and commercially produced sausages (20%) were the most common vehicles. Five deaths (three men and two women) from foodborne botulism were registered in Poland in 2002 (Przybylska, 2004).

- **Home preserved vegetables (Asia)**

- In northern Thailand 13 persons were hospitalised with symptoms of botulism. Two died. All had eaten home-canned bamboo shoots consumed without prior cooking. No other common food was identified (CDC, 1999b; Swaddiwudhipong and Wongwatcharapaiboon, 2000).

- **Milk products, some cheeses (Europe, Argentina).** Products implicated include cheese sauce stored at room temperature (Townes, *et al*., 1996), commercially produced mascarpone cheese that was temporarily stored unrefrigerated (Aureli *et al*., 2000), Brie cheese (France and Switzerland) (Billon *et al*., 1980).

- **Condiments (sauces) (N. America).** An example is consumption of a hot sauce made in a restaurant with improperly home-canned jalapeño peppers (Terranova *et al*., 1978).

- **Fruit (especially tomatoes**). Products involved are home-canned tomatoes that caused foodborne type B botulism in Ontario (Loutfy, 2003) and home canned fruit products in Austria (Eurosurveillance Weekly archives 2001,Volume 5 / Issue 15)

Outbreaks of botulism in circumstances not anticipated

- **Foil-wrapped baked potatoes subsequently made into potato salad**

  Potatoes were cooked in aluminium foil and stored unrefrigerated for several days before being incorporated into a potato salad (Seals *et al*., 1981; Solomon *et al*., 1998).

- **Vegetable soup associated with botulism**
  A case of botulism in northern Italy arose following consumption of a canned vegetable soup ('ribollita', which forms part of Tuscany's regional cuisine) which was contaminated with *C. botulinum* type A and toxin. The soup was produced by a small local factory and sterilized under no quality control measures. The patient drank the soup without heat treatment for lunch on 25 August 1998. Six hours later she developed failure of accommodation, etc. and was admitted to the local hospital with suspected botulism. Field investigations revealed that the jar had been opened one week before being used. It was noted to have a bad smell, but the patient kept it refrigerated after opening until 25 August, when she drank it.

  The incident illustrates a hazard associated with small-scale manufacture of 'home-made' style foods (Eurosurveillance Weekly archives 1998, Volume 2 / Issue 37)]

- **Egg plant in oil**

  In August and October 1993, public health officials in Italy were notified of ten cases of type B botulism. The cases were associated with sliced roasted egg-plant stored in oil.

  The company reported preparing the eggplant in the following manner: eggplant slices were washed and soaked overnight in a solution of water, vinegar, and salt; roasted in an oven; and subsequently placed in glass jars. Garlic, peppers, oregano, and citric acid were added. The mixtures then were covered with sunflower oil and sealed with screw-on lids; after being filled, the jars were boiled in water for 30 minutes. The pH of the product was not consistently monitored (CDC, 1995).

- **Fried lotus rhizomes mixed with mustard.**

  The product had been manufactured in Kumamoto prefecture. All 36 patients had eaten commercial fried lotus-rhizome solid mustard without heating and 14 died (Otofuji *et al*., 1987)

- **Sautéed unions**

  From October 15, to October 21, 1983, 28 cases of foodborne botulism occurred in Peoria, Illinois. The epidemiological investigation implicated sauteed onions served on a patty-melt sandwich as the most probably vehicle. The onions were said to have been prepared daily with fresh whole onions, margarine, paprika, garlic salt, and a chicken-base powder; they were held uncovered in a pan with a large volume of melted margarine on a warm stove (below 60°C) and were not reheated before serving (CDC, 1984; MacDonald *et al*., 1985).

- **Pickled eggs**

  On November 23, 1997, in a previously healthy 68-year-old man botulism developed. A food history revealed no exposures to home-canned products; however, the patient had eaten pickled eggs that he had prepared 7 days before onset of illness; gastrointestinal symptoms began 12 hours after ingestion. The patient recovered after prolonged supportive care (CDC, 2000a).

- **Botulism associated with home-preserved mushrooms**
  Two clinical cases of botulism, one fatal, arose in members of a British family who ate home-preserved mushrooms bottled in oil. They ate the mushrooms without heating. (Eurosurveillance Weekly archives 1998, Volume 2 / Issue 18)

- **Retail packed fresh mushrooms**

  After botulinal neurtoxin had been detected in commercially canned mushrooms (CDC, 1973), Sugiyama and Yang (1975) reported that proteolytic *C. botulinum* can grow and produce toxin in packaged fresh mushrooms. No cases of botulism have been attributed to fresh mushrooms.

8.5 Annex V: Examples of Botulism associated with home preservation, traditional preservation, or failure of a commercial process.

Example 1: Controlling *C. botulinum* (from Hauschild, 1989)

Category 1: Shelf-stable products. *C. botulinum* controlled by heat processing, e.g. low acid canned foods.

In the UK pH >4.5; in the USA pH >4.6. F_0 at least 3.0 (heating for 3 min at 121°C or an equivalent heat process).

Category 2: Shelf-stable products. *C. botulinum* controlled by heat processing, sodium chloride and nitrite; e.g. shelf-stable canned cured meats.

Category 3: Shelf-stable products. *C. botulinum* controlled by mild heat processing and acidity; e.g. acid canned foods (canned fruits, fruits with a natural low pH, acid or acidified fruit.

Category 4: Shelf-stable products. *C. botulinum* controlled by reduced water activity, sodium chloride, sometimes nitrite, and refrigeration; e.g. shelf-stable raw salted and salt-cured meats.

Category 5: Shelf-stable products. *C. botulinum* controlled by reduced water activity, acidity and nitrite; e.g. dry fermented sausages.

Category 6: Perishable products. *C. botulinum* controlled mainly be refrigeration.

Category 7: Perishable products. *C. botulinum* controlled mainly by, sodium chloride and refrigeration, often in combination with nitrite, sometimes with other factors such as sorbate, benzoate and nitrate; e.g. salted fish, lightly and semi-preserved fish products, hot-smoked fish, perishable cooked cured meats.

Category 8: Perishable or shelf-stable products. *C. botulinum* controlled by mild heat treatment, reduced water activity, acidity, and refrigeration; e.g. processed cheese and cheese spreads.

Category 9: Perishable products. *C. botulinum* controlled by mild heat treatment and refrigeration; e.g. ready-meals, "sous vide".

Example 2: Advisory Committee Microbiological Safety of Food (ACMSF, 1992)

After concern that the rapidly expanding range of foods stored under refrigeration might pose a risk, a search of the scientific literature and the use of models of growth available at that time led to conditions that would prevent multiplication of all types of *C. botulinum* Group II being defined.

(i) Storage <3.3°C unlimited shelf-life

(ii) Storage <5°C and shelf-life 10 maximum

(iii) Storage 5° to 10°C and shelf-life 5 maximum

(iv) heat at 90°C for 10 min and store < 10°C

(v) pH < 5.0 throughout and store < 10°C

(vi) NaCl > 3.5% throughout the food and store < 10°C

(vii) a_w <0.97 throughout food and store <10°C.

(viii) Other combinations that consistently give protection factor of 6.