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REVIEW ARTICLE

Clostridium botulinum and the safety of minimally heated, chilled foods: an emerging issue?

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Abstract

Foodborne botulism is caused by consumption of preformed botulinum neurotoxin, with as little as 30 ng of neurotoxin being potentially lethal. Consumption of minute quantities of neurotoxin-containing food can result in botulism. In view of the severity of foodborne botulism, it is essential that new foods be developed safely without an increase in incidence of this disease. Minimally heated, chilled foods are a relatively new type of food, sales of which are currently increasing by about 10% per annum. These products meet consumer demand for high-quality foods that require little preparation time. Their safety and quality depends on mild heat treatment, chilled storage, restricted shelf life and sometimes on intrinsic properties of the foods. The principal microbiological hazard is nonproteolytic *Clostridium botulinum*, and there is a concern that this may become an emerging issue. A considerable amount of research and development over the last 15 years has underpinned the safe production of commercial, minimally heated, chilled foods with respect to foodborne botulism, and it is essential that safe food continues to be developed. In particular, the desire to use lighter heat processes and a longer shelf life presents a challenge that will only be met by significant developments in quantitative microbiological food safety.

Clostridium botulinum and botulism

Clostridium botulinum and its neurotoxins

Clostridium botulinum is a heterogeneous species of four groups of Gram-positive spore-forming anaerobic bacteria that share the ability to form a botulinum neurotoxin. Some strains of two other clostridia, *Clostridium baratii* and *Clostridium butyricum*, also form a botulinum neurotoxin (Table 1). These six neurotoxigenic clostridia (four groups of *Cl. botulinum* and neurotoxigenic strains of *Cl. baratii* and *Cl. butyricum)* are physiologically distinct (Hatheway 1992). *Clostridium botulinum* group I (proteolytic *Cl. botulinum*) and *Cl. botulinum* group II (nonproteolytic *Cl. botulinum*) are responsible for most cases of foodborne botulism. *Clostridium botulinum* group III is responsible for avian botulism and botulism associated with animals, for example, a recent large outbreak in Finland affected 52 000 farmed foxes and mink (Lindström *et al.* 2004b).

There are seven botulinum neurotoxins (A–G), with the neurotoxin formed being dependent on the producing organism (Table 1). All seven neurotoxins comprise a heavy and light chain linked by a disulfide bridge. The heavy chains transport the light chains to the motor neuron cytosol, their site of action. The light chains possess zinc endopeptidase activity and cleave proteins involved in the release of the neurotransmitter, acetylcholine, leading to flaccid paralysis of the muscle. Symptoms of botulism are neurological and often commence with blurred vision. Flaccid paralysis of the respiratory muscles can result in death (Hatheway 1988). In many countries, foodborne botulism is treated with equine antitoxin and this with supportive therapy has led to a reduction in the fatality rate to approx. 5–10% of cases. Full recovery, however, may take many months or even longer.

Table 1 The six clostridia that produce the botulinum neurotoxin

Clostridia	Neurotoxins formed
Cl. botulinum group I (proteolytic *Cl. botulinum*)	A, B, F
Cl. botulinum group II (nonproteolytic *Cl. botulinum*)	B, E, F
Cl. botulinum group III	C, D
Cl. botulinum group IV (*Cl. argentinense*)	G
Cl. baratii (neurotoxigenic strains)	F
Cl. butyricum (neurotoxigenic strains)	E

Foodborne botulism

Foodborne botulism is an intoxication resulting from the consumption of preformed botulinum neurotoxin, with as little as 30 ng of neurotoxin sufficient to cause illness and even death (Lund and Peck 2000). The consumption of a minute quantity of food in which neurotoxin-producing clostridia have grown can result in botulism. For example, in 2002, a 35-year-old man ate a mouthful of foil-wrapped baked potatoes, found it to be foul tasting and spat it out; but he had consumed sufficient neurotoxin to require extensive medical treatment that included more than 6 months in hospital (Bhutani *et al.* 2005). As botulism is such a severe disease, a considerable effort is dedicated to ensuring the safe production of food with respect to this hazard and that botulism outbreaks are rare.

Foodborne botulism has been recognized as a disease in Europe for several hundred years. The name 'botulism' was given to this disease on the strength of a frequent association with the consumption of blood sausage and is derived from the Latin word 'botulus' meaning sausage. A causative organism was first isolated by van Ermengem in 1897 from home-made raw salted ham and the spleen of a man who later died of botulism (Hauschild 1989). During the next few decades, outbreaks were often associated with home and commercial canning processes. The identification and implementation of effective control measures has brought about a reduction in the incidence of foodborne botulism. At the present time, most outbreaks are associated with home-made foods, where known control measures have not been implemented. Outbreaks involving commercial processing are uncommon but can have significant medical and economical consequences. It has been estimated that in the United States, the cost per case of botulism is approx. $30 million, 3000 times more than for each case of listeriosis or salmonellosis (Setlow and Johnson 1997).

Recent outbreaks of foodborne botulism associated with proteolytic *Cl. botulinum* have often involved incorrectly canned foods (Table 2), while outbreaks associated with nonproteolytic *Cl. botulinum* have often involved fish (Table 3). In other recent outbreaks, the responsible organism (and sometimes toxin) has not been reported (Table 4). It is likely that those involving a failed canning process are due to proteolytic *Cl. botulinum*, while those involving smoked, dried or salted fish involve nonproteolytic *Cl. botulinum*. In Europe, outbreaks have been often associated with type B neurotoxin, and it appears that many are due to nonproteolytic *Cl. botulinum* type B (Lucke 1984; Hauschild 1992). Occasional outbreaks of foodborne botulism have also been associated with neurotoxigenic strains of *Cl. butyricum* and *Cl. baratii* (Annibalii *et al.* 2002; Harvey *et al.* 2002).

In Europe, more than 2500 cases of foodborne botulism were reported in 1999/2000 (Table 5). It should be noted that definitions and the efficiency of reporting vary from country to country (Therre 1999). Countries with a high reported incidence include Armenia, Azerbaijan, Belarus, Georgia, Poland, Russia, Turkey and Uzbekistan (Table 5). Georgia has one of the highest nationally reported rates of foodborne botulism in the world, with more than 80% of the cases attributed to home-preserved vegetables (Varma *et al.* 2004). One large outbreak at a wedding in 1994 affected 173 people and was associated with the consumption of contaminated fish (Table 4). Recent cases of foodborne botulism in Russia have been attributed to smoked, salted and dried fish and to canned vegetables, often prepared in the home (Table 4).

Minimally heated, chilled foods and guidelines for their safe production

Minimally heated, chilled foods

Minimally heated, chilled foods have been developed in response to consumer demand for high-quality convenience foods that require minimal preparation time and contain low levels of preservatives. In the United Kingdom, sales of prepared chilled foods increased by 58% in the 5 years upto 2004, to reach an estimated £7200 million (http://www.chilledfood.org/content/market_data.asp, 31 October 2005). There was a 50% increase in the European-prepared chilled food sector in the 5 years upto 1996, to an estimated £6200 million [ECFF (European Chilled Food Federation) 1998]. These foods are also known as ready-to-eat foods, ready meals, cook-chill foods, sous-vide foods and refrigerated processed foods of extended durability. These foods are not sterile, and safety and quality is dependent on a combination of a minimal heat treatment (typical maximum of 70–95°C, i.e. intended to minimize loss of sensory and organoleptic quality), storage at a chilled temperature (typically ≤8°C) under a modified low-oxygen atmosphere or vacuum, a restricted

Table 2 Examples of recent outbreaks of foodborne botulism involving proteolytic *Clostridium botulinum*

Year	Location	Product	Toxin type	Cases (deaths)	Factors	Reference
1985	Canada	Commercial garlic-in-oil	B	36	Bottled, no preservatives, temperature abuse	St. Louis *et al.* (1988)
1986	Taiwan	Commercial jars of heat-processed unsalted peanuts in water	A	9 (2)	Inadequate heat treatment	Chou *et al.* (1988)
1987	Canada	Bottled mushrooms	A	11	Underprocessing and/or inadequate acidification	CDC (Centers for Disease Control) (1987), McLean *et al.* (1987)
1989	UK	Commercial hazelnut yoghurt	B	27 (1)	Hazelnut conserve underprocessed	O'Mahony *et al.* (1990)
1993	USA	Restaurant commercial process cheese sauce	A	8 (1)	Recontamination, temperature abuse	Townes *et al.* (1996)
1993	Italy	Commercial, canned, roasted eggplant in oil	B	7	Insufficient heat treatment, improper acidification	CDC (Centers for Disease Control) (1995)
1994	USA	Restaurant; potato dip ('skordalia') and aubergine dip ('meligianoslata')	A	30	Baked potatoes held at room temperature	Angulo *et al.* (1998)
1994	USA	Commercial clam chowder	A	2	No secondary barrier, temperature abuse	Anon (1995)
1994	USA	Commercial black bean dip	A	1	No secondary barrier, temperature abuse	Anon (1995)
1996	Italy	Commercial mascarpone cheese	A	8 (1)	No competitive microflora, pH >6, temperature abuse	Franciosa *et al.* (1999), Aureli *et al.* (2000)
1997	Italy	Home-made pesto/oil	B	3	pH 5·8, a_w 0·97	Chiorboli *et al.* (1997)
1997	Iran	Traditional cheese preserved in oil	A	27 (1)	Unsafe process	Pourshafie *et al.* (1998)
1998	Argentina	Meat roll ('matambre')	A	9	Cooked and heat-shrink plastic wrap, temperature abuse	Villar *et al.* (1999)
1998	UK	Home-bottled mushrooms in oil (imported from Italy)	B	2 (1)	Unsafe process	CDSC (Communication Disease Surveillance Centre) (1998), Roberts *et al.* (1998)
2001	USA	Commercial chilli sauce	A	16	Temperature abuse at salvage store	Kalluri *et al.* (2003)
2002	South Africa	Commercial tinned pilchards	A	2 (2)	Corrosion of tin, permitted secondary contamination	Frean *et al.* (2004)
2002	Canada	Restaurant-baked potato in aluminium foil	A	1	Baked potato held at room temperature?	Bhutani *et al.* (2005)

Table 3 Examples of recent outbreaks of foodborne botulism involving nonproteolytic *Clostridium botulinum*

Year	Location	Product	Toxin type	Cases (deaths)	Factors	Reference
1987	USA and Israel	Commercial uneviscerated, salted, air-dried fish ('kapchunka')	E	8 (1)	Lack of refrigeration	Slater *et al.* (1989)
1991	Egypt	Commercial uneviscerated salted fish ('faseikh')	E	>91 (18)	Putrefaction of fish before salting	Weber *et al.* (1993)
1992	USA	Commercial uneviscerated salted fish ('moloha')	E	8	Insufficient salt	CDC (Centers for Disease Control) (1992)
1995	Canada	'Fermented' seal or walrus (four outbreaks)	E	9	Unsafe process	Proulx *et al.* (1997)
1997	Germany	Commercial hot-smoked vacuum-packed fish ('Raucherfisch')	E	2	Suspected temperature abuse	Jahkola and Korkeala (1997), Korkeala *et al.* (1998)
1997	Argentina	Home cured ham	E	6	?	Rosetti *et al.* (1999)
1997	Germany	Home-smoked vacuum-packed fish ('Lachsforellen')	E	4	Temperature abuse	Anon (1998)
1998	France	Frozen vacuum-packed scallops	E	1	Temperature abuse (?)	Boyer *et al.* (2001)
1999	Finland	Whitefish eggs	E	1	Temperature abuse	Lindström *et al.* (2004a)
1999	France	Grey mullet	E	1	Temperature abuse (?)	Boyer *et al.* (2001)
2001	Australia	Reheated chicken	E	1	Poor temperature control	Mackle *et al.* (2001)
2001	USA	Home-made fermented beaver tail and paw	E	3	Temperature abuse	CDC (Centers for Disease Control) (2001)
2001	Canada	Home-made fermented salmon roe (two outbreaks)	E	4	Unsafe process	Anon (2002)
2002	USA	Home-made 'muktuk' (from Beluga whale)	E	12	Unsafe process	McLaughlin *et al.* (2004)
2003	Germany	Home-salted air-dried fish	E	3	Temperature abuse (?)	Eriksen *et al.* (2004)

shelf life (can be up to 42 days) and in some cases also intrinsic properties of the food (e.g. pH, water activity). This process favours nonproteolytic *Cl. botulinum*, a spore-forming bacterium that grows in the absence of oxygen at chilled temperatures (Peck 1997; Peck and Stringer 2005). Proteolytic *Cl. botulinum* is a lesser concern, as it is unable to grow at chilled temperature.

Guidelines for the safe production of minimally heated, chilled foods

A number of recommendations, guidelines and a code of practice have been drawn up to ensure the safe production of these foods with respect to nonproteolytic *Cl. botulinum* (ACMSF (Advisory Committee on the Microbiological Safety of Foods) 1992, 1995; Betts 1996; ECFF (European Chilled Food Federation) 1996; Gould 1999). In 1992, the UK Advisory Committee on the Microbiological Safety of Food (ACMSF) made recommendations on the safe production of vacuum- and modified atmosphere-packed chilled foods with respect to *Cl. botulinum* and the associated foodborne botulism hazard (ACMSF (Advisory Committee on the Microbiological Safety of Foods) 1992). One of the recommendations (storage at ≤10°C and a shelf life of ≤10 days) related to foods, where chilled storage was the sole controlling factor. Later the ACMSF recommendations were revised, and this 10-day rule was changed to storage at ≤5°C and a shelf life of ≤10 days or storage at 5–10°C and a shelf life of ≤5 days (ACMSF (Advisory Committee on the Microbiological Safety of Foods) 1995). This change was made based on a review of 31 references from the literature on the production of neurotoxin by nonproteolytic *Cl. botulinum* within 10 days at ≤10°C (mostly challenge tests carried out in foods) and also predictions from a PC-based version of Food MicroModel and an unpublished industry challenge test data (ACMSF (Advisory Committee on the Microbiological Safety of Foods) 1995). Current recommendations made by the ACMSF are summarized in Table 6. It is recommended that the heat treatments or combination processes deliver a safety factor of 10^6 [a six-decimal (6D) process] with regard to spores of nonproteolytic *Cl. botulinum* (ACMSF (Advisory Committee on the Microbiological Safety of Foods) 1992; ECFF (European Chilled Food Federation) 1996).

Control of proteolytic *Clostridium botulinum* in minimally heated, chilled foods

Incidence of foodborne botulism

Strains of proteolytic *Cl. botulinum* form type A, B or F neurotoxin (or sometimes more than one toxin).

Table 4 Other examples of recent outbreaks of foodborne botulism

Year	Location	Product	Toxin type	Cases (deaths)	Reference
1993/1994	Switzerland	Commercial dry-cured ham	B*	12	Troillet and Praz (1995)
1994	Georgia	Fish consumed at wedding	nk	173	Varma *et al.* (2004)
1998	Croatia	Ham	B	20	Pavic *et al.* (2001)
1999	Morocco	Commercial Mortadella sausage	B	78 (20)	Ouagari *et al.* (2002)
1999	Azerbaijan	Restaurant, contaminated fish	nk	90 (4)	http://www.promedmail.org (21 December 1999)
2003	France	Halal sausage	B	4	Espie *et al.* (2003)
2003	South Korea	Commercial, canned sausage	nk	3	http://www.promedmail.org (29 June 2003)
2003	Ukraine	Home-canned corn	nk	6	http://www.promedmail.org (21 November 2003)
2004	Italy	Restaurant, preserved green olives in saline	B	16	Cawthorne *et al.* (2005)
2004	Russia	Commercial dried fish	nk	4 (1)	http://www.promedmail.org (26 May 2004)
2004	Russia	Smoked fish	nk	10 (1)	http://www.promedmail.org (25 August 2004)
2004	Ukraine	Commercial dried fish	nk	6	http://www.promedmail.org (30 October 2004)
2004	Kyrgyzstan	Home-canned aubergine	nk	5 (1)	http://www.promedmail.org (3 December 2004)
2005	Russia	Smoked fish	nk	9	http://www.promedmail.org (18 January 2005)
2005	Ukraine	Commercial dried fish	nk	3	http://www.promedmail.org (20 February 2005)
2005	Russia	Home-canned cucumbers	nk	16	http://www.promedmail.org (26 February 2005)
2005	Kyrgyzstan	Home-canned salad	nk	6	http://www.promedmail.org (6 April 2005)
2005	Russia	Home-canned food	nk	5	http://www.promedmail.org (5 July 2005)
2005	Russia	Canned food	nk	15 (1)	http://www.promedmail.org (12 July 2005)
2005	Russia	Home-salted fish	nk	6	http://www.promedmail.org (14 October 2005)
2005	Kazakhstan	Home-dried fish	nk	25 (1)	http://www.promedmail.org (17 October 2005)

nk, not known, toxin reported as present, but type not reported.
*Only toxin identified, unclear whether proteolytic *Clostridium botulinum* type B or nonproteolytic *Cl. botulinum* type B.

Proteolytic *Cl. botulinum* is a mesophile with growth not reported at 10°C or below; thus the correct storage of chilled products will prevent growth and neurotoxin formation by this organism. However, temperature abuse has resulted in outbreaks of botulism associated with commercially produced, chilled foods and proteolytic *Cl. botulinum*; examples include garlic-in-oil, black bean dip and clam chowder (Table 2). The chopped garlic in soybean oil was purchased by a restaurant and then stored unrefrigerated for 8 months (not refrigerated as indicated on the label). Proteolytic *Cl. botulinum* type B grew and formed neurotoxin in one bottle of garlic, which was used in sandwiches, although some employees considered it spoiled (St. Louis *et al.* 1988). This product is now acidified to ensure safety. Two separate outbreaks in California in 1994 also involved temperature abuse of chilled foods and associated growth and neurotoxin formation by proteolytic *Cl. botulinum* type A (Anon 1995). The first outbreak occurred in June and was associated with vacuum-packed clam chowder purchased from the refrigeration section of a supermarket. The chowder was labelled 'keep refrigerated' but stored at room temperature for 1 month prior to consumption. The second outbreak occurred in September and was associated with a black bean dip, also purchased from the refrigeration section of a store. The container was labelled 'perishable, keep refrigerated' but was held at room temperature for 3 weeks prior to consumption. The chowder and bean dip were consumed despite seeming spoiled (Anon 1995). Chilled storage was the only barrier to controlling neurotoxin formation by proteolytic *Cl. botulinum* in these three foods.

Outbreaks of foodborne botulism have also occurred when preformed neurotoxin has been inadvertently added to a food that is correctly stored at a chilled temperature. Examples include the large outbreaks associated with hazelnut yoghurt in the United Kingdom and *skordalia* (a yoghurt-based dip containing cooked potatoes) in the United States (Table 2). The largest recorded botulism outbreak in the United Kingdom was caused by type B neurotoxin, with 27 cases and 1 death, and involved yoghurt prepared with neurotoxin-contaminated hazelnut conserve (O'Mahony *et al.* 1990). Heat processing of the hazelnut conserve was inadequate to destroy spores of proteolytic *Cl. botulinum* type B. The spores subsequently germinated, leading to growth and neurotoxin formation. Although some of the cans exploded, as a result of gas build up, the contents of those that did not were added to the yoghurt. A large restaurant-associated botulism outbreak in the United States was caused by type A neurotoxin, with 30 cases, and involved *skordalia* prepared with neurotoxin-containing potatoes (Angulo *et al.* 1998). Potatoes wrapped in foil were baked and then stored at ambient temperature. Temperature abuse during storage permitted growth and neurotoxin formation by

Table 5 Reported number of cases of foodborne botulism in European countries in 1999/2000 [http://www.bfr.bund.de/internet/8threport/8threp_fr.htm (12 October 2005)]

Country	Number of cases
Albania	0
Armenia	176
Austria	0
Azerbaijan	181
Belgium	0
Belarus	344
Bosnia and Herzegovina	0
Bulgaria	18
Croatia	16
Czech Republic	7
Finland	1
France	60
Georgia	122
Germany	30
Greece	0
Estonia	1
Iceland	0
Italy	21
Kyrgyzstan	94
Latvia	7
Lithuania	36
The Netherlands	3
Norway	5
Poland	169
Portugal	33
Moldova	32
Romania	0
Russia	887
Slovakia	4
Slovenia	7
Sweden	0
Switzerland	3
Tajikistan	4
Turkey	114
United Kingdom	0
Uzbekistan	131

proteolytic *Cl. botulinum* type A. The neurotoxin-containing potatoes were then added to yoghurt to give the *skordalia*. In view of these outbreaks, it is essential to understand factors controlling growth and neurotoxin formation by proteolytic *Cl. botulinum* when producing chilled foods.

Control of proteolytic *Clostridium botulinum*

Proteolytic *Cl. botulinum* produces spores of high heat resistance and is the principal concern for the safe production of low-acid-canned foods (Table 7). A heat treatment at 121·1°C for 3 min (or equivalent heat treatment at another temperature) has been adopted as the minimum standard for a 'botulinum cook' for canned foods (Stumbo *et al.* 1975). Botulism outbreaks have occurred in ambient stored foods when the full heat treatment has not been appropriately delivered (Table 2). The low heat treatments applied to minimally heated foods would be unlikely to have any significant effect on spores of proteolytic *Cl. botulinum*.

The minimum temperature at which growth and neurotoxin production occurs is within the range of 10–12°C (Peck and Stringer 2005) and thus will not occur in correctly chilled products. Growth and neurotoxin formation have been reported in 3–4 weeks at 12°C from a large inoculum. Growth of proteolytic *Cl. botulinum* is prevented at pH ≤4·6, or by 10% NaCl (Table 7), and the minimum water activity permitting growth is 0·96 and 0·93 with NaCl and glycerol, respectively, as humectants (Lund and Peck 2000). The use of other factors and combinations of factors to control or prevent the growth of proteolytic *Cl. botulinum* has been described, and predictive models have been developed (Baker and Genigeorgis 1992; Dodds 1993; Lund 1993; Lund and Peck 2000). A model available in ComBase Predictor (http://www.combase.cc/predictor.html, 3 April 2006) describes the effect of temperature (14–40°C), pH (4·7–7·2) and NaCl concentration (0–7·5%) on growth from spores of proteolytic *Cl. botulinum*. According to this model, 37°C is the temperature with the shortest time for a three-decimal increase (12·6 h). Proteolytic *Cl. botulinum* is slower growing than nonproteolytic *Cl. botulinum* at 30°C and below, when other factors are not limiting (Fig. 1). Published and unpublished original growth and death curves are available free of charge in ComBase (http://www.combase.cc, 31 October 2005). Predictive models are also freely available through software packages, such as ComBase Predictor and the Pathogen Modeling Program (http://www.combase.cc/predictor.html, http://www.arserrc.gov/mfs/PMP6_start.htm). Predictions generally compare well with observed growth and neurotoxin formation in independent data sets, giving the user confidence that the models can be used to target effectively challenge tests.

Control of nonproteolytic *Clostridium botulinum* in minimally heated, chilled foods

Incidence of foodborne botulism

Nonproteolytic *Cl. botulinum* is a psychrotroph and derives energy from the degradation of sugars. Strains form type B, E or F neurotoxin. Botulism outbreaks associated with nonproteolytic *Cl. botulinum* have occurred most frequently with processed fish (e.g. salted fish, dried fish, vacuum-packed fish) and fermented marine products that are consumed in Alaska and northern Canada (Table 3). It is likely that several of the outbreaks detailed

Table 6 Recommended procedures to ensure the safety of minimally heated, chilled foods with respect to nonproteolytic *Clostridium botulinum* (ACMSF (Advisory Committee on the Microbiological Safety of Foods) 1992, 1995)

Storage at <3·0°C*
Storage at ≤5°C and a shelf life of ≤10 days
Storage at 5–10°C and a shelf life of ≤5 days
Storage at chill temperature† combined with heat treatment of 90°C for 10 min or equivalent lethality (e.g. 70°C for 1675 min, 75°C for 464 min, 80°C for 129 min, 85°C for 36 min)‡
Storage at chill temperature combined with ≤pH 5·0 throughout the food
Storage at chill temperature combined with a salt concentration ≥3·5% throughout the food
Storage at chill temperature combined with ≤a_w 0·97 throughout the food
Storage at chill temperature combined with combinations of heat treatment and other preservative factors, which can be shown consistently to prevent the growth and toxin production by *Cl. botulinum.*

*Originally 3·3°C but growth has now been demonstrated at 3·0°C (Graham *et al.* 1997).
†Chill temperature is specified as 8°C in the United Kingdom.
‡Alternative heat treatments of 80°C for 270 min, 85°C for 52 min are recommended by the European Chilled Food Federation (ECFF (European Chilled Food Federation) 1996).

Table 7 Effect of environmental factors on the growth and survival of the two clostridia most commonly responsible for foodborne botulism (modified from Lund and Peck 2000)

	Proteolytic *Cl. botulinum*	Nonproteolytic *Cl. botulinum*
Neurotoxins formed	A, B, F	B, E, F
Minimum temperature for growth	10–12°C	2·5–3·0°C
Optimum temperature for growth	37°C	25°C
Minimum pH for growth	4·6	5·0
NaCl concentration preventing growth	10%	5%
Minimum water activity for growth NaCl as humectant	0·96	0·97
Glycerol as humectant	0·93	0·94
Spore heat resistance	$D_{121°C}$ = 0·21 min	$D_{82·2°C}$ = 2·4/231 min*
Foods involved in botulism outbreaks	Home-canned foods, faulty commercial processing	Fermented marine products, dried fish, vacuum-packed fish
Potential food problems	Canned foods	Minimally heated, chilled foods

*Heat resistance data without/with lysozyme during recovery.

in Table 4 are also associated with nonproteolytic *Cl. botulinum* (e.g. those involving fish, European type B cases). An outbreak in Germany in 1997 involved suspected temperature abuse of commercial hot-smoked, vacuum-packed fish (Jahkola and Korkeala 1997; Korkeala *et al.* 1998), and several other outbreaks have also been associated with temperature abuse (Table 3). Two outbreaks associated with type E toxin and vacuum-packed, hot-smoked rainbow trout were recorded in Sweden in 1991 and 1994 (Korkeala *et al.* 1998).

Minimally heated, chilled foods have an excellent safety record with respect to foodborne botulism. It is essential, however, that safety be maintained as new types of foods are developed. A change in the aetiology of foodborne botulism in France has been reported over the last few years. There has been an increased association with type E toxin compared with type B toxin and with commercial foods compared with home-made foods (Boyer *et al.* 2001; Boyer and Salah 2002; Popoff and Carlier 2004). It has been suggested that these changes might be associated with an increased consumption of vacuum-packed foods (Boyer *et al.* 2001; Boyer and Salah 2002; Popoff and Carlier 2004).

Control of nonproteolytic *Clostridium botulinum*

Nonproteolytic *Cl. botulinum* forms spores of moderate heat resistance (Table 7). Heating spores for 5–10 min at 80–85°C inactivates the spore germination system resulting in sublethal injury (Peck *et al.* 1992; Lund and Peck 1994). However, a fraction (typically 0·1–1%) of these heat-damaged spores is permeable to lysozyme, and in/on lysozyme-containing media give growth and biphasic survival curves (Peck and Stringer 2005). Lysozyme is able to diffuse through the coat of this fraction of heat-damaged spores inducing germination by hydrolysing

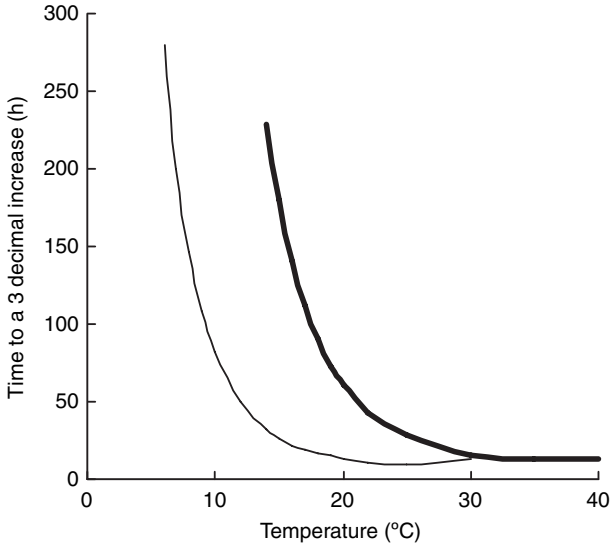

Figure 1 Effect of incubation temperature on predicted time to a three-decimal increase in viable count of proteolytic *Clostridium botulinum* and non-proteolytic *Cl. botulinum*, at pH 7·0 and 0·5% NaCl [from models in ComBase Predictor (http://www.combase.cc/predictor.html; 3 April 2006)]. — nonproteolytic *Cl. botulinum*; ▬ proteolytic *Cl. botulinum*.

peptidoglycan in the cortex. The *D* values for lysozyme-permeable spores are approx. two orders of magnitude greater than those obtained with recovery in the absence of lysozyme but are still lower than those of proteolytic *Cl. botulinum* spores (Table 7). In view of the presence of enzymes with lysozyme activity in many foods and its relatively high heat stability (Stringer *et al.* 1999; Peck and Stringer 2005), the effect of lysozyme on sublethally heat-damaged spores is potentially important in foods that rely on spore heat inactivation for safety. For example, in studies with a model food with no added lysozyme, heat treatments of 85°C for 36 min, 90°C for 10 min and 95°C for 15 min each prevented an inoculum of 10^6 spores of nonproteolytic *Cl. botulinum* leading to the growth and toxin formation at 25°C in 60 days (Peck and Fernandez 1995; Peck *et al.* 1995; Graham *et al.* 1996a; Fernandez and Peck 1997). When hen egg white lysozyme was added to this model food prior to heating (at 625–2400 U ml^{-1}), and heat treatments of 85°C for 84 min, 90°C for 34 min or 95°C for 15 min were applied, growth was observed at 25°C after 13, 14 and 32 days, respectively (Peck *et al.* 1995; Fernandez and Peck 1999). A similar effect may also have been noted with endogenous lysozyme in crabmeat, where heat treatments at 88·9°C for 65 min, 90·6°C for 65 min, 92·2°C for 35 min or 94·4°C for 15 min were required to prevent the growth and toxin formation from 10^6 spores of nonproteolytic *Cl. botulinum* at 27°C in 150 days (Peterson *et al.* 1997). A lysozyme concentration of 200 $\mu g\ g^{-1}$ has been estimated for crabmeat prior to heating (Lund and Peck 1994).

The minimum temperature at which growth and neurotoxin formation have been reported is 3·0°C (Graham *et al.* 1997). Growth has been reported at 3·0°C after 7 weeks, at 3·1°C after 6 weeks and at 3·2°C and 3·3°C after 5 weeks (Schmidt *et al.* 1961; Eklund *et al.* 1967a,b; Graham *et al.* 1997). Growth and neurotoxin formation have not been detected during incubation at 2·1–2·5°C for 90 days (Ohye and Scott 1957; Schmidt *et al.* 1961; Eklund *et al.* 1967a,b; Graham *et al.* 1997). While chilled foods could be held at <3·0°C in some circumstances (e.g. institutions, catering establishments), it is unlikely that this temperature can be maintained throughout the distribution chain, particularly in products intended for domestic use. Indeed, regulations in England and Wales require chilled foods to be held at ≤8°C. According to a model available in ComBase Predictor (http://www.combase.cc/predictor.html 3 April 2006), which describes the effect of temperature, pH and NaCl concentration on growth from spores of nonproteolytic *Cl. botulinum*, 25°C is the temperature with the shortest time to a three-decimal increase (10·4 h). Nonproteolytic *Cl. botulinum* is faster growing than proteolytic *Cl. botulinum* at 30°C and below, when other factors are not limiting (Fig. 1).

Nonproteolytic *Cl. botulinum* does not grow or form neurotoxin below pH 5·0 or at a NaCl concentration above 5%. The minimum water activity permitting growth is 0·97 and 0·94 with NaCl and glycerol, respectively, as the humectants (Hauschild 1989; Lund and Peck 2000). The effect of other single preservative and combinations of preservative factors on growth and neurotoxin formation has been described previously and predictive models have been developed (Baker and Genigeorgis 1992; Dodds 1993; Lund 1993; McClure *et al.* 1994; Graham *et al.* 1997; Whiting and Oriente 1997; Lund and Peck 2000; Fernandez *et al.* 2001). Published and unpublished original data and some of these predictive models are freely available through the Internet (as mentioned above).

Developments in predictive microbiology over the last few decades have enabled reliable and accurate predictions to be made of the growth rate of foodborne pathogens based on the environmental conditions (Baranyi 1998). Lag time is more difficult to predict reliably, however, and is dependent on the history of the spores (e.g. they might be subject to sublethal heat damage) as well as current growth conditions. The effect of history can also vary from spore to spore, and these individual biovariabilities combine to give the variability in the population lag time. This variability is particularly important when growth results from a single spore or a few spores, as is

likely to be the case with nonproteolytic *Cl. botulinum* in minimally heated, chilled foods. Here, time to neurotoxin formation is likely to be closely related to the distribution of individual lag times within a population. Prediction of growth of nonproteolytic *Cl. botulinum* in minimally heated, chilled foods would be improved by better understanding and prediction of lag time biovariability. Improved understanding would enable the development of mechanistic models rather than empirical models and could also aid identification of new intervention strategies.

Lag phase in spores can be subdivided into germination, emergence, time for maturation of one cell and time to two cells (Stringer *et al.* 2005). Spore germination is a cascade of degradative steps using preformed enzymes, and in nonproteolytic *Cl. botulinum*, it is activated by pairs of germinants (e.g. L-lactate and L-alanine, L-cysteine or L-serine) (Plowman and Peck 2002). The later stages require macromolecular synthesis. In order to better understand and predict lag time biovariability, studies have been undertaken using phase-contrast microscopy and image analysis to quantify the heterogeneity of the duration of individual stages of lag from single spores (Stringer *et al.* 2005). In tests carried out in PYGS (peptone, yeast extract, glucose, starch) medium at 22°C, the mean time to germination was 2·6 h [standard deviation (SD) = 3·2 h], emergence was 5·8 h (SD = 2·9 h), time to one mature cell was 7·6 h (SD = 2·6 h) and time to two cells was 8·7 h (SD = 2·5 h) (Fig. 2). It was established that overall lag time variability is a result of variability in each stage of lag phase and that the duration of the various stages were not correlated. Thus, it was not possible to predict the total lag time from germination time, and the first spore to germinate was not the first to reach two cells (Stringer *et al.* 2005). These initial findings have begun to explain why lag phase is so variable in nonproteolytic *Cl. botulinum*, and in the longer term can make a substantial contribution to improved predictive models, and the continued safe production of minimally heated foods with respect to nonproteolytic *Cl. botulinum*.

The microbiological safety of many minimally heated, chilled foods relies on a combination of factors, including a minimal heat treatment, chilled storage, a restricted shelf life and also occasionally intrinsic preservatives (e.g. reduced pH). Considerable effort, therefore, has been dedicated to define and develop predictive models that describe combinations of factors that provide an appropriate degree of protection against growth and toxin production by nonproteolytic *Cl. botulinum* (Fernandez and Peck 1997, 1999; Peck and Stringer 2005). In studies with a model food, the effect of heat treatment at 65–95°C combined with subsequent storage at 5–25°C on time to growth from an inoculum of 10^6 spores of a mixture of strains of nonproteolytic *Cl. botulinum* was determined. Growth was confirmed by the presence of *Cl. botulinum* neurotoxin. On their own, the heat treatments at 85°C and 90°C advocated by the ACMSF (Advisory Committee on the Microbiological Safety of Foods) (1992) and ECFF (European Chilled Food Federation) (1996) each delivered a 6D reduction in the absence of lysozyme (Table 8). However, the heat treatments recommended at 70–80°C failed to deliver a 6D reduction in the absence of lysozyme, and all the heat treatments failed to deliver this defined kill when lysozyme was present during recovery (Table 8). In many circumstances, the effective delivery of a 6D process is therefore dependent on these heat treatments being combined with chilled storage and/or a restricted shelf life. A combination of the heat treatments advocated by the ACMSF (Advisory Committee on the Microbiological Safety of Foods) (1992) and ECFF (European Chilled Food Federation) (1996) and storage for 21 days at 8°C delivered a 6D process with respect to nonproteolytic *Cl. botulinum* (Table 8). All the heat treatments, except for 70°C for 1675 min also delivered a 6D process when the storage was extended to 42 days at 8°C (Table 8). From the validated predictive models and original data, it is possible to identify effective combinations of heat treatment and storage temperature and that prevent growth and neurotoxin formation by nonproteolytic *Cl. botulinum* within a specified shelf life (Fernandez and Peck 1997, 1999). The additional effect of intrinsic factors such as a reduced pH or an elevated NaCl concentration on controlling growth of nonproteolytic *Cl. botulinum* has also been quantified (Graham *et al.* 1996b; Peck and Stringer 2005). All these data provide an important step forward with respect to the rational development of minimally heated foods, in that they identify combinations of factors that provide a 6D process with respect to nonproteolytic *Cl. botulinum*. It is likely that in some circumstances, the recommendations of the ACMSF (Advisory Committee on the Microbiological Safety of Foods) (1992) and ECFF (European Chilled Food Federation) (1996) may leave only a small margin of safety, while in other circumstances they might lead to unnecessary overprocessing. By adopting effective combination processes, it should be possible to avoid potentially dangerous situations and optimize organoleptic properties of product.

Process risk models have been developed to assess the risk of foodborne botulism presented by nonproteolytic *Cl. botulinum* using a probabilistic modelling approach (Barker *et al.* 2002, 2005). The entire process is considered as a series of linked operations and utilizes recent developments in mathematics and computing to assess the magnitude of the risks and to identify potential hazard scenarios. Rather than using a single number for the value of each variable (e.g. number of spores initially

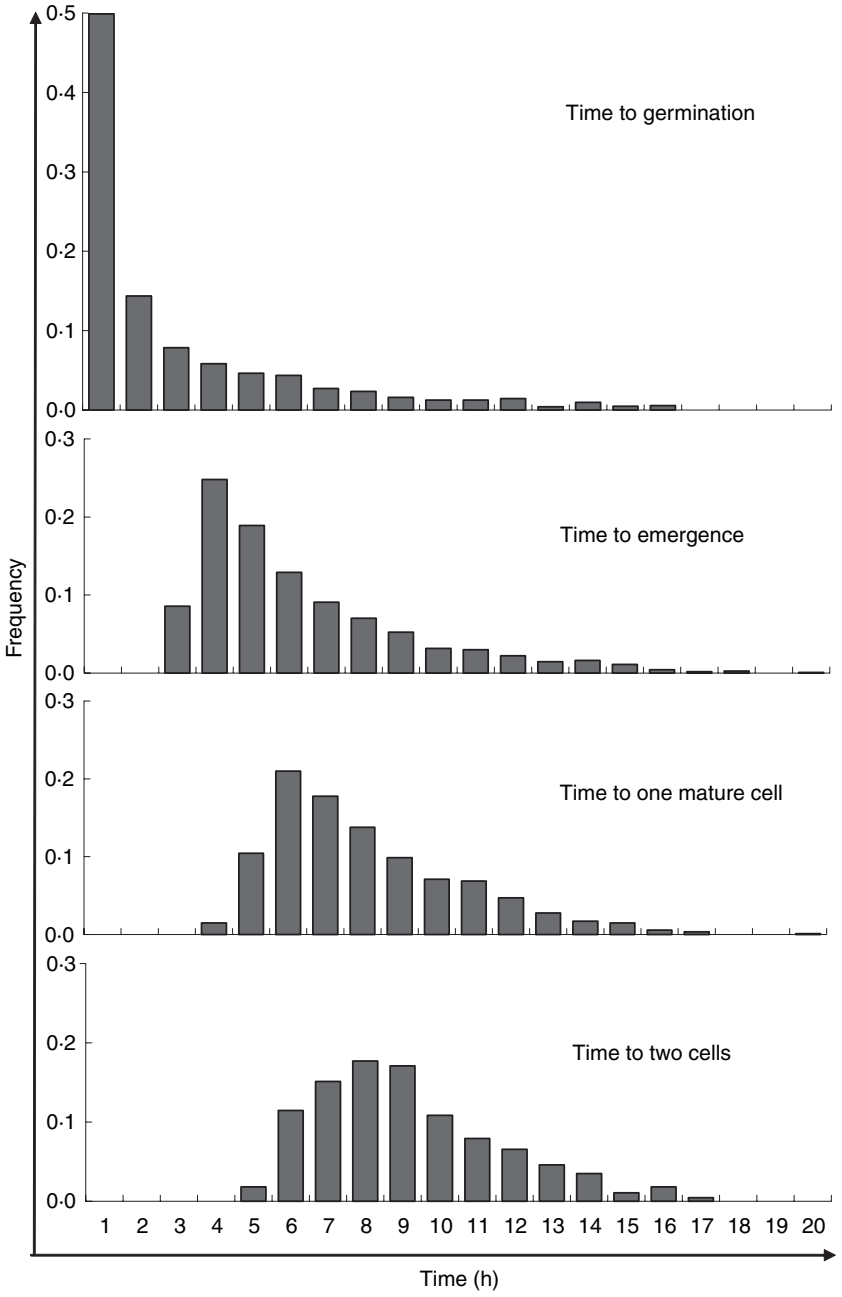

Figure 2 Frequency distributions for individual stages of lag time for single spores of nonproteolytic *Clostridium botulinum* strain Eklund 17B in PYGS (peptone, yeast extract, glucose, starch) medium at 22°C (modified from Stringer *et al.* 2005).

contaminating the product), the probabilistic approach assigns a probability distribution that incorporates an intrinsic representation of uncertainties. This framework can be incorporated in a belief network structure to give a clear picture of information dependencies and system complexity. The outputs from probabilistic risk models can be used to identify and prioritize steps that minimize the effects of detrimental events and that maximize awareness of process control options. A probabilistic representation has been developed for the exposure of consumers to nonproteolytic *Cl. botulinum* neurotoxin in gnocchi, a minimally heated, chilled potato product (Barker *et al.* 2005). The model concentrates on a simple end point, the toxicity of an individual retail unit at the point of consumer preparation, which is related to an individual risk. The production process includes mixing of the ingredients (raw potato flakes, starch and other minor ingredients), delivery of a minimal heat treatment (<90°C/10 min), cooling and packaging. Account is taken of consumer behaviour, with respect to temperature of storage and time prior to consumption. The product has an extended shelf life under chilled conditions. By

Table 8 Assessment of the ability of recommended heat treatments to give a 6D reduction in the probability of growth from spores of nonproteolytic *Clostridium botulinum* (from data in Fernandez and Peck 1997, 1999)

Recommended heat treatment	6D process by heat treatment alone		6D process at 8°C and 21 days		6D process at 8°C and 42 days	
	No lys	+lys	No lys	+lys	No lys	+lys
ACMSF (Advisory Committee on the Microbiological Safety of Foods) (1992)						
70°C/1675 min	No	No	Yes	Yes	No	No
75°C/464 min	No	No	Yes	Yes	Yes	Yes
80°C/129 min	No	No	Yes	Yes	Yes	Yes
85°C/36 min	Yes	No	Yes	Yes	Yes	Yes
90°C/10 min	Yes	No	Yes	Yes	Yes	Yes
ECFF (European Chilled Food Federation) (1996)						
80°C/270 min	No	No	Yes	Yes	Yes	Yes
85°C/52 min	Yes	No	Yes	Yes	Yes	Yes
90°C/10 min	Yes	No	Yes	Yes	Yes	Yes

No lys, no lysozyme added; +lys, hen egg white lysozyme added at 625–2400 U ml^{-1} prior to heat treatment.

combining data and information from several sources, it was demonstrated that gnocchi is particularly safe with respect to nonproteolytic *Cl. botulinum* hazards (Barker *et al.* 2005). Output from the process risk model was consistent with an assessment of the prevalence and behaviour of nonproteolytic *Cl. botulinum* in gnocchi (Del Torre *et al.* 2004).

Future developments

Foodborne botulism is a severe disease, and it is essential that all new foods be developed safely without associated illness. There must not be a repeat of the large number of botulism cases encountered when various processes were first used for the production of low-acid-canned foods. Nonproteolytic *Cl. botulinum* has been identified as the principal microbiological safety hazard for the safe production of minimally heated, chilled foods. Commercial minimally heated, chilled foods have a good safety record with respect to foodborne botulism. Occasional outbreaks have been associated with temperature abuse, and it has been suggested that a change in the aetiology of foodborne botulism in France might be associated with vacuum-packed foods. The chilled food market is currently increasing by about 10% per year, and it is likely that this trend will continue. Additionally, there is considerable interest in increasing the quality of these foods (by the use of lighter heat processes) and extending the shelf life. There is, therefore, a continuing need to develop safe chilled foods. In particular, the movement towards lighter processing and a longer shelf life will require further developments in quantitative microbiological food safety. These developments should enable that overprocessing be avoided, while ensuring that food remains safe with respect to the foodborne botulism hazard. There are several routes through which quantitative microbiological food safety can be progressed.

- Develop a better understanding of properties of the food and the effect of the applied process. Some foods undoubtedly present a greater risk than others, and it is important to identify those foods that present a greater risk and identify the mechanism. It might be reasonable to classify foods as representing a low, medium or high risk and to apply different processes. For example, in tests carried out at 8°C with 69 sous-vide-type foods inoculated with nonproteolytic *Cl. botulinum*, 17 foods became toxic within 9–16 days, while the other 52 foods were not toxic at 18 days (Baker and Genigeorgis 1992).
- Identify and apply data and predictive models that describe the effect of combinations of factors on growth and neurotoxin formation by nonproteolytic *Cl. botulinum* in minimally heated, chilled foods. Much data and several models already exist, but there would be merit in extending the range of those available. Information on the properties of the food and the applied process are important inputs.
- Deliver a more reliable prediction of time to neurotoxin formation through a better understanding and prediction of lag time and its variability. Improved understanding of lag time may also aid the development of novel intervention strategies.
- Use a probabilistic modelling approach to develop process risk models to assess the magnitude of the risks and to identify potential hazard scenarios by taking account of the entire process. Initial results appear promising (Barker *et al.* 2002, 2005).
- A recent innovation has been that of Food Safety Objectives [e.g. ICMSF (International Commission on Microbiological Specifications for Foods) 2002]. This allows account to be taken of the contamination level, in addition to any reduction or increase of the hazard, and can be described in terms of (all in log units)

$$H_0 - \sum R + \sum I \leq \text{FSO}$$

where H_0 is the initial level of hazard, $\sum R$ the total cumulative reduction of hazard, $\sum I$ the total cumulative increase of hazard and FSO the food safety objective.

At the present time, there is no specified FSO for minimally heated, chilled foods, and default criteria have been developed by expert bodies (e.g. ACMSF (Advisory Committee on the Microbiological Safety of Foods) 1992, 1995; ECFF (European Chilled Food Federation) 1996). The

default criteria embrace the idea of using a 6D process and is fail-safe and intended to control hazards under 'worst-case' situations. The default criteria assume, for example, a higher than normal level of contamination of the product (H_0). Based on the above equation, any combination of H_0, $\sum R$ and $\sum I$ can be used to deliver the required FSO. For example, it is reasonable to expect that manufacturers who can demonstrate that their raw ingredients contain low numbers of spores be permitted to apply a lower heat treatment. One difficulty with this approach, however, is that it uses single-point values. For example, just one value for H_0 is used. There would be merit in implementing distributions, as for process risk models.

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