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Hazard and control of group II (non-proteolytic) *Clostridium botulinum* in modern food processing

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Abstract

Group II (non-proteolytic) *Clostridium botulinum* poses a safety hazard in modern food processing, which consists of mild pasteurization treatments, anaerobic packaging, extended shelf lives and chilled storage. The high risk is reflected in the relatively large number of botulism cases due to group II *C. botulinum* in commercially produced foods during the past decades. Because of the high prevalence of group II *C. botulinum* in the environment, food raw materials may carry spores. Although group II spores are less heat-resistant than group I (proteolytic) spores, they can tolerate the heat treatments employed in the chilled food industry. Some food components may actually provide spores with protection from heat. Spore heat resistance should therefore be investigated for each food in order to determine the efficiency of industrial heat treatments. Group II strains are psychrotrophic and thus they are able to grow at refrigeration temperatures. Anaerobic packages and extended shelf lives provide *C. botulinum* with favourable conditions for growth and toxin formation. As the use of salt and other preservatives in these foods is limited, microbiological safety relies mainly on refrigerated storage. This sets great challenges on the production of chilled packaged foods. To ensure the safety of these foods, more than one factor should safeguard against botulinal growth and toxin production.

Keywords: *Clostridium botulinum*; Botulism; Botulinum neurotoxin; Spore heat resistance; Food safety

1. Introduction

Clostridium botulinum is an anaerobic bacterium that under non-optimal growth conditions can form heat-resistant endospores. During their late-logarithmic growth *C. botulinum* strains produce highly potent neurotoxins that cause a neuroparalytic disease known as botulism in humans and animals. Botulism may lead to death due to respiratory muscle paralysis unless treated appropriately. The most common forms of human botulism include foodborne botulism, an intoxication due to ingestion of preformed neurotoxin in foods; infant botulism, an infection due to *C. botulinum* spores germinating, outgrowing and producing neurotoxin in the infant's gastrointestinal tract, where the protective, competitive microflora is poorly developed; and wound botulism, an infection with *C. botulinum* spores growing and producing toxin in deep anaerobic wounds. Other rare forms of botulism consist of adult infectious botulism, which resembles infant botulism, inhalational botulism and iatrogenic botulism, a consequence of botulinum toxin treatment.

C. botulinum is ubiquitous in nature and its spores are naturally present in soil and water. Based on the serological properties of the toxins they produce, *C. botulinum* strains are divided into types A through G. These strains form a diverse group of organisms possessing various genetic (Lee and Riemann, 1970; Hielm et al., 1999; Keto-Timonen et al., 2005; Nevas et al., 2005) and phenotypic characteristics, and thus, have been further divided into four subgroups I to IV (Holdeman and Brooks, 1970; Lee and Riemann, 1970; Suen et al., 1988). The strains causing human botulism belong to groups I and II. Group I consists of proteolytic organisms producing type A, B, and F neurotoxins. These strains are mesophilic and grow optimally at 35–37 °C, but not at all below 10 °C (Lynt et al., 1982). Their growth-limiting pH is 4.3–4.5 (Smelt et al., 1982), and they can tolerate NaCl concentrations as high as 10% in brine. Their spores are highly resistant to heat. Group II consists of strains with non-proteolytic metabolism that produce type B, E and F toxins.

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These strains are psychrotrophic, with an optimum growth temperature of 26–30 °C. However, they may grow at temperatures as low as 3 °C (Schmidt et al., 1961; Eklund et al., 1967a,b; Graham et al., 1997). Their growth-limiting pH is 5 (Segner et al., 1966), and inhibitory NaCl concentration in brine is 5%. While group II spores are less heat-resistant than group I spores, they can still survive the heat processes employed in the food industry (Hyytiä et al., 1998; Hyytiä-Trees et al., 2000; Lindström et al., 2003).

The strains belonging to groups I and II possess different types of risks in food processing. Group I strains, the spores of which are highly heat-resistant, are frequently related to insufficiently processed home-preserved foods such as canned vegetables and cured meats. Group II strains, owing to their ability to grow at refrigerated temperatures, are a safety risk in modern industrially processed foods. These foods are processed with mild heat treatments that may allow the survival of group II spores. Hermetic sealing yields anaerobic conditions and ensures extended shelf lives, providing botulinal spores with conditions favourable for growth. Due to limited use of salt and other preservatives, the microbiological safety of refrigerated processed foods of extended durability (REPFED) relies mainly upon refrigerated storage. However, the storage temperatures commonly used at the retail level and in home refrigerators may reach 10 °C (Evans, 1998), a temperature that enables the growth and toxin formation of group II strains. This paper focuses on the safety risks posed by group II *C. botulinum* in REPFED products.

2. Foodborne botulism due to group II *C. botulinum* in industrially processed foods

Independent of the type of toxin, the clinical manifestation of foodborne botulism is always similar. Through binding to presynaptic nerve endings, the toxin blocks acetylcholine secretion to the synaptic cleft and inhibits muscle contraction, causing flaccid paralysis. The incubation period is 12–72 h, and typically the symptoms start in the cranial parts of the body causing double vision, uncontrolled salivation, blurred speech and difficulty in swallowing. This is followed by paralysis of the caudal muscles. The foodborne form may also cause gastrointestinal signs such as nausea and constipation. The condition requires urgent therapy as it may lead to death when respiratory musculature fails. The therapy consists of intravenous delivery of trivalent (types A, B and E) antitoxin and intensive supportive care including mechanical ventilation

Table 1
Human botulism due to group II *Clostridium botulinum* in commercial food products in 1980–2004

Country	Year	Number of cases	*C. botulinum* type	Vehicle food item (country of origin if known)	Reference
Algeria	1998	1400	ND[a]	Rotten poultry, processed meat, 'kashir' (Algeria)	Anonymous, 1998
Denmark	2002	1	B[b]	Ready-to-eat garlic in chilli oil dressing (Germany)	Krusell, 2003
Egypt	1991	91	E	Salted uneviscerated mullet fish, 'faseikh'	Weber et al., 1993
France	1997	1	E	Scallops	Therre, 1999
France	1999	1	E	Vacuum-packaged frozen scallops	Boyer et al. 2001
France	1999	1	E	Vacuum-packaged frozen prawns	Boyer et al. 2001
France	2003	4	B[b]	Beef and poultry sausages, 'halal'	Espié et al., 2003
Germany	1997	2	E	Smoked fish	Therre, 1999
Germany	1997	1	E	Deep-frozen fish	Therre, 1999
Germany	1997	2	E	Vacuum-packaged hot-smoked whitefish (Finland)	Korkeala et al., 1998
Italy	1997	1	B[b]	Canned truffle cream (Italy)	Therre, 1999
Italy	ND[b]	ND	ND	Ethnic cheese product (Italy)	Aureli et al., 1999
Italy	ND	ND	B[b]	Pasteurized vegetables in oil (Italy)	Aureli et al., 1999
Italy	1997	1	ND	Roast mushrooms in oil (Italy)	Therre, 1999
Italy	1983	1	E	Canned tuna fish in oil	Mongiardo et al., 1985
Japan	1998	6	B[b]	Salted olives (Italy)	Matsuki, 1998
Kyrgyzstan	2004	5	ND	Canned eggplant	Peredkov, 2004
Morocco	1999	78	B[b]	Mortadella sausage (Morocco)	Ouagari et al., 2002
Norway	1997–2003	9	E	Fermented fish product, 'rakfisk' (Norway)	Kuusi et al., 1998, 1999
Poland	2000	9	ND	Sausages	Przybylska, 2002
Poland	2001	7	ND	Canned fish	Przybylska, 2003
Republic of Georgia	1980–2002	85[c]	E	Smoked fish (Georgia)	Varma et al., 2004
Russia, Buryatia	1999	72	ND[d]	Fish	Pollack, 1999
	2004	6	ND[d]	Smoked fish, 'omul'	Anonymous, 2004a
Russia, Volgograd	2004	4	ND[d]	Dried fish	Anonymous, 2004b
Spain	1997	3	B[b]	Canned asparagus	Therre, 1999
Switzerland	1993–1994	12	B[b]	Cured ham	Troillet and Praz, 1995
Ukraine	2004	6	ND[d]	Dried fish	Melnik, 2004
USA, Hawaii	1990	3	B[b]	Sturgeon fish, 'palani' (USA)	CDC, 1991
USA, New Jersey	1992	3	E	Salted uneviscerated fish, 'moloha' (USA)	French et al., 1992
USA, Oregon	1997	1	B[b]	Burrito (USA)	Sobel et al., 2004

[a] ND, no data available.
[b] The physiological group of type B toxin-producing *C. botulinum* has not been reported.
[c] Estimated from smoked fish, accounting for 12% of the events and involving 706 persons.
[d] ND, no data available; however, botulism related to fish products is frequently due to type E toxin.

(Robinson and Nahata, 2003). As the toxin binding is irreversible, recovery follows the development of sprouting nerve endings. Depending on the toxin type and dose, recovery may take a couple of weeks to several months.

Traditionally, foodborne botulism has been associated with such home-preserved foods as cured meat and canned vegetables, where group I *C. botulinum* prevails. Disease due to commercial products has been less frequent. However, with an accelerating trend in ready-to-eat food consumption, the number of botulism cases caused by these foods has increased. Concern of botulism as a result of consumption of REPFED products first emerged in the 1960s, when large outbreaks of type E botulism from group II strains in commercial vacuum-packaged hot-smoked fish occurred in the Great Lakes district of the United States. The risk has since been established, and cases due to group II *C. botulinum* have been reported in the last 25 years all over the world (Table 1). A pitfall in the laboratory investigation of botulism outbreaks triggered by type B toxin, and more rarely also by type F toxin, is that the physiological group of the causative agent often remains unclear. As the risks possessed by the two groups of *C. botulinum* are distinct because of very different physiologies, information on the causative agent would aid in designing prevention strategies and tools against foodborne botulism in the food industry. Surveillance systems and reporting of botulism outbreaks should thus be improved to include more information on the causative organisms and vehicle foods of botulism outbreaks.

3. Prevalence of group II *C. botulinum* in foods

Because of the high prevalence of *C. botulinum* in the environment (Johannsen, 1962, 1963; Cann et al., 1965,1968; Eklund and Poysky, 1965; Bott et al., 1967; Kravchenko and Shishulina, 1967; Laycock and Loring, 1971; Smith, 1978; Notermans et al., 1979; Huss, 1980; Smith and Young, 1980; Yamakawa and Nakamura, 1992; Hielm et al., 1996,1998a,b; Dhaked et al., 2002), food raw materials may carry spores, challenging the heat processes employed in the food industry. While the prevalence of group II spores in fish and other seafoods has been studied extensively, only a few reports on

Table 2
Prevalence of group II *Clostridium botulinum* in raw foods

Sample	Country	Positive samples (%)	*C. botulinum* type	Reference
Meat	Germany	36	E	Klarmann, 1989
Fish	Denmark	65	E	Huss et al., 1974
	Finland	7.1	E	Ala-Huikku et al., 1977
	Finland	19	E	Hyytiä et al., 1998
	Indonesia	5.1	B[a], E, F[a]	Haq and Suhadi, 1981
	Japan	4.5	E, F[a]	Yamamoto et al., 1970
	Norway	11	E	Tjaberg and Håstein, 1975
	Russia	35	E	Rouhbakhsh-Khaleghdoust, 1975
	Sweden	46	E	Johannsen, 1963
	Sweden and Norway	4.8	E	Cann et al., 1966, 1967
	UK	10	B, E, F	Cann et al., 1975
	UK	1.4	B	Burns and Williams, 1975
	USA	6.3	E	Chapman and Naylor, 1966
	USA, Milwaukee	8.7	B[a], E	Pace et al., 1967a,b
Fish and seafood	USA	43	B, E, F	Baker et al., 1990
	USA	3.6	B, E, F	Baker et al., 1990
Fish viscera, roe and flesh	USA, Alaska	4.9	E	Miller, 1975
Fish gills and viscera	USA, Alaska	1.2	E	Houghtby and Kaysner, 1969
	USA, West coast	9.5	B[a], E	Craig and Pilcher, 1967
Fish skin and intestines	Finland	10	E	Hielm et al., 1998b
Fish intestines, gills and skin	Germany	30	E	Hyytiä-Trees et al., 1999
Fish intestines	Nordic countries	15	E	Huss and Pedersen, 1979
	Poland	18	E	Zaleski et al., 1978
	Thailand	2.3	E	Tanasugarn, 1979
	USA, East coast	4.5	E	Nickerson et al., 1967
	USA, Great Lakes	11	E	Bott et al., 1966
	USA, Great Lakes	17	E	Bott et al., 1968
Fish roe	Finland	7.7	E	Hyytiä et al., 1998
Crab	USA, West coast	53	B[a], E	Eklund and Poysky, 1967
Shellfish	Nordic countries	14	E	Huss and Pedersen, 1979
	USA, West coast	23	B[a], E	Craig et al., 1968
Oysters	USA, California	25	E	Taclindo et al., 1967
Vegetables	Italy	4.3	B[a]	Quarto et al., 1983
Mushrooms	Canada	NR[b]	B[a]	Hauschild et al., 1975
Potato peels	Sweden	68	E	Johannsen, 1963

[a] The physiological group of *C. botulinum* types B and F was not indicated in the original report; however, a low incubation temperature (26–30 °C) was used and/or trypsin activation was required in the detection of types B and F toxins, thus, the presence of group II organisms can not be excluded.
[b] NR, not reported.

Table 3
Heat resistance of group II *Clostridium botulinum* spores in various media

Heating medium	Group II *C. botulinum* type	Temperature (°C) for *D*-value	*D*-value (min)	Temperature range (°C) for *z*-value	*z*-value (°C)	Reference
Phosphate buffer	B	77.5	4.0–103			Smelt, 1980
		80.0	0.6–2.3[a]			Juneja et al., 1995
		85.0	2.5–51.0	85.0–90.0	6.3[a]	Smelt, 1980
		87.5	1.5–24.0			Smelt, 1980
		90.0	0.4–8.3			Smelt, 1980
	E	77.5	1.5–38.0			Smelt, 1980
		80.0	0.4–3.9[a]			Juneja et al., 1995
		80.0	1.2–36.0	80.0–87.5	8.3–9.4[a]	Smelt, 1980
		82.5	0.5–23.6			Smelt, 1980
		85.0	0.3–10.4			Smelt, 1980
		87.5	0.2–6.1			Smelt, 1980
	F	73.9	9.1–12.7	71.1–85.0	5.2–6.7[a]	Lynt et al., 1983
		76.6	1.7–6.6			Lynt et al., 1983
		79.4	0.9–2.1			Lynt et al., 1983
		82.2	0.3–0.8			Lynt et al., 1983
		85.0	0.4			Lynt et al., 1983
Pork and pea broth	B	82.2	1.5–32.3	75.0–100.0	6.5–16.5	Scott and Bernard, 1982
	E	82.2	0.3	75.0–100.0	8.7	Scott and Bernard, 1982
Phosphate buffer, L[b]	B	75.0	283			Peck et al., 1993
		80.0	2.5–4.3[a]			Juneja et al., 1995
		85.0	73.6–90	85.0–95.0	7.6	Peck et al., 1993
		90.0	18.1			Peck et al., 1993
		95.0	4.6			Peck et al., 1993
	E	80.0	1.0–4.5[a]			Juneja et al., 1995
		85.0	48.3	85.0–95.0	8.3	Peck et al., 1993
		90.0	12.6			Peck et al., 1993
		90.6	13.5			Alderton et al., 1974
		95.0	3.2			Peck et al., 1993
		93.3	3.8			Alderton et al., 1974
Distilled water, L		90.6	5.0			Alderton et al., 1974
Pork and pea broth, L	B	82.2	28.2–2224			Scott and Bernard, 1985
	E	82.2	24.2			Scott and Bernard, 1985
Crabmeat	B	88.9	12.9	88.9–94.4	8.5	Peterson et al., 1997
		90.6	8.2			Peterson et al., 1997
		92.2	5.3			Peterson et al., 1997
		94.4	2.9			Peterson et al., 1997
	E	73.9	6.2–13	73.9–85.0	6.4–8.1[a]	Lynt et al., 1977, 1983
		76.6	1.7–4.1			Lynt et al., 1977, 1983
		79.4	1.1–1.7			Lynt et al., 1977, 1983
		82.2	0.5–0.7			Lynt et al., 1977
		82.2	0.5–0.8			Lynt et al., 1983
		85.0	0.2[a]			Cockey and Tatro, 1974
		85.0	0.3			Lynt et al., 1977, 1983
	F	76.6	9.5	76.6–85.0	6.6[a]	Lynt et al., 1983
		79.4	3.6			Lynt et al., 1983
		82.2	1.2			Lynt et al., 1983
		85.0	0.5			Lynt et al., 1983
Crawfish	E	80.0	4.9–7.0	80.0–95.0	8.0–14.5	De Pantoja, 1986
		85.0	6.7–8.8			DePantoja, 1986
		90.0	2.5–3.1			DePantoja, 1986
Oyster homogenate	E	73.9	2.0–9.0	73.9–82.2	4.2–6.2	Chai and Liang, 1992
		75.0	1.3–5.3			Chai and Liang, 1992
		76.7	0.7–2.7			Chai and Liang, 1992
		79.4	0.3–1.0			Chai and Liang, 1992
		80.0	0.8	70.0–80.0	7.6	Bucknavage et al., 1990
		82.2	0.1–0.4			Chai and Liang, 1992
Cod homogenate	B	75.0	53.9	75.0–92.0	8.6	Gaze and Brown, 1990
		80.0	18.3			Gaze and Brown, 1990
		85.0	4.0			Gaze and Brown, 1990
		90.0	1.1			Gaze and Brown, 1990
		92.0	0.6			Gaze and Brown, 1990

(continued on next page)

Table 3 (*continued*)

Heating medium	Group II *C. botulinum* type	Temperature (°C) for *D*-value	*D*-value (min)	Temperature range (°C) for *z*-value	*z*-value (°C)	Reference
	E	75.0	58.5	75.0–92.0	8.3	Gaze and Brown, 1990
		80.0	15.1			Gaze and Brown, 1990
		85.0	4.8			Gaze and Brown, 1990
		90.0	0.8			Gaze and Brown, 1990
		92.0	0.6			Gaze and Brown, 1990
Whitefish chubs	E	80.0	1.6–4.3	80.0–90.0	5.7–7.6[a]	Crisley et al., 1968
Rainbow trout	E	75.0	4.6	75.0–93.0	10.4	Lindström et al., 2003
Rainbow trout, L	E	75.0	255			Lindström et al., 2003
		85.0	98			Lindström et al., 2003
		93.0	4.2			Lindström et al., 2003
Rainbow trout	E	85.0	2.0			Lindström et al., 2003
		93.0	0.4			Lindström et al., 2003
Whitefish	E	81.0	1.9	81.0–90.0	10.1	Lindström et al., 2003
		90.0	1.0			Lindström et al., 2003
Whitefish, L	E	81.9	55			Lindström et al., 2003
		90.0	7.1			Lindström et al., 2003
Turkey slurry, L	B	75.0	32.5	70.0–90.0	9.4	Juneja et al., 1995; Juneja, 1998
		80.0	15.2			Juneja et al., 1995; Juneja, 1998
		85.0	4.9			Juneja et al., 1995; Juneja, 1998
		85.0	7.8	80.0–90.0	10.8	Juneja and Eblen, 1995
		90.0	0.8			Juneja et al., 1995; Juneja, 1998
		90.0	1.1			Juneja and Eblen, 1995
	E	75.0	18.1	70.0–90.0	9.9	Juneja et al., 1995; Juneja, 1998
		80.0	13.4			Juneja et al., 1995; Juneja, 1998
		85.0	1.2			Juneja et al., 1995; Juneja, 1998
Carrot homogenate	B	75.0	19.4	75.0–92.0	9.8	Gaze and Brown, 1990
		80.0	4.2			Gaze and Brown, 1990
		85.0	1.6			Gaze and Brown, 1990
		90.0	0.4			Gaze and Brown, 1990
		92.0	0.4			Gaze and Brown, 1990
	E	75.0	18.1	70.0–90.0	9.8	Gaze and Brown, 1990
		80.0	4.3			Gaze and Brown, 1990
		85.0	0.7			Gaze and Brown, 1990
		90.0	0.5			Gaze and Brown, 1990

[a] Extrapolated from thermal destruction data reported by authors.
[b] L, heating medium containing added lysozyme.

these strains in raw meat and vegetables are available (Table 2). Furthermore, as detection and identification of *C. botulinum* have traditionally been based merely on the ability of a strain to produce botulinum neurotoxin, many earlier studies of raw foods do not indicate the physiological group of the strains. However, based on some laboratory tests employed to demonstrate the toxin formation ability of *C. botulinum* strains at low incubation temperatures and possible trypsin activation of toxin, both indicative of the isolation of group II strains, some type B and F strains included in Table 2 are assumed to belong to group II.

The prevalence of group II *C. botulinum* in food raw materials varies by food stuff and geographical location, with the highest prevalence reported in fish caught in Scandinavia, particularly in the Baltic Sea region (Johannsen, 1963; Huss et al., 1974, Zaleski et al., 1978; Hyytiä et al., 1998; Hyytiä-Trees et al., 1999), in Russia (Rouhbakhsh-Khaleghdoust, 1975) and in the United States (Eklund and Poysky, 1967; Baker et al., 1990). The prevalence in fish and other seafood may be as high as 40–70% (Johannsen, 1963; Huss et al., 1974; Baker et al., 1990). Apart from fish, a high prevalence (36%) of type E spores has been found in meat (Klarmann, 1989) and in potato peels (Johannsen, 1963) (Table 2). The latter reflects not only the high spore contamination level in soil but also the risk of natural contamination of food raw materials with spores. Group II *C. botulinum* counts in raw foods are typically not very high, varying from less than 1 spore/kg (Tanasugarn, 1979; Baker et al., 1990) to 10^2 spores/kg (Hielm et al., 1998a).

The presence of *C. botulinum* spores in food raw materials sets great challenges on the food industry. Consumers are increasingly demanding fresher foods with high nutritive and sensory qualities, and thus, minimal heat processing and limited use of preservatives are desired. These food processing practices do not, however, take into account the microbiological hazards posed by psychrotrophic spore-forming bacteria. Group II *C. botulinum* has been shown to be present in heat-processed foods such as hot-smoked fish (Pace et al. 1967a; Christiansen et al. 1968; Hayes et al., 1970; Hyytiä et al., 1998; Korkeala et al. 1998) and vegetable sausages (Lindström et al., 2001a). While a recent survey suggested a low prevalence of *C. botulinum* in chilled foods (Braconnier et al., 2001), there is evidence that vacuum-packaged frankfurters (Insalata et al., 1969), cured luncheon meat (Taclindo et al., 1967) and smoked turkey products (Abrahamsson and Riemann, 1971) may

contain group II spores. However, as stated earlier, many of these reports do not indicate the physiological group of *C. botulinum*. More extensive studies on the prevalence of group II *C. botulinum* in processed foods are therefore required for thorough risk assessment of this toxin-producing pathogen in REPFED products.

4. Thermal resistance of group II *C. botulinum* spores

Bacterial endospores are known to tolerate different types of stress such as starvation, drying and extreme temperatures. Heat resistance of bacterial spores has been widely studied using *Bacillus* sp. and *Clostridium* sp. as model organisms. Due to the fatal consequences of botulism, particular attention has been paid to the thermal resistance of *C. botulinum*. Spore destruction of group II *C. botulinum* has been researched in a range of media, including laboratory broths, meat, fish and vegetables (Table 3). The heating medium and its pH, water activity (a_w) and protein and fat content, and the natural physiological variation between bacterial strains all have a marked effect on the heat resistance of group II *C. botulinum* spores. *D*-values (decimal reduction time, the time [min] required to reduce the bacterial number by one log-cycle) in different types of foods vary considerably (Table 3).

Because of the high risk of botulism in fish and other seafoods (Table 2), these foods have frequently been used as model matrices for estimating the heat resistance of group II *C. botulinum* spores. *D*-values in oyster homogenate (Bucknavage et al., 1990; Chai and Liang, 1992) have been reported to be lower than those measured in cod homogenate (Gaze and Brown, 1990), crawfish (De Pantoja, 1986) and crabmeat (Cockey and Tatro, 1974; Lynt et al., 1977, 1983; Peterson et al., 1997). Interestingly, in crabmeat, the *D*-values measured by Peterson et al. (1997) were generally higher than those reported elsewhere (Cockey and Tatro, 1974; Lynt et al., 1977, 1983). Methodological differences certainly affect the results of different studies. However, another potential explanation for differences in heat resistance of spores in different foods is the presence or absence of such lytic enzymes as lysozymes.

When present in the recovery medium of heat-injured spores, enzymes with lytic activities increase the apparent heat resistance of group II *C. botulinum* spores (Alderton et al., 1974; Peck et al., 1992a,b,1993; Peck and Fernández, 1995). These enzymes have been postulated to permeate the heat-injured spore coat and induce germination by hydrolysing peptidoglycan in the spore cortex (Gould, 1989). From 0.1% to 20% of the group II *C. botulinum* spore population have been reported to be naturally permeable to lysozyme, possessing a higher measured heat resistance than spores not permeable to lysozyme (Peck et al., 1992a, b, 1993; Lindström et al., 2003). This explains the biphasic thermal destruction curve, with spores non-permeable to lysozyme being destroyed more often than those permeable to lysozyme (Peck et al., 1992a,b; Lindström et al., 2003). Concerns regarding the safety of minimally heat-treated foods have arisen in the food industry since lysozymes and other lytic enzymes are present in many food stuffs (Scott and Bernard, 1985; Proctor and Cunningham, 1988; Lie et al., 1989; Peck and Stringer, 1996; Stringer and Peck, 1996; Stringer et al., 1999). The impact of lysozyme on the heat resistance of spores in various media is clearly illustrated in Table 4. *C. botulinum* type E spores were shown to possess a greater heat resistance in raw fish mince than in autoclaved fish (Alderman et al., 1972), probably indicating higher activity of lytic enzymes in raw foods than in processed foods (Lund and Peck, 1994). A considerable difference in the heat resistance of type E spores in rainbow trout and whitefish was observed between samples incubated in the presence and in the absence of lysozymes (Lindström et al., 2003).

Because the *D*-values of *C. botulinum* group II spores are greatly affected by the heating medium, it is obvious that *z*-values, which are extrapolated from the thermal death-time curves formed by *D*-values measured at different temperatures, are also influenced by the matrix. This is demonstrated in Table 3, where the reported *z*-values vary from 4.2 °C in oyster homogenate to 16.5 °C in pork and pea broth. The *z*-values have been used to create mathematical predictions of process lethality of certain microorganisms, and the *z*-value chosen has a marked effect on the estimate of a safe process time–temperature combination. For example, the recommended heat treatments for processing of group II *C. botulinum* spores vary from 36 min (ACMSF, 1992) to 52 min (ECFF, 1996) at 85 °C based on reference *z*-values of 7 °C (ECFF, 1996) and 9 °C (ACMSF, 1992), respectively. Thus, it is not difficult to see how a fatal underestimation of sufficient heat processes in the food industry might occur. Toxin formation at 8 °C from group II spores has been reported to occur in fish processed at 85 °C for 44 min within 35 days, whereas meat processed for 52 min at the same temperature remained non-toxic for longer than 90 days (Table 4). Therefore, before making mathematical predictions for safe processing of foods, it is of the utmost importance that the *D*- and *z*-values used in the calculations are for the food type in question instead of averages from the literature.

The a_w of the heating medium seems to have a considerable impact on the thermal destruction of *C. botulinum* spores. In an early study by Murrell and Scott (1957) the greatest heat resistance of *C. botulinum* type E spores at 110 °C was observed at an a_w of 0.2–0.9; at an a_w of 0.998, the heat resistance of type E spores decreased drastically by a factor of 30 000. 'Moist' heat has since been employed to facilitate spore destruction in the hot-smoking processing of fish (Pace et al., 1967a; Lindström et al., 2003). A relative humidity (RH) of 70% combined with heat processing at 82 °C for 30 min, the heat treatment officially recommended for commercial hot-smoking of fish in the US in the 1960s (Anonymous, 1964; City of Milwaukee, 1964) were sufficient to eliminate *C. botulinum* type E spores in whitefish chubs by a factor of 10^5 (Pace et al., 1972). When the same heat treatment was employed in the presence of a lower RH, growth and toxin production of 10^5 to 10^6 type E spores were observed (Christiansen et al., 1968; Alderman et al., 1972; Pace et al., 1972). In a later study, RH greater than 70% combined with a heat treatment of 85 °C for 42 min inhibited growth and toxin production from 10^6 type E spores in vacuum-packaged hot-

Table 4
Time to toxin production from 10^3 to 10^6 spores of group II *Clostridium botulinum* types B, E and F in unprocessed and processed laboratory media and foods stored at 5–10 °C

Medium	Heat process		Storage temperature (°C)	Time to growth or toxicity (days)	pH	NaCl content (% v/v)	Type and number of spores	Reference
	Process temperature (°C)	Process time (min)						
Meat	–	–	8	5–7	6.5	0.6–1.5	BEF 10^6	Graham et al., 1996; Fernández et al., 2001
Peptone-yeast extract-glucose-starch (PYGS)	–	–	5	14	6.5	1.5	BEF 10^6	Fernández et al., 2001
Vacuum-packaged salmon	–	–	8	9	6.4	NR[a]	BEF 10^4	Baker and Genigeorgis, 1990
Mushroom	–	–	10	5	6.4	NR	BEF 10^3	Carlin and Peck, 1996
Asparagus	–	–	10	8	5.3	NR	BEF 10^3	Carlin and Peck, 1996
Broccoli	–	–	10	19	5.5	NR	BEF 10^3	Carlin and Peck, 1996
Meat	85	11.4	5	58	6.5	NR	BEF 10^6	Fernández and Peck, 1997
		18.1	5	104	6.5	0.6	BEF 10^6	Graham et al., 1996
		11.4	8	24	6.5	NR	BEF 10^6	Fernández and Peck, 1997
		19.2	8	53	6.1–6.3	NR	BEF 10^6	Peck et al., 1995
		17.3	8	>60	6.1–6.3	NR	BEF 10^6	Peck et al., 1995
		23.3	8	>90	6.5	NR	BEF 10^6	Fernández and Peck, 1997
		17.5	8	>91	6.5	0.6	BEF 10^6	Graham et al., 1996
Meat, L[b]	85	23.3	5	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		35.7	5	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		18.1	8	43	6.5	2.5	BEF 10^6	Graham et al., 1996
		18.1	8	64	6.5	0.6	BEF 10^6	Graham et al., 1996
		23.3	8	61	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		35.7	8	48	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		52.0	8	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
	90	10.3	5	>90	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		10.3	8	54	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		10.9	8	58	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
		15.3	8	68	6.4–6.6	NR	BEF 10^6	Fernández and Peck, 1999
PYGS, L	90	1.0	5	>161	6.8	NR	BEF 10^6	Stringer et al., 1997
		15.0	10	7	6.8	1.5	B $10^{5.1}$	Stringer and Peck, 1997
		15.0	10	14	6.8	3.0	B $10^{5.1}$	Stringer and Peck, 1997
		60.0	10	>161	6.8	NR	BEF 10^6	Stringer et al., 1997
Crab analogue	85	15.0[c]	10	>120	7.2	2.1	B 10^4	Peterson et al., 2002
Hot-smoked salmon	92.2	45.0[d]	10	>120	7.2–7.4	1.0–2.0	BE 10^6	Eklund et al., 1988
Vacuum-packaged hot-smoked rainbow trout, L	85[e]	26–34[e]	8	>35	6.4–6.9	<0.5%	E 10^6	Lindström et al., 2003
Vacuum-packaged hot-smoked whitefish, L	85[e]	44[e]	8	35	6.7–7.6	<0.5%	E 10^6	Lindström et al., 2003
Sous vide beef, L	85[e]	<0.1[e]	8	21	6.2	<0.5%	B $10^{5.3}$	Lindström et al., 2001b
Sous vide pork, L	85[e]	15[e]	8	21	6.0–6.3	0.7%	B $10^{5.3}$	Hyytiä-Trees et al., 2000

[a] NR, not reported.
[b] L, lysozyme added to the recovery medium of heated spores.
[c] Process time does not include the effect of the come-up time of 12 min to the target temperature of 85 °C.
[d] Process time includes a come-up time of 27.7 min to the target temperature of 92.2 °C.
[e] These time–temperature combinations correspond to processes employed in chilled food industry.

smoked fish stored at 8 °C for 5 weeks, whereas the same heat treatment at a low RH resulted in type E toxin production at 8 °C (Lindström et al., 2003). High RH in processing of unpackaged foods should thus be considered another tool for preventing botulism.

5. Risk of group II *C. botulinum* in modern food processing

Increased consumer demand for convenient and fresher foods with minimal preservatives and low thermal processing has led to a tremendous increase in the sales of REPFED products worldwide. These foods are treated with mild heat processes, with maximum temperatures typically reaching 65–95 °C. Whereas these heat treatments eliminate vegetative bacteria, they do not necessarily destroy bacterial spores (Hyytiä et al., 1999; Hyytiä-Trees et al., 2000). Heat treatments are followed by rapid cooling and chilled storage at 1–8 °C. The microbiological quality of REPFED foods thus relies mainly upon the heat treatment and the refrigerated storage temperature. As REPFED products are generally packaged under vacuum or in modified atmospheres (MA) to ensure anaerobic conditions, the shelf lives may be several weeks. This has raised food safety concerns with regard to anaerobic, psychrotrophic spore-forming bacteria (Genigeorgis, 1985; Del Torre et al., 1998, 2001). The three main types of REPFED products are (1) foods that are first processed and then

packaged, (2) foods that are first packaged and then processed and (3) foods that are first cooked, then packaged and pasteurized. Ingredients used in the production of REPFED foods are abundant.

As highlighted above, several factors increase the risk of group II *C. botulinum* in REPFED foods. These include (1) the raw materials applied in REPFED technology containing spores; (2) heat treatments generally being too low to eliminate spores but sufficiently high to destroy competing vegetative bacterial flora; (3) vacuum and MA packaging result in extended shelf lives, thereby allowing multiplication of facultative anaerobic and anaerobic bacteria; (4) group II *C. botulinum* growing at temperatures as low as 3 °C (Schmidt et al., 1961; Eklund et al., 1967a,b; Graham et al., 1997), while typical storage temperatures measured at retail and consumer levels often exceed 10 °C (Evans, 1998); (5) the limited use of NaCl and other preservatives in REPFED foods; and (6) the products not always being further heated, and toxin production by group II *C. botulinum* sometimes preceding the sensory spoilage of the product (Post et al., 1985; Garcia et al., 1987; Ikawa and Genigeorgis, 1987; Gorris and Peck, 1998; Reddy et al., 1999; Lawlor et al., 2000).

6. Measures to control the risk of group II *C. botulinum* in modern food processing

Ideally, the control of group II *C. botulinum* in REPFED products should employ the parallel use of multiple inhibitory factors (Peck and Stringer, 2004) such as sporicidal heat processes and factors inhibiting the germination, growth and toxin production from spores potentially surviving heat treatment (Conner et al., 1989; Gorris and Peck, 1998). According to European guidelines (ACMSF, 1992; ECFF, 1996), the safety of REPFED foods with respect to group II *C. botulinum* should be ensured by a 6D heat treatment, reducing the initial number of group II *C. botulinum* spores by a factor of 10^6. This is analogous to the 'botulinum cook' or 12D concept known in the canning industry. For products to be stored longer than 10 days, time–temperature combinations of 10 min at 90 °C, 36 min at 85 °C, and 129 min at 80 °C (*z*-value of 9 °C; ACMSF, 1992), or 10 min at 90 °C, 52 min at 85 °C, and 270 min at 80 °C (*z*-value of 7 °C; ECFF, 1996) have been proposed to ensure a 6D reduction. Equivalent time–temperature combinations with respect to spore elimination can be extrapolated from the two regression lines (ACMSF, 1992; ECFF, 1996). However, many of these time–temperature combinations have been shown to be insufficient to cause a significant number of decimal reductions for the elimination of spores existing in the product and thus preventing the potential germination growth and toxigenesis (Table 4). Furthermore, factors like a high protein and fat content or the presence of lytic enzymes in the raw material may provide spores with a higher heat resistance (Lindström et al., 2003). The use of high RH together with moderate heat processing could increase the safety of unpackaged foods (Pace et al., 1972; Lindström et al., 2003). As creating universally applicable guidelines for safe processing of REPFEDs is impossible due to the wide variation of different types of ingredients, it is essential that before being launched on the market all products and processes are tested for safety with inoculated pack studies.

If a 6-log reduction in group II spore number can not be guaranteed, the germination and outgrowth of spores must be inhibited. NaCl content of 5% (w/v), a_w below 0.97 (Ohye and Christian, 1966; Baird-Parker and Freame, 1967; Emodi and Lechowich, 1969) or pH below 5.0 (Segner et al., 1966; Lund et al., 1990) will inhibit the growth of group II strains. Alternatively, a storage temperature of under 3 °C throughout the entire storage period will effectively control the risk. However, as the germination of group II *C. botulinum* occurs at a wider temperature range than does growth, i.e. 1–50 °C, with the optimum being 9–25 °C (Strasdine, 1967; Ando and Iida, 1970; Grecz and Arvay, 1982; Evans et al., 1997; Plowman and Peck, 2002), even a slight fluctuation in storage temperature may be dangerous.

Various gas mixtures in MA packages have been applied to control botulinal growth in REPFED foods. A good antitoxigenic effect has been demonstrated with 65–100% CO_2 with or without O_2 and N_2 (Post et al., 1985; Baker and Genigeorgis, 1990; Reddy et al., 1997; Lawlor et al., 2000). Preservatives, although not extensively used in REPFED technology, have been reported to effectively control the growth of group II *C. botulinum*. These include sodium lactate (Meng and Genigeorgis, 1993, 1994) and bacteriocins (Okereke and Montville, 1991) such as subtilin (LeBlanc et al., 1953) and nisin (Scott and Taylor, 1981a,b; Taylor et al., 1985; Somers and Taylor, 1987). Nisin is added to such commercial milk products as yoghurt and cheese. However, as it is most active at an acidic pH, its usefulness in neutral pH foods, like meat, is limited (Lindström et al., 2001b). Nisin has also been reported to decrease the heat resistance of bacterial spores (Penna and Moraes, 2002). In addition, competitive microflora, e.g. lactic acid bacteria, have been shown to inhibit the growth of *C. botulinum* (Lyver et al., 1998a,b; Skinner et al., 1999).

While inhibition of group I *C. botulinum* by sodium nitrite has been extensively studied (Roberts and Ingram, 1973; Christiansen et al., 1974; Roberts, 1975; Tompkin et al., 1978; Sofos et al., 1979), reports on its effects on group II strains are scarce (Cuppett et al., 1987; Hyytiä et al., 1997; Keto-Timonen et al., 2002). In heat-processed foods, nitrite forms the Perigo factor, which is inhibitory to *C. botulinum* (Perigo et al., 1967; Christiansen et al., 1973). The use of nitrite is, however, limited in European countries due to its possible adverse health effects, and in fish products produced in the EU region its use is banned entirely. A large number of other compounds with moderate inhibitory actions against group II *C. botulinum* have been reviewed (Roberts and Gibson, 1982; Rhodehamel et al., 1992).

Mathematical models predicting the lag time to growth and toxin production from unheated and heated spores of group II *C. botulinum* in foods have been developed based on large data series obtained in laboratory media and model food media (Lindroth and Genigeorgis, 1986; Baker and Genigeorgis, 1990; Genigeorgis et al., 1991; Meng and Genigeorgis, 1993; Graham et al., 1996; Fernández and Peck, 1997, 1999; Skinner

and Larkin, 1998; Fernández et al., 2001) (Table 4). The models typically describe the estimated reduction in spore numbers and/or the probability of growth from a single spore when various spore loads are treated by different time–temperature combinations and then incubated under a range of conditions. Theoretically, these models provide a convenient tool to estimate safe shelf lives and minimum heat treatments required in the food industry. However, the commercially available microbiological models do not take into account the possible effect of lysozymes present in the foods, or the effect of other process parameters, such as RH. Moreover, as the data employed in the development of the models are often derived from studies using laboratory media that provide optimal conditions for botulinal growth, the models may generate false predictions (Meng and Genigeorgis, 1993; Gould, 1999; Hyytiä et al., 1999). Safety evaluations by inoculated pack studies are therefore essential (Hyytiä et al., 1999).

7. Conclusions

Several factors contribute to the health hazard posed by group II *C. botulinum* in modern food production. The presence of spores in REPFED foods challenges the chilled storage of foods and questions the safety of packaging minimally heat-treated foods. Botulism arising from REPFED products has been reported since the 1960s and cases continue to emerge. Fish products are a common vehicle for human botulism due to group II *C. botulinum*, but the spectrum of potential causative food items is broad.

The high concentration of lytic enzymes in some foods may enhance the apparent heat resistance of group II *C. botulinum*. This should be borne in mind when evaluating the safety of foods with ingredients that may potentially contain these enzymes. As the heat resistance of group II spores varies greatly depending on the food, parameters (*D*- and *z*-values) employed in predicting safe time–temperature combinations for processing should be individually determined for each food. Production of universal guidelines for different types of food products should be avoided, and the safety of each product should be challenge-tested by inoculated pack studies. Maximum storage temperatures of 3 °C should be enforced for all REPFED products throughout Europe.

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