University of East Anglia

IMPACT OF STRAIN VARIABILITY ON THE RISK PRESENTED TO CHILLED FOODS BY NON-PROTEOLYTIC *CLOSTRIDIUM BOTULINUM*

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Abstract

This PhD thesis aims to extend the current quantitative microbial risk assessment for minimally heated chilled foods by including information on variability of non-proteolytic *Clostridium botulinum* at strain level. The research on strain variability covers practical experiments as well as literature surveys and focuses on heat resistance properties of non-proteolytic *C. botulinum* spores, variability in growth at chill temperatures and variability in growth and neurotoxin formation in the presence of different carbohydrates. A strain classification pattern was developed and compared with published literature information on the genetic variation of non-proteolytic *C. botulinum.* Results for the growth at chill temperatures and from the carbohydrate study provide strong evidence of significant variability for strains of non-proteolytic *C. botulinum* that is associated with the type of neurotoxin formed. Although a literature review on heat resistance did not show correlation between the decimal reduction time and toxin type for non-proteolytic *C. botulinum* it provides a strong quantitative support for modelling of the thermal properties of the spores. Insights on strain variability were included in a modular quantitative microbial risk assessment (QMRA) for a model dairy-based chilled dessert using a Monte Carlo simulation technique. The model considered four steps in product manufacture that either reduced or increased the hazard associated with non-proteolytic *C. botulinum*: spore load in raw material, thermal inactivation of spores, distribution of spores in retail units and population kinetics for non-proteolytic *C. botulinum* during multi-stage storage regimes. The replacement of a commonly applied assumption on strain homogeneity with strain specific characteristics of non-proteolytic *C. botulinum* revealed that inclusion of information on strain variability has an important impact on estimated risks. The QMRA indicated that, for a minimally heated dairy-based dessert, a greater hazard was associated with type E strains than with those of type B and F. The findings in this thesis provide important information relating to food safety and public health and could be used by risk managers for verification of microbiological criteria for particular products originating from geographical locations with higher prevalence of type E non-proteolytic *C. botulinum* strains. This approach illustrates a step forward by including population details into to risk assessment which may become a significant element in the assessment of complex foodborne hazards.

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Oraz siostrom – Ani i Marioli
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Glossary and abbreviations

ACMSF – Advisory Committee on the Microbiological Safety of Food

ANOVA – analysis of variance

APPA – Anderson's pork-pea agar

ATP – adenosine-5'-triphosphate

CDC – Centers for Disease Control

CDF – normal cumulative distribution function

CFA – Chilled Food Association

CFIA – Canadian Food Inspection Agency

CFU – colony form unit

CMM – cooked meat medium

CV – critical value

D(T) – *D*-value at temperature T

DHMH – Maryland Department of Health and Mental Hygiene

DPA – dipicolinic acid

D-value – decimal reduction time

EA – Eugon agar

ESBA – extract-starch-bicarbonate agar

FEM – fortified egg-meat medium

FSA – Food Standards Agency

HACCP – Hazard Analysis and Critical Control Points

HEWL – hen egg white lysozyme

HPA – Health Protection Agency

IFR – Institute of Food Research

LCL – lower confidence interval

LD_{50} – a point on a probability curve representing the time, at which 50% of mice would die

-LYS – recovery media without addition of lysozyme

+LYS – recovery media with addition of lysozyme

MAM – Molten agar medium

MLD_{50} – minimal lethal dose for 50% of mice

MPN – Most Probable Number Method

NAD^+ – nicotinamide adenine dinucleotide

NASA – National Aeronautics and Space Administration

NCIMB – National Collection of Industrial Food and Marine Bacteria

NFPA – National Food Processors Association

NVB – Noyes veal-broth medium

PG – peptidoglycan

PIT – pork-infusion-thioglycollate-starch agar

PYG – peptone-yeast-glucose agar

PYGS – peptone-yeast-glucose-starch medium

QMRA – Quantitative Microbial Risk Assessment

RCA – reinforced clostridial agar

RCM – reinforced clostridial medium

REPFEDs – refrigerated processed foods of extended durability

S.E. – standard error

TDT – thermal death time

TPG – tripticase-peptone-glucose agar

TPGY – trypticase-peptone-glucose-yeast extract agar

TPGYT – trypticase-peptone-glucose-yeast extract agar + 0.1% trypsin

TSA – tryptic-soy agar

TYD – tryptone-yeast-dextrose

UCL – upper confidence interval

UH – University of Helsinki

UR – Unilever Research

VL – Viande-levure layered blood agar

W & F – Wynne and Foster's pork infusion broth

WHO – World Health Organization

1. Introduction

Dynamic social and economic changes over the last few decades have led to new trends in food production. These trends meet consumers demand for minimally processed, healthy products with limited chemical preservatives at an affordable price and little time needed for preparation prior to consumption. The production of this type of product presents many challenges for industry to overcome. These challenges are not limited to structural or business management, but most importantly, the implementation of an effective food safety and quality system that provides consumers with high sensory and high nutritional products without adverse health effects. This is particularly challenging due to the number of potential biological hazards arising at all stages in the food chain (McClure, 2008). Although the traditional approach to food safety has protected consumers for many decades, the increasing complexity of the food supply chain, combined with a strong sensitivity to controlling factors (e.g. in case of chilled products correct storage at refrigeration temperature through the entire shelf life), requires a sophisticated method for the assessment and control of microbiological hazards. Such method is a Quantitative Microbial Risk Assessment (QMRA) – a relatively new tool that allows for the optimal utilisation of available information to improve the efficiency and control of steps involved in the manufacture of minimally heated chilled foods, leading to better assessment and control of the corresponding hazards. One important potential hazard in these foods is non-proteolytic *C. botulinum*, a highly dangerous pathogen (Peck, 1997). Foodborne botulism outbreaks associated with this microorganism in commercially processed foods are rare, however in the case of minimally heated chilled foods there is the potential for many persons to become ill (with 10^9 packs sold each year in UK), so that associated risk has great public health importance. The health effects and medical and economic costs attributable to botulism outbreaks linked with commercially produced foods can be high (Setlow and Johnson, 1997). QMRAs have previously been presented for non-proteolytic *C. botulinum* in minimally heated chilled foods (Carlin *et al.*, 2000; Barker *et al.*, 2002; Barker *et al.*, 2005a; Malakar *et al.*, 2011; Hudson and Lake, 2012). The risks were based on an assumption of homogeneous behaviour for the pathogen in all elements of the food chain and all aspects of the pathogen response in many parts of the modular risk assessment. Nevertheless, a number of

published studies (see section 5.1.6) have revealed a high level of variability for non-proteolytic *C. botulinum* strains. The variability is known to be important during the interpretation of risk assessment in terms of decision making that can lead to underestimation of risk. Therefore, it is crucial to test the significance of strain variability in the risk assessment process for non-proteolytic *C. botulinum* for minimally heated chilled food.

1.1 *C. botulinum* – low frequency, high impact risk

In 2011 there were upwards of 70,000 cases of foodborne disease in reported outbreaks in the European Union, resulting in more than 7,000 hospitalisations and 93 deaths. The most commonly reported causative agents were *Salmonella* spp., bacterial toxins, *Campylobacter*, viruses and *Escherichia coli*. The clostridial toxins were responsible for 165 outbreaks of which 35 cases were reported to be caused by *C. botulinum* (28 hospitalised and 1 death) (EFSA/ECDC, 2013). It should be noted that since foodborne botulism and other forms of foodborne disease are under reported in different European countries, these numbers are likely to be an underestimate of the real position. According to studies of Scallan *et al.* (2011) under reporting factor for *C. botulinum* is equal to 1.1 and 2.0 for under diagnosis. Although *C. botulinum* is a relatively rare source of foodborne disease, it has serious health (extensive convalescence and high fatality rate in comparison to other foodborne pathogens) and commercial consequences (reputation and financial loss) and requires a sophisticated method of control and prevention.

1.1.1 Characteristics of *C. botulinum*

C. botulinum is a group of Gram-positive, rod-shaped organisms (Figure 1.1) that was isolated for the first time in 1895 by Emile van Ermengem (Erbguth, 2004). The bacteria form heat-resistant spores, which are widely distributed in the environment (e.g. soils, sediments of coastal waters, fruits, vegetables, viscera of fish, mammals and shellfish) (Dodds, 1993a; Dodds, 1993b; Carlin *et al.*, 2004; Lindström *et al.*, 2009; Carlin, 2011; Malakar *et al.*, 2013) and are able to germinate under anaerobic conditions. Germination leads to growth and potentially the production of a potent botulinum neurotoxin which is the cause of neuroparalytic disease with a high mortality rate (~10% cases) (Peck, 2006).

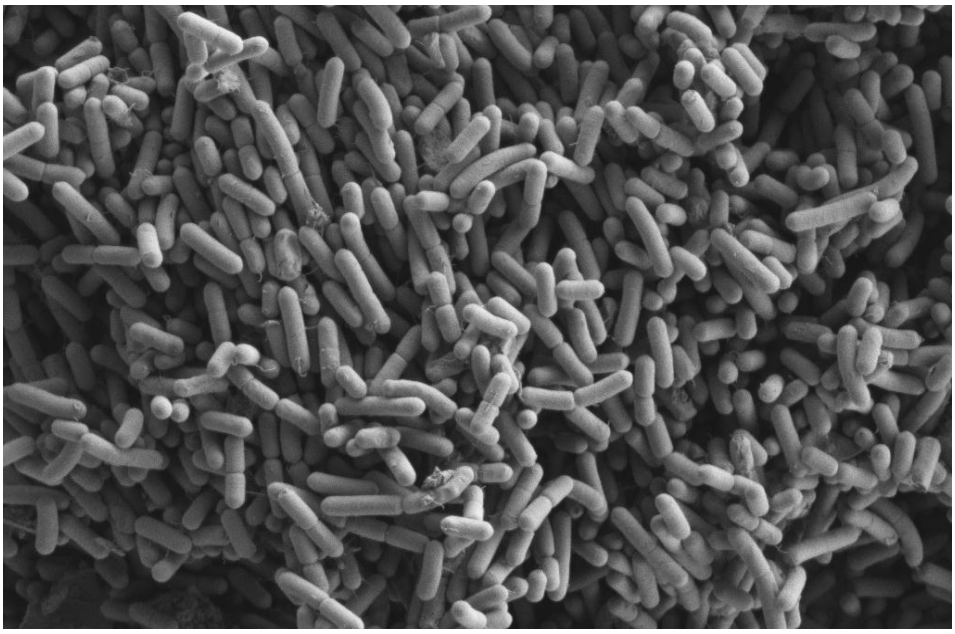

Figure 1.1 Scanning Electron Microscope photograph of non-proteolytic *C. botulinum* strain Eklund 17B
Source: Cross and Carter (2013)

Based on cultural, biochemical and physiological properties this species is classified into four (I – IV) distinct genetic and phenotypic groups with different pathogenicity for humans and animals. The groups have the ability to produce antigenically different neurotoxins. There are seven distinct botulinum neurotoxins, A through G (Table 1.1). Most strains of *C. botulinum* produce toxin of a single antigenic type, although dual-neurotoxin-forming strains have been reported. In this case, a predominant amount of one toxin is formed and a smaller amount of a second toxin e.g. Bf (Peck, 2010). Additionally, some strains of *C. baratii* and *C. butyricum* also form botulinum neurotoxin (Peck, 2009).

Group I *C. botulinum* (proteolytic *C. botulinum*) and Group II *C. botulinum* (non-proteolytic *C. botulinum*) strains form type A, B, E or F neurotoxins and are responsible for foodborne botulism (see below). These groups differ in biodiversity, with Group I *C. botulinum* consisting of more closely related strains than Group II *C. botulinum* (Keto-Timonen *et al.*, 2005; Hill *et al.*, 2007; Stringer *et al.*, 2013). Section 5.1.6 includes an extensive description of inter-strain variability of non-proteolytic *C. botulinum*.

Group of neurotoxigenic *C. botulinum*				
Characteristic	Group I *proteolytic C. botulinum*	Group II *non-proteolytic C. botulinum*	Group III	Group IV
Toxins formed	A, B, F	B, E, F	C, D	G
Genome size (Mb)	3.86 – 4.26	3.66 – 3.80	2.96	
Minimum temp. for growth	10.0 – 12.0°C	2.5 – 3.0°C	15.0°C	
Optimum temp. for growth	37.0°C	25.0°C	40.0°C	37.0°C
Minimum pH for growth	4.6	5.0	5.1	
NaCl concentration preventing growth	10%	5%		6.5%
Minimum a_w for growth[a)]	0.94/0.93	0.97/0.94		
Spore heat resistance[b)]	121°C/0.2	82°C/2.4	104°C/0.9	104°C/1.2
Spore radiation resistance[b)]	2.0 – 4.5 kGy	1.0 – 2.0 kGy		
Ferment glucose[c)]	+	+	+	-
Ferment fructose	V	+	V	-
Ferment maltose	V	+	V	-
Ferment sucrose	-	+	-	-
Lipase production	+	+	+	-
Proteolysis	+	-	-	+
Disease host	human	human	animal	
Similar atoxic organism	*C. sporogenes*	no species specified	*C. novyi*	*C.subtermi-nale*

Table 1.1 Physiological properties of four groups of *C. botulinum* that form botulinum toxin

Source: Data from Hatheway (1993), Peck (2009), Peck (2010), Peck *et al.* (2011)

[a)] a_w (water activity) – amount of water available for microbial growth; [b)] expressed in a form of *D*-value at a given temperature (the concept of *D*-value (in minutes) is described in Chapter 2); [c)] + positive for all strains, - negative for all strains, V variable response; an empty cell indicates that data were not available

Spores of non-proteolytic *C. botulinum* are less heat resistant than those of proteolytic *C. botulinum,* and have different growth requirements. Non-proteolytic *C. botulinum* is able to grow and produce toxins at refrigeration temperature (minimum growth temperature 2.5 – 3.0°C, optimum of 25.0°C), whereas growth of proteolytic *C. botulinum* has not been reported at or below 10.0°C and strains have an optimal growth temperature ~37.0°C. A pH below 5.0 or NaCl concentration above 5% prevents growth and neurotoxin formation by non-proteolytic *C. botulinum*, whereas growth of proteolytic *C. botulinum* is not observed at pH below 4.6 or at a NaCl concentration above 10%. These differences in characteristics of proteolytic and non-proteolytic *C. botulinum* influence the types of foods implicated in foodborne botulism outbreaks (Table 1.2). Dried or vacuum-packed fish and fermented marine products are often associated with non-proteolytic *C. botulinum* (Lund and Peck, 2000; Lindström *et*

al., 2006) and low acid canned foods are often associated with proteolytic *C. botulinum* (Peck, 2010). Strains of Group III *C. botulinum*, that form types C and D neurotoxin, cause most of the cases of botulism in animals, mainly birds, cattle and horses (Kelch *et al.*, 2000).

Although *C. botulinum* strains have been isolated throughout the world, the type of isolated strain tends to be associated with geographical location. For example strains that produce type A toxin are frequently detected in soils from the Western USA and Argentina. Type B strains are most common in soils of Eastern USA and sediments in Britain, Ireland and Iceland, whereas type E strains are found in sediments and coastal soils of Nordic countries, the Baltic Sea, Alaska, Northern Canada and Japan. Type F strains are of relatively low prevalence in the environment (Dodds, 1993b). Such a heterogeneous distribution of strains in different geographic areas means that information regarding the origin of food components has a crucial importance in the calculation of the risk associated with *C. botulinum* (see section 5).

1.1.2 Characteristics of botulism

Botulism is a serious but rare illness that causes flaccid paralysis of muscles. It occurs from ingestion of preformed botulinum neurotoxin or germination and growth of spores present in tissues and subsequent growth and neurotoxin formation. There are four major forms of botulism:

Foodborne botulism

This is most frequently caused by neurotoxins of type A, B, E or occasionally F. The onset of botulism generally occurs 12 to 72 hours after exposure to preformed toxin. Classic neurologic symptoms of botulism include blurred vision, double vision, dry mouth and muscle weakness. Other symptoms may include vomiting, nausea, abdominal cramps or diarrhoea (Lindström and Korkeala, 2006). Untreated, symptoms may include flaccid paralysis of arms, legs, trunk and respiratory muscles. Flaccid paralysis is caused by the neurotoxin blocking release of the neurotransmitter acetylcholine at the motor nerve terminals of the neuromuscular junctions, leading to a bilateral descending paralysis, starting with the head and neck. Severe cases may involve a long hospital stay with an

extensive period of convalescence that may take months or even years. In extremely severe cases, death may result from cardiac or respiratory muscle failure (Lund and Peck, 2000; Peck, 2010). Botulism has been caused by a diverse range of food products (Table 1.2), often home-prepared, but occasionally commercial foods are implicated (see section 1.1.3).

Wound botulism

This form of botulism is analogous to tetanus. It occurs when a wound becomes contaminated with spores, which then lead to growth and toxin production. The symptoms are similar to those in foodborne botulism, however the incubation period is typically much longer ranging from 4 to 14 days (Lindström and Korkeala, 2006; Reller *et al.*, 2006). The first cases of wound botulism were reported in the 1940s and 1950s in the USA (Davis *et al.*, 1951), and most of the time were associated with traumatic wound or post-operative injures. Since 2000 the number of reported wound botulism cases has increased and it is now mainly associated with intravenous drug abuse. The country with the highest number of reported cases is the USA with ~13 cases each year (CDC, 2011a). A few cases are reported each year in the UK (Akbulut *et al.*, 2005), Germany (Galldiks *et al.*, 2007), Sweden (Artin *et al.*, 2007) and France (Roblot *et al.*, 2006). Occasionally wound botulism can occur in a small-scale epidemic often associated with a batch of contaminated heroin (Dhaked *et al.*, 2010).

Infant botulism

Infant botulism occurs in children less than 52 weeks old when ingested spores germinate, grow and produce toxin in the gastrointestinal track. The first clinical symptoms include constipation that lasts for several days, poor feeding and progressive descending neurological deterioration (Brett *et al.*, 2005). Outbreak investigations show that infant botulism is often associated with honey consumption. Many countries recommend that honey jars should be labelled with a statement indicating that the product is not suitable for infants less than 12 months old (Lund and Peck, 2000). Other vehicles previously reported as responsible for infant botulism include infant formula (Brett *et al.*, 2005), corn syrup (Spika *et al.*, 1989) and dust (Nevas *et al.*, 2005).

Adult infectious botulism

Sporadic cases occur when *C. botulinum* colonises the adult's gut following extensive gastrointestinal surgery or intensive antibiotic therapy (Sobel, 2005).

There are also two other forms of botulism: inhalation and iatrogenic. To date few cases of inhalation botulism have been reported – these result from inhalation of toxin by laboratory personnel (Holzer, 1962). Although this form of botulism occurs very rarely it is of public health importance as it could include the use of *C. botulinum* neurotoxin as a biological weapon (Arnon *et al.*, 2001; Peck, 2010). Iatrogenic botulism is the most recently described form of botulism. It occurs as an adverse effect associated with the therapeutic or cosmetic use of botulinum neurotoxin (Coban *et al.*, 2010; Ghasemi *et al.*, 2012).

1.1.3 Epidemiology of foodborne botulism

In all European Union countries, plus Iceland and Norway, botulism is a statutory notifiable disease although the effectiveness of reporting is variable. The effective reporting of foodborne botulism is further complicated by the possibility of misdiagnosis including a number of other conditions including, anxiety, Guillain-Barré syndrome, myasthenia gravis, intoxication, stroke, and more rarely polio, tick paralysis and viral infection of the central nervous system (HPA, 2008). Nevertheless, if botulism is confirmed then due to its serious health effects, epidemiologists conduct a thorough investigation of the botulism outbreak including the identification of the contaminated product and factors supporting spore survival and subsequent growth and neurotoxin formation. Botulism outbreaks summarised in Table 1.2 indicate that botulism has been reported throughout the world, affecting from one to several victims, with various health effects and some fatal. In general, based on the number of reported cases, countries can be classified as those with no reported cases (e.g. Belgium – no reported case in the last 30 years), rare (e.g. UK – less than one per year in the last 30 years) or those where cases are more frequent. The last category includes countries where botulism outbreaks are reported more than once in a year, e.g. Poland with 22 cases reported in 2012 (PZH, 2012), Italy and Romania with 23 and 32 in 2008, respectively (ECDC, 2010). In the majority of cases, botulism is caused by type A, B, E or F toxins produced by proteolytic

C. botulinum or non-proteolytic *C. botulinum*, with rare cases also due to neurotoxigenic *C. butyricum* or *C. baratii* (Peck, 2002; Lindström and Korkeala, 2006). Since proteolytic *C. botulinum* and non-proteolytic *C. botulinum* possess different physiological properties (Table 1.1), the range of food involved in botulism outbreaks is great and can be divided into three main categories: those associated with home-preservation (meats, vegetables or fishes), commercial production or food served in restaurants. Foods involved in outbreaks originating from catering facilities or commercial productions occur relatively rarely (Table 1.2), however due to the potential for many persons to become ill, and the severity of the illness, such outbreaks are always thoroughly investigated. Such an investigation can result in changes in product composition and/or in the manufacturing process, as well as increase in awareness of the hazard amongst the population.

Year	Country	Product	Toxin type	Cases (deaths)	Factors	Reference
Proteolytic *C. botulinum*						
1985	Canada	Restaurant, chopped garlic in oil	B	36	Anaerobic garlic storage, temperature abuse, no controlling factors i.e. acid or salt	Leclair *et al.* (2013)
1985	Canada	Home-canned mushrooms	B	1	Unknown	Leclair *et al.* (2013)
1986	Taiwan	Commercial heat processed bottled peanuts	A	9	Contamination of peanuts with spores of *C. botulinum*, time and temperature abuse	Chou *et al.* (1988)
1987	Canada	Home-bottled chantarelle mushrooms	A	11	Inadequate acidification	CDC (1987b)
1987	UK	Commercial pre-packed kosher in-flight airline meal	A	1	Temperature abuse	Colebatch *et al.* (1989)
1989	UK	Commercial hazelnut yoghurt	B	27 (1)	Insufficient heat process of conserved hazelnut	O'Mahony *et al.* (1990)
1989	Canada	Home-made bean soup	A	1	Unknown	Leclair *et al.* (2013)
1989	USA	Commercial chopped garlic in oil	A	3	Anaerobic garlic storage, temperature abuse, no controlling factors i.e. acid or salt	Morse *et al.* (1990)
1991	Canada	Home-canned asparagus	A	3 (1)	Unknown	Leclair *et al.* (2013)
1993	Canada	Home-made beef and vegetable soup	A	1	Unknown	Leclair *et al.* (2013)
1993	USA	Commercial canned cheese sauce	A	8 (1)	Post-opening contamination, lack of adequate refrigeration	Townes *et al.* (1996)
1993	Italy	Commercial roasted eggplant in oil	B	7	Insufficient acidification/heat treatment	CDC (1995b)
1994	USA	Commercial potato-based and eggplant-based dips	A	30	Foil-wrapped potatoes kept at ambient temperature	Angulo *et al.* (1998)
1994	USA	Commercial clam chowder	A	2	Temperature abuse	Peck (2006)
1994	USA	Home-prepared stew containing roast beef and potatoes	A	1	Product left at ambient temperature under a cover	CDC (1995a)
1995	Canada	Commercial pâté	B	2	Temperature abuse	Leclair *et al.* (2013)
1996	Italy	Commercial acidified cream cheese	A	8 (1)	Heat process insufficient to destroy spores of *C. botulinum*, break in chill storage	Aureli *et al.* (2000)

1997	Italy	Home-prepared pesto/oil	B	3	Inadequate acidification	Peck (2009)
1997	Germany	Home-prepared beans	A	1	Poor hygiene of meal preparation	Peck (2009)
1997	Iran	Commercial traditional cheese	A	27 (1)	Unsafe process	Pourshafie *et al.* (1998)
1998	Algeria	Commercial processed meat ("kashir")	A	340 (37)	Unknown	Lund and Peck (2013)
1998	Thailand	Home-canned bamboo shoots	A	13 (2)	Unsafe process	CDC (1999)
1998	UK	Home-bottled mushrooms in oil brought back from Italy	B	2 (1)	Unknown	CDC (1998)
1998	Argentina	Commercial meat roll ("matambre")	A	9	Unsafe process/storage in heat-shrinked plastic bags, temperature abuse	Villar *et al.* (1999)
1999	France	Commercial chilled fish soup	A	1	Temperature abuse at home	Peck (2009)
1999	Canada	Home-canned tomatoes	B	3	Insufficient acidification, temperature abuse	Loutfy *et al.* (2003)
1999	Japan	Commercial curry boiled in bag	A	1	Temperature abuse	Kobayashi *et al.* (2003)
2000	France	Home-canned asparagus	B	9	Unknown	Abgueguen *et al.* (2003)
2000	Canada	Home-made sausage used to prepare spaghetti sauce	A	1	Unknown	Leclair *et al.* (2013)
2001	Canada	(Outbreak 1) Commercial cooked boneless pork/(Outbreak 2) Home-made fish	A/B	2	Temperature abuse	Leclair *et al.* (2013)
2001	USA	Commercial frozen chili sauce	A	16	Temperature abuse during storage in a shop	Kalluri *et al.* (2003)
2002	South Africa	Commercial tinned fish in tomato sauce	A	2 (2)	Corrosion of tin, which allowed for contamination of *C. botulinum* spores	Frean *et al.* (2004)
2002	Denmark	Commercial garlic in dressing	B	1	Unknown	Lindström *et al.* (2006)
2002	Canada	Restaurant, baked potato in aluminium foil	A	1	Unknown	Bhutani *et al.* (2005)
2003	France	Commercial produced beef and poultry sausage ("halal")	B	4	Unknown	Lindström *et al.* (2006)
2003	UK	Home-prepared meat product ("bigos") brought back from Poland	B	1	Possible temperature abuse during transport	McLauchlin *et al.* (2006)
2004	UK	(Outbreak 1) Commercial prepared hummus/(Outbreak 2) person returning from Georgia	Unknown/A	2	Time and temperature abuse during storage at home/unknown	McLauchlin *et al.* (2006)
2004	USA	Home-made pruno	A	5	Unsafe process	Vugia *et al.* (2009)

2005	UK	Home-preserved pork brought back from Poland	B	1	Unsafe process/time and temperature abuse	McLauchlin *et al.* (2006)
2005	Turkey	Home-made condensed yogurt ("süzme") buried under soil	A	10 (2)	Unsafe process	Akdeniz *et al.* (2007)
2006	Canada/ USA	Commercial chill carrot juice	A	6 (1)	Storage at room temperature	CDC (2006a)
2006	USA	Home-prepared fermented tofu	A	2	Unsafe process	CDC (2007)
2006	Thailand	Home-canned bamboo shoots	A	163	Unsafe process	CDC (2006b)
2006	Taiwan	Home-prepared fermented goat meat	B	5	Unsafe process	Tseng *et al.* (2009)
2006	Austria	Home-slaughtered pork and bacon	B	5	Possible contamination during slaughtering and freezing of pork in airtight bags	Meusburger *et al.* (2006)
2006	Canada	(Adult intestinal botulism) Commercial peanut butter	A	1	Undergone previous bowel surgery	Sheppard *et al.* (2012)
2007	Canada	(Adult intestinal botulism) (Outbreak 1) Commercial peanut butter/(Outbreak 2)	A/A and B	2	Undergone previous bowel surgery/Unknown	Sheppard *et al.* (2012)
2007	USA	Commercial canned hot dog chili sauce	A	8	Insufficient heat process	Juliao *et al.* (2013)
2007	Australia	Commercial nacho meal	A	1	Unknown	Peck (2009)
2007	China	Commercial produced sausage	A	66	Unknown	Lund and Peck (2013)
2008	USA	(Outbreak 1) Home-canned green bean and carrot blend/(Outbreak 2) Home-canned green beans	A	4/3	Insufficient heat process	Date *et al.* (2011)
2008	France	Commercial chicken enchiladas	A	2	Temperature abuse at home	King (2008)
2008	Turkey	Home-packed black olives affected Dutch nationals	B	8	Insufficient acidification	Swaan *et al.* (2010)
2009	USA	Home-canned asparagus	A	3	Insufficient heat process	Date *et al.* (2011)
2010	France	Home-made canned beans or salted roast pork	A	5 (1)	Unknown	Oriot *et al.* (2011)
2011	Austria	Unknown	B	4	Unknown	Vossen *et al.* (2012)
2011	France	(Outbreak 1)/(Outbreak 2) Commercial green olive paste	A	5/3	Insufficient sterilisation process applied to the product	Pingeon *et al.* (2011)
2011	Finland	Commercial conserved olives	B	2	Failure in the processing, packaging and transportation of product	Jalava *et al.* (2011)

2011	USA	Commercially produced potato soup	A	2	Time and temperature abuse	CDC (2011b)
2011	UK	Commercially produced korma sauce	A	3	Insufficient acidification	Browning *et al.* (2011)
2012	USA	Home-fermented tofu	B	2	Possible contamination of bulk tofu with *C. botulinum* spores	CDC (2013)
?	Iran	Home-pickled vegetables/Caviar fish	A and B/A	8/1	Unknown	Barari (2010)/Zadeh *et al.* (2007)
Non-proteolytic *C. botulinum*						
1987	USA/Israel	Commercial uneviscerated, salted, air-dried fish (“kapchunka”)	E	8 (1)	Lack of refrigeration	CDC (1987a)
1991	Sweden	Commercial vacuum-packed smoked rainbow trout	E	Unknown	Unknown	Peck (2009)
1991	Egypt	Commercial uneviscerated, salted fish (“faseikh”)	E	> 91	Suspected temperature abuse with possible anaerobic conditions	Weber *et al.* (1993)
1992	USA	Commercial uneviscerated, salted fish (“moloha”)	E	4	Insufficient salt	CDC (1992)
1994	Sweden	Commercial vacuum-packed smoked fish	E	Unknown	Unknown	Peck (2009)
1995	Canada	Home-made marinated and smoked fish	E	3 (1)	Unknown	Leclair *et al.* (2013)
1995	Canada	Home-made fermented walrus meat	E	9	Insufficient heat process	Peck (2009)
1997	Germany	(Outbreak 1) Commercial hot-smoked vacuum-packed fish (“raucherfisch”) imported from Canada/(Outbreak 2) Home-smoked vacuum-packed fish (“lachsforellen”)	E	6	Suspected temperature abuse	Korkeala *et al.* (1998)/Peck (2009)
1997	France	Fish	E	1	Unknown	Boyer *et al.* (2001)
1997	Argentina	Home-cured ham	E	6	Unknown	Peck (2009)
1998	France	(Outbreak 1) Commercial vacuum-packed frozen scallops imported from China/(Outbreak 2) Commercial vacuum-packed prawns from in Nigeria	E	2	Possible temperature abuse during thawing/Unknown	Boyer *et al.* (2001)

1998	Germany	Commercial smoked vacuum-packed fish	E	4	Unknown	Peck (2009)
1999	Finland	Home-prepared fish eggs	E	1	Unknown	Lindström *et al.* (2004)
1999	France	(Outbreak 1) Salmon or fish soup/(Outbreak 2) Grey mullet	E	2	Unknown	Boyer *et al.* (2001)
2001	Australia	Reheated chicken	E	1	Temperature abuse	Peck (2009)
2001	USA	Home-prepared beaver tail and paws	E	3	Prolonged storage at ambient temperature	CDC (2001)
2001	Canada	Home-made fermented salmon	E	4	Insufficient salt	Peck (2009)
2002	USA	Home-made beluga whale ("muktuk")	E	8	Possible contamination of whale carcass with *C. botulinum* spores	McLaughlin *et al.* (2004)
2003	Norway	Home-made fish ("rakfisk")	E	6	Unknown	Eriksen *et al.* (2004)
2003	Germany	Home-prepared air-dried fish	E	3	Lack of secondary barrier	Eriksen *et al.* (2004)
2004	Germany	Commercial vacuum-packed smoked salmon	E	1	Consumed after "use by date"	Peck (2009)
2005	USA	Home-salted fish	E	5	Insufficient salt	Sobel *et al.* (2007)
2006	Finland	Commercial vacuum-packed smoked whitefish	E	1	Temperature abuse	Peck (2009)
2006	Iran	Home-made soup ("ashmast")	E	11	Unknown	Vahdani (2006)
2009	France	Commercial vacuum packed hot-smoked whitefish	E	3	Temperature abuse before consumption	King *et al.* (2009)
?	Taiwan	Commercial vacuum-packed dried bean curd	E	1	Unknown	Lai *et al.* (2011)
Toxin type not identified						
2005	Kazakhstan	Home-dried fish	Unknown	25 (1)	Unknown	Peck (2006)
2011	Russia	Vegetable salad served in a canteen	Unknown	48	Unknown	Khamzina (2011)
2011	Germany	Unknown	Unknown	2	Unknown	Anon. (2011)
2012	Canada	Egyptian dish ("fesikh") prepared from grey mullet	Unknown	3	Unknown	CFIA (2012)
2012	USA	Home-canned food	Unknown	3	Unknown	DHMH (2012)
2013	Italy	Commercial produced pesto	Unknown	30	Unknown	Powell (2013)
2013	USA	Home-canned elk meat	Unknown	1	Insufficient heat process	Chapman (2013)

Table 1.2 Examples of foodborne botulism incidents in the last 30 years involving proteolytic *C. botulinum* and non-proteolytic *C. botulinum*

1.2 Chilled food in the UK

Urban development, growing industrialization and social changes have led to the development of products which require no or little preparation time prior to consumption. Often these products are minimally heated and stored under chilled conditions (at or below 8.0°C (targeting 5.0°C)) and are variously known as sous-vide, cook-chill and ready-to-eat foods, ready meals, and refrigerated processed foods of extended durability (REPFEDs) (Figure 1.2).

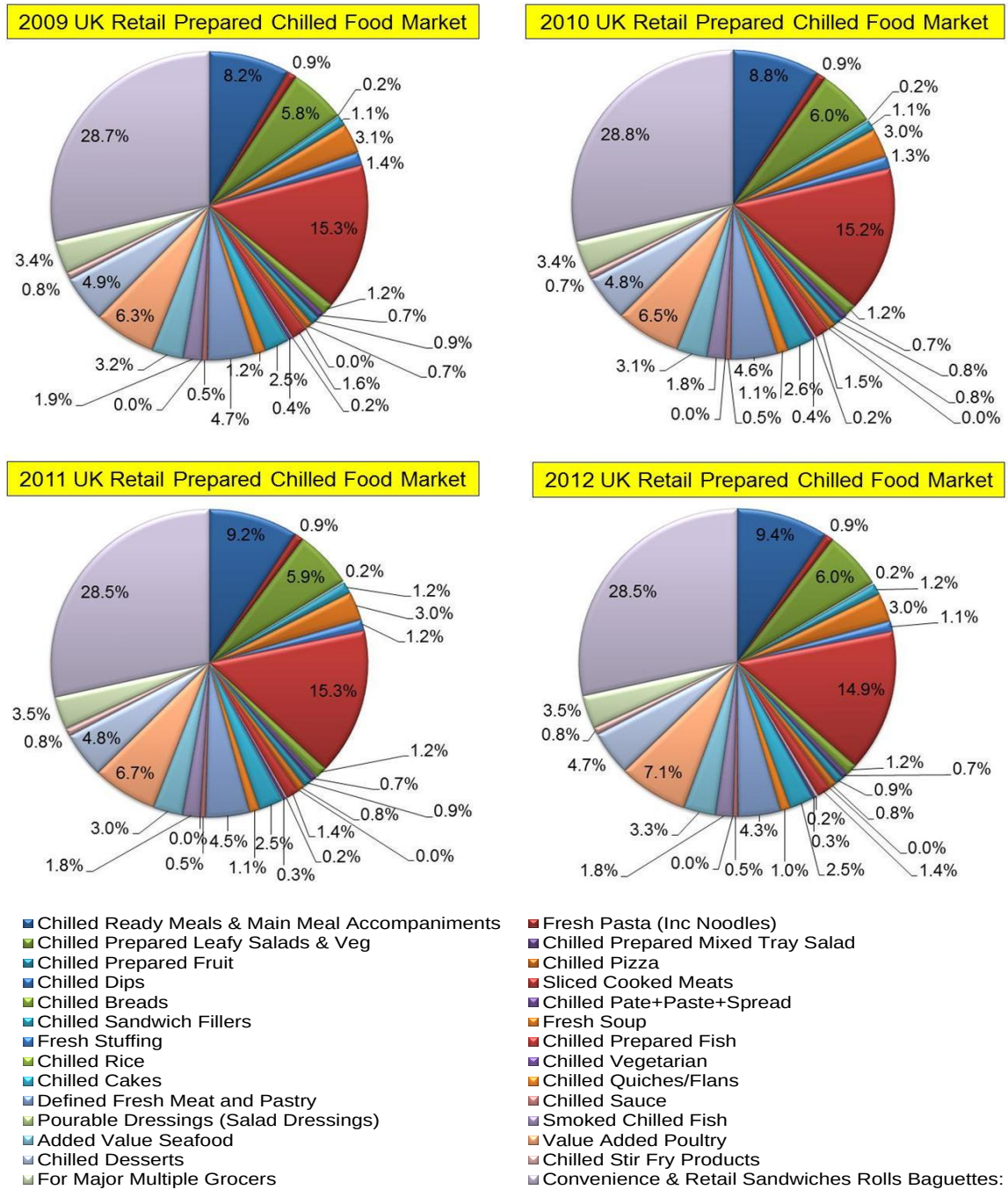

Figure 1.2 UK Retail Prepared Chilled Food Market 2009 – 2012
Source: Chilled Food Association (2013)

Chilled food products have been available since the 1960s in the form of sliced meats and pies. Over the years, the range of products has increased enormously, with over 12000 quick and easy to prepare foods such as: sandwiches, pizzas, luxury meal kits, leafy salads, dairy desserts, dips, salads available in the UK (Figure 1.2). They are based on traditional British cuisine as well as a wide range of international meals.

In the last two decades the chilled food market has been the most dynamic food sector in the UK, and possibly in the world, with 317% growth in value between 1999 and 2011. The UK market is dominated by a few multiple retailers with their own brands and is estimated to be worth £14.4 billion in 2012 (Figure 1.3)

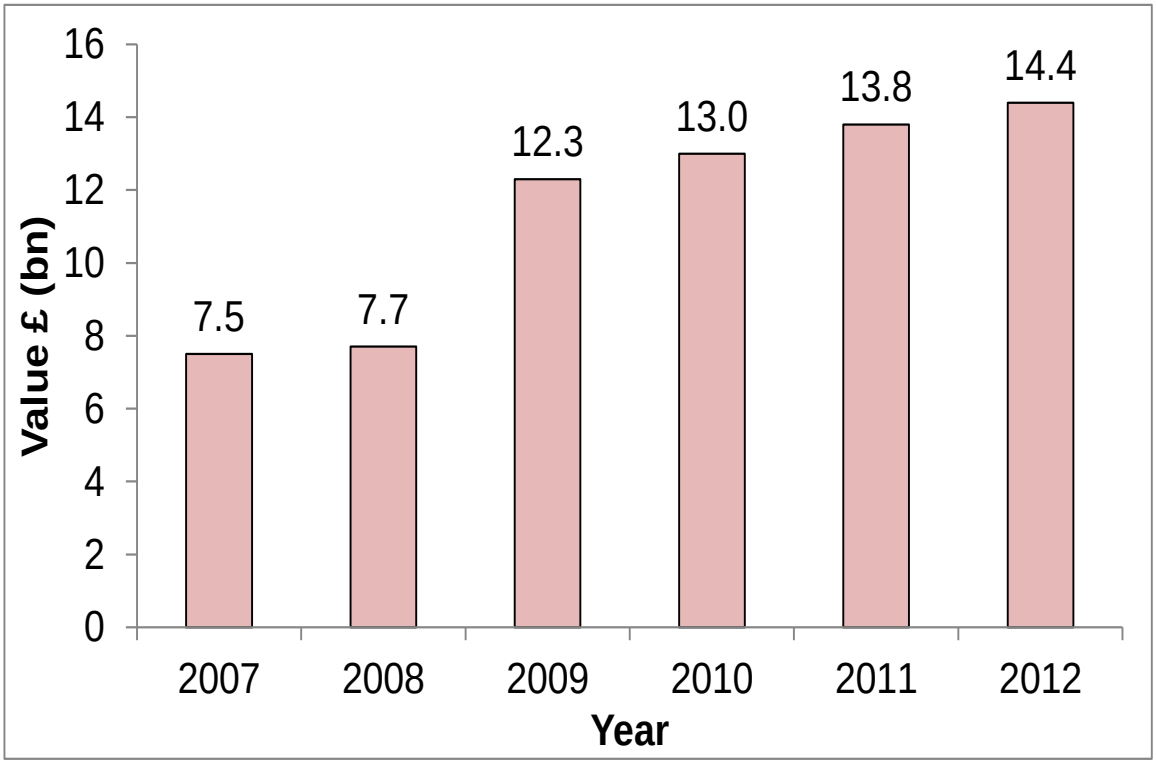

Figure 1.3 UK Retail Chilled Prepared Food Market 2007 – 2012
Source: Chilled Food Association (2013)

The largest segments of the market are convenience foods, retail sandwiches, rolls, baguettes (28.5%), sliced cooked meat (14.9%), chilled ready meals (9.5%) and value added poultry (7.5%). The remaining 40% includes fresh pasta, chilled leafy salads and prepared fruits, pizza, breads, soups, cakes, seafood and salad dressings (Figure 1.2).

The characteristic features of chilled products, apart from the short period of time needed for their preparation, are their high nutritional value, sensory quality and limited amount of preservatives. Their microbiological safety and quality relies on a moderate heat treatment (70 – 95°C) with consumer storage at chill temperature (below 8.0°C in UK). Often these foods are packed under a low-oxygen atmosphere and can have a shelf life up to 42 days.

Chilled food conditions may support growth or survival of many foodborne pathogens including *Escherichia coli, Listeria monocytogenes, Clostridium perfringens, Bacillus cereus, Staphylococcus aureus,* Salmonellae *or C. botulinum*, and this has a serious impact on consumer health and safety. Vegetative cells of pathogens such as *E. coli* or *L. monocytogenes* would not be expected to survive the heat treatment delivered, but could present a hazard if there was post-process contamination. Bacterial spores would survive many of the heat treatments and, in the case of spores of non-proteolytic *C. botulinum*, could lead to growth and neurotoxin formation at chill temperatures (Stringer *et al.*, 1997). Non-proteolytic *C. botulinum* is considered as the major concern in the production of chilled foods and has been previously implicated in foodborne botulism outbreaks (Table 1.2). Outbreaks have usually been associated with time or temperature abuse during storage (Peck *et al.*, 2008). Growth of proteolytic *C. botulinum* will be controlled by effective chilled storage but may be an issue in the case of prolonged temperature abuse, with some botulism outbreaks reported (Table 1.2).

Considering the severe adverse health effects and the serious commercial implications of foodborne botulism (the estimated cost of one foodborne botulism case in the USA is $30 million (Peck, 2009)), and the increasing consumer demand for chilled foods (Figure 1.3), the control of non-proteolytic *C. botulinum* in minimally heated chilled foods is essential and requires the identification of appropriate controls (e.g. effective heating time, product acidity, a_w) based on sound science as well as a systematic approach to inform the public concerning safety. This can be achieved by well-defined and implemented risk assessment, risk management and risk communication – the three components of risk analysis.

1.3 Risk Analysis

Chilled foods have many benefits such as convenience (easy to prepare and easy to take away), extended shelf life, adaptation to consumer's preferences (e.g. small portions for individuals) and the availability of a wide range of ethnic meals (e.g. British, Italian, Indian, oriental), but their production faces a number of safety issues. One of the most important is that they are a potential vehicle for the transmission of hazardous agents (i.e. viruses, bacteria or parasites) that may lead to foodborne disease, e.g. *E. coli* O104:H4 (outbreak associated with sprouts) (Buchholz *et al.*, 2011), *Yersinia enterocolitica* O:9 (associated with ready-to-eat salad mix) (MacDonald *et al.*, 2011) and *C. botulinum* (outbreaks associated with vacuum packed fish) (Table 1.2). Illness caused by these agents, in the "best scenario" can lead to a variety of mild symptoms and in the "worst scenario" to death.

In addition, the complexity of chilled foods, together with a developing global transport of raw materials and ingredients (Ercsey-Ravasz *et al.*, 2012) can make it difficult to trace the origins of their content. For example the most recent scandal in Europe associated with the presence of horsemeat in burgers and ready meals, revealed problems with the control and the ability to trace the source of food materials, and the complexity of processes involved in the production and distribution of many food products (Morley, 2013).

Ensuring public health protection and progressing international trade requires the setting of mutually agreed international standards, regulations and process requirements. These requirements should be scientifically justified to support fair trade. Risk analysis is one of the most recent and structured approaches which not only represents relevant scientific information in a transparent way, but also manages the policy and exchange of information concerning food safety (FAO/WHO, 1995). In addition, the application of risk analysis not only provides benefits on an international and national level, but also to industry by ensuring a consistent approach to the safety and quality of food, and an effective traceability in the food network, which minimizes recalls and protects manufacturer's reputation.

1.3.1 Historical aspects of risk analysis

The desire for safe food has a long history. The earliest method for controlling safety and protecting society from illness and deaths associated with consumption of particular products was the prohibition of manufacture and distribution. One example is a ban on the production of blood sausages by Emperor Leo VI of Byzantium in the 10th century, because of poisoning possibly caused by *C. botulinum* (Erbguth, 2004). The accumulation of scientific knowledge has replaced prohibition with guidelines, laws and recommendations which aim to control and minimize the risk associated with certain products. A significant milestone in food safety regulations appeared with the development of sterilization processes, which allowed for storage of perishable products for a long period in an ambient temperature. Work carried out by Esty and Meyer (1922) on the heat resistance of spores of *C. botulinum* in low-acid, canned foods is a classic example of research used to establish process criteria for commercially produced foods.

An important development in food science was made in the 1960s by the National Aeronautics and Space Administration (NASA) to ensure food safety for the first manned space mission. This was the development of the Hazard Analysis and Critical Control Point system (HACCP) (Ross-Nazzal, 2008). The final version of HACCP with seven principles and guidelines for its application in food safety was accepted by the Codex Alimentarius Commission (CAC) in 1997, and gave the system an international dimension.

The HACCP system is a systematic preventive approach to food safety management, which identifies potential hazards associated with each step of food manufacturing, establishes critical limits for hazards and systems for their control. Nevertheless, due to fact that originally the HACCP system was designed to different purpose than food safety, there are drawbacks in its application. One of weak points of the HACCP system is a lack of an objective and quantitative approach in estimating the risk associated with particular hazards. Moreover, HACCP itself is not an effective way to ensure consumer health at an international level (it does not allow to control the burden of foodborne diseases in the country), and this became a major concern for food

safety authorities with developing international trade. In 1995 the World Trade Organization (WTO) in order to facilitate the trade between nations introduced The Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement). The SPS Agreement required associated countries to protect human, animal and plant health by food law based on risk analysis (WTO, 1995).

1.3.2 The elements of risk analysis

Risk analysis is a structured, systematic process composed of three interconnected elements: risk assessment (see section 5), risk management and risk communication (Figure 1.4).

The process supports food safety authorities in controlling risks to human health and safety, and food manufacturers in assessing the process parameters/measures that need to be applied during product manufacture to control hazards (chemical, physical or biological). Moreover, risk analysis helps understanding and managing risks associated with particular hazards and improves food safety control systems.

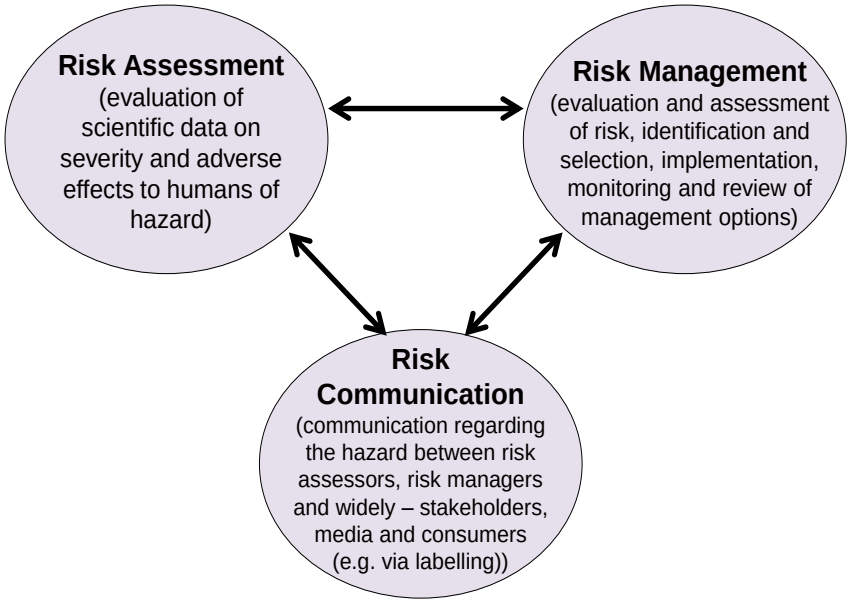

Figure 1.4 The three elements of risk analysis
Source: CAC (1999; 2007)

Risk analysis may vary in details according to the actual risk, the purpose of the analysis and available resources, but in all cases, it should be transparent and well documented.

1.4 Purpose of this research

The primary aims of this research are to:

(i) generate and collate data on the variation in physiological response between strains of non-proteolytic *C. botulinum*

(ii) to quantify the effect that strain variability has on the risk of adverse effects associated with a model dairy-based minimally heated chilled food.

Data on strain variability have been generated through experimental study and an extensive literature review. Since spore heat resistance and the ability to grow at chill temperatures are important elements of pathogenicity for non-proteolytic *C. botulinum*, these two parameters have been studied in detail. A literature survey has been carried out that describes the effect of strain variability on the heat resistance of spores of non-proteolytic *C. botulinum* (Chapter 2), and experimental data has been generated to describe the effect of strain variability on growth of non-proteolytic *C. botulinum* at chill temperatures (Chapter 3). Additionally, the effect of carbohydrate (the major carbon and energy source for non-proteolytic *C. botulinum*) source on the variation in growth from different strains of non-proteolytic *C. botulinum* has also been examined experimentally (Chapter 4). These observations on physiological variability between strains have been compared with genetic variability data available in the published literature (Chapter 5). Finally Chapter 5 also summarise the background to Quantitative Microbial Risk Assessment and places in context the information obtained in previous chapters on non-proteolytic *C. botulinum* strain variability. These data are combined in a QMRA model to describe the impact of strain variability on the risk presented by non-proteolytic *C. botulinum* in a minimally processed chilled food.

2. Heat resistance of spores of non-proteolytic *C. botulinum*: a systematic literature review

Heat treatment is one of the most important controlling factors used to prevent growth and associated neurotoxin formation by non-proteolytic *C. botulinum* in chilled foods. It is generally agreed that a heating process used to deliver a 6D reduction in spore concentration is sufficient to control non-proteolytic *C. botulinum* (ACMSF, 1992). The purpose of the present study is to review the available data on the heat resistance properties of non-proteolytic *C. botulinum* spores in a systematic manner and to obtain probability distributions for parameters describing the heat resistance of spores. Parameters are subsequently used in a QMRA model for minimally heated chilled foods. The analysis of 880 *D* and 528 *z*-values reveals that there is a significant difference in spore heat resistance properties when recovered in the presence and absence of lysozyme. A total of 505 *D* and 340 *z*-values collected at temperatures at and below 83°C were used to obtain a probability distribution representing the variability in spore heat resistance of strains recovered in media which did not contain lysozyme, and 325 *D* and 160 *z*-values were obtained at a range of 75°C – 95°C to derive a probability distribution for heated spores recovered in media that did contain lysozyme. Linear regression for the collected data suggested two *z*-values, z = 7°C and z = 9°C, for spores recovered with and without lysozyme. Using the 99% confidence limit of the fit to thermal death data suggested that the time required to effect a 6D reduction in the spore concentration when heating at 90°C was approximately 5 minutes. In addition the data were used to test hypotheses regarding the dependency of spore heat resistance on toxin type, strain, heating technique, method of *D*-value determination and testing laboratory.

2.1 Background

Spores of non-proteolytic *C. botulinum* are considerably less heat resistant than those of proteolytic *C. botulinum*, however, the mild heat treatments applied to chilled products may allow the survival of spores of non-proteolytic *C. botulinum*, and combined with the ability to grow and form toxin at chilled temperature, this makes non-proteolytic *C. botulinum* a major concern for the study of modern minimally heated chilled foods (Peck, 2006).

Currently there are a number of guidelines related to the safety of chilled foods, e.g. shelf life, salt concentration, a_w, pH, transport and storage temperature. One recommendation is that a heat treatment of 90°C for 10 minutes or equivalent, is necessary to deliver a 6D reduction for spores of non-proteolytic *C. botulinum* (Table 2.1) ("6D" is reduction of the population density of microorganisms by six orders of magnitude). This heat treatment is used for longer shelf life chilled products.

Recommendation/guidance	Recommended parameters of heat treatment	
Report on Vacuum Packaging and Associated Processes (ACMSF, 1992)	90°C/10 minutes or equivalent *z* = 9.2°C	
Code of Hygenic Practise for Refrigerated Packaged Foods with Extended Shelf Life (CAC, 1999).	not stated: factors given which need to be considered when developing scheduled heat	
Guidelines for Good Hygienic Practise in the Manufacture of Chilled Foods (CFA, 2006).	90°C/10 minutes or equivalent *z* = 7.0°C below 90°C z = 10.0°C above 90°C	
Recommendations for the Production of Prepackaged Chilled Food (ECFF, 2006).	90°C/10 minutes or equivalent *z* = 7.0°C below 90°C z = 10.0°C above 90°C	
Food Standards Agency guidance on the safety and shelf life of vacuum and modified atmosphere packed chilled foods with respect to non-proteolytic *C. botulinum* (FSA, 2008).	90°C/10 minutes or equivalent *z* = 9.2°C below 90°C z = 10.0°C above 90°C	
Fish and Fishery Products Hazards and Control Guidance (USFDA, 2011).	Fish and fishery products	90°C/10 minutes or equivalent *z* = 7.0°C below 90°C z = 10.0°C above 90°C
	Blue crabmeat	85°C/31 minutes *z* = 9.0°C
	Dungeness crabmeat	90°C/57 minutes z = 8.6°C

Table 2.1 Examples of currently recommended heat treatments related to safety of minimally heated chilled foods
Source: ACMSF (1992); CAC (1999); FSA (2004); CFA (2006); ECFF (2006); FSA (2008); USFDA (2011)

In order to protect consumers from hazards that are associated with non-proteolytic *C. botulinum* in minimally heated chilled foods, the Advisory Committee on the Microbiological Safety of Food (ACMSF), Food Standards Agency (FSA) and Chilled Food Association (CFA) recommended processes that

deliver a heat treatment of 90°C for 10 minutes or equivalent lethality when combined with storage at chill temperature (Table 2.2).

Heating temperature (°C)	Required heating time (t) (min) required and estimated *D*-value (min) according to					
	FSA		ACMSF		CFA/ECFF	
	t	*D*-value	t	*D*-value	t	*D*-value
70	-		1675	277.8	-	
75	-		464	77.5	-	
80	129	21.5	129	21.5	270	45.0
85	36	6.0	36	6.0	52	8.7
90	10	1.7	10	1.7	10	1.7
95	3.2	0.5	not indicated		3.2	0.5
100	1.0	0.2	not indicated		1.0	0.2
z-value < 90°C	*z* = 9.2°C		*z* = 9.2°C		*z* = 7.0°C	
z-value > 90°C	*z* = 10.0°C		not indicated		*z* = 10.0°C	
Recommended shelf life: 10 days to 42 days Recommended storage temperature: ≤ 8°C						

Table 2.2 Equivalent 6D time/temperature combinations for spores of non-proteolytic *C. botulinum*
Source: ACMSF (1992), CFA (2006), ECFF (2006), FSA (2008)

2.1.1 Spore heat resistance

Heat treatment as a preservation technique

A number of processing techniques are used to ensure microbiological food safety and to prevent food spoilage, allowing for prolonged shelf life by slowing or preventing the growth of microorganisms, inactivation of microorganisms or restricting the access of microorganisms to the product (Gould, 2000). Methods of processing food can be divided into two main categories: physical and chemical.

Physical methods of food preservation include the use of heat, refrigeration, freezing, frozen storage, dehydration (Peck *et al.*, 1993), new food preservation technologies (e.g. high intensity light, high voltage electric discharge, high intensity magnetic field pulses, ultrasonication) or a combination of physical and chemical methods (e.g. use of salt, benzoic acid, sorbic acid). Despite the wide range of available food preservation technologies, the most widely used method for inactivation of spoilage and pathogenic microorganisms for many years has

been heat treatment (Pflug and Gould, 2000). Heat processes such as pasteurization and sterilization allow not only for destruction of vegetative bacterial cells but may also inactivate bacterial spores in commercial food (Brown, 2000).

In order to determine the heat treatment for a particular food product, which would assure consumer safety, two main factors need to be considered: the heating characteristics of products and the heat resistance properties of the microorganism of concern in that food. In terms of minimally heated chilled foods, the spore-forming bacterium non-proteolytic *C. botulinum* is a pathogen of concern, and this chapter summarises data on its spore heat resistance.

Bacterial endospores

Since the discovery of bacterial endospores by Cohn and Koch in 1876, their resistance and dormancy have been thoroughly investigated, due to the problems that spores present to the food industry (Setlow and Johnson, 1997). A number of spore-forming bacteria, notably *C. botulinum*, *C. perfringens*, *Bacillus cereus*, and occasionally *Bacillus subtilis* and *Bacillus licheniformis* (Brown, 2000) produce toxins that can cause food spoilage, foodborne illness or even death (Del Torre *et al.*, 2004).

Bacterial spores are able to survive unfavourable conditions such as extreme temperatures, the presence of preservatives, UV light, desiccation, harsh chemicals, irradiation and physical damage. Spore resistance properties and their worldwide occurrence in soil samples (Carlin, 2011; Heyndrickx, 2011) ensure that foods are potentially contaminated, and an important aim of food processing is to reduce the number of spores in food products to a commercially acceptable level (Atrih and Foster, 2002).

In a majority of circumstances the presence of dormant spores in food does not pose a risk to the consumers. However, spore germination and outgrowth under favourable conditions can lead to the formation of vegetative cells and toxin production in the case of pathogens, or in other circumstances may lead to food spoilage (Ciarciaglini *et al.*, 2000).

Endospore structure

Endospores are produced by Gram-positive bacterial species such as anaerobic *Clostridium* and aerobic *Bacillus* (with *B. subtilis* often used as a model spore forming organism for facultative anaerobes (Errington, 2003)) and their basic structure (Figure 2.1) is similar (Atrih and Foster, 2002).

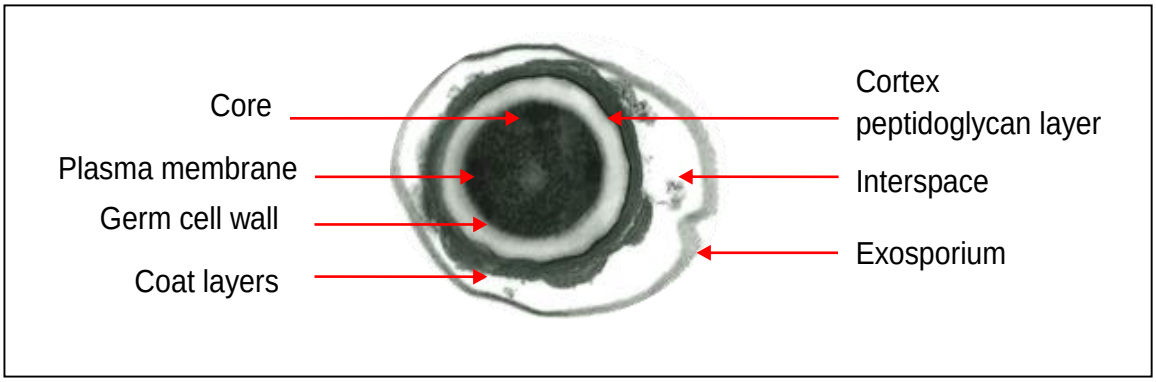

Figure 2.1 Electron micrograph of a cross-section through a non-proteolytic *C. botulinum* spore
Source: Micrograph courtesy of Dr. M. L. Parker, Institute of Food Research, Norwich, UK

The external layer of the spore, the exosporium, is strain specific with differences in its presence, structure and thickness between different sporulating species (Stevenson and Vaughn, 1972; Henriques and Moran, 2007). When present, the exosporium constitutes about 2% of the mass of the spore (Matz *et al.*, 1970) and is composed of an inner paracrystalline basal layer and external hair-like nap (Henriques and Moran, 2007) formed of proteins, lipids and carbohydrates (Matz *et al.*, 1970; Ball *et al.*, 2008). The explicit function of the exosporium is not known. However, being the outermost external layer of the spore it provides a barrier to large molecules e.g. antibodies, hydrolytic enzymes and macrophages (Kang *et al.*, 2005; Ramarao and Lereclus, 2005; Steichen *et al.*, 2005). It is also thought to play a role in spore adhesion to surfaces and resistance to cleaning (Faille *et al.*, 2007; Lequette *et al.*, 2011). The mechanism and extent to which the exosporium may have a role in spore germination is not fully understood. Two enzymes (alanine racemase and inosine hydrolase) located in the exosporium might play a role in spore germination (Todd *et al.*, 2003; Redmond *et al.*, 2004).

The exosporium is separated from the electron-dense coat by a region called the interspace. In species lacking the exosporium (e.g. *Bacillus subtilis*), the coat is the outermost layer. The coat is mainly composed of proteins from 8 to 65 kD in size (50 – 60% of the total protein within spore). There are two morphologically distinct, closely packed layers in the spore coat: an inner – lamellar-like layer, and electron-dense outer layers (Henriques and Moran, 2007). Together with the exosporium (when present), the coat plays an important role in the resistance of spores to chemical and enzymatic assault and UV light (Gould *et al.*, 1970; Zheng *et al.*, 1988; Riesenman and Nicholson, 2000). Moreover, proteins that comprise the spore coat are responsible for spore resiliency (Setlow, 2006). Interestingly, it was shown that the coat is the main barrier for the cortex against lysozyme, a peptidoglycan-hydrolysing enzyme, whose activity results in spore germination (Peck *et al.*, 1993), and might influence resistance to predatory microorganisms (e.g. *Tetrahymena thermophile*) (Zheng *et al.*, 1988).

Under the coat is a thick layer of modified peptidoglycan (PG), structurally similar to cell wall peptidoglycan, which plays an important role in spore heat resistance by maintaining the dehydrated state of the spore protoplast (Popham and Setlow, 1993). The PG layer consists of an inner, germ cell wall layer and the cortex. The cortex has a unique structure, highly conserved across species, and subtle variations in its structure have a significant effect on spore wet heat resistance and core water content (Atrih and Foster, 2001). The cortex is rapidly hydrolysed by lytic enzymes during germination, which is crucial for spore core expansion and subsequent outgrowth (Dowd *et al.*, 2008).

Between the germ cell wall and the core is the expandable plasma membrane, that is not permeable to small hydrophilic compounds (Swerdlow *et al.*, 1981; Cowan *et al.*, 2004). The plasma membrane, also called the inner forespore membrane, is sensitive to oxidizing agents triggering spore germination (Cortezzo *et al.*, 2004), but nevertheless as a strong permeability barrier it plays a significant role in spore resistance.

The centre of the spore – core (cytoplasm), contains the genetic material (DNA), metabolic components (enzymes, dipicolinic acid (DPA) and divalent cations)

and small acid soluble proteins (SASPs) (Atrih and Foster, 2002). The SASPs comprise about 20% of total core protein and are essential for spore resistance protecting its DNA (Popham *et al.*, 1995; Leyva-Illades *et al.*, 2007; Moeller *et al.*, 2009). DPA, which comprises 5 – 15% of the dry weight of spores, also plays a crucial role in spore resistance (Setlow, 2006).

Basis of spore heat resistance

The identity of targets whose damage results in heat killing of bacterial spores is crucial in preventing food spoilage, food poisoning and infectious diseases caused by spore-forming bacteria. Throughout the years, several hypotheses have been put forward to explain the mechanism responsible for spore resistance to heating e.g. core dehydration (Nakashio and Gerhardt, 1985), peptidoglycan expansion (Atrih *et al.*, 1998), changes in spore core mineral content (Beaman and Gerhardt, 1986), denaturation of macromolecular components (e.g. proteins, RNA and DNA) (Belliveau *et al.*, 1992; Setlow and Setlow, 1998). However, although the mechanism of spore resistance remains not fully understood, many factors have been identified, which modulate spore heat resistance: inherent thermal resistance (Warth, 1978), sporulation temperature and media composition (Ababouch *et al.*, 1995; Sala *et al.*, 1995; Palop *et al.*, 1999a), core water content and permeability (Beaman and Gerhardt, 1986; Popham *et al.*, 1995), spore mineral content (Bender and Marquis, 1985; Beaman and Gerhardt, 1986; Igura *et al.*, 2003) and α/β-Type SASPs properties (Fairhead *et al.*, 1993; Popham *et al.*, 1995).

Despite the considerable number of studies on endospore resistance, it is apparent that resistance does not solely rely on one factor, but it is a complex mechanism. The knowledge of factors responsible for differences in the heat resistance of spores of different organisms remains also unclear. However, understanding of this mechanism is crucial for food production in order to potentially deliver a less severe heat treatment to the product, reduce time and costs, while ensuring microbiological stability.

2.1.2 Determination of bacterial heat resistance

There are two main methods of bacterial spore heat resistance determination: establishing a survivor curve or conducting thermal death time (TDT) studies.

Survivor curve

In this method, a known concentration of spores is heat treated in a test medium for a given period of time, and the number of survivors is enumerated with time. Enumeration of the heat treated spore suspension can be determined either by a Most Probable Number (MPN) method, or by plating of appropriate aliquots onto a microbiological growth medium. The logarithm of the number of survivors plotted as a function of heating time gives the survivor curve.

From many years, the semilogarithmic survivor curve has been the most important tool used to calculate microbial survival parameters. Usually it is a straight line, but there are many examples in the literature where it is reported to be parabolic, sigmoidal, or linear but with a shoulder or tail (Figure 2.2).

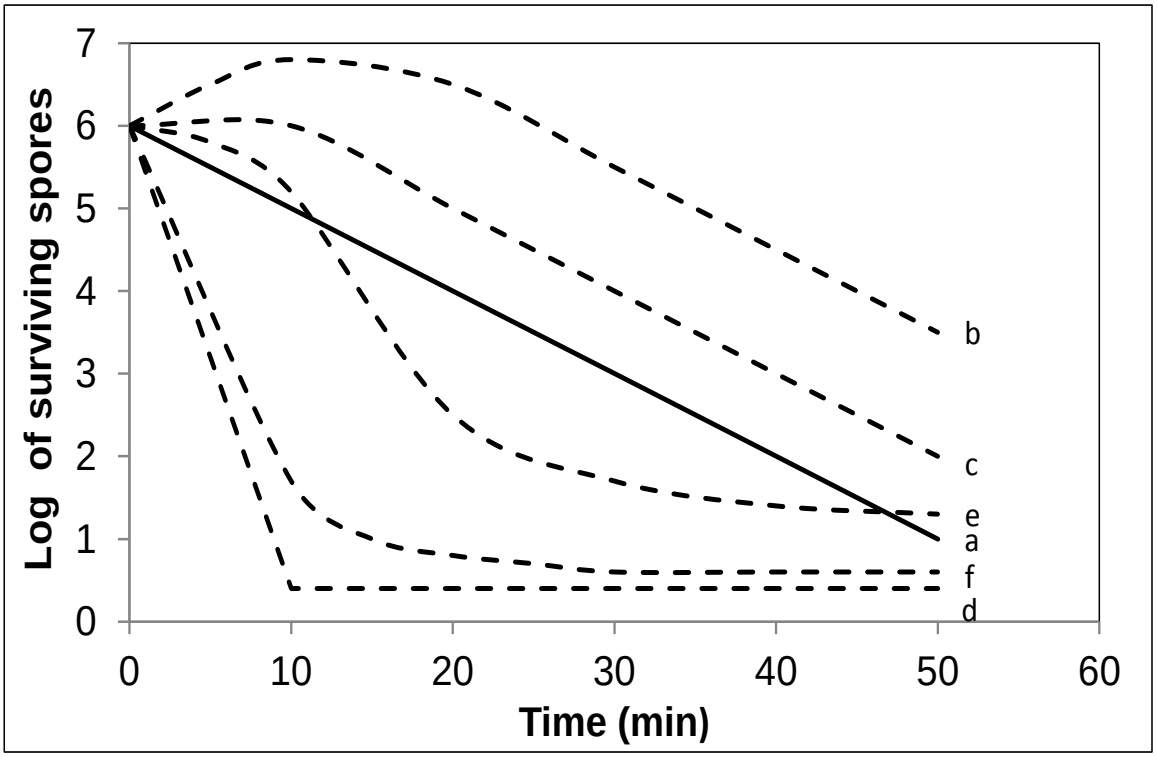

Figure 2.2 Shape of semilogarithmic survivor curve of bacterial spores after a heat treatment
a) commonly observed linear curve, b) and c) linear with shoulder (Palop et al., 1999b), d) linear with a tail – biphasic (Peck *et al.*, 1992a), e) sigmoid, f) parabolic (Licciardello and Nickerson, 1963)

The most common explanations for the differences in shape of a survivor curve are the presence of a heterogeneous population of spores, showing a different response (Pflug, 2001), the occurrence of spore activation, clumping, “heat adaptation” (Gould *et al.*, 1983), or errors associated with the experimental technique, e.g. uneven dispersion of spores in the tube, therefore occurrence of spores being subjected to dry rather than moist heat or using the open tube technique (Graikoski and Kempe, 1964).

Despite frequent examples of divergence (in some cases due to poor technique), the approach generally taken is to assume a semilogarithmic relationship, where at a given temperature and given time interval, the same fraction of the bacterial population will be destroyed regardless of the initial population size. Therefore, data on the heat resistance of a homogeneous population of spores gathered under ideal conditions (e.g. uniform lethal stress, optimal recovery media) can be described by first order reaction kinetics given by equation 2.1 (Bigelow and Esty (1920)), from which we can calculate the rate of thermal death of a specific organism

$$\frac{dN(t)}{dt} = -kN(t) \qquad 2.1$$

where, N(t) is a number of microorganisms at the time (t) and k is a thermal death rate constant (min^{-1}) expressed as

$$k = \left[\frac{LogN_0 - LogN(t)}{t}\right] \qquad 2.2$$

where N_o is an initial number of microorganisms.
A solution of the equation 2.1 is often rewritten as

$$N(t) = N_0 10^{-kt} \qquad 2.3$$

Although the survival curve technique is relatively simple, it is very time consuming, when a large number of strains are tested at a several temperatures. Through the years, several systematic experimental techniques for measuring the heat resistance of bacterial spores have been developed. The most common,

that allow for successive sampling and enumeration are: thermoresistometer (Stumbo, 1948), capillary tube method (Wilder and Nordan, 1957), screw-capped tube method (Kooiman and Geers, 1975), metal tube method (Odlaug and Pflug, 1977), flask method (Bucknavage *et al.*, 1990), universal bottle method (Gaze and Brown, 1990), and vial method (Juneja and Eblen, 1995).

Thermal Death Time

A second, common method for determining the heat resistance of bacterial spores is conducting Thermal Death Time (TDT) studies. TDT is the length of time required to kill a known number of spores in a medium at a specific temperature. The TDT is based on a growth/no growth method. Its precision is improved by increasing the number of replicates. TDT can be determined by exposing a spore suspension to heat for a fixed time. Spore survival is determined by either direct incubation or by subculturing and assessment of growth/no growth. The TDT is assumed to be between the longest heating time when a positive growth result (t_{max}) was noted and the shortest heating time when growth was not observed ($t_{minimum}$) (Bigelow and Esty, 1920; Alderman *et al.*, 1972), and can be expresses as the geometric mean of two times

$$TDT = \sqrt{t_{max} t_{minimum}} \qquad 2.4$$

Commonly used practical approaches include the TDT tube (Bigelow and Esty, 1920) and TDT can (Townsend *et al.*, 1938).

2.1.3 Treatment of heat resistance data to calculate *D*-value

In order to calculate the effect of temperature on the death rate of various microorganisms the concept of decimal reduction time (*D*-value) was introduced. The *D*-value is the time (min), at a particular temperature, required to reduce the bacterial viable count by 90% (i.e. to result in a 1-log reduction). The *D*-value depends on temperature, the type of microorganism and the food/medium in which the microbe is heated and it can be determined in several ways.

Survival curve

The most common method is to plot the log of surviving population against heating time. When a straight line is obtained, the *D*-value can be read directly from the slope of survival curve (Figure 2.3). If an initial shoulder is observed, the *D*-value can be obtained from the straight portion of the graph (Figure 2.2).

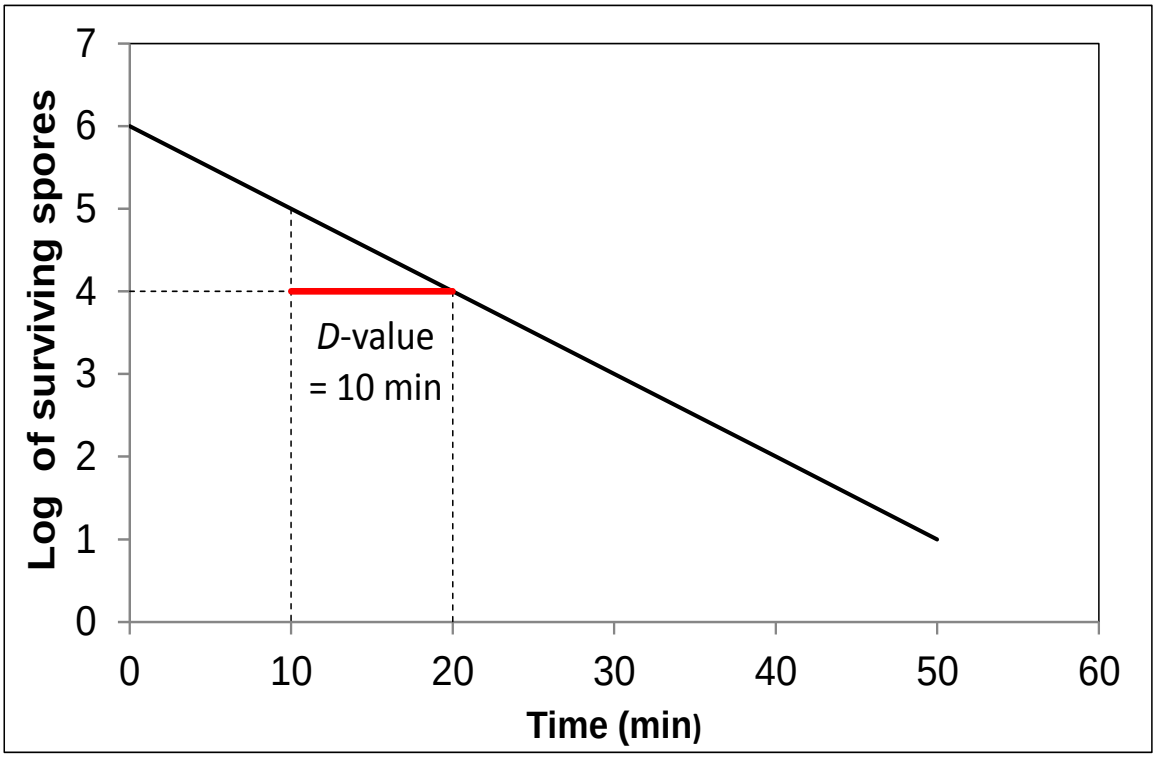

Figure 2.3 Graphical representation of survival curve and concept of *D*-value

Equally, the *D*-value can be estimated mathematically following from equation 2.2, where the *D*-value is inversely related to the constant *k* (equation 2.2)

$$D(T) = \left[\frac{1}{k}\right] = \frac{t}{[LogN_0 - LogN(t)]} \qquad 2.5$$

Thermal Death Time

In the case of Thermal Death Time studies, the *D*-value can be calculated in several ways. The most common approaches were introduced by Stumbo (1948), Stumbo *et al.* (1950) and Schmidt (1957) or originate from equation 2.4.

Stumbo (1948) used the formula

$$D(T) = \frac{t}{loga - logb} \qquad 2.6$$

where *a* is the total number of heated samples multiplied by the number of spores per sample and *b* is the number of surviving spores (calculated by assuming one surviving spore per container when less than the total number of containers showed survival) after the heating time (t).

The Stumbo-Murphy-Cochran (1950) method also used the equation 2.6 to estimate the number of survivors, except that now *a* is the total number of heated samples multiplied by the number of spores per tube, *b* is the most probable number of spores ($\bar{x}$) surviving the treatment per sample (calculated from $\bar{x} = 2.303\ log\frac{n}{q}$, where *n* = total number of replicates, *q* = number of units showing no growth) multiplied by the number of replicates after an exposure time (t).

The calculation of Schmidt (1957) is based on two assumptions:

1) “any sample not showing survivors at a given exposure time, would not show survivors at longer time of exposure”
2) “any sample showing survivors at a given exposure time would show survivors at a shorter exposure time”.

These assumptions allow for calculation of the probability of sterility ($P = \frac{n+1}{m+n+2}$, where *n* = cumulated samples not surviving each exposure time, obtained by adding the negative samples downward from the shortest exposure time to the longest and *m* = cumulated samples surviving each exposure time obtained by adding the positive samples upward from the longest exposure time to the shortest).

Therefore, the *D*-value can be calculated according to

$$D(T) = \frac{LD_{50}}{loga + 0.16} \quad 2.7$$

where *a* is the initial number of spores per tube and LD_{50} is a point on a probability curve (obtained from plotting the probability of sterility versus time), that represents the time, at which 50% of tubes will be sterile.

Alternatively a *D*-value can be estimated using equation 2.8

$$D(T) = \frac{TDT}{nD} \quad 2.8$$

where TDT is calculated according to equation 2.4 and *nD* is a reduction of orders of magnitude (e.g. if $N_0 = 10^9$ of spores, therefore *nD* = 9).

The *D*-values calculated according to above methods, based on an example with $N_0 = 10^9$ spores, T = 90°C and 10 replicate cans, are summarised in Table 2.3 and Table 2.4.

Heating time (t)	No. of containers			Stumbo (1948)				Stumbo *et al.* (1950)								Schmidt (1957)					
minutes	Tested	+ve	-ve	loga	b	logb	*D*-value	q	n/q	log(n/q)	$\bar{x}$	b	logb	*D*-value		loga	m	n	m+n	P	*D*-value
2.0	10	9	1	10	9	0.95	0.22	1	10.00	1.00	2.30	23.03	1.36	0.23		9	19	1	20	0.09	
2.5	10	7	3	10	7	0.85	0.27	3	3.33	0.52	1.20	12.04	1.08	0.28		9	10	4	14	0.31	
3.0	10	2	8	10	2	0.30	0.30	9	1.11	0.05	0.11	2.23	0.02	0.30		9	3	13	16	0.78	
3.5	10	1	9	10	1	0.00	0.36	8	1.25	0.10	0.22	1.05	0.35	0.36		9	2	21	23	0.88	
4.0	10	0	10	10												9	0	31	31	0.97	
Mean *D*-value							0.29							0.29							0.28

Table 2.3 Calculations of *D*-value according to the procedures of Stumbo (1948), Stumbo *et al.* (1950) and Schmidt (1957)

Heating time (t)	No. of containers			Replicates									
minutes	Tested	+ve	-ve	1	2	3	4	5	6	7	8	9	10
2.0	10	9	1	-	+	+	+	+	+	+	+	+	+
2.5	10	7	3	-	-	-	+	+	+	+	+	+	+
3.0	10	2	8	-	-	-	-	-	-	-	-	+	+
3.5	10	1	9	-	-	-	-	-	-	-	-	-	+
4.0	10	0	10	-	-	-	-	-	-	-	-	-	-
9D				0.00	2.24	2.24	2.74	2.74	2.74	2.74	2.74	3.24	3.74
***D*-value**				0.00	0.25	0.25	0.30	0.30	0.30	0.30	0.30	0.40	0.42
Mean *D*-value				0.31									

Table 2.4 Calculation of *D*-value according to equation 2.4
+ve – number of positive containers after heating time (t)
-ve – number of sterile containers after heating time (t)

2.1.4 Calculation of *z*-value

A *D*-value refers to a specific temperature. In order to calculate equivalent thermal processes and indicate the relative impact of heating at different temperatures on a microorganism, the concept of *z*-value was introduced. It is a measure of the change in death rate with a change in temperature. The *z*-value is a change of temperature (°C), for which the *D*-value is reduced/increased by a factor of 10, and may be expressed as follows

$$z = \frac{T_2 - T_1}{logD(T_1) - logD(T_2)} \qquad 2.9$$

in which $logD(T_1)$ and $logD(T_2)$ are logD values corresponding to T_1 and T_2. Smaller *z*-values indicate greater sensitivity to increasing heating treatment. The *z*-value can also be determine by plotting (at least two) logD values versus temperature (Figure 2.4).

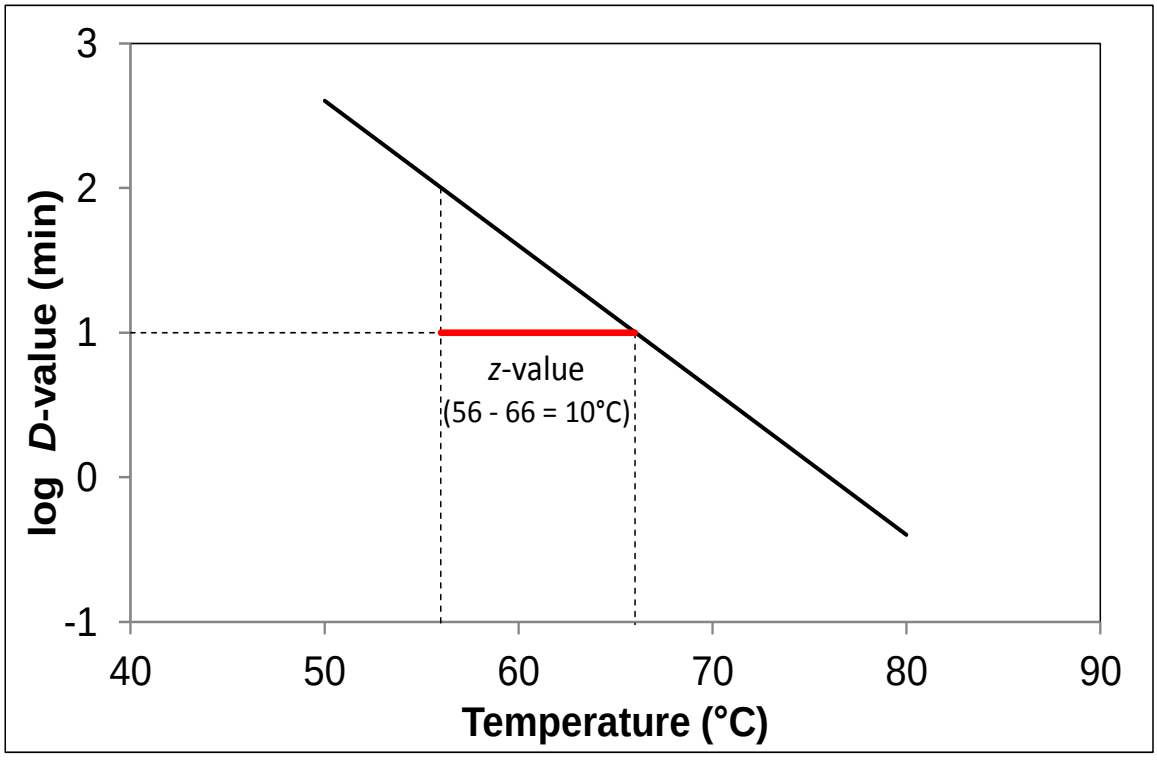

Figure 2.4 Graphical representation of the concept of *z*-value

D and *z*-values are the most common parameters use in the food industry for describing the impact of heat treatment (and other technologies) on the reduction of a microbial population. Together these are used for the development of food preservation processes to ensure the safety of food for consumers.

2.1.5 Alternatives and limitations of *D*-values

_F_-value

In order to determine the effect of a heat process for a particular food, the *F*-value may be used (Pflug, 1987). The *F*-value (min) is often a sterilization process, which aims to inactivate bacterial spores and is generally used at temperatures greater than 100°C. It can be converted to other temperatures (using a specific *z*-value) and is a characteristic value for each type of food, due the influence of food properties on the destruction of spores. The *F*-value is a central concept in the canning industry, where it is referred to as F_0, the "reference unit of lethality". Heating at 121°C for 3 min is known as $F_o = 3$ (with *z*-value = 10°C), which aims to provide 10^{12} reduction of population density of proteolytic *C. botulinum* spores in low acid canned foods (Hoxey *et al.*, 2006). The *F*-value can be calculated from the equation (Pflug and Gould, 2000)

$$D(T) = \frac{F(T)}{(logN_0 - logN(F))} \qquad 2.10$$

where, N(F) is the endpoint of preservation process, the number of surviving microorganisms per unit.

Alternatives to *D*-values

As mentioned above, many deviations from the straight line of semilogarithmic survivor curves have been reported. Therefore, in order to model the microbial survival curves, alternative approaches to first order reaction kinetics have been proposed by Linton *et al.* (1996), Anderson *et al.* (1996), Xiong *et al.* (1999) and Stone *et al.* (2009). These and other authors have used alternative models to describe thermal death data and to calculate death rate parameters.

A substitute for *D*-value has been suggested by Mossel (1977); as $MPED_n$-values (most probable effective heat dissipation to achieve *n* overall decimal reduction of CFU under certain conditions), which solved the problem of shoulders and tailing. Also Valdramidis, Bernaerts, Van Impe and Geeraerd (2005) defined an alternative parameter denoted as t_{xD} (time required for *x* log reductions in the microbial population (or xD)).

Limitations of *D*-value

Despite the utility of *D* and *z*-values in designing the heat process to ensure the microbial safety and quality of food, there are certain limitations that should be considered. First of all, the *D*-value is a specific parameter for a particular microorganism, therefore care should be taken when it is used for identification and comparison of heat resistance of different microorganisms. Limitations of *D*-value are also associated with the experimental procedure itself. Many factors such as age of culture, estimate of initial number of spores, pH of suspension, dimensions of test tubes, thickness of glass in tubes and experience of the experimenter have a large impact on the value of heat resistance parameters. Pflug (2001) indicated that using the initial number of organisms as 10^6 in calculations instead of a true value of 0.5×10^6 causes underestimation of *D*-value by 5%. Therefore it is important to determine the N_0 precisely. A challenge also arises from temperature control and temperature changes during the heat treatment. Also care should be taken when comparing the data generated by different laboratories. Pflug (2005) has shown that there is a variability in *D*-values measured by various laboratories, even using the same testing system.

In spite of evidence of deviations from the first order kinetics model, with consequent doubts in the accuracy of *D* and *z*-values, there is no universally accepted alternative approach, therefore these parameters still have wide use for determining the heat process for novel formulation of food products.

2.1.6 Rationale for this review

An extensive literature survey has been carried out in order to provide a comprehensive description of the thermal death of spores of non-proteolytic *C. botulinum,* and to identify and characterise strain variability. Very often the presence of lysozyme in the recovery medium and other recovery conditions are responsible for variability of *D* and *z*-values between strains of non-proteolytic *C. botulinum*. However, previously such data have not been collected in a systematic and extensive manner. Therefore the outputs of this review can provide information for updating microbiological risk assessment and helping to ensure the microbiological safety of new minimally heated chilled food products.

Previous reviews

Five other reviews of the heat resistance of spores of non-proteolytic *C. botulinum* have been carried out (Lund and Notermans, 1993; ICMSF, 1996; Lindström *et al.*, 2006; Membrè and McClure, 2006; Stringer and Peck, 2008). Lund and Notermans (1993) reported 12 sources, ICMSF (1996) collected *D* and *z*-values from 9 sources, Lindström *et al.* (2006) summarised data from 18 studies, Membré and McClure (2006) described 24 sources, and Stringer and Peck (2008) collected data from 20 studies.

Some conclusions from these reviews are:

i. heat resistance of spores depends on many factors (type of strain tested, composition of heating menstruum and recovery conditions),
ii. spores of non-proteolytic *C. botulinum* type B strains are more resistant than those of type E and F,
iii. in most of these studies thermal inactivation appeared to follow the first-order reaction kinetics, from which *D*-values were calculated,
iv. presence of lytic enzymes, e.g. lysozyme, in recovery medium increase the recovery of viable, sublethally damaged spores

The above reviews provide valuable information about the range of *D* and *z*-values of non-proteolytic *C. botulinum* in different heating mentruum at different temperatures. Nevertheless, they do not (except Membré and McClure (2006)) analyse/model these data to determine the most appropriate target heat process and *z*-value to allow the setting of equivalent process for the manufacture of microbiologically safe minimally heated chilled foods.

Objectives

The purpose of this study was to collect and summarize literature data on the thermal inactivation of spores of non-proteolytic *C. botulinum,* in a systematic manner, and to use this information to derive probabilistic distributions for parameters of thermal inactivation models for inclusion in QMRA. Additionally, the validity of current recommendations for food processing and the effect of toxin type on spore heat resistance is considered.

The contribution of this review

According to the most recent QMRA guidelines (USDA/FSIS and EPA, 2012) risk assessments should be established on systematically developed scientific evidence, presented in a clear, transparent way, which would be easily communicated to interested bodies. In this thesis, a systematic review of literature data has been carried out in a transparent and reproducible manner, following a standard set of stages, to ensure that relevant and reliable data are available for a QMRA model. Hence, this provides a comprehensive description of knowledge on the heat resistance of non-proteolytic *C. botulinum* spores in foods.

2.2 Methods of literature search and data analysis

2.2.1 Search methods for identification of studies

A systematic review on the heat resistance of non-proteolytic *C. botulinum* spores has been performed. Resources included online databases (Web of Science, SCOPUS, PubMed and Google Scholar), published reviews, personal literature collections, unpublished Institute of Food Research data and references cited in eligible articles.

Electronic searches

The search was performed on the following electronic databases:

- Web of Science (1977 to February 2011) (Figure 2.5)

Advanced search (ts-subject)

1. ts = botulinum (#1)
2. ts = spores (#2)
3. ts = heat (#3), ts = temperature (#4), ts = inactivation (#5), ts = thermal (#6)
4. #3 OR #4 OR #5 OR #6 (#7)
5. #1 AND #2 AND #7

- SCOPUS (1973 – 2011)

Advanced search

1. ALL (“botulinum”) (#1)
2. ALL (“spores”) (#2)
3. ALL (“heat treatment”) OR (“temperature”) OR (“inactivation”) OR (“thermal”) (#3)
4. #1 AND #2 AND #3 (#4)
5. ALL(“type B”) OR (“type E”) OR (“type F”) (#5)
6. #4 AND #5

- PubMEd

1. All fields (“botulinum”)
2. All fields (“spores”)
3. All fields (“heat or inactivation or thermal or survival”)
4. #1 AND #2 AND #3

- Google Scholar (1920 – 2011)

Advanced Scholar Search

1. articles with the exact phrase “botulinum spores”

2. with at least one of the words “treatment” OR “heat” OR “thermal” OR “inactivation”
3. words occur: anywhere in the article
4. articles only in: Biology, Life Science, Environmental Science, Medicine, Pharmacology, Veterinary Science

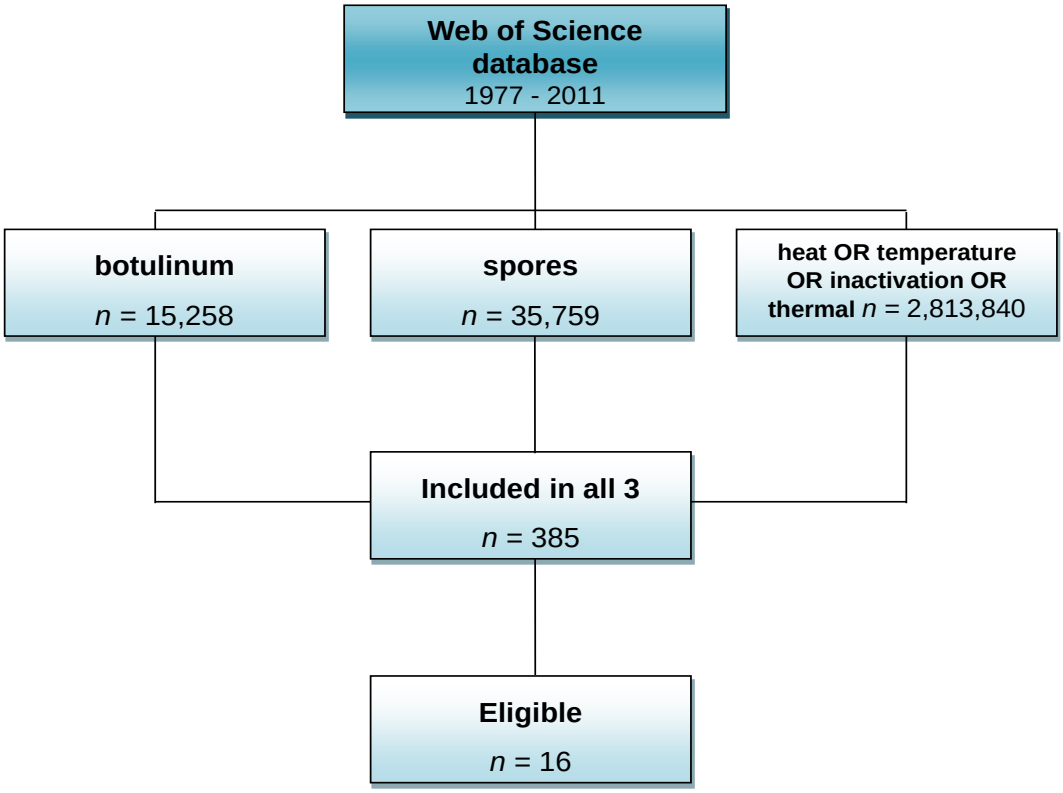

Figure 2.5 Number of references identified from example key words from electronic searches in Web of Science

Searching other resources

Manual searching

Private databases of Dr Terry A. Roberts, Dr Barbara M. Lund and Prof Michael W. Peck and the Institute of Food Research lab books were searched manually.

Personal contacts

The review includes data originating from private organizations e.g. Unilever. One academic dissertation was obtained throughout personal contact with Dr Miia Lindström. Personal contact with Dr Sandra C. Stringer, Dr Barbara M. Lund, Prof Michael W. Peck and Dr Gary C. Barker ensured that important articles have not been missed.

Searches were not restricted by country or language. The references of the eligible articles were also searched, a process that was conducted until no new references were identified. Previous reviews were also searched for possible references. Non-peer reviewed articles were also included.

Criteria and quality assessment of studies

Studies, which were considered as eligible, were assessed against quality criteria in order to minimize the risk of bias. There is no standard agreement on assessing the quality of research. According to the guidance of CRD (2009) for conducting systematic reviews, so far two tools have been developed for assessing quality, such us: checklists of quality items and scales with summary scores. Since, a checklist is more flexible and can be specifically designed for particular studies; it was used in this review.

Each study was assessed against methodological quality criteria according to whether:

a) the aim of the study was measuring the heat resistance of *C. botulinum* spores
b) non-proteolytic *C. botulinum* strains were tested
c) thermal treatment was performed by wet thermal process, but does not include high pressure processing, pulse electric field process or microwave energy
d) spore suspensions were used in studies
e) tests were performed in the range 50 – 95˚C
f) thermal death point clearly present heat treatment (e.g. come up time)
g) the strain name or toxin type was defined
h) there was an adequate description of heating menstruum used
i) there was an adequate description of heating method
j) there was an adequate description of recovery method
k) there was an adequate description of recovery media
l) there was an adequate description of recovery conditions
m) *D* and *z*-values were given by the author
n) there was an adequate description of *D* and *z*-value method calculation
o) *D*-value measures more than 1 log reduction of initial spore concentration
p) there are no inhibitory factors in heating menstruum or during recovering (e.g. low pH)

q) if D and *z*-values were not given, it was possible to calculate them from surviving data by plotting and fitting the best straight line by linear regression. In case of TDT method, where at least two points were given (the last positive and the first negative) the *D*-value of non-proteolytic *C. botulinum* was calculated using equation 2.4

Data with lysozyme (+LYS) in a recovery medium:

r) kinetic data with LYS clearly separates heat sensitive and heat resistant fraction and shows an example curve

Studies did not have to meet all 16 or 17 (in the case of publications with lysozyme in recovery media) criteria to be included in the analysis. The mandatory criteria are those from 'a' to 'e' and 'm or q' (points 'm' and 'q' are treated as interchangeable). The remaining points take account of the precision and methodological reliability of the study. Studies that met fewer than 8 of the above criteria and did not meet the mandatory criteria were considered as 'low' quality, those meeting 8 – 12 (13 for data with lysozyme) criteria were of 'medium' quality, and those meeting 13 – 16 (17 for data with lysozyme) criteria were of 'high' quality. Only studies of medium and high quality were included in the final analysis.

When the TDT method was used in studies with lysozyme, the document was only assessed against criteria but no quality rank was given. These data were analysed separately, since it was impossible to distinguish heat sensitive and heat resistant fractions. This rule was applied to the following publications: Notermans *et al.* (1990), Peterson *et al.* (1997), Scott and Bernard (1985), Juneja and Eblen (1995), Juneja *et al.* (1995a and 1995b). Although in the last three studies the heat resistance of spores was determined using the survivor curve, there was no graphical representation of an example curve and the authors calculated only one *D*-value.

Collecting the data from the included studies

Extracted data were labelled by strain and type of toxin produced, heating menstruum, temperature of heat treatment, *D* (min) and *z*-values (˚C) and its method of calculation, heating method, recovery medium, and conditions. Where

possible pH, a_w and addition of various nutrients to heating mentruum were also recorded. All data were included in an Microsoft Excel spreadsheet (Microsoft Excel, 2010). If the *D*-value was not reported, but the source provided a temperature at which the heat treatment was performed, starting inoculum and surviving spores, the *D*-value was determined from given tables or figures by fitting the best straight line using linear regression. When the heat treatment was conducted using the TDT method, the *D*-value was calculated according to equation 2.4. Some z-values were determined from reported or calculated *D*-values. In some publications the original authors questioned the quality of either the methodology or the way the experiment was conducted. In these cases such data were not included. In many cases the eligibility of articles retrieved by the electronic and manual searches was discussed with a senior scientist at IFR. Endnote X6 (2012) software was used for managing the bibliographic records.

An overview of the statistical and mathematical approach

Thermal properties of non-proteolytic *C. botulinum* spores are represented by a complex set of *D* and *z*-values, often labelled by categorical variables that included: strain name, toxin type, heating menstruum, heating technique and method of *D*-value calculation. The aim of the mathematical approach is to present the probability distribution for parameters *D* and *z*. In order to obtain a distribution for *D*, that describes the whole collected dataset, known *D*-values measured at various temperatures are transformed to one particular temperature using a known *z*-value. Additionally, a statistical analysis is used to evaluate the full database and to identify factors that influence the magnitude of the parameters above. Differences between groups of *D*-values are examined by a standard *t*-test. In the case of two or more groups the difference between mean *D*-values is tested using one-way analysis of variance (ANOVA), by SPSS Statistics software (IBM (version 21), 2012). The assumption of homogeneity of population variance is tested by Levene's test. When the homogeneity is assumed, the Tukey test was used in *post-hoc* analysis. If the assumption regarding the equal variance was violated, an adjusted *F*- statistic by Welch statistic was used with Games-Howell (which allows for comparison of groups with unequal variance and sample size) as the *post-hoc* test. The differences were tested at a 5% significance level.

2.2.2 Statistical and mathematical analysis

A number of authors e.g. Scott and Bernard (1985), Peck *et al.* (1993) observed that the presence of lytic enzymes during recovery had a large effect on the measured decimal reduction time for spores of non-proteolytic *C. botulinum*. Therefore the collected data from 48 eligible sources was divided into two major subsets. Firstly, -LYS (recovery of spores in the absence of lysozyme) and secondly +LYS (recovery of spores in the presence of lysozyme). The lysozyme was either deliberately added to medium in which spores were recovered, or the heat treatment and recovery was conducted in a food substrate, where the activity of lytic enzymes has previously been observed.

Due to the occurrence of biphasic survival curves for recovery in the presence of lysozyme, *D*-values were determined for each part of the curve: i.e. for the fraction of spores not permeable to lysozyme (heat sensitive (+LYS HS)) and for the fraction of spores permeable to lysozyme (heat resistant (+LYS HR)). When the heat resistance of spores was determined by the TDT method (+LYS TDT), the data were treated separately (this subset also contains the publications of Juneja *et al.* as mentioned above).

The number of reports for both parameters *D* and *z* were plotted against temperature to illustrate the minimum, maximum and dominant temperatures at which experiments were performed for all strains and for particular toxin types.

The average value of logD(T), at every fixed temperature, T, was plotted (for both subsets of data (-LYS) and (+LYS)) against the temperature to illustrate *D*-values and to fit a thermal destruction line using the method of least squares.

Throughout the analysis the following expressions were used: all strains – *D*-values for all strains (irrespectively on toxin type), type B toxin strains, type E toxin strains, type F toxin strains and mixed strains – i.e. a “cocktail” of strains, which corresponds to a mixture of more than one toxin type.

2.2.2.1 Probability distribution of D(T)

Under particular conditions *D*-values are fixed but uncertain. A distribution used to represent uncertain *D*-values should correspond with all non-negative values. Moreover, although *D*-values are finite in general extreme (high) values cannot be ignored and this should be considered when developing the uncertainty distributions.

As lognormal distribution meets these requirements, and is a well-known distribution for microbiologists and this was used to express the uncertainty concerning the measured *D*-value at fixed temperature (T)

$$p(D(T)) = \left(\frac{1}{\sqrt{2\pi}\sigma D(T)}\right) e^{\frac{-(\ln(D(T))-\mu)^2}{2\sigma^2}} \qquad 2.11$$

where μ and σ are the mean and standard deviation of the natural logarithm of *D*. Collected *D*-values vary over a wide range; therefore the lognormal distribution is consistent with the collected data.

The statistical descriptors of the data, <D> and σ_D, are related to the lognormal parameters according to

$$\mu = \ln(< D >) - \frac{\ln(\left(\frac{\sigma_D}{< D >}\right)^2 + 1)}{2} \qquad 2.12$$

and

$$\sigma = \sqrt{(\ln((\frac{\sigma_D}{< D >})^2) + 1)} \qquad 2.13$$

The transformation of parameters from ln<D> to log<D> involves a simple change of base: log<D> = ln<D>/ln(10).

2.2.2.2 Probability distribution of *z*-value

Similarly to *D*-values, under particular conditions, the *z*-value is fixed but uncertain. It is normal to assume that *z*-value is bounded by minimal and maximal values. In this case distribution for the uncertainty associated with

z-value is limited to distributions which cover real values e.g. beta, triangular or uniform. In this work the uncertainty associated with *z*-value was represented by a beta distribution. The distribution of *z*-values was fitted to a beta distribution with four parameters (a, c, α_1 and α_2)

$$p(z) = \frac{(z-a)^{\alpha_1-1}(c-z)^{\alpha_2-1}}{\beta(\alpha_1,\alpha_2)(c-a)^{\alpha_1+\alpha_2-1}} \quad 2.14$$

where a and c are the minimum and maximum values of the range, α_1 and α_2 are continuous shape parameters and β is the Beta Function

$$\beta(\alpha_1,\alpha_1) = \int_0^1 x^{\alpha_1-1}(1-x)^{\alpha_2-1}dx \quad 2.15$$

The mean, <z>, of the beta distribution is given by

$$< z >= a + \frac{\alpha_1}{\alpha_1+\alpha_2}(c-a) \quad 2.16$$

The cumulative beta probability density function, CDF, can be used in the add-in package Solver® for Microsoft Excel (2010) to establish the fitted parameters.
The fit was achieved by minimizing the sum of squared deviations, SS, of the differences between the actual data and the fit (least squares fitting)

$$SS = \sum_{i=1}^{n}[y_i - y_{fit}]^2 \quad 2.17$$

where, y_i is the data point, and y_{fit} is the value of the fitted distribution. The initial estimates of distribution parameters (a, c, α_1 and α_2) were changed several times and the fit recalculated in order to obtain the smallest SS value. When the same *z*-value was recorded on more than one occasion, only one point is used in the cumulative distribution fitting process. Therefore, the model gives a conservative bias to predicted values. Fitted parameters were subjected to some constraints such as e.g. $\alpha > 0$.

The upper and lower confidence interval, UCL and LCL, for the parameter, and the assessment of goodness of fit were calculated according to method described by Brown (2001). The confidence interval of $\hat{\beta}$ were calculated according to the formula

$$CL = \hat{\beta} \pm t_{\alpha,n-p} S.E. \quad 2.18$$

where $\hat{\beta}$ represents one of four parameters, *n* is the number of observations, p is the number of parameters, $t_{\alpha,n-p}$ is the 100(1 - α/2) percentile of the *t*-distribution with n - p degrees of freedom, α is confidence level and S.E. is an estimate of parameter's standard error (which expresses the variability arising from the sampling process).

2.2.2.3 Probability distribution of D'(T_{ref})

The data concerning *D*-values reflect measurements at several temperatures. Nevertheless, when using a *z*-value it is possible to convert the information about D(T) to one reference temperature, T_{ref}. In this case 80°C was used as T_{ref}, since it is a dominant temperature at which the measurement of *D*-values were conducted. Moreover, it is a commonly applied temperature in production of minimally heated chilled foods. The transformation of D(T) into D'(T_{ref}) can be made following the definition of *z* (equation 2.9)

$$D'(T_{ref}) = D(T)10^{(T - T_{ref})/z} \quad 2.19$$

All D(T) are converted to equivalent D'(80) using two approaches: when *z*-value is dependent on and independent of temperature. In the first case, the *z*-value used in calculations corresponds to the actual temperature range at which the heat resistance was conducted. When a *z*-value is not given for the particular experiment for -LYS data, the corresponding mean value for a toxin type is used (for type B, E, F and mixed strains <*z*> = 6.9°C, 6.9°C, 6.5°C and 6.7°C respectively). If the information about the *z*-value was missing for +LYS data, the mean value of z = 7.5°C, 9.6°C and 11.4°C for HS, HR and TDT respectively were used.

When the independence of *z*-value on T is assumed, the beta distribution of *z*-value as described above is used. The transformation of probability distribution can be written symbolically as

$$p\left(logD'\left(T_{ref}\right)\right) = p\left(logD(T)\right) - \frac{T_{ref} - T}{p(z)} \qquad 2.20$$

where logD(T) is the logarithm of the *D*-value at fixed T. The computation of uncertainty regarding the *z*-value in equation 2.20 can be performed using a Monte Carlo simulation process (described in details in section 5.1.4) with @RISK – risk analysis add-in for Microsoft Excel software (2010).

The parameters of p(logD'(80)) are obtained for ten subsets of D'(80) corresponding to T in different temperature ranges: 50 – 79°C, 50 – 80°C, 50 – 81°C, 50 – 82°C, 50 – 83°C, 50 – 85°C, 50 – 88°C, 50 – 90°C, 50 – 92°C and 50 – 93°C. To build the final distribution, in each case the distribution of p(logD'(80)) is weighted according to the number of experiments conducted at each temperature. For a graphical presentation of logD'(80), a box plot was used, with minimum, maximum – whiskers, mean – vertical line inside the box and 95th and 5th percentiles – top and bottom of box.

2.2.2.4 Anderson-Darling test

To test if logD(T) values transformed to logD'(80) to build the distribution of p(logD'(80)) are consistent across the temperature range the Anderson-Darling test is used. The test compares the distribution of empirical data with a theoretical normal distribution, and its parameter is defined as

$$A^2 = -n - S \qquad 2.21$$

where *n* is the sample size and *S* is calculated according to

$$S = \sum_{i}^{n} \frac{(2i-1)}{n} [\ln F(Y_i) + \ln(1 - F(Y_{n+1-i}))] \qquad 2.22$$

where the $F(Y_i)$ is a cumulative distribution function of the particular p(logD'(80)). The Anderson-Darling statistic tests whether data is normally distributed. The hypothesis regarding the distributional form is rejected at a significance level, if the calculated value, A^2, is greater than the critical value, given in Table 2.5.

Distribution	$\alpha = 0.15$	$\alpha = 0.10$	$\alpha = 0.05$	$\alpha = 0.025$	$\alpha = 0.01$
Critical value	1.610	1.933	2.492	3.070	3.857

Table 2.5 The critical values for Anderson-Darling test for normal distribution *Source:* Stephens (1974)

In order to conduct the Anderson-Darling test, each D(T) collected from the literature is converted to logD'(80) with assumed dependency of *z* on T (as described above). Ten subsets of logD'(80) have been created corresponding to different temperature ranges. For each subset <logD'(80)> and $\sigma_{logD'(80)}$ have been calculated and an Anderson-Darling test conducted. The analysis has two purposes: to test if the normal distribution is a good representation of the data for logD'(80), and to assess if the form of the *D*-value distribution changes as successively higher temperatures are included in the range: e.g. if *D*-value measured at higher T transformed to logD'(80) belongs to the same population.

2.2.2.5 Testing the significance of T and *z*-value dependence

An independent sample *t*-test with unequal variance was conducted to compare the differences in logD'(80) based on two assumptions: a *z*-value dependent on and independent of T (a significance level 5% was used).

2.2.2.6 Testing the validity of temperature transformation

In order to verify the validity of the D'(80) transformation, a one-way ANOVA was conducted. The ANOVA compares the means of logD'(80) evaluated from data in temperature ranges from 50 to 83°C and logD'(80) evaluated from one particular temperature. The test was conducted for data at four dominant temperatures: T = 70°C, 75°C, 77°C, 82°C. Additionally the mean of logD'(80) was compared with logD values measured at a fixed temperature of 80°C (D(80)). In total six pairwise comparisons were made. The logD'(80) with assumed dependence of *z*-value on T was used in the analysis.

2.2.2.7 Testing the dependence of spore heat resistance on toxin type

The hypothesis stating that there is no significant difference in spore heat resistance for spores with different toxin type was tested at four temperatures. For D(75) an independent sample *t*-test was performed for type B and E toxin type strains. A one-way ANOVA was conducted for pairwise comparison of D(80), D(82) and D'(80) converted from temperatures at 50 – 83°C for type B, E, F and mixed strains.

2.2.2.8 Testing the dependence of spore heat resistance on heating menstruum

The complete set of logD(T) values was plotted against T, and linear regression computed

$$logD(T) = \alpha - \beta T \qquad 2.23$$

where parameters α is intercept and β is the slope of the linear regression which is equivalent to inverse of the *z*-value, β = 1/z. With logD(T) as the response variable and T as the factor, a linear regression procedure using MS Excel was performed to estimate α and β. Estimated parameters are used to calculate the *z*-value and corresponding D'(80) for culture media and food matrices. The 95th and 99th UCL of predicted response and 95th UCL and LCL of the fit are estimated. Based on equation 2.23 the 99th UCL of the model was used to calculate the heating time at 80°C needed for a 6D reduction of spore concentration as a function of T in order to review current process recommendations.

In order to determine whether there is a difference in heat resistance of spores measured in culture media and food matrices a standard *t*-test and a 5% significance level was used.

2.2.2.9 Testing the dependence of spore heat resistance on heating technique

To examine whether spore heat resistance is dependent on the heating technique used, a one-way ANOVA was conducted. The analysis was carried

out for logD'(80) measured using nine different heating techniques reported in 48 eligible studies.

2.2.2.10 Testing the dependence of spore heat resistance on method of *D*-value calculation

A one-way ANOVA was used to compare the mean of logD'(80) derived from data according to four methods of *D*-value calculation.

2.2.2.11 Testing the dependence of spore heat resistance on strain of non-proteolytic *C. botulinum*

An unsupervised hierarchical clustering algorithm, with dissimilarity metric based on Euclidean distance, was used to indicate a strain classification pattern based on their heat resistance. Clustering was conducted using SPSS Statistics software. The hypothesis that there is a significant difference in spore heat resistance between different strains of non-proteolytic *C. botulinum* was tested for seven strains for which there were at least 20 *D*-values in the database. The analysis was conducted based on logD'(80) values using a one-way ANOVA.

2.2.2.12 Testing the dependence of spore heat resistance on testing laboratory

In order to test whether *D*-values are significantly different in relation to laboratory (or authors) in which they were generated, the one-way ANOVA was use. The analysis was conducted for six laboratories, from which the largest number of *D*-values was recorded.

2.2.2.13 Testing the similarity of +LYS HS and +LYS HR fractions

The significant difference in heat resistance of spores permeable and not permeable to lysozyme was tested using a standard *t*-test with assumed unequal variance. The analysis was performed for +LYS HS and HR fractions of *D*-values measured at T = 75°C, 78°C and 80°C and D'(80). For HS fraction a D'(80) transformed from the temperature range 75 – 83°C, and for HR fraction D'(80) in a temperature range between 75 – 95°C, was used. A similar procedure was applied in order to compare the *D*-value of the +LYS HS fraction with -LYS data at T = 78°C, 80°C and D'(80). A significance level of 5% was used.

2.3 Results

2.3.1 Description of studies on spore heat resistance

2.3.1.1 Output of literature search

The searches identified a total of 15,037 records. Figure 2.6 describes the flow of these records through the map.

A manual search of private databases yielded 16 eligible publications (Figure 2.6).

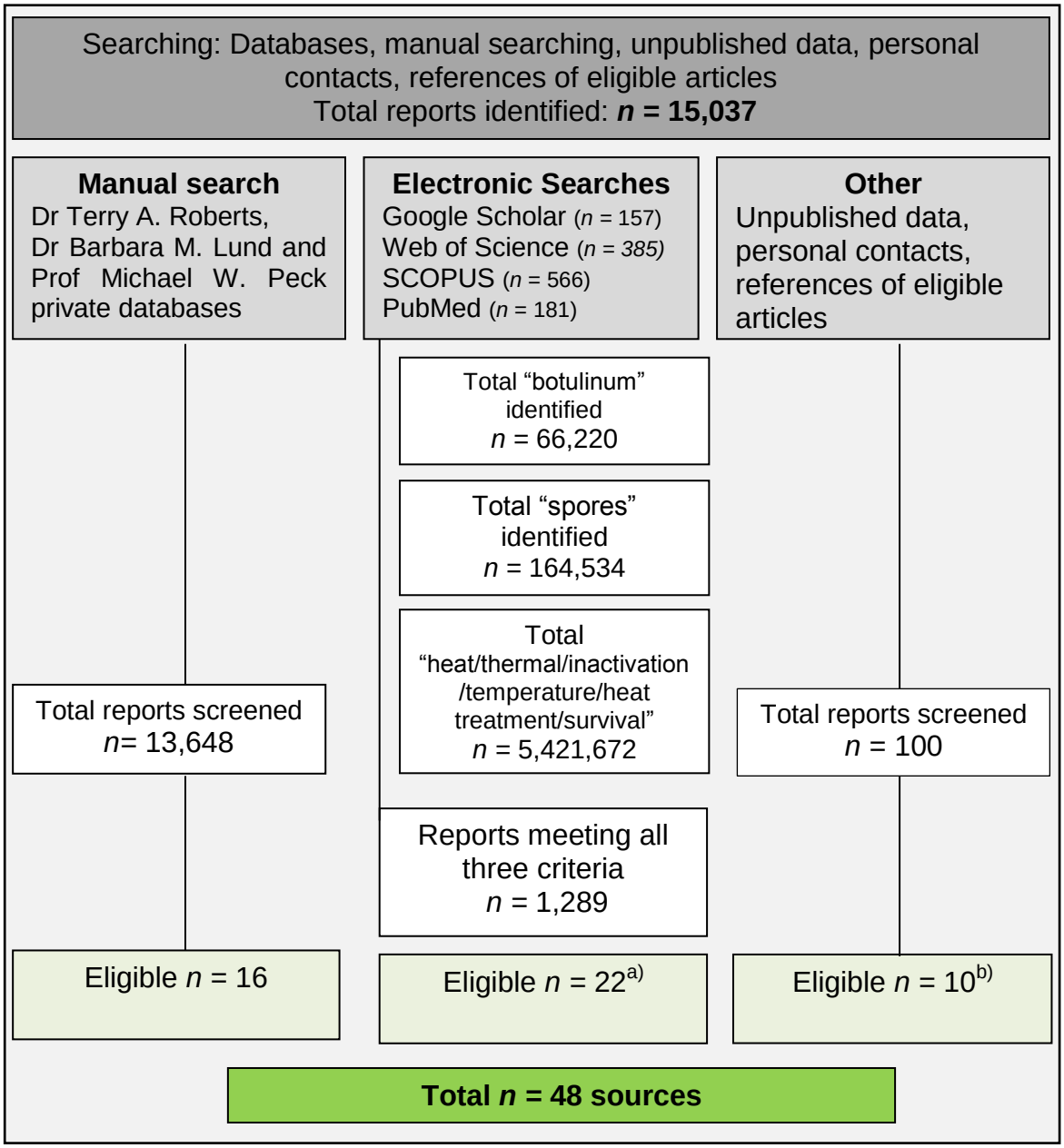

Figure 2.6 Flow literature search, for non-proteolytic *C. botulinum* spores heat resistance, through the map

a) 22 eligible articles not found in previous manual search

b) 10 eligible articles not found in previous searches

Keywords for electronic searches of public databases varied according to database. A search of article's title in Google Scholar for "botulinum" (with of all the words), "spores" (with the exact phrase), "heat"/"thermal"/"inactivation"/"temperature"/"heat treatment"/"survival" (with at least one of the words), yielded 157 records. A search of the 1977 – 2011 Web of Science for "botulinum" (ts = topic) yielded 15,258 citations, search for "spores" (ts) yielded 35,759 citations, for "heat"/"temperature"/"inactivation"/"thermal" (ts) yielded 2,813,840 citations. There were 385 citations that were included in all three searches (Figure 2.6). A search of the 1973 – 2011 SCOPUS for "botulinum" (all fields) yielded 37,071 citations, for "spores" 99,377, for "heat treatment"/"temperature"/"inactivation"/ "thermal" 1,194,345 references. Combining all three citations yielded 955 sources. Due to the large number of citations further search (type B strain/type E/type F) yielded 4,358,280 citations. The combination of four searches generated 566 references. Advanced search for "botulinum" in PubMed yielded 13,891 citations, for "spores" 29,398 and "heat"/"temperature"/"inactivation"/"thermal"/"survival" 1413,487 citations. There were 181 sources that were included in all three searches. In total from electronic searches twenty-two publications were judged eligible.

Other sources yielded a further ten eligible articles, to give a total of 48 eligible articles (Figure 2.6).

2.3.1.2 Analysis of electronic search (Web of Science and SCOPUS example)

Further analysis has been carried out of results from searches of Web of Science and SCOPUS. A search of the Web of Science database yielded 385 citations that include all three searches (botulinum AND spores AND (heat OR temperature OR inactivation OR thermal)). The most common sources were the Journal of Food Protection (83 records), Applied and Environmental Microbiology (37 records) and International Journal of Food Microbiology (32 records). Similar results were obtained in searching of SCOPUS, with Journal of Food Protection showing 76 records, International Journal of Food Microbiology 66 records, and Applied and Environmental Microbiology 37 records (Table 2.6).

Source	Web of Science		SCOPUS	
	Record count	% of 385	Record count	% of 566
Journal of Food Protection	83	22	76	13
Applied and Environmental Micro.	37	10	37	7
International Journal Food Micro.	32	8	66	12
Journal of Food Science	25	6	24	4
Food Microbiology	19	5	24	4
Letters in Applied Microbiology	17	4	12	2
Journal of Applied Microbiology	16	4	30	5
Journal of Food Safety	12	3	12	2
Food Control	6	2	9	2
Journal of Food Engineering	6	2	11	2
Critical Reviews in Food Science	2	0	12	2
Other sources	130	34	313	45

Table 2.6 Number of records for non-proteolytic *C. botulinum* spore heat resistance, rank by source in Web of Science and SCOPUS search

The most common records appearing in both databases that met all searching criteria included M. W. Peck as an author, with 41 in Web of Science and 46 records in SCOPUS (Figure 2.7). The second most common author was V. M. Balasubramaniam with 20 in Web of Science and 16 records in SCOPUS database. Records of V. K. Juneja, T. J. Montville and S. C. Stringer appeared 12 times each in Web of Science search, and 13, 5 and 13 respectively in SCOPUS (Figure 2.7).

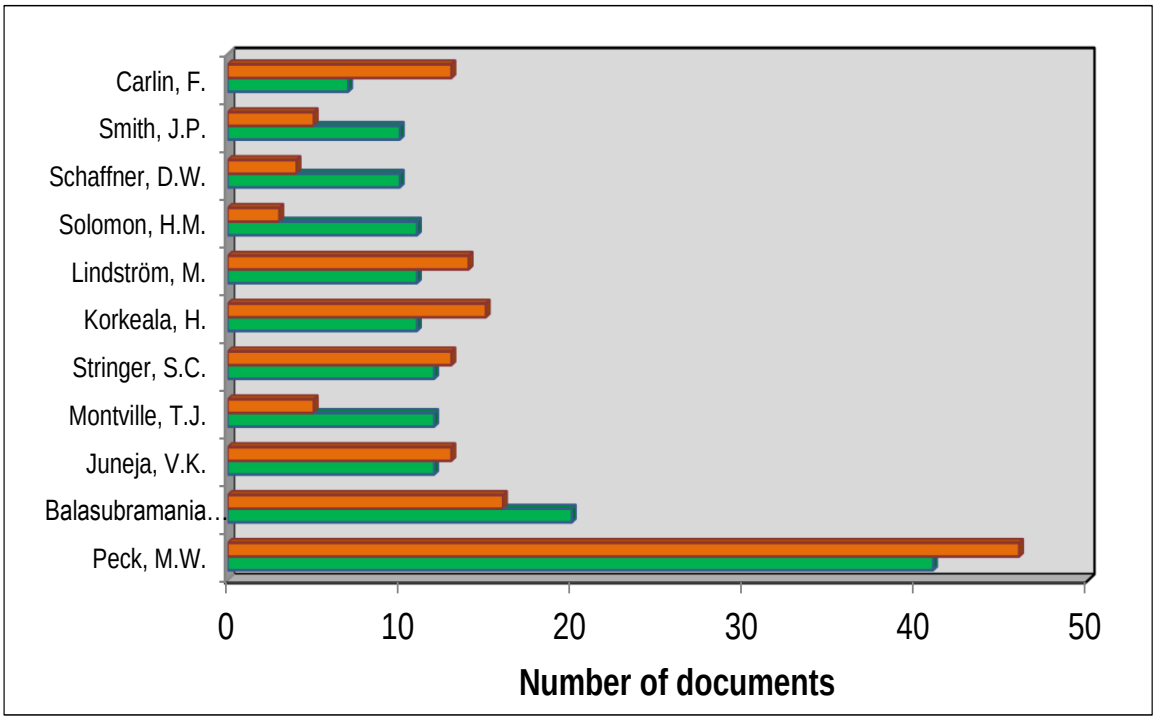

Figure 2.7 Summary of the eleven authors, who have published most articles on non-proteolytic *C. botulinum* spore heat resistance (by number of articles found in SCOPUS and Web of Science databases)
SCOPUS database (orange bars), Web of Science (green bars)

Approximately 41% (Web of Science) and 32% (SCOPUS) of records were from authors based in USA, 18% (Web of Science) and 17% (SCOPUS) from UK-based authors, and 5% (Web of Science and SCOPUS) were from authors located in Canada (Table 2.7). A majority of the documents were published in the period between 2000 and 2011. The number of published recorded in SCOPUS database increased from only six before 1995, to on average of 31 documents per year between 1997 and 2009, and 65 records in 2010 (Figure 2.8).

Source	Web of Science		SCOPUS	
	Record count	% of 385	Record count	% of 566
USA	159	41	183	32
UK	71	18	94	17
Canada	20	5	27	5
France	18	5	44	8
Australia	17	4	24	4
Netherlands	16	4	29	5
Spain	16	4	41	7
Finland	13	3	18	3
New Zealand	11	3	19	3
Germany	10	3	19	3
Other sources	34	9	68	12

Table 2.7 Number of records for non-proteolytic *C. botulinum* spore heat resistance by country of laboratory in Web of Science and SCOPUS search

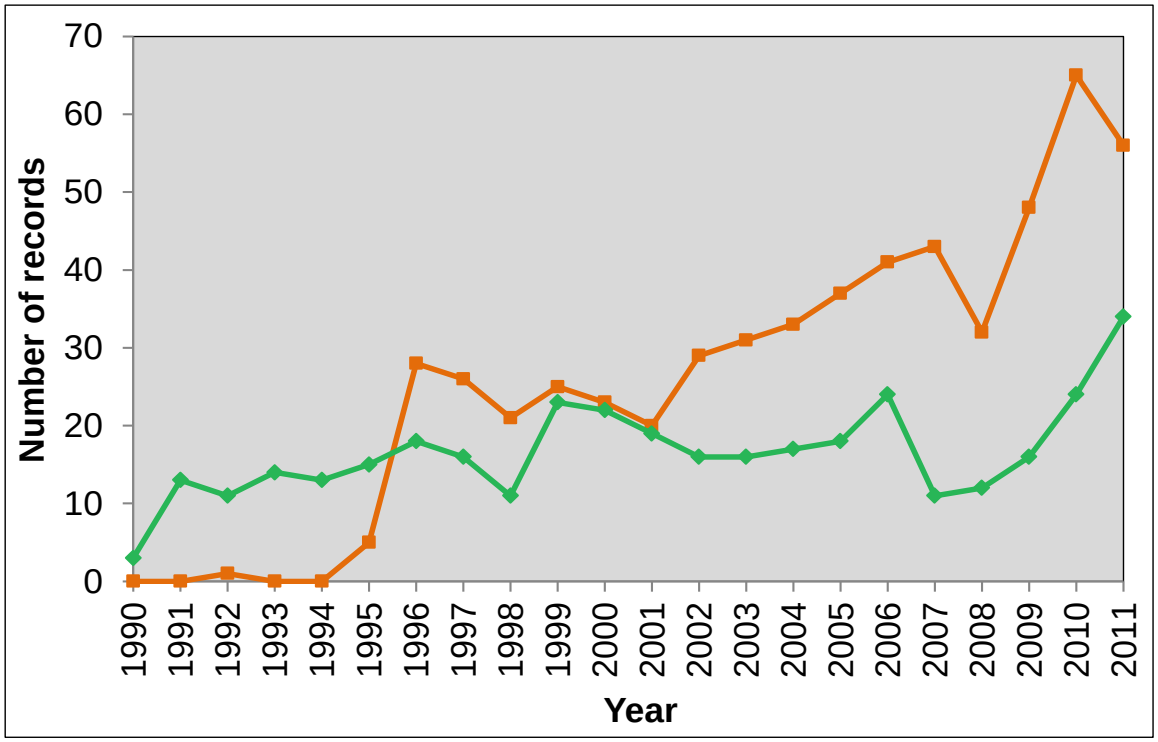

Figure 2.8 Number of records for non-proteolytic *C. botulinum* spore heat resistance search, published annually in SCOPUS and Web of Science between 1990 and 2011
SCOPUS database (orange line), Web of Science (green line)

2.3.1.3 Eligible records

The most common reason for exclusion of studies was testing of proteolytic *C. botulinum* strains. Further reports were excluded as tests were performed on vegetative cells or heat resistance of toxins was tested. Many publications described inactivation of non-proteolytic *C. botulinum* by high pressure processing, radiation or chemical treatment; therefore they were also excluded from the analysis. As of September 2011, 48 eligible studies were identified. Appendix 1 provides a summary of included studies.

Although differences in the physiological properties of certain toxin types of *C. botulinum* were observed in the early decades of 20th century (Gunnison and Meyer, 1929), the first study on the heat resistance of non-proteolytic *C. botulinum* spores was reported in 1950's. Nine reports were published before 1969, ten between 1970 – 1979 and six between 1980 – 1989. The majority had publication dates between 1990 and 1999, with only three reports dated between 2000 and 2011 (Figure 2.9).

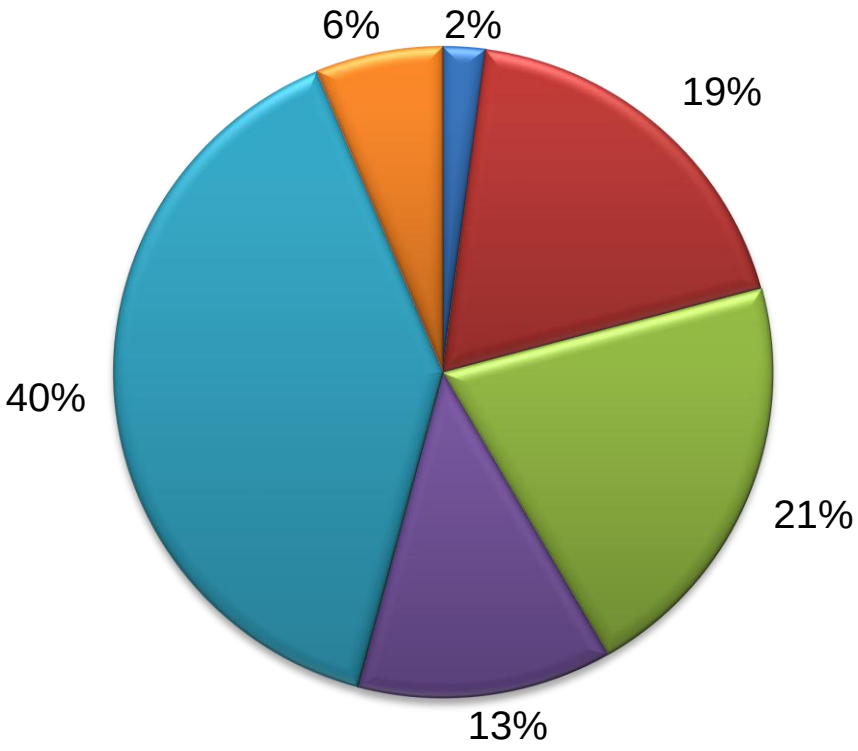

Figure 2.9 Eligible records of non-proteolytic *C. botulinum* spore heat resistance studies by publication date (*n* = 48)
Pre – 1959 (dark blue), 1960 – 1969 (red), 1970 – 1979 (green), 1980 – 1989 (violet), 1990 – 1999 (light blue), 2000 – 2011 (orange)

The largest numbers of eligible studies were published in the Journal of Food Protection (9), Journal of Food Science (6), Applied and Environmental Microbiology (4), Letters in Applied Microbiology (4), Journal of Applied Microbiology (2), Journal of Food Safety (2) and Journal of General Microbiology (2). Other journals, e.g. Journal of Milk and Food Technology, Marine Fisheries Review, Food Science (TAIPEI) and Australian Journal of Biological Science published one study each. Nine studies were published in Conference Proceedings (3), Academic dissertations (3) and Reports (3). Two studies were reported in Technical Memoranda, and single studies were published in a book and an abstract of annual meeting and a research bulletin. The database also contains unpublished data from the Institute of Food Research (Figure 2.10).

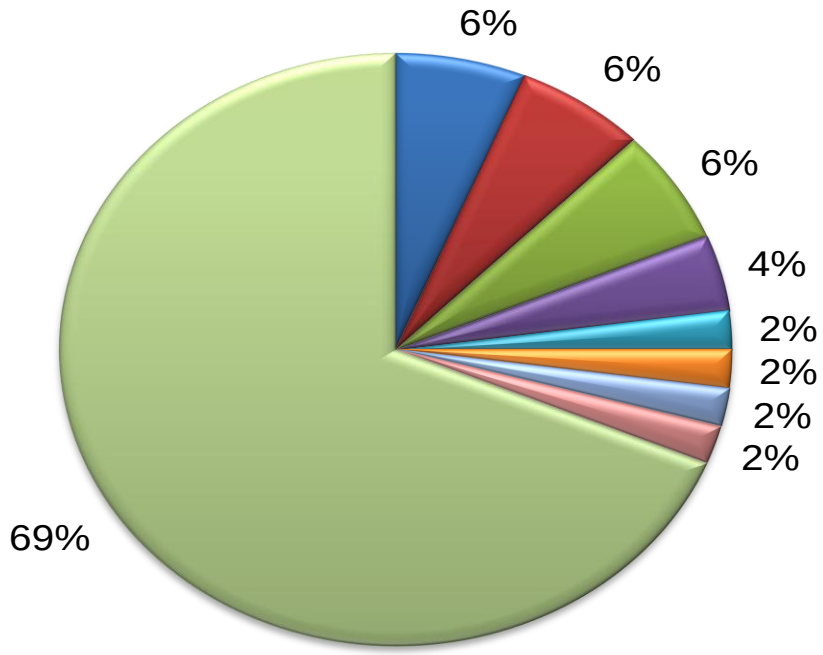

Figure 2.10 Eligible records of non-proteolytic *C. botulinum* spore heat resistance studies by published source (*n* = 48)
Conference Proceedings (dark blue), Academic dissertations (red), Reports (dark green), Technical Memorandum (violet), Book chapter (turquoise), Abstract of annual meeting (orange), Research bulletin (light blue), Unpublished data (pink), Scientific Journals (light green)

Altogether a total of 880 *D*-values were collected, with the greatest number (205) originating from the Institute of Food Research (23.2%). Approximately 6.0% of data points were derived from Bohrer *et al.* (1973), 5.9% from Chai and Liang (1992) and 10.2% from two publications by Juneja and colleagues (Juneja *et al.*,

1995a; Juneja *et al.*, 1995b). Between 10 and 38 *D*-values were from each of a further 19 documents (which together constituted 45% of collected data) and less than 9 *D*-values were from each of 24 sources (which constituted approximately 10%) out of 880 *D*-values (Figure 2.11).

Approximately 30% of *z*-values were derived from studies by Bohrer *et al.* (1973), Chai and Liang (1992) and the Institute of Food Research. In the case of 16 eligible articles the *z*-value was not given by authors and was impossible to calculate it from the presented data.

2.3.1.4 Results of quality assessment

In total, 48 eligible studies were included, from which relevant information detailed in section 2.2.1 was extracted. Details of included data are summarised in Appendix 2.

The included studies were assessed against the quality criteria specified in section 2.2.1. The purpose of the quality rating was to provide a descriptive overview of the methodological robustness of the included studies. Details of quality ranking are summarised in Table 2.8. Thirty-five sources were ranked as “high” quality, 7 as “medium” and 6 documents were not given the quality rank. The data without a quality rank were related to experiments in which the TDT method was used to determine the heat resistance of spores in the presence of lysozyme in a recovery medium, or studies, where a survival curve was not presented. These data were treated separately, since it was believed that there were heat resistant and heat sensitive fractions, but only a single *D*-value was reported by the authors. Data considered as “low” quality were not included.

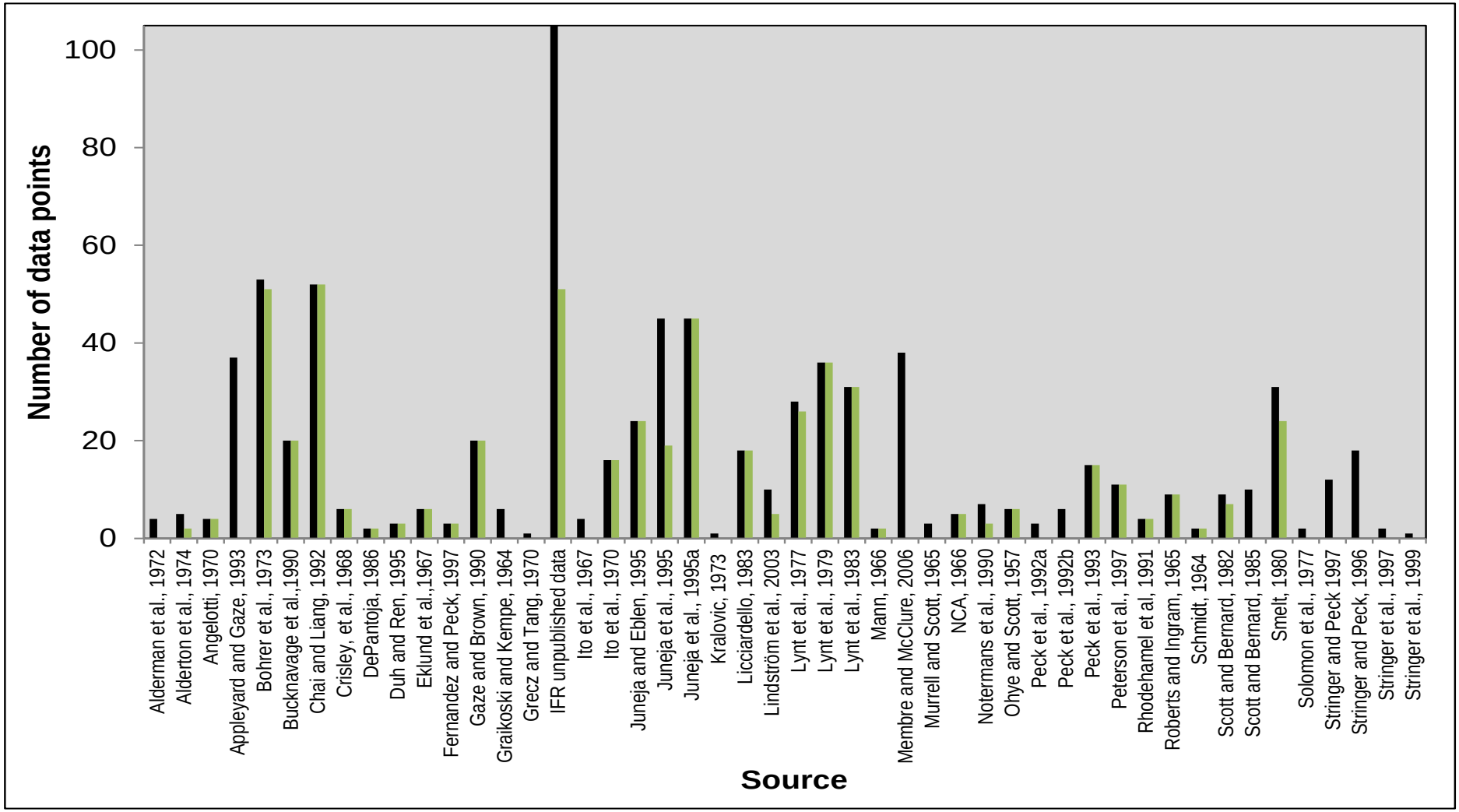

Figure 2.11 Number of *D* and *z*-values collected for non-proteolytic *C. botulinum* spore heat resistance from eligible studies (*n* = 48) *D*-values (dark bars), *z*-values (green bars)

Study ID	Alderman *et al.*, 1972	Angelotti, 1970	Appleyard & Gaze, 1993	Bohrer *et al.*, 1973	Bucknavage *et al.*, 1990	Chai & Liang, 1992	Crisley, *et al.*, 1968	DePantoja, 1986	Duh & Ren, 1995	Eklund *et al.*, 1967	Fernandez & Peck, 1997	Gaze & Brown, 1990	Graikoski & Kempe, 1964	Grecz & Tang, 1970	Ito *et al.*, 1967	Ito et al., 1970	Kralovic, 1973	Licciardello, 1983	Lynt *et al.*, 1977	Lynt *et al.*, 1979	Lynt *et al.*, 1983	Mann, 1966	Membre & McClure, 2006	Murrell & Scott, 1965	NCA, 1966	Ohye & Scott, 1957	Rhodehamel *et al*, 1991	Roberts & Ingram, 1965	Schmidt, 1964	Scott & Bernard, 1982	Solmon *et al.*, , 1977	Stringer *et al.*, 1999	Alderton *et al.*, 1974*	IFR unpublished, 1980-2011*	Juneja & Eblen, 1995	Juneja *et al.*, 1995a	Juneja *et al.*, 1995b*	Lindström *et al.*, 2003	Notermans *et al.*, 1990	Peck *et al.*, 1992a	Peck *et al.*, 1992b	Peck *et al.*, 1993	Peterson *et al.*, 1997	Scott & Bernard, 1985*	Smelt, 1980	Stringer & Peck 1997*	Stringer & Peck, 1996	Stringer *et al.*, 1997
Quality criteria	**-LYS**																															**+LYS**														
a	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
b	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
c	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
d	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
e	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
f	✓	-	✓	✓	✓	✓	✓	✓	-	-	✓	✓	-	-	-	-	-	-	✓	✓	✓	-	-	-	-	-	-	-	-	✓	✓	✓	-	✓	-	✓	✓	✓	✓	-	-	✓	✓	✓	✓
g	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
h	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
i	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	-	✓	✓	-	✓	-	-	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
j	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
k	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	-	✓	-	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
l	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	-	-	✓	✓	✓	✓	-	-	-	-	✓	✓	✓	-	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓
m	-	✓	✓	✓	✓	✓	✓	✓	✓	-	-	✓	-	✓	✓	✓	✓	-	✓	✓	✓	✓	✓	-	-	✓	✓	✓	✓	✓	-	-	✓	-	✓	✓	✓	-	-	✓	✓	-	✓	-	-	-
n	x	-	✓	✓	✓	✓	✓	✓	✓	x	x	✓	x	✓	x	-	-	x	✓	✓	✓	-	-	x	x	✓	✓	✓	-	✓	✓	✓	✓	x	✓	✓	✓	✓	-	x	x	✓	✓	✓	✓	✓	✓
o	✓	-	✓	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	-	-	✓	✓	✓	-	-	✓	✓	-	✓	-	-	-	✓	✓	✓	✓	-	-	-	✓	✓	✓	✓	✓	✓	✓	✓	✓
p	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	-	✓	✓	✓	✓	-	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
q	✓	x	x	x	x	x	x	x	x	✓	✓	x	✓	x	x	x	x	✓	x	x	x	x	x	✓	✓	x	x	x	x	x	✓	✓	x	x	✓	x	✓	✓	✓	x	✓	x	✓	✓	✓	✓	✓
r	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	✓	✓	-	-	-	✓	-	✓	✓	✓	-	-	✓	✓	✓	✓
Total	15	10	16	16	15	16	16	16	15	14	15	16	14	14	14	10	10	13	16	16	16	8	10	12	13	13	15	13	10	15	15	16	15	16	X	X	X	17	X	16	16	17	X	X	16	17	17	17
Quality	**H**	**M**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**M**	**M**	**H**	**H**	**H**	**H**	**M**	**M**	**M**	**H**	**H**	**H**	**H**	**M**	**H**	**H**	**H**	**H**	**X**	**X**	**X**	**H**	**X**	**H**	**H**	**H**	**X**	**X**	**H**	**H**	**H**	**H**

Table 2.8 Quality rank of eligible studies on the heat resistance of spores of non-proteolytic *C. botulinum* (*n* = 48)
✓ quality criteria was met; - the information was missing, not given or not clear from the text; x the information does not apply; X source did not undergo the quality ranking; *source describe both -LYS and +LYS data, H – high quality, M – medium quality
(see Appendix 1 for more information)

2.3.2 Statistical and mathematical analysis

2.3.2.1 Summary of collected data

Thermal destruction of non-proteolytic *C. botulinum* spores has been studied in foods, buffer and media under different recovery conditions at a temperature range of 50 – 95°C. In the case of -LYS data, a temperature of 80°C was most frequently tested (151 *D*-values). Other dominant temperatures at which *D*-values were determined were 70°C, 75°C, 77°C, 79°C and 82°C, with 61, 66, 50, 37 and 57 data points at these temperatures, respectively. When lysozyme was added to a recovery medium, the temperature at which the heat resistance of spores was determined was generally higher, with 105 *D*-values reported at 90°C, 52 at 80°C and 67 at 85°C (Figure 2.12).

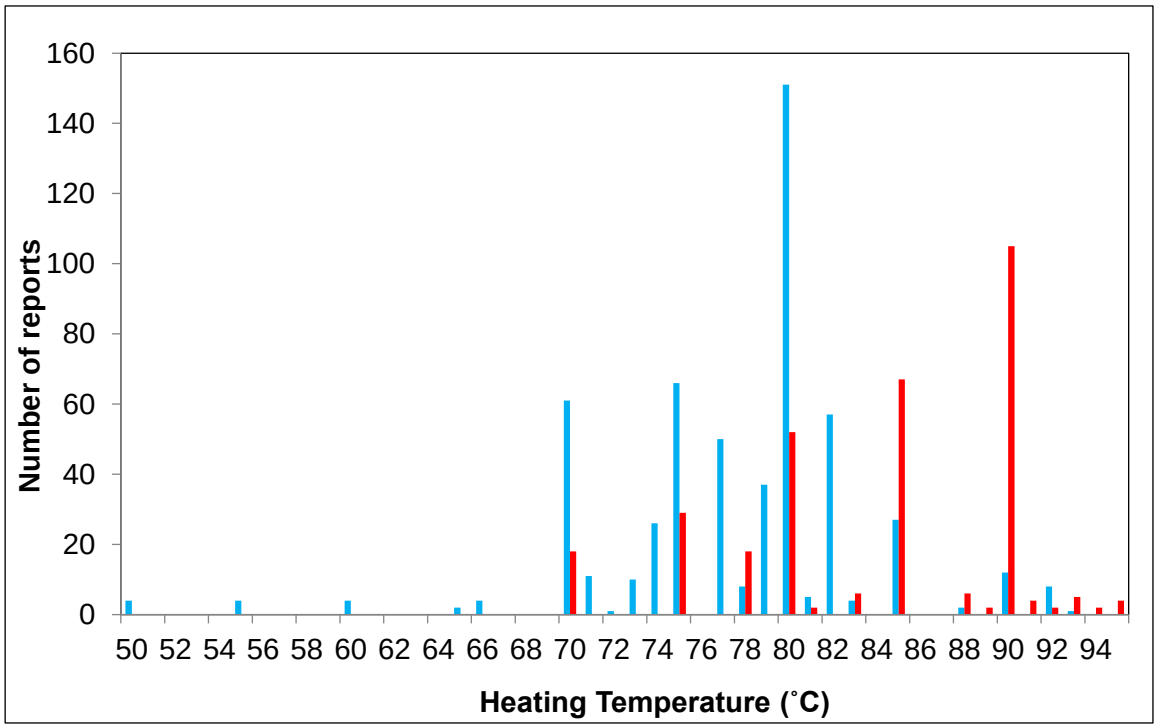

Figure 2.12 Summary of temperatures at which *D*-values for spores of non-proteolytic *C. botulinum* were measured
-LYS data (blue bars), +LYS data (red bars)

Reported number of *D* and *z*-values for all strains, for particular toxin types and for mixed strains are summarised in Table 2.9. In total 880 *D*-values and 528 *z*-values were generated from the 48 eligible sources, with 308 *D*-values for type B strains, 386 for type E strains, 67 for type F strains and 119 for mixed strains (i.e. strains of more than one toxin type). The database was separated into two

major subsets with 551 *D*- and 368 *z*-values from -LYS studies, and 325 *D*- and 160 *z*-values from +LYS studies.

Parameter	*D*-value				*z*-value			
Strains	**Total**	**%**	**-LYS**	**+LYS**	**Total**	**%**	**-LYS**	**+LYS**
All strains	880	100	551	329	528	100	368	160
Type B strains	308	35	103	205	139	26	48	91
Type E strains	386	44	330	59	297	56	258	39
Type F strains	67	8	63	4	59	11	59	0
Mixed strains	119	13	55	61	33	7	3	30

Table 2.9 Summary of published *D* and *z*-values for spores of non-proteolytic *C. botulinum* from 48 eligible sources

Approximately 70% of collected *z*-values were generated from -LYS experiments. In general, smaller *z*-values could be observed for -LYS, with <z> = 6.7°C (for all strains) and higher *z*-values were reported for +LYS, with <z> = 10.3°C (Figure 2.13).

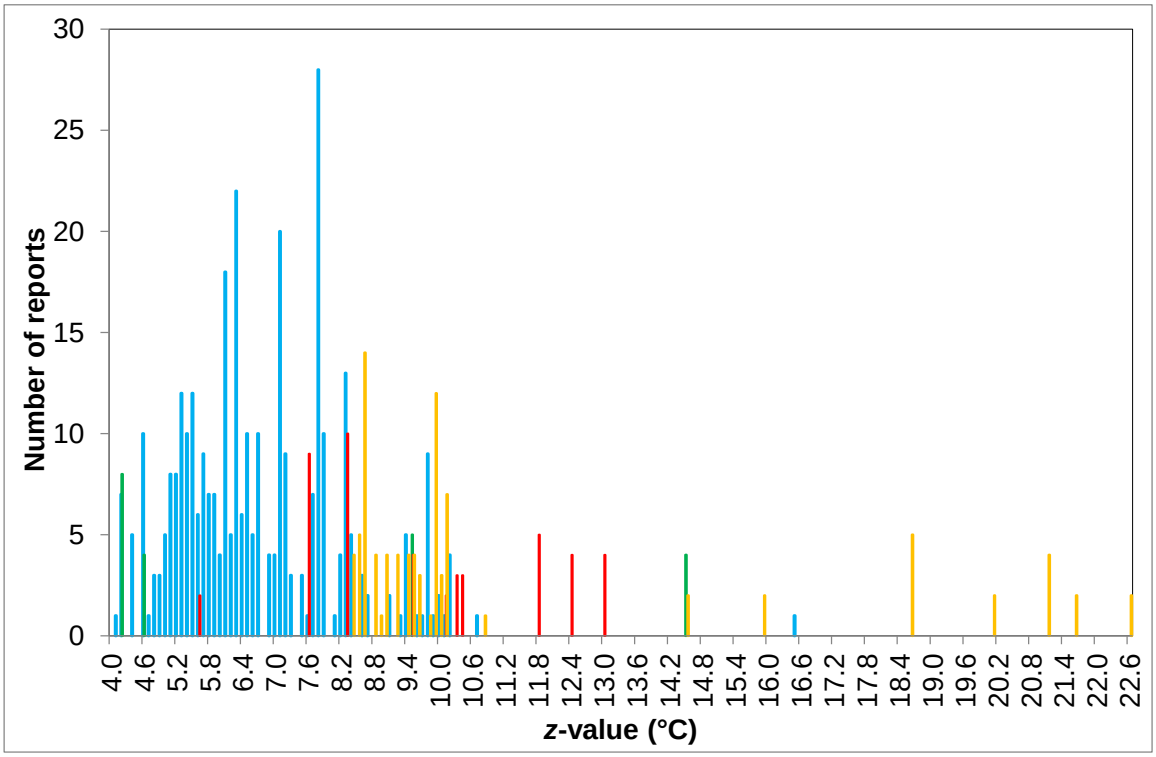

Figure 2.13 Summary of published *z*-values for spores of non-proteolytic *C. botulinum* from 48 eligible sources
-LYS data (blue bars), +LYS HS fraction (green bars), +LYS HR fraction (red bars), +LYS TDT data (yellow bars)

The highest reported *z*-value was 22.6°C for mixed spores (type B and E toxin) (Juneja *et al.*, 1995a) and the lowest reported value was 4.1°C for strain 8E heated in water and enumerated on RCM (Roberts *et al.*, 1965).

2.3.2.2 Heat resistance of spores of non-proteolytic *C. botulinum* with recovery in the absence of lysozyme (-LYS)

Summary of *D*-values

The average *D*-value at each heating temperature was plotted against temperature and two lines were fitted by linear regression (Figure 2.14). For one line all data were used, for the other, only data corresponding to temperatures at and below 83°C were used (see section 2.2.2.4 for explanation).

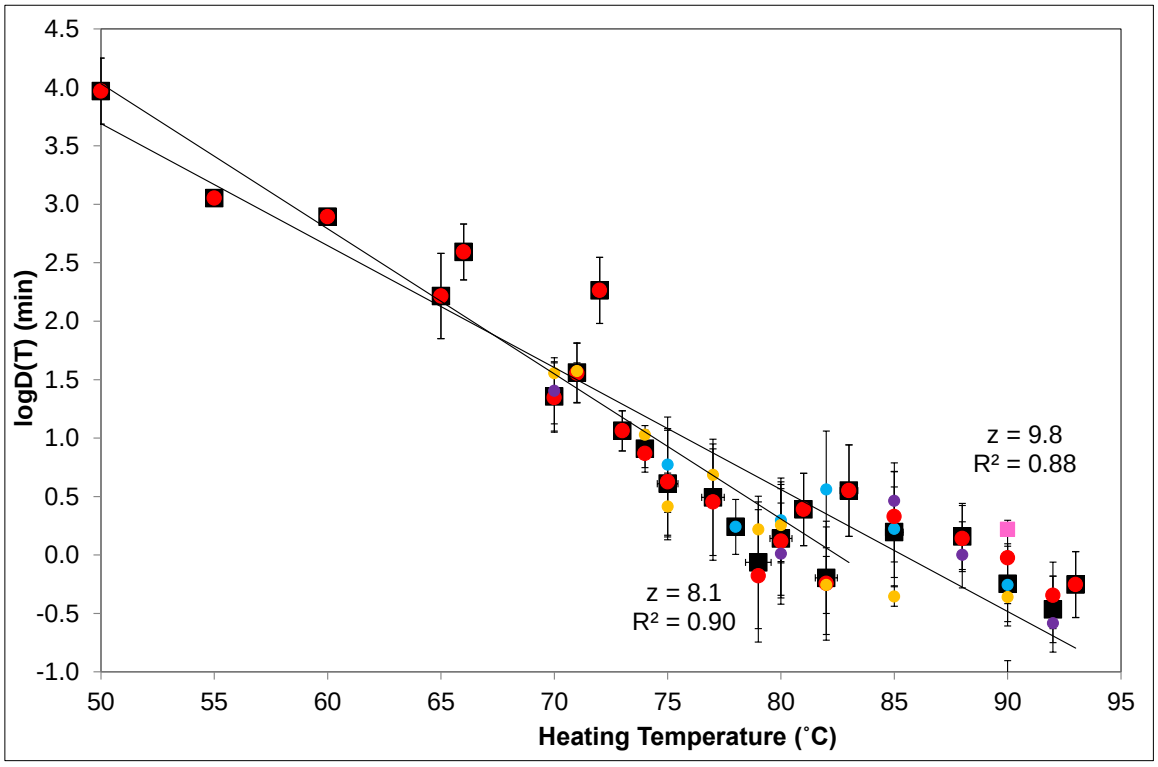

Figure 2.14 Mean and standard deviation of *D*-values (min) for spores of non-proteolytic *C. botulinum* at fixed heating temperatures (-LYS)
All strains (black square), type B strains (blue circle), type E strains (red circle), type F strains (yellow circles), mixed strains (violet circles), ACMSF recommended heat treatment of chilled food at 90°C/10 minutes (pink square)

Figure 2.14 is a visualisation of 551 data points. The error bars represent one standard deviation of the mean and reveal the uncertainty in reported *D*-values at each temperature. The mean *D*-values at 75°C, 79°C, 80°C and 82°C have the widest error bar, which is an effect of combining the large number of *D*-values that were measured at these temperatures (Figure 2.12). The size of error bars in Figure 2.14 reflects variability in data obtained from different laboratories in many different experiments. The value of logD(T) calculated for lower temperatures (50 – 65°C), which present very small error bars, involve only a few data points, determined in one laboratory by Bucknavage *et al.* (1990).

Probability distribution of D(T)

In order to obtain the parameters of uncertainty distributions for D(T), a logarithmic transformation is used. The parameters for a normal distribution for logD(T) and the corresponding parameters for a lognormal distribution of D(T) at each heating temperature are summarised in Table 2.10. The fitting procedure is described in section 2.2.2.1.

T (°C)	Normal distribution of logD(T)		Lognormal distribution of D(T)	
	<logD(T)> (min)	$\sigma_{logD(T)}$ (min)	<D(T)> (min)	$\sigma_{D(T)}$ (min)
50	3.99	0.26	11690	7675
55	3.05	0.04	1126	104
60	2.89	0.05	781	90
65	2.21	0.55	361	720
66	2.53	0.46	593	854
70	1.36	0.28	28.20	20.25
71	1.54	0.30	44.02	34.42
72	2.36	0.20	254	123
73	1.07	0.14	12.38	4.10
74	0.90	0.21	8.93	4.58
75	0.71	0.49	9.69	15.54
77	0.50	0.42	5.05	6.28
78	0.22	0.30	2.11	1.65
79	0.01	0.39	1.53	1.71
80	0.16	0.41	2.26	2.71
81	0.40	0.32	3.30	2.80
82	-0.21	0.55	1.37	2.74
83	0.54	0.45	5.93	8.23
85	0.04	0.60	2.85	6.82
88	0.16	0.10	1.48	0.35
90	-0.27	0.43	0.88	1.13
92	-0.50	0.33	0.42	0.37
93	-0.16	-0.01	0.69	0.02

Table 2.10 Parameters for normal distribution of logD(T) (min) and corresponding lognormal distribution of D(T) for heat resistance of non-proteolytic *C. botulinum* spores (-LYS)

The highest average value <logD(T)> was recorded for experiments performed at 50°C, whereas the lowest at 92°C. According to equation 2.1 the *D*-value is a representation of the time-temperature relationship: the higher the heating temperature, the shorter time needed to reduce the concentration of spores. In general this relationship is reflected in the recorded data, but occasionally fluctuations can be observed and attributed to natural experimental variability. This can be observed for example for *D*-values measured at 72°C and 81°C (Table 2.10).

Probability distribution of z-values

Beta distributions for *z*-value were obtained by fitting to the observed data (which are summarised in Table 2.11 – see section 2.2.2.2 for more details). The fit was not obtained for mixed strains, because only two z-values were recorded for this subset.

Subset of data	No. of collected *z*-values	No. of *z*-values used to build p(z)*
All strains	368	340
Type B strains	48	42
Type E strains	258	245
Type F strains	59	51
Mixed strains	3	2

Table 2.11 Number of collected *z*-values from eligible studies and number of *z*-values (°C) used to build distribution of p(z) for heat resistance of non-proteolytic *C. botulinum* spores (-LYS)
*see section 2.2.2.4 for explanation on why all *z*-values were not included

Based on empirical *z*-values, parameters of a beta distribution were fitted using MS Excel Solver® (see section 2.2.2.2). An example fit obtained for all strains is illustrated in Figure 2.15.

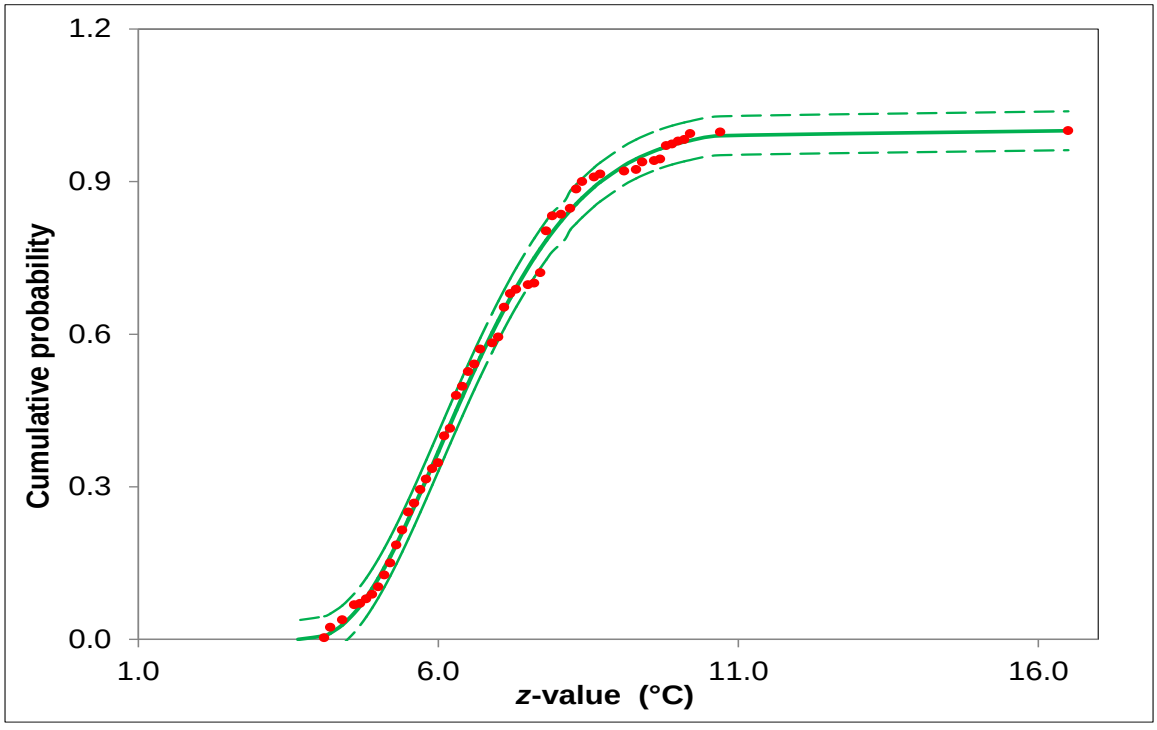

Figure 2.15 Cumulative beta probability density function fit of *z*-values (°C) for spores of non-proteolytic *C. botulinum* (all strains)
Experimental data points (filled circles), fit based on the initial parameter estimates (thick line), 95% confidence interval around the fit (dashed lines)

The best fit was obtained for all strains with one constraint ($c \geq 16.5$). A fit was obtained for remaining subsets of *z*-values, but data were subjected to further constraints. The fitted lines represented the data and often they extend to high *z*-values; therefore the estimated parameters are more difficult to interpret. The UCL and LCL calculated for each of the fitted parameters are based on an estimate of the standard error (S.E.). In some cases the negative values for LCL are non-physical and represent the difficulty of the fitting procedure. When the fit was considered poor, due to a small number of data points, the estimated parameter values for type B, E and F strains are less reliable.

Parameters presented in Table 2.12 describe beta distributions for *z*-value. The mean *z*-value was calculated according to equation 2.16. Distributions of *z*-values for all strains, type B strains, type E strains and type F strains, based on parameters in Table 2.12, are illustrated in Figure 2.16. The calculated mean *z*-value for all strains is 6.7°C with 95% confidence interval [4.4, 10.0].

Toxin type	Parameter	Estimate	S.E.	UCL	LCL	Constraint
All strains	a	3.7	0.4	4.5	2.9	
	c	16.5	5.2	27.0	6.0	$c \geq 16.5$
	α_1	2.9	1.0	5.0	0.8	
	α_2	9.6	7.2	24.0	-4.8	
	<z>	6.7				
Type B strains	a	4.0	1.1	6.1	1.3	a = 4.0
	c	30.0	162	382	-322	$c \leq 30.0$
	α_1	1.1	1.5	4.3	-2.2	$\alpha_1 \geq 1.0$
	α_2	10.0	71	165	-146	
	<z>	6.4				
Type E strains	a	3.5	0.8	5.1	2.0	$a \geq 3.5$
	c	11.2	1.6	14.5	8.0	
	α_1	3.4	1.7	6.9	-0.1	
	α_2	4.6	2.9	10.5	-1.3	
	<z>	6.8				
Type F strains	a	4.0	11.8	34.3	-26.3	a = 4.0
	c	30.0	1414	3665	-3605	$c \leq 30.0$
	α_1	3.9	61.8	162	-155	$\alpha_1 \geq 1.2$
	α_2	49.5	3385	8751	-8652	
	<z>	5.9				

Table 2.12 Parameters of beta distribution for *z*-values (°C) for non-proteolytic *C. botulinum* spores obtained from MS Excel Solver® fit with specified constraints, and calculated UCL and LCL (-LYS)
a is minimum and c maximum of fitted *z*-value, α_1 and α_2 are continuous shape parameters and <z> is mean *z*-value

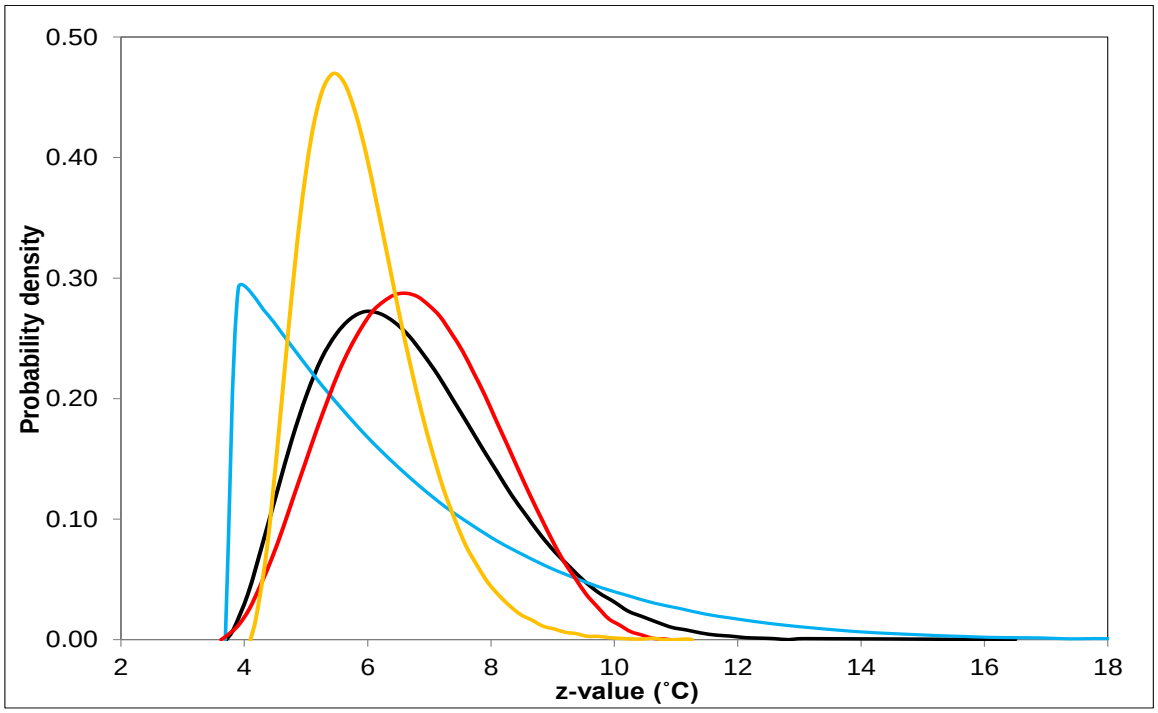

Figure 2.16 Beliefs concerning z-value (°C) for non-proteolytic *C. botulinum* spores (-LYS)
All strains (black line), type B strains (blue line), type E strains (red line), type F strains (yellow line)

Probability distribution of D'(80)

The conversion of logD(T) into equivalent logD'(80) was developed in two ways: with an assumed dependency and independency of *z*-value on T (see section 2.2.2.3 for details). The parameters of the lognormal distributions for logD(T) and the corresponding normal distributions for D(T) are summarised in Table 2.13. It is apparent from Figure 2.14, that logD(T) values measured at higher T (above ~80°C) have a different behaviour pattern compared with logD(T) values measured at lower T. The same pattern was observed, when a conversion of logD(T) into logD'(80) included the logD(T) values in the higher T range (Table 2.13). When *D*-values measured at higher temperatures (above ~80°C) were included in the calculation of D'(80), the uncertainty associated with D'(80) was greater.

When a transformation of logD(T) into logD'(80) was conducted in two different ways, $\sigma_{logD'(80)}$ was slightly greater when *z*-value was assumed to be independent of T. This is associated with the fact that the transformation includes greater variability associated with the distribution of p(z) and probability distribution of logD(T). Similar observations apply to parameters of the normal distribution.

D'(80) (min) assumed *z* dependent on T										
T range (°C)	**50-79**	**50-80**	**50-81**	**50-82**	**50-83**	**50-85**	**50-88**	**50-90**	**50-92**	**50-93**
n	288	439	444	501	505	532	534	542	550	551
<D'(80)>	1.36	1.67	1.71	1.79	1.87	2.16	2.27	2.32	2.43	2.56
$\sigma_{D'(80)}$	1.57	2.08	2.13	2.31	2.51	3.22	3.50	3.59	3.90	4.25
<logD'(80)>	-0.05	0.02	0.03	0.04	0.05	0.08	0.09	0.10	0.11	0.12
$\sigma_{logD'(80)}$	0.40	0.42	0.42	0.43	0.44	0.47	0.48	0.48	0.49	0.50
D'(80) (min) assumed *z* independent on T										
<D'(80)>	1.61	1.97	1.97	2.12	2.23	2.82	2.82	3.15	3.52	3.52
$\sigma_{D'(80)}$	3.09	3.64	3.64	4.07	4.44	6.52	6.52	7.84	9.45	9.45
<logD'(80)>	-0.13	-0.03	-0.03	-0.01	0.00	0.05	0.05	0.07	0.09	0.09
$\sigma_{logD'(80)}$	0.54	0.53	0.53	0.54	0.55	0.59	0.59	0.61	0.63	0.63

Table 2.13 The parameters for the normal distribution of logD′(80) (min) for spores of non-proteolytic *C. botulinum* at different temperature ranges (-LYS)

A simple visualisation (Figure 2.17) shows that although the mean values for logD'(80) are very close, there is a small shift to higher values, and the range of the box and the whiskers are wider, suggesting increasing uncertainty in p(log(D'(80)) as *D*-values recorded at higher heating T are included.

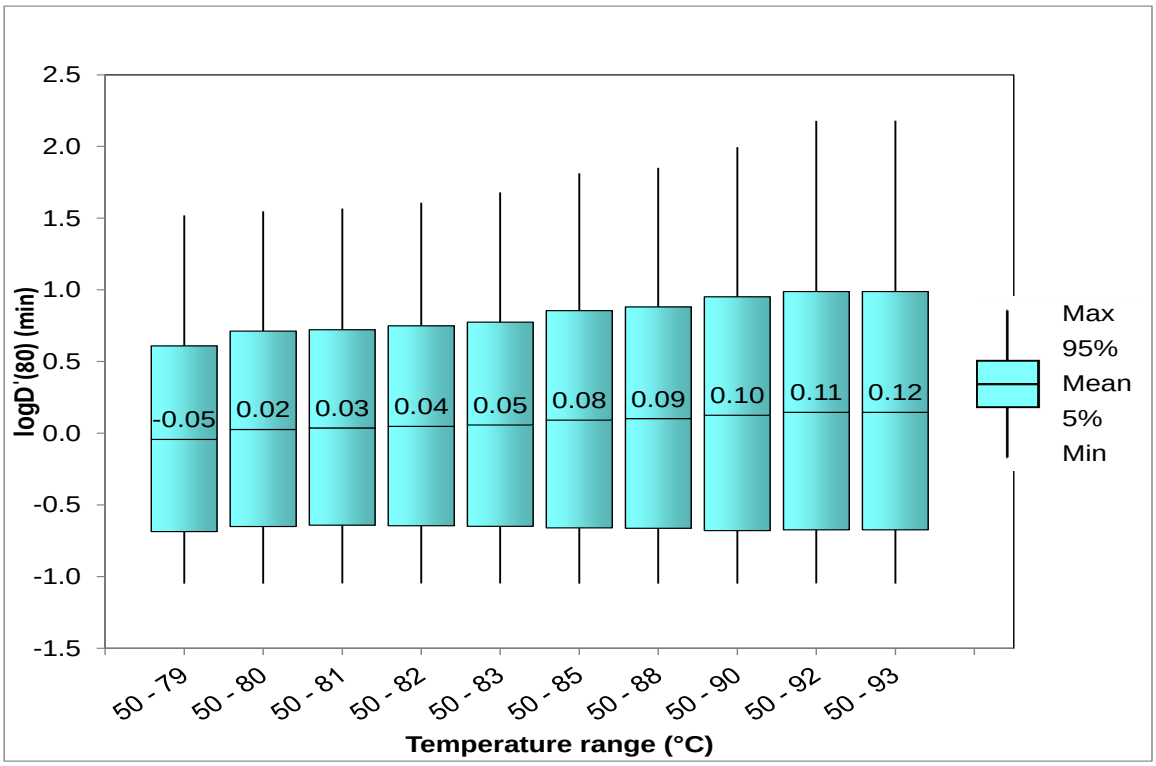

Figure 2.17 A box plot for p(log(D'(80)) for spores of non-proteolytic *C. botulinum* converted from log(D(T)) at different heating temperature ranges (*z*-value assumed to be dependent on T) (-LYS)
The upper and lower border of the box is the value of the 95th and 5th percentiles. The vertical 'whiskers' represent the min and max values. The mean estimate for each temperature range is represented by the horizontal line within the box

Anderson-Darling test

The Anderson-Darling test was conducted with logD'(80) for data collected for ten different temperature ranges (see section 2.2.2.4). The Anderson-Darling parameter, A^2, was calculated for the different ranges of temperatures specified in Table 2.13. The values of A^2 were used to indicate which *D*-values could be considered part of the same population, and if the transformed data were normally distributed.

The calculated A^2 value for each of the different subsets of data ranged from 1.79 to 5.94 (Table 2.14). The variability of calculated A^2 for the subsets of temperature range indicates that a change in the distribution of *D*-value is detected when data from experiments conducted at higher temperatures were included.

A^2	Temperature range (°C)									
	50-79	**50-80**	**50-81**	**50-82**	**50-83**	**50-85**	**50-88**	**50-90**	**50-92**	**50-93**
	1.79	1.44	1.41	1.98	2.18	4.06	4.28	4.91	5.39	5.94

Table 2.14 Calculated values of Anderson-Darling for p(logD'(80)) for spores of non-proteolytic *C. botulinum* at different temperature ranges (-LYS)

The critical value of 2.492 for a 5% level of significance, as given in Table 2.5, is less than calculated values (4.06, 4.28, 4.91, 5.39 and 5.94) for logD'(80) converted from data including temperatures above 83°C. Therefore, the Anderson-Darling test rejects the hypothesis that the *D*-values converted from T > 83°C follow the normal distribution.

Since the critical value is greater than $A^2 = 2.18$, for logD'(80) in the range of 50 – 83°C, the Anderson-Darling test supports the hypothesis that *D*-values are sampled from a normal distribution. Therefore, it is accepted that data (n = 505) with estimated parameters <logD'(80)> = 0.05 min and $\sigma_{logD'(80)}$ = 0.44 min (see Table 2.13) corresponding to *D*-values in the range of 50 – 83°C are normally distributed.

Testing the significance of temperature and *z*-value dependence

The two-sample *t*-test showed that there was no significant difference between the normal distribution for logD'(80) with parameters <logD'(80)> = 0.05 min and $\sigma_{logD'(80)}$ = 0.44 min, and logD'(80) distributed normally with parameters <logD'(80)> = 0.00 min and $\sigma_{logD'(80)}$ = 0.55 min (*t*-statistic = -1.56, degree of freedom: 957, *p* = 0.12) (Figure 2.18). This shows that using either a single *z*-value corresponding to actual measurement of *D*-value at the heating T, or a distribution of *z*-values does not have a significant effect on the parameters of p(logD'(80)). This result confirms the validity of using the p(z) in risk assessment.

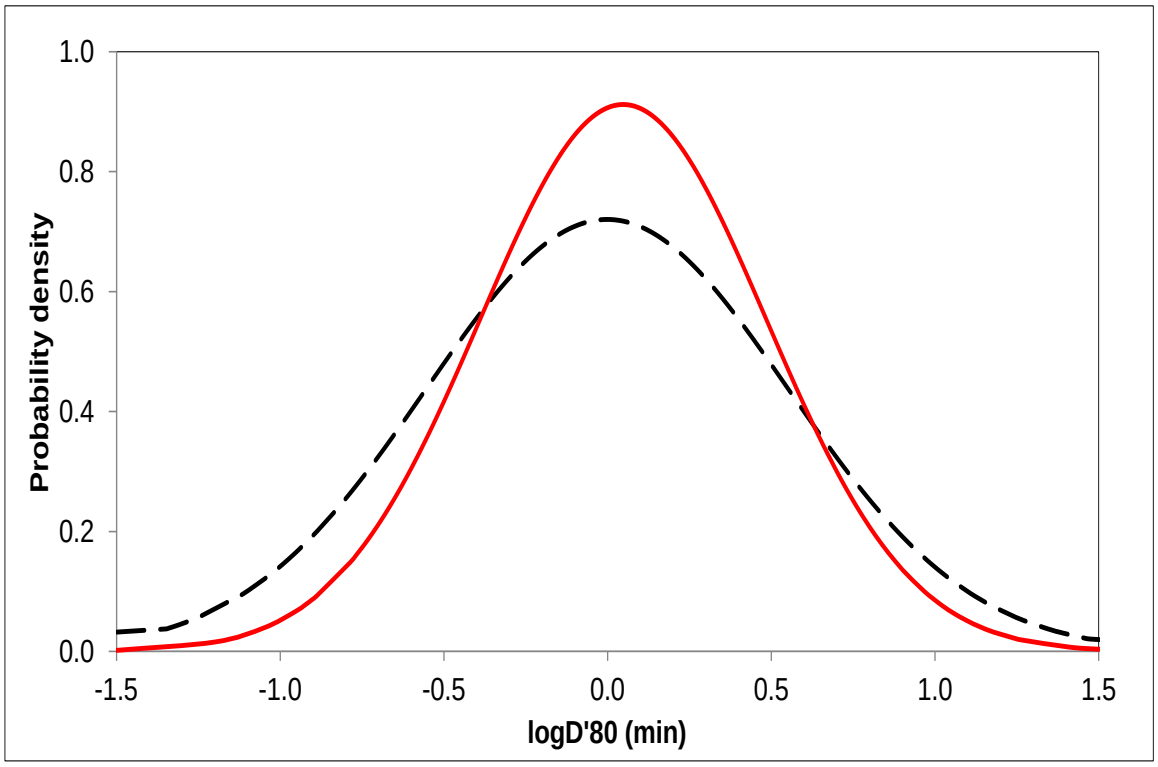

Figure 2.18 Belief concerning logD'(80) (min) value for spores of non-proteolytic *C. botulinum* obtained using two approaches: *z*-value is dependent on and independent of temperature

Assuming independency *z*-value of T (the distribution of p(z) was used in transformation of logD(T) into logD'(80)) (black line), assuming dependency *z*-value of T (*z*-values or mean of reported *z*-values were used in transformation of logD(T) into logD'(80) (red line)

The uncertainty distribution for logD'(80) converted with the p(z) has a greater standard deviation than logD'D(80) converted using reported *z*-value (or mean of *z*-values) (Figure 2.18). Clearly, including uncertainty in the *z*-value represents a conservative factor in the evaluation of the heat treatment of spores. Therefore, the normal distribution for logD'(80) with parameters <logD'(80)> = 0.00 min and

$\sigma_{logD'(80)}$ = 0.55 min and corresponding parameters for a lognormal distribution equal to <D'(80)> = 2.23 and $\sigma_{D'(80)}$ = 4.44 min will be used in a model to represent the uncertainty in *D*-value in QMRA (Chapter 5).

Testing the validity of temperature transformation

A one-way ANOVA was used to determine whether the conversion of log*D*-values to logD'(80) is consistent across all heating temperatures. This analysis revealed that the logD'(80) transformation is not consistent across all tested temperatures (*F*-statistic = 3.48, between groups degree of freedom: 5 and within groups: 884, $p < 0.01$). The Tukey test indicated that only D'(80) measured at 70°C and 75°C are not consistent.

D'(80) converted from T	*n*	logD'(80) (min)			
		Min	Max	<D'(80)>	$\sigma_{D'(80)}$
D'(80) (70°C)	61	-0.79	0.70	-0.04	0.33
D'(80) (75°C)	66	-0.95	1.59	-0.05	0.51
D'(80) (77°C)	50	-0.89	1.25	-0.01	0.45
D(80)	151	-0.70	1.41	0.16	0.41
D'(80) (82°C)	57	-0.74	1.74	0.12	0.52
D'(80) (50-83°C)	505	-1.05	1.74	0.05	0.44

Table 2.15 Summary of transformed *D*-values (min) for spores of non-proteolytic *C. botulinum* measured at different heating temperatures

Most importantly there is no significant difference (Tukey test) in *D*-values originating from experiments measuring spore heat resistance at 80°C (<logD(80)> = 0.16 min, $\sigma_{logD(80)}$ = 0.41 min) and *D*-values transformed from temperatures in a range of 50°C and 83°C (<logD'(80)> = 0.05 min, $\sigma_{logD'(80)}$ = 0.44 min). Also no significant difference was observed in the distribution of D'(80) transformed from T in a range of 50 – 83°C and D'(80) converted from 70°C (<logD'(80)> = -0.04, $\sigma_{logD'(80)}$ = 0.33), 75°C (<logD'(80)> = -0.05, $\sigma_{logD'(80)}$ = 0.51), 77°C (<logD'(80)> = -0.01, $\sigma_{logD'(80)}$ = 0.45) and 82°C (<logD'(80)> = 0.12, $\sigma_{logD'(80)}$ = 0.52) (Table 2.15). However, the mean for D(80) is significantly higher than that for D'(80) converted from 70°C and for D'(80) converted from 75°C.

As shown above, with the exception of heating at 70°C and 75°C, the temperature transformation of *D*-values appears to be consistent, thus it can concluded that the normal distribution of logD'(80) with parameters (<logD'(80)>

= 0.05, $\sigma_{logD'(80)}$ = 0.44) and the corresponding lognormal distribution with parameters (<D'(80)> = 1.87, $\sigma_{D'(80)}$ = 2.51) is a good representation of the data.

Consequently, as indicated in the previous section, both distributions (<logD'(80)> = 0.00, $\sigma_{logD'(80)}$ = 0.55 and <logD'(80)> = 0.05, $\sigma_{logD'(80)}$ = 0.44) and its corresponding lognormal distribution (<D'(80)> = 1.87, $\sigma_{D'(80)}$ = 2.51 and <D'(80)> = 2.23, $\sigma_{D'(80)}$ = 4.44) can be used to present the uncertainty associated with *D*-values of non-proteolytic *C. botulinum* spores, for example in risk assessment. Figure 2.19 is a visual representation of all tested distributions with parameters given in Table 2.15.

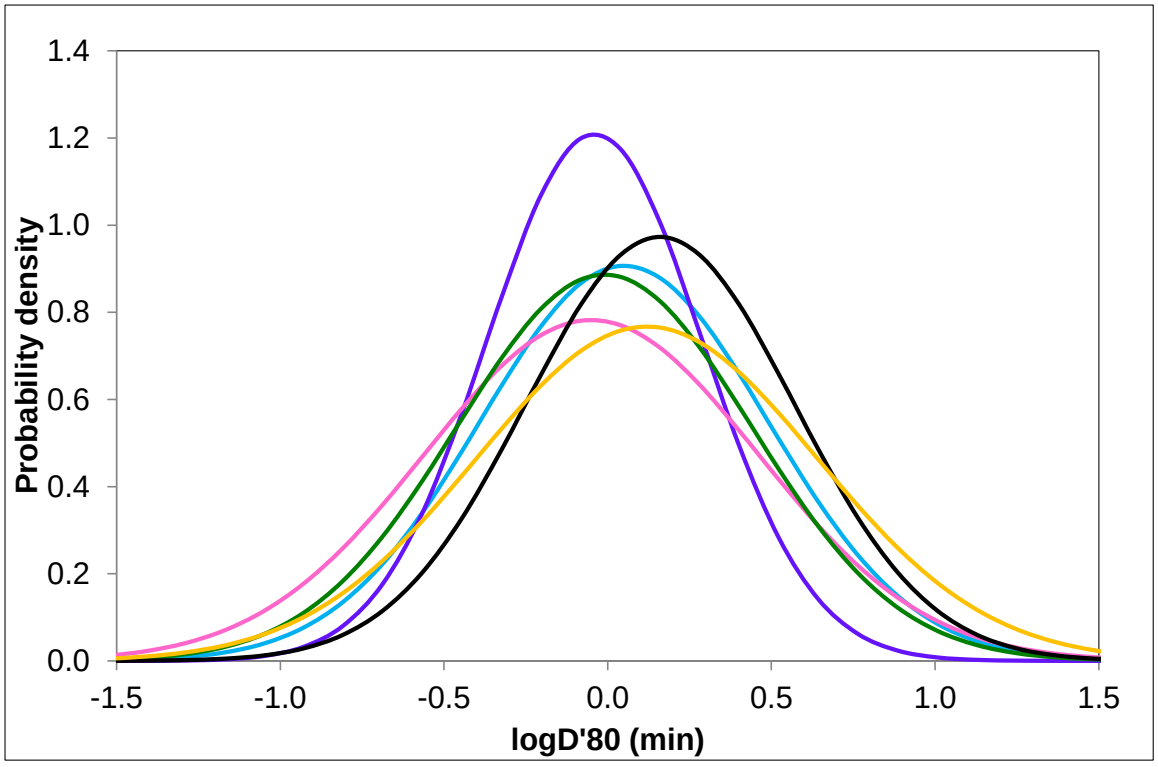

Figure 2.19 Belief concerning the distribution of logD'(80) converted from different heating temperatures for spores of non-proteolytic *C. botulinum*
p(D'(80)) (70°C) (violet line), p(D'(80)) (75°C) (pink line), p(D'(80)) (77°C) (green line), p(D(80)) (black line), p(D'(80)) (82°C) (yellow line) and p(D'(80)) (50 – 83°C) (blue line)

Testing the dependence of spore heat resistance on toxin type

Ever since the publication of data by Scott and Bernard (1982), which indicated higher spore heat resistance of type B strains than type E strains, there has been a debate whether the heat resistance properties of *C. botulinum* spores are associated with the type of toxin formed by the organism. Statistical analysis of the data collected in this thesis shows that there is no significant difference in

heat resistance of type B, E and F strains based on *D*-values measured at three different heating temperatures (75°C, 80°C, 82°C), and D'(80) converted from all data collected at 50 – 83°C. Only in one case did a one-way ANOVA of *D*-values reveal a significant difference in heat resistance for different toxin types (*F*-statistic = 5.64, between groups degree of freedom: 3 and within groups: 147, $p < 0.01$), where the mean *D*-value measured at 80°C for mixed strains appeared to be significantly lower than the mean *D*-value for type B strains (Table 2.16). Figure 2.20 is a visualisation of normal distributions for logD for different toxin types, with parameters given in Table 2.16 for three heating temperatures and logD'(80) transformed from 50 – 83°C.

Toxin type	B		E		F		Mixed	
T (°C)/min	<logD>	σ_{logD}	<logD>	σ_{logD}	<logD>	σ_{logD}	<logD>	σ_{logD}
75	0.71	0.50	0.68	0.58				
80	0.32	0.34	0.18	0.38	0.21	0.45	-0.03	0.45
82	0.20	0.85	-0.28	0.51	-0.27	0.29		
50 – 83	0.15	0.50	0.17	0.43	0.08	0.30	0.00	0.50

Table 2.16 Summary of *D*-values (min) for type B, E, F and mixed strains of non-proteolytic *C. botulinum* spores at different temperatures
An empty cell indicate that there were no reported data

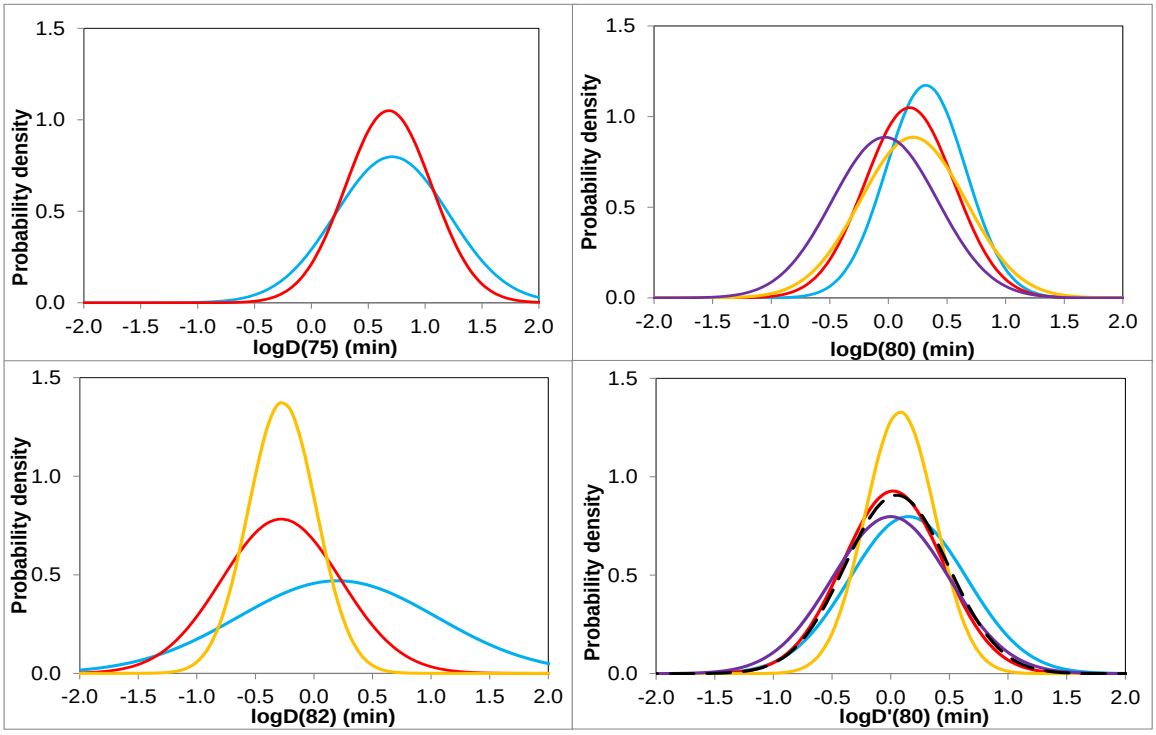

Figure 2.20 Belief concerning the logD value (min) for different toxin types of non-proteolytic *C. botulinum* spores measured at 75°C, 80°C, 82°C, and logD'(80) measured at 50 – 83°C
Toxin type B (blue line), toxin type E (red line), toxin type F (yellow line), mixed strains (violet line), all strains (black dashed line)

Testing the dependence of spore heat resistance on heating menstruum

In total 505 data points, from 48 eligible studies corresponding to 39 different heating menstruum were collected, with 261 *D*-values determined in culture media or buffer and 244 *D*-values in food matrices.

The output of the linear regression analysis for all *D*-values (Table 2.17) indicates that the model is a good fit to data ($p < 0.01$), i.e. there is a strong correlation between heating temperature and measured *D*-value.

Source of variation	***df***	***Sum of squares***	***F***	***p***
Model	1	235	1279	$<<10^{-10}$
Residual error	503	92		
Total error	504	327		
Variable	***Coefficients***	***Standard error***	***t***	***p***
α	10.51	0.28	37.67	$<<10^{-10}$
β = 1/z	0.13	0.00	-35.77	$<<10^{-10}$

Table 2.17 Analysis of variance and parameter estimates for linear regression of *D*-values (min) for non-proteolytic *C. botulinum* spores (*n* = 505) fitted to equation 2.23
df – degree of freedom, *F* – ratio of the between groups variance and the within variance
p – probability that the result observed in a study could have occurred by a chance

The validity of the model was checked by a graphical observation of the residual error distribution (data not presented). The normality of residual error can be assumed. The data and line of best fit with its 95% UCL and LCL, and predicted values (with its 95% and 99% upper limits) are plotted on Figure 2.21.

The best correlation between heating temperature and measured *D*-value was observed for food matrices, with an R^2 (% of variation of the data explained by the fitted line) of 0.76. Good correlation was also observed for all menstrua (R^2 = 0.72), whereas the lowest correlation for media/buffer (R^2 = 0.61) (Table 2.18).

Menstruum	**Parameters of lines of best fit (50 – 83°C)**				**Mean *z*-value from published data**	
	Equation for fitted line	R^2	***z*-value**	**D(80)**		
All menstrua	LogD=10.506-0.1299T	0.72	7.7	1.29	6.7	(S.D.=1.54, *n* = 340)
Media/buffer	LogD=10.680-0.1323T	0.61	7.6	1.24	6.9	(S.D.=1.77, *n* = 144)
Food matrices	LogD=10.407-0.1285T	0.76	7.8	1.35	6.6	(S.D.=1.32, *n* =196)

Table 2.18 Summary of published data on the effect of different heating menstrua on the heat resistance of non-proteolytic *C. botulinum* spores
S.D. – standard deviation

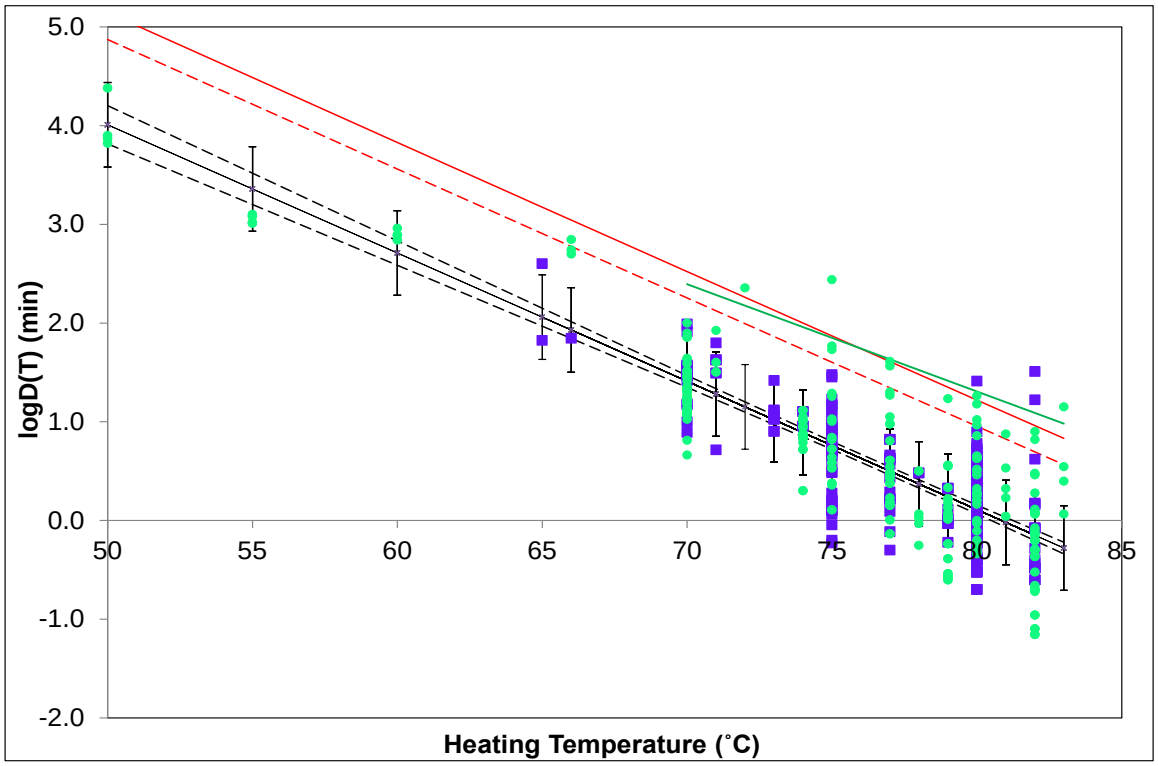

Figure 2.21 Summary of published heat resistance data for non-proteolytic *C. botulinum* spores heated in all menstrua
Food matrices (green circles), media/buffer (blue squares), line of best fit (black solid line) (see Table 2.17 for parameters of this line), 95% UCL and LCL of the mean (black dashed line), 95% UCL of predicted response (red dashed line), 99% UCL (red dashed line) and current recommendation of heat treatment for chilled food (ACMSF, 90°C/10min, z = 9.2°C) (green solid line)

From the line of best fit for all menstrua D(80) is 1.29 min (16.60 min when using 99% upper limit of the model), which is in agreement with reported *D*-values from the literature review measured at 80°C (0.20 – 25.84 min).

The mean reported *z*-value for all menstrua was 6.7°C, with a standard deviation of 1.54°C (Table 2.18), and it is very close to the mean *z*-value reported for media/buffer (z = 6.9°C) and food matrices (z = 6.6°C). Slightly higher *z*-values were obtained from estimated parameter of the line of best fit, with a *z*-value of 7.7°C for all menstrua.

Current recommendation of the FSA, ACMSF and CFA/ECFF for the heat treatment of minimally heated chilled foods includes heating for 10 min at 90°C or equivalent (Table 2.2). This treatment is considered to provide a 6D reduction of non-proteolytic *C. botulinum* spores concentration. The line derived from the current ACMSF recommendation (90°C/10 min, z = 9.2°C) plotted on Figure 2.21

gives higher logD values than the 99% upper limit given by the thermal death model. However, the ACMSF recommendation was based on relatively little data (Gaze and Brown, 1990).

From Figure 2.21 it is apparent that almost all of the reported *D*-values lie below the 99% UCL prediction line. There are six *D*-values from five different studies that are higher than those predicted by the thermal death model and the current recommendation. Two *D*-values are from Scott and Bernard (1982) who reported D(82) of 32.30 min and D(83) = 16.70 min. Other points that fall above this line corresponds to tuna (in oil) with D(72) = 226.50 min (Bohrer *et al.*, 1973) and D(83) = 14.16 min for raw egg white calculated from data given by Alderman *et al.* (1972). Although the latter authors (Alderman *et al.*, 1972) reported coagulation of the egg white, the carryover of trace amount of active hen egg white lysozyme to the recovery medium (TPGY broth) cannot be excluded. Further *D*-values that fall above the line are: D(80) = 25.84 min calculated from data given by Murrell and Scott (1966) (the heating menstruum and incubation time were not given) and D(75) = 274.76 min calculated from studies of Fernandez and Peck (1997).

Four out of six of the above *D*-values were obtained when incubation of heated test tubes was carried out for prolonged time e.g. Scott and Bernard (1982) – 30°C/168 days, Bohrer *et al.* (1973) – 29°C/168 – 336 days, Fernandez and Peck (1997) – 25°C/90 days. It was observed by many authors e.g. by Lynt *et al.* (1983) that an extended period of incubation permits germination of damaged spores, thereby increasing measured spore heat resistance.

From the analysis of collected data the highest average *D*-value was noted for raw egg white with *D* = 32.61 min (*n* = 1) and the lowest for 0.05M phosphate buffer, 0.34 min (*n* = 2). Bohrer *et al.* (1973) reported that the heat resistance of spores depends on heating menstrum. The *D*-values reported by authors for spores of type E strains were greater for foods high in fat and protein content than for phosphate buffer. However, a *t*-test analysis of data summarized in this thesis (*n* = 505) for 39 different heating menstrua (Table 2.19) has shown that there is no significant difference in the heat resistance of spores measured in

media/buffer (n = 13) (<logD'(80) = 0.05, $\sigma_{logD'(80)}$ = 0.40) and food matrices (n = 27) (<logD'(80) = 0.05, $\sigma_{logD'(80)}$ = 0.48); (t-statistic = -0.02, degree of freedom: 472, p = 0.98).

Heating menstruum	*n*	logD'(80) (min)			
		Min	Max	<D'(80)>	$\sigma_{D'80}$
0.017M phosphate buffer	13	-0.03	0.18	0.05	0.08
0.03M phosphate buffer	4	-0.55	-0.14	-0.32	0.17
0.05M phosphate buffer	2	-0.50	-0.45	-0.47	0.04
0.067M phosphate buffer	133	-1.05	1.74	0.05	0.38
0.1M phosphate buffer	12	-0.41	0.59	0.14	0.38
autoclaved chub fish	2	0.76	0.91	0.83	0.10
béchamel sauce	1	0.86	0.86	0.86	
blue crabmeat	27	-0.09	0.80	0.10	0.17
bolognaise sauce	1	0.97	0.97	0.97	
broccoli puree	10	-0.47	0.16	-0.19	0.24
carrot homogenates	5	0.50	0.78	0.66	0.11
clam liquor	9	-0.27	1.25	0.14	0.60
cod	4	1.15	1.26	1.19	0.05
corn brine	4	0.41	0.56	0.49	0.06
crabmeat	25	-0.23	0.64	0.23	0.27
distilled water	2	-0.31	0.07	-0.12	0.27
fine carrot	2	0.20	0.26	0.23	0.04
haddock slurry	4	-0.30	-0.10	-0.20	0.11
meat medium	2	0.63	1.59	1.11	0.68
menhaden Surimi	4	0.21	0.32	0.28	0.05
milk (evaporated)	4	0.22	0.33	0.27	0.05
oyster homogenates	72	-0.74	0.70	-0.26	0.38
peas	2	0.52	0.59	0.55	0.05
physiological saline	4	0.58	0.60	0.59	0.02
potato puree	9	-0.53	0.09	-0.24	0.21
pre-coagulated egg white	1	0.43	0.43	0.43	
PY broth	40	-0.95	0.78	0.21	0.45
raw egg white	1	1.51	1.51	1.51	
salmon	1	0.46	0.46	0.46	
sardines (in tomato sauce)	4	0.65	0.82	0.75	0.08
shrimp	1	0.28	0.28	0.28	
tomato homogenates	37	-0.79	0.19	-0.21	0.20
TPB broth	1	0.26	0.26	0.26	
TPG or TYD	38	-0.70	0.71	-0.16	0.32
TPGY broth	1	0.49	0.49	0.49	
tuna	1	0.01	0.01	0.01	
tuna (in oil)	6	1.02	1.18	1.08	0.06
water	9	-0.48	0.10	-0.13	0.16
whitefish chubs	5	0.20	0.63	0.32	0.18
not given	2	0.18	1.41	0.80	0.87

Table 2.19 Summary of *D*-values (min) for spores of non-proteolytic *C. botulinum* measured in different heating menstrua

Testing the dependence of spore heat resistance on heating technique

For decades researchers have debated whether heating technique influences measured spore heat resistance. In order to test if heating technique has an

impact on magnitude of measured *D*-value a one-way ANOVA with logD'(80) as the dependent variable and heating techniques as the independent variable was conducted.

Heating technique	*n*	logD'(80) (min)			
		Min	Max	<D'(80)>	$\sigma_{D'80}$
bottles in water bath	46	-0.79	1.26	0.01	0.49
flasks in water bath	20	-0.26	0.70	0.17	0.32
sealed ampules in water bath	4	0.58	0.60	0.59	0.02
sealed capillaries in oil bath	16	-1.05	1.25	-0.15	0.61
sealed capillaries in water bath	7	-0.22	0.10	-0.07	0.10
TDT cans in water bath	25	0.22	1.18	0.65	0.30
TDT tubes in water bath	231	-0.74	1.74	0.00	0.38
unsealed TDT tubes in water bath	3	-0.35	0.43	-0.06	0.43
screw-cap vials in water bath	12	-0.41	0.59	0.14	0.38
screw-cap tube in water bath	98	-0.95	1.59	0.09	0.48
method not given by author	43	-0.70	0.71	-0.15	0.30

Table 2.20 Summary of *D*-values (min) for spores of non-proteolytic *C. botulinum* measured using different heating techniques

Levene's *F*-test revealed that the homogeneity of variance assumption was not met ($p < 0.01$), in this case Welch's *F*-test was used. The analysis shows that *D*-values differ depending on heating technique applied (*F*-statistic = 105.64, between groups degree of freedom: 10 and within groups: 494, $p < 0.01$). From the Games-Howell test and from Table 2.20 it is apparent that *D*-values, where sealed ampules and TDT cans in water bath were used as heating techniques have mean values of logD'(80) greater than remaining heating techniques.

Testing the dependence of spore heat resistance on method of *D*-value determination

Several authors have suggested that the method of *D*-value determination and calculation is an important factor influencing measured spore heat resistance. For example Chai and Liang (1992) have suggested that the *D*-value measured according to the TDT method can be higher than that measured using survival curves. Within 48 eligible studies, five main methods were employed in the measurement of the *D*-value (see section 2.1.2). The majority of *D*-values were determined or calculated according to the survivor curve method, and for 23

reported data points information about the method of *D*-value determination was not given (Table 2.21).

Method of *D*-value measurement	*n*	logD'(80) (min)			
		Min	Max	<D'(80)>	$\sigma_{D'(80)}$
Survivor curve	251	-1.05	1.59	0.01	0.45
TDT	23	-0.03	1.51	0.51	0.41
TDT/Stumbo(1948)	53	-0.06	1.18	0.36	0.35
TDT/Stumbo(1950)	13	-0.55	1.74	0.28	0.71
TDT/Schmidt(1957)	142	-0.74	0.54	-0.11	0.33
Method not given by author	23	-0.10	0.29	0.13	0.09

Table 2.21 Summary of *D*-values (min) for spores of non-proteolytic *C. botulinum* according to method of measurement

A one-way ANOVA was used to test the differences in *D*-value determined or calculated according to different methods. Since Levene's statistic shows that the equal variance assumption was violated ($p < 0.01$) Welch's *F*-test was applied, which indicated that the mean *D*-values differed significantly across all measurement methods (*F*-statistic = 18.14, between groups degree of freedom: 5 and within groups: 499, $p < 0.01$).

The Games-Howell statistic indicated that the *D*-values calculated by TDT and TDT/Stumbo(1948) are not significantly different, $p = 0.82$, the same result was observed for TDT/Stumbo(1950) and TDT/Schmidt(1957). Similarly *D*-values obtained from survivor curves are in agreement with *D*-values calculated according to TDT/Stumbo(1950). The mean value of logD'(80) calculated according to TDT and TDT/Stumbo (1948) is significantly greater than the mean of logD'(80) obtained from survivor curves. But the logD'(80) calculated according to TDT/Schmidt(1957) is significantly smaller than the one obtained from survivor curves.

Although the method of *D*-value measurement has a significant effect on its magnitude, this is not substantial from a risk assessment point of view. The distribution parameters for D'(80) allows for inclusion of uncertainty associated with experimental procedures.

Testing the dependence of spore heat resistance on strain of non-proteolytic *C. botulinum*

Spore heat resistance of non-proteolytic *C. botulinum* was measured for 35 strains of different toxin types. Table 2.22 summarizes strains for which the *D*-value was measured at least three times. The most common strains tested were Eklund 17B (*n* = 70) and Beluga (*n* = 52). The highest average D'(80) was noted for strain Nanaimo (3.10 min), whereas the lowest was for strain Crab 25V-2 (0.26 min).

Strains*	Toxin type	logD'(80) (min)					logD(80) (min)		
		n	Min	Max	<D'(80)>	$\sigma_{D'(80)}$	*n*	<D(80)>	$\sigma_{D(80)}$
CBW25	B	4	-0.24	0.44	0.12	0.30	2	0.13	0.21
Eklund 17B	B	70	-0.95	1.56	0.18	0.49	33	0.35	0.36
Kap B2	B	10	-0.53	0.41	-0.21	0.29	3	0.04	0.37
Kap B5	B	3	0.18	0.42	0.32	0.12	3	0.32	0.12
1304E	E	13	-0.34	0.27	0.09	0.20	3	0.22	0.05
8E	E	14	-1.05	0.26	-0.31	0.39	2	0.08	0.25
Alaska	E	39	-0.72	1.51	0.01	0.45	7	0.28	0.30
ATCC 17786	E	37	-0.79	0.19	-0.21	0.20			
ATCC 9564	E	11	-0.77	1.41	0.40	0.73	5	0.60	0.76
Beluga	E	52	-0.51	0.70	0.10	0.25	12	-0.04	0.19
Crab 25 V-1	E	11	-0.45	0.11	-0.27	0.16			
Crab 25 V-2	E	11	-0.74	0.01	-0.64	0.22			
Crab G21-5	E	27	-0.72	0.80	-0.20	0.37			
Detroit	E	6	-0.27	1.25	0.34	0.70			
Iwanai	E	3	-0.35	0.20	-0.05	0.28	2	0.10	0.14
Minneapolis	E	5	-0.28	0.36	0.01	0.29	3	0.19	0.22
Minnesota	E	12	-0.14	0.76	0.02	0.24			
Mixed strains E	E	4	0.21	0.32	0.28	0.05			
Nanaimo	E	3	0.47	0.52	0.49	0.03	1	0.52	
Saratoga	E	47	-0.23	1.18	0.42	0.35	15	0.40	0.28
Vancouver Herring	E	4	-0.40	0.23	-0.18	0.29	1	-0.40	
Whitefish	E	3	-0.41	-0.08	-0.30	0.19	2	-0.41	0.00
190	F	14	-0.39	-0.07	-0.19	0.11			
Craig 610	F	11	-0.58	0.42	0.16	0.28	1	-0.12	
Eklund 202F	F	28	-0.23	0.60	0.15	0.27	2	0.19	0.59
Mixed strains		45	-0.70	1.59	0.00	0.50	44	-0.03	0.45
Not specified		3	0.01	0.18	0.09	0.09	1	0.18	

Table 2.22 Summary of *D*-values (min) for spores of different strains of non-proteolytic *C. botulinum* measured at heating temperature of 80°C and transformed to D'(80) from 50 – 83°C

*table presents only strains for which *D*-values were noted at least three times, the remaining 11 strains (for which *D*-value was measured once or twice) were not presented

For strains for which *D*-values were determined at least 20 times (Eklund 17B, Alaska, ATCC 17786, Beluga, Crab G21-5, Saratoga and Eklund 202F) a one-

way ANOVA was conducted to test for evidence of a significant difference in spore heat resistance of *D*-value between strains. The homogeneity of variance assumption was violated ($p < 0.01$ in Levene's test), therefore the Welch test was used to assess the difference. The analysis revealed that the *D*-values significantly differed between strains (*F*-statistic = 14.02, between groups degree of freedom: 6 and within groups: 293, $p < 0.01$). Significantly more heat resistant compared to other strains appeared to be strain Saratoga ($p < 0.01$). The *D*-values for strains ATCC 17786 and Crab G21-5 are significantly smaller compared to *D*-values recorded for strains Eklund 17B, Beluga, Saratoga and Eklund 202F ($p < 0.01$). No significant difference in *D*-values was observed for strains Eklund 17B, Alaska and Beluga. The *D*-value for strain Alaska is not significantly different to that of all strains (except Saratoga). Interestingly according to statistical analysis the heat resistance of spores of Eklund 17B and Eklund 202F are exactly the same ($p < 1.0$).

The clustering analysis revealed (results not presented) that there is no observed pattern based on heat resistance properties of tested strains, and it is subjected to many variable factors e.g. recovery media, recovery conditions, heating method and heating conditions.

Evaluating the dependence of spore heat resistance on testing laboratory

All the collected data points ($n = 505$) were obtained from experiments carried out in 23 different laboratories (Table 2.23). In order to compare the heat resistance of spores tested by different laboratories/authors a one-way ANOVA was performed. Data collected from six laboratories with the largest number of reported *D*-values: Campden BRI ($n = 46$), Chai & Liang ($n = 52$), Food and Drug Administration (FDA) ($n = 92$), Institute of Food Reseach (IFR) ($n = 92$), National Canners Association (NCA) ($n = 78$), Unilever ($n = 38$) were used in an analysis with logD'(80) as the dependent variable and laboratory/author as the independent variable (Table 2.23).

The data failed Levene's *F*-test for homogeneity of variance ($p < 0.01$), therefore Welch's *F*-test with a significance level of 1% was used. The results of a one-way ANOVA revealed statistical significance, which indicates that not all

D-values collected by different laboratories/authors are similar (*F*-statistic = 44.62, between groups degree of freedom: 5 and within groups: 392, $p < 0.01$).

Laboratory/author	*n*	logD'(80) (min)			
		Min	Max	<D'(80)>	$\sigma_{D'(80)}$
Alderman *et al.*	4	0.43	1.51	0.90	0.45
Alderton *et al.*	2	-0.31	0.03	-0.14	0.24
Angelotti	4	0.04	0.16	0.10	0.06
Bucknavage *et al.*	20	-0.26	0.70	0.17	0.32
Campden BRI	46	-0.79	1.26	0.01	0.49
Chai & Liang	52	-0.74	0.03	-0.43	0.25
Crisley *et al.*	6	-0.10	0.63	0.25	0.24
Duh & Ren	2	0.20	0.26	0.23	0.04
Eklund *et al.*	4	0.58	0.60	0.59	0.02
FDA	92	-0.39	0.80	0.08	0.22
Graikoski & Kempe	4	-0.35	0.43	-0.12	0.36
Grecz&Tang	1	0.23	0.23	0.23	
IFR	92	-0.95	1.59	0.09	0.49
Kralovic	1	0.18	0.18	0.18	
Licciardello	16	-1.05	1.25	-0.15	0.61
Mann	2	0.01	0.07	0.04	0.04
NCA	78	-0.55	1.18	0.26	0.34
NFPA	12	-0.50	1.74	0.45	0.77
Ohye & Scott	6	-0.40	0.52	0.09	0.45
Roberts & Ingram	9	-0.48	0.10	-0.13	0.16
Schmidt	2	0.26	0.36	0.31	0.08
Unilever	38	-0.70	0.71	-0.16	0.32
USDA	12	-0.41	0.59	0.14	0.38

Table 2.23 Summary of *D*-values (min) for spores of non-proteolytic *C. botulinum* measured in different laboratories

From a *post-hoc* Games-Howell comparison test, the values of D'(80) converted from *D*-values reported by Chai & Liang (1992) are significantly lower than D'(80) from other laboratories/authors. The D'(80) converted from data obtained in IFR are not significantly different from data reported by Campden, FDA and NCA and Unilever. A significant difference was also not observed between data collected by Campden, FDA, NCA, and Unilever. A significant difference in D'(80) was observed between data reported by FDA and NCA ($p = 0.01$) and Unilever ($p = 0.01$). The D'(80) originating from *D*-values reported by NCA are significantly different from those reported by Unilever ($p < 0.01$).

Final comments

Spore heat resistance does not solely depend on a single factor. There are several sources of variability determining heat resistance properties. The clustering technique have failed to demonstrate a clear pattern of resistance of spores in respect of strain or type of neurotoxin formed. The most likely explanation for the failure to demonstrate such a pattern is the considerable variation in experimental approaches used by different authors. The effect of a number of these factors has been shown to be significant. The generation of a high quality dataset on the heat resistance of individual strains of non-proteolytic *C. botulinum* requires the use of a consistent and rigorous experimental approach and the testing of a number of strains (data for 35 strains are reported in this review) in triplicate. This is a substantial undertaking, requiring the preparation and testing of a substantial number of spore crops. Considering the time required this is unlikely to be carried out.

2.3.2.3 Heat resistance of spores of non-proteolytic *C. botulinum* with recovery in the presence of lysozyme (+LYS)

Summary of *D*-values

Data on spore heat resistance with recovery in the presence of lysozyme or data originating from experiments where recovery was conducted in seafood (see section 2.2.2), was divided into three subsets: heat sensitive fraction (+LYS HS fraction), heat resistant fraction (+LYS HR fraction) and data where the *D*-value was measured using the TDT method (+LYS TDT) (this also contain studies by Juneja *et al.*). Due to the relatively small number of +LYS *D*-values in the dataset (compared with -LYS dataset), an analysis was not conducted separately for different toxin type.

Heat resistance of spores was measured in different heating menstrua and in the temperature range of 70 – 95°C, with 80°C, 85°C and 90°C being the most frequently tested temperatures. For the +LYS HS subset there were 27 *D*-values, for the +LYS HR subset there were 105 *D*-values, and for +LYS TDT subset there were 125 *D*-values reported, with 16, 46 and 60 *z*-values, respectively. Figure 2.22 is a visualisation of average *D*-values for each subset of data.

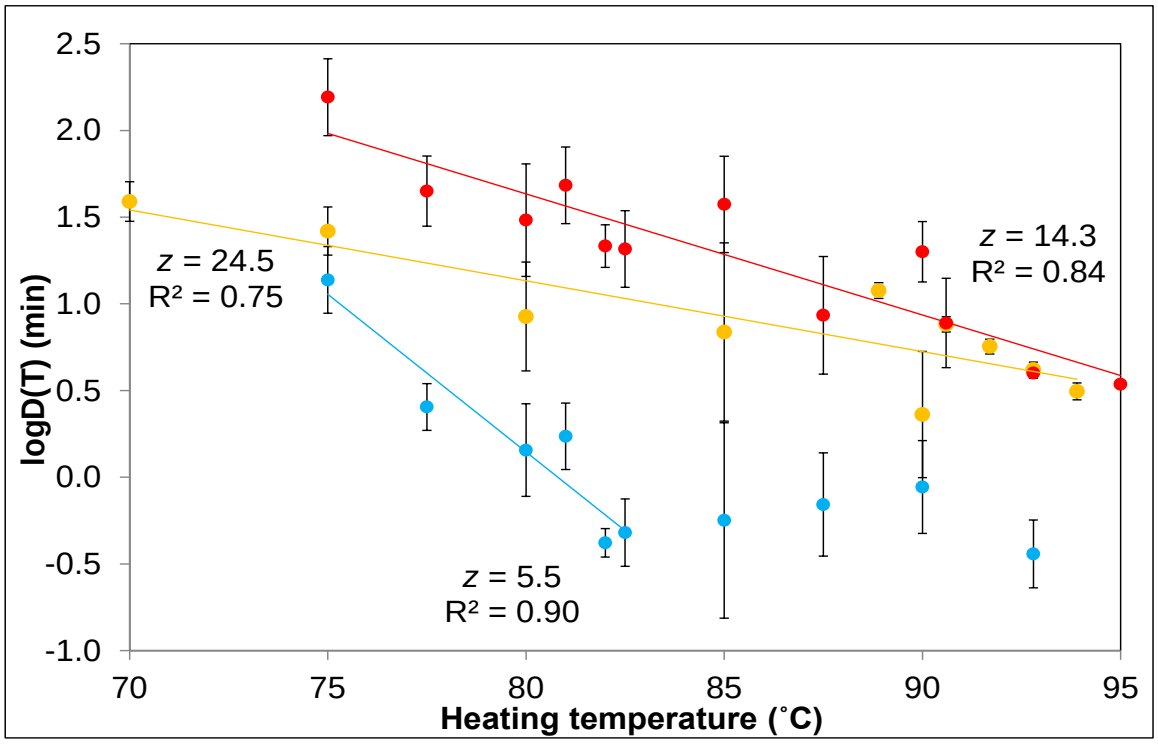

Figure 2.22 Mean and standard deviation of *D*-values (min) for spores of non-proteolytic *C. botulinum* at fixed heating temperatures (+LYS)
+LYS HS (blue circles), +LYS HR (red circles), +LYS TDT (yellow circles)

It is apparent from the Figure 2.22 that *D*-values corresponding with the +LYS HR fraction are much higher than those obtained for the +LYS HS fraction. The heat resistance determined by TDT method gives *D*-values which are between those for the HS and HR fractions. The small error bars for the +LYS TDT subsets at 70°C, 89°C, 92°C and 94°C correspond with only a few data points determined in one laboratory. The largest error bars are observed for +LYS HS at 85°C and represent data obtained by four different authors.

Probability distribution of D(T)

For the three subsets of data (HS, HR and TDT) the parameters of the normal distribution for logD(T) and corresponding lognormal distribution (see section 2.2.2.1) at each heating temperature are included in Table 2.24. The *D*-values recorded for the HR fraction are substantially higher (<D> = 186 min at T = 75°C) than those for the HS fraction (<D> = 16.73 min at T = 75°C). The lowest average *D*-value for the HS fraction corresponded to <D> = 0.43 min at T = 82°C and for the HR fraction to <D> = 3.45 min at 85°C. The lowest average *D*-value for the TDT subset of data was calculated when spores were heated at 94°C with <D> = 3.18 min and the highest when heated at 70°C, with <D> = 40.69 min.

Subset	HS				HR				TDT			
T (°C)	$<logD(T)>$	$\sigma_{logD(T)}$	$<D(T)>$	$\sigma_{D(T)}$	$<logD(T)>$	$\sigma_{logD(T)}$	$<D(T)>$	$\sigma_{D(T)}$	$<logD(T)>$	$\sigma_{logD(T)}$	$<D(T)>$	$\sigma_{D(T)}$
70									1.59	0.13	40.69	12.46
75	1.12	0.30	16.73	13.09	2.18	0.28	186	133	1.41	0.20	28.58	13.89
78	0.41	0.14	2.71	0.90	1.65	0.21	50	25.77				
80	0.16	0.26	1.73	1.14	1.37	0.64	69	194	0.87	0.43	12.10	15.62
81	0.28	0.19	2.10	0.96	1.74	0.22	62	33.79				
82	-0.37	0.08	0.43	0.08	1.34	0.13	22.88	7.01				
83	-0.28	0.19	0.58	0.27	1.37	0.22	26.65	14.42				
85	-0.35	0.57	1.06	2.27	1.50	0.39	3.45	0.90	0.74	0.61	14.74	36.37
88	-0.22	0.53	1.27	2.35	0.95	0.37	12.81	13.23				
89									1.08	0.05	12.10	1.40
90	-0.03	0.21	1.05	0.54	1.29	0.20	21.68	10.54	0.22	0.57	3.93	8.42
91					0.91	0.31	10.49	8.55	0.88	0.04	7.62	0.70
92									0.75	0.04	5.65	0.52
93	-0.40	0.20	0.44	0.22	0.60	0.03	3.99	0.28	0.62	0.04	4.19	0.39
94									0.50	0.05	3.18	0.37
95					0.54	0.13	3.63	1.11				

Table 2.24 Parameters for normal distribution of logD(T) (min) and corresponding lognormal distribution of D(T) for non-proteolytic *C. botulinum* spores for the HS, HR and TDT subsets (+LYS)
empty cells indicate that there were no data collected at particular temperature

Probability distribution of z-values

As described in section 2.2.2.2, distributions of *z*-values, p(z), were obtained for HR and TDT subsets of data. There were too few data for the HS fraction and it was not possible to obtain reliable parameters for the distribution of p(z). There were 46 *z*-values obtained for the HR fraction and 90 *z*-values for TDT subset (Table 2.25).

Subset of data	No. of collected *z*-values	No. of *z*-values taken to build p(z)*
HS fraction	21	-
HR fraction	46	46
TDT data	90	90

Table 2.25 Number of collected *z*-values (°C) from eligible studies and number of *z*-values used to build distribution of p(z) for non-proteolytic *C. botulinum* spores (+LYS)
*see section 2.2.2.4 for explanation on why all *z*-values were not included

Table 2.26 summarises the estimated parameters with the standard error, UCL and LCL that are used to build the beta distribution. The parameters for TDT subset have large S.E., therefore care should be taken when the distribution of p(z) is used. The mean value, <z>, was calculated according to equation 2.16.

Subset	Parameter	Estimate	S.E.	UCL	LCL	Constraint
HR	a	4.0	5.2	16.4	-8.4	a = 4.0
	c	19.6	24.4	77	-38.2	c ≥ 13.0
	α_1	3.1	7.4	20.6	-14.4	$\alpha_2 = 6.0$
	α_2	6.0	25.6	59	-47.3	
	<z>	9.3				
TDT	a	7.5	4.6	17.1	-2.1	a ≥ 7.5
	c	25.0	375	814	-764	22 ≤ c ≤ 25
	α_1	2.9	19.0	42.8	-36.97	$\alpha_1 \geq 1.0$
	α_2	20.2	582	1243	-1202	
	<z>	9.7				

Table 2.26 Parameters of beta distribution for *z*-values (°C) for non-proteolytic *C. botulinum* spores obtained from MS Excel Solver® fit with specified constraints, and calculated UCL and UCL (+LYS)
(a is minimum and c maximum of fitted *z*-value, α_1 and α_2 are continuous shape parameters and <z> is mean *z*-value)

Distributions of *z*-values for TDT dataset and the HR fraction of the dataset, based on the parameters in Table 2.26 are illustrated in Figure 2.23. The calculated value of <z> for the HR fraction is 9.3°C with a 95% confidence

interval [5.4, 14.3] and for TDT dataset is 9.7°C with a 95% confidence interval [7.9, 12.4].

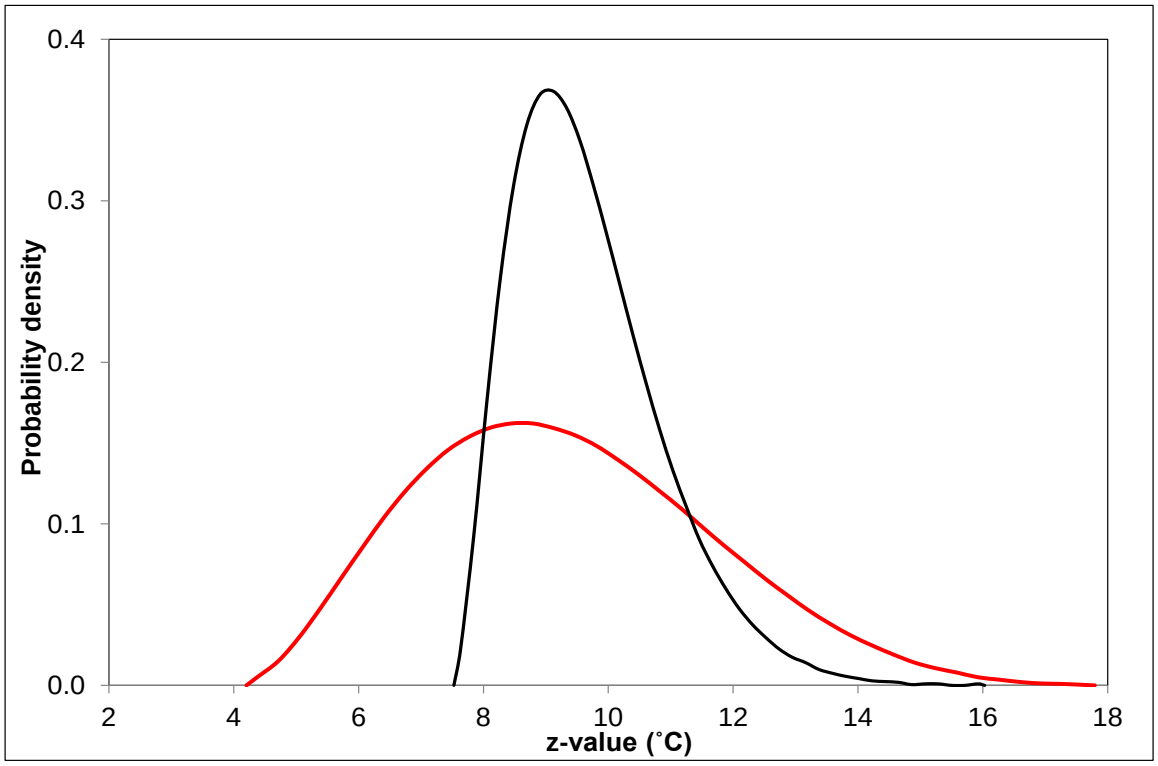

Figure 2.23 Beliefs concerning z-value (°C) for spores of non-proteolytic *C. botulinum* (+LYS)
TDT subset of data (black line), HR fraction (red line)

Probability distribution of D'(80)

The conversion of logD(T) into equivalent logD'(80) was developed in the two ways as used previously for the -LYS data (see section 2.2.2.3). Parameters for log normal and corresponding normal distributions of logD'(80) of HS and HR fractions corresponding to *D*-values measured at different heating temperature ranges are summarised in Table 2.27.

When the transformation of the *D*-value into D'(80) for the HS fraction contains the *D*-values measured at higher heating temperatures there is an increase uncertainty for p(D'(80)) and the mean value of *D* is higher. For the HR fraction added *D*-values originating from higher heating temperatures increase the uncertainty for p(D'(80)), but the mean value of *D* does not increase by the same magnitude.

Since the +LYS TDT subset does not distinguish the HS and HR spore fraction it was not used in further analysis. For the +LYS TDT subset, the distribution of p(D'(80)) was built only for one subset of temperatures, corresponding to all collected *D*-values in the range of 70 – 94°C (*n* = 125). The normal distribution of logD'(80) is parameterized with <logD'(80)> = 1.28 min and $\sigma_{logD'(80)}$ = 0.78 min. The corresponding parameters of lognormal distribution are equal to <D'(80)> = 96 min and $\sigma_{D'(80)}$ = 470 min.

HS fraction								
D'(80) (min) assumed *z* dependent on T								
T range (°C)	**75-80**	**75-81**	**75-82**	**75-83**	**75-85**	**75-88**	**75-90**	**75-93**
n	22	23	26	27	45	48	88	89
<D'(80)>	1.40	1.48	1.42	1.42	2.22	2.42	14.51	14.84
$\sigma_{D'(80)}$	0.68	0.76	0.65	0.65	2.38	2.80	44.44	41.39
<logD'(80)>	0.10	0.12	0.11	0.11	0.18	0.20	0.69	0.70
$\sigma_{logD'(80)}$	0.20	0.21	0.19	0.19	0.38	0.40	0.64	0.64
D'(80) (min) assumed *z* independent of T								
<D'(80)>	1.20	1.33	1.29	1.32	6.43	8.94	387.36	396
$\sigma_{D'(80)}$	0.86	1.04	0.98	0.95	16.01	26.93	8048	8236
<logD'(80)>	-0.01	0.02	0.02	0.03	0.38	0.45	1.27	1.28
$\sigma_{logD'(80)}$	0.28	0.30	0.28	0.28	0.61	0.66	1.07	1.07

HR fraction										
D'(80) (min) assumed *z* dependent on T										
T range (°C)	**75-80**	**75-81**	**75-82**	**75-83**	**75-85**	**75-88**	**75-90**	**75-91**	**75-93**	**75-95**
n	23	24	27	28	50	53	97	99	101	105
<D'(80)>	47.08	48.18	47.16	46.15	106	101	199	203	214	213
$\sigma_{D'(80)}$	60	62	56	53	177	162	330	338	368	354
<logD'(80)>	1.46	1.47	1.48	1.48	1.74	1.73	2.01	2.02	2.03	2.04
$\sigma_{logD'(80)}$	0.43	0.43	0.41	0.40	0.50	0.49	0.50	0.50	0.51	0.50
D'(80) (min) assumed *z* independent of T										
<D'(80)>	36.88	37.74	38.04	38.04	101	101	246	246	240	245
$\sigma_{D'(80)}$	27.65	28.29	27.31	27.31	135	135	440	440	413	423
<logD'(80)>	1.47	1.48	1.49	1.49	1.78	1.78	2.08	2.08	2.08	2.09
$\sigma_{logD'(80)}$	0.29	0.29	0.28	0.28	0.44	0.44	0.52	0.52	0.51	0.51

Table 2.27 The parameters for normal distribution of logD'(80) (min) for spores of non-proteolytic *C. botulinum* at different temperature ranges (+LYS)

Anderson-Darling test

As in the case of -LYS data the Anderson-Darling test was used to determine if all collected *D*-values represent a single population. The Anderson-Darling test was conducted for the HS and HR fractions of spores, according to the method

described in section 2.2.2.4. The only difference lies in the range of temperatures, and therefore the number of subsets and calculated A^2 values. Eight A^2 values were calculated for the HS fraction and ten for the HR fraction. The values were calculated based on parameters <logD'(80)> and $\sigma_{logD'(80)}$ with assumed dependency of *z*-value on T (Table 2.28).

A^2	Fraction	Temperature range (°C)									
		75-80	75-81	75-82	75-83	75-85	75-88	75-90	75-91	75-93	75-95
	HS	1.22	1.17	1.32	1.44	3.27	3.24	4.43		4.52	
	HR	1.33	1.29	1.46	1.53	1.04	1.15	2.24	1.95	1.72	1.87

Table 2.28 Calculated values of Anderson-Darling for p(logD'(80)) for spores of non-proteolytic *C. botulinum* at different temperature ranges (+LYS)
empty cells indicate there were no data in this range

For the HS subset the lowest A^2 was calculated at the temperature range 75 – 81°C (A^2 = 1.17), whereas the highest for logD'(80) at 75 – 93°C (A^2 = 4.52). Across the tested subsets the most significant change in A^2 value was observed between logD'(80) converted from 75 – 83°C and logD'(80) converted from 75 – 85°C. The critical value of 2.492 for the 5% level of significance, as given in Table 2.5, is less than the calculated values of 3.27, 3.24, 4.43 or 4.52 for logD'(80) converted from data at above 83°C. Therefore the results of the Anderson-Darling test indicate that inclusion of the transformation into logD'(80) *D*-values measured at temperatures above 83°C will cause a deviation. Therefore, we can assume that data (n = 27) with estimated parameters <logD'(80)> = 0.11 min and $\sigma_{logD'(80)}$ = 0.19 min represent the same population of data. The corresponding lognormal distribution parameters are <D'(80)> = 1.42 min and $\sigma_{D'(80)}$ = 0.65 min (Figure 2.24).

The “cut off” point above 83°C for the HS fraction is in agreement with results of the Anderson-Darling analysis for -LYS data. Since the +LYS HS fraction represents spores not affected by the presence of lysozyme, it is not unexpected that they show the same property as the -LYS spores.

The calculated A^2 (Table 2.28) for the HR fraction reveals homogeneous data across all heating temperature ranges, with the lowest for logD'(80) in the range of 75 – 85°C (A^2 = 1.04). The highest A^2 was calculated for logD'(80) for heating

temperatures in the range of 75 – 90°C (A^2 = 2.24). Since the calculated A^2 value of 1.87 for logD'(80) transformed from temperatures in the range 75 – 95°C is smaller than the critical value of 2.492, there is no reason to reject the null hypothesis. It is accepted that all logD values measured at each of heating temperature for the HR fraction represent a single population. The belief concerning the heat resistance of the HR fraction of spores has normal parameterisation with <logD'(80)> = 2.09 min and $\sigma_{logD'(80)}$ = 0.51 min, and corresponding lognormal <D'(80)> = 245 min and $\sigma_{D'(80)}$ = 423 min (Figure 2.24).

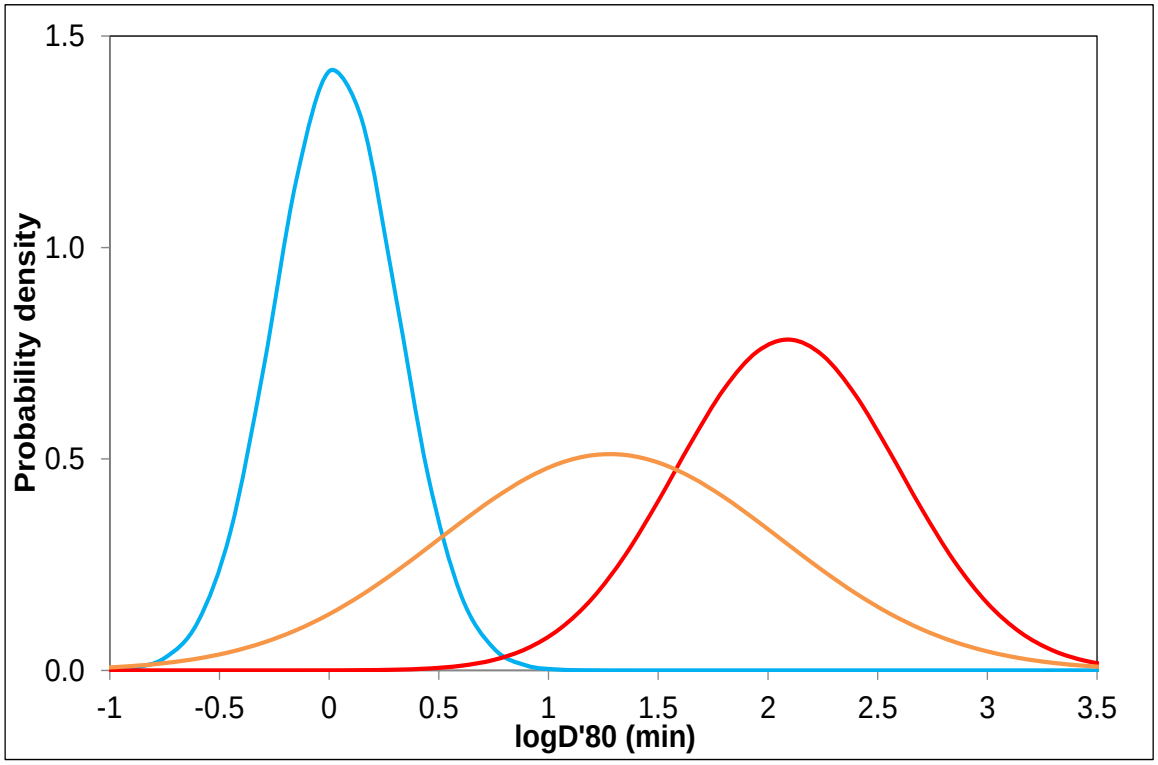

Figure 2.24 Belief concerning logD'(80) value for spores of non-proteolytic *C. botulinum* for HS and HR fractions and TDT data (+LYS)
HS fraction (blue line), TDT data (yellow line), HR fraction (red line)

Testing the similarity of +LYS HS and +LYS HR fractions

Collected *D*-values for +LYS data has shown that addition of lysozyme during recovery following a heat treatment affects their measured heat resistance. Based on 34 separate tests it was estimated that the HR fraction constitutes approximately 0.02 to 3.12% of the initial spore population. Thus, the HS fraction predominates.

Figure 2.25 illustrates probability distributions of the *D*-values for the HS and HR fractions obtained at three temperatures and converted to logD'(80). It is noted from the graphs that the distribution for the HR fraction is significantly shifted to higher values.

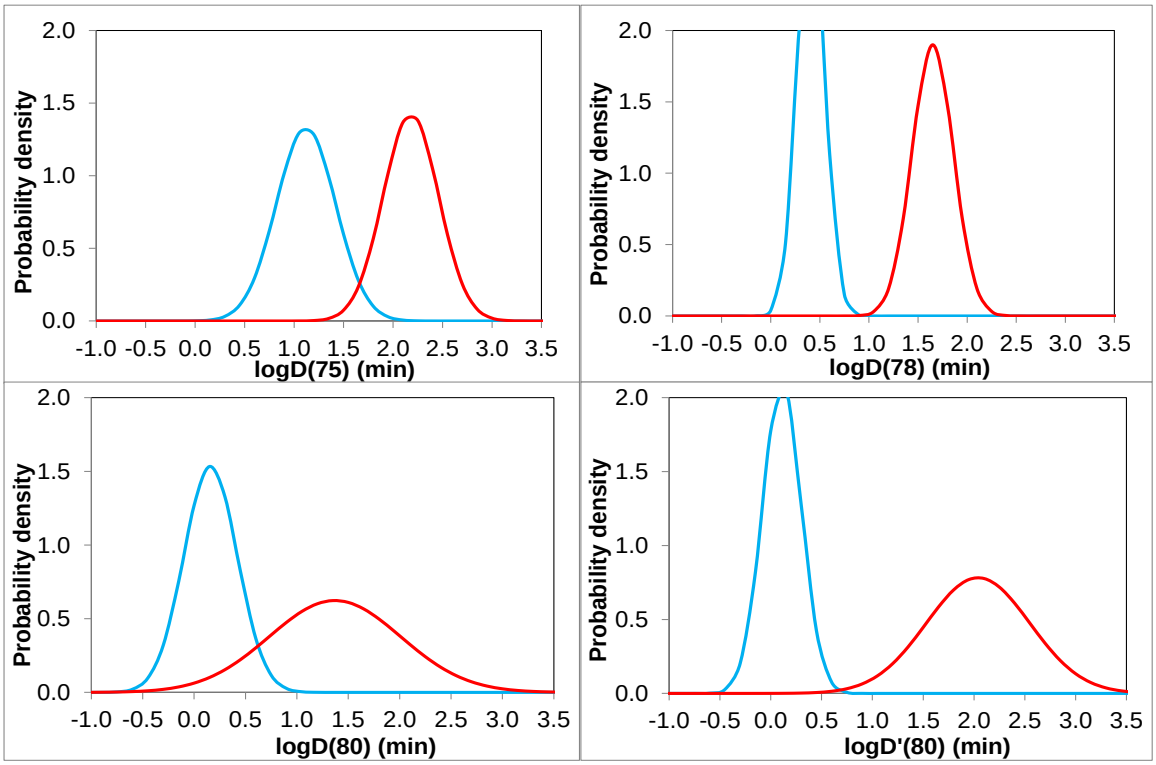

Figure 2.25 Beliefs concerning the distribution of *D*-values at different temperatures for spores of non-proteolytic *C. botulinum* (+LYS)
Heat sensitive (+LYS HS) fraction (blue line), heat resistant (+LYS HR) fraction (red line)

The Anderson-Darling analysis and the distribution parameters for logD'(80) indicates that there is a difference in *D*-values for these two fractions. This hypothesis is confirmed by a standard *t*-test analysis. The spore heat resistance of the HR fraction at four temperatures is significantly greater than that of the HS fraction (Table 2.29).

***D*-value measured at T**	***t***	***df***	***p***
D(75)	1.90	7	< 0.01
D(78)	1.75	16	< 0.01
D(80)	1.80	11	< 0.01
D'(80)	1.66	112	< 0.01

Table 2.29 Results of *t*-test analysis for heat sensitive (+LYS HS) and heat resistant (+LYS HR) fractions for spores of non-proteolytic *C. botulinum*

Heat resistance of spores recovered in absence (-LYS) or presence (+LYS) of lysozyme

A *t*-test analysis of the +LYS HS fraction and the -LYS data revealed that there is no statistical significant difference in logD values for these two subsets of data at 78°C, 80°C and logD'(80) transformed from temperatures in a range 50 – 83°C for -LYS and 75 – 83°C for +LYS HS fraction (Figure 2.26).

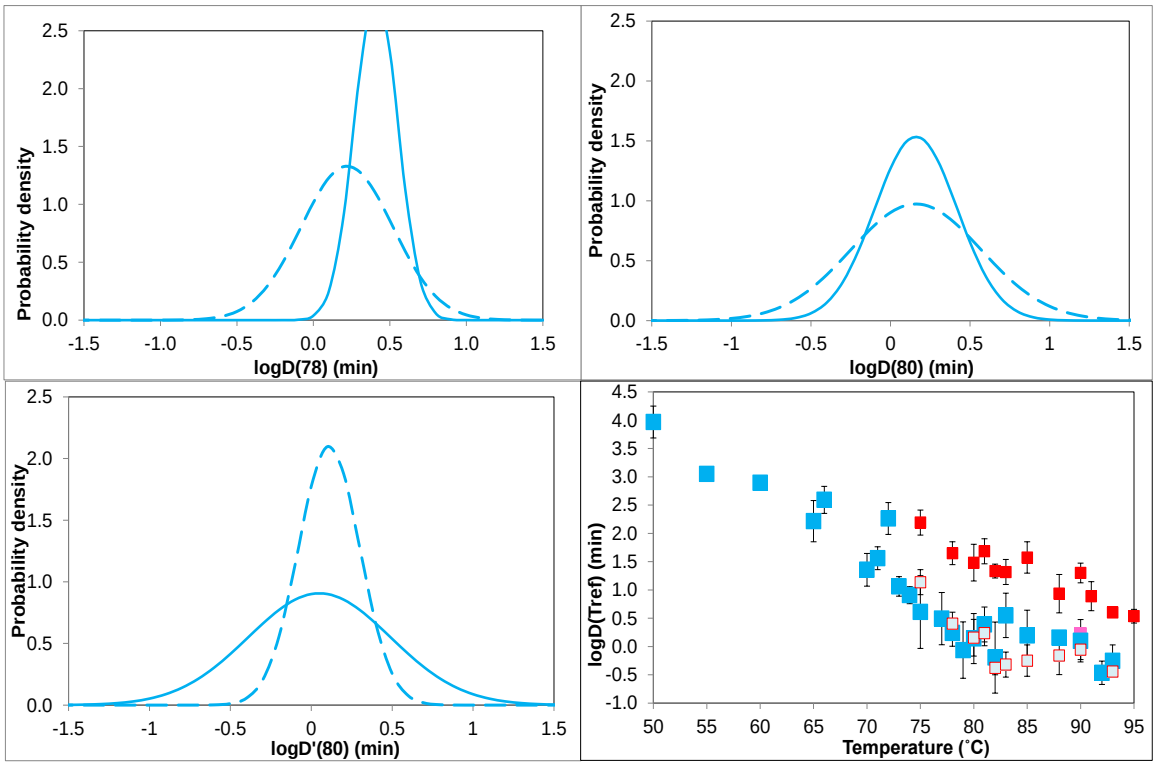

Figure 2.26 Comparison of heat resistance of spores of non-proteolytic *C. botulinum* recovered in presence and absence of lysozyme
-LYS data (blue full line and dark blue squares), +LYS HS fraction (blue dashed line and light blue squares), +LYS HR fraction (red squares)

Since the distribution of logD'(80) for -LYS is built from much more data, then the +LYS HS data are not considered further. The analysis of the dependence of spore heat resistance on heating menstruum (+LYS HR fraction) revealed similar results to the -LYS subset, i.e. there is no significant difference in *D*-values measured in media/buffer and food matrices (data not shown).

Practical application of heat treatment parameters

Table 2.30 and Table 2.31 summarise *D* and *z*-values calculated using different methods. For the -LYS subset, the calculated *z*-values ranged from 6.8 – 9.8°C. A *z*-value of 6.8°C (Figure 2.13) is the mean of all reported values in eligible studies and a value of 9.8°C is obtained from the plot in Figure 2.14. Both *z*-values include data from experiments performed at temperatures above 83°C. As shown from the Anderson-Darling analysis (see section 2.3.2.2), these data should not be included; therefore these *z*-values are of limited value. By taking account of data generated at 83°C and below, z-values of 8.1°C and 7.7°C are obtained from the slope of the line of best fit (Figure 2.14 and Figure 2.21). The former *z*-value was fitted to the average logD values (single point) at each heating temperature whereas the latter, was fitted to all logD values (multiple points) reported at each heating temperature. The beta distribution of *z*-value with parameters a = 3.7, c = 16.5, α_1 = 2.9 and α_2 = 9.6 (with <z> = 6.7°C) (Table 2.12) includes the range of all above values.

Data for the +LYS HR subset were collected in the range of 75 – 95°C, with the majority of *D*-values generated at 85°C or 90°C. The *z*-values were higher than reported for the -LYS data with a mean of 9.6°C (Figure 2.13). From the slope of the line of best fit (fitted to the average of logD values) (Figure 2.22) a *z*-value of 14.3°C was obtained. The highest recorded *z*-value in the literature for the +LYS HR fraction was 13.0°C (Figure 2.13), therefore 14.3°C could be an artefact due to the limited number of data available for fitting procedure. For logD the R^2 value of the fitted line is very small (R^2 = 0.3), therefore the *z*-value calculated from the slope is likely to be imprecise (Figure 2.22).

Based on this analysis, a *z*-value for -LYS data should be in the range of 6.7 – 8.1°C, and for +LYS HR data a *z*-value should be in the range of 9.3 – 9.6°C. A single integer temperature is generally used for the *z*-value, thus *z*-values of 7°C and 9°C may be appropriate for these two datasets.

As shown in Table 2.31 for -LYS data, exclusion of *D*-values generated above 83°C had an important effect on D'(80), which decreased from 2.76 to 1.87 minutes (Table 2.13). The mean value of D(80) = 2.26 min (Table 2.10) is in

agreement with the mean value for the fitted distribution of D'(80) (<D> = 2.23 min) (Table 2.13). The lowest *D*-value at 80°C (1.29 min) was calculated from the thermal death model (Table 2.18). Using the 95th and 99th UCL of the thermal death model, calculated values of *D* are respectively 9.02 and 16.60 minutes (Figure 2.21).

Data	Calculation method	Source (Table/ Figure)	Mean (SD*/R^2)[a)]	T (°C)	*n*
-LYS	Raw data	Figure 2.13	6.8 (1.6*)	50-93	368
	Fitted line (average logD value)	Figure 2.14	9.8 (0.9)	50-93	--
	Fitted line (average logD value)	Figure 2.14	8.1 (0.9)	50-83	--
	Fitted line (logD values)	Figure 2.21	7.7 (0.7)	50-83	--
	Fitted distribution	Table 2.12	6.7 (1.5*)	50-83	340
+LYS HR	Raw data	Figure 2.13	9.6 (2.1*)	75-95	46
	Fitted line (average logD value)	Figure 2.22	14.3 (0.8)	75-95	--
	Fitted line (logD values)	not shown	21.3 (0.3)	75-95	--
	Fitted distribution	Table 2.26	9.3 (2.3*)	75-95	46
+LYS TDT	Raw data	Figure 2.13	11.4 (4.3*)	70-94	90
	Fitted line (average logD values)	Figure 2.22	24.5 (0.8)	70-94	--
	Fitted line (logD values)	not shown	17.2 (0.5)	70-94	--
	Fitted distribution (all z-values)	Table 2.26	9.3 (8.5*)	70-94	90

Table 2.30 Summary of *z*-values (°C) for spores of non-proteolytic *C. botulinum* calculated by different methods

[a)] number in brackets present R^2, which assess the "goodness" of fitted line, and standard deviation calculated from data (as indicated with asterisk)

Higher values were calculated for the +LYS HR fraction, with a mean value of D'(80) = 213 min (calculated from raw data), and a mean value distribution of *D*-value <D'(80)> = 245 min (Table 2.27). A smaller value was noted for *D* measured at 80°C (D(T) = 69 min) (Table 2.24), but this is calculated from only nine data points (compare with 105 for D'(80)). Also the *D*-value of 45.71 min calculated from the thermal death model (data not shown) is small compared to other recorded *D*-values. The small R^2 = 0.3 for +LYS HS (fitted line – logD values) indicates that the fitted trendline does not provide a very good approximation for the data (Table 2.30).

Data	Calculation method	Source (Table/ Figure)	logD value (min)	<D> ± (σ_D)* (min)	T (°C)	*n*
-LYS	Raw data D'(80)	Table 2.13	0.13 (0.5)	2.76 ± (4.9)	50-93	551
	Raw data D'(80)	Table 2.13	0.05 (0.4)	1.87 ± (2.5)	50-83	505
	Raw data D(80)	Table 2.10	0.16 (0.4)	2.26 ± (2.7)	80	151
	Fitted line (thermal death model)	Table 2.18	0.11	1.29	80	--
	Fitted distribution D'(80)	Table 2.13	0.00 (0.5)	2.23 ± (4.4)	50-83	505
+LYS HR	Raw data D'(80)	Table 2.27	2.04 (0.5)	213 ± (354)	75-95	105
	Raw data D(80)	Table 2.24	1.37 (0.6)	69 ± (194)	80	9
	Fitted line (thermal death model)	not shown	1.66	45.71	80	--
	Fitted distribution D'(80)	Table 2.27	2.09 (0.5)	245 ± (423)	75-95	105
+LYS TDT	Raw data D'(80)	not shown	1.08 (0.5)	23.33 ± (38.8)	70-94	129
	Raw data D(80)	Table 2.24	0.87 (0.4)	12.10 ± (15.62)	80	30
	Fitted line (thermal death model)	not shown	1.00	9.94	80	--
	Fitted distribution D'(80)	sec. 2.3.2.3	1.28 (0.8)	96 ± (470)	70-94	129

Table 2.31 Summary of calculated *D*-values (min) at 80°C and D'(80) for spores of non-proteolytic *C. botulinum* for different subsets of data
*data are given as mean ± standard deviations

Based on the 95% and 99% UCL of the thermal death model (-LYS data) (see section 2.3.2.2 – testing the dependence of spore heat resistance on heating menstruum), time to a 1D and 6D reduction in the non-proteolytic *C. botulinum* spore population, at different temperatures can be calculated (Table 2.32). According to the model predictions heating for 54 and 100 minutes at 80°C and 2.68 and 4.91 minutes at 90°C would be required to achieve a 6D reduction using 95% and 99% UCL, respectively (Table 2.32). Due to low R^2 value heating time required to provide the 1D and 6D reduction for +LYS HR fraction was not calculated.

T (°C)	Time (min)					
	1D			6D		
	Expected value	95% UL	99% UL	Expected value	95% UCL	99% LCL
70	25.88	182	337	155	1092	2020
75	5.80	40.50	75	34.81	243	449
80	1.29	9.02	16.60	7.80	55	100
85	0.29	2.01	3.69	1.75	12.04	22.11
90	0.07	0.45	0.82	0.39	2.68	4.91

Table 2.32 Equivalent heat treatments for 1D and 6D reduction of non-proteolytic *C. botulinum* spores based on the fitting of thermal inactivation data published in the literature (*n* = 505) using 95% and 99% upper confidence limits (-LYS)

2.4 Discussion

As indicated in section 2.1.6 five other reviews of the heat resistance for spores of non-proteolytic *C. botulinum* have been carried out previously. The output of this review not only provides information about the range of reported *D* and *z*-values, but also confirms existing hypothesis regarding the heat resistance of non-proteolytic *C. botulinum* spores and puts forward new ones.

This review found several key results. First of all, -LYS *D*-values measured at above 83°C do not change significantly with heating temperature. The statistical analysis indicates that data for T > 83°C are not consistent with the data obtained from measurement at lower heating temperatures. In the studies of De Pantoja (1986) the decimal reduction time of spores measured at 85°C was slightly higher than the *D*-value observed at 80°C. Similarly, in a report published by Membrè and McClure (2006) *D*-values measured at above 90°C were not included in a model and were analysed separately. There are two possible explanations: firstly – a change in spore heat resistance might be associated with experimental factors (uneven dispersion of spores in the test tube, evaporation of water – exposure to dry heat rather than moist heat). Moreover, *D*-values at higher temperatures (~90°C) take values in a range of 0.01 – 1 min. Physical measurement of values of this magnitude present many problems. Secondly – changes in heat resistance might be associated with change in physical properties of spores (late activation of heat resistant spores present in the population).

A second key finding is that this review of the current literature did not demonstrate that heat resistance of spores is associated with toxin type, which was believed to be the case by many researchers. For example, in the studies of Scott and Bernard (1982) the heat resistance of spores of type B strains appeared to be greater than that of spores of type E strains. Nevertheless, these authors acknowledged that results for the tested strains were not consistent with reports by other researchers. The authors explained that this could possibly be caused by different batches of recovery media and the age of spores. Other studies which tested strains of two different toxin types in the same heating system were published by Lynt *et al.* (1977; 1979; 1983). These authors

evaluated the spore heat resistance of five strains of type E and three strains of type F in crabmeat. The study revealed that the heat resistance of spores of type F strains was similar to type E strains. The only difference lay in the slope of line obtained from plotting *D*-value against temperature. Consequently, spores of type F strains had lower *z*-values. This finding agreed with the results of this review, where the *z*-value for type E spores is 6.8°C, and for spores of type F strains the mean *z*-value = 5.6°C. Similarly, a study of spore heat resistance for type B and E strains in cod and carrot homogenates at 70 – 92°C, revealed that there was no difference in spore heat resistance for strains of different toxin type within the same heating menstruum (Gaze and Brown, 1990). The results were in agreement with Juneja *et al.* (1995a), who reported on the heat resistance of type B and E strains tested in two different recovery media. According to the studies of Peck *et al.* (1993) spores of strain Eklund 17B (type B) recovered in +LYS medium were more heat resistant than spores of strain Beluga (type E). Nevertheless, further testing of ten strains (four type B, four type E and two type F) at 85°C, revealed no association between toxin type and apparent spore resistance.

In this review, only in one case, following heating at 80°C, the mean of *D*-value for mixed strains appeared to be lower than the *D*-value of type B and E strains. The data at this temperature for mixed strains originates from a report by Membrè and McClure (2006) for which some information was missing because the heat treatment experiment was not conducted by the authors. There was no information about the strains used, except general information e.g. “type B (6), type E (15) and type F (3) were used”. Since there was no possibility to retrieve the original data, strains were treated as mixed strains. This could lead to bias in the analysis. As the difference was observed only for one heating temperature and only for mixed strains, this finding is not significant for an overall analysis. Thus, the overall evidence from the literature is that spore heat resistance is not associated with the type of neurotoxin formed. However, the possibility still arises for subtle differences, but that are not evident from the literature data. This could only be revealed by testing of a large number of strains in a well-controlled study.

The test carried out in this study failed to demonstrate a relationship between the heat resistance of spores and the heating menstruum. Data published in a report by Bohrer *et al.* (1973) show that the measured *D*-value was higher in food than phosphate buffer. Moreover, a correlation was observed between spore heat resistance and the amount of proteins and fat. Strain Saratoga exhibited a greater heat resistance in foods high in fat and protein such as tuna in oil and sardines in tomato sauce, with D(77) = 40.9 min and 20.0 min respectively, whereas *D*-value measured in 0.067M phosphate buffer was 4.1 min. There was no correlation observed between thermal heat resistance of spores and carbohydrate content in food, with D(77) = 11.2 min measured for corn in brine. Similar results were reported by Lynt *et al.* (1979), where the heat resistance of the strain Eklund 202F was greater in crabmeat (D(77) = 9.5 min, D(79) = 3.5 min, D(82) = 1.2 min) than in 0.067M phosphate buffer (D(77) = 4.3 min, D(79) = 3.5 min, D(82) = 0.3 min). The *D*-values calculated for strain Crab G 21-5 by Solomon *et al.* (1977) appeared to be lower in TPGY broth as a heating menstruum (with D(82) = 1.5 min) compared with crabmeat (D(82) = 3.0 min). These are the only studies which were directly designed to test the dependence of the heat resistance parameters on heating menstruum, i.e. where at least two different menstrua were used in one experiment. Nevertheless, considering the large number of collected data in this review (*n* = 505 *D*-values) the heat resistance of spores measured in different food matrices (*n* = 27) does not appear to be significantly different from that measured in media\buffer (*n* = 13). This finding agreed with the analysis of 269 collected *D*-values by Membrè and McClure (2006). However, the average *D*-value appears to be slightly higher, when determined in meat products or seafoods (Table 2.19). Since only a few data points correspond with these heating menstrua it is very difficult to draw significant conclusions. Thus, while individual studies have shown that measured spore heat resistance can appear greater in foods than in media\buffer, the overall dataset does not reveal a relationship. It might be that the great variety of techniques used by researchers in different laboratories has obscured this relationship.

In the studies of Alderman *et al.* (1972), it was shown that not only the heating menstruum itself but the physical state (i.e. the water activity (a_w)) of the

menstruum is important in the heat resistance of spores. Spores heated in raw fish had a greater *D*-value than those heated in autoclaved fish. Similar results were observed for raw egg white (D(83) = 14.2 min) and pre-coagulated egg white (D(83) = 1.2 min). Addition of sodium chloride to the heating menstruum sometimes lead to an increase of *D*-value (Bucknavage *et al.*, 1990). When the heat resistance of spores was tested by Murrell and Scott (1966) in menstruum with a_w = 0.90 a value of D(80) = 25.8 min was obtained, whereas at a_w = 1.0 the D(80) = 0.3 min. Appleyard and Gaze (1993) suggested that a prior sublethal heat treatment increases the measured *D*-value. They also concluded that the time after which the *D*-value was determined following the heat treatment is a significant factor. These authors reported that the greatest *D*-value was determined immediately after heat shocking at 50°C for 60 min (D(70) = 43.8 min). Holding samples after the heat treatment for 4 hours at room temperature decreases the *D*-value (D(70) = 32.8 min) and storage of samples for 24 hours at 6°C lowered the *D*-value further (D(70) = 22.4 min). Therefore it can be concluded that not only the heating menstruum itself, but its physical state (lower a_w), and the time for which the medium is kept before determining the *D*-value have an impact on the decimal reduction time.

This review confirms that spore heat resistance depends on heating technique. Although there are not many publications which directly compare the impact of at least two different heating techniques on *D*-value for the same non-proteolytic *C. botulinum* strain. An attempt was made by Graikoski and Kempe (1964) but the results were not very reliable, as in many cases the reduction in spore concentration was small. Of the nine heating techniques considered, TDT cans and sealed ampoules in a water bath appeared to give the highest mean value for logD'(80) with <logD'(80)> = 0.65 min and 0.59 min, respectively. The *D*-values obtained in experiments with sealed ampoules were from a study by Eklund *et al.* (1967a) and as this method appears to be very tedious this is an isolated report. The TDT can technique is more commonly employed in studies of heat resistance for spores at high temperatures (> 100°C). Moreover, the mean value of logD'(80) determined using TDT cans technique was mainly calculated from *D*-values reported by Bohrer *et al.* (1973), with products high in fat and proteins as heating medium.

According to De Pantoja (1986), the method of calculation can affect the *D*-value. This is in agreement with findings from this review. Analysis of 505 *D*-values revealed that *D*-values calculated according to methods of TDT and TDT/Stumbo(1948) are significantly higher than those calculated according to the survivor curve and TDT/Schmidt(1957) method. Similar results were obtained in a hypothetical example presented in Table 2.3 and Table 2.4. In the results of De Pantoja (1986), a comparison of decimal reduction times obtained by three different methods of calculation, the TDT/Stumbo(1948) method appeared to give the highest *D*-values. The inconsistency arising from differences in *D*-values calculated from survivor curve and TDT methods causes a variation in *z*-value. This was reported by Lynt *et al.* (1977; 1979), Licciardello (1983) and Chai and Liang (1992). In all studies *z*-values calculated from TDT curves were lower than *z*-values calculated from survivor curve data.

This review indicates that strain ATCC 9564, with <D'(80)> = 10.3 min (*n* = 11) and strain Detroit (<D'(80)> = 8.0 min (*n* = 6)) are the most heat resistant. Strains Crab 25 V-2 (*n* = 11) and Whitefish (*n* = 3) had lowest mean value of D'(80) (0.3 and 0.5 min) (all were type E strains). Nevertheless, this information should be interpreted with caution. In spite of such a large database it is difficult to distinguish the most and least heat resistant strains since they seem to vary with heating temperature as indicated for example by Bohrer *et al.* (1973) and Ito *et al.* (1970). Additionally, difficulty to determine the most heat resistant strain can arise from variability in spore crop (Blocher and Busta, 1983) and number of factors (as shown above) determining spore heat resistance. Thus it is not easy to compare strains from studies that have used different methods (e.g. heating menstruum, heating technique). The results from testing a number of strains in a very reproducible way would be of great interest.

It is apparent from this review that recovery media, especially addition of lysozyme, has a large effect on measured *D*-value, resulting in biphasic survivor curves, which represents a mix of two fractions of spores with different heat resistance properties. The first part of the survival curve describes heat sensitive spores (HS) and the second heat resistant spores (HR). It was estimated that the heat resistant fraction can constitute approximately 0.02 – 3.1% of the

population. Lindström *et al.* (2003) estimated the heat resistant fraction as ~0.1% of spores, and Peck *et al.* (1993) estimated the fraction as ~0.2 – 1.4%, with the fraction dependent on the strain. The biphasic survivor curve, and consequently the increased number of surviving spores, was observed when as little as 0.1µg lysozyme ml^{-1} was added to the recovery medium. In studies by Peck *et al.* (1992a) the maximum spore recovery was achieved at concentrations ~5 – 10 µg lysozyme ml^{-1}. Furthemore, addition of vegetable juices to the plating medium or recovery of heated spores in a medium containing crabmeat can also result in a biphasic curve (Stringer and Peck, 1996; Peterson *et al.*, 1997). An explanation of the biphasic curve is included in section 2.1.2.

The main purpose of this systematic literature review was to collect data describing the heat resistance parameters of spores of non-proteolytic *C. botulinum.* As a result the lognormal distribution of D'(80) with parameters $\langle D'(80)\rangle$ = 2.23 min and $\sigma_{D'(80)}$ = 4.44 min was obtained in order to represent belief concerning the heat resistance of non-proteolytic *C. botulinum* spores (-LYS). When the activity of lysozyme is included the parameters for a lognormal distribution of HR fraction are $\langle D'(80)\rangle$ = 245 min and $\sigma_{D'(80)}$ = 423 min. The beta distribution for *z*-values (-LYS data) has parameters a = 3.7, c = 16.5, α_1 = 2.9, α_2 = 9.6 with $\langle z\rangle$ = 6.7°C.

Thermal inactivation parameters for non-proteolytic *C. boutlinum* spores were also determined by Van Asselt and Zwietering (2006), with $\langle \log D(120)\rangle$ = -1.47 min, $\sigma_{\log D(120)}$ = 0.71 min and *z* = 18.6°C. The parameters were estimated from the relationship of log transformed *D*-values and temperature. Parameters transformed to 80°C are $\langle \log D(80)\rangle$ = 0.68 min and $\sigma_{\log D(80)}$ = 0.71 min and corresponding parameters for a lognormal distribution are $\langle D(80)\rangle$ = 18.21 min and $\sigma_{D(80)}$ = 66.86 min. The parameters are significantly higher than the ones obtained in this review. The main reason is that data used by Van Asselt and Zwietering (2006) included *D*-values from experiments when recovery of spores was conducted in media which contained lysozyme. The estimate of parameters was based on 175 data points. Probability distributions for D'(80) from this review, from the ACMSF recommendation (1992) and from Van Asselt and Zwietering (2006) are illustrated in Figure 2.27.

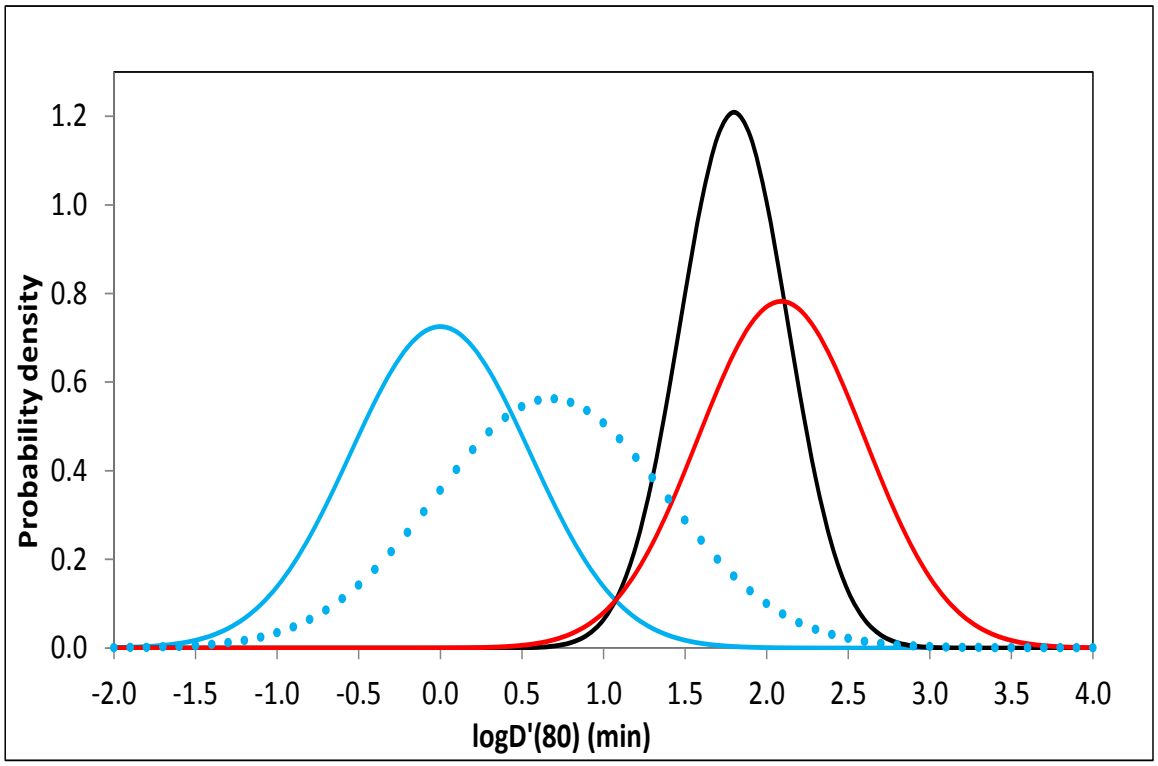

Figure 2.27 Belief concerning the distribution of D'(80) for spores of non-proteolytic *C. botulinum* according to different authors
This review (-LYS) (blue line), this review (+LYS) (red line), Van Asselt and Zwietering (2006) (blue dotted line), ACMSF "90/10" rule, converted to D'(80) using beta distribution obtained in present study for *z*-value (2.9, 9.6, 3.7, 16.5) (black line)

Collected data from this review was also used to review the current recommendations that a heat treatment of 10 min at 90°C is needed to reduce the concentration of non-proteolytic *C. botulinum* spores by six orders of magnitude in respect of minimally heated chilled foods products (ACMSF, 1992). The model (-LYS) presented in this thesis predicted that heating at 90°C for ~5 min is needed (based on 99% upper limit of prediction) for a 6D inactivation of spores. Thus, the current ACMSF recommendation provides a good level of safety. There are six studies (Murrell and Scott, 1966; Alderman *et al.*, 1972; Bohrer *et al.*, 1973; Scott and Bernard, 1982; Scott and Bernard, 1985; Fernandez and Peck, 1997) in which *D*-values are reported that are higher than those predicted by the model presented in this thesis. Four of these studies used the TDT method and the inactivation kinetics were not reported. Moreover, in these particular studies the heated spores were transferred to nutrient-rich medium and incubated for prolonged time. This procedure is known to result in larger *D*-values as a combination of prolonged storage and subculturing of spores into new nutrient medium, favours their germination and outgrowth. The

two remaining *D*-values (D(80) = 25.8 min and D(75) = 274 min from the studies of Murrell and Scott (1966) and Fernandez and Peck (1997) have been calculated from data given in the publication. However, despite these points, there is no reason to reject this model.

Using the 99% UCL for the time required for a 6D reduction of non-proteolyic *C. botulinum* spores heated at 80°C gives longer heating time than that calculated by Membrè and McClure (2006), but it is shorter than the time recommended by ACMSF and CFA (Table 2.33). The equivalent heating time at 90°C for the 99% UCL, using the *z*-value of 7°C is ~5 min which is in agreement with results of Notermans (1990) where heating at 90°C for 4 min is sufficient to provide a 5D safety factor. However, since the model from this review did not include data above 83°C, there is not enough evidence to question the validity of the ACMSF recommendation.

T (°C)	Time (min)					
	ACMSF (1992) "90/10" (z = 9.2°C)	CFA (2006) "90/10" (*z* = 7°C)	Membrè and McClure (2006) "90/7" (*z* = 9.2°C)*	Model for current literature review		
				Expected value	95% UCL	99% UCL
70	1675	--	--	155	1092	2019
75	464	--	283	34.81	243	448
80	129	270	81	7.80	54	100
85	36	52	23.5	1.75	12.04	22.11
90	10	10	6.9	0.39	2.68	4.91

Table 2.33 Heating time required at 70 – 90°C to provide a 6D safety factor respect to spores of non-proteolytic *C. botulinum* according to ACMSF (1992), CFA (2006), Membrè and McClure (2006) and the model obtained in this thesis from a review of current literature (-LYS)
* time calculated from 99th upper confidence limits of prediction

From the data summarised in this study two *z*-values are recommended; *z* = 7°C for data where there is no evidence for lysozyme activity, otherwise *z* = 9°C. So far, current recommendations do not consider the influence of lysozyme present in heated products. In the summary by Membrè and McClure (2006) any data from studies with lysozyme present in the recovery medium were excluded from the data set. Significantly higher *D*-values, and *z*-values in heating system with lysozyme means that manufacturers need to ensure that the food product is free of lytic enzyme activity before setting the thermal processing. Two *z*-values

recommended by the CFA, ECFF and USFDA (Table 2.1) related to the safety of minimally heated chilled foods are made with respect to heating temperature with a lower *z*-value (7.0°C or 9.2°C) below 90°C and a higher *z*-value (10.0°C or 10.2°C) above 90°C. A similar approach should be taken in the presence of lysozyme.

From the analysis presented in this chapter it can be concluded that the heat inactivation parameters for spores of non-proteolytic *C. botulinum* depend on many factors, e.g. variation of strains, experimental conditions, heating method and recovery conditions. There are a large number of reported *D* and *z*-values. The range of reported D(80) values was 0.20 to 25.84 min (-LYS data) and 0.69 to 111 min (+LYS) and z-values ranged from 4.1°C to 22.4°C. The choice of appropriate single parameters when estimating the effect of heat inactivation for a particular product is considerable. A poor choice may result in either over processing (which affects the sensory and nutritional value of the food) or, in the worst case scenario, under processing affecting consumer safety.

2.5 Conclusions

From an extensive literature survey, a total of 880 *D*-values and 528 *z*-values determined at 27 different temperatures were obtained. An in-depth analysis allowed the following conclusions:

i. Established data provide a strong quantitative support for modelling of the thermal properties of the spores of non-proteolytic *C. botulinum* as they allow for including uncertainty and variability into QMRA

ii. Recovery media, especially the addition of lysozyme has a large impact on measured thermal properties of spores, resulting in biphasic curves, with significantly different *D*-values observed for the +LYS HS and +LYS HR fractions. Therefore these data need to be treated separately

iii. The distribution of *D*-values for the -LYS data was not significantly different to that for the +LYS HS

iv. *D*-values for the -LYS and +LYS HS obtained at high temperatures (> 83˚C) include uncertainty arising from experimental method and these data should not be included in the analysis

v. Examination of data for B, E and F strains indicates that there are no significant differences between probability distributions of the *D*-values, demonstrating that the heat resistance of spores is not associated with their toxin type

vi. Indication of the strains that form the most and least heat resistant spores is difficult to assess due to the large number of factors that influence the *D*-value variability

vii. Spore heat resistance does not depend strongly on heating menstruum, nevertheless slightly higher *D*-values were observed in meat products

viii. Spore heat resistance depends on heating technique, with higher *D*-values obtained when TDT cans and sealed ampoules were used

ix. The *D*-value depends on the method of calculation, with the calculated decimal reduction time according to TDT and TDT/Stumbo(1948) being higher than from survivor curves

x. A lognormal distribution of D'(80) with parameters <D'(80)> = 2.23 min and $\sigma_{D'(80)}$ = 4.44 min represents belief concerning the heat resistance of non-proteolytic *C. botulinum* spores (-LYS). When the activity of lysozyme

is confirmed the parameters for a lognormal distribution are <D'(80)> = 245 min and $\sigma_{D'(80)}$ = 423 min

xi. The beta distributions of *z*-values for -LYS data were described with parameters a = 3.7, c = 16.5, α_1 = 2.9, α_2 = 9.6 and <z> = 6.7°C. For +LYS HR a = 4.0, c = 19.6, α_1 = 3.1, α_2 = 6.0 and <z> = 9.3°C

xii. The time needed for a six order of magnitude reduction in concentration of non-proteolytic *C. botulinum* spores in the absence of lysozyme at 90°C is ~5 min (based on 99% UCL)

xiii. A suitable z-value for spores of non-proteolytic *C. botulinum* might be 7°C (based on the -LYS data). While for the +LYS HR fraction, a suitable z-value is 9°C

Heat treatment is one of major factors used to control the *C. botulinum* hazard in food; therefore this process should be optimized in order to deliver sufficient microbial inactivation. The use of probability distribution of appropriate parameters in designing the heat treatment allows for the inclusion of variability (physiological property of spores) and uncertainty (associated with technical errors of conducted tests) into calculations. Therefore, the distribution parameters for p(z) and p(logD'(80)) obtained in this chapter not only support the decision-making process regarding the choice of optimal heat treatment but also allow for a calculation of the level of confidence in QMRA.

3. Variability in growth of non-proteolytic *C. botulinum* strains at chill temperatures

The variability in growth of non-proteolytic *C. botulinum* type B, E and F strains has been examined at chill temperatures (3.0°C – 10.0°C). The results demonstrate that the growth response from spores varies with inoculum concentration and incubation temperature, and is strain specific. A classification analysis reduced the variability of growth response by assigning strains to homogeneous groups according to their physiological response. These clusters are used to establish distributions of growth times – crucial information in quantifying the risk with respect to *C. botulinum*. Interestingly clusters of strains of non-proteolytic *C. botulinum* are associated with the type of toxin produced.

3.1 Background

Non-proteolytic *C. botulinum* spores can survive the moderate heat treatment that is applied to chilled foods, and thus remain viable, subsequently germinate, outgrow and produce neurotoxin (type B, E or F) during storage. Neurotoxin formation by non-proteolytic *C. botulinum* has been reported at a temperature as low as 3.0°C (Peck *et al.*, 2006). Protecting chilled foods from growth or toxin production by pathogenic microorganisms frequently relies on two steps in a HACCP system: heat treatment and storage at refrigeration temperature. Complying with EU Regulation No 852/2004 (2004) the chilled foods manufacturers shall implement the principles of HACCP system and following the Food Hygiene (England) Regulations (2006) producers must ensure that these foods are kept at or below 8°C throughout the whole supply chain. Although it should be possible to maintain these temperatures in commercial storage, there is a chance of temperature abuse during purchasing, transport, or storage in domestic conditions.

According to a study of UK domestic storage practices by George *et al.* (2010), approximately 87.5% of consumers stored chilled ready meals in a fridge, 0.3% at ambient temperature and 9.8% in a freezer (the remaining interviewees declared that they do not purchase chilled food). The same study showed that 3% of people expected to store chilled ready to eat meals for more than seven days prior to consumption and 4% did not know the product shelf life.

The above report also indicated that 7.0°C was the dominant mean operating temperature of domestic fridges; however 29% of tested fridges operated with a mean temperature at or above 9.0°C (Figure 3.1). It should be noted that the average air temperature varied in different parts of a fridge, being 9.6°C in the bottom compartment, 5.9°C at the top and 4.7°C on the central shelf (George *et al.*, 2010). Other factors, such as cleaning, loading, seals and fridge age also affected the refrigerator temperature.

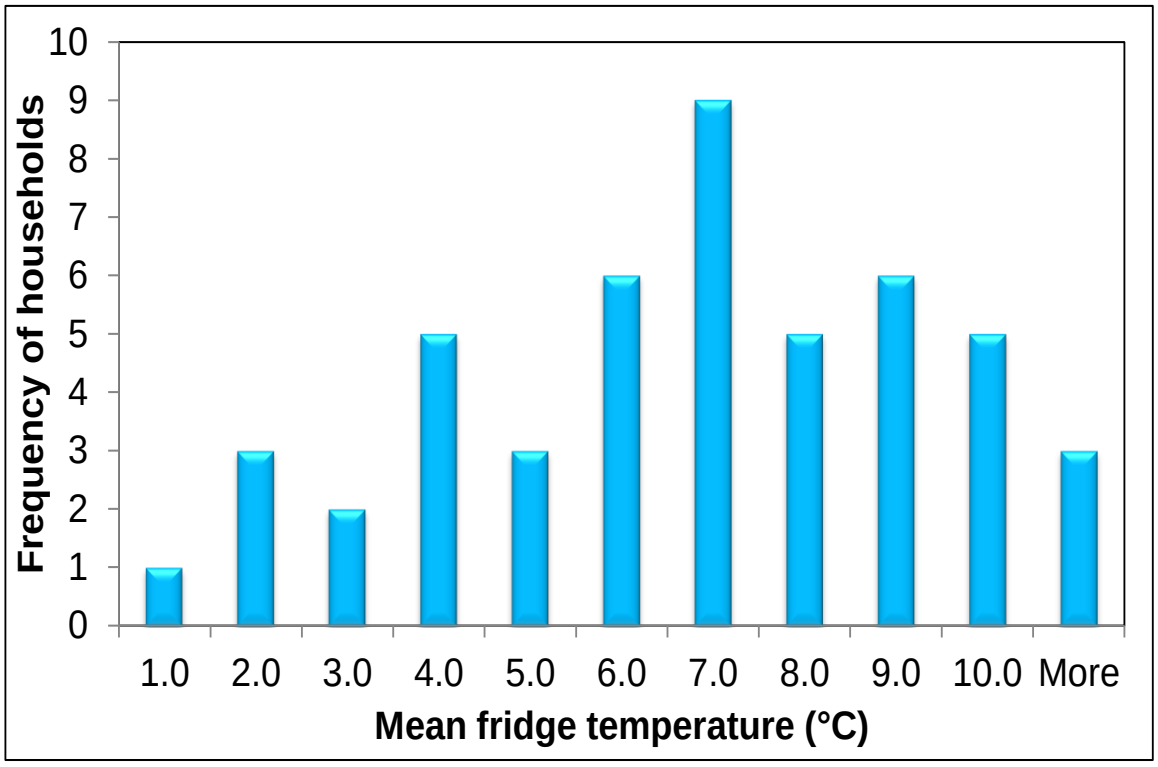

Figure 3.1 Frequency distribution of mean domestic fridge temperatures in the UK
Source: George *et al.* (2010)

The storage temperature is one of the crucial elements in quantitative risk assessment when evaluating the safety of chilled foods (see Chapter 5), as it is generally a significant source of uncertainty and sensitivity. Given the fact that only 15% of UK families use a thermometer to monitor the fridge temperature (Laguerre *et al.*, 2002), food manufacturers need to consider consumer behaviour when assessing product shelf life as it can influence the potential for growth of foodborne pathogens. All bacteria have a particular temperature growth range, and this range with its growth rate, is a crucial factor in food safety. In order to ensure the safety of chilled foods, it is vital to understand the

impact of chill temperatures on bacterial growth, and there is immense benefit in understanding its relationship with strain variability.

It is well-known that not all bacterial strains respond in the same way to environmental conditions. It is a natural property of bacteria that for two strains of the same species, one will have more rapid and another slower growth even in identical circumstances. Previously published studies for several foodborne pathogens including *E. coli* O157:H7 (Nauta and Dufrenne, 1999), *Salmonella enterica* (Lianou and Koutsoumanis, 2011), *Bacillus cereus* (Carlin *et al.*, 2013) demonstrated intra-species variability in growth properties under different environmental conditions. Although, predictive microbiology models, have been applied successfully in prediction of bacterial growth and consequently in production of safe food, they only provide one deterministic prediction of growth, rather than the probability distribution for population growth at a given time. These models do not account the variability within each species (Nauta and Dufrenne, 1999). Thus, in order to understand the variability of growth for non-proteolytic *C. botulinum* a probabilistic model for the time to growth is used, which allows it implementation in a QMRA (Nauta, 2002).

The growth of non-proteolytic *C. botulinum* at chill temperatures has been examined previously, published studies were mainly focused on determination of growth rate (Graham and Lund, 1993), the probability of growth from a single spore (Jensen *et al.*, 1987), and the effect of pH and NaCl on growth (Graham *et al.*, 1997). None of the published studies assessed strain-dependent growth properties at chill temperatures. Inclusion of the variable growth response of different strains of non-proteolytic *C. botulinum* at chill temperatures in the form of probability distribution will allow for better quantification of hazard characterisation and improved risk assessment. Moreover, generated data will be used in an estimation of the time preceding observation of growth of non-proteolytic *C. botulinum* – a dominant component of risk presented to chilled foods (see section 5.2.4.4).

3.2 Material and methods

Assessment of the variability in growth response of non-proteolytic *C. botulinum* strains during incubation at chill temperatures is based on two independent experiments. Experiment one ("Minimal growth temperature of non-proteolytic *C. botulinum* strains") was carried out previously by Stringer and Webb at IFR (unpublished data), whereas experiment two was carried out as part of these studies. The methodologies in both experiments are similar. In experiment one, the growth of 40 strains at ten incubation temperatures (in the range of 3.0°C – 10.0°C) for one spore concentration, s, ($s = 10^6$ spores $tube^{-1}$) was tested. Whereas in experiment two, the growth of 24 non-proteolytic *C. botulinum* strains from seven different inoculum concentrations was examined at a single temperature of 6.0°C. Clustering analysis was used to assess strain variability. Tested strains were assigned to particular clusters based on the parameter τ calculated from growth tests. The parameter describes the time needed for half of inoculated tubes to show growth. In order to express uncertainty in the τ-value, a normal cumulative distribution function was fitted for each identified cluster of non-proteolytic *C. botulinum* strains. The effect of non-proteolytic *C. botulinum* strain variability on growth at chill temperature was assessed based on data generated in both experiments. Additionally data from experiment two were analysed in terms of probability of growth of non-proteolytic *C. botulinum* at 6.0°C.

3.2.1 Strains

The strains used are listed in Table 3.1. In total the growth at chill temperature was tested for forty-two strains of non-proteolytic *C. botulinum*. Experiment one was performed using forty strains (13 type B, 22 type E and 5 type F). In experiment two, twenty-four strains were tested (11 type B, 9 type E and 4 type F). Chosen strains were isolated in different laboratories from various locations. These strains were also used in the tests described in Chapter 4.

IFR no.	Chill temperature incubation test		Carbohydrate utilization test		Strain name	Isolation details			Received from**
	Exp. 1*	Exp. 2	Exp. 1	Exp. 2		Source	Location	Date	
Type B strains									
81-23	✓	✓	✓	✓	Hobbs FT50	Herring	UK	1960s?	UR
81-30	✓	✓	✓	✓	Eklund 17B	Pacific sediments	USA	1965	NCIMB
83-01	✓	✓	✓	✓	Eklund 2B	Pacific sediments	USA	1965	UR
86-17	✓	✓	✓	✓	Colworth BL15	Haddock	Norway	1960s?	UR
87-02	✓	✓	✓	✓	CDC 3875	Human stool from botulism case	Iceland	1981	CDC
87-04	nt	✓	nt	✓	CDC 4672 U-1	Whey – botulism case	Iceland	1983	CDC
90-04	✓	nt	nt	✓	2129B	Unknown	France	1950s?	NFPA
93-06	✓	✓	nt	✓	CDC 5900	Human stool from botulism case	Italy	1986	CDC
93-10	✓	nt	nt	✓	Kapchunka B2	Dried salted whole whitefish [Kapchunka]	USA	1981	NFPA
93-11	✓	✓	nt	✓	Kapchunka B5	Dried salted whole whitefish [Kapchunka]	USA	1981	NFPA
02-51	✓	✓	nt	✓	ATCC 17844	Unknown	Unknown	Unknown	UH
05-20	✓	✓	nt	✓	IFR 05/020	Scallops	Canada	2005	IFR
05-25	✓	✓	✓	✓	IFR 05/025	Dried egg pasta (“Fettucine”)	UK	2005	IFR
05-29	✓	nt	nt	✓	IFR 05/029	Dried egg pasta (“Trucioli”)	UK	2005	IFR
Type E strains									
81-26	✓	nt	✓	✓	Beluga	Fermented Beluga whale [Muktuk]	USA	1950	UR
81-27	nt	nt	nt	✓	Foster B96	Smoked fish	USA	1981	UR
81-31	✓	✓	✓	✓	Hazen 36208	Labrador smoked salmon	USA	1934	NCIMB
86-21	✓	✓	✓	✓	Prevot P34	Pond-reared freshwater perch	France	1951	UR
87-01	✓	✓	nt	✓	Dolman VH	Pickled herring	Canada	1949	CDC
93-07	✓	nt	✓	✓	CDC 7854	Dried salted whole mullet (“Faseikh”)	Egypt	1991	CDC
93-08	✓	nt	nt	✓	CDC 8073	Human stool from botulism case	USA	1991	CDC
02-06	✓	nt	nt	nt	CB-K-3E	Rainbow trout surface	Finland	1995	UH
02-07	nt	✓	nt	✓	CB-K-9E	Rainbow trout intestines	Finland	1995	UH
02-09	✓	✓	nt	nt	CB-K-18E	Lake trout intestines	Finland	1995	UH
02-10	nt	nt	nt	✓	CB-K-19E	Whitefish roe	Finland	1996	UH
02-13	✓	nt	nt	nt	CB-K-22E	Burbot skin	Finland	1996	UH
02-14	✓	nt	nt	nt	CB-K-23E	Burbot intestines	Finland	1996	UH

02-15	✓	✓	nt	✓	CB-K-24E	Burbot surface	Finland	1996	UH
02-21	✓	nt	nt	nt	CB-K-31E	Vendace	Finland	1996	UH
02-22	✓	nt	nt	✓	CB-K-32E	Vendace	Finland	1996	UH
02-24	✓	nt	nt	nt	CB-K-34E	Frozen whitefish roe	Finland	1996	UH
02-25	✓	✓	✓	nt	CB-K-36E	Vacuum-packed cold smoked rainbow trout	Finland	1996	UH
02-26	✓	✓	nt	nt	CB-K-37E	Vacuum-packed hot smoked whitefish	Finland	1996	UH
02-29	✓	✓	nt	nt	CB-K-40E	Vacuum-packed cold smoked rainbow trout	Finland	1996	UH
02-32	✓	nt	nt	nt	CB-K-44E	Trout intestine	Finland	1998	UH
02-33	✓	nt	nt	nt	CB-K-45E	Trout skin	Finland	1996	UH
02-35	✓	nt	nt	nt	CB-S-3E	Sediment mud	Finland	1995	UH
02-43	nt	nt	nt	✓	CB-S-20E	Fishfarm sediment	Finland	1997	UH
02-47	✓	nt	nt	nt	CB-S-27E	Fishfarm sediment	Finland	1997	UH
02-50	✓	nt	nt	nt	CB-R-24	Unknown	Finland	Unknown	UH
08-02	nt	nt	✓	nt	NCTC 8266	Local home canned salmon (Nanaimo)	Canada	1944	HPA
Type F strains									
86-32	✓	✓	✓	✓	Eklund 202F	Pacific sediments	USA	1965	UR
86-33	✓	✓	✓	✓	Hobbs FT10	Atlantic Herring	UK	1970s	UR
86-34	✓	✓	nt	✓	Craig 610	Salmon from Columbia River	USA	1965	UR
06-01	✓	nt	✓	nt	IFR 06/001	Scallops	Canada	2006	IFR
06-05	✓	✓	nt	✓	IFR 06/005	Scallops	Canada	2006	IFR

Table 3.1 Details of non-proteolytic *C. botulinum* strains used in chill temperature incubation studies (Chapter 3) and carbohydrate assessment studies (Chapter 4)

*****(Chill temperature incubation test) Exp. 1: Minimal growth temperature of non-proteolytic *C. botulinum* strains (Stringer and Webb, unpublished data), Exp. 2: Variability in growth of strains of non-proteolytic *C. botulinum* at refrigeration temperatures; (Carbohydrate utilization test) Exp. 1: Initial experiment – Effect of carbohydrate source on growth and neurotoxin formation by non-proteolytic *C. botulinum* (API® strips), Exp. 2: Main test – Effect of carbohydrate source on growth and neurotoxin formation by non-proteolytic *C. botulinum* (tests conducted in bottles)

**Originally from: CDC = C. Hatheway, Centers for Disease Control, USA; HPA = K. Grant, Health Protection Agency, UK, IFR = isolated at IFR; NCIMB = National Collection of Industrial Food and Marine Bacteria, UK; NFPA = V. Scott, National Food Processors Association, USA; UH = M. Lindström, University of Helsinki, Finland; UR = J. Crowther, Unilever Research, UK, ✓ – strains tested, nt – strains not tested

3.2.2 Culture medium

In both experiments, cultures of non-proteolytic *C. botulinum* strains were maintained in double-strength Robertson's Cooked Meat Medium (CMM) with added glucose (Southern Group Laboratory, Corby, UK). Growth of strains at chill temperatures was determined in PYGS broth (Lund *et al.*, 1990). Viande-levure (VL) layered blood agar, Reinforced Clostridial Medium containing 5% (w/v) skim milk (RCM) (Lund *et al.*, 1985), and Tryptic Soya Agar (TSA) (Difco) plates were used to test cultures for purity.

3.2.3 Spore preparation

For spore-forming pathogens, the bacteria are commonly present in the environment in the spore form, and spores are the most common source of food contamination (Carlin, 2011). Thus it was appropriate to use spores as the inoculum in these experiments. In order to minimise additional variables, spores were not heat shocked before inoculation.

Different crops of spores of each strain were prepared for use in experiment one and experiment two. Spores were produced in 20 ml of CMM. The medium was inoculated with 100 µl of an actively growing culture in PYGS broth using strict anaerobic technique. After incubation for 8 days at 20°C, the spore-containing medium was filtered through a sterile 50 ml disposable vacuum filtration system with 20 µm nylon net filter (Steriflip-NY20, Millipore) and the spores harvested from the filtrate by centrifugation (10 000 x *g*, 4.0°C, 20 min). The spores were then washed five-times in 10 ml sterile ice-cold 0.85% (w/v) NaCl using the same centrifugation conditions, and resuspended in 1 ml of ice-cold 0.85% NaCl and stored at 4.0°C until required.

Purity and the absence of significant proteolytic activity were checked on VL agar plates and RCM + milk plates. To verify that the spore preparations were not contaminated with aerobic bacteria, spores were plated on TSA plates and incubated at ambient temperature for 2 days. The viable count was determined on PYGS agar incubated for 2 days at 30°C. All plates (except TSA) were incubated anaerobically under headspace of H_2/CO_2 (90:10, v/v). Before use, spores were diluted to the desired concentration using a 10-fold dilution series.

3.2.4 Experimental design

In experiment one, Stringer and Webb (unpublished data) measured the growth of 40 non-proteolytic *C. botulinum* strains at 10 different incubation temperatures (3.0°C, 4.0°C, 5.0°C, 5.5°C, 6.0°C, 6.5°C, 7.0°C, 8.0°C, 9.0°C and 10.0°C) using one spore concentration (s = 10^6 spores $tube^{-1}$). Three replicate tubes each containing 20 ml PYGS medium were used at each temperature. Inoculated tubes were examined at regular intervals for turbidity and gas production for 90 days.

In experiment two, growth of 24 strains at different inoculum concentrations (s = 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 spores $tube^{-1}$) was measured. Based on the results from Stringer and Webb, it was decided to conduct these tests at a temperature of 6.0°C. Five replicate tubes each containing 20 ml of PYGS medium were inoculated and examined for turbidity and gas production periodically for up to 60 days. In order to test the sterile handling and for visual comparison, uninoculated tubes were incubated in both experiments.

3.2.5 Experimental procedure

In experiment one, spores were diluted to one concentration, s = 10^7 ml^{-1}, whereas in experiment two, there was a series of seven concentrations, from s = 10^7 ml^{-1} to a final concentration of s = 10^1 ml^{-1}. Dilution series were prepared in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK). Growth tests were conducted in 20 ml of anaerobic PYGS medium in glass tubes (18 x 150 mm, BellCo Glass, USA) sealed with a butyl rubber stopper crimped closed with an aluminium cap. Each of the tubes contained a Durham tube (10 x 75 mm) (Laboratory Sales, UK) to capture gas formed thereby permitting observation of growth. An aliquot of 100 µl of appropriate spore suspension was injected into three or five replicates of PYGS broth, using a sterile 1 ml Terumo syringe with sterile 27Gx½ inch needle (Figure 3.2).

To ensure anaerobic conditions before injection, the syringes were flushed with N_2/H_2 (90:10, v/v) using the Hungate technique. Two dilutions, s = 10^3 ml^{-1} and s = 10^2 ml^{-1} were also spread plated, in duplicate, onto PYGS agar and incubated anaerobically at 30°C for 2 days in an atmosphere of H_2/CO_2 (90:10, v/v) to confirm the inoculated spore concentration. Spore suspensions, diluent

and culture medium were maintained on ice during these procedures. The above technique was used in both experiment one and experiment two.

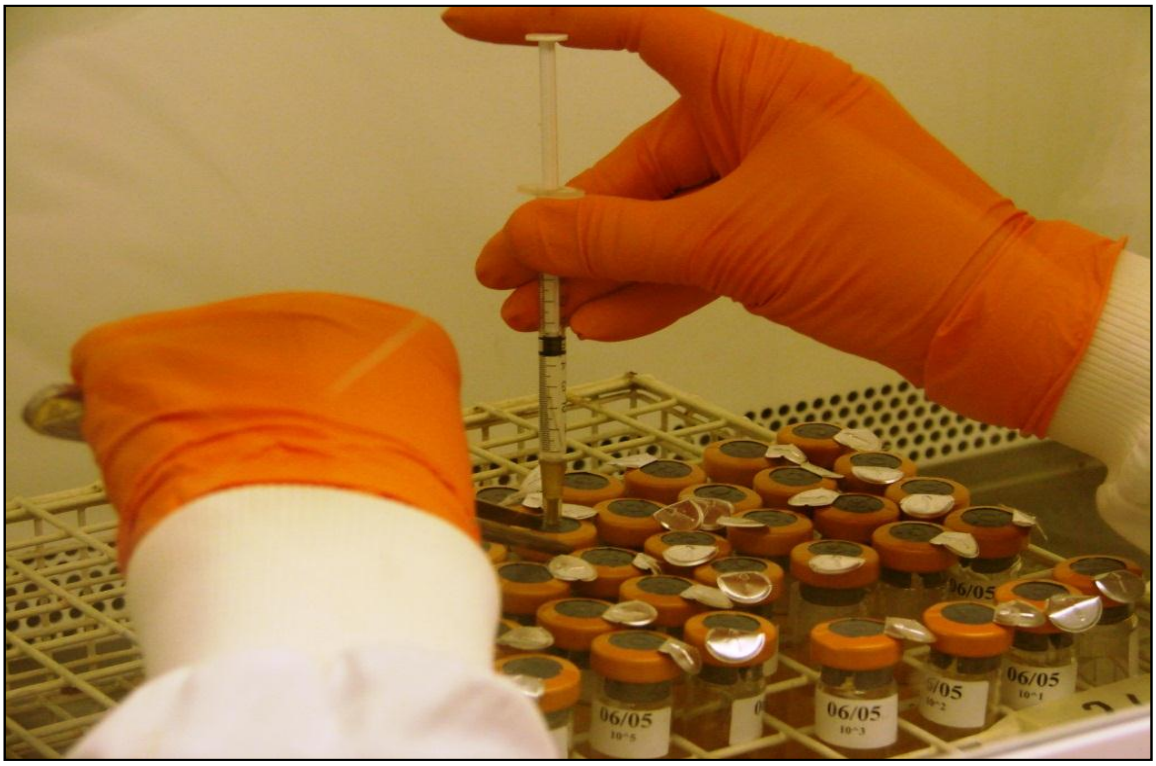

Figure 3.2 Inoculation of test tubes with non-proteolytic *C. botulinum* spore suspension

3.2.6 Temperature monitoring

In both experiments inoculated tubes were incubated in racks in a water-bath in low-temperature incubators (Figure 3.3). In experiment two, one standard probe (Type I) connected to a wireless transmitter (MD5031, Don Whitley Scientific, Shipley, UK) was placed in each rack in a tube containing 20 ml of test media. The temperature was recorded at intervals of 20 min using a Labguard2 Monitoring System (Don Whitley Scientific, Shipley, UK). Temperature probes were calibrated against a reference mercury thermometer certified by the National Physical Laboratory. At the end of the 60-day incubation period, the data were analysed, and the mean temperature and variation in temperature were determined.

Figure 3.3 Incubation of inoculated tubes in water-bath, with inserted probes

3.2.7 Probability of growth for non-proteolytic *C. botulinum* strains at chill temperatures

For both experiments the probability of growth, P(s,t), at time, t, at a particular inoculum level, s, was estimated based on the observed fraction of growth

$$f = \frac{N_p}{N_t} \qquad 3.1$$

where N_p is the number of tubes showing growth and N_t is the total number of tubes.

The probability of growth as a function of storage time was fitted to a monotonically increasing function (Whiting and Oriente, 1997) using the MS Excel SOLVER® add-in

$$P\,(s,t) = \frac{P_{max}}{(1 + e^{k(\tau - t)})} \qquad 3.2$$

where P_{max} is the maximum probability of growth after 90 days (experiment one) or 60 days (experiment two) ($0 \leq P_{max} \leq 1$), k is a rate at which tubes show growth (days^{-1}), t is the incubation time (days), and τ is the time (days) corresponding with the midpoint of the above function, i.e. time to probability of growth equal to 0.5 ($0 \leq \tau \leq 60$ or 90 days). The UCL and LCL for parameter τ were calculated according to equation 2.18.

Data points, where the number of positive tubes did not increase from a previous observation were not used in the fitting procedure model which makes the estimated τ-value slightly smaller than if all observations are used. Therefore, the model predicts growth sooner and provides a conservative bias to the prediction.

The parameter τ is the calculated value that takes account of the time when first visible growth was observed, t_1, and can be used to estimate the time that precedes the observation of growth, t_{min} (G. C. Barker, personal communication)

$$t_{min} = \tau(\min(f_\tau)) \qquad 3.3$$

where $f_\tau = \frac{t_1}{\tau}$ is a fraction of the τ-value which corresponds with the first observation of growth at time, t_1. A minimal fraction, $\min(f_\tau)$ = 0.58 obtained from many observations of t_1, is used with the obtained value of τ to establish t_{min} for each case. The minimal fraction corresponds with a value from G.C. Barker and M.W. Peck (personal communication) which is estimated for relatively small spore inoculum and so represents a conservative approach at high spore concentrations (which is crucial in calculating the risk associated with non-proteolytic *C. botulinum* spores in relation to chilled foods). The t_{min} is an important parameter that will be used in calculation of risk presented to minimally heated chilled foods by non-proteolytic *C. botulinum* (see section 5.2.4.4).

Additionally, for each strain the time, t_p, at which the probability of growth reached a particular value $P_{(\alpha)}$, can be calculated as

$$t_P = \tau - (2.31 \log(\frac{\left(\frac{P_{max}}{P_{(\alpha)}}\right) - 1}{k})) \qquad 3.4$$

where τ, k, P_{max} are the estimated parameters.

The above procedure for calculation of the probability of growth for non-proteolytic *C. botulinum* strains at chill temperatures was only applied to results for experiment two.

Based on MPN counts (Most Probable Number) the probability, P_1, for one spore to initiate growth at 6.0°C at each observation time was calculated from (Lund *et al.*, 1990)

$$P_1 = \frac{MPN\ of\ spores\ resulting\ in\ observed\ growth}{number\ of\ inoculated\ spores\ (measured\ by\ plate\ count)} \qquad 3.5$$

Calculation of the probability of growth from a single spore was based only on data from experiment two, as only the experimental design applied in this study included different inocula concentrations.

3.2.8 Variability in growth of strains of non-proteolytic *C. botulinum* at chill temperatures

Assessment of non-proteolytic *C. botulinum* strain variability is based on a single parameter, τ, that characterizes growth from different inocula at different incubation temperatures. The τ-value was calculated for data from experiment one and experiment two according to equation 3.2.

The τ-value is a consistent way to represent the dynamics of growth in particular conditions (depending on for example: inoculum concentration, salt concentration, pH, temperature) and is a fitted parameter that describes the probability of observing growth with time (measured in days). The τ-value is an estimate of the time when half of the replicates show growth.

An unsupervised hierarchical clustering algorithm, with a dissimilarity metric based on Euclidean distance, was used to show strain classification patterns based on the τ parameter. Clustering was conducted using the heatmap.2 function in the gplot package of the R Statistics Environment (Team, 2012). The

clustering was conducted for the τ-value calculated from results of both experiments. In the heat map, colours are assigned to represent the τ-value for each strain at the particular incubation temperature (experiment one) or spore inoculum concentration (experiment two).

The clustering procedure for data originating from experiment one was based on the parameter τ calculated for temperatures of 6.0°C, 6.5°C, 7.0°C, 8.0°C, 9.0°C and 10.0°C for one inoculum concentration (s = 10^6 spores tube^{-1}). Since not all strains grew at the lower incubation temperatures (3.0°C, 4.0°C, 5.0°C and 5.5 °C) these data were not included in the analysis.

In the second experiment, the parameter τ was calculated for all spore concentrations, however only τ-values calculated for higher inoculum concentrations were used in the clustering analysis. Whiting and Oriente (1997) indicated that parameter values obtained for lower spore concentration were not always reliable (i.e. sometimes negative values for P_{max} and *k* or UCL of τ greater than incubation time), therefore the clustering technique was conducted on τ-values corresponding with s = 10^6, 10^5 and 10^4 spores tube^{-1}. Strains 81-23, 02-51 and 81-31 showed poor growth at chill temperatures and were excluded from the analysis. In order to allow comparisons between experiment one and experiment two, clustering of strains based on the τ-value from experiment one was conducted only for strains used in experiment two.

Based on the results of experiment two, the mean (μ_τ) and the standard deviation (σ_τ) of τ-value for cluster members corresponding to an inoculum of s = 10^6, 10^5, 10^4 spores tube^{-1} were calculated and a normal cumulative distribution function, CDF, was used to represent the strain variability

$$f(\tau) = \frac{1}{2}\left[1 + \mathrm{erf}\left(\frac{\tau - \mu_\tau}{\sigma_\tau\sqrt{2}}\right)\right] \qquad 3.6$$

where erf is the “error function”.

3.2.9 Statistical analysis

In order to assess whether the τ-values differ significantly between variables of the cluster, the MANOVA was used. The multiple comparison of clusters centroids (means of the cluster scores for the τ-value) was conducted using the Games – Howell test. The significant difference was tested based on variables from experiment two (s = 10^6, 10^5 and 10^4 spores $tube^{-1}$). The statistical analysis was performed by SPSS Statistics software (IBM). The differences were tested at a 5% significance level.

In addition, the MANOVA test was used to assess whether the cluster membership of non-proteolytic *C. botulinum* strains is associated with isolation source. The analysis was performed on τ-values calculated for type E strains from experiment one, for three temperatures (5.5°C, 6.0°C and 6.5°C).

3.3 Results

3.3.1 Temperature variation during incubation

The actual temperatures at which strains were incubated in experiment two are summarised in Table 3.2. Temperature monitoring data for experiment one are not available. There were slight variations in mean incubation temperature between strains stored in the four different incubators. The mean incubation temperatures over the 60 day period ranged from 5.8°C to 6.2°C, with a typical standard deviation of 0.2°C (Table 3.2).

Temperature (°C)											
Toxin type	**Strain**	**Min**	**Max**	**Mean**	**S.D.**	**Toxin type**	**Strain**	**Min**	**Max**	**Mean**	**S.D.**
Type B	81-30 83-01 86-17 05-20	4.9	6.4	5.8	0.2	**Type B**	81-23 87-02 87-04 93-06	4.8	6.8	5.9	0.2
	93-11 02-51 05-25	5.4	6.8	6.1	0.1	**Type E**	86-21 87-01 02-07	5.2	6.8	6.1	0.2
Type E	81-31						02-09				
	02-15 02-25 02-26 02-29	4.2	6.6	6.0	0.2	**Type F**	86-32 86-33 86-34 06-05	5.0	6.8	6.2	0.2

Table 3.2 Temperature variation for each strain of non-proteolytic *C. botulinum* during observations of growth at 6.0°C for 60 days in experiment two

3.3.2 Growth at chill temperatures

Full details of incubation temperatures, different inoculum concentrations and growth responses from spores in PYGS broth measured in experiments one and two are given in Appendix 3 and Appendix 4.

3.3.2.1 Experiment one

The number of days prior to the first observation of growth, at each refrigeration temperature, was recorded for each tested strain (Table 3.3). All strains showed growth at 8.0°C, 9.0°C and 10.0°C. Only one strain (81-31) did not grow at temperatures below 8.0°C (Table 3.3). The same strain also showed weak or no growth when tested in experiment two (only one tube showing growth after 40 days of incubation at the concentration of s = 10^6 spores $tube^{-1}$, no additional

growth observed after 60 days). Weak growth at chill temperature was also observed for strain 81-23 (Table 3.3).

Toxin type	Strain	Time to first observation of growth (days) at specified incubation temperature (°C)								
		4.0	5.0	5.5	6.0	6.5	7.0	8.0	9.0	10.0
Type B	81-23	> 90	> 90	> 90	> 90	39	25	14	7	5
	81-30	> 90	77	39	25	18	14	8	7	5
	83-01	> 90	42	32	18	14	11	8	6	5
	86-17	> 90	> 90	28	18	14	11	8	6	4
	87-02	> 90	> 90	35	21	14	11	7	6	5
	90-04	> 90	> 90	> 90	28	18	14	11	7	5
	93-06	> 90	32	25	18	11	8	5	5	4
	93-10	> 90	39	25	14	11	11	6	6	5
	93-11	> 90	39	25	14	11	11	7	5	4
	02-51	> 90	> 90	56	25	14	11	11	6	5
	05-20	> 90	35	25	18	14	11	8	6	5
	05-25	> 90	46	70	21	14	11	7	5	5
	05-29	> 90	> 90	35	25	14	11	7	6	5
Type E	81-26	> 90	67	32	18	18	11	8	6	5
	81-31	> 90	> 90	> 90	> 90	> 90	> 90	25	14	11
	86-21	> 90	25	18	11	11	11	6	5	4
	87-01	> 90	> 90	35	18	14	14	8	6	5
	93-07	> 90	> 90	> 90	21	18	11	7	5	4
	93-08	> 90	28	14	11	8	8	6	5	4
	02-06	> 90	> 90	25	14	11	11	8	5	5
	02-09	> 90	56	21	14	11	11	7	5	5
	02-13	> 90	32	14	11	8	8	7	5	4
	02-14	> 90	> 90	> 90	49	18	11	7	6	4
	02-15	67	21	14	11	11	8	6	5	4
	02-21	> 90	39	28	11	11	8	6	5	4
	02-22	> 90	> 90	> 90	35	14	11	6	5	4
	02-24	> 90	> 90	46	25	14	11	6	5	4
	02-25	> 90	> 90	32	14	11	8	7	5	4
	02-26	> 90	81	39	21	14	11	8	5	5
	02-29	> 90	25	21	11	11	11	6	5	4
	02-32	> 90	32	21	14	11	11	7	5	4
	02-33	> 90	32	21	14	11	11	7	5	5
	02-35	> 90	39	21	14	11	8	7	5	4
	02-47	> 90	> 90	> 90	18	11	11	6	5	4
	02-50	> 90	42	21	18	11	11	7	5	4
Type F	86-32	> 90	42	35	21	18	11	7	5	5
	86-33	> 90	53	28	18	14	11	6	5	4
	86-34	> 90	> 90	35	21	18	11	6	5	4
	06-01	> 90	> 90	39	25	18	14	7	6	5
	06-05	> 90	> 90	42	25	18	14	8	6	5

Table 3.3 Time to first visible growth (days) (t_1) of non-proteolytic *C. botulinum* strains in PYGS at different incubation temperatures from 10^6 spores $tube^{-1}$ during 90 days of incubation (experiment one) [data of Stringer and Webb]
Tests were also carried out at 3.0°C, but growth was not observed in 90 days

At an incubation temperature of 3.0°C growth was not observed after 90 days, and at 4.0°C only one strain (02-15) showed visible growth on day 67. At 5.0°C and 5.5°C, some strains did not show visible growth within 90 days of incubation,

while others formed gas after 21 days of incubation (e.g. strain 02-15 at 5.0°C). At 6.0°C, all strains (with the exception of 81-23 and 81-31) grew, with the first tubes being turbid at day 11 (strains 86-21, 93-08, 02-13, 02-15, 02-21 and 02-29). The longest period prior to visible growth was noted on day 56 for strain 02-14. One tube inoculated with spores of this strain did not show growth for the remaining incubation period. A temperature of 6.0°C was used in experiment two with different spore inoculum concentrations.

At a lower incubation temperatures the time to visible growth was greater (Table 3.3). Growth was first observed after 4, 5, 5, 8, 8, 11, 14, 21 and 67 days at 10.0°C, 9.0°C, 8.0°C, 7.0°C, 6.5°C, 6.0°C, 5.5°C, 5.0°C and 4.0°C, respectively.

Within each set of incubation conditions, tubes showed growth in narrow bands. For example, at 7.0°C, 93% of tubes in which growth occurred were first positive between days 8 and 14, whereas at 10.0°C, 97% of positive tubes showed growth between days 4 and 6 (Table 3.4).

Although most growth was initiated within a short time period, occasionally growth was delayed by several days. At 4.0°C only one strain showed growth at day 67. None of the remaining strains tested showed visible growth within 90 days of incubation. At 5.0°C and 5.5°C only one out of three tubes showed growth for some strains e.g. 81-30, 02-26 and 86-32.

3.3.2.2 Experiment two

The number of days prior to the first observation of growth (turbidity and for gas production) for each spore inoculum concentration is presented in Table 3.5.

For experimental convenience, a smaller number of strains were tested in experiment two compared to experiment one. All 24 strains used in experiment two grew at 6.0°C, from s = 10^6 spores $tube^{-1}$. Exceptionally, for strains 81-23 and 81-31 only two and one tube (out of five) were positive for growth, with the first growth observed on day 37 and 40, respectively. When tubes were inoculated with approximately one spore, growth was observed in more than half of the tested strains, with strains 02-09 and 02-25 growing in all five tubes.

Day of incubation	Temperature (°C)																	
	4.0		5.0		5.5		6.0		6.5		7.0		8.0		9.0		10.0	
	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	59	49
5	0	0	0	0	0	0	0	0	0	0	0	0	1	1	57	48	55	46
6	0	0	0	0	0	0	0	0	0	0	0	0	25	21	50	42	3	3
7	0	0	0	0	0	0	0	0	0	0	0	0	46	38	8	7	0	0
8	0	0	0	0	0	0	0	0	3	3	18	14	34	28	2	2	0	0
11	0	0	0	0	0	0	8	7	45	37	72	60	8	7	0	0	2	2
14	0	0	0	0	3	3	30	24	31	26	21	18	3	3	3	3	1	1
18	0	0	0	0	9	8	26	22	29	24	3	3	0	0	0	0	0	0
21	0	0	3	3	15	13	12	9	5	4	0	0	0	0	0	0	0	0
25	0	0	4	3	14	11	22	18	0	0	3	3	1	1	0	0	0	0
28	0	0	3	3	8	7	3	3	1	1	0	0	2	2	0	0	0	0
32	0	0	11	9	7	6	4	3	0	0	0	0	0	0	0	0	0	0
35	0	0	2	2	7	6	3	3	0	0	0	0	0	0	0	0	0	0
39	0	0	5	4	11	9	1	1	1	1	0	0	0	0	0	0	0	0
42	0	0	7	6	2	1	1	1	1	1	0	0	0	0	0	0	0	0
46	0	0	2	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0
49	0	0	1	1	4	3	1	1	1	1	0	0	0	0	0	0	0	0
53	0	0	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	1	1	3	3	1	1	0	0	0	0	0	0	0	0	0	0
60	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
63	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
67	2	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
74	0	0	5	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
77	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
81	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
84	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
88	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total (out of 120 tubes)	2	2	52	43	87	73	112	93	117	98	117	98	120	100	120	100	120	100

Table 3.4 Total number of tubes and percentage of tubes showing visible growth from 10^6 spores $tube^{-1}$ of non-proteolytic *C. botulinum* when incubated at chill temperatures for 90 days (experiment one)

+ve – number of tubes showing visible growth, % – percentage of tubes showing visible growth

Toxin type	Strain	Time to first observation of growth (days) at specified spore inoculum concentration (spores tube^{-1})						
		10^6	10^5	10^4	10^3	10^2	10^1	10^0
Type B	**81-23**	37	> 60	> 60	> 60	> 60	> 60	> 60
	81-30	16	17	20	25	25	31	> 60
	83-01	12	14	17	20	23	24	37
	86-17	12	14	17	23	30	37	34
	87-02	12	16	21	28	26	28	28
	87-04	16	20	24	30	34	> 60	> 60
	93-06	12	16	18	18	23	24	> 60
	93-11	11	12	14	16	17	19	> 60
	02-51	20	59	60	> 60	> 60	> 60	> 60
	05-20	11	12	16	20	23	25	32
	05-25	17	18	20	23	25	> 60	> 60
Type E	**81-31**	40	59	40	> 60	> 60	> 60	> 60
	86-21	11	11	12	13	16	18	18
	87-01	11	12	13	16	17	19	20
	02-07	11	11	12	14	16	17	21
	02-09	11	11	12	13	16	18	20
	02-15	11	11	12	14	16	18	19
	02-25	11	11	12	13	16	17	18
	02-26	11	11	14	16	21	26	54
	02-29	11	11	11	14	16	18	20
Type F	**86-32**	12	14	19	20	27	23	26
	86-33	16	17	19	20	23	25	37
	86-34	13	17	19	23	25	32	> 60
	06-05	17	20	23	26	> 60	> 60	> 60

Table 3.5 Time to first visible growth (days) (t_1) of non-proteolytic *C. botulinum* strains in PYGS at different inoculum concentrations at 6.0°C during 60 days of incubation (experiment two)

At higher spore inoculum concentration the time at which growth was first observed (at 6.0°C) was shorter. The first positive tubes were observed at 11 day when inoculated with s = 10^6, 10^5 and 10^4 spores tube^{-1} and 13, 16, 17 and 18 days when inoculated with s = 10^3, 10^2, 10^1 and 10^0 spores tube^{-1}, respectively. As tubes were observed at discrete times, it is possible that the same time observed for the three largest inoculum concentrations is associated with the experimental procedure. Growth may have been first visible between days when growth was examined.

In a similar manner to experiment one, tubes showed growth in narrow bands. With s = 10^6 spores tube^{-1}, 77% of positive tubes at day 16, showed growth between day 11 and 16 (Table 3.6). Moreover for the following strains: 93-11, 05-20, 86-21, 87-01, 02-07, 02-09, 02-15, 02-25, 02-26, 02-29 all five tubes first showed growth on day 11.

Day of incubation	Inoculum concentration (spores tube^{-1})													
	10^6		10^5		10^4		10^3		10^2		10^1		10^0	
	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
11	50	41	34	26	5	4	0	0	0	0	0	0	0	0
12	9	7	8	7	25	21	0	0	0	0	0	0	0	0
13	8	7	7	6	1	1	7	6	0	0	0	0	0	0
14	9	7	9	8	9	8	22	18	0	0	0	0	0	0
16	15	13	6	5	7	6	9	8	29	23	0	0	0	0
17	8	7	5	4	4	3	4	3	3	2	3	2	0	0
18	4	3	14	11	6	5	5	4	5	4	12	11	2	2
19	1	1	6	5	9	8	1	1	2	2	13	11	4	3
20	3	2	8	7	7	6	11	9	2	2	7	6	8	7
21	1	1	2	2	3	2	3	2	1	1	0	0	8	7
23	1	1	2	2	9	8	8	7	10	7	5	4	2	2
24	1	1	2	2	4	3	2	2	1	1	3	2	1	1
25	0	0	0	0	4	3	9	8	8	7	3	2	0	0
26	0	0	0	0	1	1	3	2	2	2	2	2	1	1
27	0	0	0	0	0	0	2	2	3	2	1	1	0	0
28	0	0	0	0	0	0	3	2	2	2	4	3	1	1
30	0	0	1	1	2	2	3	2	8	7	6	5	0	0
31	0	0	0	0	1	1	0	0	5	4	1	1	0	0
32	0	0	0	0	2	2	1	1	2	2	1	1	1	1
34	0	0	0	0	3	2	1	1	2	2	1	1	1	1
37	1	1	0	0	0	0	1	1	5	4	3	2	2	2
40	1	1	0	0	1	1	0	0	2	2	3	2	1	1
42	0	0	0	0	0	0	0	0	0	0	2	2	1	1
44	0	0	0	0	0	0	2	2	1	1	0	0	0	0
48	0	0	0	0	1	1	1	1	1	1	2	2	2	2
51	0	0	0	0	0	0	0	0	0	0	1	1	0	0
54	0	0	0	0	0	0	1	1	1	1	2	2	1	1
59	1	1	2	2	0	0	0	0	0	0	0	0	1	1
60	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Total (out of 120 tubes)	113	95	106	89	105	87	99	82	95	79	75	63	37	34

Table 3.6 Total number of tubes and percentage of tubes showing visible growth from different inoculum concentrations of non-proteolytic *C. botulinum* spores during incubation at 6.0°C for 60 days (experiment two)
+ve – number of tubes showing visible growth, % – percentage of tubes showing visible growth

3.3.3 Probability of growth for non-proteolytic *C. botulinum* strains at chill temperatures

The procedure for calculation of growth for non-proteolytic *C. botulinum* strains is described in section 3.2.7. An example visualisation for strain 83-01 as a function of seven different inocula concentrations is presented in Figure 3.4. In this case P_{max} is unity for 10^1 to 10^6 spores tube^{-1}, and is 0.4 for s = 10^0 spores tube^{-1}.

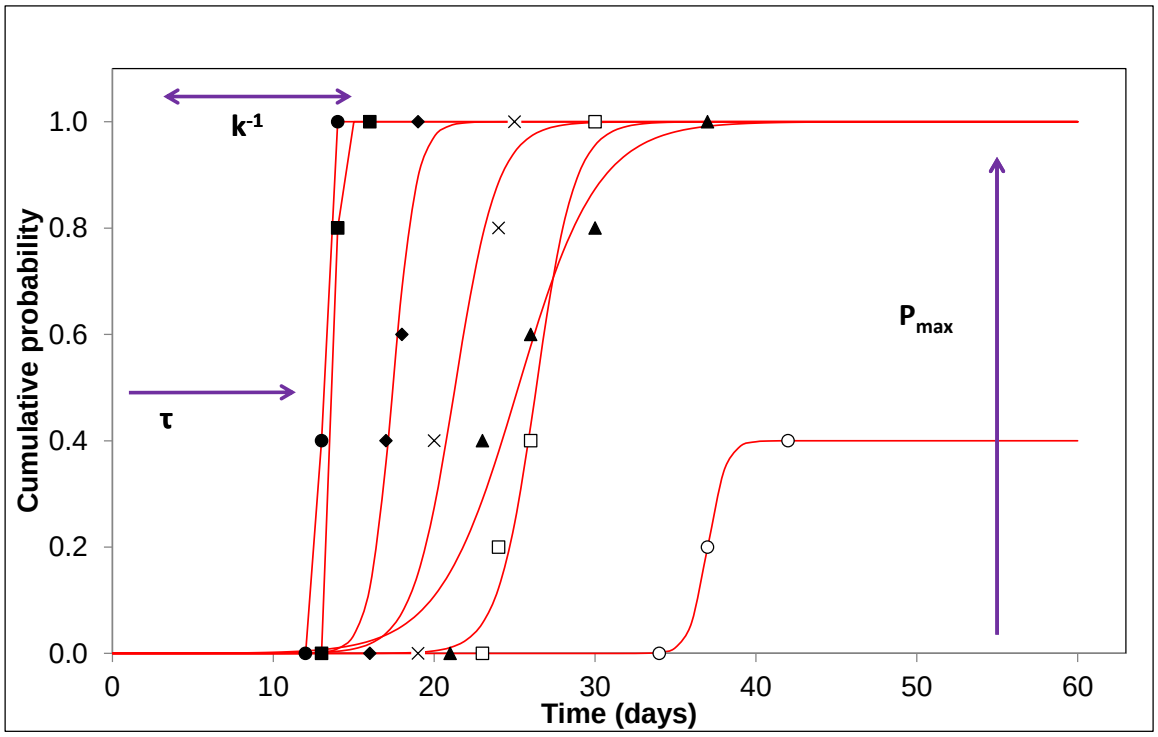

Figure 3.4 Increasing probability of growth with time for strain 83-01 during 60 days of incubation at 6.0°C
Symbols represent data (with following spore concentrations, s: ● 10^6, ■ 10^5, ♦ 10^4, x 10^3, ▲ 10^2, □ 10^1, ○ 10^0 spores tube^{-1}), lines represent the fit of data to continuous function. P_{max} is a maximum probability of growth, τ – estimate of time when half of the replicates show growth and *k* is the rate at which tubes show growth.

The parameter values of the fitted model (see section 3.2.7) for the highest three spore inoculum concentrations for 24 strains (tested at 6.0°C in the second experiment) are given in Table 3.7. When there is no growth after 60 days of incubation the cells in Table 3.7 are empty. P_{max} for a majority of strains at all three inoculum concentrations (s = 10^6, 10^5 and 10^4 spores tube^{-1}) was equal to one after 60 days of incubation. The only exceptions were noted for strains: 81-23 for which P_{max} = 0.4 at s = 10^6 spores tube^{-1}; 86-17, with P_{max} = 0.8 at s = 10^5 spores tube^{-1}; 02-51 with P_{max} = 0.2 at s = 10^5 spores tube^{-1}; and 81-31 with P_{max} = 0.2 for all three inocula concentrations. These strains gave limited growth in experiment two.

Strain*	Inoculum concentration (spores tube^{-1})																							
	10^6								10^5								10^4							
	t_1 [a]	t_{min} [b]	k [c]	τ [d]	f_τ [e]	LCLτ	UCLτ [f]	$t_{0.1}$ [g]	t_1	t_{min}	k	τ	f_τ	LCLτ	UCLτ	$t_{0.1}$	t_1	t_{min}	k	τ	f_τ	LCLτ	UCLτ	$t_{0.1}$
81-23																								
81-30	16	9	6.6	15.9	100	15.9	15.9	15.6	17	10	1.8	17.8	96	17.0	18.5	16.6	20	12	0.3	21.4	94	20.1	22.7	15.0
83-01	12	8	17.9	13.0	92	13.0	13.0	12.9	14	8	8.8	13.8	100	13.8	13.8	13.6	17	10	1.4	17.4	98	17.4	17.5	15.8
86-17	12	7	8.9	12.0	100	12.0	12.0	11.8	14	8	0.5	13.2	100	12.7	13.6	8.7	17	10	9.6	17.0	100	17.0	17.0	16.8
87-02	12	8	2.3	13.1	92	13.0	13.1	12.1	16	10	1.1	16.5	97	16.2	16.9	14.5	21	14	0.7	23.5	89	23.1	23.9	20.5
87-04	16	9	18.1	15.0	100	15.0	15.0	14.9	20	12	2.1	20.3	99	20.2	20.3	19.2	24	14	1.9	24.8	97	24.4	25.1	23.6
93-06	12	7	18.4	12.0	100	12.0	12.0	11.9	16	10	1.4	17.2	93	17.1	17.2	15.6	18	11	1.6	18.9	95	18.8	18.9	17.4
93-11	11	6	18.5	10.5	100	10.5	10.5	10.4	12	7	8.8	12.2	99	12.2	12.2	11.9	14	8	8.1	14.1	100	14.0	14.1	13.8
02-51	20	12	1.1	20.7	97	20.6	20.8	18.6																
05-20	11	6	20.7	10.5	100	10.5	10.5	10.4	12	7	2.2	12.3	98	12.2	12.3	11.2	16	9	1.2	15.9	101	15.8	16.0	14.1
05-25	17	10	8.0	16.9	100	16.9	16.9	16.7	18	10	8.7	18.0	100	18.0	18.0	17.8	20	12	1.1	21.5	93	21.4	21.6	19.5
81-31																								
86-21	11	6	21.0	10.5	100	10.5	10.5	10.4	11	6	21.0	10.5	100	10.5	10.5	10.4	12	7	18.0	11.5	104	11.5	11.5	11.4
87-01	11	6	18.5	10.5	100	10.5	10.5	10.4	12	7	9.2	11.8	100	11.8	11.8	11.6	13	8	1.3	13.9	93	13.6	14.3	12.2
02-07	11	6	18.5	10.5	100	10.5	10.5	10.4	11	6	18.5	10.5	100	10.5	10.5	10.4	12	7	18.0	11.5	104	11.5	11.5	11.4
02-09	11	6	18.5	10.5	100	10.5	10.5	10.4	11	6	18.6	10.9	100	10.9	10.9	10.8	12	7	17.6	11.5	104	11.5	11.5	11.4
02-15	11	6	18.5	10.5	100	10.5	10.5	10.4	11	6	18.0	10.5	100	10.5	10.5	10.4	12	7	17.6	11.5	104	11.5	11.5	11.4
02-25	11	6	18.5	10.5	100	10.5	10.5	10.4	11	6	18.0	10.5	100	10.5	10.5	10.4	12	7	17.6	11.5	104	11.5	11.5	11.4
02-26	11	8	18.5	13.0	84	10.5	10.5	12.9	11	6	18.0	10.5	100	10.5	10.5	10.4	14	8	17.5	13.5	104	13.5	13.5	13.4
02-29	11	6	18.5	10.5	100	10.5	10.5	10.4	11	6	18.0	10.5	100	10.5	10.5	10.4	11	6	20.7	10.5	105	10.5	10.5	10.4
86-32	12	7	7.4	12.1	100	11.7	12.4	11.8	14	9	0.9	16.3	86	16.2	16.4	13.8	19	11	1.4	19.6	97	19.3	19.9	18.0
86-33	16	9	7.0	15.9	100	15.9	15.9	15.6	17	10	2.8	17.5	97	17.5	17.5	16.7	19	13	0.6	22.3	85	22.2	22.4	18.3
86-34	13	8	2.8	13.5	96	13.4	13.6	12.7	17	10	2.8	17.5	97	17.4	17.6	16.7	19	11	2.2	19.7	96	19.7	19.8	18.7
06-05	17	10	1.6	17.8	95	17.8	17.9	16.5	20	12	7.7	19.9	100	19.7	20.2	19.7	23	16	1.3	24.0	83	27.5	28.0	19.5

Table 3.7 Estimated values for k, τ with its upper and lower 95% confidence limits, t_1, f_τ and $t_{0.1}$ calculated for growth of non-proteolytic *C. botulinum* strains when incubated at 6.0°C for 60 days with different spore concentrations (experiment two)

* Strains ordered according to toxin type produced, [a] t_1 – time when the first growth was observed (see Table 3.3 and Table 3.5); [b] t_{min} – time preceding observation of growth; [c] k – rate of the increase of positive tubes (days^{-1}); [d] τ – time when growth in half of the tubes is observed; [e] f_τ – fraction of τ-value that gives minimum time for observed growth; [f] LCLτ and UCLτ are lower and upper 95% confidence intervals; [g] time at which the probability of growth reached 0.1

The parameter *k* is highly variable with some dependence on spore inoculum concentration. Greater values were observed at s = 10^6 spores tube^{-1} (ranging from 1.1 to 21.0 days^{-1}) compared with an inocula of s = 10^4 spores tube^{-1} (from 0.3 to 20.7 days^{-1}). Nevertheless, Whiting and Oriente (1997) considered, the estimated value for *k* as difficult to interpret and not the most important parameter in the model. Also for the purpose of this study, the emphasis was on parameter τ and not the rate at which tubes showed growth.

From the risk assessment point of view the parameter τ is of crucial importance. It is a parameter obtained from fitting a mathematical function to a set of observed data, i.e. time leading to bacterial growth. The parameter τ increased with decreasing inoculum concentration, ranging from 10.5 to 20.7 (mean value $\mu_\tau = 13.0$) days for an inoculum of s = 10^6 spores tube^{-1}, from 10.5 to 20.3 (mean value $\mu_\tau = 14.2$) days for s = 10^5 spores tube^{-1} and from 10.5 to 27.8 (mean value $\mu_\tau = 17.1$) days for an inoculum of s = 10^4 spores tube^{-1}. Moreover, a lower concentration of spores (when s = 10^4, s = 10^5 or s = 10^6 spores tube^{-1}) generally increased the confidence interval about the τ-value with the highest interval observed for strain 81-30 [20.1; 22.7] when s = 10^4 spores tube^{-1}.

From the time when the first growth was observed, t_1, (Table 3.5) the fraction of τ, giving the minimal time for observed growth, f_τ, can be calculated. For the main experiment, the smallest f_τ was 84%, 86% and 83% for s = 10^6, 10^5 and 10^4 spores tube^{-1} respectively, and individual values varied for different strain, different inoculum concentration and temperature (Table 3.7).

Both the τ-value and t_1 are parameters which inform about the time prior to the observation of bacterial growth (as indicated by turbidity or gas production). Previous experience in our laboratory had indicated that turbidity or gas production can be detected when the concentration of cells is typically ~10^6 ml^{-1} (M. W. Peck, personal communication). One requirement of risk assessment is to assess the time, before bacterial growth can be initiated. Due to a complex combination of several variables (e.g. strain, incubation conditions, spore load) it is not possible to indicate the absolutely safe time. Nevertheless, the concept of t_{min} (time preceding observation of growth) estimates the shortest time when any

growth can be expected (G. C. Barker, personal communication). Time, t_{min} is a safety boundary; a time before the product safety is highly probable (Figure 3.5).

Calculation of the minimal time for expected growth, t_{min}, follows from equation 3.3. A value of min(f_τ) = 58% (G. C. Barker and M. W. Peck, personal communication) was used to calculate values for t_{min} based on data from the second experiment (Table 3.7). For different strains the smallest t_{min} for s = 10^6 and 10^5 spores tube^{-1} was calculated to be 6 days and the highest 12 days (mean value $\mu_{\tau_{min}} = 8$). For s = 10^4 spores tube^{-1} these times were 6 and 16 days respectively (mean value $\mu_{\tau_{min}} = 11$).

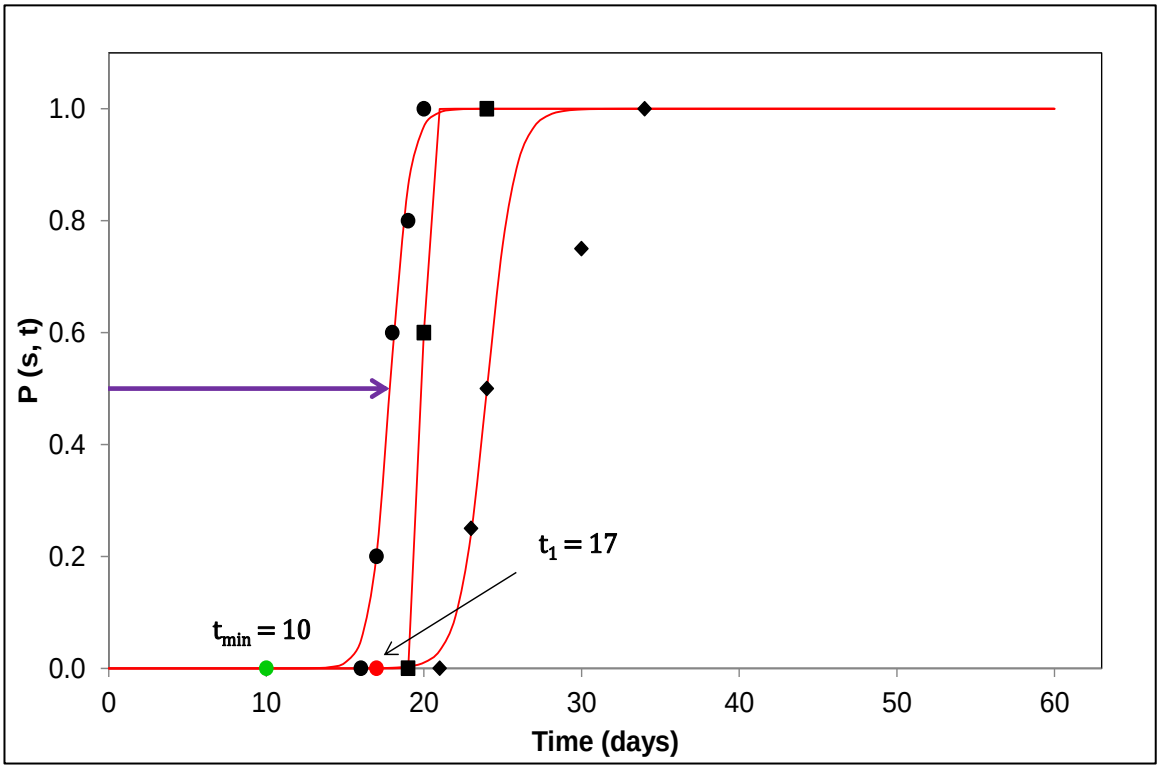

Figure 3.5 Observed time of growth, t_1; time preceding observation of growth, t_{min} and time when half of tubes show growth, τ, for non-proteolytic *C. botulinum* strain 06-05 (from s = 10^6, 10^5 and 10^4 spores tube^{-1} incubated at 6.0°C for 60 days)
Symbols represent data (with following spore concentrations, s: ● 10^6, ■ 10^5, ♦ 10^4 spores tube^{-1}) lines represent the fit of data to continuous function, t_{min} (●), t_1 (●)

The estimated parameters of *k*, τ and P_{max} were also used to calculate the time, $t_{0.1}$ (Table 3.7), at which the probability of growth is 0.1. Due to the small range of time when all replicates become positive, $t_{0.1}$ is often only 0.1 – 2.1 days smaller

than the estimated value of τ. The difference was much greater with decreasing spore concentration.

Strain variability is also expressed in the relationship between the probability of growth from a single spore, P_1, and the incubation time at 6.0°C (Table 3.8). P_1 for growth at 6.0°C of twenty-two strains ranged from 3.5×10^{-7} to 5.1×10^{-1} when incubated for 20 days, 1.9×10^{-7} to 1.0 when incubated for 30 days and 3.8×10^{-7} to 1.0 after 60 days of incubation. In relation to toxin type, the probability of growth after 60 days of incubation was: from 3.8×10^{-7} (strain 02-51) to 5.2×10^{-2} (strain 05-20) for ten type B strains, from 1.2×10^{-4} (strain 87-01) to 1.0 (strains 86-21 and 02-09) for eight type E strains, and 1.6×10^{-4} (strain 06-05) to 1.3×10^{-2} (strain 86-32) for four type F strains. For two strains (81-23 and 81-31) the probability of growth from a single spore was less than 2.0×10^{-7}. Although in many cases the calculated P_1 is very small, there is still a possibility of growth that cannot be ignored.

		$-\log_{10} P_1$[a] of growth		
	Strain	**Incubation day 20**	**Incubation day 30**	**Incubation day 60**
Type B	81-23	> 6.70	> 6.70	6.70
	81-30	4.33	3.22	1.13
	83-01	3.48	1.56	0.48
	86-17	5.27	4.94	4.63
	87-02	5.28	3.58	2.22
	87-04	5.46	4.11	3.81
	93-06	3.45	1.17	1.17
	93-11	1.31	0.64	0.64
	02-51	6.42	5.71	5.42
	05-20	3.29	1.95	0.29
	05-25	4.84	3.01	1.99
Type E	81-31	> 6.70	> 6.70	6.70
	86-21	0.29	0.00	0.00
	87-01	3.02	2.92	2.92
	02-07	1.79	1.68	1.68
	02-09	0.39	0.00	0.00
	02-15	0.73	0.58	0.58
	02-25	0.70	0.51	0.51
	02-26	4.47	2.05	1.26
	02-29	1.43	1.08	1.08
Type F	86-32	3.91	1.74	0.88
	86-33	4.33	3.23	2.42
	86-34	4.12	2.32	1.97
	06-05	5.57	4.30	3.80

Table 3.8 The effect of incubation time on the probability (P_1) of growth from a single spore of 24 strains of non-proteolytic *C. botulinum* in PYGS during 60 days of incubation at 6.0°C

[a] P_1 was calculated as the proportion of spores that resulted in growth (as indicated by MPN) at 6.0°C compared to number of inoculated spores (measured by plate count)

3.3.4 Variability in growth of strains of non-proteolytic *C. botulinum* at chill temperatures

The time when half of the tubes become positive (τ-value) is shorter at higher incubation temperatures (Figure 3.6), and with higher spore inoculum concentrations (Figure 3.7). Interestingly, the estimated τ-values for type E strains is generally lower than for those of type B and F strains when tested at 6.0°C (Figure 3.7). A similar but weaker pattern was observed when τ-values were measured at one spore concentration (s = 10^6 spores $tube^{-1}$) and different incubation temperatures (Figure 3.6). Thus under these chill temperature test conditions non-proteolytic *C. botulinum* type E strains generally grew faster than type B or F strains.

Moreover, it is apparent from both Figure 3.6 and Figure 3.7 that the uncertainty associated with τ-value decreased with higher incubation temperature and with higher inoculum concentration.

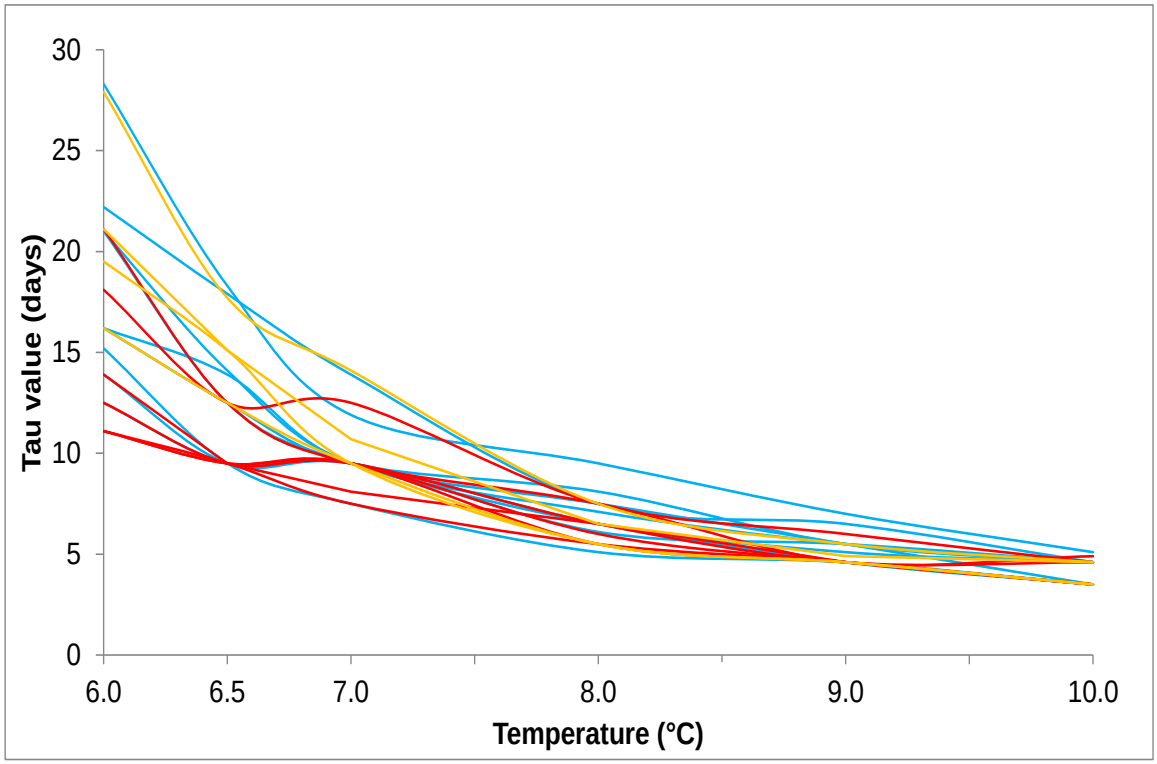

Figure 3.6 Estimates of τ-value for non-proteolytic *C. botulinum* strains incubated at chill temperatures with 10^6 spores $tube^{-1}$ for 90 days (data from experiment one)
Type B strains (blue line), type E strains (red line), type F strains (yellow line)

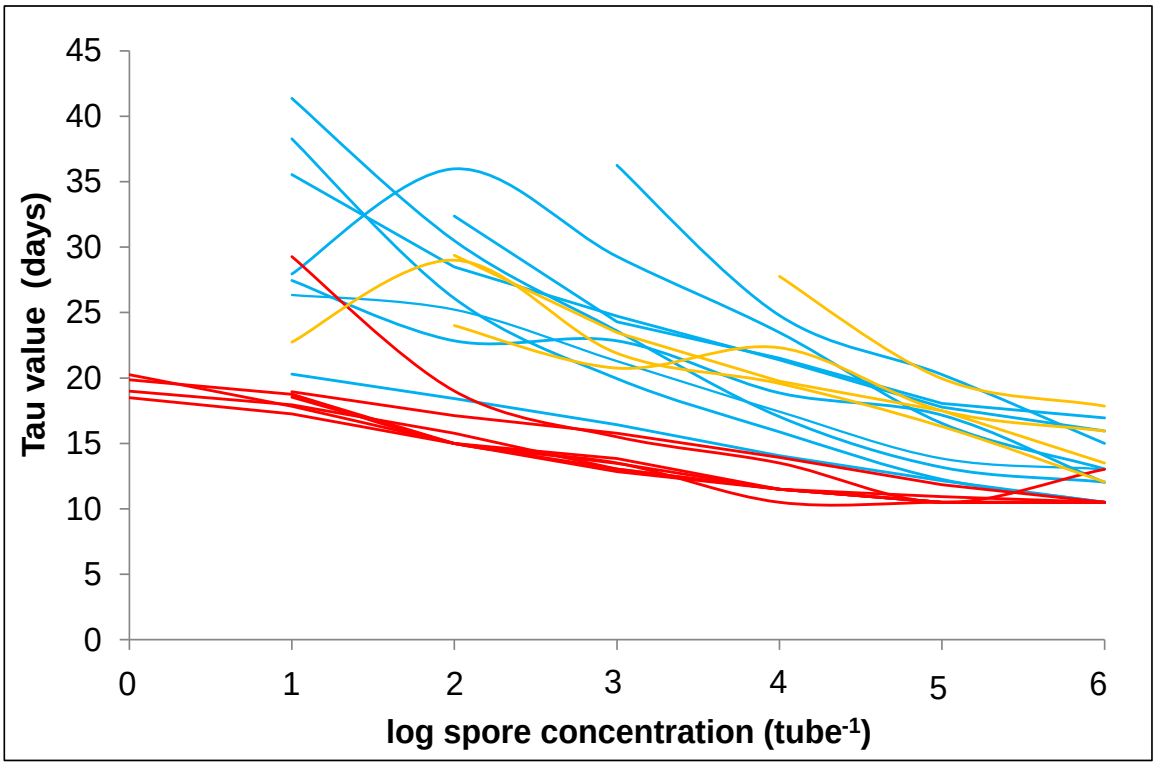

Figure 3.7 Estimates of τ-value for non-proteolytic *C. botulinum* strains incubated at 6.0°C for 60 days with various spore inoculum concentrations (data from experiment two)
Type B strains (blue line), type E strains (red line), type F strains (yellow line)

The clustering of non-proteolytic *C. botulinum* strains using heat maps (Figure 3.8 and Figure 3.9) was based on τ-values (days) calculated in experiments one and two (see section 3.2.8). The columns are spore concentrations (spores tube^{-1}) or temperatures (°C), and the rows represent 19 strains (experiment one) or 21 strains (experiment two). The τ-values are summarised in Table 3.9.

In the heat maps, colours at a particular point are assigned to represent the τ-value for the strain at the particular inoculum concentration or incubation temperature, with red corresponding to a small number (< 9 in experiment one and < 15 in experiment two) of days needed for half of tested tubes to show growth, green corresponds to a high number (> 20 in experiment one and > 25 in experiment two) of days, and black corresponds to an intermediate number of days (Figure 3.8 and Figure 3.9).

Toxin type	Strain	Temperature (°C)						Inoculum concentration (spores tube^{-1})						
		6.0	6.5	7.0	8.0	9.0	10.0	10^6	10^5	10^4	10^3	10^2	10^1	10^0
Type B	81-30	22.2	17.9	13.9	7.5	6.5	4.6	15.9	17.8	21.4	24.7	28.5	35.5	
	83-01	22.6	14.1	11.1	7.5	5.5	4.6	13.0	13.8	17.4	21.3	25.2	26.3	
	86-17	16.2	13.9	9.5	7.5	5.5	3.5	12.0	13.2	17.0	23.6	30.5	41.4	
	87-02	21.0	14.1	9.5	7.1	5.5	4.6	13.1	16.5	23.5	29.3	36.0	27.9	
	87-04							15.0	20.3	24.8	36.2			
	93-06	15.2	9.5	7.5	5.1	4.6	3.5	12.0	17.2	18.9	22.8	22.8	27.5	
	93-11	13.9	9.5	9.5	6.5	4.6	3.5	10.5	12.2	14.1	16.4	18.4	20.3	
	05-20	16.2	12.5	9.5	8.1	5.5	4.6	10.5	12.3	15.9	19.9	26.1	38.3	
	05-25	21.0	12.5	9.5	6.5	5.1	4.6	16.9	18.0	21.5	24.3	32.4		
Type E	86-21	11.1	9.5	9.5	5.5	4.6	3.5	10.5	10.5	11.5	13.0	15.8	17.9	19.0
	87-01	18.1	12.5	12.5	7.5	6.0	4.6	10.5	11.8	13.9	15.8	17.1	18.9	
	02-07							10.5	10.5	11.5	13.5	15.0	18.5	
	02-09	12.5	9.5	9.5	6.5	4.6	4.6	10.5	10.9	11.5	13.0	15.0	17.8	20.3
	02-15	11.1	9.5	7.5	5.5	4.6	3.5	10.5	10.5	11.5	13.8	15.0	18.7	19.9
	02-25	13.9	9.5	8.1	6.5	4.6	3.5	10.5	10.5	11.5	12.8	15.0	17.3	18.5
	02-26	21.1	12.5	9.5	7.5	4.6	4.9	13.0	10.5	13.5	15.5	19.0	29.3	
	02-29	11.1	9.5	9.5	6.0	4.6	3.5	10.5	10.5	10.5	13.5	15.0	18.7	
Type F	86-32	21.1	15.1	10.7	6.5	4.9	4.6	12.1	16.3	19.6	21.9	29.0	22.7	
	86-33	16.2	12.5	9.5	5.5	4.6	3.5	15.9	17.5	22.3	20.8	24.0		
	86-34	19.5	15.1	9.5	5.5	4.6	3.5	13.5	17.5	19.7	23.5	29.4		
	06-05	27.9	17.7	14.1	7.5	5.5	4.6	17.8	19.9	24.0				

Table 3.9 Tau (τ) value calculated for non-proteolytic *C. botulinum* strains incubated at chill temperatures with 10^6 spores tube^{-1} for 90 days (experiment one) and incubated at 6.0°C for 60 days with different spore inoculum concentrations (experiment two)
An empty cell indicates that growth was not observed at the tested conditions (except strains 87-04 and 02-07, which were not tested in experiment one)

In general the estimated τ-value for all strains at 8.0°C, 9.0°C and 10.0°C are very close (Figure 3.8). The variability of 19 tested strains primarily arises from growth at lower temperatures (6.0°C, 6.5°C and 7.0°C), which are more typical refrigeration temperatures. Four clusters have been identified. The cluster 3 is predominant and mainly composed of type E strains (five out of seven strains are of type E). It contains the fastest growing strains with a small τ-value at higher temperatures and a not significantly higher τ-value at lower temperatures. Clusters 1 and 2 contain the next fastest growing strains, and are closely linked (Figure 3.8). Cluster 1 is characterized by strains with low τ-value at higher temperatures, moderate at 6.5°C and 7.0°C, and higher τ-values at 6.0°C. This cluster is composed of 2 type F strains, 3 type B strains and one type E strain. The τ-values in the cluster 2 are of moderate magnitude at 6.0°C and lower at 6.5°C and 7.0°C, and the cluster is composed of two type B strains, one type F and one type E strain. Finally, two slowly growing strains, 81-30 (type B) and 06- 05 (type F), with a lower τ-value at 8.0°C, 9.0°C and 10.0°C, higher at 6.0°C and moderate at 6.5°C and 7.0°C are in the cluster 4.

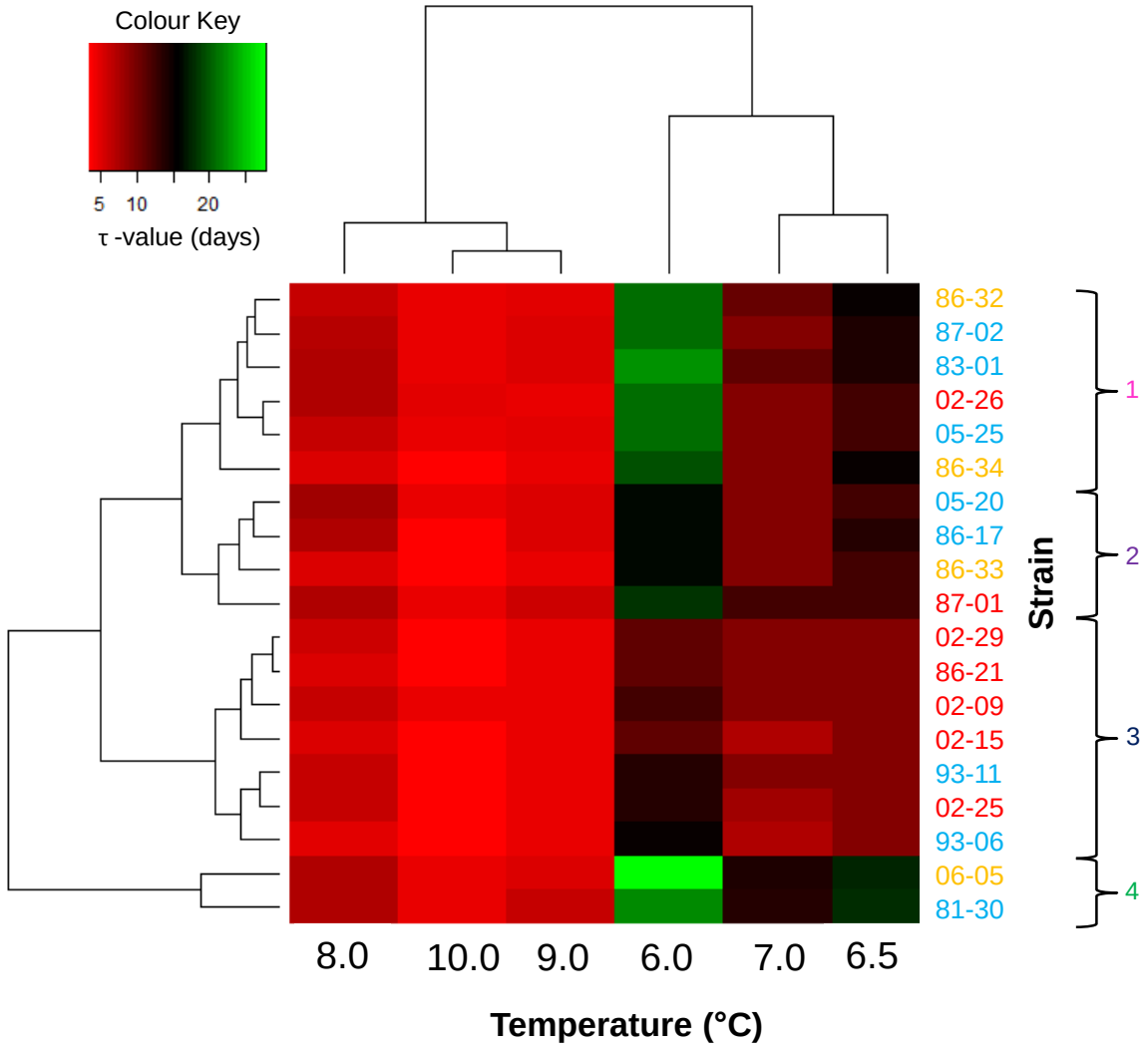

Figure 3.8 Hierarchical clustering of τ-values calculated for non-proteolytic *C. botulinum* strains incubated at chill temperatures for 90 days with 10^6 spores $tube^{-1}$ (experiment one)
Different strain number colour corresponds to toxin type: type B strains (blue colour), type E strains (red colour), type F strains (yellow colour). Tested strains were classified into four clusters: cluster 1 (*n* = 6), cluster 2 (*n* = 4), cluster 3 (*n* = 7) and cluster 4 (*n* = 2)

Figure 3.9 summarises the effect of spore inoculum concentration on the τ-value. Strain variability is lower at a higher inoculum concentration. The τ-value calculated for 21 strains was grouped into four clusters. Cluster 4 contains the fastest growing strains, and is the most homogeneous cluster characterised by type E strains with low τ-value across three inoculum concentrations (Figure 3.9). Cluster 3 contains the next fastest growing strains and is composed of four type B and two type E strains with low (at s = 10^6 spores $tube^{-1}$) and moderate (at s = 10^5 and 10^6 spores $tube^{-1}$) τ-values. Clusters 1 and 2 contain the slowest growing strains. Cluster 1 is predominant and composed of type B and F strains with a moderate τ-value at s = 10^5 spores $tube^{-1}$ and a high τ-value at s = 10^4

spores tube^{-1}. Two strains 06-05 and 87-04 form cluster 2 which is characterised by a high τ-value calculated for s = 10^4 spores tube^{-1}.

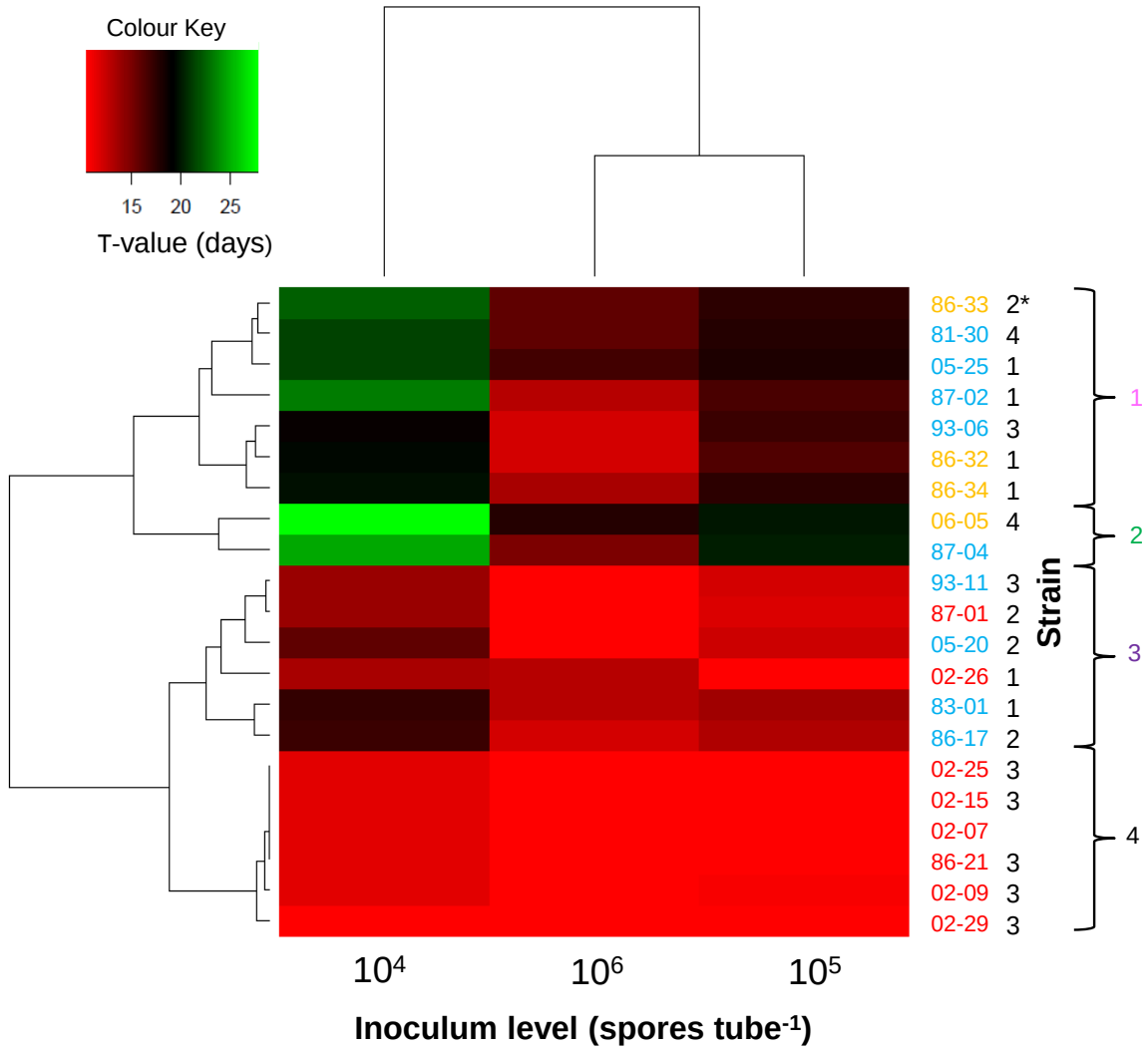

Figure 3.9 Hierarchical clustering of τ-value calculated for non-proteolytic *C. botulinum* strains incubated at 6.0°C for 60 days with 10^6, 10^5 and 10^4 spores tube^{-1} (experiment two)
Different strain number colour corresponds to toxin type: type B strains (blue colour), type E strains (red colour), type F strains (yellow colour). Tested strains were classified into four clusters: cluster 1 (*n* = 7), cluster 2 (*n* = 2), cluster 3 (*n* = 6) and cluster 4 (*n* = 6)
* Assigned cluster membership based on analysis of data in experiment one

One very important feature of the output of the clustering analysis is the agreement of cluster membership determined in the two independent datasets. In particular, strains in cluster 3 in experiment one and cluster 4 in experiment two are characterised by the most rapid growth (smallest τ-values). These two clusters contain similar (primarily type E) strains. Strains of cluster 4 (experiment one) and cluster 2 (experiment two) are those with the slowest growth (highest

τ-values). Strains in cluster 1 (both experiments), cluster 2 (experiment one) and cluster 3 (experiment two) (with τ-value greater in cluster 1) are those with moderate growth (Table 3.10), with strains assigned to cluster one showing faster growth.

Para-meter	Cluster	Experiment one						Cluster	Experiment two		
		Temperature (°C)							Inoculum concentration (spores tube^{-1})		
		6.0	6.5	7.0	8.0	9.0	10.0		10^6	10^5	10^4
Min	Cluster 1 (n = 6)	19.5	12.5	9.5	5.5	4.6	3.5	Cluster 1 (n = 7)	12.0	16.3	18.9
Max		22.6	15.1	11.1	7.5	5.5	4.9		17.0	18.1	23.5
μ_τ		21.1	13.9	10.0	6.8	5.0	4.5		14.2	17.3	21.0
σ_τ		1.0	1.2	0.7	0.8	0.4	0.5		2.0	0.6	1.7
Min	Cluster 4 (n = 2)	22.2	17.7	13.9	7.5	5.5	4.6	Cluster 2 (n = 2)	15.0	20.0	24.8
Max		27.9	17.9	14.1	7.5	6.5	4.6		17.9	20.3	27.8
μ_τ		25.1	17.8	14.0	7.5	6.0	4.6		16.4	20.1	26.3
σ_τ		4.0	0.1	0.1	0.0	0.7	0.0		2.0	0.2	2.1
Min	Cluster 2 (n = 4)	16.2	12.5	9.5	5.5	4.6	3.5	Cluster 3 (n = 6)	10.5	10.5	13.5
Max		18.1	13.9	12.5	8.1	6.0	4.6		13.0	13.8	17.4
μ_τ		16.7	12.9	10.3	7.2	5.4	4.1		11.6	12.3	15.3
σ_τ		1.0	0.7	1.5	1.1	0.6	0.6		1.3	1.2	1.7
Min	Cluster 3 (n = 7)	11.1	9.5	7.5	5.1	4.6	3.5	Cluster 4 (n = 6)	10.5	10.5	10.5
Max		15.2	9.5	9.5	6.5	4.6	4.6		10.5	10.9	11.5
μ_τ		12.7	9.5	8.7	5.9	4.6	3.7		10.5	10.3	11.3
σ_τ		1.7	0.0	1.0	0.6	0.0	0.4		2.1	0.2	0.4
Significant differences between clusters (p < 0.05)									1 > 3, 2 > 3, 1 > 4, 2 > 4	2 > 1, 2 > 3, 2 > 4, 1 > 3, 1 > 4, 3 > 4	2 > 1, 2 > 3, 2 > 4, 1 > 3, 1 > 4, 3 > 4

Table 3.10 Comparisons of τ-values calculated for clusters of non-proteolytic *C. botulinum* strains from 10^6 spores tube^{-1} incubated at chill temperatures for 90 days (experiment one) and from three inoculum concentration incubated at 6.0°C for 60 days (experiment two)

A MANOVA analysis indicated a significant difference between clusters (F-statistic = 14.3, p < 0.01) based on Wilk's Lambda. The differences among clusters were observed in the mean τ-value of three spore concentrations with s = 10^6 spores tube^{-1} having the smallest impact in assigning strains into clusters (Table 3.10).

Although the heat map procedure was conducted for a single parameter (τ-value), its actual determination is based on two different and independent factors: incubation temperature and inoculum concentration. The consistency in the strain classification pattern shows a natural biological relationship between these strains (based on the ability to growth at chill temperatures).

Interestingly, in general, the strain clustering pattern is closely associated with toxin type. In both experiment one and experiment two, strains that belong to the slowest growing (cluster 2 for experiment two) are of type B and F, whereas those strains showing fastest growth are in cluster 4 and are of type E (cluster 4 for experiment two – Figure 3.8). There were a few inconsistences in this pattern e.g. strains 87-01 and 02-26 (type E) clustered with type B strains (experiment two). This could be associated with strain property (e.g. variability in spore germination) or experimental error (e.g. unequal number of inoculated spores). Cluster membership (including its association with toxin type) was also obtained when cluster analysis was conducted on raw data i.e. all tubes showing growth as a function of incubation time (data not shown). A closer analysis of the results of the clustering procedure showed that most of the type E strains that showed the fastest growth originated from one geographical location. Therefore, to test whether rapid growth at chill temperature was a general property of type E strains or restricted to strains from Finland, the MANOVA test was used. The analysis was conducted on results for type E strains from experiment one, as strains used in this experiment represent a wider range of isolation source compared with strains used in experiment two. For type B and F strains sources were too diverse (13 type B strains isolated from nine different locations and five type F – three locations), therefore these strains were not analysed. The τ-value calculated for type E strains listed in Table 3.3 (except strain 81-31) and only for temperatures: 5.5°C, 6.0°C and 6.5°C were used in analysis (since these gave most variation in strain response). The statistical analysis (based on Wilks' Lambda) revealed that there is no significant difference in τ-value calculated for strains isolated from different countries (Table 3.11). Therefore it can be concluded that the more rapid growth of non-proteolytic *C. botulinum* type E strains at chill temperature is not associated with their source of isolation.

Toxin type	Isolated in	Number	*p*-value	*F*-value
Type E	Canada	1	0.31	1.61
	Finland	15		
	France	1		
	USA	2		
	Egypt	1		

Table 3.11 Results of MANOVA analysis for τ-value for type E strains of non-proteolytic *C. botulinum* isolated from different geographical localisations.
p – probability that the result observed in a study could have occurred by a chance
F– ratio of the between groups variance and the within variance

In addition to the aforementioned time to growth differences between toxin types, there is also a relationship between cluster membership and the probability of growth from a single spore (Table 3.12).

Cluster number	Toxin type	Strain	$-log_{10}P_1$
Cluster 1	F	86-33	2.42
	B	81-30	1.13
	B	05-25	1.99
	B	87-02	2.22
	B	93-06	1.17
	F	86-32	0.88
	F	86-34	1.97
Cluster 2	F	06-05	3.80
	B	87-04	3.81
Cluster 3	B	93-11	0.64
	E	87-01	2.92
	B	05-20	0.29
	E	02-26	1.26
	B	83-01	0.48
	B	86-17	4.63
Cluster 4	E	02-25	0.51
	E	02-15	0.58
	E	02-07	1.68
	E	86-21	0.00
	E	02-09	0.00
	E	02-29	1.08

Table 3.12 The probability of growth from a single spore of non-proteolytic *C. botulinum* at 6.0°C for 60 days in relationship to cluster membership and toxin type (Clusters from experiment two – Figure 3.9)

The strains belonging to cluster 2 are those with the lowest probability of growth from a single spore ($P_1 = 1.58 \times 10^{-4}$). Cluster 1 consists of strains for which P_1 ranges from 3.8×10^{-3} to 1.3×10^{-1}. For the majority of strains in cluster 3, P_1 ranges from 5.5×10^{-2} to 5.1×10^{-1}. Nevertheless the probability of growth from a single spore of two strains (87-01 and 86-17) is much lower, 1.2×10^{-3} and 2.3×10^{-5} respectively. The highest value of P_1 is observed for strains belonging to cluster 4 and it ranges from 8.3×10^{-2} to 1.0, with the exception of strain 02-07 for which the $P_1 = 2.1 \times 10^{-2}$. The above relationship with the clusters is not a great surprise, as the probability of spore growth is also calculated from the dataset generated in experiment two. The observed deviation (e.g. for strains 86-32, 87-01, 86-17 and 02-07) could be due to fact that the calculation of P_1 was based on the exact number of inoculated spores as measured by plate count.

However the overall close relationship between the two clustering variables strongly indicates that any deviation in number of added spores did not have a measurable effect on growth at chill temperature.

Based on the strain clustering pattern in experiment two, the mean and standard deviation of the τ-value for the three inoculum concentrations were calculated within each (experiment two) cluster (Table 3.10), and a cumulative distribution function, CDF, of a normal distribution was used to represent the variability.

The influence of spore concentration on the distribution of the τ-value within each cluster is shown in Figure 3.10. As expected, the time needed for observed growth is higher with lower spore inoculum concentration. This is due to the fact that within a larger population there is a greater probability that at least one spore will initiate growth and that fewer multiplications are necessary for visible growth. In addition for each inoculum concentration the variability associated with each cluster is smaller than the variability that would be associated with the whole population of non-proteolytic *C. botulinum* strains (Figure 3.10). Similarly, at the lower inoculum concentrations, there is greater strain variability in response to incubation conditions. With a high concentration of spores ($s = 10^6$ spores tube^{-1}) turbidity is expected within 10.5 – 16.4 days, whereas with a lower inoculum ($s = 10^4$ spores tube^{-1}) it is expected within 11.3 – 26.3 days (Table 3.10). Interestingly, the inoculum concentration has a relatively small effect on strains of cluster 1 as the distribution of τ-value for $s = 10^4$ and 10^5 spores tube^{-1} does not differ significantly. The biggest changes are observed for strains belonging to the slowest growing strains in cluster 2, where the distribution of τ-value is affected by spore inoculum concentration.

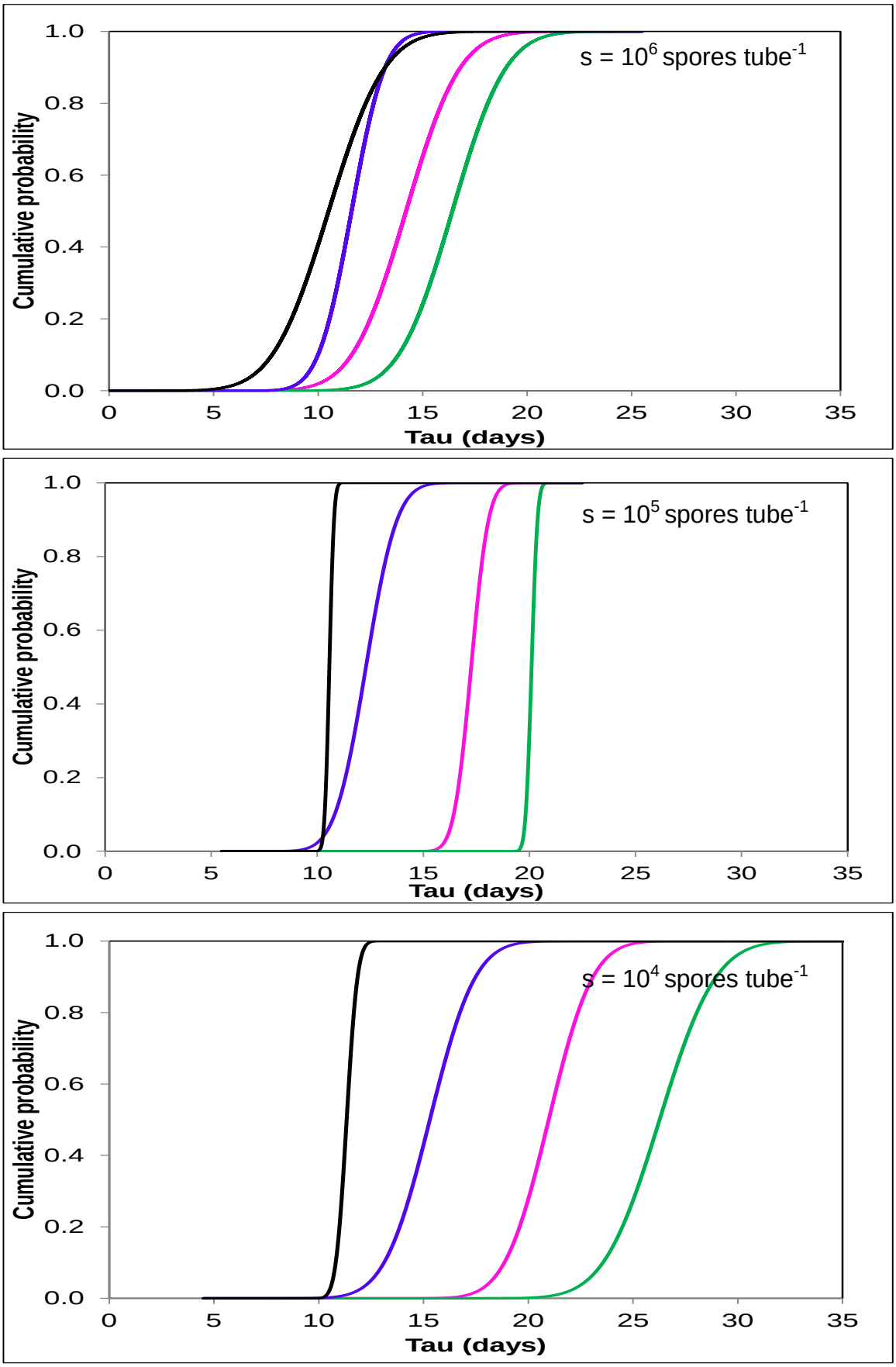

Figure 3.10 The cumulative probability, CDF, of the τ-value based on incubation of non-proteolytic *C. botulinum* strains assigned to four clusters at 6.0°C for 60 days with different concentrations of spores
Cluster 4 (black line), cluster 3 (violet line), cluster 1 (pink line), cluster 2 (green line)

3.4 Discussion

Since the discovery of the ability of non-proteolytic *C. botulinum* to produce different types of toxin, it has been suggested that the toxin type may be aligned with other phenotypic properties of strains. Nevertheless, few studies have tested this hypothesis.

This chapter describes the observation of time to initiate growth at chill temperature for 40 strains (experiment one) and 24 strains (experiment two) as a function of incubation temperature and spore inoculum concentration. Cluster analysis based on growth properties indicated that there is a classification pattern which can contribute to organisation of risk assessment for groups of strains. Moreover the clustering analysis revealed that the cluster membership aligns with toxin type.

The analysis of two independent datasets indicated that, in general terms type E strains showed more rapid grow at chill temperatures forming one cluster. Three other clusters were principally composed of type B and type F strains with less rapid growth in the same conditions.

There are no previous studies for direct comparison of the clustering patterns presented in this chapter. Derman *et al.* (2011) previously investigated the genetic diversity of non-proteolytic *C. botulinum* strains using AFLP analysis, but found no association of growth properties with assigned AFLP cluster. The discrepancy with current studies could be due to the different clustering variable used, different strains examined, or experimental design. Interestingly, a *t*-test analysis of growth rates given in the Derman study, revealed that there is no significant difference in growth rates of strains of different toxin type at 10.0°C, although at 37.0°C, type E strains had significantly higher growth rates than type B and F strains.

Graham *et al.* (1997) previously reported that type F strains grow faster than type B or E strains. In the same study, growth of non-proteolytic *C. botulinum* was observed in 5 – 6 weeks at 3.0°C, but not at 2.1°C. Also growth at 4.0°C, 5.0°C, 8.0°C and 10.0°C was observed after 3, 2, 1 and 1 weeks of incubation. Such

differences in observations of time to growth in comparison with the results of this chapter are perhaps unexpected, and may reflect a low but variable likelihood of growth at chill temperatures.

The observations of time to growth presented here are comparable with previously published studies in Table 3.13. In general, the results obtained in experiment one agreed with previously published data. The greatest discrepancy is at 3.0°C and 4.0°C. A number of previous authors have reported growth at 4.0°C and below, while in experiment one only one strain (02-15) grew at 4.0°C and no strains grew at 3.0°C. For example Schmidt *et al.* (1961) reported growth of strains VH (87-01), Iwanai, Beluga (81-26) and 8E after 45, 31, 31 and 45 days of incubation at 3.3°C. It should be noted that, the precise concentration of spores used for each strain was not given (giving the range of concentrations) and that growth was tested in food substrate (beef stew). Furthermore Eklund *et al.* (1967a) observed growth at 3.3°C from strains 86-32 and 288F after 39 and 53 days of incubation, respectively (from s = 10^6 spores tube^{-1}). Different results obtained from these experiments could be associated with the different culture media used. For example Carlin *et al.* (2000) showed that the ability of spores to lead to growth was affected by medium composition. When s = 10^7 spores tube^{-1} were inoculated into PYGS broth, potato purée or broccoli purée the first visible growth was observed after 8, > 56 and 56 days when incubated at 6.0°C, respectively.

Strain	Observation from previous studies					Observation from present study		
	s (spores tube^{-1})	Medium	Temp (°C)	Time to growth (days)[a)]	Reference	s (spores tube^{-1})	Temp (°C)	Time to growth (days)[b)]
87-01	4 x 10^6 – 1 x 10^7	beef stew	3.3	45	Schmidt *et al.* (1961)	10^6	3.0	> 90
Iwanai	4 x 10^6 – 1 x 10^7	beef stew	3.3	31	Schmidt *et al.* (1961)	nt	nt	nt
81-26	4 x 10^6 – 1 x 10^7	beef stew	3.3	31	Schmidt *et al.* (1961)	10^6	3.0	> 90
8E	4 x 10^6 – 1 x 10^7	beef stew	3.3	45	Schmidt *et al.* (1961)	nt	nt	nt
86-32	2 x 10^6	CMM	3.3	39	Eklund *et al.* (1967a)	10^6	3.0	> 90
86-32	2 x 10^5	CMM	3.3	39	Eklund *et al.* (1967a)	10^6	3.0	> 90
288 F	2 x 10^6	CMM	3.3	53	Eklund *et al.* (1967a)	nt	nt	nt
288 F	2 x 10^5	CMM	3.3	68	Eklund *et al.* (1967a)	nt	nt	nt
81-30	2 x 10^6	CMM	3.3	> 85	Eklund *et al.* (1967b)	10^6	3.0	> 90
81-30	2 x 10^5	CMM	3.3	> 109	Eklund *et al.* (1967b)	10^6	3.0	> 90
mixed B strains	10^5	PYGS	4.0	91	Graham *et al.* (1997)	nt	nt	nt
G 21-5	2 x 10^5	TPGY	4.0	52	Solomon *et al.* (1977)	nt	nt	nt
mixed E strains	10^5	PYGS	4.0	70	Graham *et al.* (1997)	nt	nt	nt
mixed F strains	10^5	PYGS	4.0	> 91	Graham *et al.* (1997)	nt	nt	nt
86-32	2 x 10^6	CMM	4.4	18	Eklund *et al.* (1967a)	10^6	4.0	> 90
86-32	2 x 10^5	CMM	4.4	22	Eklund *et al.* (1967a)	10^6	4.0	> 90
288 F	2 x 10^6	CMM	4.4	23	Eklund *et al.* (1967a)	nt	nt	nt
288 F	2 x 10^5	CMM	4.4	25	Eklund *et al.* (1967a)	nt	nt	nt
81-30	2 x 10^6	CMM	4.4	24	Eklund *et al.* (1967b)	10^6	4.0	> 90
81-30	2 x 10^5	CMM	4.4	33	Eklund *et al.* (1967b)	10^6	4.0	> 90
mixed strains	3 x 10^8	PYGS	5.0	28	Stringer *et al.* (1997)	10^6	5.0	21
mixed B strains	10^5	PYGS	5.0	28	Graham *et al.* (1997)	10^6	5.0	32
mixed E strains	10^5	PYGS	5.0	12	Graham *et al.* (1997)	10^6	5.0	21
mixed F strains	10^5	PYGS	5.0	12	Graham *et al.* (1997)	10^6	5.0	42
mixed strains	3 x 10^8	PYGS	5.0	28	Stringer *et al.* (1997)	10^6	5.0	21
86-32	2 x 10^6	CMM	5.6	14	Eklund *et al.* (1967a)	10^6	6.0	21/12
86-32	2 x 10^5	CMM	5.6	17	Eklund *et al.* (1967a)	10^5	6.0	14
288 F	2 x 10^6	CMM	5.6	15	Eklund *et al.* (1967a)	nt	nt	nt

288 F	2×10^5	CMM	5.6	19	Eklund *et al.* (1967a)	nt	nt	nt
81-30	2×10^6	CMM	5.6	17	Eklund *et al.* (1967b)	10^6	5.5	39
81-30	2×10^5	CMM	5.6	21	Eklund *et al.* (1967b)	10^6	5.5	39
mixed strains	10^6	meat medium	6.0	8	Peck *et al.* (1995)	10^6	6.0	11
mixed strains	10^6	meat medium	6.0	7	Peck *et al.* (1995)	10^6	6.0	11
mixed strains	10^6	meat medium	8.0	5	Peck *et al.* (1995)	10^6	6.0	6
mixed strains	10^6	meat medium	8.0	4	Peck *et al.* (1995)	10^6	6.0	6
G 21-5	2×10^4	TPGY broth	8.0	14	Solomon *et al.* (1977)	nt	nt	nt
Minneapolis	2×10^6	TPG	8.0	7	Segner *et al.* (1966)	nt	nt	nt
mixed strains	10^6	meat medium	10.0	4	Peck *et al.* (1995)	10^6	10.0	4
Minneapolis	2×10^6	TPG	10.0	4	Segner *et al.* (1966)	nt	nt	nt
mixed strains	10^6	meat medium	10.0	5	Peck *et al.* (1995)	10^6	10.0	4
mixed strains	3×10^8	PYGS	10.0	7	Stringer *et al.* (1997)	10^6	10.0	4

Table 3.13 Comparisons of results from previous studies on growth of non-proteolytic *C. boutlinum* at chill temperatures with results obtained in this thesis

[a)] noted as t_1 – time when the first growth was observed, [b)] time presented as (experiment one/experiment two), results noted for "mixed strains" in present study corresponds to shortest time when visible growth was observed at given temperature, CMM – cooked meat medium, TPGY – tryptone-peptone-glucose-yeast extract, TPG – trypticase-peptone-glucose medium, nt – strain was not tested

The first signs of growth for non-proteolytic *C. botulinum* type B, E and F strains at 6.0°C were observed on day 11, 11 and 18, respectively (results of experiment one). Similar results were obtained in experiment two, with the only difference that for type F strains the first observation of growth was recorded on day 12. Peck *et al.* (1995) first observed growth from the same inoculum concentration of mixed strains in a meat medium after 4 and 7 days at 10.0°C and 6.0°C, respectively. This is close to results of Stringer *et al.* (1997), where growth from the same spore inoculum at 10.0°C and 5.0°C was first observed after 7 and 28 days of incubation. Visible growth and toxin production at 8.0°C were detected from spores of type E strains (Solomon *et al.*, 1977) after 14 days of incubation. Growth of non-proteolytic *C. botulinum* type E was detected in the current study at 8.0°C on day 7. The discrepancy in the results is very likely associated with the lower inoculum concentration used by Solomon *et al.* (1977). Nevertheless, the results of experiment one in the present study were in agreement with those reported by Segner *et al.* (1966) where turbidity of type E strains at 8.0°C was reported on day 7 and at 10.0°C on day 4.

The cluster analysis based on τ-values was reflected in the probability of growth from a single spore as a function of time. The P_1 for a single type B spore at 6.0°C ranged from 3.8×10^{-7} to 5.2×10^{-2}, from 1.2×10^{-4} to 1.0 for type E and of 1.6×10^{-4} to 1.3×10^{-2} for type F (data from experiment two). Lower P_1 values for a single type E spore were noted by Jensen *et al.* (1987) with a range from 5.8×10^{-7} to 5.8×10^{-2}, and Ikawa *et al.* (1986), with a range of 4.6×10^{-8} to 2.2×10^{-7} for type B, but in both these previous studies growth was tested at the lower temperature of 4°C. Stringer *et al.* (1997) showed, in culture media, that the probability of growth from a single spore for mixture of six different strains within 23 weeks was 8.2×10^{-4}, 3.3×10^{-1} and 1, when incubated at 5.0°C, 10.0°C and 30.0°C respectively. Lund *et al.* (1990) reported a higher probability of growth from a single spore (type B strain), between 0.01 and 1, after 20 days incubation at 6.0°C. The studies of Lund *et al.* (1990) and Jensen *et al.* (1987) also reported that the probability of growth from a single cell is much higher than the probability of growth from a single spore.

Since the discovery that strains of non-proteolytic *C. botulinum* form one of three different botulinum neurotoxins, there has been speculation as to whether the type of neurotoxin formed is associated with physiological properties such as growth at chilled temperatures. Previous studies have failed to prove/disprove the hypothesis that growth at chill temperature is associated with the type of toxin formed. Combining data on growth of different strains at chill temperature from multiple studies has also failed to resolve this issue, as it is difficult to obtain reproducible results using different experimental systems. For example there might be differences in spore crop properties (which could be attributed to slight variations in conditions during sporulation, harvesting and washing of spores, and length and conditions of storage) or growth test conditions (such as pretreatment of spores, growth medium, test system, degree of anaerobiosis).

In the present study, two high quality datasets has been considered and demonstrated that type of neurotoxin formed is associated with growth at chill temperature. Strains that form type E neurotoxin show faster growth at chilled temperatures than strains that form type B or type F neurotoxin.

3.5 Conclusions

There is no doubt that growth of non-proteolytic *C. botulinum* at chill temperatures in foods presents a significant hazard. Therefore incubation temperature is a vital factor in the control and minimization of risk associated with non-proteolytic *C. botulinum*.

From the results in this chapter the following conclusions can be drawn:

i. Strain variability of non-proteolytic *C. botulinum* increased as the test conditions became more difficult (i.e. lower inoculum concentration, lower incubation temperature)
ii. The finding of this study can be used in strain selection for exploitation in food safety challenge studies considering growth through identification of strains which show high growth capability (e.g. 86-21, 02-09 and 02-15)
iii. The probability of growth from a single spore of non-proteolytic *C. botulinum* at 6.0°C after 60 days of incubation ranged from 3.5×10^{-7} to 1 depending on tested strain
iv. The τ-value is a convenient parameter that is used to assess strain variability based on observed growth at a) different temperatures from a high spore inoculum and b) different spore inoculum concentrations at 6.0°C
v. Variability of the growth kinetic behaviour among non-proteolytic *C. botulinum* strains is affected by the incubation temperature and spore inoculum concentration
vi. The clustering of non-proteolytic *C. botulinum* strains based on τ-value determined in two independent experimental datasets groups the tested strains into four clusters; clusters 1, 2 and 3 are generally associated with toxin type B and F and cluster 4 is generally associated with toxin type E, with strains in cluster 4 showing the most rapid growth, meaning that growth of type E strains occurs faster than for type B and F strains
vii. A combination of information from the probability of growth from a single spore and clustering pattern provides a strong support for the homogeneous cluster of strains

viii. The statistical analysis for type E strains revealed that the cluster membership was not affected by country from which the isolate was sourced

Cluster analysis is a very powerful method, which identifies behaviour patterns within the tested strains. Treating strains in terms of groups rather than individually reduces the number of risk assessments that are required when considering the large number of naturally occurring strains. In this study the classification of non-proteolytic *C. botulinum* strains was performed as a function of many variables: type of toxin formed, concentration of inoculum, temperature and time of incubation leading to growth in the model system. The four clusters obtained can be utilized when assessing the risk of non-proteolytic *C. botulinum* in a minimally heated dairy-based dessert (see section 5). It should be noted that this study has focused on testing unheated spores. A future study could consider whether heated spores that are incubated at chill temperature show a similar clustering pattern. In addition, analysis was based only on limited number of strains used in both experiments.

4. Effect of carbohydrate source on variability in growth and neurotoxin formation by strains of non-proteolytic *C. botulinum*

The variability in carbohydrate utilization by non-proteolytic *C. botulinum* type B, E and F strains has been examined. The results show distinct patterns in fermentation of carbohydrates. Analysis of this variability leads to the clustering of non-proteolytic *C. botulinum* strains according to their metabolic capability and may reveal important information about corresponding hazards. The data are used to establish a classification model to indicate the significance for risk assessment.

4.1 Background

Microorganisms are frequently exposed to a changing physical and chemical environment, and need to respond to these changes. This response might involve the formation of new enzymes to reflect a change in the available carbon source. A greater understanding of the metabolic capacity of microbes may suggest mechanisms that could be used to control or inhibit bacterial growth, and give an opportunity to extend the shelf life of food products.

The metabolism of microorganisms is dependent on their growth environment and genome-encoded enzymes. The minimum requirement for microbial growth is a source of nitrogen, carbon, energy (Dawes, 1964), various minerals (Southam, 2012), water and oxygen (in the case of aerobic bacteria). Bacteria frequently obtain carbon from organic compounds (such as proteins, lipids, or carbohydrates). Strains of non-proteolytic *C. botulinum*, are defined as chemoorganotrophic, deriving both energy and carbon from organic compounds such as glucose, xylose, sucrose, maltose (Holt and Sneath, 1985). Strains of non-proteolytic *C. botulinum* are highly saccharolytic, and possess the ability to degrade a range of carbohydrates (Lund and Peck, 2000).

One of the most fundamental biochemical processes in many living organisms is carbohydrate metabolism. Carbohydrates or carbohydrate derivatives (e.g. sugar alcohols, sugar amines, sugar acids, deoxy sugars, glycolsylamines, sugar

phosphates) serve as structural elements in living cells and as a source of energy for the growth of many microorganisms. Carbohydrates are organic compounds that consist of carbon, hydrogen and oxygen (with 2:1 ratio of hydrogen to oxygen). Chemically they are divided into simple (monosaccharides and disaccharides) and complex (polysaccharides, oligosaccharides).

One starting point for carbohydrate catabolism is the oxidation of glucose ($C_6H_{12}O_6$) to pyruvic acid. This metabolic pathway, known as glycolysis is common to both prokaryotic and eukaryotic organisms, with glucose used by many cell types. The chemical steps involved in glucose breakdown are always the same; differences lie in the involved enzymes. In glycolysis, also known as the Embden-Meyerhof-Parnas pathway, glucose is split into two pyruvate molecules with the generation of adenosine-5'-triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Glycolysis is a 10-step pathway divided into two stages (Figure 4.1). In the first stage, reactions 1-5, α-D-glucose is phosphorylated to glyceraldehyde-3-phosphate with the consumption of two ATP molecules. In the second stage, reactions 6 to 10, glyceraldehyde-3-phosphate is converted into pyruvate. Moreover, at this stage per molecule of glucose, four ATP and two NADH molecules are produced (Figure 4.1).

In the presence of oxygen, aerobic microorganisms catabolise glucose, in a process called aerobic respiration, where pyruvate is oxidized to CO_2 and H_2O. Anaerobic organisms convert pyruvate to products such as ethanol, acetic acid or lactic acid, depending on the species. In the case of strains of non-proteolytic *C. botulinum*, the end-products of metabolism are typically acetate and butyrate (Lund and Peck, 2000).

Given that carbohydrates are some of the most widely distributed organic compounds, being important constituents of plants and animals, and that non-proteolytic *C. botulinum* grows and persists in a wide variety of environments rich in decaying plant and animal material, sediments and soil (the main source of food contamination) (Carlin, 2011), it is vital from a food safety point of view to identify carbohydrates that can be a source of energy and carbon.

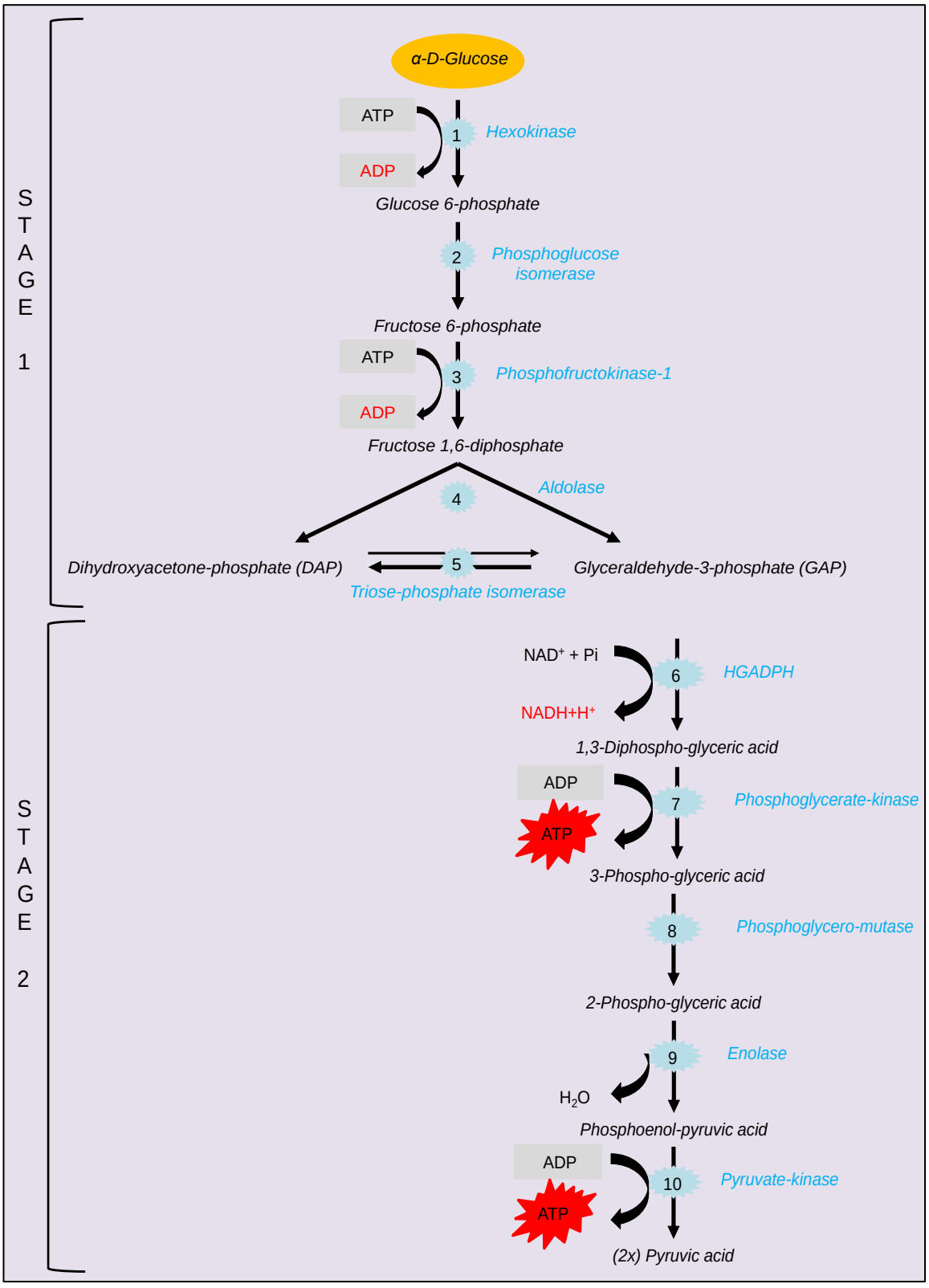

Figure 4.1 The glycolysis pathway
Source: Adopted from Stanier *et al.* (1977)

Since the ability of bacteria to utilize carbohydrate is an important part of their metabolism, certain carbohydrates have long been used for the cultural

differentiation and classification of microorganisms. Although the phenotypic properties of non-proteolytic *C. botulinum* strains have been described previously (Hobbs *et al.*, 1965; Moore *et al.*, 1966; Eklund *et al.*, 1967a; Holdeman and Brooks, 1970; Holdeman *et al.*, 1977; Holt and Sneath, 1985) tests were previously conducted on a limited number of strains, which does not allow for assessing strain variability.

This chapter describes the classification of non-proteolytic *C. botulinum* strains based on the ability to ferment different carbohydrates and considers the importance of this information for quantitative risk assessment.

4.2 Materials and methods

The variation in carbohydrate utilization by non-proteolytic *C. botulinum* strains has been determined in a two-part study. In the initial experiment, a greater number of carbohydrates and small number of strains were tested. Whereas in the main experiment, the utilization pattern of fewer carbohydrates by a greater number of strains was determined. In order to verify whether the ability of a strain to utilise a particular carbohydrate is coupled with toxin production, an amplified enzyme-linked immunosorbent assay (ELISA) test was conducted.

Strain classification using a clustering procedure was based on the formation of acid (measured as a fall in pH) and/or gas when strains were incubated with different carbohydrate. Results are used to establish a classification model to indicate the significance for risk assessment in relation to non-proteolytic *C. botulinum* in food products.

4.2.1 Strains

Experiments were performed using 33 strains of non-proteolytic *C. botulinum* type B, E and F selected from the Institute of Food Research culture collection, and summarised in Table 3.1 (section 3.2.1). The purity of cultures was tested using the procedures given in section 3.2.2.

4.2.2 Culture medium

Anaerobic peptone-yeast extract-glucose-starch (PYGS) broth, as described previously (Lund *et al.*, 1990) was used as the subculture medium. For carbohydrate utilization tests, an anaerobic Carbohydrate Medium Base (CMB) was prepared using strict anaerobic technique and contained the following: Oxoid Peptone Bacteriological (LP 0037), 10.0 gL^{-1}; Oxoid Yeast Extract (LP0021) 5.0 gL^{-1}; Sodium chloride, 5.0 gL^{-1}; Cysteine HCl, 0.5 gL^{-1}; Hemin solution (0.05%) 10.0 mlL^{-1}; Vitamin K_1 solution (0.0001%) 0.2 mlL^{-1}; Resazurin solution (0.02%) 5.0 mlL^{-1}; and glass-distilled water to 1 kg. The unadjusted pH of the medium was between 5.8 and 6.0. The hemin and vitamin K_1 solutions were prepared according to Holdeman *et al.* (1977) and were added to boiled, oxygen-free medium. Before sterilization by autoclaving, the pH of the medium

was adjusted under anaerobic conditions by the addition of an appropriate volume of 5 M KOH, to pH 6.8 – 7.0.

4.2.3 Carbohydrates used

To test the variability in carbohydrate utilization, in the initial experiment, the API® 50CH standardized system (bioMérieux, Basingstoke, UK) was used (Figure 4.2). This test kit is more generally used in conjunction with the API® 50 CHL Medium for the identification of *Lactobacillus*, or with API® 50 CHB/E Medium for the identification of *Bacillus*, Enterobacteriaceae and Vibrionaceae.
The API® strip (Figure 4.2) consists of plastic strips of 10 individual, miniaturized tests tubes (cupules) used to test the fermentation of 49 carbohydrates and their derivatives (Table 4.1).

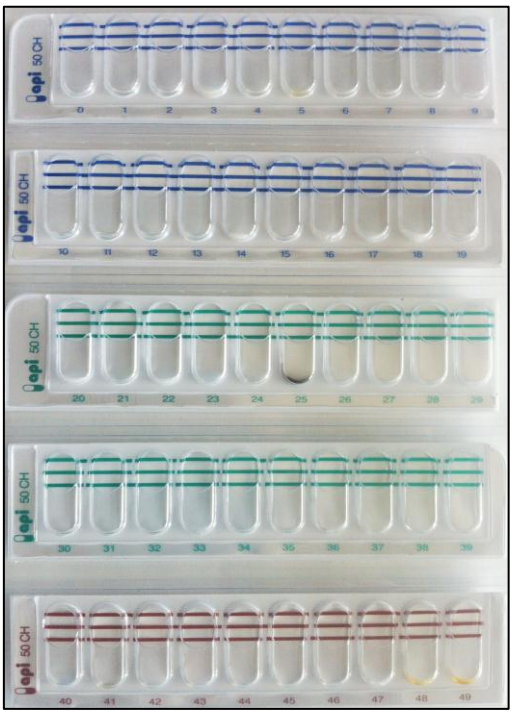

Figure 4.2 The API® 50 CH standardized system used in initial carbohydrate experiment

In the main experiment, a greater number of strains were tested with a smaller number of carbohydrates: *myo*-Inositol (Fluka 57570), D-(+)-Melezitose monohydrate (Fluka 63620), Amylopectin from maize (Sigma 10120), Amylose, from potato (Fluca A0512), Glycogen from bovine liver – Type IX (Fluca G0885), Xylitol, ≥ 99% (Fluca X3375), Levan from *Erwinia herbicola* (Sigma L8647), Pullulan from *Aureobasidium pullulans* (Sigma P4516) and Chitosan (Aldrich 448877). These tests were conducted in bottles.

Cupule	Carbohydrate	QTY (mg tube^{-1})
0	Control	-
1	Glycerol	1.64
2	Erythritol	1.44
3	D-arabinose	1.40
4	L-arabinose	1.40
5	D-ribose	1.40
6	D-xylose	1.40
7	L-xylose	1.40
8	D-adonitol	1.36
9	Methyl-βD-Xylopyranoside	1.28
10	D-galactose	1.40
11	D-glucose	1.56
12	D-fructose	1.40
13	D-mannose	1.40
14	L-sorbose	1.40
15	L-rhamnose	1.36
16	Dulcitol	1.36
17	Inositol	1.40
18	D-mannitol	1.36
19	D-sorbitol	1.36
20	Methyl-αD-Mannopyranoside	1.28
21	Methyl-αD-Glucopyranoside	1.28
22	N-acetylglucosamine	1.28
23	Amygdalin	1.08
24	Arbutin	1.08
25	Esculin	1.16
26	Salicin	1.04
27	D-cellobiose	1.32
28	D-maltose	1.40
29	D-lactose (bovine origin)	1.40
30	D-melibiose	1.32
31	D-saccharose (sucrose)	1.32
32	D-trehalose	1.32
33	Inulin	1.28
34	D-melezitose	1.32
35	D-raffinose	1.56
36	Amidon (starch)	1.28
37	Glycogen	1.28
38	Xylitol	1.40
39	Gentiobiose	0.50
40	D-turanose	1.32
41	D-lyxose	1.40
42	D-tagatose	1.40
43	D-fucose	1.28
44	L-fucose	1.28
45	D-arabitol	1.40
46	L-arabitol	1.40
47	Potassium Gluconate	1.84
48	Potassium 2-Ketogluconate	2.12
49	Potassium 5-Ketogluconate	1.80

Table 4.1 The carbohydrates included in the API® 50 CH strip
Source: bioMérieux (2002)

4.2.4 ELISA for botulinum neurotoxin type B

An ELISA assay was conducted to test whether the growth of non-proteolytic *C. botulinum* strains in the presence of tested carbohydrates was confirmed by neurotoxin formation. The test was conducted only for type B strains.

4.2.4.1 Neurotoxin and antibody biotinylation

Purified botulinal neurotoxin type B, derived from the Okra strain, with a measured potency of 2 x 10^7 MLD_{50} mg^{-1} was obtained from Metabiologics (Madison, Wisconsin, USA). The potency of botulinum neurotoxin was determined by Metabiologics using a mouse test.

A polyclonal antibody against botulinum neurotoxin type B was obtained from Metabiologics. Antibodies were biotinylated using the EZ-Link Micro Sulfo-NHS-LC-Biotin kit (21935) according to the manufacturer's instructions. Antibody was dissolved in phosphate-buffered-saline (PBS) to a final concentration of 200 $\mu g ml^{-l}$ (0.18 mM) and sulfo-NHS-LC-biotin solution (9 mM) was added to give a molar ratio of 50:1 (biotin:antibody). The reaction was incubated on ice for one hour and then desalted using a column. Purified protein sample, was collected into the column and stored at -20°C.

4.2.4.2 Buffers and diluents

Carbonate-bicarbonate buffer, at pH = 9.6, contained: Na_2CO_3, 1.59 gL^{-1} and $NaHCO_3$, 2.93 gL^{-1} of glass-distilled water. Tris-buffered saline-Tween 20 (TBST), pH = 7.5, contained: one sachet of TBS (Sigma, T6664) with 0.05 ml of Tween 20 (Sigma) L^{-1} of glass-distilled water; the pH was adjusted with concentrated HCl. Casein assay buffer, pH = 7.8 contained: 1% (w/v) vitamin-free casein (Sigma), 25mM Na_2HPO_4 and 150 mM NaCl in glass-distilled water. The buffer was filtered through a 0.45 µm membrane to reduce the background signal. PBS/BSA buffer (2% in glass-distilled water) contained 1 tablet of PBS (Sigma) and 10 gL^{-1} of Bovine serum albumin (BSA).

4.2.4.3 Measurement of neurotoxin formation

A Nunc Maxisorb microtitre plate (Thermo Fisher, Loughborough, UK) was coated with 100 µl $well^{-1}$ of anti-B toxin capture antibody at a concentration of

1 µg ml^{-1} in carbonate-bicarbonate buffer (pH = 9.6) and incubated at 4.0°C over night. Unreacted sites were blocked by adding 200 µl of filtered casein assay buffer to each well and incubating at 37°C for 90 minutes. Coated plates were prepared in advance and stored at -18°C until required.

Botulinum neurotoxin complex standard was diluted in PBS/BSA to three concentrations (0.25 – 5 $ngnl^{-1}$). Samples were diluted in 1:2 PBS/BSA and 100 µl of standard neurotoxin solution or test sample was added to duplicate wells of prepared plates. The negative control received 100 µl of PBS/BSA. Plates were incubated at 37°C for 2 hours then washed five times with Tris-buffered saline with 0.05% Tween 20 (TBST), pH = 7.5 (300 µl). Next, 100 µl of a 1:200 dilution of 1 μlml^{-1} biotinylated detector antibody in PBS/BSA was added to each well. Plates were incubated for 60 min at 37°C and washed.

Streptavidin-horseradish peroxidase enzyme conjugate (Prozyme, Europa Bioproducts, Ely, UK) was diluted 1:5000 in PBS/BSA and 100 µl pipetted into each well. Plates were incubated at 37°C for 60 minutes. Following further washing, 100 µl of 3,3',5'5'-Tetramethylbenzidine (TMB, Europa Bioproducts, Ely, UK) was added into each well. After a defined period of time (dependent on colour development), 50 µl $well^{-1}$ of stop solution (0.3 M H_2SO_4) was added. The absorbance within the wells was measured at 450 nm. The detection limit was calculated from the mean plus three standard deviations of the absorbance value of the negative control. All the tests were carried out in duplicate.

4.2.5 Experimental design

Carbohydrate utilization by non-proteolytic *C. botulinum* strains was tested in two experiments. Initially, the API® 50CH system was evaluated for the identification of the biochemical profile of 15 strains. Based on the obtained results, further experiments examined the utilization of nine carbohydrates by 30 strains. In order to confirm observations of growth, for chosen strains, neurotoxin formation was measured using an ELISA assay for type B toxin. Due to a discrepancy between pH measurement and ELISA test results, additional investigations were undertaken using an INFORS Multifors fermenter (Reigate, UK).

4.2.6 Experimental procedure – carbohydrate utilization by strains of non-proteolytic *C. botulinum*

Stock cultures of each strain were prepared by transferring 100 µl of culture into new anaerobic CMM with glucose and incubated at 30°C over night, then maintained at +4°C until required. For testing the ability to utilize carbohydrate an aliquot of stock cultures (100 µl) of each strain were transferred into 10 ml of PYGS broth and incubated at 30°C over night or at 15°C over a weekend.

In the initial experiment (Figure 4.3), actively growing populations were cultured on PYGS agar and incubated at 30°C in an anaerobic cabinet under H_2/CO_2 (90:10, v/v). Simultaneously the purity of the strains was checked (see section 3.2.2). An inoculum was prepared by picking a colony from the plate using a sterile loop and suspended in 10 ml of CMB medium. The turbidity of the prepared suspension was equivalent to 2 McFarland Standard units (opacity standard allowing estimation of the density of a bacterial suspension). Using a sterile pastette the bacterial suspension was distributed into API® strips, containing 49 carbohydrate substrates (plus a control). After incubation in humidity chamber in an anaerobic cabinet for 30°C/48h the pH was measured (pH meter 3-star Thermo Scientific Orion, Waltham, USA, equipped with Eutech Instruments, Wallerstraat, Netherlands, pH electrode).

In the main experiment, 100 µl of actively growing culture was transferred into 80 ml of double strength anaerobic CMB. An aliquot of 5 ml of inoculated CMB was transferred into bottles containing 5 ml of sterile anaerobic water with 0.1 g of a particular carbohydrate. A Durham tube (10 x 75 mm) was placed in the bottom of the medium to observe gas production. After incubation at 30°C for 48 hours in an anaerobic cabinet, the bottles were observed for gas production and the pH and toxin concentration were measured (toxin for type B strains only). Each strain was tested in duplicate (Figure 4.4).

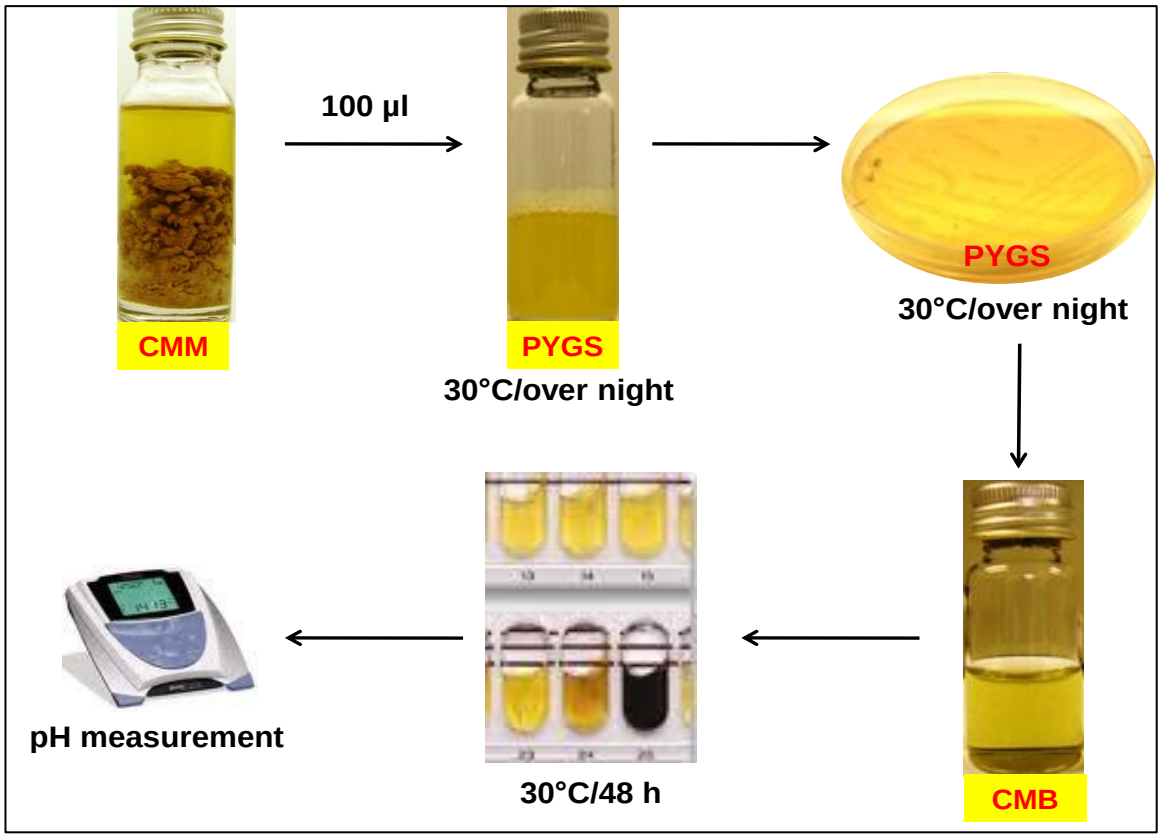

Figure 4.3 The work flow of the initial experiment of carbohydrate utilization by strains of non-proteolytic *C. botulinum* (using API® strips)

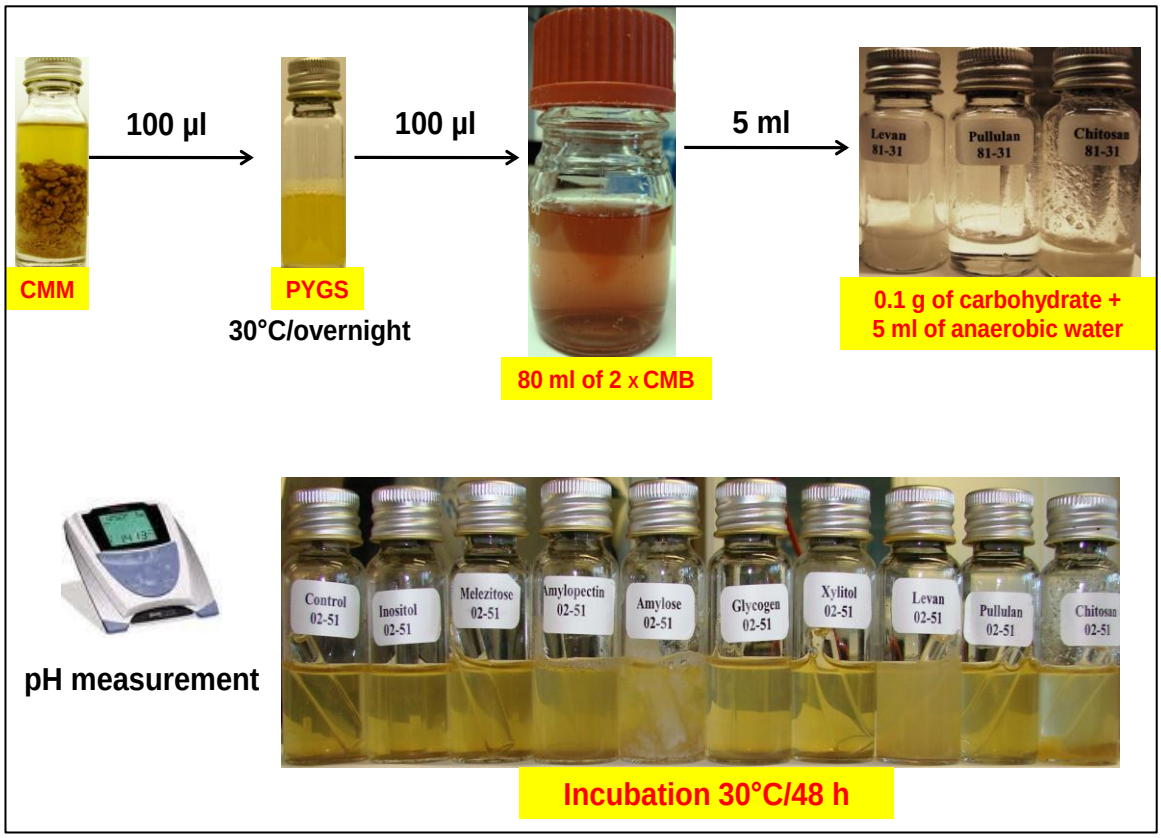

Figure 4.4 The work flow of the main experiment of carbohydrate utilization by strains of non-proteolytic *C. botulinum* (using bottles)

4.2.7 Experimental procedure – growth and neurotoxin formation by strains of non-proteolytic *C. botulinum* type B within a fermenter system

A detailed study of growth and toxin formation by non-proteolytic *C. botulinum* in the presence of glucose was tested using a fermenter system. The test was conducted using strains 86-17 and 93-06. Strains were selected based on the substantial amount of toxin produced as indicated by ELISA assay.

The tested strains were grown at 30°C over night in PYGS. Aliquots of 50 µl of actively growing cultures were transferred into 50 ml of CMB and incubated at 30°C over night in an anaerobic cabinet.

The fermenter system used consisted of paired 1L vessels, each containing 800 ml of CMB (control) or CMB with 10 gL^{-1} of glucose. After autoclaving at 121°C for 20 min the fermenters were constantly sparged with N_2/H_2 (90:10, v/v) (0.5 L min^{-1}) to maintain anaerobic conditions within the medium. Fermenters were equipped with pH (PHS-EFP-K8-225, Finesse, Switzerland) and redox (Pt805-DPAS-SC-K8S/200, Mettler-Toledo, Switzerland) electrodes, and IRIS NT5 software (Version 5, Infors) was used to monitor and control fermenter parameters: temperature (°C), pH, stirrer speed (rpm) and redox potential (mV). Fermenters were stired at 100 rpm. The temperature during the fermenter experiments was maintained at 30°C. Before inoculation, the fermenter pH was adjusted to 7.1 by addition of 1.5 M NaOH. Experiments were initiated by inoculating the fermenters with 50 ml of cultures of strain 86-17 or strain 93-06. Fermenters were sampled after inoculation and at 30 minutes intervals, until stationary phase was reached. A small quantity (5 ml) of culture was removed from each fermenter to determine growth by measurement of optical density at 600 nm, and for toxin production measured by ELISA. Figure 4.5 illustrates the fermenter system.

Figure 4.5 Fermenter system used for testing the growth and neurotoxin formation by non-proteolytic *C. botulinum* strains in the absence and presence of glucose (Multifors, INFORS, Reigate, UK)

4.2.8 Analysis of data on the effect of non-proteolytic *C. botulinum* strain variability on carbohydrate utilization pattern

The ability of strains to utilise a carbohydrate was determined by pH measurement. The two-step experimental design allowed for testing of a large number of carbohydrates, and also of a large number of strains.

4.2.8.1 Initial experiment

In the initial experiment the fermentation of 49 carbohydrates by 15 non-proteolytic *C. botulinum* strains (6 type B, 6 type E and 3 type F strains) was determined by pH measurement. A carbohydrate was considered to be fermented if a pH drop in relation to the control treatment (CMB without carbohydrate) was observed. A drop more than 1.0 pH unit below the control was considered as strong fermentation, whereas a drop of between 0.5 and 1.0 pH units was weak fermentation.

The pH data were subject to cluster analysis to classify isolates. A graphical visualisation of the main carbohydrate experiment results is presented as a heatmap (unsupervised hierarchical clustering algorithm, with a dissimilarity metric based on Euclidean distance). The distance between members of different clusters was calculated based on the pH measurements. The most physiologically homogeneous strains are placed at the lowest hierarchical level. Strain heterogeneity increases with higher hierarchical level within a given cluster. The procedure was conducted using the “heatmap.2” function in the gplot package of the R Statistics Environment (Team, 2012).

4.2.8.2 Main experiment

In the main experiment, the fermentation of nine carbohydrates by a larger number of strains (30) was examined. Substrates were classified as to whether or not they produce acid, gas, toxins or all three. Carbohydrate fermentation was demonstrated by a change in the pH as indicated in section 4.2.8.1. The criteria for gas production were based on the amount of gas produced in a Durham tube: -, no gas production; +, minimal gas production (tube 20 – 50% full), ++, high gas production (tube 50 – 80% full); -/+, one sample negative, one sample with minimal gas production, +/++, one sample with minimal gas production, one

sample with moderate gas production. The data (pH/gas) for each carbohydrate are expressed as the average of duplicate tubes.

Cluster analysis was performed using measured pH in the presence of carbohydrates following the procedure applied in initial experiment (section 4.2.8.1).

The pH profiles for thirty strains incubated in the presence of nine carbohydrates were also analysed by principal component analysis (PCA) and displayed as a biplot diagram to visualize strains location in the classified clusters. The PCA was conducted on a dataset representing the difference in pH of control (basal medium without carbohydrate) and medium with a particular carbohydrate. The PCA compressed the information from the measured pH profile to a small number of dimensions, and they were plotted as points in a two-dimensional display, of which the x and y axes represent the first and second principal components, respectively, and the original variables (carbohydrates) were indicated by arrows. The direction and length of the arrows indicate how each carbohydrate contributed to the first two components in the biplot. PCA was performed using the “princomp” function in the stats package of the R Statistics Environment (Team, 2012).

4.2.9 Statistical analysis

To test whether the measured pH of the medium containing carbohydrates (dependent variable) varied across clusters (independent variable), multivariate analysis of variance (MANOVA) with Games – Howell test for multiple comparison was used. The statistical analysis was performed using the SPSS Statistics software. The differences were tested at a 5% significance level.

4.3 Results

The results of pH measurement from both experiments allowed first of all, for clustering of carbohydrate in terms of their use by non-proteolytic *C. botulinum* strains, and secondly to distinguish homogeneous groups of strains in terms of their carbohydrate metabolism.

4.3.1 Initial experiment

Based on the API® 50 CH profiles of 15 non-proteolytic *C. botulinum* strains the phenotypic diversity of the tested isolates is summarised in Table 4.2. Thirty-two carbohydrates were not fermented by any strain (except strain 86-21, which fermented D-mannitol). A majority of cultures fermented glycerol, D-ribose, D-glucose, D-fructose, D-mannose, D-sorbitol, N-acetylglucosamine, D-maltose, D-saccharose, D-trehalose and D-turanose. Interestingly the fermentation of six carbohydrates; inositol, D-melezitose, amidon (starch), glycogen, xylitol and D-adonitol varied with the tested strain (Table 4.2). All type E strains fermented D-melezitose (except strain 86-21, but see main experiment) and showed weak ability to ferment D-adonitol, but did not ferment amidon (except strain 08-02), glycogen and xylitol. Type F strains fermented amidon, glycogen and D-adonitol, but not D-melezitose and xylitol. Type B strains fermented amidon and glycogen. The fermentation of xylitol and D-adonitol by type B strains was strain dependent.

In general the pH of a medium with a utilized carbohydrate was in the region of pH = 5.20. In some cases the pH was below 5.00, e.g. strains 81-30 (D-glucose), 86-21 (D-glucose, D-fructose, D-mannose, D-sorbitol) and 81-31 (D-ribose, D-fructose). The lowest recorded pH value was 4.81, for strain 81-31 when growth was in the presence of D-fructose. Some carbohydrates e.g. potassium 2-ketogluconate, potassium 5-ketogluconate appeared to increase the pH of the basal media (in a relation to the pH of the control) in the presence of non-proteolytic *C. botulinum* strains. Detailed results of the pH measurements are summarised in Appendix 5.

Toxin type		B						E						F		
Carbohydrate		81-23	81-30	83-01	86-17*	87-02	05-25*	81-26	81-31*	86-21	93-07	02-25*	08-02	86-32	86-33	06-01*
Carbohydrates not substantially utilized by non-proteolytic *C. botulinum* strains	Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	L-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methyl-βD-xylopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	L-sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	L-rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-mannitol	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-
	Methyl-αD-mannopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methyl-αD-glucopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Arbutin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Esculin ferric citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-lactose (bovine origin)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Inullin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Gentiobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D-fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	L-fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	D-arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	L-arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Potassium gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Potassium 2-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Potassium 5-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrates generally utilized by non-proteolytic *C. botulinum* strains	Glycerol	++	++	+	+	+	+	+	+	-	+	+	-	+	++	+
	D-ribose	++	++	++	++	++	+	+	++	++	++	++	++	++	++	++
	D-glucose	++	++	++	++	++	+	++	++	++	++	++	++	++	++	++
	D-fructose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	D-mannose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	D-sorbitol	++	++	++	++	++	+	+	++	++	+	++	++	++	++	++
	N-acetylglucosamine	++	++	+	++	++	+	+	++	++	+	++	++	++	+	++
	D-maltose	-	++	++	+	++	+	+	++	++	++	++	++	++	++	++
	D-saccharose	++	++	++	++	+	-	++	++	++	+	++	++	++	++	++
	D-Trehalose	++	++	++	++	+	+	+	++	++	++	++	++	++	++	++
	D-Turanose	++	++	++	++	+	+	+	++	++	++	++	++	++	++	++
Carbohydrates variably utilized by non-proteolytic *C. botulinum* strains	Inositol	+	-	++	+	-	+	+	-	++	+	++	++	++	+	-
	D-melezitose	-	-	-	-	-	-	+	++	-	++	++	++	-	-	-
	Amidon (starch)	++	++	++	++	+	+	-	-	-	-	-	++	++	+	++
	Glycogen	++	++	++	++	++	+	-	-	-	-	-	-	++	++	++
	Xylitol	-	++	++	++	-	-	-	-	-	+	-	-	-	-	-
	D-adonitol	-	+	-	++	-	-	-	-	+	+	+	+	++	+	+

Table 4.2 Carbohydrate utilization by strains of non-proteolytic *C. botulinum* type B, E, F (as indicated by fall in pH), the results of the initial experiment

- no pH drop observed

+ weak fermentation (pH reduction of 0.5 – 1.0 unit)

++ strong fermentation (pH reduction of > 1.0 unit)

* results present the average of two independent growth tests

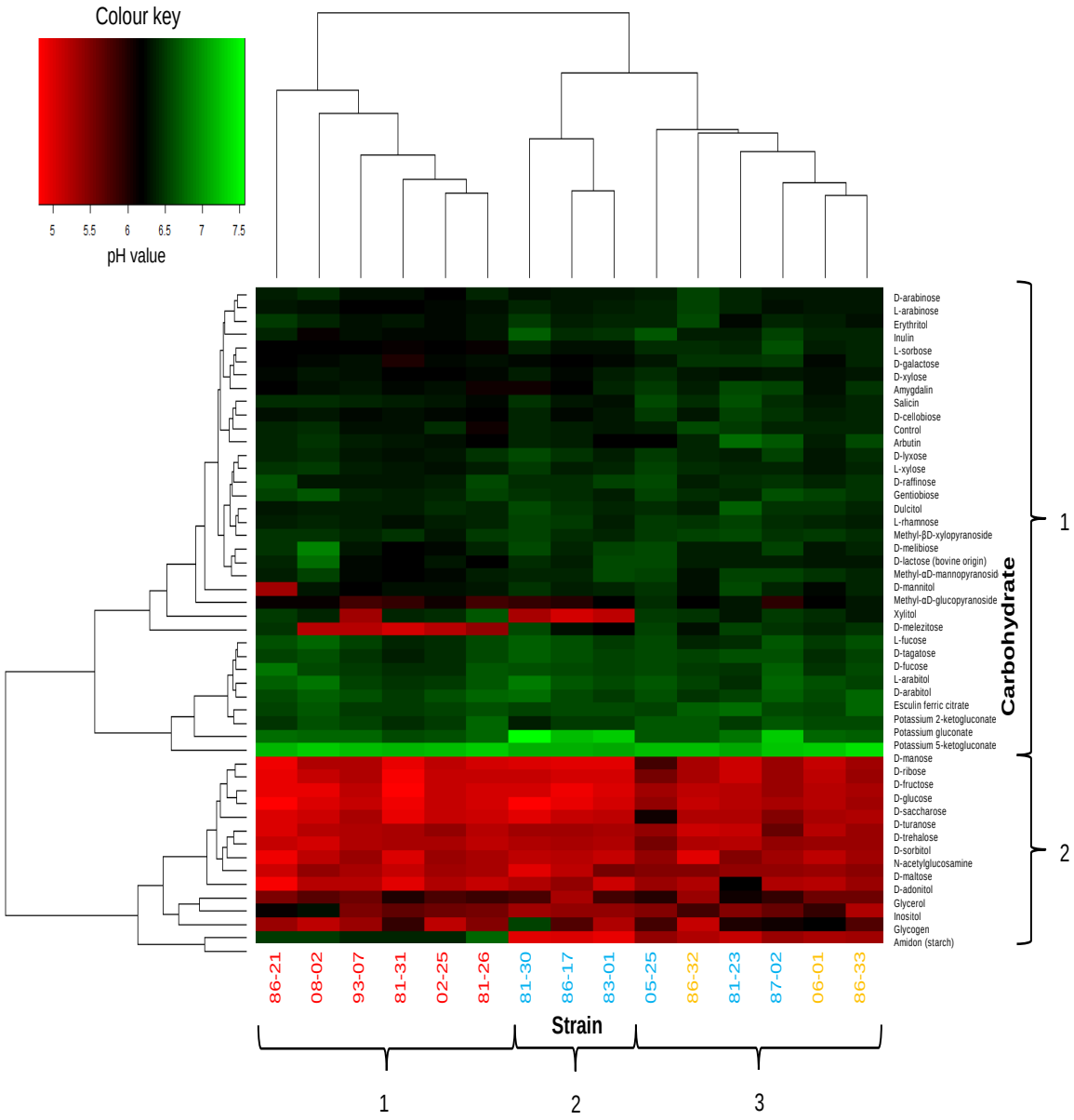

Figure 4.6 The heatmap of pH measurement results obtained from the incubation of non-proteolytic *C. botulinum* strains in the presence of different carbohydrates (initial experiment)
Different strain number colour corresponds to toxin type: type B strains (blue colour), type E strains (red colour), type F strains (yellow colour). Tested carbohydrates were classified into two clusters: cluster 1 (n = 35) and cluster 2 (n = 14) and strains into three clusters: cluster 1 (n = 6), cluster 2 (n = 3), and cluster 3 (n = 6)

Graphical visualisation of the measured pH is illustrated using a heatmap. Unsupervised hierarchical clustering, as shown in Figure 4.6, segregated the 49 carbohydrates into two distinguishable clusters. The first cluster includes carbohydrates for which (apart from a few exceptions) the pH value did not change substantially in relation to the control when inoculated with non-proteolytic *C. botulinum* strains. The second cluster consists of carbohydrates for

which the pH drops at least 0.5 units below the control (except for a few carbohydrates). Carbohydrates that were variably utilised by different strains were present in both clusters. Interestingly those carbohydrates presenting different fermentation properties, correlated with the strain toxin type. For example, xylitol appears to be utilised by some type B strains (but not by type E and F) and D-melezitose is utilised by type E strains (but not by B and F strains). Inositol, amidon and glycogen show a similar response.

Most interestingly, the classification of strains in relation to the ability to utilize different carbohydrates distinguishes strains that form type E toxin from those that form B and F toxin (Figure 4.6).

4.3.2 Main experiment

In the main experiment, the ability of thirty strains of non-proteolytic *C. botulinum* to utilise a smaller number of carbohydrates was tested. This was based on pH measurement, gas formation and also by toxin production. Additionally, to investigate the influence of carbohydrate on the behaviour of two strains a fermenter system was used.

4.3.2.1 Carbohydrate utilization

Selected carbohydrates (inositol, D-melezitose, glycogen, xylitol and two components of starch (amylopectin and amylose)), which in the initial experiment appeared to be fermented only by certain strains of non-proteolytic *C. botulinum*, were tested in the main experiment with greater number of strains. Additionally three new carbohydrates (levan, pullulan and chitosan) were also tested, as according to a genomic study of Stringer *et al.* (2013) may be fermented by some, but not all, strains.

Results from the main experiment supported observations made in the initial experiment regarding the phenotypic diversity of non-proteolytic *C. botulinum* strains (Table 4.3).

Toxin type	Carb.	Control		Inositol		D-Melezitose		Amylopectin		Amylose		Glycogen		Xylitol		Levan		Pullulan		Chitosan	
	Strain	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas
Type B	Control	6.58	-	6.57	-	6.60	-	6.61	-	6.65	-	6.53	-	6.59	-	7.48	-	6.59	-	7.07	-
	81-23	5.99	+	5.57	+	5.87	+/++	5.06	+/++	5.12	+/++	5.04	+/++	5.90	+	6.75	+	5.64	+	6.63	+
	81-30	5.85	+	5.85	+	5.69	+	4.74	+/++	5.02	+/++	4.76	+/++	5.03	+/++	6.78	+	5.88	+	6.67	+
	83-01	5.95	+	5.44	+	5.43	+/++	4.81	+	4.98	+/++	4.87	++	5.00	++	6.72	+	5.42	+/++	6.55	+
	86-17	6.14	+	5.84	+	5.91	+	4.79	+/++	4.88	++	4.79	++	4.99	++	6.74	+	5.56	+	6.10	+
	87-02	6.10	+	5.68	+	5.94	+	5.05	+	5.25	++	5.03	+	5.93	+	6.86	+	5.98	+	6.66	+
	87-04	5.97	+	5.51	+	5.88	+/++	5.22	+/++	5.27	++	5.17	++	5.87	+	6.77	+	5.95	+	6.55	-/+
	90-04	5.96	+	5.11	+/++	5.93	+/++	5.30	++	5.50	+	5.40	++	5.97	+	6.84	+	6.00	+	6.95	+
	93-06	6.08	+	5.54	+	5.95	+/++	5.44	-/++	5.36	+	5.46	++	6.00	+	6.81	+	6.01	+	6.69	+
	93-10	5.86	+	5.54	+	5.72	+	5.84	+	5.87	+	5.83	+/++	5.78	+	6.30	+	5.88	+	6.68	+
	93-11	6.13	+	5.59	++	5.93	++	6.02	+	6.10	+	6.05	+	5.99	+	6.82	+	5.90	+	6.53	+
	02-51	5.90	+	5.53	+/++	5.79	+	5.17	++	5.26	++	5.20	++	5.77	+	6.35	+	5.87	+	6.70	-/+
	05-20	6.00	+	5.95	+	5.86	+	4.69	+/++	4.86	++	4.80	++	5.02	++	6.88	+	6.01	+	6.77	+
	05-25	5.86	+	5.75	+/++	5.78	+/++	5.25	+/++	5.43	+	5.15	+/++	5.84	+	6.69	+	5.85	+	6.82	+
	05-29	5.87	+	5.60	+	5.74	+	5.28	+/++	5.47	+/++	5.29	++	5.81	+	6.74	+	5.83	+	6.84	+
Type E	81-26	6.12	+	5.03	+/++	4.83	+/++	6.08	+	6.11	+	6.13	+	6.05	+	6.87	+	6.09	+	6.66	+
	81-27	5.99	+	4.73	+/++	4.81	+/++	6.01	+	6.05	-/+	6.03	+	6.00	+	6.89	+	6.10	+	6.64	-/+
	81-31	5.88	+	4.97	++	4.75	+/++	5.83	+	5.88	+	5.82	+	5.76	+	6.72	+	5.85	+	6.52	-/+
	86-21	6.07	+	4.94	++	4.67	++	6.06	-/+	6.11	+	6.07	+	6.25	+	6.83	+	6.08	+	6.70	+
	87-01	5.95	+	5.46	+/++	4.80	++	5.90	+	5.92	+	5.88	+	5.80	+	6.68	+	5.91	+	6.56	+
	93-07	6.24	+	4.71	++	4.94	++	6.16	+	6.25	+	6.20	+	4.96	++	6.85	+	6.20	+	6.66	+
	93-08	5.99	+	4.71	++	4.74	++	5.97	+	6.00	+	5.98	+	5.90	+	6.84	+	6.01	+	6.60	+
	02-07	6.16	+	4.74	+/++	4.80	++	6.16	+	6.17	+	6.09	+	6.05	+	6.81	+	6.16	+	6.68	+
	02-10	5.99	+	4.76	++	4.83	++	6.00	+	6.03	+	5.97	+	4.84	++	6.77	+	5.99	+	6.55	+
	02-15	6.10	+	5.07	+	4.84	++	6.05	+/++	6.04	+	6.02	+	5.97	+	6.76	+/++	6.00	+	6.58	+
	02-22	5.92	+	4.97	++	4.63	++	5.87	+	5.92	+	5.89	+	5.80	+	6.75	+	5.92	+	6.62	+
	02-43	6.07	+	4.89	+/++	4.70	++	6.05	+	6.14	+	6.13	+	6.09	+	6.82	+	6.14	+	6.64	+
Type F	86-32	5.92	+	5.24	+	5.79	+	4.93	++	5.15	++	4.90	++	5.83	+	6.81	+	5.91	+	6.60	+
	86-33	5.97	+	5.43	+	5.80	+/++	5.14	++	5.14	+/++	5.10	++	5.80	+	6.77	+	5.85	+	6.56	-/+
	86-34	6.01	+	5.44	+	5.89	+	5.00	++	4.99	++	5.04	++	5.86	+	6.71	+	6.03	+	6.63	+
	06-05	5.99	+	5.70	+	5.86	+	4.98	++	5.11	++	4.97	++	5.83	+	6.75	+	5.98	+	6.62	+

Table 4.3 Carbohydrate utilization by strains of non-proteolytic *C. botulinum* type B, E, F (as indicated by a fall in pH) (results of the main experiment)

The data (pH/gas) for each carbohydrate are expressed as the average of duplicate bottles at the end of the incubation period; weak fermentation: pH drop between 0.5 – 1.0 units (light brown shaded boxes), strong fermentation: pH drop > 1.0 unit (dark brown shaded boxes), - no gas production, + minimal gas production (tube 20 – 50% full), ++ high gas production (tube 50 – 80% full), -/+ one sample negative, one sample with minimal gas production, +/++ one sample with minimal gas production, one sample with moderate gas production

The pH profiles of the tested strains (Table 4.3) were compared in a dendrogram generated by hierarchical cluster analysis (Figure 4.7). The strains were divided into three groups (cluster 1, 2, and 3) which contained 12, 4 and 14 strains, respectively. Striking differences in the ability to utilize carbohydrates by strains were observed between the three clusters.

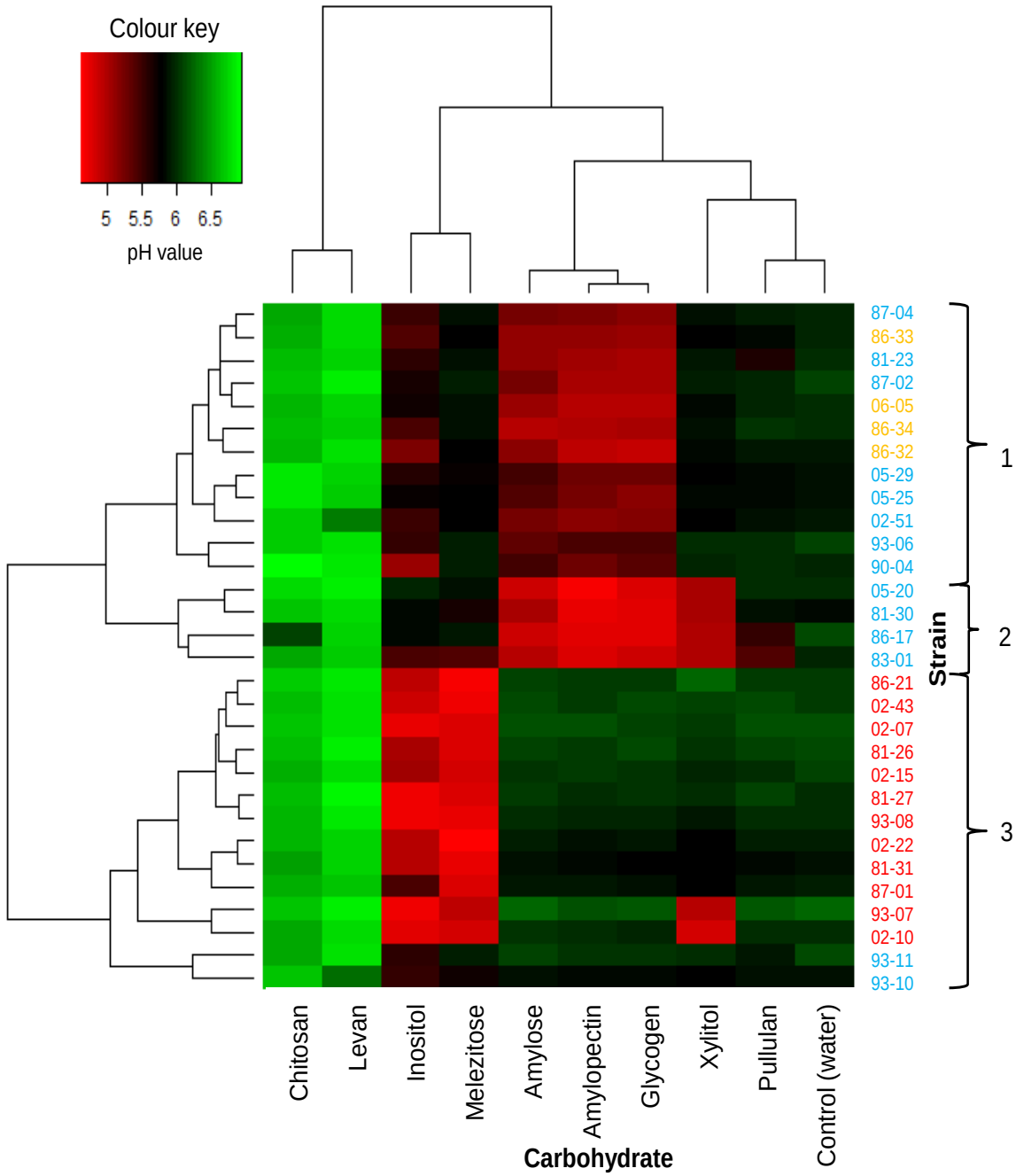

Figure 4.7 The heatmap of pH measurement results obtained from the incubation of non-proteolytic *C. botulinum* strains in the presence of different carbohydrates (main experiment)
Different strain number colour corresponds to toxin type: type B strains (blue colour), type E strains (red colour), type F strains (yellow colour). Tested strains were classified into three clusters: cluster 1 (*n* = 12), cluster 2 (*n* = 4), and cluster 3 (*n* = 14)

Cluster 1 is composed of strains which show an ability to utilize amylopectin, amylose and glycogen. Only a few strains produced acid from inositol and the pH was reduced only by 0.5 units. Cluster 2 consists of strains which produce substantial quantities of acid from amylopectin, amylose, glycogen and xylitol. Exceptionally strain 83-01 appears to produce small amounts of acid from inositol, D-melezitose and pullulan. Cluster 3 includes strains which utilize inositol and D-melezitose (except strains 93-10 and 93-11). These strains do not produce acid from amylopectin, amylose, glycogen, levan, pullulan, chitosan and from xylitol (except strains 93-07 and 02-10). There were two sub-clusters in cluster 3. The first sub-cluster comprised all type E strains, and the second sub-cluster contained two type E strains (93-07 and 02-10) and two type B strains (93-10 and 93-11). These four strains were divergent from other type B and type E strains (Figure 4.7).

The characteristics of each cluster in terms of the pH measurement obtained from the incubation of strains in the presence of nine carbohydrates are shown in Table 4.4. In general cluster membership was associated with the type of toxin produced. Cluster 3 is composed of type E strains (except strains 93-10 and 93-11, which are of type B), cluster 2 of type B strains, and cluster 1 of type B and F strains (Figure 4.7). In most of the cases a pH drop was associated with gas production with the largest quantity of gas produced in the presence of inositol and D-melezitose (type E strains), amylopectin, amylose and glycogen (type B and F strains) (Table 4.3).

A MANOVA analysis indicated significant differences between the clusters (*F*-statistic = 19.3, $p = 0.01$), based on Wilk's Lambda. The differences between clusters were observed in the mean pH in the presence of D-melezitose, amylopectin, amylose, glycogen, xylitol and pullulan and these carbohydrates appear to have the greatest impact when assigning strains into clusters.

Carbohydrate	Cluster 1 (*n* = 12)	Cluster 2 (*n* = 4)	Cluster 3 (*n* = 14)	Significant differences between clusters (*p* < 0.05)
Control	5.97 ± (0.07)*	5.99 ± (0.12)	6.03 ± (0.11)	NS
Inositol	5.51 ± (0.19)	5.77 ± (0.22)	5.01 ± (0.31)	1 > 3, 2 > 3, 2 > 1
D-Melezitose	5.85 ± (0.07)	5.72 ± (0.22)	4.93 ± (0.39)	1 > 3, 2 > 3
Amylopectin	5.15 ± (0.15)	4.76 ± (0.06)	6.00 ± (0.11)	3 > 2, 3 > 1, 3 > 2
Amylose	5.25 ± (0.16)	4.94 ± (0.08)	6.04 ± (0.11)	3 > 2, 3 > 1, 1 > 2
Glycogen	5.14 ± (0.17)	4.81 ± (0.05)	6.00 ± (0.12)	3 > 2, 3 > 1, 1 > 2
Xylitol	5.87 ± (0.07)	5.01 ± (0.02)	5.80 ± (0.41)	3 > 2, 1 > 2
Levan	6.74 ± (0.13)	6.78 ± (0.07)	6.76 ± (0.15)	NS
Pullulan	5.91 ± (0.11)	5.72 ± (0.27)	6.02 ± (0.11)	3 > 2, 3 > 1, 3 > 2
Chitosan	6.69 ± (0.12)	6.52 ± (0.30)	6.62 ± (0.06)	NS

Table 4.4 Comparisons of pH measurement obtained from three clusters of non-proteolytic *C. botulinum* strains in the presence of nine carbohydrates (main experiment)

* data are given as mean pH ± standard deviation, NS indicates lack of significant difference between variables among clusters. Weak fermentation: pH drop between 0.5 – 1.0 units (light brown shaded boxes), strong fermentation: pH drop > 1.0 unit (dark brown shaded boxes)

To visualize the differences in the pH profiles among the three clusters, they were analysed by PCA and represented as points with three colours on a PCA biplot diagram of the first two principal components (PC1 and PC2). These two components explain 74.4% of the total variance.

Cluster 3 is localized on the left, cluster 1 on the right, and cluster 2 in upper and lower-right areas of the diagram. Of the nine carbohydrates, amylopectin, amylose and glycogen had large loadings in the direction in which clusters 2 and 1 were positioned (Figure 4.8). Cluster 3 was localized in the negative direction, corresponding with large negative loadings for D-melezitose and inositol (Table 4.5). The other four carbohydrates (levan, chitosan, xylitol, pullulan) had a much less effect on strain clustering, as indicated by arrow's length (Figure 4.8).

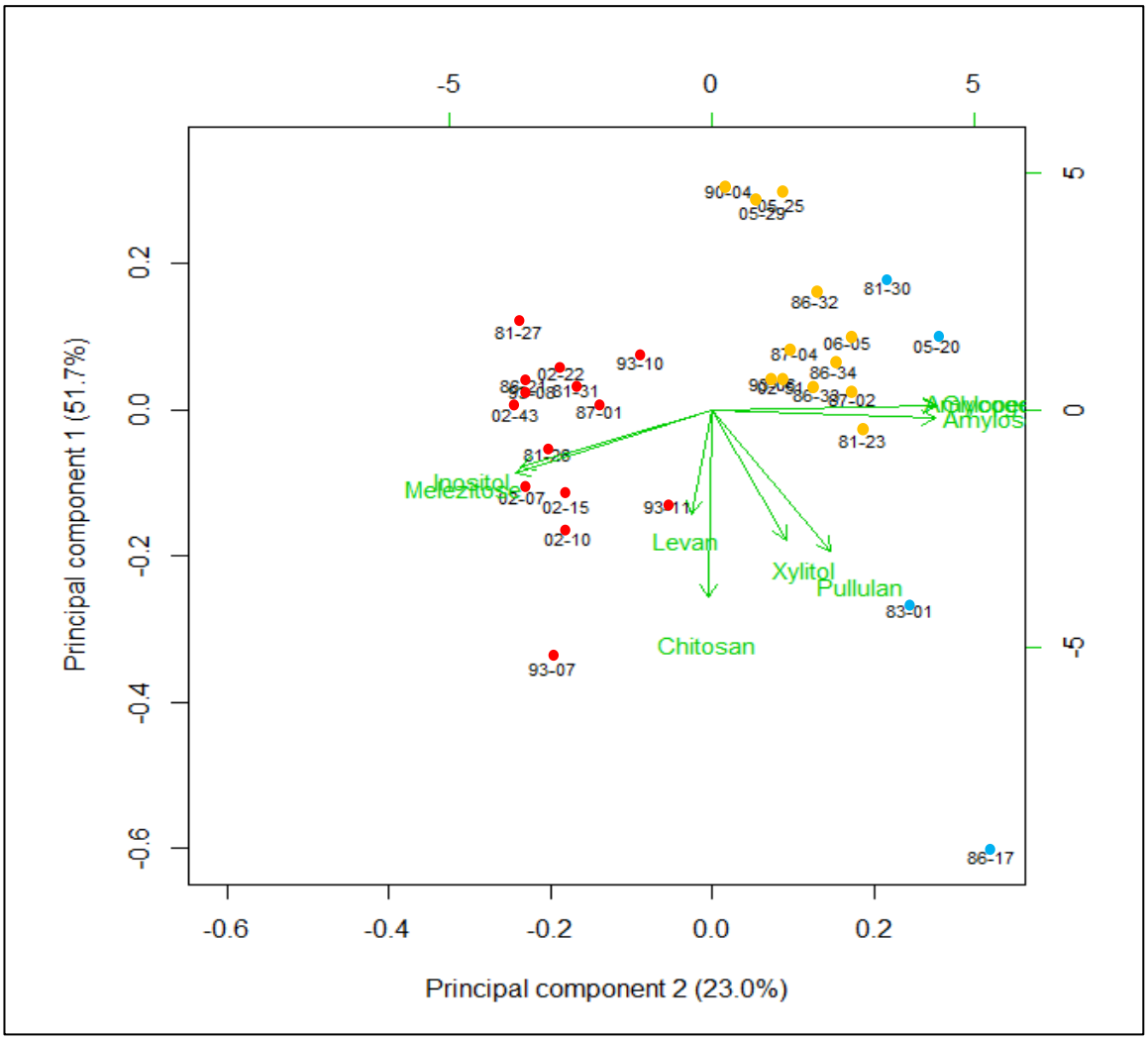

Figure 4.8 Principal component analysis (PCA) biplot diagram showing the relationship between variables (each carbohydrate; arrows) and 30 strains of non-proteolytic *C. botulinum* classified into three clusters by hierarchical cluster analysis

Different points colours correspond to cluster member: cluster 3 – red (type E strains), cluster 2 – blue (type B strains), cluster 1 – yellow (type B and F strains)

Carbohydrate	Loading of PC		% of contribution of variable to each PC	
	PC 1	PC 2	PC 1	PC 2
Inositol	-0.34	-0.20	15.0	
D-Melezitose	-0.40	-0.21	15.4	
Amylopectin	0.45		17.6	
Amylose	0.45		17.4	
Glycogen	0.45		17.6	
Xylitol	0.15	-0.43		18.6
Levan		-0.35		14.9
Pullulan	0.24	-0.47		20.2
Chitosan		-0.62		26.8

Table 4.5 Comparison of loadings and contribution of variables to first two components in PCA in each cluster of non-proteolytic *C. botulinum* strains

4.3.2.2 Neurotoxin formation by strains of non-proteolytic *C. botulinum* in the presence of tested carbohydrates

In order to confirm observations of growth, neurotoxin formation was measured using an ELISA for the type B strains. The effect of nine carbohydrates on the toxicity of non-proteolytic *C. botulinum* cultures is shown in Table 4.6. Unexpectedly, the ability of strains to form acid from the carbohydrates is not consistent with the production of toxin (Table 4.6). Strains fermenting a specific carbohydrate (e.g. amylopectin, glycogen) did not seem to produce more toxin in comparison to growth in basal CMB medium. From these tests, it would appear as if growth and neurotoxin formation had been uncoupled. Furthermore, carbohydrates that had led to more growth, as measured by a greater fall in pH, had not led to more toxin being formed. The tests are useful, however, in identifying high and low toxin forming strains. The amount of toxin produced ranged from 0.0 – 33.1 $ngml^{-1}$ depending on tested strain and carbohydrate. The weakest toxin producers appeared to be strains 81-23 and 87-04. These two strains did not produce a significant amount of toxin in the presence of any carbohydrate. The greatest quantity of toxin was produced by strains 87-02 and 93-06 with amylose being the most favourable carbohydrate for toxin production.

Due to the discrepancy between the measurement of pH and neurotoxin formation it was decided not to measure neurotoxin formation by type E and F strains. Instead further tests were undertaken to investigate the relationship between growth and neurotoxin formation by two type B strains (86-17 and 93-06) using a fermenter system.

Strain	Carbohydrate																			
	Control		Inositol		D-Melezitose		Amylopectin		Amylose		Glycogen		Xylitol		Levan		Pullulan		Chitosan	
	pH	toxin*	pH	toxin	pH	toxin	pH	toxin	pH	toxin	pH	toxin	pH	toxin	pH	toxin	pH	toxin	pH	toxin
Control	6.58	0.0	6.57	0.0	6.60	0.0	6.61	0.0	6.65	0.0	6.53	0.0	6.59	0.0	7.48	0.0	6.59	nt	7.07	0.0
81-23	5.99	0.5	5.57	0.5	5.87	0.4	5.06	0.0	5.12	0.0	5.04	0.0	5.90	0.0	6.75	0.0	5.64	0.1	6.63	0.0
81-30	5.85	1.3	5.85	1.2	5.69	1.6	4.74	0.6	5.02	0.4	4.76	13.4	5.03	3.5	6.78	1.6	5.88	0.3	6.67	0.0
83-01	5.95	4.8	5.44	5.5	5.43	2.2	4.81	7.1	4.98	6.6	4.87	12.0	5.00	10.6	6.72	1.2	5.42	4.1	6.55	0.4
86-17	6.14	8.0	5.84	11.6	5.91	5.3	4.79	1.4	4.88	10.8	4.79	13.9	4.99	2.0	6.74	7.4	5.56	5.7	6.10	8.7
87-02	6.10	9.8	5.68	13.9	5.94	11.3	5.05	6.1	5.25	22.5	5.03	9.1	5.93	12.2	6.86	8.0	5.98	9.7	6.66	10.0
87-04	5.97	0.0	5.51	0.4	5.88	nt	5.22	0.0	5.27	0.0	5.17	0.0	5.87	0.0	6.77	0.0	5.95	0.3	6.55	3.3
90-04	5.96	8.8	5.11	4.1	5.93	7.4	5.30	6.5	5.50	20.3	5.40	6.6	5.97	8.2	6.84	nt	6.00	6.2	6.95	9.4
93-06	6.08	13.3	5.54	5.6	5.95	5.6	5.44	17.5	5.36	33.1	5.46	17.2	6.00	12.4	6.81	nt	6.01	nt	6.69	nt
93-10	5.86	5.8	5.54	1.9	5.72	nt	5.84	3.5	5.87	4.1	5.83	13.5	5.78	2.1	6.30	nt	5.88	nt	6.68	nt
93-11	6.13	9.3	5.59	2.8	5.93	nt	6.02	3.1	6.10	2.9	6.05	5.5	5.99	5.2	6.82	nt	5.90	nt	6.53	nt
02-51	5.90	3.3	5.53	1.0	5.79	nt	5.17	6.3	5.26	14.6	5.20	5.9	5.77	3.1	6.35	nt	5.87	nt	6.70	nt
05-20	6.00	1.2	5.95	1.1	5.86	2.8	4.69	0.8	4.86	2.2	4.80	12.5	5.02	2.5	6.88	1.9	6.01	2.2	6.77	7.8
05-25	5.86	6.8	5.75	4.2	5.78	nt	5.25	2.9	5.43	5.9	5.15	7.6	5.84	9.5	6.69	nt	5.85	nt	6.82	nt
05-29	5.87	7.9	5.60	3.7	5.74	nt	5.28	4.1	5.47	10.2	5.29	3.5	5.81	9.4	6.74	nt	5.83	nt	6.84	nt

Table 4.6 Type B neurotoxin formation by non-proteolytic *C. botulinum* strains in the presence of tested carbohydrates (results of main experiment)

* expressed as $ngml^{-1}$, nt – not tested

The data (pH/toxin) for each carbohydrate are expressed as the average of duplicate tubes; weak fermentation: pH drop between 0.5 – 1.0 units (light brown shaded boxes), strong fermentation: pH drop > 1.0 unit (dark brown shaded boxes)

4.3.2.3 Growth and neurotoxin formation by strains of non-proteolytic *C. botulinum* within a fermenter system

Growth and formation of neurotoxin was determined in the presence and absence of glucose (for strain 86-17), and only in the presence of glucose (for strain 93-06).

The addition of carbohydrate to CMB appears to have a substantial effect on growth and toxin production by non-proteolytic *C. botulinum* strains (Figure 4.9 and Figure 4.10). Strain 86-17 appeared to growth better in CMB with added glucose, as indicated by the OD measurement.

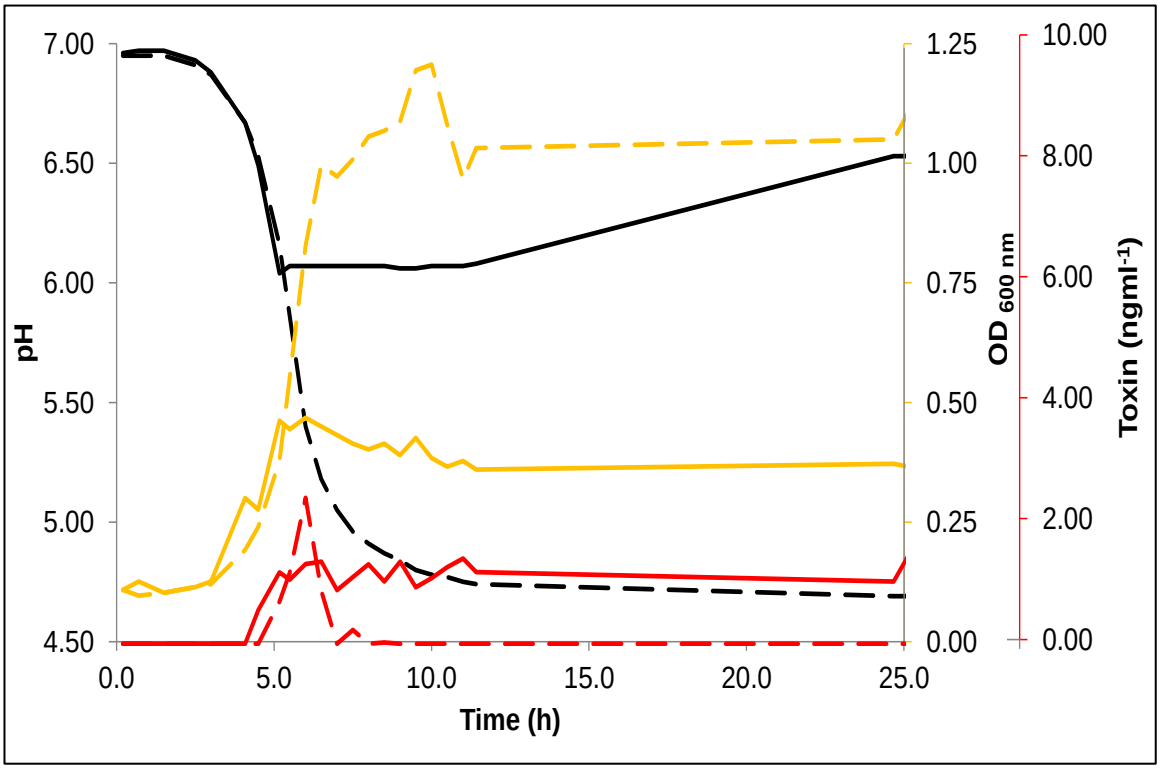

Figure 4.9 Growth, changes in pH and toxin production by non-proteolytic *C. botulinum* strain 86-17 in CMB and CMB containing glucose
pH of CMB without carbohydrate (black solid line), pH of CMB containing glucose (black dashed line), OD of CMB without carbohydrate (orange solid line), OD of CMB containing glucose (orange dashed line), toxin produced ($ngml^{-1}$) in CMB without carbohydrate (red solid line), toxin produced ($ngml^{-1}$) in CMB containing glucose (red dashed line)

For strain 86-17 in control medium (without glucose), stationary phase was reached when the OD_{600} was ~0.4 after five hours of incubation. A greater OD_{600}, ~1.0 was noted in the presence of glucose after seven hours of incubation, and in both cases it was constant during the remaining incubation time. Whereas in

the case of strain 93-06, growth in the presence of glucose the OD_{600} reached ~0.8 after ~10 hours, but the OD_{600} then fell to 0.1 (growth was not observed in the CMB in the absence of glucose due to sample lost).

Both strains lowered the pH of CMB containing glucose from 6.95 to 4.69 (strain 86-17) and 5.05 (strain 93-06). In contrast the pH of control did not fall below 6.00 for strain 86-17 (Figure 4.9 and Figure 4.10).

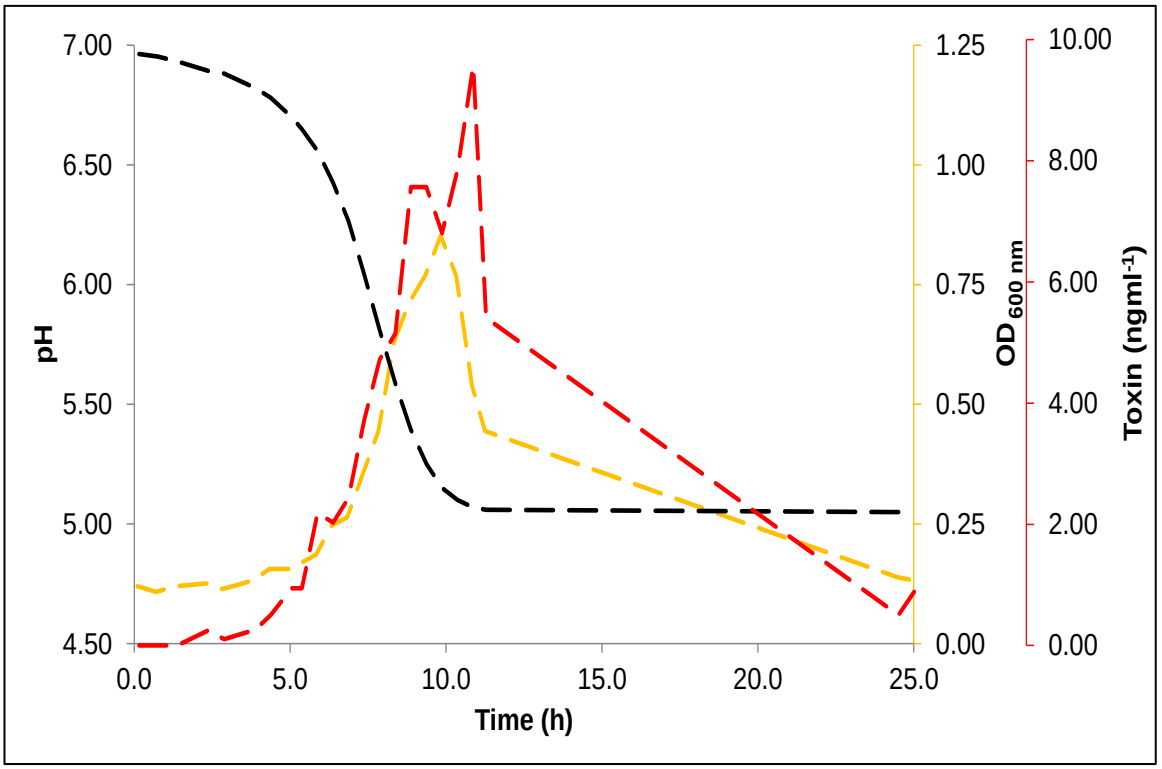

Figure 4.10 Growth, changes in pH and toxin production by non-proteolytic *C. botulinum* strain 93-06 in CMB containing glucose
[test also done in absence of glucose but the test failed – results not shown]
pH of CMB containing glucose (black dashed line), OD of CMB containing glucose (orange dashed line), toxin produced ($ngml^{-1}$) in CMB containing glucose (red dashed line)

Interestingly, addition of glucose to CMB also affected toxin production. In the control medium the maximum concentration produced by strain 86-17 was 1.19 $ngml^{-1}$ and the final concentration was ~1 $ngml^{-1}$ throughout stationary phase. The presence of carbohydrate in medium appears to not only stop the production of toxin but was also associated with its degradation. For example the maximal concentration of toxin produced by strain 86-17 was 2.39 $ngml^{-1}$, and was reduced to 0 $ngml^{-1}$, whereas for strain 93-06 the maximal was 9.58 $ngml^{-1}$ and dropped to 0.5 $ngml^{-1}$.

In conclusion, in the presence of glucose strains 86-17 appeared to degrade toxin that it have formed, and therefore growth and toxin formation had become uncoupled. Although, for strain 93-06 growth was not measured in CMB without glucose, it is suspect to show a similar behaviour to strain 86-17. Thus, in the carbohydrate fermentation tests, a drop in pH and visual observation of gas formation, rather than toxin formation, was taken as evidence of ability to ferment the test carbohydrate. Further studies could be dedicated to investigating this observation in detail. It may be an unusual feature of this medium since toxin survives in PYGS medium (M. W. Peck, personal communication), meat slurry (Peck *et al.*, 1995) and many different foods to cause foodborne botulism.

4.4 Discussion

The ability of microorganisms to ferment carbohydrates have been widely used for their identification and classification for decades. Previous studies (e.g. Eklund *et al.*, 1967a; Holdeman and Brooks, 1970), described the use of carbohydrate utilisation patterns to differentiate between proteolytic (Group I) *C. botulinum* and non-proteolytic (Group II) *C. botulinum*. Nevertheless, this property has not been used so far to distinguish strains within these Groups.

The work presented in this chapter, conducted on 33 strains (14 type B, 14 type E and 5 type F strains) and 54 different carbohydrates, was used to describe the variability of non-proteolytic *C. botulinum* strains. The results of this analysis indicate that the tested strains can be classified according to carbohydrate utilisation.

In general terms, the carbohydrates can be separated into a) those that are utilised by most/all tested strains, b) those that are not utilised by most/all tested strains, and c) those carbohydrates (e.g. xylitol, D-melezitose, inositol, starch, amylopectin, amylose, glycogen) for which the fermentation pattern varied between strains, with the pattern of utilisation associated with toxin type.

Biochemical properties such as the fermentation of monosaccharides, polysaccharides and carbohydrate derivatives by non-proteolytic *C. botulinum* have been summarised previously (Hobbs *et al.*, 1965; Moore *et al.*, 1966; Eklund *et al.*, 1967a; Holdeman and Brooks, 1970; Holdeman *et al.*, 1977; Holt and Sneath, 1985). The results (Table 4.7) generated in this study (as indicated by pH measurement and gas production) are in general agreement with previous published data.

Fermentation of carbohydrate													
Carbohydrate	Toxin type												
	B	B	B	E	E	E	E	F	F	F	F	B, E, F	B, E, F
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	†
Maltose	V	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	†	+	+	+	†	+	+	+	†	+	+	+
Sorbitol	+	†	+	V	V	†	+	+	+	†	+	V	V
Glycerol	–	–	V	V	–	+	V	+	–	V	+	V	†
Lactose	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannose	+	†	+	V	+	†	+	†	+	†	+	+	+
Mannitol	–	–	–	–	–	–	–	–	–	–	+	V	–
Starch	V	†	+	–	V	†	–	†	+	†	–	V	V
Xylose	–	†	–	–	–	†	–	†	–	†	–	–	–
Rhamnose	–	†	–	–	–	†	–	†	–	†	–	–	–
Raffinose	–	†	–	–	–	†	–	–	–	†	–	–	–
Arabinose	–	†	–	–	–	†	–	†	–	†	–	V	–
Cellulose	†	†	†	–	†	†	†	†	†	†	†	†	†
Glycogen	+	†	+	–	–	†	–	†	–	†	+	V	V
Salicin	–	–	–	–	–	–	–	–	–	–	–	–	–
Dulcitol	–	†	–	–	–	†	–	†	–	†	–	–	†
Inulin	–	†	–	–	–	†	–	†	–	†	–	–	V
Galactose	V	†	–	†	–	†	–	+	–	†	–	–	V
Dextrin	V	†	†	†	–	†	†	+	V	†	†	†	†
Trehalose	+	†	+	†	V	†	+	+	+	†	+	+	+
Melibiose	–	†	–	†	–	†	–	–	–	†	–	V	–
Inositol	–	†	V	†	–	†	V	–	+	†	+	V	V
Ribose	V	†	+	†	+	†	+	+	+	†	+	+	V
Amygdalin	–	†	–	†	–	†	–	†	V	†	–	V	V
Cellobiose	–	†	–	†	–	†	–	†	–	†	–	–	–
Esculin	V	†	–	†	–	†	–	†	V	†	–	–	–
D-Melezitose	–	†	–	†	+	†	+	†	–	†	–	V	V
Pectin	V	†	†	†	V	†	†	†	V	†	†	†	†
Adonitol	–	†	V	†	–	†	–	†	V	†	+	V	†
Erythritol	–	†	–	†	–	†	–	†	–	†	–	–	†
Sorbose	–	†	–	†	–	†	–	†	–	†	–	–	†
N-acetylglucosamine	†	†	+	†	†	†	+	†	†	†	+	†	†
D-turanose	†	†	+	†	†	†	+	†	†	†	+	†	†
Xylitol	†	†	V	†	†	†	–	†	†	†	–	†	†
Source	Holdeman and Brooks (1970)	Moore *et al.* (1966)	Current study	Hobbs *et al.* (1965)	Holdeman and Brooks (1970)	Moore *et al.* (1966)	Current study	Eklund *et al.* (1967a)	Holdeman and Brooks (1970)	Moore *et al.* (1966)	Current study	Holdeman *et al.* (1977)	Holt and Sneath (1985)

Table 4.7 Comparison of carbohydrate utilization by strains of non-proteolytic *C. botulinum* type B, E and F with previously published studies and results presented in this thesis

+ positive for all strains; – negative for all strains; V – variable or weak response; † – not tested

The current study also found that in some circumstances non-proteolytic *C. botulinum* strains lowered the pH of the growth medium below the generally accepted minimum growth pH for this bacterium (pH = 5.0; Lund and Peck, 2000). The lowest recorded pH was 4.81. This observation raises the question as to whether non-proteolytic *C. botlinum* can growth at pH 4.81 – 5.0, or whether the low pH might be associated with release of acids on cell lysis or another feature of experimental system. A number of published reports (for example; Segner *et al.*, 1966; Baird-Parker and Freame, 1967; Graham *et al.*, 1996; Graham *et al.*, 1997), considered that non-proteolytic *C. botulinum* strains do not grow and produce toxin in foods at pH = 5.0 or below. Some authors have previously speculated that the ability of bacteria to grow at low pH could be associated with a high concentration of proteins in the studied medium, the presence of moulds or bacilli that increase the pH, media acidified with citric or acetic acid and the acid moving into the oil phase, high inoculum concentration, or incubation temperature (Segner *et al.*, 1966; Baird-Parker and Freame, 1967; Smelt *et al.*, 1982; McClure *et al.*, 1994). The observed findings of possible growth of non-proteolytic *C. botulinum* at 4.81 – 5.0, has not been reported elsewhere and may reflect that tests at this pH range have not been widely done.

The main experiment with nine carbohydrates provided evidence that the additional growth (as indicated by pH drop/gas formation) associated with the addition of a carbohydrate was not always associated with increased toxin formation by type B strains. Further tests with a fermenter system demonstrated that although a larger concentration of toxin was initially formed in the presence of glucose, this subsequently disappeared from the culture medium, and may have been degraded by the bacterium. Although some inconsistencies in growth and toxin production by non-proteolytic *C. botulinum* have been occasionally previously reported (Graham *et al.*, 1997), the observed results in the present work were unexpected. The observations made in this study have not been previously described in relation to non-proteolytic *C. botulinum*, and may be a novel finding for toxin forming microorganisms.

One possible explanation for toxin degradation is that this protein is being used by non-proteolytic *C. botulinum* as a source of essential nutrients possibly

nitrogen and/or carbon. Since this has not been reported previously, it may be that the medium is nitrogen-limited, and is an unusual situation for non-proteolytic *C. botulinum* to find itself. Alternatively since growth in the presence of glucose lowers the pH, toxin degradation could be a consequence of acidification of the medium. Although, if this is the explanation it is surprising that it had not been reported previously. When Siegel and Metzger (1980) studied the effect of various fermentation conditions on the appearance and quantity of toxin production by proteolytic *C. botulinum* in a fermenter system (with different medium composition), they did not report any disappearance of toxin. With glucose concentrations of 1.5, 1.0, 0.5, and 0.25%, the authors observed a better growth of *C. botulinum,* with the greatest amount of toxin produced in the presence of 1.5 and 0.5% of glucose. Potentially, this finding has a massive implication, but further investigation of factors influencing toxin degradation by non-proteolytic *C. botulinum* is an area for future research.

The clustering analysis based on pH measurement of 30 strains (main experiment) divides the non-proteolytic *C. botulinum* strains into three groups with specific, significantly different substrate utilization profiles ($p < 0.05$) (Table 4.8). The first group consisted of strains 86-32, 86-34, 87-02, 06-05, 81-23, 87-04, 86-33, 90-04, 93-06, 02-51, 05-25, and 05-29. These strains utilise amylopectin, amylose and glycogen. The second group consisted of strains 81-30, 05-20, 83-01, and 86-17, utilising amylopectin, amylose, glycogen and xylitol. The third was composed of strains 81-27, 93-08, 81-26, 02-15, 02-07, 86-21, 02-43, 87-01, 81-31, 02-22, 93-07, 02-10, 93-10, and 93-11. Those strains produced acid from inositol and D-melezitose. There was a relationship between the classification and toxin produced by strains assigned to each cluster. The majority of strains in cluster 3 are of type E toxin type strains, in cluster 2 of type B strains, whereas cluster 1 of type B and F strains. This clustering pattern aligns with genomic content (Stringer *et al.*, 2013).

Only one previous publication appears to have applied cluster analysis to carbohydrate utilization profiles of an organism (Draganova *et al.*, 2011). The analysis was conducted on fungal isolates, which divided *Beauveria bassiana* into two groupings that were associated with the source of isolation. In the case

of non-proteolytic *C. botulinum* strains there was no correlation between strain cluster membership and geographic source of strain.

Carbohydrate	Cluster 1	Cluster 2	Cluster 3
Inositol	-	-	+
D-Melezitose	-	-	+
Amylopectin	+	+	-
Amylose	+	+	-
Glycogen	+	+	-
Xylitol	-	+	-
Levan	-	-	-
Pullulan	-	-	-
Chitosan	-	-	-
Strains	81-27 93-08 81-26 02-15 02-07 86-21 02-43 87-01 81-31 02-22 93-07 02-10 93-10 93-11	81-30 05-20 83-01 86-17	86-32 86-34 87-02 06-05 81-23 87-04 86-33 90-04 93-06 02-51 05-25 05-29

Table 4.8 Classification of non-proteolytic *C. botulinum* strains based on the ability to acid production in the presence of carbohydrates
+ strains produce acid in the presence of given carbohydrate, - strains do not produce acid in the presence of given carbohydrate

The carbohydrate utilization study was used to characterise phenotypic properties of non-proteolytic *C. botulinum* strains. The results demonstrate diversity between their metabolism, showing that non-proteolytic *C. botulinum* form three groups according to carbohydrates utilisation. Interestingly group membership is associated with type of toxin produced.

4.5 Conclusions

In this chapter it was demonstrated, for the first time, that strains of non-proteolytic *C. botulinum* can be separated into three groups according to their pattern of carbohydrate utilisation, and that the groups are associated with type of toxin produced. Such information is important for strain identification and also has a crucial importance in risk assessment for minimally heated chilled foods.

From the results in this chapter the following conclusions can be drawn:

i. A total 54 carbohydrates were tested. Most were either degraded by all strains or not degraded at all. A small number of carbohydrates were variably utilized by non-proteolytic *C. botulinum* strains
ii. The ability to utilize carbohydrates led to strain classification into three groups: Cluster 1 (composed of type B and F strains), which utilize amylopectin, amylose and glycogen; Cluster 2 (composed of type B strains), which ferment amylopectin, amylose, glycogen and xylitol; Cluster 3 (mainly composed of type E strains), which produce substantial amount of acid from inositol and D-melezitose
iii. The ability of a strain to utilize a given carbohydrate was indicated by a pH fall and gas formation, but the production of toxin could not be used to show which carbohydrates had been degraded as growth in a presence of carbohydrates was uncoupled from production of toxin
iv. In the presence of glucose toxin formed by non-proteolytic *C. botulinum* 86-17 strain was subsequently degraded

The presented results not only show the clear relationship between strains and carbohydrate utilisation but also identify new research questions, including:

- What mechanisms (i.e. enzymes, genes) determine the ability of a strain to utilize a certain carbohydrate?
- What are the carbohydrate utilization pathways in non-proteolytic *C. botulinum* strains, and is there any variability?
- What are the mechanisms responsible for toxin degradation in the presence of glucose and other carbohydrates?

This information can lead to better control of hazard associated with non-proteolytic *C. botulinum* in food.

5. Adding strain variability information to a QMRA model for non-proteolytic *C. botulinum* in minimally heated chilled food

Although non-proteolytic *C. botulinum* is a pathogenic microorganism of public health importance and many risk assessments have previously been published, none of them takes into account strain variability and its impact on risk presented to minimally heated chilled foods. The common "default" assumption in food safety research follows "worst case scenario" which represents all strains of a given species that can possibly be found in food. Incorporating the information on strain variability in the form of probability distributions may lead to more precise analysis and greater flexibility. This chapter describes a modular process risk model for a relatively simple model dairy-based minimally heated chilled dessert and the hazards arising from non-proteolytic *C. botulinum*. The estimate of risk is based on data describing spore loads in raw materials, spore inactivation during thermal processing, distribution of spores in retail units and variability in growth of non-proteolytic *C. botulinum* strains during multi-stage storage prior to consumption. The model was used to estimate the probability of toxicity of an individual retail unit of dairy-based dessert at point of preparation. The probabilistic analysis was built using a Monte Carlo simulation technique. The strengths of the approach as well as data gaps and further research have been identified.

5.1 Background

The first step in risk analysis (see section 1.3) is risk assessment. Originally systematic risk assessment was first introduced in 1986 to evaluate human cancer risks (NRC, 2009), but the methodology has been refined for use in other fields e.g. microbial and chemical contamination of food (Fabiansson, 2001). In terms of food safety a "hazard" is defined by the CAC (2001) as an "agent or condition of food with the potential to cause an adverse health effect", whereas a "risk" is defined as "a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s)". Risk assessment is usually carried out by independent experts, and it commonly involves collecting, analysing and modelling of information (e.g. from the published literature,

challenge tests, assumptions or experts' knowledge) related to a hazard. Risk assessment identifies the hazards and estimates the potential adverse health effects for consumers but also is a decision support tool for risk managers. The assessment of risk can be conducted qualitatively, semi-quantitatively, quantitatively or as a combination of these.

In qualitative risk assessment the likelihood of an adverse event is identified and ranked according to its effect on human health by semantic "scores" (e.g. "very low" – very rare but cannot be excluded, "high" – occurs very often). Although qualitative risk assessment is relatively quick and easy to conduct there is potential for ambiguity. A step forward is a semi-quantitative risk assessment, which assigns indicative values to the scales from qualitative risk assessment, and is generally conducted when limited data are available. A quantitative risk assessment is often more complex, requires a multidisciplinary team and also specific technical knowledge (e.g. statistics, biology, mathematics).

5.1.1 Quantitative Microbial Risk Assessment (QMRA)

QMRA assesses human health risks associated with exposure to hazards for pathogenic microorganisms or their toxins, and generally consists of four steps: hazard identification, exposure assessment, hazard characterisation and risk characterisation (Figure 5.1) (CAC, 1999). A QMRA based on scientific evaluation or evidence, estimates the severity and likelihood of illness or detriment resulting from exposure to a specific hazard in food. QMRA is a tool, which can be used to support a HACCP management system and can assist in facilitating international trade (by setting up a sound scientifically supported food safety policy) (Nauta *et al.*, 2007).

5.1.2 QMRA and the HACCP system

The QMRA and HACCP system can be used together to control microbiological hazards in food, and a crucial step in their implementation is hazard identification. The HACCP system is a management tool which is used to achieve control of particular hazard but which does not provide additional quantitative information about the nature of this hazard under different conditions (e.g. temperature and time heating/storage, addition of preservatives) during

manufacturing. Therefore QMRA integrated with HACCP can be used as a tool: first of all, in setting up the HACCP scheme; secondly, in establishing the effective critical limits in a HACCP plan; third, in the identification of points, which require greater control and in the optimisation of production parameters. Moreover, QMRA models that use a probabilistic approach can estimate confidence associated with control and can allow for the inclusion of variability and uncertainty arising from properties of a microbial population and from imprecise measurements. Most importantly, sensitivity analysis can be included in a probabilistic risk assessment to identify the steps or production parameters that are of most importance for product safety and what additional measurement provides the most valuable information.

5.1.3 Deterministic vs. probabilistic risk assessment

Calculation of the risk associated with a hazard can be conducted in different ways. The most commonly used methods of risk assessment are deterministic and probabilistic approaches. The deterministic approach uses a single-point, e.g. minimal, maximal, modal or average values for each model parameter, to establish the “worst-case”, “best-case” or “most likely” scenarios based on an equal weight for all scenarios. Parameters are usually developed by linear regression or non-linear curve fitting procedures. The impact of the input parameters on the overall model output can be assessed by sensitivity analysis. The drawback of this approach is that the model can be over simplified by ignoring the variability and uncertainty associated with parameters (see section 5.1.5).

The second approach in quantitative risk assessment is a probabilistic approach. In a probabilistic risk assessment, each model variable is defined as a probability distribution rather than as a single value. A crucial advantage of this approach, in comparison with a deterministic risk assessment, is the consideration of variability and/or uncertainty (section 5.1.5) in risk estimates (Vose, 2008). These help in the identification of extreme events. In addition, probabilistic risk assessments provide estimates for the confidence limits associated with risks, which is an important factor in risk or policy decision making. In many ways probability is a consistent way to express and combine beliefs.

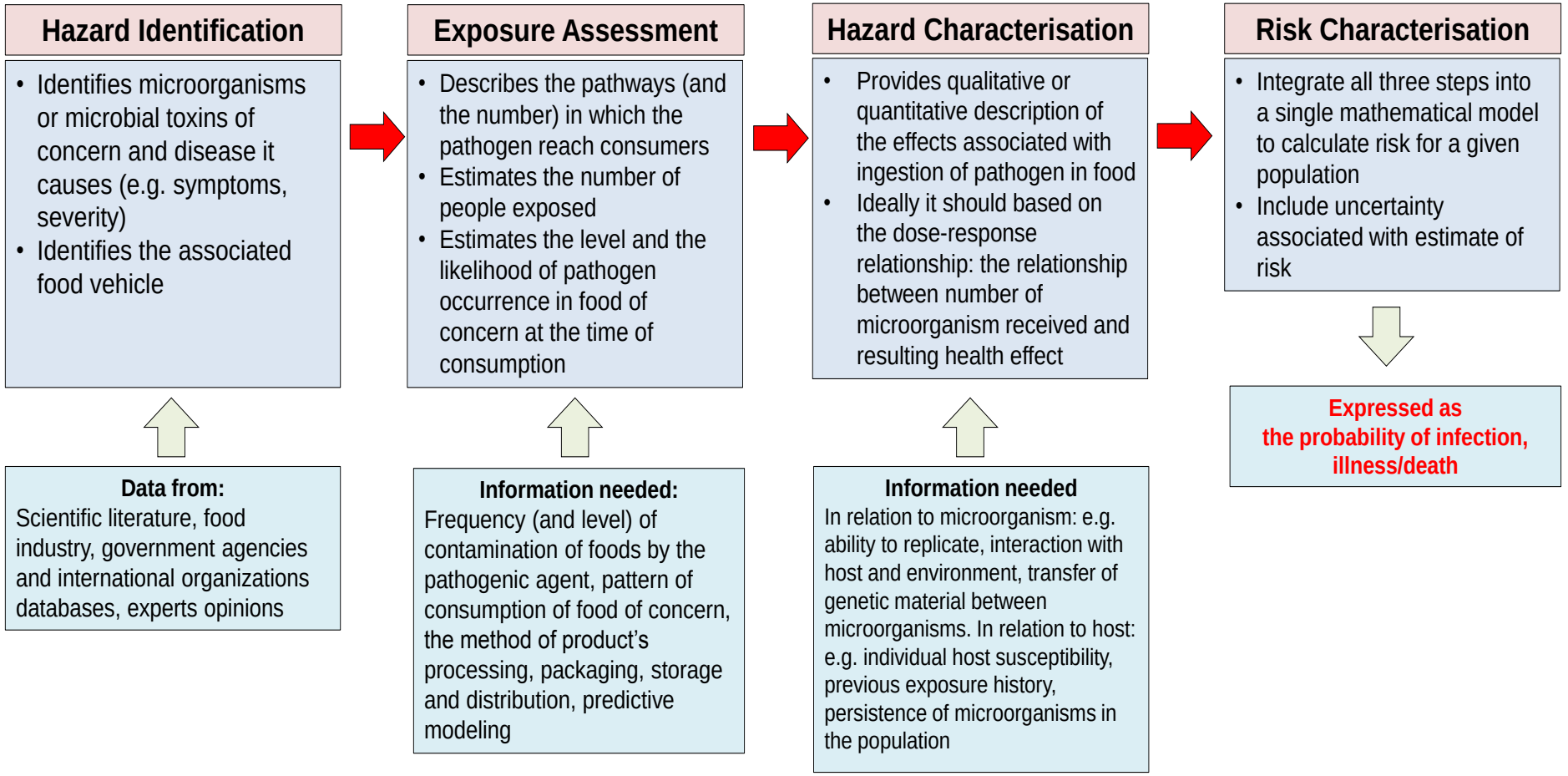

Figure 5.1 Organisation of risk assessment for hazard arising from foodborne pathogens (CAC, 1999)

5.1.4 Monte Carlo simulation in QMRA

The most common technique applied to construct probability distributions used in QMRA is a Monte Carlo simulation. The technique builds the QMRA model by generating (e.g. 1000 or 10000 times) a random sample for values of the model parameters; the sample is identified with a probability distribution of possible outcomes. Therefore it is possible to calculate not only what values a parameter can take, but how likely they will occur. An increased number of interations (number of samplings) can increase the precision of a calculated probability. A Monte Carlo simulation generates samples from the probability distribution and combines them according to the dependency of the variables. Other advantages of Monte Carlo simulation are: graphical representation of simulation's outputs (an important factor when communicating the risk to risk managers and other interested bodies); the relatively simple mathematics and the number of commercially available softwares with user-friendly interfaces which makes it a widely recognised and used technique (Vose, 2008). The essential factor in a Monte Carlo simulation is that it combines probability distributions systematically and consistently to give a new distributions.

Monte Carlo methods require random numbers to choose elements of the sample (without bias). There are many alternatives to generate random numbers, including: Latin Hypercube sampling (LHS), Mid-point LHS or importance sampling (Vose, 2008).

5.1.5 Variability and uncertainty in QMRA

Variability and uncertainty are two important properties of probabilistic variables that have an impact on a model (Hartnett *et al.*, 2001) and can be included in a calculation of risk by using probability distributions. These two concepts are very different, and therefore should be clearly separated in risk assessment (when possible) (Nauta, 2000). In terms of QMRA, variability relates to the natural variation of a particular property within a (statistical) population (e.g. variability of heat resistance in a population of strains, variability in growth from single spore (Figure 5.2), variability in processing conditions and product composition and variable consumer behaviour (e.g. purchasing pattern)). The variability cannot be reduced through further measurements.

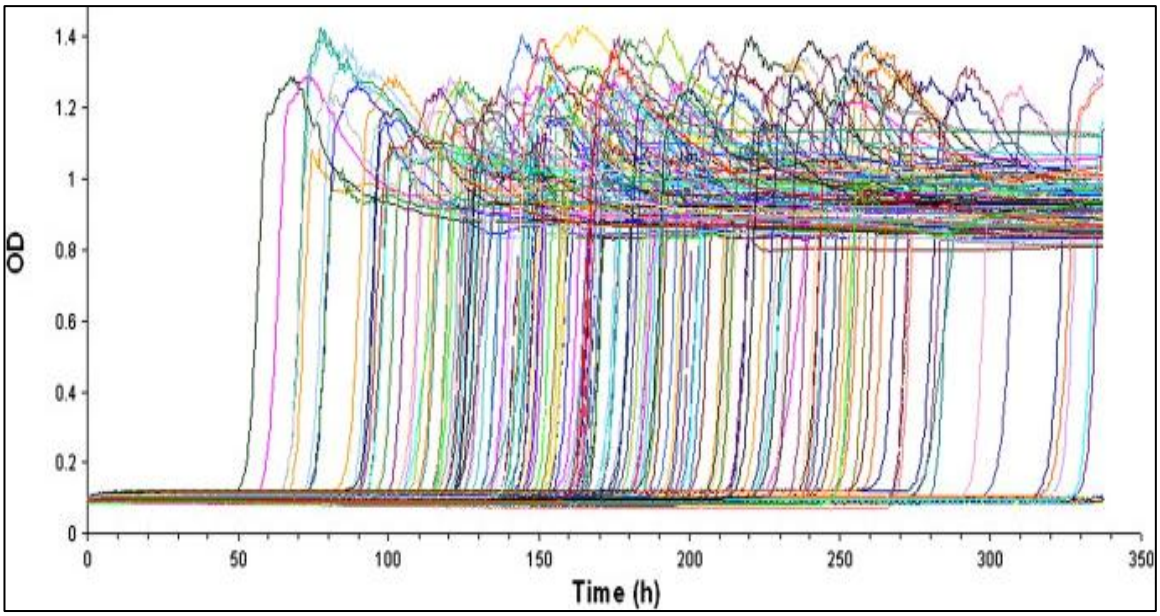

Figure 5.2 Variable growth of non-proteolytic *C. botulinum* Eklund 17B initiated from a single spore following a heat treatment of 1 min at 80°C (a 3D reduction)(observed for 338h)
Source: Reprinted from "Lag time variability in individual spores of *Clostridium botulinum"* by Stringer et al. (2011) *Food Microbiology*, 28, p.229. Copyright 2013 by Elsevier B.V. Adapted with permission.

Uncertainty arises from lack of sufficient data and an inability to be precise about the value of a given parameter. The important difference in dealing with variability and uncertainty, is that uncertainty can be reduced e.g. by additional tests, study or expert consultations. For example, the final comments in section 2.3.2.2 were that the uncertainty associated with the *D*-value of non-proteolytic *C. botulinum* strains could be reduced by testing of at least 35 strains (in triplicate).

5.1.6 Variability of non-proteolytic *C. botulinum* strains

As introduced in the above sections, an important component of a well-defined risk assessment is the identification of variables that have an impact on the calculated probability of an adverse event e.g. probability of toxic pack at point of consumption. A more sophisticated approach in QMRA it to take account of the variability of model parameters, e.g. strain variability.

Strain variability can be a function of several factors, e.g. growth conditions (e.g. pH, NaCl concentration, temperature), type of media used and presence of competing microorganisms, and generally it increases as the conditions become

less favourable (Derman *et al.*, 2011; Lianou and Koutsoumanis, 2012). Although a variable response of strains to environmental conditions is frequently reported, many risk assessment models for foodborne pathogens omit this property by assuming their homogeneous behaviour, which may lead to miscalculation of the risk (Nauta, 2000). The failure to include strain variability in a microbial risk assessment often results from a lack of available data on strain variation, or resulting from time and work required in its acquisition.

Different characteristics of strains of *C. botulinum* types A, B, E and F led to the separation of strains into two distinct groups (proteolytic *C. botulinum* and non-proteolytic *C. botulinum*) decades ago (Lynt *et al.*, 1982). Although the variability between strains of these two groups is very clear e.g. different spore heat resistance, different temperature growth range, different salt and pH tolerance (Table 1.1) and different foods implicated in foodborne botulism (Table 1.2), limited information is available regarding the variability of strains within these two groups. Considering the fact, that the distinction between strains of proteolytic *C. botulinum* and non-proteolytic *C. botulinum* has contributed substantially to control of the risk presented by *C. botulinum*, further investigation of the physiological and phylogenetic groupings of non-proteolytic *C. botulinum* strains can provide important information for a risk assessment, for pathogen control and also in the investigation of foodborne botulism outbreaks.

The present study has quantified the variable physiological response, (heat resistance of spores, growth at chill temperatures and effect of carbohydrate source on growth) of almost fifty strains of non-proteolytic *C. botulinum*, and described significant variability between strains of different toxin types (Table 5.1).

Toxin formed					
Characteristic		**Type B**	**Type E**	**Type F**	**Source**
Geographical distribution[a)]		North America, Europe, Australia	Northern Canada, Alaska, Nordic countries	North America	Dodds (1993b)
Genome size (bp)		3 781 509 (strain Eklund 17B)	3 659 644 (strain Alaska E43)		Stringer *et al.* (2013)
Ferment D-glucose[b)]		+	+	+	Chapter 4
Ferment D-fructose		+	+	+	
Ferment D-maltose		+	+	+	
Ferment D-saccharose		+	+	+	
Ferment D-melezitose		-	+	-	
Ferment starch		+	-	+	
Ferment glycogen		+	-	+	
Ferment xylitol		V	-	-	
Ferment D-adonitol		V	V	+	
Ferment amylopectin		+	-	+	
Ferment amylose		+	-	+	
Time to growth at chill temperatures (days)[c)]	4.0°C	> 90	67	> 90	Chapter 3
	5.0°C	32	21	42	
	5.5°C	25	14	28	
	6.0°C	14	11	18	
Maximum growth temperatures[d)]		36.6 ± (1.9°C)	39.0 ± (0.8°C)	37.8 ± (0.6°C)	Derman *et al.* (2011)
Spore heat resistance[e)] (minutes)	-LYS	$D^{'}(80)$ = 2.74 ± (4.56)	$D^{'}(80)$ = 2.41 ± (3.12)	$D^{'}(80)$ = 1.53 ± (1.19)	Chapter 2
	+LYS	$D^{'}(80)$ = 225 ± (401)	$D^{'}(80)$ = 194 ± (323)		

Table 5.1 Physiological properties of different toxin types of non-proteolytic *C. botulinum*

[a)] based on percentage of type identified; [b)] + positive for all strains; - negative for all strains; V variable or weak response; [c)] Time to growth at chill temperatures determined in PYGS broth; [d)] Maximum growth temperature determined in TPGY broth; [e)] *D*-value without/with lysozyme in a recovery media (data given as mean ± standard deviation of *D*-values measured at temperatures in a range 50°C – 83°C (for -LYS) and in a range 75°C – 95°C (for +LYS) converted to reference temperature T = 80°C); empty cell indicates that data were not reported

Tests conducted on growth properties of non-proteolytic *C. botulinum* at chill temperatures (Chapter 3) distinguished four clusters of strains. Although a small amount of variation was observed, these clusters were consistent across two independent datasets, generated using different experimental designs. Strain clustering correlated with type of neurotoxin formed and revealed groups of strong growing and weak growing strains at chill temperatures, with type E strains showing the most rapid growth compared to type B and F strains (Table 5.1). Moreover, the variation between strains of different toxin types was greater when test conditions were more demanding e.g. low incubation temperature or small inoculum concentration of spores.

Interestingly, the clustering of strains based on growth properties at chill temperatures agreed with clusters derived from growth tests in the presence of different carbohydrates (Chapter 4). Variability between strains of non-proteolytic *C. botulinum* for both of these growth measures correlates with type of neurotoxin formed. Clustering analysis of carbohydrate utilisation was conducted on 33 strains in two independent experiments and distinguished three groups of non-proteolytic *C. botulinum*.

The assessment of strain variability based on a literature review of heat resistance of non-proteolytic *C. botulinum* spores (Chapter 2) failed to distinguish homogeneous groups of strains in terms of their heat resistance properties (see section 2.3.2.2 – testing the dependence of spore heat resistance on strain of non-proteolytic *C. botulinum*). The analysis of collected *D*-values also revealed that there is no significant different between strains of different toxin type (see section 2.3.2.2 – testing the dependence of the spore heat resistance on toxin type). Nevertheless, summarised data on inactivation kinetic parameters (*D* and *z*-values) provided valuable information on variability and uncertainty associated with those parameters including well-defined probability distributions that are suitable for incorporation into inactivation models applied in QMRA, such as a model for minimally heated model dairy-based chilled dessert (see section 5.2.4.2).

Several authors have used a range of molecular methods to investigate the phylogenetic relationship of *C. botulinum* strains. Sequencing of the 16s rRNA gene by Hutson *et al.* (1993a; 1993b) supported the physiological grouping (Table 1.1) and confirmed that non-proteolytic *C. botulinum* strains are phylogenetically distant from those of proteolytic *C. botulinum*. Similar results were obtained by Hielm *et al.* (1999), Keto-Timonen *et al.* (2005), Kirkwood *et al.* (2006), Keto-Timonen *et al.* (2006), Hill *et al.* (2007) using ribotyping, pulse field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) analysis.

Investigations on the genetic variability between non-proteolytic *C. botulinum* strains has been performed by various authors using a range of methods e.g. PFGE (Hielm *et al.*, 1998a; Hielm *et al.*, 1998b; Leclair *et al.*, 2006; Kirkwood *et al.*, 2006), randomly amplified polymorphic DNA (RAPD) (Leclair *et al.*, 2006), AFLP (Keto-Timonen *et al.*, 2005; Keto-Timonen *et al.*, 2006; Derman *et al.*, 2011; MacDonald *et al.*, 2011), multilocus sequence typing (MLST), variable-number tandem-repeat (VNTR) analysis (MacDonald *et al.*, 2011) and DNA microarray (Stringer *et al.*, 2013). The results of these test revealed large variability and details of the relationships between strains of non-proteolytic *C. botulinum*, which seemed to be associated with toxin serotype. The analysis of 17 strains (3 type B, 14 type E and 2 type F) using the ribotyping by Hielm *et al.* (1999) roughly indicated two clusters within non-proteolytic *C. botulinum*. One cluster consists of type E isolates, and the second cluster contains type B and type F strains. A very clear separation of type E strains from those of type B and F was also observed using AFLP with 15 strains tested (4 type B, 8 type E and 3 type F) (Keto-Timonen *et al.*, 2006), 24 strains tested (3 type B, 16 type E and 5 type F) (Derman *et al.*, 2011) and with 37 strains (7 type B, 26 type E and 4 type F) (Keto-Timonen *et al.*, 2005). The same strain classification pattern was obtained using PFGE with 34 strains (4 type B, 24 type E and 6 type F) (Kirkwood *et al.*, 2006) and 39 strains (6 type B, 27 type E and 6 type F) (Leclair *et al.*, 2006). The classification pattern obtained by Leclair *et al.* (2006) using PFGE was also confirmed with RAPD. One feature of all these studies is a tendency to test large number of type E strains, but few type B or type F strains.

The most recent study by Stringer *et al.* (2013) examined the relationship between 43 strains of non-proteolytic *C. botulinum* (14 type B, 24 type E and 5 type F) using a DNA microarray. The results from this study not only confirmed previous observations, but also indicated a genetic biodiversity between type B and type F strains (Figure 5.3), where four type B strains (Eklund 17B (81-30), Colworth BL151 (86-17), IFR 05/020 (05-20) and Eklund 2B (83-01)) formed a separate clade from other type B and F strains. Interestingly the ability to grow on different carbohydrates revealed a similar pattern (Chapter 4). Stringer *et al.* (2013) describes the only study where a substantial number of type B strains have been tested, therefore a direct comparison with findings of other authors is not possible. The DNA microarray used for this comparative genomic indexing study was composed of probes based on the genome of the Eklund 17B (type B) strain and did not reveal details of the genetic variability between type E strains that had been described previously (Hielm *et al.*, 1998b; MacDonald *et al.*, 2011).

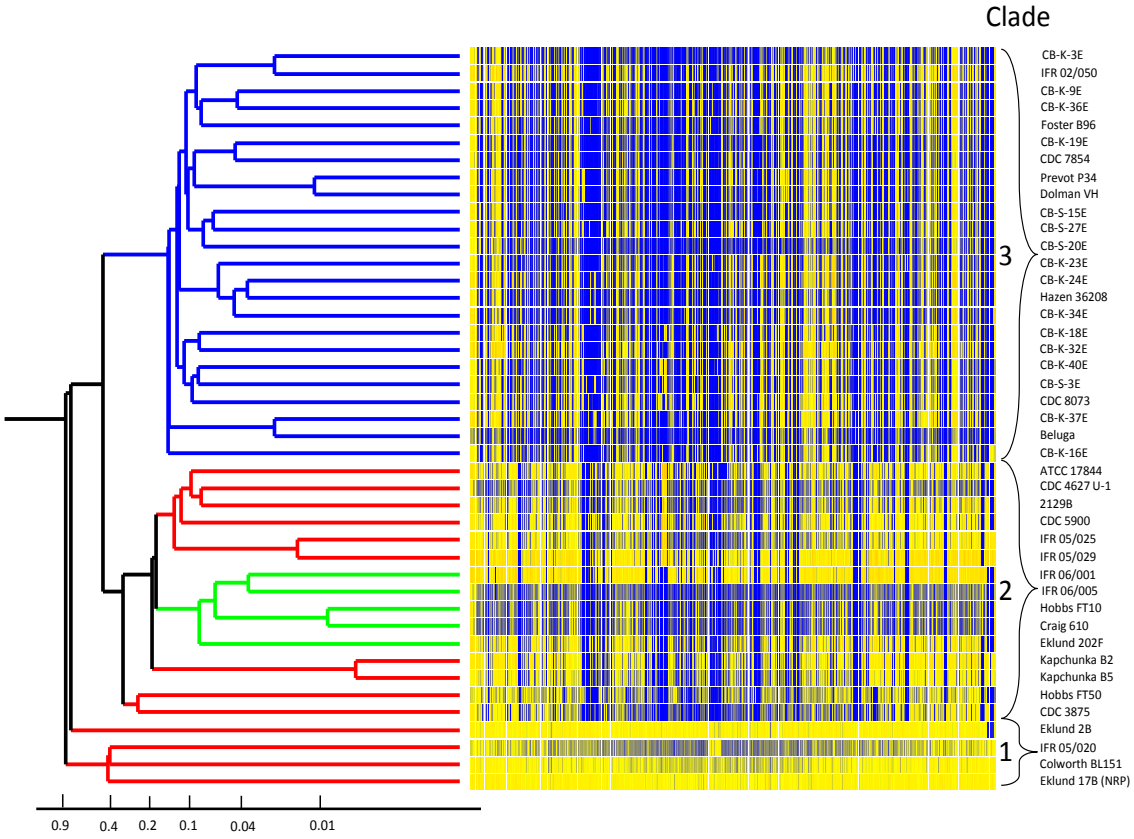

Figure 5.3 Genomic indexing of 43 strains of non-proteolytic *C. botulinum*
Source: Reprinted from "Genomic and physiological variability within Group II (non-proteolytic) *Clostridium botulinum*" by Stringer *et al.* (2013) *BMC Genomics* 2013, 14, p.333. Copyright 2013 by BioMed Central. Adapted with permission.

In conclusion, genomic analysis based on published data, shows a large diversity within non-proteolytic *C. botulinum* strains which is associated with the type of neurotoxin formed. Type B and F strains cluster together and are distinct from type E strains. The work presented in Chapters 3 and 4 reveals that strains from these different phylogenetic groups exhibit different growth properties at chill temperatures and also a different ability to utilise various carbohydrates. The strong correlation between the genomic groupings and the physiological groupings is likely to reflect the real relationship between strains of non-proteolytic *C. botulinum* and provides a basis for testing the effect of strain variability on a risk assessment for minimally processed chilled foods.

5.1.7 QMRA model

A QMRA model describes the path of a food product (from farm to fork) or a particular part (e.g. steps involved solely in manufacturing) in terms of mathematics. In this way QMRA evaluates the effects of different process components on the risks associated with hazards from pathogens and also indicates the impact of interventions.

The first steps in building a QMRA model involves the identification of hazards and the definition of production steps, and how they influence the hazard strength. The model input parameters are described in the form of probability distributions: continuous or discrete. In a continuous probability distribution a random variable can take any value in a finite or infinite range, while a discrete probability distribution describes a variable which can have a finite set of values. Examples of commonly used probability distributions in the calculation of risk in relation to food safety are: normal, lognormal (section 2.2.2.1 and 5.2.4.1), beta (section 2.2.2.2), BetaPert (section 5.2.4.1), uniform (section 5.2.4.1) or Poisson (section 5.2.4.3). Simple simulations can be conducted in commonly used spreadsheets (e.g. Microsoft Excel). For more complex models, a number of Microsoft Excel add-in routines, based on stochastic simulation algorithms, have been developed, e.g. @RISK (www.palisade.com), Crystal Ball (www.oracle.com) and ModelRisk (www.vosesoftware.com). Alternative methods of constructing probabilities in QMRA models are influence diagrams (using e.g.

Analytica (www.lumina.com) or Bayesian Belief Networks (www.hugin.com)), event trees, decision trees, fault trees or discrete event simulations (Vose, 2008).

Recently, the number of publications that describe QMRA models has increased. Some models are from food safety authorities, international organisations, academia and industry, and they have been developed for a range of microorganisms and food products. Examples are shown in Table 5.2. Due to serious health consequences and commercial implications several risk assessments for non-proteolytic *C. botulinum* in minimally heated chilled foods have been conducted previously (see section 1). Nevertheless, these models do not take account of variability arising from strain behaviour.

Product	Microorganism	Reference*
Dairy	*Staphylococcus aureus*	Barker and Goméz-Tomé (2013)
Egg and egg products	*Salmonella enteritidis*	EFSA (2010)
Ready-to-eat food	*Listeria monocytogenes*	Ross *et al.* (2009)
Seafood	*Vibrio parahaemolyticus*	WHO/FAO (2011)
Poultry meat	*Campylobacter spp.*	WHO/FAO (2009)
Water	*Cryptosporidium*	Pintar *et al.* (2012)

Table 5.2 Examples of quantitative microbial risk assessment models for different microorganisms and food products
* More published risk assessments are available on www.foodrisk.org

5.1.8 Scope of the risk assessment

This chapter describes an example quantitative risk assessment for hazards arising from spores of non-proteolytic *C. botulinum*. The assessment concerns a model dairy-based product, but with realistic parameters. The assessment includes a detailed analysis of spore loads in raw materials, thermal inactivation of spores during manufacture, distribution of spores into individual retail units and spore germination and growth during multi-stage storage. Dose-response modelling falls outside the scope of this model, as the presence of any botulinum neurotoxin is considered unacceptable. The chemical environment within the food is considered in the model (i.e. assumed to be optimal for growth). The purpose of this risk assessment is to estimate the risk from toxicity for a single retail unit of a chilled dairy product at the point of consumption. Additionally, the impact of strain variability (as a function of growth) of non-proteolytic *C. botulinum* on the calculated risk has been included. The assessment includes quantification of uncertainties and identification of sensitivity.

5.2 Methods

The risk assessment has been conducted and presented according to CAC (1999) principles and structure for microbiological risk assessment. A probabilistic approach was chosen for the QMRA model, and developed using @Risk simulation software (@Risk version 5.5.1, Palisade Corporation, Ithaca, USA). The @Risk tool performs risk analysis using Monte Carlo simulation as an add-in for Microsoft Excel (2010).

The model was structured according to a modular process risk model (MPRM) (Nauta, 2001). According to this approach factors influencing the risk, and effectivness of potential interventions, follow the food pathway, which is separated into smaller units (modules). In this QMRA for a model dairy-based product, four modules were considered: product formation, non-isothermal heating process, packing into retail units and multi-stage storage. In terms of hazards arising from non-proteolytic *C. botulinum* spores, these steps corresponds to: spore load in raw materials, thermal inactivation of spores, distribution of spores in individual product units, spore germination and outgrowth. A schematic diagram of the QMRA modules is presented in Figure 5.4. The following process can be neutral or contribute to a reduction or an increase of risk arising from non-proteolytic *C. botulinum* strains. The model input parameters and distribution functions are provided in Table 5.3 and they are derived from experimental data, literature review and/or expert elicitation. The model allows for changing the input parameters (except the temperature of storage) to examine their effect on estimated risk.

Probability distributions were generated from 1000000 interations using a Monte Carlo (section 5.1.4) simulation method with Latin hypercube sampling (LHS) to predict the impact of model parameters on the level of non-proteolytic *C. botulinum* in a model chilled dairy-based product. In this situation LHS is an effective method of generating random numbers for probability distributions that include tails.

Input parameter description	Value	Distribution	Source
Spore load, S_{batch} (spores kg^{-1})		Lognormal (<S>, σ_S)	
Mean load, <S> (spores kg^{-1})		BetaPert ($<S>_{min}$, $<S>_{mod}$, $<S>_{max}$)	
$<S_A>_{min}$	0.0001		Expert opinion
$<S_A>_{mod}$	0.1		Expert opinion
$<S_A>_{max}$	10.0		Expert opinion
$<S_B>_{min}$	0.001		Assumed
$<S_B>_{mod}$	1		Assumed
$<S_B>_{max}$	100		Assumed
Standard deviation for load σ_S (spores kg^{-1})		Uniform [σ_{Smin}, σ_{Smax}]	
σ_{Smin}	5.6		Expert opinion
σ_{Smax}	562		Expert opinion
Decimal reduction time, D(T) (min)		Lognormal (<D(T)>, σ_D)	
$<D(80)>_{-LYS}$	2.23		Chapter 2
$\sigma_{D\ -LYS}$	4.44		Chapter 2
$<D(80)>_{+LYS}$	245		Chapter 2
$\sigma_{D\ +LYS}$	423		Chapter 2
z-value (°C)		Beta (a, c, α_1, α_2)	
a_{-LYS}	3.7		Chapter 2
c_{-LYS}	16.5		Chapter 2
$\alpha_{1\text{-}LYS}$	2.9		Chapter 2
$\alpha_{2\text{-}LYS}$	9.6		Chapter 2
a_{-LYS}	4.0		Chapter 2
c_{-LYS}	19.6		Chapter 2
$\alpha_{1\text{-}LYS}$	3.1		Chapter 2
$\alpha_{2\text{-}LYS}$	6.0		Chapter 2
Spore load in volume, V, s (spores/unit)		Poisson (sV)	
V (kg)	0.1		
Time preceding the observation of growth, t_{min} (hrs)		Normal ($<t_{min}>$, σ_{tmin})	
Mean time preceding observation of growth, $<t_{min}>$ (hrs)			
Variable for strains in clusters 1 – 4	Table 5.4		Chapter 3
Coefficient of variation for spore expected growth, $\sigma_{tmin/<\sigma tmin>}$ (hrs)		Uniform [$(\sigma_{tmin/<\sigma tmin>})_{min}$, $(\sigma_{tmin/<\sigma tmin>})_{max}$]	
$(\sigma_{tmin/<\sigma tmin>})_{min}$	0.06		Chapter 3
$(\sigma_{tmin/<\sigma tmin>})_{max}$	0.21		Chapter 3

Table 5.3 Model specification and parameterization for calculation of risk associated with non-proteolytic *C. botulinum* in a model dairy-based food product

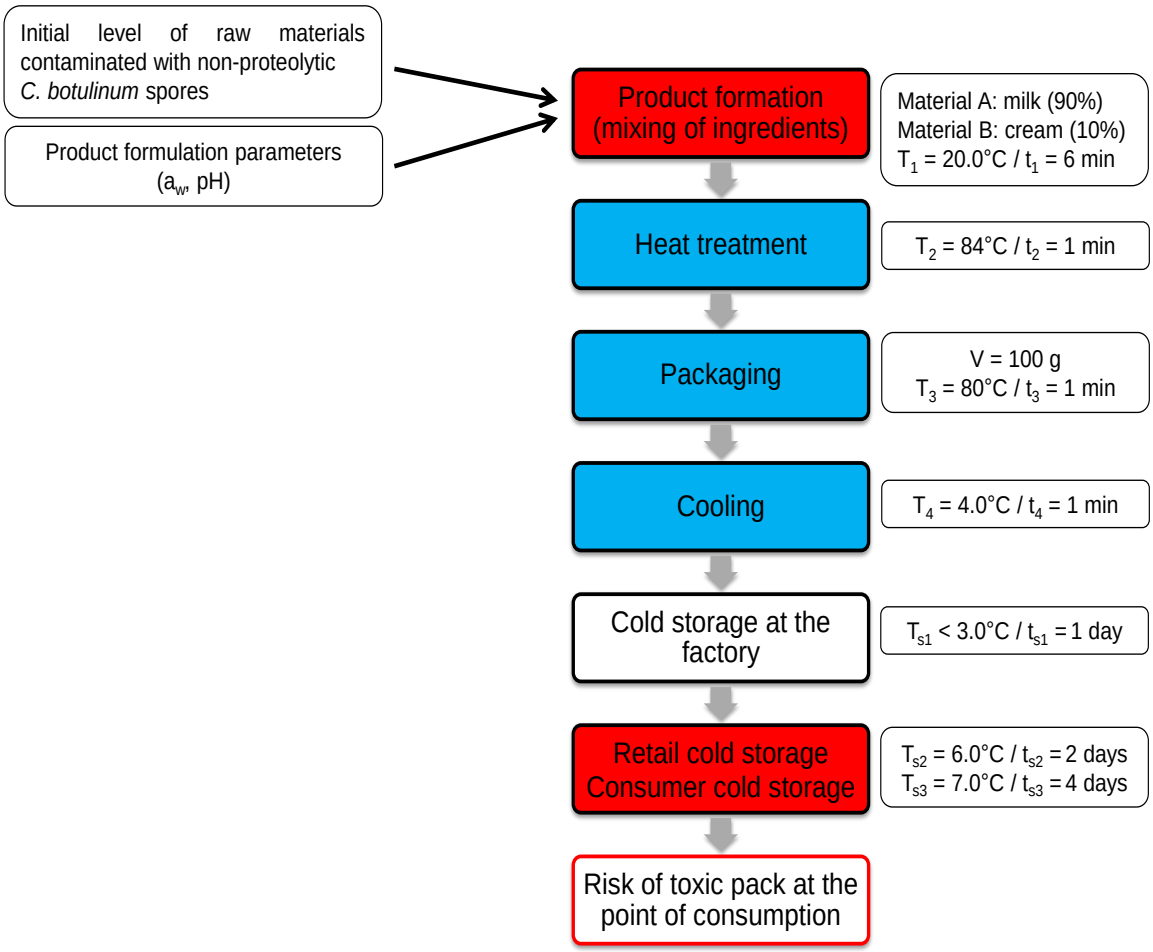

Figure 5.4 A risk assessment scheme for hazards arising from spores of non-proteolytic *C. botulinum* in a model dairy-based chilled food product
The diagram shows stages of product manufacture considered in the model with corresponding inputs and parameters. At each stage the level of non-proteolytic *C. botulinum* spores is calculated. Colours of boxes indicate the impact of manufacture phase on the loading of non-proteolytic *C. botulinum* spores: neutral (white colour), decrease (blue colour), increase (red colour)

This QMRA model for non-proteolytic *C. botulinum* includes for the first time estimates of the impact of strain variability and also lytic enzyme activity on the calculated risk. The variability of non-proteolytic *C. botulinum* strains is described by data generated in Chapters 2, 3 and 4. The response of the tested strains to environmental conditions is summarised in section 5.1.6 and indicates that variability is associated with the type of neurotoxin. In general, type E strains show the ability to utilize different carbohydrates compared to type B and F strains. Moreover, clustering of strains in terms of their growth at chill temperatures indicates that strains forming type E neurotoxin show more rapid growth compared with strains forming type B and F neurotoxin. Since time to growth is identified as a significant source of strain variability, the calculation of

probability of toxicity at the point of consumption is based on a classification of strains into the four clusters described in section 3.3.4 (Figure 3.9). Statistical analysis of data on heat resistance for non-proteolytic *C. botulinum* spores revealed that there is no significant difference in decimal reduction time for strains of different toxin types (section 2.3.2.2 – testing the dependence of the spore heat resistance on toxin type). Nevetheless, the same data indicated significantly greater *D* and *z*-values for spores recovered in the presence of lysozyme (section 2.3.2.3 – testing the similarity of +LYS HS and +LYS HR fractions). For comparison, the effect of heat treatment applied to the product with and without egg yolk is considered (egg yolk being potentially contaminated with egg white – a rich source of lysozyme).

5.2.1 Hazard identification

The risk assessment considers the risk posed by spores of non-proteolytic *C. botulinum.* Although botulism (the foodborne disease caused by consumption of preformed *C. botulinum* neurotoxin) occurs relatively rarely, it has a high impact on consumer safety, between 5 – 10% of cases are fatal. Full details of the hazard presented to consumers by non-proteolytic *C. botulinum* are summarised in section 1.1. In contrast to many other foodborne pathogens, the hazard arising from non-proteolytic *C. botulinum* is associated with the ability of dormant spores to survive in harsh conditions. Those spores are commonly present in the environment and can potentially enter the unprocessed food materials or a food product at any stage of the manufacturing process (particularly in manufacturing facilities where spore contaminated materials are handled). The heat treatment applied may not always be sufficient for inactivation of spores, and this can allow to subsequent germination, growth and botulinum neurotoxin formation, which is identified as a hazard.

5.2.2 Exposure assessment

Chilled food product sales in the UK have been summarised in detail in section 1.2, based on data from the Chilled Food Association. It is estimated that 10^9 packs are sold in the UK annually and sales of chilled foods continue to rise at an average of 10% per annum (Peck *et al.*, 2008). The sale of chilled prepared desserts in 2012 was estimated to be worth £ ~0.7 billion pounds and it constituted 4.6% of the total chilled market (Figure 1.2). Consumer enthusiasm

to purchase and consume convenient and fresher foods with minimal preservatives makes it likely that the increase in consumption of minimally heated chilled foods can be expected to continue.

There are very few data describing the frequency and concentration of non-proteolytic *C. botulinum* spore contamination of dairy based chilled products. Nevertheless, since non-proteolytic *C. botulinum* spores are widely distributed in the environment (Dodds, 1993b; Lund and Peck, 2000; Carlin, 2011; Heyndrickx, 2011) it can be assumed that any ingredient/food might be contaminated. Information regarding the spore load in dairy based products was derived from the literature and based on expert opinion.

5.2.3 Hazard characterisation

A description of clinical forms of botulism: incubation times, vehicles, symptoms, treatment and long term effects are summarised in section 1.1.2. There is no particular group identified at risk in terms of botulinum neurotoxin – the mechanism of action is similar irrespectively on victim's gender, age or health conditions. Even a single spore of non-proteolytic *C. botulinum* can lead to growth, gas production and neurotoxin formation at chill temperatures (Chapter 3). Lund and Peck (2000) considered that death can be caused by consumption of as little as 30 to 100 ng of botulinum neurotoxin, thus from a risk assessment perspective, any amount of toxin is considered as hazard, and this is the end point of concern.

5.2.4 Risk characterisation

An overview of the steps considered in the mathematical model is presented in Figure 5.4. The probabilistic modelling (with variables presented as probability distributions) was applied for evaluating: 1) spore load in raw materials (product formation), 2) thermal inactivation of spores (heat treatment), 3) distribution of spores in individual units (volume partition), and 4) germination and growth of non-proteolytic *C. botulinum* (storage).

Product description

The model dairy-based chilled product is prepared from multiple materials in standard 10000 kg batches. All batches are assumed to be independent. The formulation includes two separate components – milk (material A) and cream (material B) constituted in a single vessel (at ambient temperature of ~20.0°C) (an alternative product contains egg yolk). Following cooking at 84°C for 1 min the batches are transferred to a filling line and hot filled into ~100000 x 100 g sealed cartons. The sealed units are cooled and stored before distribution into the retail and consumer chain. Values for water activity and pH throughout the process step are constant, a_w = 0.997 and pH ~6.65. The same procedure and parameters are applied to a product with added egg yolk. Throughout the calculations the following notation is applied: -LYS – product without added egg yolk and assumed not to contain lysozyme; and +LYS – product with added egg yolk and associated lysozyme.

5.2.4.1 Spore load in raw material

Knowledge of spore concentration variability in raw materials is essential to quantify hazards, as it affects the strength of treatment required to ensure product safety.

This model assumes a clear separation of variability and uncertainty in spore load. The spore concentration in a batch of raw materials, S_{batch}, spores kg^{-1}, is assumed to have a lognormal distribution (equation 2.11), to allow for small probabilities of large loads. The variability in the concentration of spores is parameterized by mean value <S>, and by the standard deviation, σ_s, of S_{batch}. The parameters are uncertain and reflect the lack of precise knowledge on the number of spores present in the raw materials used to make dairy-based desserts. The uncertainty in the mean concentration, <S>, is represented by a BetaPert distribution, a special form of the beta distribution (equation 2.15), which is commonly used for representing expert opinions. The BetaPert distribution requires three parameters: minimum, modal and maximum values for the expected mean concentration of spores ($<S>_{min}$, $<S>_{mod}$ and $<S>_{max}$).

A uniform probability distribution defined by range values σ_{Smin} and σ_{Smax}, is chosen to represent uncertainty in the standard deviation for S_{batch}

$$\mathrm{p}(\sigma_S) = \frac{1}{\sigma_{Smax} - \sigma_{Smin}} \qquad 5.1$$

where $\sigma_{Smin} \leq \sigma_S \leq \sigma_{Smax}$. The uniform distribution expresses an equal probability of occurrence for every value in the range, and in many cases is used when limited information is available.

The spore concentration is the sum of the defined component concentrations weighted by their volume fraction. In this assessment, 90% of material is type A, and 10% of material is type B. A group of experts were used to provide estimates for parameters of the uncertainty distribution for the mean concentration of spores, <S>, in type A material, and 95% UCL and LCL of spore concentration, S_{batch}, (Appendix 6). The 95% UCL and LCL were used to estimate the σ_S. Spore loads are insensitive to any parameters that describe the loads in egg yolk.

Expert opinion established values $\langle S_A \rangle_{min} \approx 0.0001$, $\langle S_A \rangle_{mod} \approx 0.1$ and $\langle S_A \rangle_{max} \approx 10$ spores kg^{-1} for type A material. These values are consistent with values published by the ACMSF (2005) for spore loads of *C. botulinum* in infant formula. Previous published studies by Hussong and Hammer (1930), Fooladi *et al.* (2010) and Meshref (2013) indicated that cream is more contaminated with microorganisms than milk. Thus in this risk assessment for a model dairy-based chilled dessert it is assumed that the concentration of spores in type B material is ten times greater than in material A. A single value of $\sigma_S \approx 56$ (calculated from elicited 95% UCL and LCL for S_{batch}) was used to estimate the uncertainty distribution for σ_S, completed by $\sigma_{S\text{min}} = \frac{\sigma_S}{10}$ and $\sigma_{S\text{max}} = 10\sigma_S$. It is assumed that the standard deviation of the spore concentration is independent of the material. For a complex product with many components a similar distribution could be constructed.

5.2.4.2 Thermal inactivation of spores

A thermal process is the primary method for reduction of spore numbers in food products, therefore it is an important component of the risk assessment model. It is assumed that for each batch of dairy-based dessert all ingredients are mixed and heat treated in a single vessel (the materials are not considered to be preheated before mixing). During the heat treatment the thermal inactivation of spores is assumed to be uniform through the volume. In the model product the temperature of the system varies with time during heating and cooling phases. Initially the temperature of the product rises from ambient temperature T_1 = 20.0°C, towards a holding temperature T_2 = 84°C. During this period (Δt_1 ~6 min) the temperature of product increases uniformly. After reaching T_2, the cooking phase has duration Δt_2 = 1 min. Following cooking the product is hot filled into sealed cartons with a final temperature, T_3 = 80°C, this process takes time Δt_3 ~1 min (the time to fill a complete batch of units). Finally a cooling phase reduces the temperature to T_4 = 4.0°C over a period Δt_4 ~1 min (Figure 5.5).

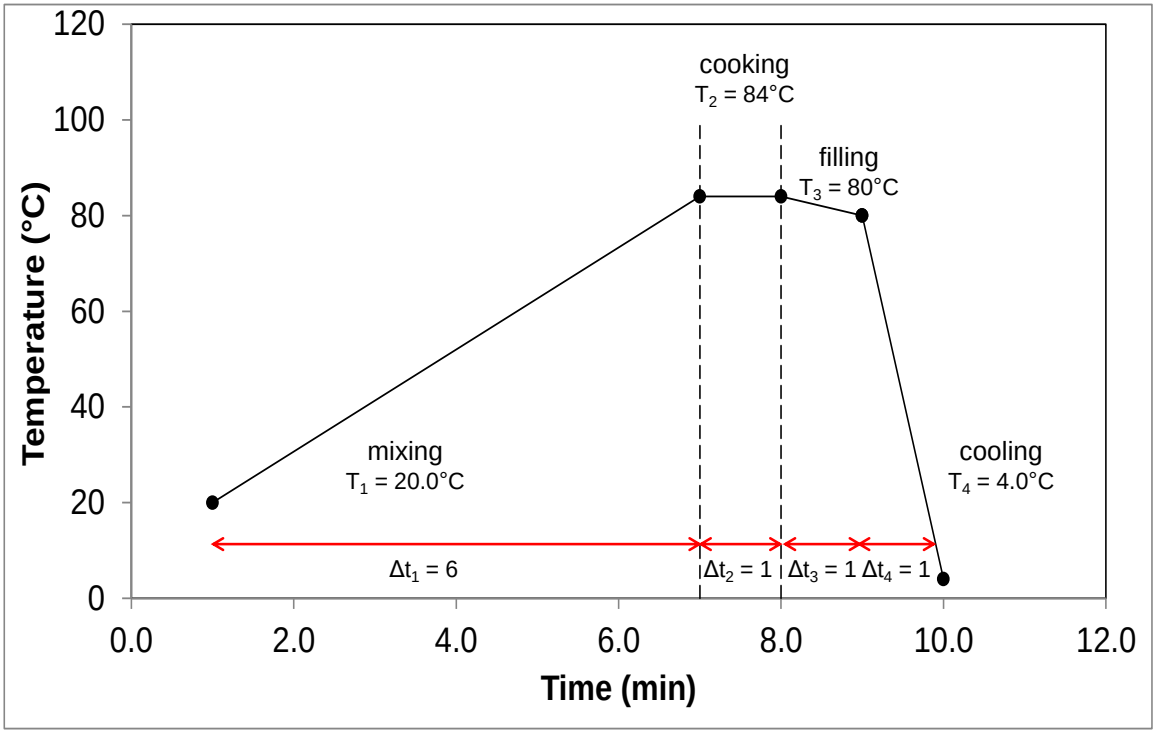

Figure 5.5 Parameterised schematic for the thermal history of model dairy-based chilled dessert

As summarised in section 2.1.2, the bacterial heat resistance can be described by first order reaction kinetics (equation 2.3), which in terms of spore *D*-value can be expressed as

$$S(t) = S_o 10^{-t/(D(T))} \quad 5.2$$

It is assumed that the *D*-value does not vary between batches. Nevertheless, different *D* and *z*-values are applied when the presence of lytic enzymes is considered in the product. The uncertainty concerning the *D*-value at temperature T is represented by a lognormal distribution, parameterized by <D> and σ_D (see section 2.2.2.1).

A reduction of the number of spores in a batch corresponds to a non-isothermal process equivalent to a corresponding "effective" isothermal process, at a reference temperature, T_{ref}, for an effective time, $t_{eff}(T_{ref})$. When the temperature variation is linear (straight line) the effective heating time is

$$\Delta t_{eff}(T_{ref}) = \frac{z}{ln10}\left(\frac{\Delta t}{\Delta T}\right)\left(10^{\frac{T_2 - T_{ref}}{z}} - 10^{\frac{T_1 - T_{ref}}{z}}\right) \quad 5.3$$

where $\Delta T = T_2 - T_1$, $\Delta t = t_2 - t_1$ (T_1, T_2, t_1 and t_2 are the initial and final heating temperature and time) (G. C. Barker, personal communication).

A beta distribution (see section 2.2.2.2), parameterized by extreme values a, c and shape parameters α_1 and α_2, was used to represent uncertainty concerning the *z*-value. Reference temperature, T_{ref} = 80°C (a commonly applied temperature in a manufacturing of the chilled products) was used.

Each batch with a particular number of spores has an uncertain *D*-value. The range of *D*-values for non-proteolytic *C. botulinum* spores in different laboratory media and food matrices, obtained from a literature review, is presented in Figure 2.14 in Chapter 2. The parameters (mean and standard deviation) for lognormal distribution of *D*-value at reference temperature T_{ref} = 80°C, are respectively $<D'(80)>$ = 2.23 min and $\sigma_{D'(80)}$ = 4.44 min (for -LYS data), and $<D'(80)>$ = 245 min and $\sigma_{D'(80)}$ = 423 min (for +LYS data) (Figure 2.24).

The temperature profile for dairy-based chilled product is created from a set of time-temperature slopes with a high temperature isothermal hold for 1 min.

Transformation of uncertain knowledge of *z*-value (with parameters a = 3.7, c = 16.5, α_1 = 2.9, α_2 = 9.6 (for -LYS) and a = 4.0, c = 19.6, α_1 = 3.1, α_2 = 6.0 (for +LYS)) into uncertainty about equivalent heating time, t_{eff} at reference temperature (T_{ref} = 80°C) using the relationship between t_{eff} and real time, gives uncertainty for effective time with $\langle t_{eff} \rangle$ = 6.99 and σ_{teff} = 1.95 for the product without lysozyme activity and $\langle t_{eff} \rangle$ = 5.02 and σ_{teff} = 1.25 for the product with egg yolk added. Two distributions for t_{eff} corresponding to the presence and absence of lytic enzyme activity are illustrated in Figure 5.6.

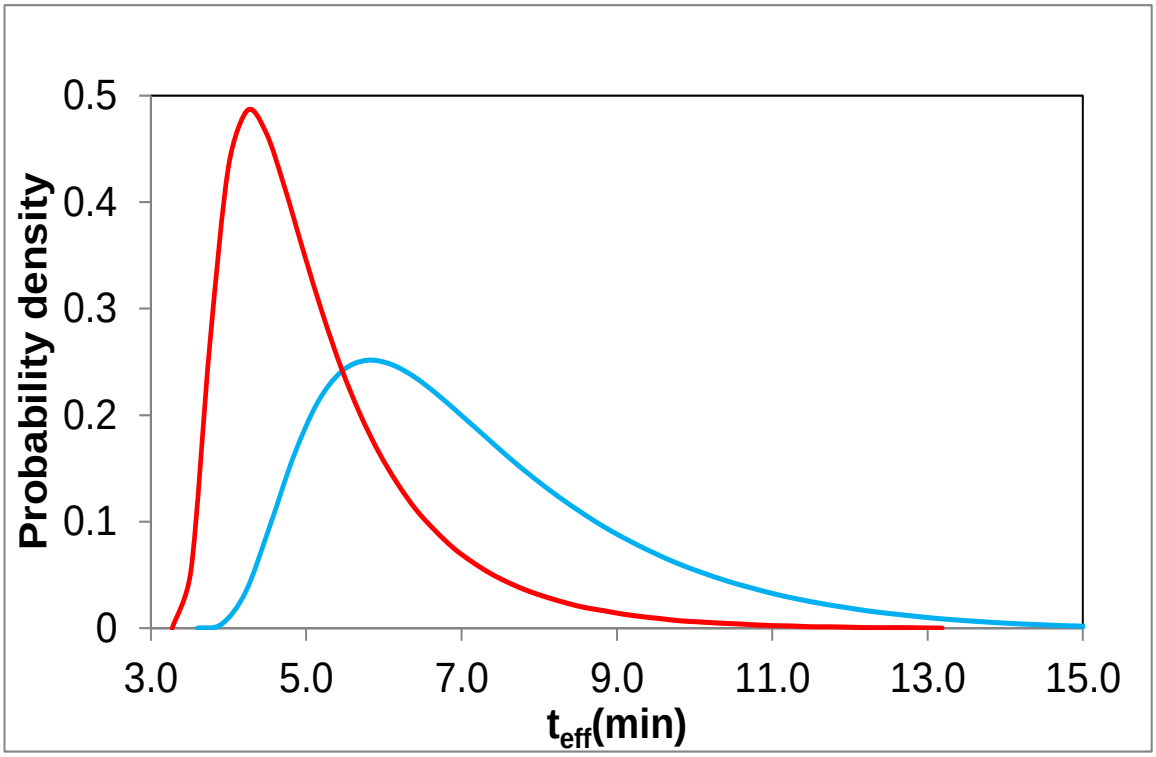

Figure 5.6 Distributions representing belief concerning the effective heating time, $t_{eff}(80)$, for batches of dairy-based dessert
Distributions obtained using uncertain *z*-values (represented by beta distribution) for dairy-based dessert without added egg yolk; a = 3.7, c = 16.5, α_1 = 2.9, α_2 = 9.6 (blue line) and for dairy-based dessert in presence of egg yolk; a = 4.0, c = 19.6, α_1 = 3.1, α_2 = 6.0 (red line)

Combining beliefs for the effective heating time and for decimal reduction time for spores of non-proteolytic *C. botulinum*, gives beliefs concerning the logarithmic population reduction achieved by the thermal process for the model dairy-based chilled dessert

$$\text{kill} = \log\left(\frac{N_0}{N(t)}\right) = \frac{t_{eff}}{D(T_{reference})} \qquad 5.4$$

5.2.4.3 Distribution of spores in individual units

Following the heating process the product is transferred to a filling step, hot filled and sealed, into individual 100 g sealed cartons. The partitioning process is an important step in QMRA that redistributes spores over the units of food product. Considering a batch consists of 10^5 units, the distribution of spores can be either regular – in which each individual unit contains the same number of spores, clustered – meaning that the remaining spore load in post-processed batch of dairy-based dessert may be contained in a single unit and all the other units are free of contamination, or random – where some units may be spore free and some can contain variable number. A random distribution of spores within independent units, s_{unit}, is assumed in the model and is described by a Poisson process

$$p(s_{unit}|\lambda) = \frac{\lambda^s e^{-\lambda}}{s!} \quad 5.5$$

where s_{unit} is the number of spores and λ is the expected number of spores in the individual unit. The exposure of product to the air during dispensing process is not considered as a potential contamination source.

5.2.4.4 Germination and growth of non-proteolytic *C. botulinum* during multi-stage storage

Following volume partition the model dairy-based dessert is stored within the factory facilities under controlled conditions at low temperature T_{s1} < 3.0°C for time t_1 ~1 day prior to distribution. Subsequently the product is transferred to retailers at temperature T_{s2} = 6.0°C for a maximal period t_2 ~2 days. The final stage of the product pathway is consumer storage. This is the most variable storage phase during the product lifetime, and there are very limited data available regarding time-temperature history of food during consumer storage. George *et al.* (2010) indicated that the dominant mean operating temperature of domestic fridges in the UK is 7.0°C (see section 3.1) and this temperature T_{s3}, for a period of t_3 ~4 days is used to represent consumer storage. The fluctuations due to transport conditions and due to variable fridge temperatures in different parts of a fridge are not considered in the model. In this assessment it is assumed that the pH and the water activity of the dairy-based chilled product are

optimal (pH = 6.65 and a_w = 0.997) and constant during the total period of storage.

In the case of *C. botulinum* in food growth can be uncertain (Barker *et al.*, 2005b) and initiated from a very low spore concentration, even from a single spore but it can be variable within a population of spores (Ikawa *et al.*, 1986; Jensen *et al.*, 1987; Stringer *et al.*, 2009). The food unit is considered to be free of hazard before any individual germinated spore has entered the growth phase that may lead to subsequent neurotoxin formation (this is very often referred to as a delay time (Malakar *et al.*, 2011)). The evaluation of the time, before a spore has entered the growth phase is of primary interest in assessment of foodborne botulism. It depends on the storage temperature and chemical composition of food matrices, and can be highly variable and uncertain (Stringer *et al.*, 2005; Stringer *et al.*, 2011).

In the model, the assessment of time, during which the product is free of hazard is based on results summarised in Chapter 3. The calculated τ parameter, used to indicate variable growth of non-proteolytic *C. botulinum* strains at chill temperatures (see section 3.2.8), is used to calculate t_{min}, the time preceding observation of growth at temperatures 6.0°C (storage at retailer) and 7.0°C (consumer storage). The relationship between the τ parameter and t_{min} is expressed in equation 3.3. As presented in section 3.3.2.1, during 90 days of incubation at 3.0°C, growth from non-proteolytic *C. botulinum* spores was not observed, therefore this stage is not considered in the calculation of risk. Previously Graham *et al.* (1997) reported growth at 3.0°C, and these data could be added to an updated risk assessment.

For the purpose of this assessment the normal distribution (see section 3.2.8) with mean, $<t_{min}>$, and coefficient of variation, $\sigma_{t_{min}}/<t_{min}>$, is used to parameterise the variability distribution of the time preceding observation of growth from a small population of spores, t_{min}, at chill temperatures. A uniform distribution, defined by extreme values $(\sigma_{t_{min}}/< t_{min}>)_{min}$, $(\sigma_{t_{min}}/< t_{min}>)_{max}$, is used to represent uncertainty in the coefficient of variation for t_{min}.

The value of $<t_{min}>$ at 6.0°C for a small population of spores (s = 100, 10 and 1 spores tube^{-1}) is obtained from extrapolation of $<t_{min}>$ calculated from measured τ for large population of spores (s = 10^6, 10^5 and 10^4 spores tube^{-1}) (Figure 5.7). The value of $<t_{min}>$ is calculated for the four clusters of non-proteolytic *C. botulinum* strains (see Figure 3.9 for strain classification based on parameter τ). Extrapolation is used because not all strains tested in Chapter 3 show growth from low spore concentrations (Appendix 4). The extrapolation of $<t_{min}>$ is validated by comparison of the values with those calculated for the subset of strains that show growth from small concentration of spores (marked as red points in Figure 5.7).

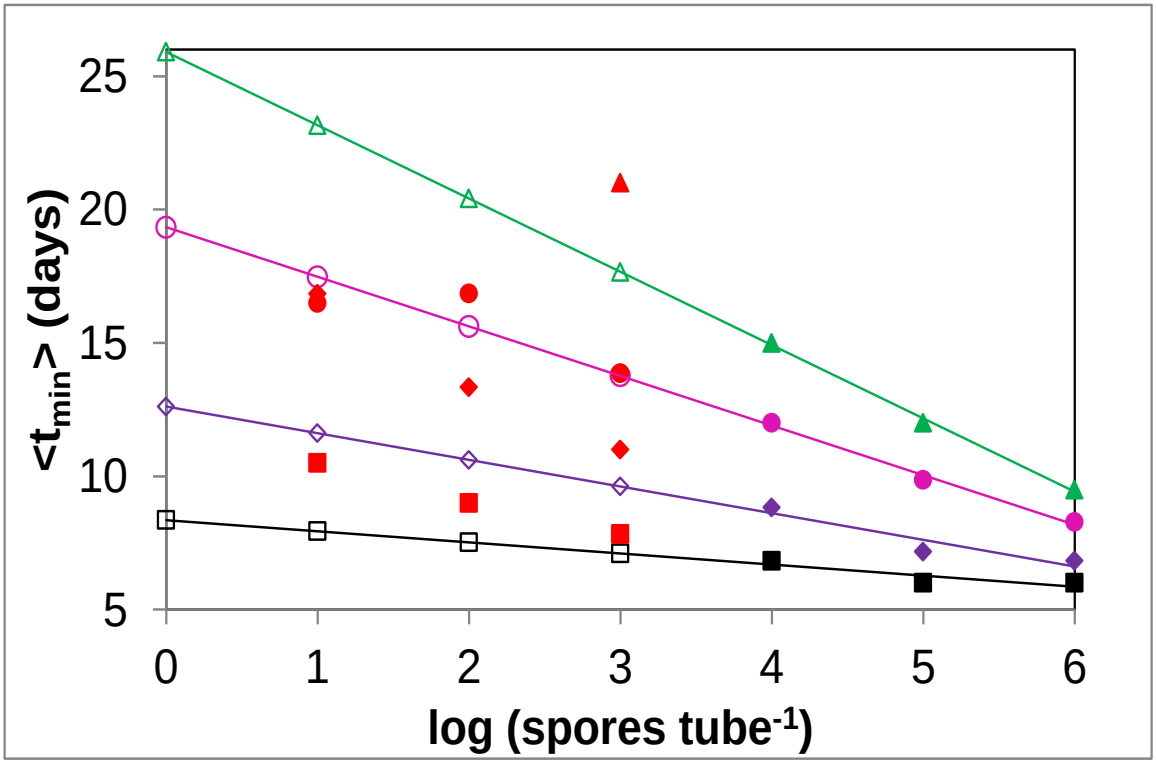

Figure 5.7 Calculated mean time preceding observation of growth $<t_{min}>$ (in days) for non-proteolytic *C. botulinum* at 6.0°C from large population of spores and corresponding $<t_{min}>$ at small concentration of spores obtained by extrapolation

The figure presents $<t_{min}>$ for four clusters of non-proteolytic *C. botulinum* strains. $<t_{min}>$ measured (full symbols) extrapolated (open symbols) for strains assigned to cluster 1: (●) and (○), cluster 2: (▲) and (△), cluster 3: (♦) and (◊), cluster 4: (■) and (□). The red coloured marks values calculated for $<t_{min}>$ for strains which show growth from low concentration of spores (see Chapter 3) and symbols for clusters 1 – 4

Figure 5.7 indicates that the extrapolated value for $<t_{min}>$ takes smaller values than $<t_{min}>$ obtained from measurements. Thus extrapolation ensures a conservative approach for estimation of time preceding observation of growth,

t_{min}. The value for $<t_{min}>$ at 7.0°C is extrapolated using the same gradient as the line obtained at 6.0°C. The values of $<t_{min}>$ (hrs) for strains assigned to clusters 1, 2, 3 and 4 at 6.0°C and 7.0°C from a low number of spores (s = 100, 10 and 1 spores unit^{-1}) are summarised in Table 5.4.

Cluster	$<t_{min}>$ at 6.0°C (hrs)			$<t_{min}>$ at 7.0°C (hrs)		
	s = 100	s = 10	s = 1	s = 100	s = 10	s = 1
1	375	419	464	318	362	407
2	490	556	622	460	526	592
3	255	279	303	239	263	287
4	181	191	201	163	173	183
Average	325	361	397	295	331	367

Table 5.4 Extrapolated mean value of t_{min} for a low number of spores of non-proteolytic *C. botulinum* at 6.0°C and 7.0°C

The range of calculated $<t_{min}>$ values quantifies the uncertainty in the variability of shortest time when any growth could be expected with $(\sigma_{t_{min}}/<t_{min}>)_{min}$ = 0.06 and $(\sigma_{t_{min}}/<t_{min}>)_{max}$ = 0.21. The $\sigma_{t_{min}}/<t_{min}>$ value is considered to be independent of growth conditions and is calculated from the average standard deviation of t_{min} at 6.0°C over all clusters.

The impact of multiple sequential periods of isothermal storage (t_i at T_i i = 1,3) on t_{min} is expressed by considering exhausted fractions $f_i = \frac{t_i}{t_{min}(T_i)}$ where t_{min} is the time preceding observation of growth, at temperature T_i and t_i is incubation time for the isothermal storage period. It is assumed that a spore exceeds the time preceding observation when the cumulative fraction, $f = \sum f_i$, for a spore is greater than one (Malakar *et al.*, 2011).

By combining the probability for a finite number of spores in single retail unit with the probability of spores exceeding t_{min} (i.e. f > 1) the probability of a hazardous pack is calculated, as a product, and this is the main measure of risk used for risk characterisation.

5.3 Results

All factors affecting the risk of intoxication from consumption of dairy-based chilled products are incorporated into a mathematical model. Its implementation is presented in Figure 5.4 and values for a probabilistic representation of the parameters used are summarised in Table 5.3. The probabilistic model was built using a Monte Carlo simulation tool (Figure 5.8) with 1000000 interations for products with and without egg yolk added.The simulation process was repeated five times and the calculated risk is an average of all simulations. The average and standard deviation of the risk estimate was calculated.

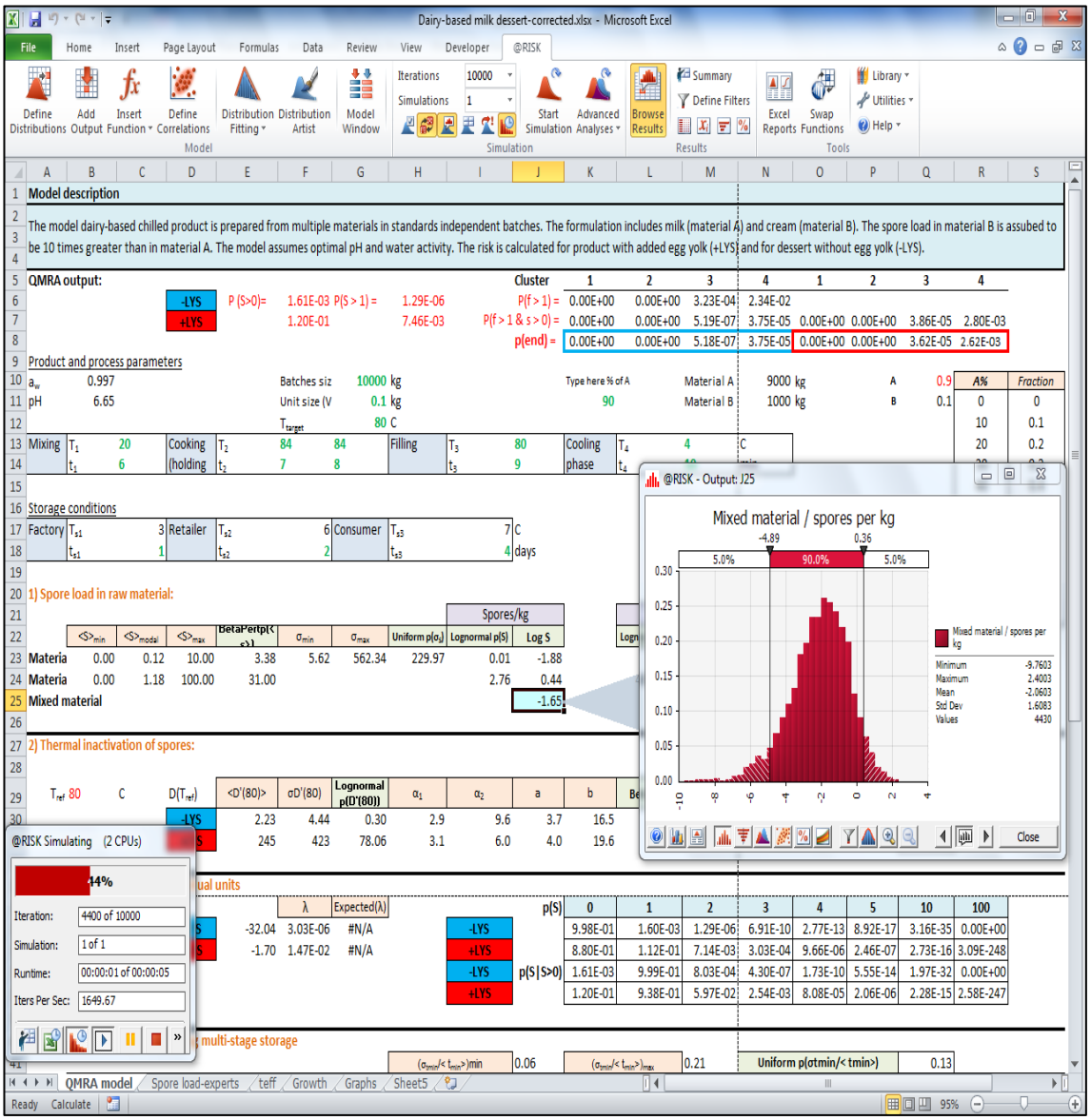

Figure 5.8 A screen-shot presenting a mathematical model for calculation of risks associated with non-proteolytic *C. botulinum* in dairy-based chilled product

The product, consisting of raw materials from different sources, may contain large number of spores, S. Distributions representing the spore loads for non-proteolytic *C. botulinum* in both materials and in the mixed product (90% of A and 10% of B) based on the parameterizations (Table 5.3), are illustrated in Figure 5.9.

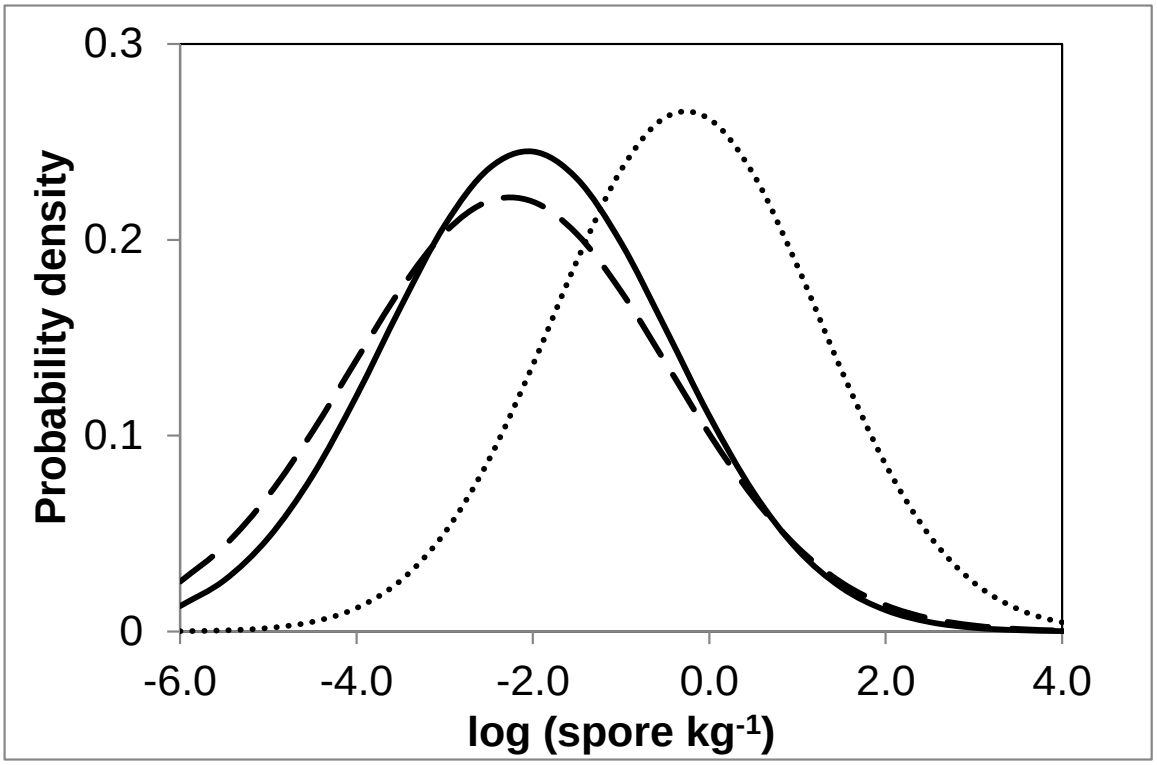

Figure 5.9 Distributions representing belief concerning spore loads, for non-proteolytic *C. botulinum*, in raw materials of type A and type B materials, and mixed material
material A – milk (dash) and material B – cream (dotted line), mixed material – 90% of A and 10% of B (solid line)

It was assumed in the model that cream was more contaminated than milk, and that it could be the major source of spores in model dairy-based product, but the total spore load, S_{batch}, in the mixed product is dependent on the proportion of the ingredients. Figure 5.10 illustrates the distribution of batch spore loads in mixed product, with expected value ~5 x 10^4 spores, prior to heat treatment and the sensitivity of this distribution with respect to the $<S_A>_{max}$ parameter.

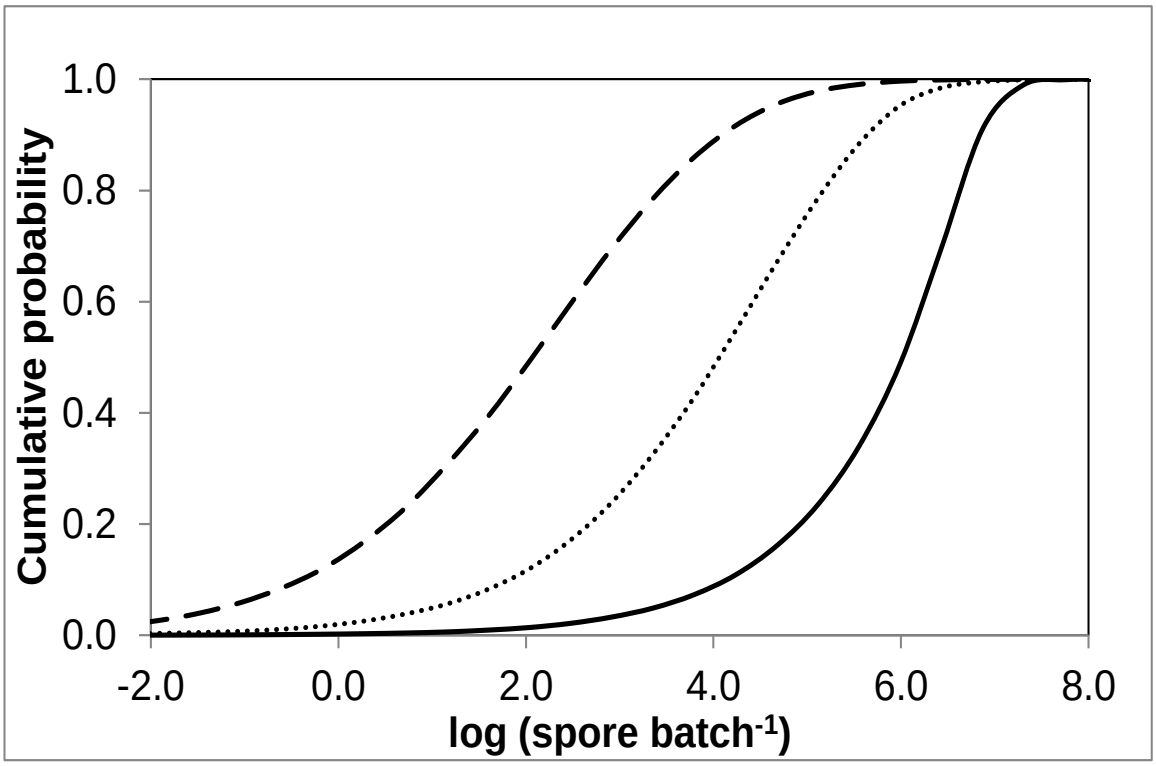

Figure 5.10 The cumulative probability of the spore load for a batch of dairy dessert (prior to heat treatment)
The full, dotted and broken lines corresponds to parameter values $<S_A>_{max}$ = 1000, 100 and 10 spores kg^{-1}

Belief concerning the population reduction achieved by the heat treatment process is represented in Figure 5.11. It is apparent from the graph that the effect of heat treatment on reduction of spores, largely depends on the composition of the product. The model predicts that effective heating time t_{eff} = 6.99 min and $<D'(80)>$ = 2.23 min, assumed for product without egg yolk, provides at least 0.2 order of magnitude population reduction and there is a probability ~0.54 for batches to receive a heat treatment that is stronger than a 6 log kill. There is also ~0.38 probability that the reduction of spores exceeds 10 orders of magnitude. The effective heating time t_{eff} = 5.02 min and $<D'(80)>$ = 245 min, assumed for product with added egg yolk, would provide only one order of magnitude reduction of spores with probability ~0.99. There are very small probabilities, ~3.0 x 10^{-3}, ~4.0 x 10^{-4} and ~1.2 x 10^{-4} that a batch experiences a two, three or four orders of magnitude in population reduction. Use of a single *D*-value D'(80)~1.29 min (see section 2.3.2.3 on practical application of heat treatment parameters) gives a probability ~0.85 that the population reduction exceeds six orders of magnitude.

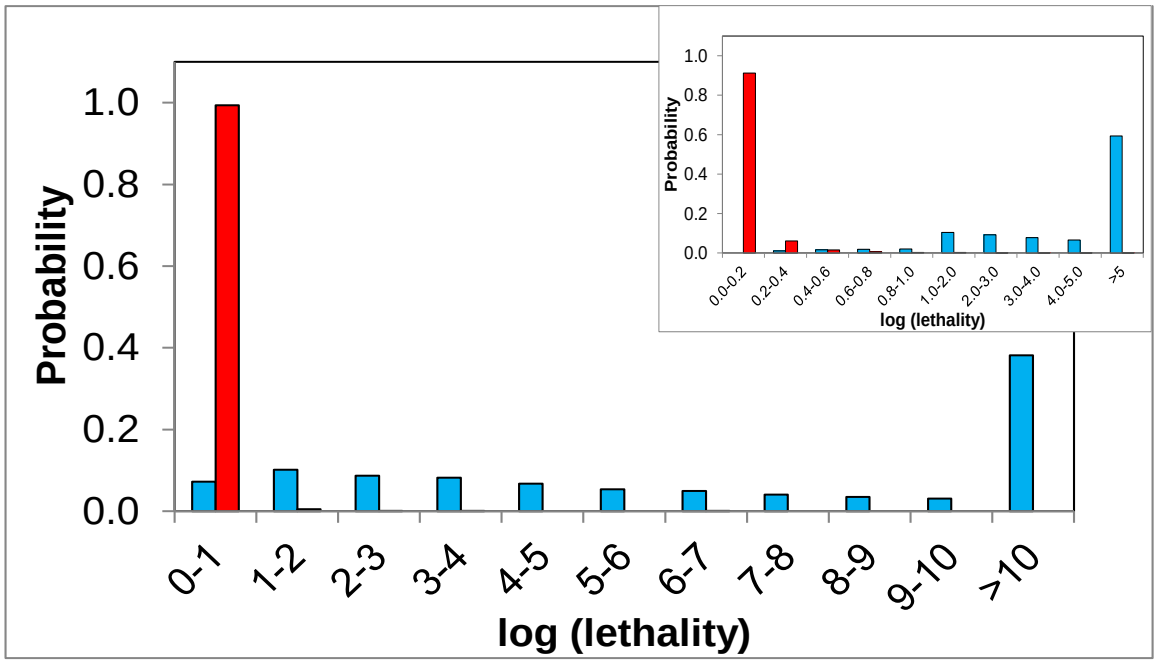

Figure 5.11 Reduction of non-proteolytic *C. botulinum* spores, achieved by the thermal process applied to model dairy-based dessert
The bars corresponds to distinct belief concerning the *D* and *z*-values for dessert without egg yolk ($<D'(80)>_{-LYS}$ = 2.23 min; p(z) with parameters a = 3.7, c = 16.5, α_1 = 2.9, α_2 = 9.6) (blue bars), and with egg yolk ($<D'(80)>_{+LYS}$ = 245 min; p(z) with parameters a = 4.0, c = 19.6, α_1 = 3.1, α_2 = 6) (red bars). The insert graph represents the distribution for reduction of spores corresponding to less than five orders of magnitude

The heat treatment applied to the dairy-based dessert reduces the spore load, but this is only significant in the absence of egg yolk in the product. Lysozyme activity provides protection to spores, and there is a probability ~0.99 that a pasteurized batch is not spore free, with expected value of the spore load, S_{batch} ~9600. For batches of dessert without egg yolk with $<D'(80)>$ = 2.23 and $\sigma_{D'(80)}$ = 4.44 the probability that processed batches contain finite numbers of non-proteolytic *C. botulinum* spores is ~0.18 with a mean load S_{batch} ~244 spores in an occupied batch. The batch spore load is very sensitive to parameters for D'(80) (Table 5.5) and it is essential to take into account the variability associated with *D*-value. A single value for D'(80) = 1.29 increased the probability of a spore free batch (~0.96) with expected spore load S_{batch} ~2. Slightly greater standard deviation or mean value for the *D*-value decreases the probability that a pasteurised batch is spore free and the corresponding spore concentration of contaminated batches.

<D'(80)>*	$\sigma_{D'(80)}$*	Expected load (spore batch^{-1}) S_{batch}	97.5% load (spore batch^{-1}) S_{batch}	Probability of spore free batch	Probability batch load exceeds 10 spores	Probability batch load exceeds 100 spores
2.23	4.44	395	404	0.82	0.11	0.05
2.76	4.90	332	688	0.78	0.13	0.06
1.87	2.50	206	229	0.84	0.09	0.04
2.26	2.70	149	383	0.79	0.12	0.05
1.29	-	0.57	2	0.96	0.01	0.00

Table 5.5 Distribution of spore loads of non-proteolytic *C. botulinum* in heat treated batches of dairy-based chilled dessert (-LYS)
*Mean and standard deviation of *D*-values derived from literature review on spore heat resistance summarised in Table 2.31

The probability that a processed retail unit contains spores is very small and clearly depends on the expected spore concentration in a pasteurised batch. The details of the distribution of spore loads for non-proteolytic *C. botulinum* in units, s_{unit}, of the chilled dairy-based dessert are illustrated in Figure 5.12. The insert graph presents a distribution corresponding to non-zero spore load.

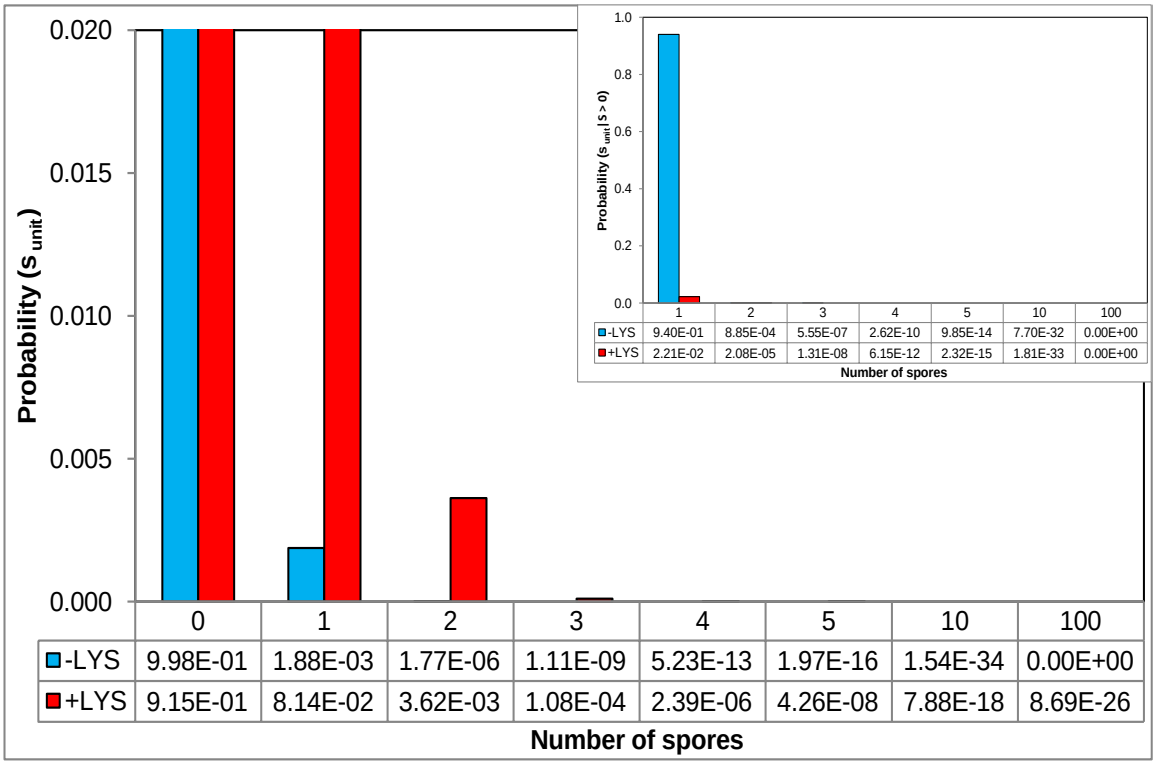

Figure 5.12 Belief concerning probability distribution of the number of spores of non-proteolytic *C. botulinum* in a heat treated retail unit of model dairy-based dessert.
A dairy-based chilled dessert units without addition of egg yolk (blue bars) and with addition of egg yolk (red bars). An insert graphs represent distribution for non-zero spore load (numeric values are added for clarity)

The greatest contribution to risk arises from a unit with a single spore, which for -LYS is ~1.9 x 10^{-3} (with the <D'(80)> = 2.23 and $\sigma_{D'(80)}$ = 4.44) and for +LYS

~8.1 x 10^{-2}. Probabilities decrease for higher unit loads. Unit spore loads higher than one are very rare with probability ~1.8 x 10^{-6} for a product without egg yolk. Addition of egg yolk to a product increases the probability of more than one spore in a retail unit to ~3.7 x 10^{-3}. These probabilities indicate an expectation that 180 and 3700 units in one batch (1 batch = 100000 units) of a chilled dairy-based dessert carry finite loads of non-proteolytic *C. botulinum.* If the mean and standard deviation for the decimal reduction time is reduced $\langle D'(80)\rangle$ = 1.87 and $\sigma_{D'(80)}$ = 2.50, the probability of finite unit loads is reduced to ~8.9 x 10^{-4} for (-LYS), and for a single *D*-value with $D'(80)$ = 1.29 the probability of a unit with a single spore is reduced by three orders of magnitude (~5.0 x 10^{-6}). In this case approximately 99% of individual units of chilled dessert are free from spores of non-proteolytic *C. botulinum*. The probability of finite spore loads in individual units of dairy-based chilled dessert is also sensitive to the $\langle S_A\rangle_{max}$ parameter. For $\langle S_A\rangle_{max}$ = 1000 spores kg^{-1} with $\langle D'(80)\rangle$ = 2.23 and $\sigma_{D'(80)}$ = 4.44 min, the probability of non-zero spore loads increases to ~3.4 x 10^{-1}. Table 5.6 indicates the sensitivity of probability for a finite number of spores with respect to thermal inactivation parameters and spore load of material A.

Process parameters		Probability unit is non-zero	Probability unit load exceeds 1 spore	Process parameters		Probability unit is non-zero	Probability unit load exceeds 1 spore
$D'(80)$	2.23	2.0 x 10^{-3}	1.8 x 10^{-6}	$D'(80)$	1.87	8.9 x 10^{-4}	4.0 x 10^{-7}
$\sigma_{D'(80)}$	4.44			$\sigma_{D'(80)}$	2.5		
$\langle S_A\rangle_{max}$	10			$\langle S_A\rangle_{max}$	10		
$D'(80)$	2.23	3.7 x 10^{-2}	6.9 x 10^{-4}	$D'(80)$	1.87	2.0 x 10^{-2}	2.0 x 10^{-4}
$\sigma_{D'(80)}$	4.44			$\sigma_{D'(80)}$	2.5		
$\langle S_A\rangle_{max}$	100			$\langle S_A\rangle_{max}$	100		
$D'(80)$	2.23	3.4 x 10^{-1}	6.5 x 10^{-2}	$D'(80)$	1.87	2.3 x 10^{-1}	2.7 x 10^{-2}
$\sigma_{D'(80)}$	4.44			$\sigma_{D'(80)}$	2.5		
$\langle S_A\rangle_{max}$	1000			$\langle S_A\rangle_{max}$	1000		
$D'(80)$	2.79	2.4 x 10^{-3}	2.8 x 10^{-6}	$D'(80)$	1.29	5.0 x 10^{-6}	1.3 x 10^{-11}
$\sigma_{D'(80)}$	4.9			$\sigma_{D'(80)}$			
$\langle S_A\rangle_{max}$	10			$\langle S_A\rangle_{max}$	10		
$D'(80)$	2.79	4.0 x 10^{-2}	8.0x 10^{-4}	$D'(80)$	1.29	8.0 x 10^{-5}	3.2 x 10^{-9}
$\sigma_{D'(80)}$	4.9			$\sigma_{D'(80)}$			
$\langle S_A\rangle_{max}$	100			$\langle S_A\rangle_{max}$	100		
$D'(80)$	2.79	4.5x 10^{-1}	1.2 x 10^{-1}	$D'(80)$	1.29	1.0 x 10^{-3}	5.4 x 10^{-7}
$\sigma_{D'(80)}$	4.9			$\sigma_{D'(80)}$			
$\langle S_A\rangle_{max}$	1000			$\langle S_A\rangle_{max}$	1000		

Table 5.6 Sensitivity to process parameters of distribution of spore loads in individual units of dairy-based chilled dessert without added egg yolk

An important component of this risk assessment is the probability of the time preceding observation of growth from spores of non-proteolytic *C. botulinum*, t_{min} during multi-stage storage. It was shown in Chapter 3 that growth at chill temperatures is highly variable and is associated with strain toxin type. At 6.0°C, the time preceding observation of growth, t_{min}, from a single spore ranges from ~8 – 26 days (with expectation t_{min} ~17 days). This time is smaller for increasing concentration of spores (Table 5.4 and Figure 5.13).

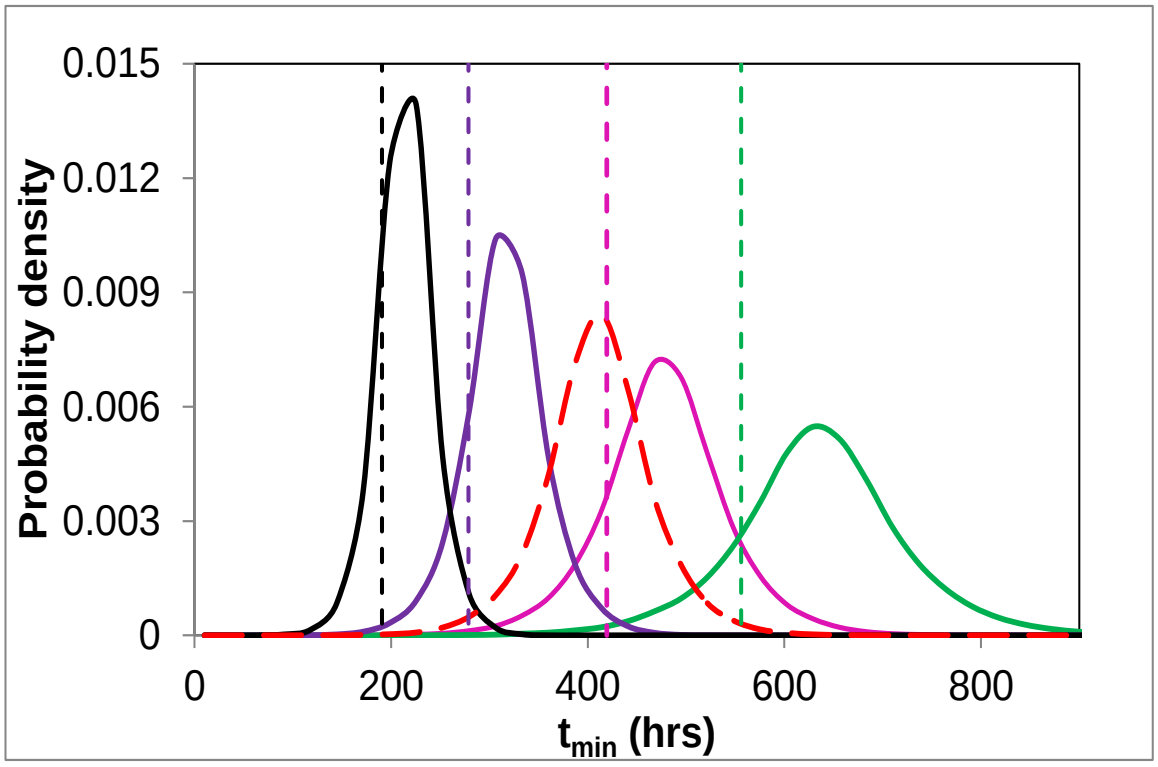

Figure 5.13 Distributions representing belief concerning time preceding observation of growth, t_{min} from a single spore of non-proteolytic *C. botulinum* at T_{s2} = 6.0°C
A distribution of t_{min} for strains belonging to cluster 1 (pink line), cluster 2 (green line), cluster 3 (violet line), cluster 4 (black line), average value of $<t_{min}>$ for all clusters (Table 5.4) (red dashed line). Dashed lines indicate the expected value for probability distribution for t_{min} from s = 10 spores tube^{-1} (colours for clusters 1 – 4)

For isothermal storage at 7.0°C, typical domestic storage fridge temperature, time preceding the observation of growth is in a similar range (~8 – 25 days) but with smaller expectation $<t_{min}>$ ~15 days. The classification of non-proteolytic *C. botulinum* strains based on τ parameter, produced homogeneous clusters of strains in terms of the growth properties at chill temperatures. The biggest t_{min} is calculated for strains belonging to cluster 2: ~26 and ~25 days and cluster 1: ~19 and ~17 days, whereas shorter times are estimated for strains in cluster 3: ~13

and ~12 days (for each cluster the values are calculated at temperatures at 6.0°C and 7.0°C). Growth from strains belonging to cluster 4 is expected to be the shortest: ~8 days at both temperatures.

From the cumulative distributions of t_{min} it is possible to estimate the probability when a spore will exceed the time preceding the observation of growth. Table 5.7 shows that the probability varies across clusters and it increases with time and number of spores.

t (days)	Cluster 1			Cluster 2			Cluster 3			Cluster 4		
	s=1	s=10	s=100	s=1	s=10	s=100	s=1	s=10	s=100	s=1	s=10	s=100
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.003	0.005
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.012	0.019	0.034
6	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.003	0.005	0.060	0.102	0.175
7	0.000	0.001	0.002	0.000	0.000	0.000	0.005	0.009	0.021	0.251	0.406	0.608
8	0.001	0.002	0.006	0.000	0.000	0.000	0.014	0.030	0.073	0.666	0.817	0.911
9	0.002	0.005	0.016	0.000	0.000	0.001	0.039	0.088	0.216	0.914	0.961	0.985
10	0.005	0.013	0.041	0.000	0.001	0.002	0.105	0.235	0.516	0.983	0.994	0.998
11	0.011	0.030	0.099	0.000	0.001	0.004	0.254	0.514	0.803	0.997	0.999	1.000
12	0.023	0.065	0.220	0.001	0.002	0.008	0.513	0.783	0.934	1.000	1.000	1.000
13	0.047	0.137	0.439	0.002	0.005	0.015	0.766	0.919	0.981	1.000	1.000	1.000
14	0.093	0.272	0.687	0.003	0.009	0.030	0.904	0.973	0.995	1.000	1.000	1.000
15	0.177	0.479	0.855	0.006	0.017	0.055	0.964	0.992	0.999	1.000	1.000	1.000

Table 5.7 The probability that a number of spores s = 1, s = 10, s = 100 spores tube^{-1} will exceed the time preceding observation of growth during isothermal storage for time t (days) at T_{s3} = 7.0°C

The probability distributions for t_{min} (Figure 5.13 and Table 5.7) indicate that at certain storage conditions germination and growth from non-proteolytic *C. botulinum* is possible, and strain variability is an important factor in the estimate of risk associated with a product that is stored at chill temperatures.

Risks associated with non-proteolytic *C. botulinum* are considered when the storage period exceeds the time preceding the observation of growth. This is expressed in a form of a cumulative fraction of time preceding observation of growth, t_{min}, consumed by three storage regimes (T_{s1} = 3.0°C, T_{s2} = 6.0°C, T_{s3} = 7.0°C for a period of t_{s1} = 1, t_{s2} = 2 and t_{s3} = 4 days). The fraction can take the values from 0 to infinity, but values below one are considered as safe "boundary". In another words, if the exhausted fraction of the time preceding observation of growth is less than one, the population of spores is still in the time

prior to growth. Figure 5.14 shows the cumulative probability distribution for the fraction of the time preceding observation of growth for a single spore during a non-isothermal storage regime with T_{s1} = 3.0°C, T_{s2} = 6.0°C and T_{s3} = 7.0°C and t_{s1} = 1 day, t_{s2} = 2 days, t_{s3} = 4 days. Coloured lines correspond to strains belonging to four clusters. It is apparent from the figure that the greatest risk arises from strains that belong to cluster four. Interestingly, a common assumption of many published risk assessments, that treat strains as a homogeneous population, would not indicate hazards arising from some regimes that are appropriated for dairy-based desserts.

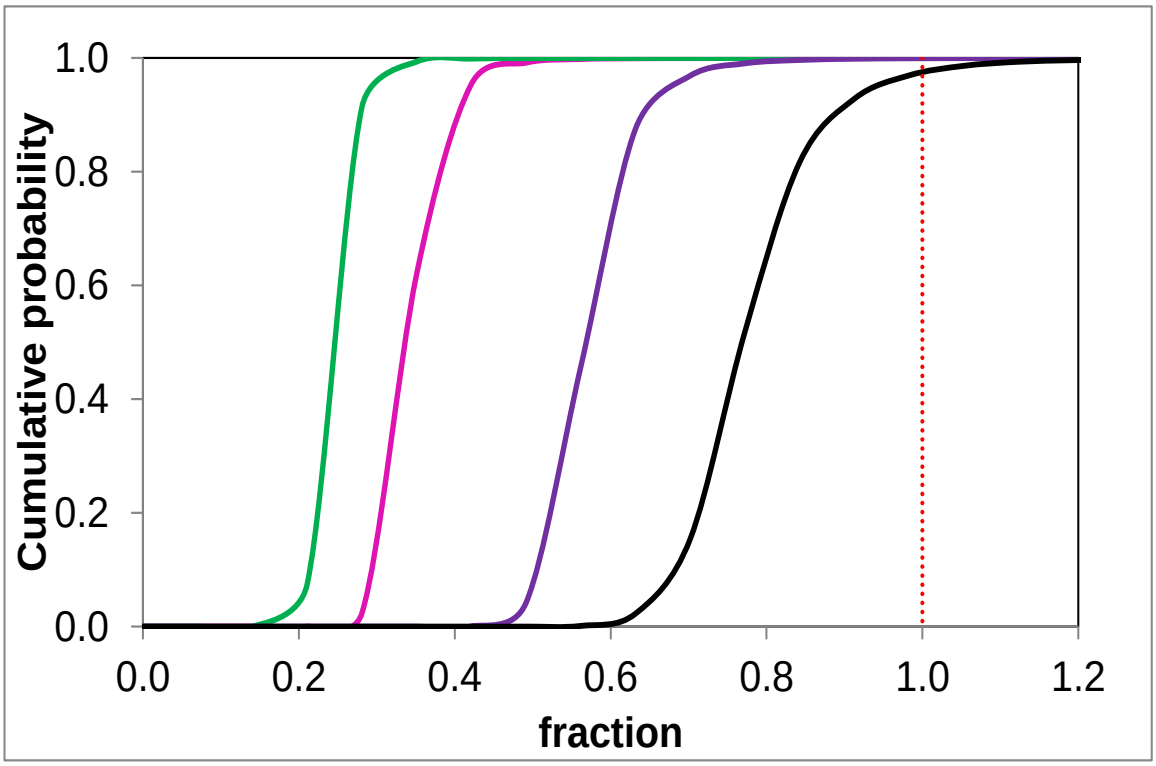

Figure 5.14 The cumulative probability of the fraction of time preceding observation of growth, t_{min}, from s = 1 spore tube^{-1} during non-isothermal storage at temperatures T_{s1} = 3.0°C, T_{s2} = 6.0°C and T_{s3} = 7.0°C for a period of t_{s1} = 1 day, t_{s2} = 2 days, t_{s3} = 4 days
A distribution of fraction for strains belonging to cluster 1 (pink line), cluster 2 (green line), cluster 3 (violet line), cluster 4 (black line). The red dotted line indicates fraction of the time preceding observation of growth equal to 1

Storage regimes can be variable (due to temperature elevation, time abuse) during the product's lifetime which can affect product safety. Table 5.8 lists a series of storage regimes which represent three isothermal periods. The first isothermal period, with temperature T_{s1} < 3.0°C is considered as neutral to product safety for any duration t_1. Therefore the effect of storage period for two

remaining regimes, t_{s2} and t_{s3} (retail and consumer storage) on exhaustion of time preceding observation of growth from three inocula s = 1, 10 and 100 spores tube^{-1} was considered. In each regime, the fluctuation of the time preceding observation of growth that is exhausted by the storage in the presence of strain variability is expressed and added to the cumulative value. Additionally, Table 5.8 lists the probability that the storage time will exceed the time preceding observation of growth, p(f > 1), and the maximum value of the exhausted fraction of time preceding observation of growth for a variable population of strains, f_{max}.

Spore concentration (spores tube^{-1})				s = 1			s = 10			s = 100		
Regime	T_s	t_s	Cluster	<f>	p(f > 1)	f_{max}	<f>	p(f > 1)	f_{max}	<f>	p(f > 1)	f_{max}
	°C	day										
Factory	3	1	1	0.35	0.00	1.00	0.39	0.00	0.83	0.44	0.00	0.98
Retail	6	2	2	0.24	0.00	0.48	0.27	0.00	0.76	0.31	0.00	0.73
Consumer	7	4	3	0.50	1.8×10^{-4}	1.08	0.55	4.7×10^{-4}	1.55	0.60	1.8×10^{-3}	1.63
			4	0.78	2.6×10^{-2}	1.76	0.82	4.4×10^{-2}	2.19	0.87	9.2×10^{-2}	1.97
Factory	3	1	1	0.57	9.6×10^{-4}	1.25	0.64	2.6×10^{-3}	1.51	0.72	0.00	2.38
Retail	6	4	2	0.41	0.00	0.81	0.46	2.8×10^{-5}	1.03	0.52	2.0×10^{-4}	1.27
Consumer	7	6	3	0.84	5.1×10^{-2}	1.61	0.91	1.4×10^{-1}	2.57	1.00	4.4×10^{-1}	2.73
			4	1.29	9.9×10^{-1}	2.51	1.37	9.9×10^{-1}	3.29	1.45	1.00	2.87
Factory	3	1	1	0.17	0.00	0.41	0.19	8.1×10^{-6}	1.05	0.22	0.00	0.58
Retail	6	1	2	0.12	0.00	0.33	0.14	0.00	0.30	0.16	0.00	0.38
Consumer	7	2	3	0.25	0.00	0.76	0.27	2.3×10^{-5}	1.13	0.30	0.00	0.53
			4	0.39	0.00	0.97	0.41	0.00	0.94	0.44	0.00	0.99

Table 5.8 The exhaustion of the time preceding observation of growth, t_{min}, from s = 1, s = 10 and s = 100 spore tube^{-1} during multi-stage isothermal storage

At certain storage conditions a single spore for strains belonging to clusters 3 and 4 give a high probability of exhaustion of the time preceding observation of growth ~0.05 and ~0.99 (T_{s2} = 6.0°C, t_{s2} = 4 days and T_{s2} = 7.0°C, t_{s2} = 6 days). As expected the probability for a hazard increases with increasing number of spores. The development of the probability that a single spore will exceed the time preceding the observation of growth as a function of time is presented in Figure 5.15. The example includes fixed conditions for factory and retail storage and shows the progression of the exhaustion probability, p(f > 1), as a function of consumer storage time at 7.0°C. After times ~3 days the probability for strains belonging to cluster 4 increases rapidly from ~0.02 (on day 4) to 1.0 (on day 9), with the most rapid rise between day 5 and 7. For some strains (cluster 2) the exhausted probability p(f > 1) does not increase significantly before 14 days of

storage. Clearly the total time for which the probability that a single spore exceeds the time preceding observation of growth can be reduced by extending storage in factory or by the retailer.

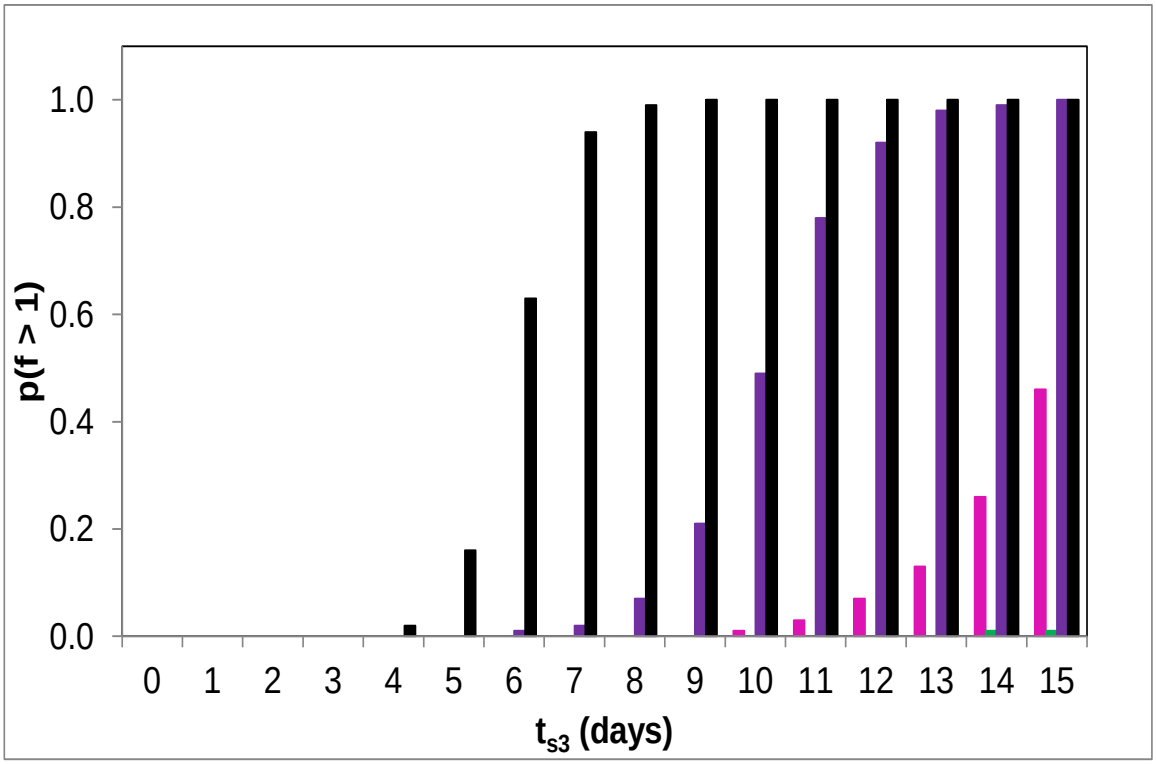

Figure 5.15 The probability for a single spore to exceed the time preceding observation of growth during non-isothermal storage
The storage regime includes t_{s1} = 1 day at T_{s1} < 3.0°C (factory), t_{s2} = 2 days at T_{s2} = 6.0°C (retail) and a variable duration for consumer storage for t_{s3} at T_{s3} = 7.0°C
Bars colours corresponds to strains belonging to cluster 1 (pink bar), cluster 2 (green line), cluster 3 (violet line), cluster 4 (black line)

From Table 5.8 and Figure 5.15 is clear that spore variability is an important factor in the determination of storage regimes and consequently product shelf-life. Clearly, use of predictive models in estimation of the period preceding growth and toxin production would treat the population of spores as a uniform, and may under estimate those strains for which growth can be expected faster. The end point measure for non-proteolytic *C. botulinum* in dairy-based chilled dessert is a product of two independent probabilities: the probability for a finite number of spores in a retail unit and the probability that spores of non-proteolytic *C. botulinum* will exceed the time preceding observation of growth during multi-stage storage period. The values listed in Table 5.8 are sensitive to product composition (addition of egg yolk) and strain variability. The sensitivity was summarised by evaluating the exhausted fraction of the time preceding

observation of growth for different consumer storage duration (Table 5.10). The model for the time preceding observation of growth from a given number of spores includes a dependency on spore population size after heat treatment (in general larger populations exhaust time preceding observation of growth quicker). Nevertheless, the dominant hazard arises from retails units containing a single spore (Table 5.9).

Cluster	Product without added egg yolk			Product with added egg yolk		
	s = 1	s = 10	s = 100	s = 1	s = 10	s = 100
	Hazard probability*					
1	1.42×10^{-8} (1.53×10^{-8})	6.11×10^{-11} (5.30×10^{-11})	0.00	5.33×10^{-7} (5.58×10^{-7})	9.82×10^{-8} (8.24×10^{-8})	1.49×10^{-21} (2.99×10^{-21})
2	0.00	0.00	0.00	0.00	0.00	0.00
3	4.96×10^{-7} (1.45×10^{-7})	1.05×10^{-9} (5.31×10^{-10})	0.00	1.98×10^{-5} (4.35×10^{-6})	1.79×10^{-6} (6.49×10^{-7})	3.73×10^{-20} (7.45×10^{-20})
4	4.85×10^{-5} (8.61×10^{-6})	9.24×10^{-8} (3.10×10^{-8})	0.00	1.94×10^{-3} (1.10×10^{-4})	1.61×10^{-4} (2.52×10^{-5})	2.47×10^{-18} (4.94×10^{-18})

Table 5.9 Probabilities for end point measures in a quantitative risk assessment of model dairy-based chilled dessert
The results indicate the probability for the multi-stage storage time (t_{s1} = 1 day at T_{s1} < 3.0°C (factory), t_{s2} = 2 days at T_{s2} = 6.0°C (retail) and t_{s3} = 4 days at T_{s3} = 7.0°C) to exceed time preceding observation of growth * data given as mean (standard deviation of five simulations)

Table 5.10 indicates the end point measures, for non-proteolytic *C. botulinum* with the parameters corresponding to Table 5.3 and an additional period of consumer storage with duration up to 21 days. For the smallest periods of domestic storage strains belonging to cluster 2 will not make a contribution to the end point measure. The main hazard arises from strains belonging to clusters 3 and 4. Additional storage for a period of 21 day increases the hazard which becomes uniform across all clusters.

Cluster	Product without added egg yolk				Product with added egg yolk			
	Hazard probability							
	t_{s3} = 5	t_{s3} = 7	t_{s3} = 14	t_{s3} = 21	t_{s3} = 5	t_{s3} = 7	t_{s3} = 14	t_{s3} = 21
1	1.59×10^{-7}	1.63×10^{-6}	5.52×10^{-4}	2.28×10^{-3}	4.14×10^{-6}	5.39×10^{-5}	2.11×10^{-2}	8.91×10^{-2}
2	0.00	8.60×10^{-8}	1.43×10^{-5}	6.06×10^{-4}	0.00	2.83×10^{-6}	5.46×10^{-4}	2.36×10^{-2}
3	3.58×10^{-6}	5.26×10^{-5}	2.09×10^{-3}	2.30×10^{-3}	9.34×10^{-5}	1.74×10^{-3}	7.99×10^{-2}	8.97×10^{-2}
4	5.16×10^{-4}	2.22×10^{-3}	2.11×10^{-3}	2.30×10^{-3}	1.32×10^{-2}	7.32×10^{-2}	8.04×10^{-2}	8.97×10^{-2}

Table 5.10 Probabilities for end point measures in a quantitative risk assessment of model dairy-based chilled dessert (s = 1 spore tube^{-1})
The results indicate the probability for the multi-stage storage time that includes t_{s1} = 1 day at T_{s1} < 3.0°C (factory), t_{s2} = 2 days at T_{s2} = 6.0°C (retail) and a variable duration for consumer storage for t_{s3} at T_{s3} = 7.0°C

5.4 Discussion

This assessment considered hazards associated with non-proteolytic *C. botulinum* in a model dairy-based minimally heated chilled dessert. The assessment is based on data summarised in chapters 2, 3 and 4 with implementation of Monte Carlo simulation. The strength of the hazard depends on spore loads in raw materials, on thermal properties of spores and on the definition of t_{min}, time that represents a period before non-proteolytic *C. botulinum* can produce toxin. The quantification of time t_{min} was based on a τ parameter (determined in predictive models for the probability of growth). The assessment explicitly includes an evaluation of strain variability associated with time preceding the observation of growth.

The assessment is quantitative and represents the probability that a product's storage time, does not exceed the time preceding observation of growth from spore inocula in a retail units of dairy-based dessert. For a model corresponding to the parameters given in Table 5.3 and storage with T_{s2} = 6.0°C for t_{s2} = 2 days and T_{s3} = 7.0°C for t_2 = 4 days, the probabilities for exceeding the time preceding observation of growth are very small for strains belonging to clusters 1 and 2, 2.6 x 10^{-2} for strains from cluster 3 and ~1.8 x 10^{-4} for strains belonging to cluster 4. The combined end point measure ranges from 0 to 4.85 x 10^{-5} (s = 1 spore $tube^{-1}$), from 0 to 9.24 x 10^{-8} (s = 10 spores $tube^{-1}$) and very small for s = 100 spore $tube^{-1}$. These results relate to strains 1 – 4 (no egg) and show that the dominant contribution to risk is associated with single spore occupancy. In the presence of egg yolk the values are increased and range from 5.33 x 10^{-7} to 1.94 x 10^{-3} (s = 1 spore $tube^{-1}$), from 1.61 x 10^{-4} to 9.82 x 10^{-8} (s = 10 spore $tube^{-1}$) and very small for s = 100 spore $tube^{-1}$. Longer periods of storage add to the values of the end point measures.

The statistical analysis performed on the model variables measured the correlation coefficient between the input variables and the end point measure. Figure 5.16 shows a tornado chart of the Spearman rank correlation of the main uncertain input parameters and the end point measure. In this model *D*-value (that determines the heat treatment applied) is the main control measure for ensuring a safe product. The *D*-value chosen influences one of two components

of end point measure – a probability that an individual unit of dairy-based chilled dessert contains a finite number of spores. A big value for σ_D leads to an end point measure, for non-proteolytic *C. botulinum* that is bigger than the one in the model, whereas use of single *D*-value leads to smaller end point measure. The product that contains egg yolk can be protective to spores of non-proteolytic *C. botulinum*, due to activity of lytic enzymes, leading to increase in the number of spores that survive heating. The information summarised in chapter 2 on spore inactivation kinetics in the presence of lysozyme, indicates a 100-fold greater heating required to reduce population of spores, compared to spores heated in the absence of lysozyme.

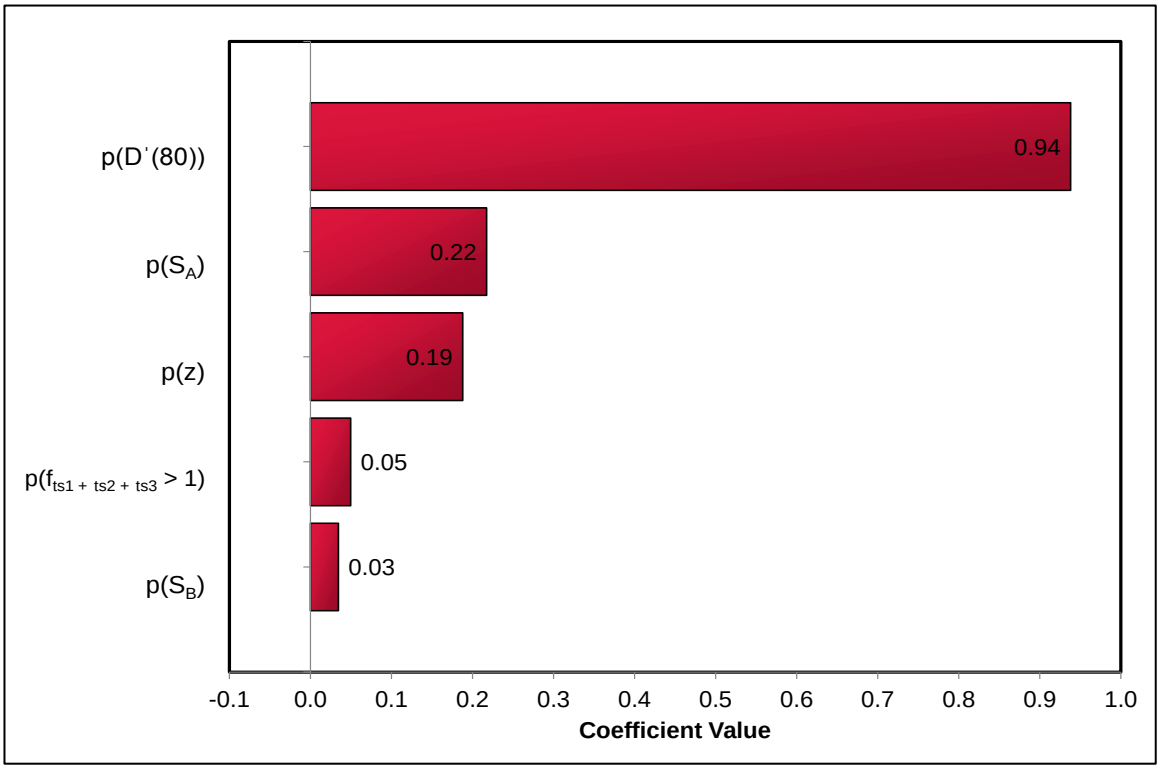

Figure 5.16 Spearman rank correlation coefficients of uncertain input variables with toxicity of a dairy-based chilled dessert
The length of the bar indicates the impact of particular input variable on the analysed output. A positive coefficient (bar extending to the right – the input has a positive impact, bar extending to the left – the input has a negative impact, e.g. increase the input will reduce the output)

Variable risk for non-proteolytic *C. botulinum* was based on identification of appropriate clusters of strains as a function of growth at chilled temperatures – one of the crucial elements for the end point measure. The strain variability in

relation to growth is consistent with variability in carbohydrate utilisation and that is associated with toxin type.

The risk of a toxic pack at the point of preparation for a model dairy-based chilled dessert is almost two orders of magnitude higher for strains belonging to cluster 4 (4.85×10^{-5}) – type E strains, than strains belonging to cluster 3 (4.96×10^{-7}), three orders of magnitude higher than strains in cluster 1 (1.42×10^{-8}) and much higher than for strains in cluster 2 (for which risk is very small) – mainly type B strains. The output of this risk assessment is consistent with epidemiology for non-proteolytic *C. botulinum* summarised in Table 1.2 which shows many more reported outbreaks of botulism associated with non-proteolytic *C. botulinum* type E compared to non-proteolytic C*. botulinum* type B. Nevertheless, it should be noted that non-proteolytic type B cases are under reported, since in many cases investigators fail to distinguish between proteolytic and non-proteolytic *C. botulinum* type B hazards. A majority of type B outbreaks associated with canned foods are probably associated with proteolytic type B, while some type B outbreaks in Europe are associated with non-proteolytic *C. botulinum* type B (Lücke, 1984; Hauschild, 1993; Peck, 2006). An additional contributory factor for greater incidence of outbreaks associated with strains of type E might be that type E strains are more common in the environment than non-proteolytic *C. botulinum* type B strains (Dodds, 1993b).

Despite this, the development of a probabilistic risk assessment model, that includes integration of strain variability and associated effects can be used by product manufacturers to define an acceptable level of risk and to distinguish different hazards. The assessment is modular and therefore can be adopted to addition of more raw materials, different products and alternative process parameters.

5.5 Conclusions

The main challenge for this risk assessment of model dairy-based chilled desserts was the inclusion of information regarding strain variability of non-proteolytic *C. botulinum* summarised in this thesis. This approach reflects the variability of strain response to different factors present in their environment and therefore helps to give a better representation of the hazard.

Additionally, the calculation of risk allowed the following conclusions:

i. The inherent high level of phenotypic variability observed between non-proteolytic *C. botulinum* strains has an important impact on calculated risk for minimally heated dairy-based chilled dessert
ii. Strains forming type E neurotoxin present greater hazard for minimally-heated chilled dessert, than strains forming type B and F neurotoxins
iii. The results of risk assessment for non-proteolytic *C. botulinum* are consistent with foodborne botulism outbreaks (more cases reported due to non-proteolytic *C. botulinum* type E strains)
iv. Extended storage time decreases the impact of strain variability on estimated risk
v. The probability of spores in post-pasteurized retail units of model dairy-based dessert is sensitive to spore heat resistance, given by a *D*-value and in consequence to product ingredients e.g. lysozyme
vi. A single spore is the dominant hazard in the calculation of risk presented by a model dairy-based chilled dessert
vii. Strain variability makes a considerable impact on the assessment of the time preceding observation of growth

The model product chosen for this assessment includes relatively simple composition, a minimal heat process and storage regime dominantly at two temperatures. Each of these elements could be expanded, and could include more details, within the current model framework. In this case additional data supplies, such as growth at 5.0°C or 8.0°C, would be required to reduce corresponding uncertainty. Although additional elements in the process model would change the numeric values of the end point measure, the conclusions which point to an ability to quantify the hazard and identify variability associated with strain types would remain unchanged. The estimate of end point value could

be enhanced by improved description of spore loads in raw materials, by taking into account spore damage, by including temperature fluctuations for the consumer storage period and transport and by integration of information regarding carbohydrate fermentation e.g. ability of type B and F strains but not type E strains to utilise amylose, amylopectin (two components of starch) or glycogen. Moreover, the model could be improved by modification of the assumption regarding the product's physico-chemical characteristics e.g. optimal and constant pH and a_w or by inclusion of information on the effects of preservatives that inhibit growth of non-proteolytic *C. botulinum*.

6. Summary and conclusions

The ability of non-proteolytic *C. botulinum* to survive moderate heat treatments and the ability for growth and neurotoxin formation at refrigeration temperatures makes this pathogen a major safety concern for minimally heated chilled foods. Although outbreaks of foodborne poisoning associated with the presence of botulinum neurotoxin in commercially manufactured products are rare, their serious consequences for consumers and food manufacturers requires use of the most sophisticated methods to assess and control the risk. One suitable method is Quantitative Microbial Risk Assessment (QMRA), and one way to make a QMRA for non-proteolytic *C. botulinum* in minimally heated chilled foods more sophisticated is to take account of the effect of strain variability. Based on their physiological properties (growth at chill temperatures and growth on different carbohydrate source) and their phylogenetic relationship, strains of non-proteolytic *C. botulinum* can be separated into distinct two groups, one of strains that form type B or type F neurotoxin, and one of strains that form type E neurotoxin. Account has been taken of strain variability in a QMRA for non-proteolytic *C. botulinum* in a model minimally heated chilled dairy-based dessert.

One of the factors important in controlling non-proteolytic *C. botulinum* in minimally heated chilled foods is heat treatment. Chapter 2 of this thesis presents the main findings of an extensive systematic literature review on the heat resistance of spores of non-proteolytic *C. botulinum*. The survey yielded 48 eligible sources from which 880 *D* and 528 *z*-values were extracted. Analysis of the kinetic inactivation parameters indicated that: (i) activity of lytic enzymes is an important factor when applying a heat treatment, as it significantly affects the thermal properties of non-proteolytic *C. botulinum* spores; (ii) two *D*-values were identified $<D'(80)> = 2.23$ min (for spores recovered in absence of lysozyme in recovery media) and $<D'(80)> = 245$ min (for product in presence of lysozyme in recovery media); (iii) two *z*-values should be considered: $z = 7.0$°C (for product without lytic enzyme activity) and $z = 9.0$°C (for product with lytic enzyme activity); (iv) there was no relationship between spore heat resistance and toxin type, and strains forming exceptionally heat resistant spores were not identified; (v) the survey provided valuable information regarding the variability of *D* and *z*-values that is suitable for use in QMRA, and this has been used in the

quantification of risk for a model dairy-based minimally heated chilled product (Chapter 5). Moreover, the outputs of this review have been applied by companies producing chilled foods in the SUSSLE project (CFA, 2013). It is planned to publish the major findings as a review on spore heat resistance of non-proteolytic *C. botulinum*.

Another major factor important in controlling non-proteolytic *C. botulinum* in minimally heated chilled foods is effective chilled storage. Chapter 3 of this thesis focuses on the variability in growth between different strains of non-proteolytic *C. botulinum* at chill temperatures. The variability of 42 strains was assessed in respect of two independent variables: incubation temperature and spore inoculum concentration. In the first experiment, the variable response of 40 strains was tested at 3.0 – 10.0°C (using one spore concentration s = 10^6 spores $tube^{-1}$), whereas in the second experiment, 24 strains were tested at one incubation temperature (6.0°C) from seven spore inocula concentrations (from s = 10^6 to 10^0 spores $tube^{-1}$). These two independent experiments produced similar results. A clustering technique indicated an extensive variation in response among non-proteolytic *C. botulinum* strains, and most interestingly, the variability in growth was associated with type of neurotoxin formed, with type E strains generally showing more rapid growth at chilled temperatures than type B and F strains. Based on their growth response at chilled temperatures, strains of non-proteolytic *C. botulinum* were assigned to one of four clusters. Probabilistic models were developed that described the variability in growth response within these four clusters, and these were used in a risk assessment for a model dairy-based minimally heated chilled dessert. It is planned to publish the major findings of this Chapter.

Carbohydrates are the major source of carbon and energy for strains of non-proteolytic *C. botulinum*. Chapter 4 describes tests to determine the ability of 33 strains of non-proteolytic *C. botulinum* to utilize 54 different carbohydrates. Growth was determined by acidification of the culture medium, turbidity and gas formation. Most carbohydrates were either fermented or not fermented by all strains. However, a number of important differences were identified, and strains could be classified into three groups, with each group fermenting different

carbohydrates. Interestingly, strain classification was associated with type of neurotoxin formed. Strain clustering appeared to be consistent with the clustering pattern obtained in growth tests at chill temperatures, therefore these data support the validity of the classification pattern for non-proteolytic *C. botulinum*. The major findings of this chapter have been included in a publication on the genomic and physiological variability within non-proteolytic *C. botulinum* (Stringer *et al.*, 2013). One unexpected finding was that in this test system observations of growth were not correlated with the presence of neurotoxin. Tests conducted in a fermenter system indicated that strains of non-proteolytic *C. botulinum* seemed able to degrade toxin that they had previously formed. This observation is worthy of further study.

Generated and collected information in Chapters 2, 3 and 4 on strain variability of non-proteolytic *C. botulinum* were used as input information to the quantitative microbial risk assessment model for minimally heated chilled foods. The modular process risk model described in Chapter 5 considers hazards arising from raw materials, spore inactivation during thermal processing (based on a distribution for parameters *D* and *z* obtained in Chapter 2), distribution of spores in retail units and variability of strains during multi-stage storage regimes (based on strain variability tested in Chapter 3). The model is constructed using a Monte Carlo simulation technique and calculates the risk of a toxic retail unit of dairy dessert at point of preparation. The assessment includes the following findings: (i) inclusion of information on strain variability of non-proteolytic *C. botulinum* has an important impact on calculated risk; (ii) the greater hazard for model minimally-heated chilled dessert was associated with type E strains rather than those of type B and F; (iii) the output of the risk assessment agreed with the epidemiology of non-proteolytic *C. botulinum*, were more outbreaks are associated with type E strains; (iv) heat treatment parameters are significant elements of QMRA model and should be precisely defined when the activity of lytic enzymes in manufactured product is considered. The results of this risk assessment can be used for example when estimating the risks associated with food products originating from distinct geographical locations (e.g. with higher prevalence of type E strains). Information regarding the effect of carbohydrate source on variability of growth and neurotoxin formation by strains of non-

proteolytic *C. botulinum* were not directly implemented into the QMRA model. Nevertheless, this information strongly supports the variability between strains of non-proteolytic *C. botulinum* that is associated with the type of neurotoxin formed. This information can be also used in a future risk assessment for products that contain e.g. starch (that appeared in tests to be utilized by B and F strains but not by type E strains). The main observations of this chapter will be published as a model risk assessment.

The present study was designed to determine the impact of strain variability on risks presented by non-proteolytic *C. botulinum* in chilled foods. The data obtained in two independent experiments (growth at chill temperatures and carbohydrate utilisation) indicated a significant variability between strains of types B and F and those of type E, and this appeared to have an impact on calculated risk. Nevertheless, additional research could be undertaken in order to test whether strain variability is related with combination of factors including the toxin formed. These could include: (i) testing the spore heat resistance of strains used in experiments described in Chapters 3 and 4 in well-controlled manner; (ii) tests different NaCl concentrations; (iii) tests the effect of pH and water activity on heat resistance; (iv) testing the strain variability as a function of the combined effect of heat treatment and storage at chill temperatures. Although the particular risk assessment presented in Chapter 5 is limited by model process, the QMRA technique and implementation can be extended to other, more realistic situations. The QMRA results clearly show the ability to encapsulate complexities that are associated with strain variability for non-proteolytic *C. botulinum*. In addition the QMRA illustrates opportunities for progression and future work concerning improved appreciation of *C. botulinum* hazards that includes partition of the hazards according to cluster of strains and potential complex behaviour involving toxin degradation in the presence of glucose.

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Appendix 1 Brief summary of included studies on heat resistance of non-proteolytic *C. botulinum* spores

Studies of recovery in absence of lysozyme (-LYS) *[ordered alphabetically]*

Alderman *et al.* (1972)

Toxin type	E	
No. of strains tested	2	
Temperature [a)]	83°C	
Heating menstruum	autoclaved chub fish and egg white, raw egg white,	
No. of D/z-values [b)]	4/-/-	
Notes	The influence of moist and dry environment on spore heat resistance was tested. Spores heated in raw fish had significantly greater *D*-value than those heated in autoclaved fish. The same finding was observed using egg white (which coagulates rapidly at high temperatures). Authors concluded that the physical state of the menstruum is important in the heat tolerance of spores. Lower a_w increases the resistance of spores.	
Quality	***High***	*Quality criteria not met*

Angelotti (1970)

Toxin type	E	
No. of strains tested	4	
Temperature	80°C	
Heating menstruum	0.067M phosphate buffer	
No. of D/z-values	4/4/4	
Notes	The paper summaries current state of knowledge on heat resistance of type E strains. Four *D*-values originate from the experiment performed by another author (that could not be retrieved) and there is no detailed description of the system.	
Quality	***Medium***	*Quality criteria not met*
		f, k, l, n, o, p

Appleyard and Gaze (1993)

Toxin type	E	
No. of strains tested	1	
Temperature	70°C	
Heating menstruum	tomato homogenates	
No. of D/z-values	37/-/-	
Notes	Heat resistance was determined following a range of sublethal heat treatments. The sublethal heating (heat shock) at all conditions (except one: 60 min/40°C) increased the *D*-value. Simultaneously the influence of conditions at which the heating menstruum was kept before determining the *D*-value was examined. The *D*-value obtained immediately after heat treatment was the highest (to compare to those obtained after holding the samples for 4h/room temp. and 24h/6°C). Sublethal heating activates spores and induces higher spore heat resistance (which might have a crucial role in food that is heat treated slowly with long come up time).	
Quality	***High***	*Quality criteria not met*

Bohrer *et al.* (1973)

Toxin type	E
No. of strains tested	4
Temperature	70, 72, 73, 75, 77, 79, 80, 81, 82°C
Heating menstruum	0.07M and 0.067M phosphate buffer, corn brine, crabmeat, milk (evaporated), peas, salmon, sardines (in tomato sauce), shrimp, tuna (in oil)
No. of D/z-values	53/12/51
Notes	The heat resistance of only one strain, Saratoga, was tested in food. As a reference heating menstruum phosphate buffer was used, where at 80°C a *D*-value of 1.1 minute was calculated. Much higher values were obtained in tuna, sardines, crabmeat, peas and corn, which show that food is more protective to heat than buffer. Authors also presented the percentage composition of tested food. There was no correlation in heat resistance and content of carbohydrates in food. Tuna – high in protein and fat content gave the highest *D*-value. Sardines in tomato sauce – high in fat in protein content – gave second highest *D*-value. Both tuna and sardines are low percentage of water. Shrimp – low in fat and high in protein gave little protection from heat. A comparison of heat resistance of 4 strains was conducted in phosphate buffer, and it was difficult to distinguish the most heat resistant strain, since it varied with T. The most heat resistant strain at 70°C was Alaska, at 73°C and 75°C Saratoga, at 77°C Beluga and 80°C 1304E.

Quality	*Quality criteria not met*
High	

Bucknavage *et al.* (1990)

Toxin type	E
No. of strains tested	1
Temperature	50, 55, 60, 70, 80°C
Heating menstruum	oyster homogenates
No. of D/z-values	20/4/20
Notes	Initially the heat resistance of 5 strains was tested, of which Beluga was the most heat resistant. The impact of addition of salt and/or potassium sorbate (KS) to heating menstruum was tested. Salt increased the *D*-value only at certain T (50, 70 and 80°C). KS had no effect on heat resistance of spores. Authors calculated *z*-values for two different temperatures range according to better fit found. The *z*-values calculated for the curve between 70°C and 80°C ranged from 6.9°C to 7.6°C, while *z*-value calculated between 55°C and 70°C were 12.1 to 14.7°C. In the analysis the *z*-values were calculated for all temperatures. (50 – 80°C), and ranged from 7.7°C to 7.9°C.

Quality	*Quality criteria not met*
High	o

Chai and Liang (1992)

Toxin type	E	
No. of strains tested	5	
Temperature	74, 75, 77, 79, 82°C	
Heating menstruum	oyster homogenates	
No. of D/z-values	52/10/52	
Notes	From 5 strains tested the most heat resistant was strain Minnesota. Authors calculated *z*-values using two different methods (TDT curve and survivor curve). The *z*-values were higher when determined from TDT curve as compared to survivor curve. Although authors gave 21 *D*-values, 52 were noted, since for each *D*-value two *z*-values were calculated, which is important when all *D*-values are converted to D'(80).	
Quality	***High***	*Quality criteria not met*

Crisley *et al.* (1968)

Toxin type	E	
No. of strains tested	5	
Temperature	80°C	
Heating menstruum	whitefish chubs	
No. of D/z-values	6/6/6	
Notes	The test was conducted at four T. Data were presented as TDT was converted to *D*-value, with strain Alaska showing the highest heat resistance.	
Quality	***High***	*Quality criteria not met*

De Pantoja (1986)

Toxin type	E	
No. of strains tested	1	
Temperature	80, 85, 90, 95°C	
Heating menstruum	crawfish paste	
No. of D/z-values	2/1/2	
Notes	*D*-values calculated by three methods (Stumbo, 1948; Stumbo *et al.*, 1950; Schmidt, 1957) were inconsistent, causing a variation in *z*-values. Since *D*-values at 80 and 95°C did not confirm earlier findings of other researchers, the author excluded them from further analysis. Only the *D*-value calculated by the method of Stumbo (1948) gave the *z*-value consistent with results of previous studies therefore these data were included in database. The author observed that heat resistance of spores seemed to be greater at higher T. This might be attributed to: possible activation of more heat resistant spores present in population or problems with the experimental procedure itself - sedimentation of crawfish paste along sides of the test tubes - providing “dry” environment or loss of water, that might occur during heating at high T due to evaporation.	
Quality	***High***	*Quality criteria not met*

Duh and Ren (1995)

Toxin type	E	
No. of strains tested	1	
Temperature	70, 80, 90°C	
Heating menstruum	fine carrot	
No. of D/z-values	3/1/3	
Notes	The paper was written in Chinese. The publication was translated by visiting student in IFR.	
Quality	***High***	*Quality criteria not met*
		f

Eklund *et al.* (1967a)

Toxin type	F	
No. of strains tested	2	
Temperature	70, 80, 90°C	
Heating menstruum	physiological saline	
No. of D/z-values	6/2/6	
Notes	The heat resistance was included in tests examining the properties of a type F strain. The *D* and z-values were not given in the publication and parameters were calculated from TDT data.	
Quality	***High***	*Quality criteria not met*
		f

Fernandez and Peck (1997)

Toxin type	mixed strains (3 type B, 3 type E and 2 type F)	
No. of strains tested	mixture of 8 strains	
Temperature	70, 75, 80, 85°C	
Heating menstruum	meat medium	
No. of D/z-values	3/1/3	
Notes	The purpose of this study was to develop a predictive model that described the effect of heating and incubation on growth and toxin production by *C.botulinum* strains. The *D*-values were not given by authors. MPN results were used to fit the best straight line and calculated the parameters of heat treatment. It was impossible to calculate the *D*-value at 90°C since rapid death. The *D*-value at 70°C was excluded from analysis. The low *z*-value (5.9°C) might be a result of including the *D*-value in a range of 70-85°C, since it gave better fit that the *D*-values in a range 75 – 85°C.	
Quality	***High***	*Quality criteria not met*

Gaze and Brown (1990)

Toxin type	B and E	
No. of strains tested	2	
Temperature	70, 75, 80, 85, 90, 92°C	
Heating menstruum	cod and carrot homogenates	
No. of D/z-values	20/4/20	
Notes	This study is a basis for ACMSF recommendation (heat treatment of 10 minutes at 90°C) to deliver a 6*D* reduction of *C. botulinum* spores. Precisely, authors recommended heating of "sous-vide" products at 90°C for 7 minutes with *z*-value equal to 9.84°C. For both strains at all measured temp. *D*-values were higher in cod than in carrot homogenate. There was no significant difference in heat resistance of different toxin type strains within the same heating menstruum.	
Quality	***High***	*Quality criteria not met*

Graikoski and Kempe (1964)

Toxin type	E	
No. of strains tested	6	
Temperature	65, 70, 85°C	
Heating menstruum	phosphate buffer,	
No. of D/z-values	6/-/-	
Notes	This vast report summarises the resistance of spores to heat and radiation. The authors conducted a comparison of five counting media (beef-infusion broth, pork-infusion agar, Mossel's media, pork-pea extract and peptone media). As a result, beef infusion agar was used for spore count in further tests. Two techniques of spores heating were employed (open tube and sealed ampules). In case of two strains (Forks and Nanaimo), using the open tube technique heated at 70°C and 75°C, slightly higher viable count was obtained compared to two other strains – those strains were stored for several weeks after preparation. Authors suggested that this might be attributed to spore aging due to storage. With both techniques a tailing effect was observed. Spores surviving heating were tested for the ability to produce toxin. The results showed that it varies across different strains and different temperatures. In the database only results of 6 tests were included due to tailing effect. The tailing effect could be a consequence of error associated with technique: only 1/3 of the top of the tubes were immersed in a water bath, tubes were inoculated before they reached the test temperature. In few cases there was less than 1 log reduction of initial number of spores during heat resistance. therefore these data were not included in database	
Quality	***High***	*Quality criteria not met* f

Grecz and Tang (1970)

Toxin type	E
No. of strains tested	5
Temperature	75°C
Heating menstruum	0.067M phosphate buffer
No. of D/z-values	1/-/-
Notes	The publication describes a correlation between the spore heat resistance and content of dipicolinic acid (DPA). Authors tested type A, B and E strains (but only results of type E strains were included in database) and concluded that there was no correlation between the DPA-content of spores and their heat resistance, but a correlation was observed between the rate of loss of spore viability and the rate of loss of DPA.

Quality	*Quality criteria not met*
High	f, l

Ito *et al.* (1967)

Toxin type	E
No. of strains tested	4
Temperature	77°C
Heating menstruum	0.03M phosphate buffer
No. of D/z-values	4/-/-
Notes	The paper presents the study of thermal and chlorine resistance of spores. Authors concluded that there is a good correlation between heat resistance and chlorine resistance. Comparison of heat resistance of proteolytic type A, B strains and non-proteolytic type E toxins revealed that type A and B spores appears to be generally more chlorine resistant than type E strains.

Quality	*Quality criteria not met*
High	f

Ito *et al.* (1970)

Toxin type	E
No. of strains tested	4
Temperature	70, 73, 75, 80°C
Heating menstruum	0.067M phosphate buffer
No. of D/z-values	16/4/16
Notes	The paper summaries the state of knowledge regarding the heat resistance of *C. botulinum* spores and specifies factors influencing the *D*-value. Additionally the authors present results on heat treatment type E strains. From all four strains the most heat resistant at 70°C appeared to be strain Alaska, at 73 and 75°C strain Saratoga and at 80°C strain 1304E.

Quality	*Quality criteria not met*
Medium	f, k, l, n, o, p

Kralovic (1973)

Toxin type	E	
No. of strains tested	not specified	
Temperature	80°C	
Heating menstruum	not specified	
No. of D/z-values	1/-/-	
Notes	The data were derived from an abstract of the annual meeting of ASM. The test was designed to investigate the influence of lysozyme treatment on HR of spores. Since, it was not clear if the lysozyme was added to the recovery media or heating menstruum, only the control (no lysozyme) test was included in the analysis.	
Quality	***Medium***	*Quality criteria not met*
		f, h, l, n, o, p, r

Licciardello (1983)

Toxin type	E	
No. of strains tested	2	
Temperature	66, 71, 77, 82, 88, 93°C	
Heating menstruum	0.067M phosphate buffer, clam liquor, haddock slurry	
No. of D/z-values	18/5/18	
Notes	The author compares *z*-values determined by two different methods. The *z*-value calculated from TDT curve for spores in clam liquor was higher than the *z*-value determined from plotting survivor curves. The author explained that this could be caused by the difference in incubation time or medium used during the recovery of spores. The author only gives the *z*-value. *D*-values were not given; therefore they were read from survivor curve and TDT curve. The parameter was higher in experiments, where food was used as heating medium, rather than phosphate buffer.	
Quality	***High***	*Quality criteria not met*
		f, o

Lynt *et al.* (1977)

Toxin type	E	
No. of strains tested	5	
Temperature	74, 77, 79, 82, 85 °C	
Heating menstruum	blue crabmeat	
No. of D/z-values	28/6/26	
Notes	A comparison of two methods of *z*-value calculation was conducted. TDT curve and survival curve were obtained by plotting the log of *D*-value, and show different slopes for the same strain. The greater slope was found for the *D*-curve data; therefore the *z*-value was smaller. This was particularly notable for strain Beluga. The most resistant strain at all tested T was Beluga.	
Quality	***High***	*Quality criteria not met*

Lynt *et al.* (1979)

Toxin type	F
No. of strains tested	3
Temperature	71, 74, 77, 79, 82, 85°C
Heating menstruum	0.067M phosphate buffer, crabmeat
No. of D/z-values	36/8/36
Notes	Similarly, as in the previous paper (Lynt *et al.*, 1977), the authors compared the methods of *z*-value calculation. TDT curve and survival curve obtained by plotting the log of *D*-value. It shows different slopes for the same strain. The greater slope was found for the *D*-curve data; therefore the *z*-value was smaller. The study also reported that the heat resistance of type F strains was similar to type E strains, but with some differences (the slope of TDT and *D*-curves are steeper). Strains 190 and 202 had similar heat resistance patterns. The most resistant strain at all temperatures was 610 with the greatest *z*-value. At all temperature the crabmeat presented protection to spores of all type F strains tested.

Quality	***High***	*Quality criteria not met*

Lynt *et al.* (1983)

Toxin type	E and F
No. of strains tested	6
Temperature	71, 74, 77, 79, 82, 85°C
Heating menstruum	0.067M phosphate buffer, crabmeat
No. of D/z-values	31/8/31
Notes	The effect of delayed germination by heat damaged spores on estimates of heat resistance of type E and F spores was determined. The *D*-values were calculated for all strains during different periods of incubation. Parameters extracted to the database were calculated at the final period. The lowest delays in germination were reported for two type E strains (Alaska and Beluga). The delay was greater for the type F strains (190, 202 and 610). The delayed incubation influences the measured heat resistance of spores. Both type E and F showed an apparent increase in *D*-values during the incubation period, with type F more clearly reflecting the delayed germination. The *D*-value of those strains measured in phosphate buffer increased from 2- to 4-fold. As reported previously (Lynt *et al.*, 1979) crabmeat offered greater protection to spores. The *z*-value of type F strains showed a slight increase over the period of incubation.

Quality	***High***	*Quality criteria not met*

Mann (1966)

Toxin type	E	
No. of strains tested	1	
Temperature	71°C	
Heating menstruum	distilled water, tuna	
No. of D/z-values	2/2/2	
Notes	The data were presented in abstract of a thesis; therefore much information regarding the heating procedure is missing. Nevertheless, *D* and *z*-values given by author were added to database.	
Quality	***Medium***	*Quality criteria not met*
		f, i, j, k, l, n, o, p

Membrè and McClure (2006)

Toxin type	mixed strains	
No. of strains tested	6 (type B), 15 (type E), 3 (type F)	
Temperature	80°C	
Heating menstruum	TPG, TYD	
No. of D/z-values	38/-/-	
Notes	The report was the most recent literature review in the given field. Data were obtained by other authors; therefore information regarding heating method and recovery conditions are missing. The original data could not be retrieved from their original studies.	
Quality	***Medium***	*Quality criteria not met*
		f, i, l, n, o, p

Murrell and Scott (1966)

Toxin type	E	
No. of strains tested	1	
Temperature	70, 80°C	
Heating menstruum	0.05M phosphate buffer	
No. of D/z-values	3/-/-	
Notes	The heat resistance of spores of six bacterial species at various a_w was tested. The *D*-value was not given by authors – it was calculated from survivor curves. Authors concluded that the heat resistance of spores increases as the a_w value decreases.	
Quality	***Medium***	*Quality criteria not met*
		f, k, l

NCA (1966)

Toxin type	E	
No. of strains tested	2	
Temperature	70, 75, 80°C	
Heating menstruum	0.017M phosphate buffer	
No. of D/z-values	5/2/5	
Notes	Two strains were heated in phosphate buffer at three temperature regimes. At 75 and 80°C strain Beluga appeared to more heat resistant.	
Quality	***High***	*Quality criteria not met*
		f, l

Ohye and Scott (1957)

Toxin type	E
No. of strains tested	2
Temperature	70, 75, 80°C
Heating menstruum	0.067M phosphate buffer
No. of D/z-values	6/2/6
Notes	The paper is one of the first publications showing studies in physiology of type E strains. The heat resistance of spores was tested among other properties, such us: temperature of growth, toxin production and heat resistance of toxin. Strain 103 was more heat resistant than strain 108, simultaneously showing lower heat resistance in comparison with proteolytic *C. botulinum* type A and B strains.

Quality	***High***	*Quality criteria not met*
		f, i, o

Rhodehamel *et al.* (1991)

Toxin type	E
No. of strains tested	mixture of five strains
Temperature	74, 77, 79, 82°C
Heating menstruum	menhaden Surimi
No. of D/z-values	4/1/4
Notes	The incidence of type E spores in menhadeb surimi was determined. The results shown that the incidence is relatively low (1.2%). Reported *D*-value at 74 and 77°C agreed with values reported by other authors. At 79 and 82°C values were slightly higher for those previously reported. A slightly higher *z*-value compared with other reported for type E, could be a result of high protein content of a surimi or inclusion in spore “cocktail” strain of unknown heat resistance properties.

Quality	***High***	*Quality criteria not met*
		f

Roberts *et al.* (1965)

Toxin type	E
No. of strains tested	9
Temperature	80°C
Heating menstruum	water
No. of D/z-values	9/9/9
Notes	The most resistant was strain 1957/61. The heat resistance of remaining strains appeared to be very similar. The most heat sensitive strain was 16/63. The *D*-values were given by the authors, but it was not clear what method of heat treatment was used for the specified *D*-values.

Quality	***High***	*Quality criteria not met*
		f, i, o

Schmidt (1964)

Toxin type	E	
No. of strains tested	1	
Temperature	80°C	
Heating menstruum	TPB broth, 0.067M phosphate buffer	
No. of D/z-values	2/2/2	
Notes	The thesis summaries updated knowledge about the heat resistance of type E strains. One of the main conclusions is that spores of proteolytic *C. botulinum* types A and B are more heat resistant that spores of type E. Two *D*-values were given by the author, but there is very little information about the experiment.	
Quality	***Medium***	*Quality criteria not met*
		f, i, k, l, n, o

Scott and Bernard (1982)

Toxin type	B and E	
No. of strains tested	7	
Temperature	77, 82°C	
Heating menstruum	0.067M phosphate buffer	
No. of D/z-values	9/5/7	
Notes	Spores of type B strains had greater thermal resistance than type E strains and less than of proteolytic *C. botulinum* type B strains. Authors noted differences in heat resistance obtained in their study from data reported by other researchers, e.g. for strain Saratoga and Minnesota. Nevertheless the authors commented that their results were not consistent, and might be possibly caused by different batches of recovery media and age of spore	
Quality	***High***	*Quality criteria not met*
		o

Solomon *et al.* (1977)

Toxin type	E	
No. of strains tested	1	
Temperature	82°C	
Heating menstruum	crabmeat, TPGY broth	
No. of D/z-values	2/-/-	
Notes	The purpose of the experiment was to investigate the effect of low temperatures on growth of *C. botulinum* in a autoclaved creabmeat. The *D*-values were not given by authors, but were calculated from the initial and final spore concentration given in publication.	
Quality	***High***	*Quality criteria not met*
		l, m

Stringer *et al.* (1999)

Toxin type	B
No. of strains tested	1
Temperature	80°C
Heating menstruum	0.067M phosphate buffer
No. of D/z-values	1/-/-
Notes	The *D*-value was not given by the authors; it was calculated from given data. The purpose of the study was to test growth from spores of non-proteolytic *C. botulinum* in heated vegetable juice. Unheated spores were able to growth in all tested juices (broccoli, spring green, turnip, potato, helda bean) but the probability of growth was lower than in PYGS or PYGSL broth. A greater probability of growth in bean or broccoli juice than in culture media was observed after heating spores at 80°C for 10 minutes (incubation in heated media). Heating at 80°C for 100 minutes prevented the growth of spores both in juices and culture media. Nevertheless, the shortest time leading to growth was observed in PYGS broth (incubated at 30°C). Vegetable juice heated at 75°C for 10 min gave counts similar to those obtained with unheated juice; therefore heating increased the measured heat resistance of the spores, despite the fact that no lysozyme activity was detected.

Quality	***High***	*Quality criteria not met*
		m

Studies of recovery in presence of lysozyme [+LYS] *[ordered alphabetically]*

Alderton *et al.* (1974)

Toxin type	E	
No. of strains tested	1	
Temperature	79, 91, 93°C	
Heating menstruum	distilled water, phosphate buffer	
Nr. of D/z-values	5/1/2	
Notes	No data regarding inoculum size and heating method was given. The *D*-value was not calculated for the heat sensitive fraction. This part of survivor curve was ignored. The heat resistance of proteolytic and non-proteolytic *C. botulinum* was compared in a presence of lysozyme. Addition of enzyme to recovery media increases heat resistance both type A and type E strains, but with a greater impact on heat resistance of type E strains. Lysozyme also affected the colony outgrowth time of type A strains - shortening it by approximately half. This phenomenon was not observed in case of type E strains. Authors stated also, that the magnitude of *D*-values depended on individual batches of recovery medium.	
Quality	***High***	*Quality criteria not met*
		f, i

Institute of Food Research (IFR), 1980-2011

Toxin type	B, E, F and mixed strains	
No. of strains tested	9 [11]	
Temperature	75, 78, 80, 82, 85, 88, 92°C	
Heating menstruum	0.067M phosphate buffer, PY broth, béchamel sauce, bolognaise sauce, broccoli puree, cooked pasta potato puree,	
No. of D/z-values	108/6/24 [96/9/27]	
Notes	The data are a summary of manual search of laboratory books. Experiments were conducted in different heating menstruum with recovery media in absence and presence of lysozyme, HEWL or vegetable juices in varius media. In most cases the parameters were not given, therefore they were obtained by fitting the best straight line. Majority of the data were collected at 90°C and the resistance of strains depended on heating menstruum used. The most resistant strains of heat sensitive and heat resistant fraction at 80 and 90°C were Foster B96 and Eklund 17B. In case of -LYS tests the most resistant strain at 75 and 80° was Eklund 17B.	
Quality	***High***	*Quality criteria not met*

Juneja and Eblen (1995)

Toxin type	B
No. of strains tested	9
Temperature	75, 80, 85, 90°C
Heating menstruum	turkey slurry
No. of D/z-values	24/6/24
Notes	The Authors determined the effect of salt in turkey slurry and recovery medium on the heat resistance of spores. They concluded that increasing concentration of salt both in heating menstruum and recovery medium reduced the heat resistance. There were no kinetic data presented, therefore it was impossible to verify, whether the tailing effect in spore recovery was observed.

Quality	*Quality criteria not met*
	f, o, r

Juneja *et al.* (1995a)

Toxin type	B and E
No. of strains tested	7
Temperature	70, 75, 80, 85, 90°C
Heating menstruum	0.1M phosphate buffer, turkey slurry
No. of D/z-values	33/33/33 [12/12/12]
Notes	The data showed that the *D*-value obtained in turkey slurry was higher than in phosphate buffer and that the heat resistance of spores is higher in media +LYS to compare to -LYS. The most resistant strains at 70°C in a phosphate buffer at 75°C were CBW25 and KapB5, at 80 and 85°C – Saratoga. There were no kinetic data presented, therefore it was impossible to verify, whether the tailing effect in spore recovery was observed.

Quality	*Quality criteria not met*
	o, r

Juneja *et al.* (1995b)

Toxin type	B, E
No. of strains tested	mixture of six strains
Temperature	70, 75, 80, 85, 90°C
Heating menstruum	turkey slurry
No. of D/z-values	45/7/19
Notes	The authors examined the effect and interactions of heating temperature, pH, sodium chloride and sodium pyrophosphate on the heat resistance of *C. botulinum*. The data suggested that a lower pH resulted in a greater heat resistance, particularly at high temperatures. The parameters of heat resistance can be reduced by combining all four factors. There were no kinetic data presented, therefore it was impossible to verify, whether the tailing effect in spore recovery was observed.

Quality	*Quality criteria not met*
	o, r

Lindström *et al.* (2003)

Toxin type	E	
No. of strains tested	1	
Temperature	75, 81, 85, 90, 93°C	
Heating menstruum	rainbow trout, whitefish	
No. of D/z-values	10/2/5	
Notes	The authors estimated that the heat resistant fraction constitutes of approximately 0.1% of spores.	
Quality	***High***	*Quality criteria not met*

Notermans *et al.* (1990)

Toxin type	B and E	
No. of strains tested	3	
Temperature	70, 80, 90°C	
Heating menstruum	0.07M phosphate buffer, different media	
No. of D/z-values	7/1/30	
Notes	The authors concluded that spores of non-proteolytic *C. botulinum* can lead to growth and toxin production in REPFEDs, but heating at 90°C for 4 minutes was sufficient to provide a 5D safety factor. Strains CDI-1 and CDI-2 presented a very similar heat resistance pattern both at 70 and 80°C. There are no example kinetic data; therefore it was impossible to state if tailing effect was observed.	
Quality		*Quality criteria not met*
		f, n, r

Peck *et al.* (1992a)

Toxin type	B	
No. of strains tested	1	
Temperature	85°C	
Heating menstruum	0.067M phosphate buffer	
No. of D/z-values	3/-/-	
Notes	The effect of recovery medium on the heat resistance of spores was tested. The authors shown that inclusion of as little as 0.1µl lysozyme ml^{-1} is sufficient to increases the number of surviving spores. The maximum spore recovery was achieved at a concentration of 5 – 10 µg/ml of lysozyme. The *D*-values were not given by authors and they were extrapolated from survivor curve given only for one strain. The authors estimate also that 0.1 – 1.0% of heated spores are permeable to lysozyme.	
Quality	***High***	*Quality criteria not met*

Peck *et al.* (1992b)

Toxin type	B and E	
No. of strains tested	2	
Temperature	85°C	
Heating menstruum	0.067M phosphate buffer	
No. of D/z-values	6/-/-	
Notes	All strains had a higher *D*-value in +LYS media compared with the control. The effect of alkaline thioglycolate treatment of heated spores on their inactivation kinetics was tested. Spores of Eklund 17B untreated with alkaline thioglycolate before plating gave an estimated 4.1 decimal kill in 120 min. Treatment of spores gave an estimated 1.6 decimal kill in 120 min. The authors suggested that a marked increase in the number of heated spores recovered on +LYS medium was due to their increased permeability to lysozyme.	
Quality	***High***	*Quality criteria not met*

Peck *et al.* (1993)

Toxin type	B and E	
No. of strains tested	2	
Temperature	75, 85, 90, 95°C	
Heating menstruum	0.067M phosphate buffer	
No. of D/z-values	15/2/15	
Notes	In +LYS media, spores of strain Beluga were more sensitive to heating than those of strain Eklund 17B. Heated spores of a further 10 strains (type E and F) at 85°C and recovered in +LYS, resulted in *D*-values of 29 – 53 min (heat resistant fraction). Within those 10 strains there was no relationship between toxin type and *D*-value. The authors estimated that 0.2 – 1.4% of heated spores were permeable to lysozyme with temperature having little effect on its size. The effect of alkaline thioglycolate treatment of heated spores on their inactivation kinetics was tested. Similar curves for the heat resistant fraction were obtained for spores both treated/untreated with alkaline thioglycolate.	
Quality	***High***	*Quality criteria not met*

Peterson *et al.* (1997)

Toxin type	mixed strains	
No. of strains tested	3	
Temperature	89, 91, 92, 93, 94°C	
Heating menstruum	crabmeat	
No. of D/z-values	11/1/11	
Notes	The authors showed that recovery of spores in a medium containing crabmeat increased the *D*-value, suggesting that crabmeat provide protection to the spores against the heat. The effect of two methods determination (TDT and survivor curve) of *D*-value on its magnitude was explained.	
Quality		*Quality criteria not met*

Scott and Bernard (1985)

Toxin type	B and E
No. of strains tested	5
Temperature	85°C
Heating menstruum	0.067M phosphate buffer
No. of D/z-values	5/-/- [5/-/-]
Notes	The *D*-values were given by authors; unfortunately they did not agree with presented graphs for *z*-values. Therefore *D*-values extracted to the database were read directly from graphs. The authors concluded that the effect of lysozyme on the heat resistance of spores varied for different strains. The heat resistance of strain ATCC 17844 was about 60-fold higher for spores recovered without lysozyme, but this difference was smaller at higher temperatures. The difference in heat resistance of strain Eklund 17B in medium with and without lysozyme was significant, but it was not the case for strain 2129B. The higher heat resistance due to lysozyme of type B strains was similar to type E strains. *D*-values obtained in this study varied from values reported by other authors, which could be the result of different medium used.

Quality	*Quality criteria not met*
	f, l, o, r

Smelt (1980)

Toxin type	B and E
No. of strains tested	2
Temperature	78, 80, 83, 85, 88, 90°C
Heating menstruum	0.067M phosphate buffer, 0.1M citrate buffer, 0.1M citrate buffer + 30% (w/w) sucrose
No. of D/z-values	31/6/24
Notes	The heat resistance of spores of type B and E strains at neutral and low pH were tested. The survival curves spores heated in citrate buffer or phosphate buffer showed tailing. The author dispute the theory that tailing is caused by clumping of spores. In the case of the two tested strains, the pH of heating medium had little effect on the heat resistance of the heat sensitive fraction. At a higher pH there was a greater *D*-value of the heat resistant fraction, which was very noticeable for strain Eklund 17B. The thermal destruction of spores of strain Beluga was different in neutral phosphate buffer and citrate buffer. The biphasic curve was not observed. When spores were heated in the presence of glucose+fructose or glucose alone survival curves were characterised by a shoulder, the length of which depends on temperature and carbohydrate concentration (*D*-values not calculated by author).

Quality	***High***	*Quality criteria not met*
		f

Stringer and Peck (1997)

Toxin type	B	
No. of strains tested	1	
Temperature	75, 90°C	
Heating menstruum	PY broth	
No. of D/z-values	10/-/- [2/-/-]	
Notes	The effect of sodium chloride on the recovery of heat treated spores was tested. Heat treatment at 75°C had little effect on the heat resistance of spores, when recovered in media containing 1.4%, 3.0% or 4.0% NaCl at 10°C. In the presence of lysozyme in the recovery medium the *D*-value increased with an increasing concentration of NaCl (both for heat sensitive and heat resistant fractions). Incubation of inoculated heated broths at 10°C for 147 days, or for further 28 days at 30°C resulted in additional growth.	
Quality	***High***	*Quality criteria not met* m

Stringer and Peck (1996)

Toxin type	B	
No. of strains tested	1	
Temperature	85°C	
Heating menstruum	PY broth	
No. of D/z-values	18/-/-	
Notes	The effect of vegetable juices in the plating medium on recovery of heated spores was tested. The results showed that turnip, swede, potato, flat bean or cabbage juice increased the measured heat resistance of spores – resulting in biphasic curve. The *D*-values were not given by authors.	
Quality	***High***	*Quality criteria not met* m

Stringer *et al.* (1997)

Toxin type	B and E	
No. of strains tested	mixture of six strains	
Temperature	90°C	
Heating menstruum	PY broth	
No. of D/z-values	2/-/-	
Notes	The *D*-values were not given in the paper. Heating at 90°C resulted in a rapid fall in the first 2 min. of heating. Further heat treatment gave less rapid decline.	
Quality	***High***	*Quality criteria not met* m

[a)] Temperatures from which *D*-values were extracted to the database and analysed

[b)] the *D* and *z*-values are presented in a form of three numbers (a/b/c), where:

i. total number of *D*-values extracted to the database from a given source
ii. *z*-values originating from the particular test (e.g. the same strain, the same inoculum size, recovery conditions, at particular temperature range)
iii. total number of *z*-values that were used to calculate the D'(80) (this could be due e.g. two different methods of *z*-value calculation conducted by authors)

Data in [ ] the –LYS data

Appendix 2 Details of collected *D* and *z*-values for non-proteolytic *C. botulinum* spores

No.	Strain	Toxin type	Heating menstruum	pH	Addition of (aw)	SH	T(°C)	*D*-value (min)	logD (min)	D'(80)	HS HR	Method of *D*-value calculation	*z*-value (°C)	Heating technique	Recovery method	Recovery media	Recovery conditions	Reference
1	Nanaimo	E	0.067M phosphate buf	7.0			70	36.00	1.56	3.11		Survivor curve	9.4	TDT tubes in wate	counts	PIT	25°C/14 days	Ohye and Scott, 1957
2	Nanaimo	E	0.067M phosphate buf	7.0			75	10.00	1.00	2.94		Survivor curve	9.4	TDT tubes in wate	counts	PIT	25°C/14 days	Ohye and Scott, 1957
3	Nanaimo	E	0.067M phosphate buf	7.0			80	3.30	0.52	3.30		Survivor curve	9.4	TDT tubes in wate	counts	PIT	25°C/14 days	Ohye and Scott, 1957
4	Vancouver He	E	0.067M phosphate buf	7.0			70	7.80	0.89	0.41		Survivor curve	7.8	TDT tubes in wate	counts	PIT	25°C/14 days	Ohye and Scott, 1957
5	Vancouver He	E	0.067M phosphate buf	7.0			75	3.10	0.49	0.71		Survivor curve	7.8	TDT tubes in wate	counts	PIT	25°C/14 days	Ohye and Scott, 1957
6	Vancouver He	E	0.067M phosphate buf	7.0			80	0.40	-0.40	0.40		Survivor curve	7.8	TDT tubes in wate	counts	PIT	25°C/14 days	Ohye and Scott, 1957
7	Eklund 17B	B	cod	6.8			75	53.90	1.73	14.13		Survivor curve	8.6	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
8	Eklund 17B	B	cod	6.8			80	18.30	1.26	18.30		Survivor curve	8.6	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
9	Eklund 17B	B	cod	6.8			85	4.00	0.60	15.26		Survivor curve	8.6	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
10	Eklund 17B	B	cod	6.8			90	1.10	0.04	16.00		Survivor curve	8.6	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
11	Eklund 17B	B	cod	6.8			92	0.62	-0.21	15.41		Survivor curve	8.6	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
12	ATCC 9564	E	cod	6.8			75	58.50	1.77	14.61		Survivor curve	8.3	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
13	ATCC 9564	E	cod	6.8			80	15.10	1.18	15.10		Survivor curve	8.3	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
14	ATCC 9564	E	cod	6.8			85	4.75	0.68	19.02		Survivor curve	8.3	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
15	ATCC 9564	E	cod	6.8			90	0.79	-0.10	12.66		Survivor curve	8.3	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
16	ATCC 9564	E	cod	6.8			92	0.56	-0.25	15.63		Survivor curve	8.3	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
17	Eklund 17B	B	carrot homogenates	5.7			75	19.39	1.29	5.99		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
18	Eklund 17B	B	carrot homogenates	5.7			80	4.24	0.63	4.24		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
19	Eklund 17B	B	carrot homogenates	5.7			85	1.57	0.20	5.08		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
20	Eklund 17B	B	carrot homogenates	5.7			90	0.43	-0.37	4.51		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
21	Eklund 17B	B	carrot homogenates	5.7			92	0.36	-0.44	6.04		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
22	ATCC 9564	E	carrot homogenates	5.7			70	32.80	1.52	3.13		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
23	ATCC 9564	E	carrot homogenates	5.7			75	18.05	1.26	5.58		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
24	ATCC 9564	E	carrot homogenates	5.7			80	4.33	0.64	4.33		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
25	ATCC 9564	E	carrot homogenates	5.7			85	0.73	-0.14	2.36		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
26	ATCC 9564	E	carrot homogenates	5.7			90	0.48	-0.32	5.03		Survivor curve	9.8	bottles in water ba	counts	EA+0.1% starcl	30°C/6 days	Gaze and Brown, 1990
27	Beluga	E	oyster Homogenates	6.2			50	7943.00	3.90	1.27		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
28	Beluga	E	oyster Homogenates	6.2	0.13% KS		50	7413.00	3.87	1.06		Survivor curve	7.8	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
29	Beluga	E	oyster Homogenates	6.2	1.0 % NaCl		50	23990.00	4.38	3.05		Survivor curve	7.7	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
30	Beluga	E	oyster Homogenates	6.2	1.0% NaCl + 0.13% K		50	6607.00	3.82	1.05		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
31	Beluga	E	oyster Homogenates	6.2			55	1259.00	3.10	0.86		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
32	Beluga	E	oyster Homogenates	6.2	0.13% KS		55	1202.00	3.08	0.75		Survivor curve	7.8	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
33	Beluga	E	oyster Homogenates	6.2	1.0 % NaCl		55	1047.00	3.02	0.59		Survivor curve	7.7	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
34	Beluga	E	oyster Homogenates	6.2	1.0% NaCl + 0.13% K		55	1023.00	3.01	0.70		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
35	Beluga	E	oyster Homogenates	6.2			60	776.00	2.89	2.28		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
36	Beluga	E	oyster Homogenates	6.2	0.13% KS		60	912.00	2.96	2.49		Survivor curve	7.8	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
37	Beluga	E	oyster Homogenates	6.2	1.0 % NaCl		60	692.00	2.84	1.75		Survivor curve	7.7	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
38	Beluga	E	oyster Homogenates	6.2	1.0% NaCl + 0.13% K		60	776.00	2.89	2.28		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
39	Beluga	E	oyster Homogenates	6.2			70	72.00	1.86	3.90		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
40	Beluga	E	oyster Homogenates	6.2	0.13% KS		70	72.00	1.86	3.76		Survivor curve	7.8	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
41	Beluga	E	oyster Homogenates	6.2	1.0 % NaCl		70	100.00	2.00	5.03		Survivor curve	7.7	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
42	Beluga	E	oyster Homogenates	6.2	1.0% NaCl + 0.13% K		70	79.00	1.90	4.28		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
43	Beluga	E	oyster Homogenates	6.2			80	0.78	-0.11	0.78		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
44	Beluga	E	oyster Homogenates	6.2	0.13% KS		80	0.63	-0.20	0.63		Survivor curve	7.8	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990

45	Beluga	E	oyster Homogenates	6.2	1.0 % NaCl		80	1.00	0.00	1.00		Survivor curve	7.7	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
46	Beluga	E	oyster Homogenates	6.2	1.0% NaCl + 0.13% K		80	0.55	-0.26	0.55		Survivor curve	7.9	flasks in water bat	counts	PYG	30°C/2 days	Bucknavage *et al.*, 1990
47	Alaska	E	oyster Homogenates				74	7.56	0.88	0.48		TDT/Schmidt(1957)	5.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
48	Alaska	E	oyster Homogenates				74	7.56	0.88	0.27		TDT/Schmidt(1957)	4.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
49	Minnesota	E	oyster Homogenates				74	8.96	0.95	1.07		TDT/Schmidt(1957)	6.6	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
50	Minnesota	E	oyster Homogenates				74	8.96	0.95	0.96		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
51	Crab G21-5	E	oyster Homogenates				74	5.23	0.72	0.72		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
52	Crab G21-5	E	oyster Homogenates				74	5.23	0.72	0.41		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
53	Crab 25 V-1	E	oyster Homogenates				74	7.13	0.85	0.88		TDT/Schmidt(1957)	6.7	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
54	Crab 25 V-1	E	oyster Homogenates				74	7.13	0.85	0.55		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
55	Crab 25 V-2	E	oyster Homogenates				74	2.00	0.30	0.21		TDT/Schmidt(1957)	6.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
56	Crab 25 V-2	E	oyster Homogenates				74	2.00	0.30	0.18		TDT/Schmidt(1957)	5.9	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
57	Alaska	E	oyster Homogenates				75	3.85	0.59	0.40		TDT/Schmidt(1957)	5.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
58	Minnesota	E	oyster Homogenates				75	5.28	0.72	0.92		TDT/Schmidt(1957)	6.6	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
59	Minnesota	E	oyster Homogenates				75	5.28	0.72	0.85		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
60	Crab G21-5	E	oyster Homogenates				75	2.38	0.38	0.47		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
61	Crab G21-5	E	oyster Homogenates				75	2.38	0.38	0.29		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
62	Crab 25 V-1	E	oyster Homogenates				75	3.40	0.53	0.61		TDT/Schmidt(1957)	6.7	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
63	Crab 25 V-2	E	oyster Homogenates				75	1.28	0.11	0.20		TDT/Schmidt(1957)	6.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
64	Crab 25 V-1	E	oyster Homogenates				75	3.40	0.53	0.42		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
65	Crab 25 V-2	E	oyster Homogenates				75	1.28	0.11	0.18		TDT/Schmidt(1957)	5.9	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
66	Alaska	E	oyster Homogenates				75	3.85	0.59	0.25		TDT/Schmidt(1957)	4.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
67	Alaska	E	oyster Homogenates				77	1.53	0.18	0.34		TDT/Schmidt(1957)	5.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
68	Alaska	E	oyster Homogenates				77	1.53	0.18	0.25		TDT/Schmidt(1957)	4.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
69	Minnesota	E	oyster Homogenates				77	2.69	0.43	0.85		TDT/Schmidt(1957)	6.6	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
70	Crab G21-5	E	oyster Homogenates				77	1.01	0.00	0.35		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
71	Crab 25 V-1	E	oyster Homogenates				77	1.42	0.15	0.46		TDT/Schmidt(1957)	6.7	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
72	Crab 25 V-1	E	oyster Homogenates				77	1.42	0.15	0.36		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
73	Crab 25 V-2	E	oyster Homogenates				77	0.73	-0.14	0.21		TDT/Schmidt(1957)	6.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
74	Crab 25 V-2	E	oyster Homogenates				77	0.73	-0.14	0.20		TDT/Schmidt(1957)	5.9	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
75	Alaska	E	oyster Homogenates				79	0.41	-0.39	0.31		TDT/Schmidt(1957)	5.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
76	Minnesota	E	oyster Homogenates				77	2.69	0.43	0.81		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
77	Minnesota	E	oyster Homogenates				79	1.03	0.01	0.73		TDT/Schmidt(1957)	6.6	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
78	Crab G21-5	E	oyster Homogenates				77	1.01	0.00	0.29		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
79	Crab G21-5	E	oyster Homogenates				79	0.29	-0.54	0.21		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
80	Crab G21-5	E	oyster Homogenates				79	0.29	-0.54	0.19		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
81	Crab 25 V-1	E	oyster Homogenates				79	0.58	-0.24	0.47		TDT/Schmidt(1957)	6.7	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
82	Crab 25 V-1	E	oyster Homogenates				79	0.58	-0.24	0.45		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
83	Alaska	E	oyster Homogenates				79	0.41	-0.39	0.30		TDT/Schmidt(1957)	4.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
84	Alaska	E	oyster Homogenates				82	0.07	-1.15	0.19		TDT/Schmidt(1957)	5.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
85	Minnesota	E	oyster Homogenates				79	1.03	0.01	0.83		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
86	Minnesota	E	oyster Homogenates				82	0.43	-0.37	0.93		TDT/Schmidt(1957)	6.6	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
87	Crab G21-5	E	oyster Homogenates				82	0.11	-0.96	0.22		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
88	Crab 25 V-1	E	oyster Homogenates				82	0.20	-0.70	0.43		TDT/Schmidt(1957)	6.7	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
89	Crab 25 V-1	E	oyster Homogenates				82	0.20	-0.70	0.50		TDT/Schmidt(1957)	5.5	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
90	Crab 25 V-2	E	oyster Homogenates				79	0.25	-0.60	0.20		TDT/Schmidt(1957)	6.2	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
91	Crab G21-5	E	oyster Homogenates				82	0.11	-0.96	0.22		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGYT	28°C/62 days	Chai and Liang, 1992
92	Crab G21-5	E	oyster Homogenates				79	0.27	-0.57	0.22		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGYT	28°C/14 days	Chai and Liang, 1992
93	Alaska	E	oyster Homogenates				82	0.07	-1.15	0.23		TDT/Schmidt(1957)	4.2	TDT tubes in wate	TDT	TPGYT	28°C/14 days	Chai and Liang, 1992

94	Minnesota	E	oyster Homogenates				82	0.43	-0.37	0.96		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT		TPGYT	28°C/14 days	Chai and Liang, 1992
95	Crab G21-5	E	oyster Homogenates				79	0.27	-0.57	0.22		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT		TPGYT	28°C/14 days	Chai and Liang, 1992
96	Crab 25 V-2	E	oyster Homogenates				79	0.25	-0.60	0.20		TDT/Schmidt(1957)	5.9	TDT tubes in wate	TDT		TPGYT	28°C/62 days	Chai and Liang, 1992
97	Crab 25 V-2	E	oyster Homogenates				82	0.08	-1.10	0.18		TDT/Schmidt(1957)	6.2	TDT tubes in wate	TDT		TPGYT	28°C/62 days	Chai and Liang, 1992
98	Crab 25 V-2	E	oyster Homogenates				82	0.08	-1.10	0.19		TDT/Schmidt(1957)	5.9	TDT tubes in wate	TDT		TPGYT	28°C/62 days	Chai and Liang, 1992
99	Beluga	E	crabmeat				74	12.97	1.11	2.39		TDT/Schmidt(1957)	8.3	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
100	Beluga	E	crabmeat				77	4.07	0.61	1.63		TDT/Schmidt(1957)	8.3	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
101	Beluga	E	crabmeat				79	1.65	0.22	1.40		TDT/Schmidt(1957)	8.3	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
102	Beluga	E	crabmeat				82	0.74	-0.13	1.36		TDT/Schmidt(1957)	8.3	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
103	Beluga	E	crabmeat				85	0.29	-0.54	1.16		TDT/Schmidt(1957)	8.3	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
104	Beluga	E	crabmeat				74	12.97	1.11	1.55		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
105	Beluga	E	crabmeat				77	4.07	0.61	1.26		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
106	Beluga	E	crabmeat				79	1.65	0.22	1.33		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
107	Beluga	E	crabmeat				82	0.74	-0.13	1.61		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
108	Beluga	E	crabmeat				85	0.29	-0.54	1.70		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
109	Alaska	E	crabmeat				74	10.39	1.02	1.40		TDT/Schmidt(1957)	7.0	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
110	Alaska	E	crabmeat				77	3.04	0.48	1.03		TDT/Schmidt(1957)	7.0	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
111	Alaska	E	crabmeat				79	1.35	0.13	1.11		TDT/Schmidt(1957)	7.0	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
112	Alaska	E	crabmeat				82	0.51	-0.29	1.05		TDT/Schmidt(1957)	7.0	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
113	Alaska	E	crabmeat				74	10.39	1.02	1.04		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
114	Alaska	E	crabmeat				77	3.04	0.48	0.87		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
115	Alaska	E	crabmeat				79	1.35	0.13	1.08		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
116	Alaska	E	crabmeat				82	0.51	-0.29	1.17		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
117	Crab G21-5	E	crabmeat				74	6.80	0.83	1.31		TDT/Schmidt(1957)	8.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
118	Crab G21-5	E	crabmeat				77	2.38	0.38	0.96		TDT/Schmidt(1957)	8.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
119	Crab G21-5	E	crabmeat				79	1.10	0.04	0.93		TDT/Schmidt(1957)	8.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
120	Crab G21-5	E	crabmeat				82	0.63	-0.20	1.15		TDT/Schmidt(1957)	8.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
121	Crab G21-5	E	crabmeat				74	6.80	0.83	0.97		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
122	Crab G21-5	E	crabmeat				77	2.38	0.38	0.82		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
123	Crab G21-5	E	crabmeat				79	1.10	0.04	0.91		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
124	Crab G21-5	E	crabmeat				82	0.63	-0.20	1.29		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
125	Crab 25 V-1	E	crabmeat				82	0.62	-0.21	1.29		TDT/Schmidt(1957)		TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
126	Crab 25 V-2	E	crabmeat				82	0.49	-0.31	1.02		TDT/Schmidt(1957)		TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1977
127	190	F	0.067M phosphate buf	7.0			71	31.08	1.49	0.85		TDT/Schmidt(1957)	5.7	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
128	190	F	0.067M phosphate buf	7.0			74	9.07	0.96	0.77		TDT/Schmidt(1957)	5.7	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
129	190	F	0.067M phosphate buf	7.0			77	1.66	0.22	0.44		TDT/Schmidt(1957)	5.7	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
130	190	F	0.067M phosphate buf	7.0			79	1.03	0.01	0.81		TDT/Schmidt(1957)	5.7	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
131	190	F	0.067M phosphate buf	7.0			82	0.25	-0.60	0.61		TDT/Schmidt(1957)	5.7	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
132	190	F	0.067M phosphate buf	7.0			71	31.08	1.49	0.70		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
133	190	F	0.067M phosphate buf	7.0			74	9.07	0.96	0.67		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
134	190	F	0.067M phosphate buf	7.0			77	1.66	0.22	0.41		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
135	190	F	0.067M phosphate buf	7.0			79	1.03	0.01	0.80		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
136	190	F	0.067M phosphate buf	7.0			82	0.25	-0.60	0.64		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
137	202	F	0.067M phosphate buf	7.0			71	42.41	1.63	1.24		TDT/Schmidt(1957)	5.8	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
138	202	F	0.067M phosphate buf	7.0			74	12.68	1.10	1.13		TDT/Schmidt(1957)	5.8	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
139	202	F	0.067M phosphate buf	7.0			77	4.29	0.63	1.16		TDT/Schmidt(1957)	5.8	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
140	202	F	0.067M phosphate buf	7.0			79	0.93	-0.03	0.73		TDT/Schmidt(1957)	5.8	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
141	202	F	0.067M phosphate buf	7.0			82	0.33	-0.48	0.79		TDT/Schmidt(1957)	5.8	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979
142	202	F	0.067M phosphate buf	7.0			71	42.41	1.63	0.89		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT		TPGY	26°C/14 days	Lynt *et al.*, 1979

143	202	F	0.067M phosphate buf	7.0			74	12.68	1.10	0.90		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
144	202	F	0.067M phosphate buf	7.0			77	4.29	0.63	1.02		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
145	202	F	0.067M phosphate buf	7.0			79	0.93	-0.03	0.72		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
146	202	F	0.067M phosphate buf	7.0			82	0.33	-0.48	0.86		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
147	Craig 610	F	0.067M phosphate buf	7.0			77	6.64	0.82	2.63		TDT/Schmidt(1957)	8.2	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
148	Craig 610	F	0.067M phosphate buf	7.0			79	2.12	0.33	1.79		TDT/Schmidt(1957)	8.2	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
149	Craig 610	F	0.067M phosphate buf	7.0			82	0.84	-0.08	1.56		TDT/Schmidt(1957)	8.2	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
150	Craig 610	F	0.067M phosphate buf	7.0			85	0.37	-0.43	1.51		TDT/Schmidt(1957)	8.2	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
151	Craig 610	F	0.067M phosphate buf	7.0			77	6.64	0.82	1.99		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
152	Craig 610	F	0.067M phosphate buf	7.0			79	2.12	0.33	1.70		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
153	Craig 610	F	0.067M phosphate buf	7.0			82	0.84	-0.08	1.88		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
154	Craig 610	F	0.067M phosphate buf	7.0			85	0.37	-0.43	2.30		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
155	202	F	crabmeat	7.0			77	9.50	0.98	3.45		TDT/Schmidt(1957)	7.5	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
156	202	F	crabmeat	7.0			79	3.55	0.55	2.95		TDT/Schmidt(1957)	7.5	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
157	202	F	crabmeat	7.0			82	1.16	0.06	2.28		TDT/Schmidt(1957)	7.5	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
158	202	F	crabmeat	7.0			85	0.53	-0.28	2.46		TDT/Schmidt(1957)	7.5	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
159	202	F	crabmeat	7.0			77	9.50	0.98	2.90		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
160	202	F	crabmeat	7.0			79	3.55	0.55	2.86		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
161	202	F	crabmeat	7.0			82	1.16	0.06	2.56		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
162	202	F	crabmeat	7.0			85	0.53	-0.28	3.20		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/14 days	Lynt *et al.*, 1979
163	Beluga	E	rainbow trout	6.6			75	4.60	0.66	0.99	HS	Survivor curve		metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
164	Beluga	E	rainbow trout	6.6			75	255.00	2.41	84.29	HR	Survivor curve	10.4	metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
165	Beluga	E	rainbow trout	6.6			85	2.00	0.30	9.28	HS	Survivor curve		metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
166	Beluga	E	rainbow trout	6.6			85	98.00	1.99	296.48	HR	Survivor curve	10.4	metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
167	Beluga	E	rainbow trout	6.6			93	0.40	-0.40	21.65	HS	Survivor curve		metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
168	Beluga	E	rainbow trout	6.6			93	4.20	0.62	74.69	HR	Survivor curve	10.4	metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
169	Beluga	E	whitefish	7.0			81	1.90	0.28	2.58	HS	Survivor curve		metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
170	Beluga	E	whitefish	7.0			81	55.00	1.74	69.08	HR	Survivor curve	10.1	metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
171	Beluga	E	whitefish	7.0			90	1.00	0.00	21.54	HS	Survivor curve		metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
172	Beluga	E	whitefish	7.0			90	7.10	0.85	69.40	HR	Survivor curve	10.1	metal cap tubes in	counts + LYS	TPGY + LYS	30°/90 days	Lindström *et al.*, 2003
173	Mixed strains	E	menhaden Surimi				74	8.66	0.94	2.07		TDT/Schmidt(1957)	9.8	TDT tubes in wate	TDT	TPGY	26°C/14 days	Rhodehamel *et a*l, 1991
174	Mixed strains	E	menhaden Surimi				77	3.49	0.54	1.61		TDT/Schmidt(1957)	9.8	TDT tubes in wate	TDT	TPGY	26°C/14 days	Rhodehamel *et a*l, 1991
175	Mixed strains	E	menhaden Surimi				79	2.15	0.33	1.87		TDT/Schmidt(1957)	9.8	TDT tubes in wate	TDT	TPGY	26°C/14 days	Rhodehamel *et a*l, 1991
176	Mixed strains	E	menhaden Surimi				82	1.22	0.09	2.05		TDT/Schmidt(1957)	9.8	TDT tubes in wate	TDT	TPGY	26°C/14 days	Rhodehamel *et a*l, 1991
177	Alaska	E	water				80	0.60	-0.22	0.60		Survivor curve	5.8		counts	RCM	25°C/5-7 days	Roberts and Ingram, 1965
178	Beluga	E	water				80	0.75	-0.12	0.75		Survivor curve	5.3		counts	RCM	25°C/5-7 days	Roberts and Ingram, 1965
179	Iwanai	E	water				80	1.00	0.00	1.00		Survivor curve	5.6		counts	RCM	25°C/5-7 days	Roberts and Ingram, 1965
180	Minneapolis	E	water				80	0.87	-0.06	0.87		Survivor curve	5.8		counts	RCM	25°C/5-7 days	Roberts and Ingram, 1965
181	8E	E	water				80	0.80	-0.10	0.80		Survivor curve	4.1		counts	RCM	25°C/5-7 days	Roberts and Ingram, 1965
182	1957/61	E	water				80	1.25	0.10	1.25		Survivor curve	6.0		counts	RCM	25°C/5-7 days	Roberts and Ingram, 1965
183	1537/62	E	water				80	0.83	-0.08	0.83		Survivor curve	6.0		counts	RCM	25°C/5-7 days	Roberts and Ingram, 1965
184	16/63	E	water				80	0.33	-0.48	0.33		Survivor curve	5.0		counts	RCA	25°C/5-7 days	Roberts and Ingram, 1965
185	4318/63	E	water				80	0.66	-0.18	0.66		Survivor curve	5.6		counts	RCA	25°C/5-7 days	Roberts and Ingram, 1965
186	Eklund 17B	B	0.067M phosphate buf	7.0			75	283.00	2.45	62.21	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
187	Eklund 17B	B	0.067M phosphate buf	7.0			85	73.60	1.87	334.79	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
188	Eklund 17B	B	0.067M phosphate buf	7.0	Alkaline thioglycolate a		85	79.00	1.90	359.35	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
189	Eklund 17B	B	0.067M phosphate buf	7.0			85	90.00	1.95	409.39	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
190	Eklund 17B	B	0.067M phosphate buf	7.0	Alkaline thioglycolate a		85	111.00	2.05	504.91	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
191	Eklund 17B	B	0.067M phosphate buf	7.0			90	18.10	1.26	374.51	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993

192	Eklund 17B	B	0.067M phosphate buf	7.0	Alkaline thioglycolate a	90	19.40	1.29	401.41	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
193	Eklund 17B	B	0.067M phosphate buf	7.0		95	4.57	0.66	430.13	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
194	Eklund 17B	B	0.067M phosphate buf	7.0	Alkaline thioglycolate a	95	4.23	0.63	398.13	HR	Survivor curve	7.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
195	Beluga	E	0.067M phosphate buf	7.0		85	48.30	1.68	193.36	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
196	Beluga	E	0.067M phosphate buf	7.0	Alkaline thioglycolate a	85	42.80	1.63	171.34	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
197	Beluga	E	0.067M phosphate buf	7.0		90	12.60	1.10	201.92	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
198	Beluga	E	0.067M phosphate buf	7.0		90	11.00	1.04	176.28	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
199	Beluga	E	0.067M phosphate buf	7.0		95	3.17	0.50	203.37	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
200	Beluga	E	0.067M phosphate buf	7.0		95	2.34	0.37	150.12	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°	Peck *et al.*, 1993
201	Mixed strains (Ham,		crabmeat			89	12.90	1.11	139.79		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
202	Mixed strains (Ham,		crabmeat			89	11.10	1.05	138.99		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
203	Mixed strains (Ham,		crabmeat			90	9.50	0.98	138.20		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
204	Mixed strains (Ham,		crabmeat			91	8.20	0.91	140.08		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
205	Mixed strains (Ham,		crabmeat			91	7.10	0.85	139.03		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
206	Mixed strains (Ham,		crabmeat			92	6.10	0.79	138.77		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
207	Mixed strains (Ham,		crabmeat			92	5.30	0.72	138.96		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
208	Mixed strains (Ham,		crabmeat			93	4.50	0.65	137.80		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
209	Mixed strains (Ham,		crabmeat			93	3.90	0.59	138.38		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
210	Mixed strains (Ham,		crabmeat			94	3.40	0.53	140.15		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
211	Mixed strains (Ham,		crabmeat			94	2.90	0.46	137.03		TDT/Stumbo(1948)	8.6	packaged samples	TDT+crabmeat	TPGY + crabme	27°/21 days	Peterson *et al.*, 1997
212	Beluga	E	0.07M phosphate buffe	7.2		70	20.00	1.30	2.00		Survivor curve	10.0	metal cap tubes in	counts + egg yolk	FEM + egg yolk	30°C/5 days	Notermans *et al.* , 1990
213	Beluga	E	0.07M phosphate buffe	7.2		80	1.70	0.23	1.70		Survivor curve	10.0	metal cap tubes in	counts + egg yolk	FEM + egg yolk	30°C/5 days	Notermans *et al.* , 1990
214	Beluga	E	0.07M phosphate buffe	7.2		90	0.20	-0.70	2.00		Survivor curve	10.0	metal cap tubes in	counts + egg yolk	FEM + egg yolk	30°C/5 days	Notermans *et al.* , 1990
215	CDI-1	B	different media			80	5.20	0.72	5.20		Survivor curve		metal cap tubes in	counts + egg yolk	FEM + egg yolk	30°C/5 days	Notermans *et al.* , 1990
216	CDI-1	B	different media			90	0.60	-0.22	4.52		Survivor curve		metal cap tubes in	counts + egg yolk	FEM + egg yolk	30°C/5 days	Notermans *et al.* , 1990
217	CDI-2	B	different media			80	5.10	0.71	5.10		Survivor curve		metal cap tubes in	counts + egg yolk	FEM + egg yolk	30°C/5 days	Notermans *et al.* , 1990
218	CDI-2	B	different media			90	0.60	-0.22	4.52		Survivor curve		metal cap tubes in	counts + egg yolk	FEM + egg yolk	30°C/5 days	Notermans *et al.* , 1990
219	8E	E	0.03M phosphate buffe	7.0		77	0.77	-0.11	0.28		TDT/Stumbo(1950)		TDT tubes in wate	TDT	Liver broth with	30°C	Ito *et al.*, 1967
220	1304E	E	0.03M phosphate buffe	7.0		77	1.23	0.09	0.45		TDT/Stumbo(1950)		TDT tubes in wate	TDT	Liver broth with	30°C	Ito *et al.*, 1967
221	Minneapolis	E	0.03M phosphate buffe	7.0		77	1.55	0.19	0.57		TDT/Stumbo(1950)		TDT tubes in wate	TDT	Liver broth with	30°C	Ito *et al.*, 1967
222	Saratoga	E	0.03M phosphate buffe	7.0		77	1.95	0.29	0.72		TDT/Stumbo(1950)		TDT tubes in wate	TDT	Liver broth with	30°C	Ito *et al.*, 1967
223	CBW25	B	0.1M phosphate buffer	7.0		70	46.03	1.66	3.14		Survivor curve	8.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
224	CBW25	B	0.1M phosphate buffer	7.0		80	1.90	0.28	1.90		Survivor curve	10.0	screw-capped vials	counts	RCM	28°C/6 days	Juneja *et al.*, 1995a
225	CBW25	B	0.1M phosphate buffer	7.0		80	4.09	0.61	4.09		Survivor curve	8.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
226	CBW25	B	0.1M phosphate buffer	7.0		80	0.96	-0.02	0.96		Survivor curve	8.2	screw-capped vials	counts	TSA	28°C/6 days	Juneja *et al.*, 1995a
227	CBW25	B	0.1M phosphate buffer	7.0		80	3.73	0.57	3.73		Survivor curve	8.6	screw-capped vials	counts + LYS	TSA + LYS	28°C/6 days	Juneja *et al.*, 1995a
228	Eklund 17B	B	0.1M phosphate buffer	7.0		70	27.16	1.43	2.11		Survivor curve	9.0	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
229	Eklund 17B	B	0.1M phosphate buffer	7.0		75	5.45	0.74	1.52		Survivor curve	9.0	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
230	Eklund 17B	B	0.1M phosphate buffer	7.0		80	0.60	-0.22	0.60		Survivor curve	8.4	screw-capped vials	counts	RCM	28°C/6 days	Juneja *et al.*, 1995a
231	Eklund 17B	B	0.1M phosphate buffer	7.0		80	3.22	0.51	3.22		Survivor curve	9.0	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
232	Eklund 17B	B	0.1M phosphate buffer	7.0		80	0.67	-0.17	0.67		Survivor curve	8.6	screw-capped vials	counts	TSA	28°C/6 days	Juneja *et al.*, 1995a
233	Eklund 17B	B	0.1M phosphate buffer	7.0		80	3.22	0.51	3.22		Survivor curve	8.9	screw-capped vials	counts + LYS	TSA + LYS	28°C/6 days	Juneja *et al.*, 1995a
234	Eklund 17B	B	0.1M phosphate buffer	7.0		85	0.41	-0.39	1.47		Survivor curve	9.0	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
235	Kap B5	B	0.1M phosphate buffer	7.0		75	27.42	1.44	6.91		Survivor curve	8.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
236	Kap B5	B	0.1M phosphate buffer	7.0		80	1.53	0.18	1.53		Survivor curve	9.1	screw-capped vials	counts	RCM	28°C/6 days	Juneja *et al.*, 1995a
237	Kap B5	B	0.1M phosphate buffer	7.0		80	4.31	0.63	4.31		Survivor curve	8.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
238	Kap B5	B	0.1M phosphate buffer	7.0		80	2.25	0.35	2.25		Survivor curve	9.3	screw-capped vials	counts	TSA	28°C/6 days	Juneja *et al.*, 1995a
239	Kap B5	B	0.1M phosphate buffer	7.0		80	2.53	0.40	2.53		Survivor curve	8.5	screw-capped vials	counts + LYS	TSA + LYS	28°C/6 days	Juneja *et al.*, 1995a
240	Kap B5	B	0.1M phosphate buffer	7.0		85	0.89	-0.05	3.53		Survivor curve	8.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a

241	Kap B5	B	0.1M phosphate buffer	7.0			90	0.46	-0.34	7.25		Survivor curve	8.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
242	Whitefish	E	0.1M phosphate buffer	7.0			70	25.50	1.41	2.32		Survivor curve	9.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
243	Whitefish	E	0.1M phosphate buffer	7.0			80	0.39	-0.41	0.39		Survivor curve	9.1	screw-capped vials	counts	RCM	28°C/6 days	Juneja *et al.*, 1995a
244	Whitefish	E	0.1M phosphate buffer	7.0			80	1.03	0.01	1.03		Survivor curve	9.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
245	Whitefish	E	0.1M phosphate buffer	7.0			80	0.39	-0.41	0.39		Survivor curve	9.6	screw-capped vials	counts	TSA	28°C/6 days	Juneja *et al.*, 1995a
246	Whitefish	E	0.1M phosphate buffer	7.0			80	0.98	-0.01	0.98		Survivor curve	9.9	screw-capped vials	counts + LYS	TSA + LYS	28°C/6 days	Juneja *et al.*, 1995a
247	Whitefish	E	0.1M phosphate buffer	7.0			85	0.68	-0.17	2.26		Survivor curve	9.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
248	Saratoga	E	0.1M phosphate buffer	7.0			70	44.58	1.65	4.34		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
249	Saratoga	E	0.1M phosphate buffer	7.0			80	3.61	0.56	3.61		Survivor curve	9.9	screw-capped vials	counts	RCM	28°C/6 days	Juneja *et al.*, 1995a
250	Saratoga	E	0.1M phosphate buffer	7.0			80	4.51	0.65	4.51		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
251	Saratoga	E	0.1M phosphate buffer	7.0			80	3.77	0.58	3.77		Survivor curve	10.1	screw-capped vials	counts	TSA	28°C/6 days	Juneja *et al.*, 1995a
252	Saratoga	E	0.1M phosphate buffer	7.0			80	3.80	0.58	3.80		Survivor curve	9.8	screw-capped vials	counts + LYS	TSA + LYS	28°C/6 days	Juneja *et al.*, 1995a
253	Saratoga	E	0.1M phosphate buffer	7.0			85	1.03	0.01	3.30		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
254	Alaska	E	0.1M phosphate buffer	7.0			70	30.63	1.49	3.12		Survivor curve	10.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
255	Alaska	E	0.1M phosphate buffer	7.0			80	3.91	0.59	3.91		Survivor curve	10.0	screw-capped vials	counts	RCM	28°C/6 days	Juneja *et al.*, 1995a
256	Alaska	E	0.1M phosphate buffer	7.0			80	4.35	0.64	4.35		Survivor curve	10.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
257	Alaska	E	0.1M phosphate buffer	7.0			80	2.60	0.41	2.60		Survivor curve	10.7	screw-capped vials	counts	TSA	28°C/6 days	Juneja *et al.*, 1995a
258	Alaska	E	0.1M phosphate buffer	7.0			80	4.31	0.63	4.31		Survivor curve	10.8	screw-capped vials	counts + LYS	TSA + LYS	28°C/6 days	Juneja *et al.*, 1995a
259	Alaska	E	0.1M phosphate buffer	7.0			85	0.80	-0.10	2.51		Survivor curve	10.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
260	Alaska	E	turkey slurry				70	51.89	1.72	5.07		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
261	Alaska	E	turkey slurry				75	18.06	1.26	5.65		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
262	Alaska	E	turkey slurry				80	13.37	1.13	13.37		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
263	Alaska	E	turkey slurry				85	1.18	0.07	3.78		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
264	Kap B5	B	turkey slurry				75	32.53	1.51	9.60		Survivor curve	9.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
265	Kap B5	B	turkey slurry				80	15.21	1.18	15.21		Survivor curve	9.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
266	Kap B5	B	turkey slurry				85	4.85	0.69	16.44		Survivor curve	9.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
267	Kap B5	B	turkey slurry				90	0.80	-0.10	9.19		Survivor curve	9.4	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995a
268	Beluga	E	0.017M phosphate buf	7.0			70	26.10	1.42	0.93		TDT/Stumbo(1948)	6.9	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
269	Beluga	E	0.017M phosphate buf	7.0			73	11.60	1.06	1.05		TDT/Stumbo(1948)	6.9	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
270	Beluga	E	0.017M phosphate buf	7.0			75	5.00	0.70	0.94		TDT/Stumbo(1948)	6.9	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
271	Beluga	E	0.017M phosphate buf	7.0			80	1.00	0.00	1.00		TDT/Stumbo(1948)	6.9	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
272	Saratoga	E	0.017M phosphate buf	7.0			70	81.60	1.91	1.34		TDT/Stumbo(1948)	5.6	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
273	Saratoga	E	0.017M phosphate buf	7.0			73	26.20	1.42	1.36		TDT/Stumbo(1948)	5.6	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
274	Saratoga	E	0.017M phosphate buf	7.0			75	11.20	1.05	1.43		TDT/Stumbo(1948)	5.6	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
275	Saratoga	E	0.017M phosphate buf	7.0			80	1.10	0.04	1.10		TDT/Stumbo(1948)	5.6	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
276	Alaska	E	0.067M phosphate buf	7.0			70	37.50	1.57	1.21		TDT/Stumbo(1948)	6.7	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
277	Alaska	E	0.067M phosphate buf	7.0			73	11.80	1.07	0.99		TDT/Stumbo(1948)	6.7	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
278	Alaska	E	0.067M phosphate buf	7.0			75	5.30	0.72	0.95		TDT/Stumbo(1948)	6.7	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
279	Alaska	E	0.067M phosphate buf	7.0			77	2.70	0.43	0.87		TDT/Stumbo(1948)	6.7	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
280	Alaska	E	0.067M phosphate buf	7.0			80	1.60	0.20	1.60		TDT/Stumbo(1948)	6.7	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
281	Beluga	E	0.067M phosphate buf	7.0			70	29.30	1.47	1.53		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
282	Beluga	E	0.067M phosphate buf	7.0			73	8.00	0.90	0.96		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
283	Beluga	E	0.067M phosphate buf	7.0			75	5.10	0.71	1.17		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
284	Beluga	E	0.067M phosphate buf	7.0			77	4.60	0.66	1.74		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
285	Beluga	E	0.067M phosphate buf	7.0			80	1.40	0.15	1.40		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
286	Saratoga	E	0.067M phosphate buf	7.0			70	33.80	1.53	1.38		TDT/Stumbo(1948)	7.2	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
287	Saratoga	E	0.067M phosphate buf	7.0			73	13.10	1.12	1.31		TDT/Stumbo(1948)	7.2	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
288	Saratoga	E	0.067M phosphate buf	7.0			75	8.20	0.91	1.66		TDT/Stumbo(1948)	7.2	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
289	Saratoga	E	0.067M phosphate buf	7.0			77	4.10	0.61	1.43		TDT/Stumbo(1948)	7.2	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973

290	Saratoga	E	0.067M phosphate buf	7.0			80	1.40	0.15	1.40		TDT/Stumbo(1948)	7.2	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
291	1304E	E	0.067M phosphate buf	7.0			70	29.60	1.47	1.55		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
292	1304E	E	0.067M phosphate buf	7.0			73	10.60	1.03	1.27		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
293	1304E	E	0.067M phosphate buf	7.0			75	5.60	0.75	1.28		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
294	1304E	E	0.067M phosphate buf	7.0			77	3.70	0.57	1.53		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
295	1304E	E	0.067M phosphate buf	7.0			80	1.80	0.26	1.80		TDT/Stumbo(1948)	7.8	TDT tubes in wate	TDT	W & F	29.4°C/21 days	Bohrer *et al.*, 1973
296	Saratoga	E	tuna (in oil)				72	226.50	2.36	11.92		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	tuna (in oil)	29.4°C/168-336	Bohrer *et al.*, 1973
297	Saratoga	E	tuna (in oil)				77	40.90	1.61	13.18		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	tuna (in oil)	29.4°C/168-336	Bohrer *et al.*, 1973
298	Saratoga	E	tuna (in oil)				79	17.10	1.23	11.29		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	tuna (in oil)	29.4°C/168-336	Bohrer *et al.*, 1973
299	Saratoga	E	tuna (in oil)				80	10.50	1.02	10.50		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	tuna (in oil)	29.4°C/168-336	Bohrer *et al.*, 1973
300	Saratoga	E	tuna (in oil)				81	7.50	0.88	11.36		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	tuna (in oil)	29.4°C/168-336	Bohrer *et al.*, 1973
301	Saratoga	E	tuna (in oil)				82	6.60	0.82	15.14		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	tuna (in oil)	29.4°C/168-336	Bohrer *et al.*, 1973
302	Saratoga	E	crabmeat				80	4.40	0.64	4.40		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	crabmeat	29.4°C/168-336	Bohrer *et al.*, 1973
303	Saratoga	E	crabmeat				81	2.10	0.32	3.14		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	crabmeat	29.4°C/168-336	Bohrer *et al.*, 1973
304	Saratoga	E	crabmeat				82	1.90	0.28	4.25		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	crabmeat	29.4°C/168-336	Bohrer *et al.*, 1973
305	Saratoga	E	corn brine				77	11.20	1.05	3.61		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	corn brine	29.4°C/168-336	Bohrer *et al.*, 1973
306	Saratoga	E	corn brine				80	3.20	0.51	3.20		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	corn brine	29.4°C/168-336	Bohrer *et al.*, 1973
307	Saratoga	E	corn brine				81	1.70	0.23	2.57		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	corn brine	29.4°C/168-336	Bohrer *et al.*, 1973
308	Saratoga	E	corn brine				82	1.30	0.11	2.98		TDT/Stumbo(1948)	6.1	TDT cans in water	TDT	corn brine	29.4°C/168-336	Bohrer *et al.*, 1973
309	Saratoga	E	milk (evaporated)				77	6.40	0.81	2.14		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	milk (evaporated	29.4°C/168-336	Bohrer *et al.*, 1973
310	Saratoga	E	milk (evaporated)				80	1.90	0.28	1.90		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	milk (evaporated	29.4°C/168-336	Bohrer *et al.*, 1973
311	Saratoga	E	milk (evaporated)				81	1.10	0.04	1.64		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	milk (evaporated	29.4°C/168-336	Bohrer *et al.*, 1973
312	Saratoga	E	milk (evaporated)				82	0.80	-0.10	1.79		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	milk (evaporated	29.4°C/168-336	Bohrer *et al.*, 1973
313	Saratoga	E	sardines (in tomato sauce)				77	20.00	1.30	6.68		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	sardines (in tom	29.4°C/168-336	Bohrer *et al.*, 1973
314	Saratoga	E	sardines (in tomato sauce)				80	4.50	0.65	4.50		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	sardines (in tom	29.4°C/168-336	Bohrer *et al.*, 1973
315	Saratoga	E	sardines (in tomato sauce)				81	3.40	0.53	5.08		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	sardines (in tom	29.4°C/168-336	Bohrer *et al.*, 1973
316	Saratoga	E	sardines (in tomato sauce)				82	2.90	0.46	6.48		TDT/Stumbo(1948)	6.3	TDT cans in water	TDT	sardines (in tom	29.4°C/168-336	Bohrer *et al.*, 1973
317	Saratoga	E	shrimp				80	1.90	0.28	1.90		TDT/Stumbo(1948)		TDT cans in water	TDT	shrimp	29.4°C/168-336	Bohrer *et al.*, 1973
318	Saratoga	E	peas				77	18.60	1.27	3.87		TDT/Stumbo(1948)	4.4	TDT cans in water	TDT	peas	29.4°C/168-336	Bohrer *et al.*, 1973
319	Saratoga	E	peas				80	3.30	0.52	3.30		TDT/Stumbo(1948)	4.4	TDT cans in water	TDT	peas	29.4°C/168-336	Bohrer *et al.*, 1973
320	Saratoga	E	salmon				80	2.90	0.46	2.90		TDT/Stumbo(1948)		TDT cans in water	TDT	salmon	29.4°C/168-336	Bohrer *et al.*, 1973
321	ATCC 17786	E	tomato homogenates	4.1			70	6.50	0.81	0.23		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
322	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	4.61	0.66	0.16		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
323	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	20.00	1.30	0.71		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
324	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	38.81	1.59	1.38		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
325	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	16.80	1.23	0.60		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
326	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	17.50	1.24	0.62		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
327	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	11.90	1.08	0.42		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
328	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	43.80	1.64	1.56		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
329	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	43.00	1.63	1.53		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
330	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	11.80	1.07	0.42		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
331	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	20.20	1.31	0.72		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
332	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	22.60	1.35	0.80		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
333	ATCC 17786	E	tomato homogenates	4.1		Immedia	70	21.50	1.33	0.76		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
334	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	29.40	1.47	1.04		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
335	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	21.90	1.34	0.78		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
336	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	13.50	1.13	0.48		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
337	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	28.60	1.46	1.02		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993
338	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	18.24	1.26	0.65		Survivor curve		bottles in water ba	counts	EA+0.1% starcl	37°C/5 days	Appleyard and Gaze, 1993

339	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	15.95	1.20	0.57		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
340	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	32.80	1.52	1.17		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
341	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	13.60	1.13	0.48		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
342	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	12.04	1.08	0.43		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
343	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	11.70	1.07	0.42		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
344	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	13.12	1.12	0.47		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
345	ATCC 17786	E	tomato homogenates	4.1		4 h at ar	70	15.15	1.18	0.54		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
346	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	15.60	1.19	0.55		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
347	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	10.60	1.03	0.38		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
348	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	20.80	1.32	0.74		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
349	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	16.10	1.21	0.57		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
350	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	12.80	1.11	0.45		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
351	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	17.30	1.24	0.61		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
352	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	22.40	1.35	0.80		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
353	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	28.20	1.45	1.00		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
354	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	15.18	1.18	0.54		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
355	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	17.70	1.25	0.63		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
356	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	12.20	1.09	0.43		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
357	ATCC 17786	E	tomato homogenates	4.1		24 h hol	70	25.80	1.41	0.92		Survivor curve		bottles in water ba	counts	EA+0.1% starch	37°C/5 days	Appleyard and Gaze, 1993
358	Eklund 17B	B	0.067M phosphate buf	7.0			82	16.70	1.22	36.41		TDT/Stumbo(1950)	6.5	TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
359	Eklund 17B	B	0.067M phosphate buf	7.0			82	0.83	-0.08	1.81		TDT/Stumbo(1950)	6.5	TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
360	CBW25	B	0.067M phosphate buf	7.0			82	1.49	0.17	2.74		TDT/Stumbo(1950)	8.3	TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
361	2129B	B	0.067M phosphate buf	7.0			82	32.30	1.51	54.45		TDT/Stumbo(1950)	9.7	TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
362	ATCC 17844	B	0.067M phosphate buf	7.0			82	4.17	0.62	5.67		TDT/Stumbo(1950)	16.5	TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
363	Minnesota	E	0.067M phosphate buf	7.0			82	0.52	-0.28	1.08		TDT/Stumbo(1950)		TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
364	Whitefish	E	0.067M phosphate buf	7.0			82	0.40	-0.40	0.83		TDT/Stumbo(1950)		TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
365	Saratoga	E	0.067M phosphate buf	7.0			82	0.33	-0.48	0.59		TDT/Stumbo(1950)	8.7	TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
366	Saratoga	E	0.067M phosphate buf	7.0			77	1.44	0.16	2.58		TDT/Stumbo(1950)	8.7	TDT tubes in water	TDT	APPA	30°C/168 days	Scott and Bernard, 1982
367	CBW25	B	0.067M phosphate buf	7.0			85	0.65	-0.19	3.45		TDT/Stumbo(1950)		TDT tubes in water	TDT	APPA		Scott and Bernard, 1985
368	CBW25	B	0.067M phosphate buf	7.0			85	30.00	1.48	82.36		TDT/Stumbo(1950)		TDT tubes in water	TDT + LYS	APPA + LYS		Scott and Bernard, 1985
369	2129B	B	0.067M phosphate buf	7.0			85	15.00	1.18	79.57		TDT/Stumbo(1950)		TDT tubes in water	TDT	APPA		Scott and Bernard, 1985
370	2129B	B	0.067M phosphate buf	7.0			85	25.00	1.40	68.63		TDT/Stumbo(1950)		TDT tubes in water	TDT + LYS	APPA + LYS		Scott and Bernard, 1985
371	ATCC 17844	B	0.067M phosphate buf	7.0			85	3.00	0.48	15.91		TDT/Stumbo(1950)		TDT tubes in water	TDT	APPA		Scott and Bernard, 1985
372	ATCC 17844	B	0.067M phosphate buf	7.0			85	100.00	2.00	274.53		TDT/Stumbo(1950)		TDT tubes in water	TDT + LYS	APPA + LYS		Scott and Bernard, 1985
373	Eklund 17B	B	0.067M phosphate buf	7.0			85	0.25	-0.60	1.33		TDT/Stumbo(1950)		TDT tubes in water	TDT	APPA		Scott and Bernard, 1985
374	Eklund 17B	B	0.067M phosphate buf	7.0			85	85.00	1.93	233.35		TDT/Stumbo(1950)		TDT tubes in water	TDT + LYS	APPA + LYS		Scott and Bernard, 1985
375	Saratoga	E	0.067M phosphate buf	7.0			85	0.15	-0.82	0.80		TDT/Stumbo(1950)		TDT tubes in water	TDT	APPA		Scott and Bernard, 1985
376	Saratoga	E	0.067M phosphate buf	7.0			85	15.00	1.18	41.18		TDT/Stumbo(1950)		TDT tubes in water	TDT + LYS	APPA + LYS		Scott and Bernard, 1985
377	1304E	E	0.067M phosphate buf	7.0			79	1.30	0.11	1.08		Survivor curve			counts	T-Best agar	30°C/21 days	Alderton *et al.*, 1974
378	1304E	E	0.067M phosphate buf	7.0			91	13.50	1.13	1079.04	HR	Survivor curve	5.6		counts + LYS	T-Best agar + L	30°C/14 days	Alderton *et al.*, 1974
379	1304E	E	0.067M phosphate buf	7.0			93	3.80	0.58	958.49	HR	Survivor curve	5.6		counts + LYS	T-Best agar + L	30°C/14 days	Alderton *et al.*, 1974
380	1304E	E	distilled water				79	0.60	-0.22	0.49		Survivor curve			counts	T-Best agar	30°C/21 days	Alderton *et al.*, 1974
381	1304E	E	distilled water				91	5.00	0.70	62.95	HR	Survivor curve			counts + LYS	T-Best agar + L	30°C/14 days	Alderton *et al.*, 1974
382	Mixed strains: B (Col		PY broth	6.8			90	0.58	-0.24	12.50	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/28 days	Stringer *et al.*, 1997
383	Mixed strains: B (Col		PY broth	6.8			90	27.25	1.44	299.94	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/28 days	Stringer *et al.*, 1997
384	Eklund 17B	B	PY broth	6.8			75	30.00	1.48	5.66		Survivor curve		screw-cap tube in	counts	PYGS	10°C/28 days	IFR unpublished data
385	Eklund 17B	B	PY broth	6.8			90	0.69	-0.16	14.87	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/28 days	Stringer and Peck 1997
386	Eklund 17B	B	PY broth	6.8			90	8.92	0.95	98.18	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/28 days	Stringer and Peck 1997
387	Eklund 17B	B	PY broth	6.8			90	0.78	-0.11	16.80	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/7 days	Stringer and Peck 1997

388	Eklund 17B	B	PY broth	6.8			90	27.70	1.44	304.89	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/7 days	Stringer and Peck 1997
389	Eklund 17B	B	PY broth	6.8	3.0 % NaCl		75	28.39	1.45	5.35		Survivor curve		screw-cap tube in	counts	PYGS	10°C/147 days t	Stringer and Peck 1997
390	Eklund 17B	B	PY broth	6.8	4.0 % NaCl		75	13.00	1.11	2.45		Survivor curve		screw-cap tube in	counts	PYGS	10°C/147 days t	Stringer and Peck 1997
391	Eklund 17B	B	PY broth	6.8	1.5 % NaCl		90	0.81	-0.09	304.89	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/147 days t	Stringer and Peck 1997
392	Eklund 17B	B	PY broth	6.8	1.5 % NaCl		90	8.30	0.92	17.45	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/147 days t	Stringer and Peck 1997
393	Eklund 17B	B	PY broth	6.8	3.0 % NaCl		90	1.39	0.14	91.36	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/147 days t	Stringer and Peck 1997
394	Eklund 17B	B	PY broth	6.8	3.0 % NaCl		90	13.35	1.13	29.95	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/147 days t	Stringer and Peck 1997
395	Eklund 17B	B	PY broth	6.8	4.0 % NaCl		90	1.08	0.03	146.94	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/147 days t	Stringer and Peck 1997
396	Eklund 17B	B	PY broth	6.8	4.0 % NaCl		90	35.59	1.55	23.27	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/147 days t	Stringer and Peck 1997
397	Eklund 17B	B	0.067M phosphate buf	7.0			85	0.09	-1.05	391.74	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C	Peck *et al.,* 1992a
398	Eklund 17B	B	0.067M phosphate buf	7.0			85	9.31	0.97	30.89	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C	Peck *et al.,* 1992a
399	Eklund 17B	B	0.067M phosphate buf	7.0			85	0.18	-0.74	0.95		Survivor curve		screw-cap tube in	counts	PYGS	30°C	Peck *et al.,* 1992a
400	Eklund 17B	B	0.067M phosphate buf	7.0	2 M sodium thioglycola		85	84.92	1.93	281.74	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	Peck *et al.,* 1992b
401	Eklund 17B	B	0.067M phosphate buf	7.0			85	3.85	0.59	17.87	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	Peck *et al.,* 1992b
402	Eklund 17B	B	0.067M phosphate buf	7.0			85	70.42	1.85	233.63	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	Peck *et al.,* 1992b
403	Beluga	E	0.067M phosphate buf	7.0	2 M sodium thioglycola		85	41.15	1.61	136.52	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	Peck *et al.,* 1992b
404	Beluga	E	0.067M phosphate buf	7.0			85	11.48	1.06	53.29	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	Peck *et al.,* 1992b
405	Beluga	E	0.067M phosphate buf	7.0			85	50.76	1.71	168.40	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	Peck *et al.,* 1992b
406	Alaska	E	whitefish chubs				80	4.30	0.63	4.30		TDT	7.3	TDT tubes in wate	TDT	NVB	30°/7 days & 25	Crisley, *et al.,* 1968
407	Beluga	E	whitefish chubs				80	2.10	0.32	2.10		TDT	7.3	TDT tubes in wate	TDT	NVB	30°/7 days & 25	Crisley, *et al.,* 1968
408	8E	E	whitefish chubs				80	1.80	0.26	1.80		TDT	5.7	TDT tubes in wate	TDT	NVB	30°/7 days & 25	Crisley, *et al.,* 1968
409	Iwanai	E	whitefish chubs				80	1.60	0.20	1.60		TDT	7.6	TDT tubes in wate	TDT	NVB	30°/7 days & 25	Crisley, *et al.,* 1968
410	Tenno	E	whitefish chubs				80	1.60	0.20	1.60		TDT	7.3	TDT tubes in wate	TDT	NVB	30°/7 days & 25	Crisley, *et al.,* 1968
411	Minneapolis	E	TPB broth				80	1.80	0.26	1.80		TDT	7.8	TDT tubes in wate	TDT			Schmidt, 1964
412	Minneapolis	E	0.067M phosphate buf	7.0			80	2.30	0.36	2.30		TDT	8.3	TDT tubes in wate	TDT			Schmidt, 1964
413	Tenno	E	0.067M phosphate buf	7.0			80	0.80	-0.10	0.80			5.3					Crisley, *et al.,* 1968
414	Minnesota	E	autoclaved chub fish				83	2.50	0.40	5.76		TDT		TDT tubes in wate	TDT	TPGY	25°C/7 days	Alderman *et al.,* 1972
415	Alaska	E	autoclaved chub fish				83	3.50	0.54	8.06		TDT		TDT tubes in wate	TDT	TPGY	25°C/7 days	Alderman *et al.,* 1972
416	Alaska	E	raw egg white				83	14.16	1.15	32.61		TDT		TDT tubes in wate	TDT	TPGY	25°C/7 days	Alderman *et al.,* 1972
417	Alaska	E	pre-coagulated egg white				83	1.16	0.06	2.67		TDT		TDT tubes in wate	TDT	TPGY	25°C/7 days	Alderman *et al.,* 1972
418	8E	E	0.067M phosphate buf	7.0			66	70.00	1.85	0.13		Survivor curve	5.1	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
419	8E	E	0.067M phosphate buf	7.0			71	5.20	0.72	0.09		Survivor curve	5.1	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
420	8E	E	0.067M phosphate buf	7.0			77	0.50	-0.30	0.13		Survivor curve	5.1	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
421	8E	E	clam liquor				66	550.00	2.74	1.12		Survivor curve	5.2	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
422	8E	E	clam liquor				71	40.00	1.60	0.74		Survivor curve	5.2	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
423	8E	E	clam liquor				77	2.90	0.46	0.77		Survivor curve	5.2	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
424	8E	E	clam liquor				82	0.30	-0.52	0.73		Survivor curve	5.2	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
425	8E	E	haddock slurry				66	500.00	2.70	0.79		Survivor curve	5.0	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
426	8E	E	haddock slurry				71	32.00	1.51	0.51		Survivor curve	5.0	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
427	8E	E	haddock slurry				77	3.00	0.48	0.75		Survivor curve	5.0	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
428	8E	E	haddock slurry				82	0.20	-0.70	0.50		Survivor curve	5.0	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
429	Detroit	E	clam liquor				66	700.00	2.85	0.63		Survivor curve	4.6	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
430	Detroit	E	clam liquor				77	2.40	0.38	0.53		Survivor curve	4.6	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
431	Detroit	E	clam liquor				82	0.20	-0.70	0.54		Survivor curve	4.6	sealed capillaries i	counts	MAM	30°C/2 days	Licciardello, 1983
432	Detroit	E	clam liquor				77	36.76	1.57	17.63		TDT	9.4	sealed capillaries i	TDT	TPG broth	30°C/14 days	Licciardello, 1983
433	Detroit	E	clam liquor				82	7.95	0.90	12.98		TDT	9.4	sealed capillaries i	TDT	TPG broth	30°C/14 days	Licciardello, 1983
434	Detroit	E	clam liquor				88	1.71	0.23	12.14		TDT	9.4	sealed capillaries i	TDT	TPG broth	30°C/14 days	Licciardello, 1983
435	Detroit	E	clam liquor				93	0.69	-0.16	16.67		TDT	9.4	sealed capillaries i	TDT	TPG broth	30°C/14 days	Licciardello, 1983
436	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	75	42.10	1.62	13.44		Survivor curve	10.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995

437	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	80	17.10	1.23	17.10		Survivor curve	10.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
438	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	85	7.80	0.89	24.44		Survivor curve	10.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
439	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	90	1.10	0.04	10.80		Survivor curve	10.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
440	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	75	25.70	1.41	6.97		Survivor curve	8.8	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
441	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	80	15.10	1.18	15.10		Survivor curve	8.8	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
442	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	85	5.50	0.74	20.29		Survivor curve	8.8	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
443	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	90	0.60	-0.22	8.16		Survivor curve	8.8	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
444	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	75	17.70	1.25	4.55		Survivor curve	8.5	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
445	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	80	13.10	1.12	13.10		Survivor curve	8.5	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
446	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	85	3.20	0.51	12.46		Survivor curve	8.5	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
447	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	90	0.50	-0.30	7.58		Survivor curve	8.5	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja and Eblen, 1995
448	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	75	27.40	1.44	8.52		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS + 1	28°C/6 days	Juneja and Eblen, 1995
449	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	80	13.20	1.12	13.20		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS + 1	28°C/6 days	Juneja and Eblen, 1995
450	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	85	5.00	0.70	16.07		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS + 1	28°C/6 days	Juneja and Eblen, 1995
451	Kap B5	B	turkey slurry		1% NaCl	60˚C/10	90	0.80	-0.10	8.27		Survivor curve	9.9	screw-capped vials	counts + LYS	RCM + LYS + 1	28°C/6 days	Juneja and Eblen, 1995
452	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	75	19.90	1.30	5.70		Survivor curve	9.2	screw-capped vials	counts + LYS	RCM + LYS + 2	28°C/6 days	Juneja and Eblen, 1995
453	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	80	12.60	1.10	12.60		Survivor curve	9.2	screw-capped vials	counts + LYS	RCM + LYS + 2	28°C/6 days	Juneja and Eblen, 1995
454	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	85	4.30	0.63	15.01		Survivor curve	9.2	screw-capped vials	counts + LYS	RCM + LYS + 2	28°C/6 days	Juneja and Eblen, 1995
455	Kap B5	B	turkey slurry		2% NaCl	60˚C/10	90	0.40	-0.40	4.87		Survivor curve	9.2	screw-capped vials	counts + LYS	RCM + LYS + 2	28°C/6 days	Juneja and Eblen, 1995
456	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	75	16.90	1.23	5.04		Survivor curve	9.5	screw-capped vials	counts + LYS	RCM + LYS + 3	28°C/6 days	Juneja and Eblen, 1995
457	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	80	8.20	0.91	8.20		Survivor curve	9.5	screw-capped vials	counts + LYS	RCM + LYS + 3	28°C/6 days	Juneja and Eblen, 1995
458	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	85	2.60	0.41	8.72		Survivor curve	9.5	screw-capped vials	counts + LYS	RCM + LYS + 3	28°C/6 days	Juneja and Eblen, 1995
459	Kap B5	B	turkey slurry		3% NaCl	60˚C/10	90	0.30	-0.52	3.38		Survivor curve	9.5	screw-capped vials	counts + LYS	RCM + LYS + 3	28°C/6 days	Juneja and Eblen, 1995
460	Mixed strains	B,E	turkey slurry	5.0			70	45.10	1.65	14.34		Survivor curve	20.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
461	Mixed strains	B,E	turkey slurry	5.0	1.5% NaCl + 0.15% So		70	33.80	1.53	9.80		Survivor curve	18.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
462	Mixed strains	B,E	turkey slurry	6.0			70	51.30	1.71	6.81		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
463	Mixed strains	B,E	turkey slurry	6.0	0.3% Sodium phyropho		70	33.90	1.53	4.50		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
464	Mixed strains	B,E	turkey slurry	6.0	1.5% NaCl + 0.15% So		70	38.60	1.59	12.96		Survivor curve	21.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
465	Mixed strains	B,E	turkey slurry	6.0	3.0% NaCl		70	31.10	1.49	4.13		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
466	Mixed strains	B,E	turkey slurry	6.5			70	57.70	1.76	7.66		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
467	Mixed strains	B,E	turkey slurry	6.5	1.5% NaCl + 0.15% So		70	40.10	1.60	5.32		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
468	Mixed strains	B,E	turkey slurry	7.0	1.5% NaCl + 0.15% So		70	44.10	1.64	5.85		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
469	Mixed strains	B,E	turkey slurry	5.0	1.5% NaCl + 0.15% So		75	28.90	1.46	15.56		Survivor curve	18.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
470	Mixed strains	B,E	turkey slurry	5.5	1% NaCl + 0.10% Sod		75	36.10	1.56	16.32		Survivor curve	14.5	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
471	Mixed strains	B,E	turkey slurry	5.5	1% NaCl + 0.20% Sod		75	28.50	1.45	13.82		Survivor curve	15.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
472	Mixed strains	B,E	turkey slurry	5.5	2% NaCl + 0.1% Sodiu		75	28.70	1.46	10.45		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
473	Mixed strains	B,E	turkey slurry	5.5	2% NaCl + 0.2% Sodiu		75	20.90	1.32	7.61		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
474	Mixed strains	B,E	turkey slurry	6.0	1.5% NaCl + 0.15% So		75	36.40	1.56	21.09		Survivor curve	21.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
475	Mixed strains	B,E	turkey slurry	6.25	1% NaCl + 0.10% Sod		75	39.10	1.59	22.95		Survivor curve	21.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
476	Mixed strains	B,E	turkey slurry	6.25	1% NaCl + 0.20% Sod		75	32.90	1.52	19.77		Survivor curve	22.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
477	Mixed strains	B,E	turkey slurry	6.25	2% NaCl + 0.1% Sodiu		75	38.70	1.59	14.10		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
478	Mixed strains	B,E	turkey slurry	6.25	2% NaCl + 0.2% Sodiu		75	30.40	1.48	11.07		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
479	Mixed strains	B,E	turkey slurry	5.0	1.5% NaCl + 0.15% So		80	13.50	1.13	13.50		Survivor curve	18.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
480	Mixed strains	B,E	turkey slurry	6.0	0.15% Sodium phyropl		80	32.10	1.51	32.10		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
481	Mixed strains	B,E	turkey slurry	6.0	1.5% NaCl		80	26.20	1.42	26.20		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
482	Mixed strains	B,E	turkey slurry	6.0	1.5% NaCl + 0.15% So		80	23.00	1.36	23.00		Survivor curve	21.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
483	Mixed strains	B,E	turkey slurry	6.0	1.5% NaCl + 0.3% Sod		80	21.00	1.32	21.00		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
484	Mixed strains	B,E	turkey slurry	6.0	3% NaCl + 0.15% Sod		80	25.10	1.40	25.10		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
485	Mixed strains	B,E	turkey slurry	6.5	1.5% NaCl + 0.15% So		80	28.20	1.45	28.20		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b

486	Mixed strains	B,E	turkey slurry	5.0	1.5% NaCl + 0.15% S	85	7.00	0.85	13.00		Survivor curve	18.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
487	Mixed strains	B,E	turkey slurry	5.5	1% NaCl + 0.10% Sod	85	7.40	0.87	16.37		Survivor curve	14.5	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
488	Mixed strains	B,E	turkey slurry	5.5	1% NaCl + 0.20% Pho	85	6.70	0.83	13.82		Survivor curve	15.9	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
489	Mixed strains	B,E	turkey slurry	5.5	2% NaCl + 0.1% Sodiu	85	7.40	0.87	20.32		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
490	Mixed strains	B,E	turkey slurry	5.5	2% NaCl + 0.2% Sodiu	85	4.00	0.60	10.98		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
491	Mixed strains	B,E	turkey slurry	6.25	1% NaCl + 0.10% Sod	85	13.50	1.13	23.00		Survivor curve	21.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
492	Mixed strains	B,E	turkey slurry	6.25	1% NaCl + 0.20% Sod	85	11.90	1.08	19.81		Survivor curve	22.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
493	Mixed strains	B,E	turkey slurry	6.25	2% NaCl + 0.2% Sodiu	85	9.70	0.99	26.63		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
494	Mixed strains	B,E	turkey slurry	5.0		90	5.00	0.70	15.72		Survivor curve	20.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
495	Mixed strains	B,E	turkey slurry	5.0	1.5% NaCl + 0.15% S	90	3.10	0.49	10.69		Survivor curve	18.6	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
496	Mixed strains	B,E	turkey slurry	6.0		90	8.80	0.94	66.32		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
497	Mixed strains	B,E	turkey slurry	6.0	0.3% Sodium phyroph	90	5.40	0.73	40.70		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
498	Mixed strains	B,E	turkey slurry	6.0	1.5% NaCl + 0.15% S	90	4.80	0.68	14.29		Survivor curve	21.1	screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
499	Mixed strains	B,E	turkey slurry	6.0	3% NaCl	90	5.60	0.75	42.21		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
500	Mixed strains	B,E	turkey slurry	6.25	2% NaCl + 0.2% Sodiu	90	3.50	0.54	26.38		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
501	Mixed strains	B,E	turkey slurry	6.50		90	9.30	0.97	70.09		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
502	Mixed strains	B,E	turkey slurry	6.50	1.5% NaCl + 0.15% S	90	7.30	0.86	55.02		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
503	Mixed strains	B,E	turkey slurry	6.5		70	53.30	1.73	7.07		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
504	Mixed strains	B,E	turkey slurry	5.0		70	49.50	1.69	6.57		Survivor curve		screw-capped vials	counts + LYS	RCM + LYS	28°C/6 days	Juneja *et al.*, 1995b
505	Vancouver He	E	0.067M phosphate buf	7.0		75	9.00	0.95	1.70		Survivor curve		screw-cap tube in	counts	ESBA		Grecz and Tang, 1970
506	Saratoga	E	0.017M phosphate buf	7.0		70	98.00	1.99	1.17		TDT	5.2	TDT tubes in wate	TDT	W & F		NCA, 1966
507	Saratoga	E	0.017M phosphate buf	7.0		75	13.89	1.14	1.52		TDT	5.2	TDT tubes in wate	TDT	W & F		NCA, 1966
508	Saratoga	E	0.017M phosphate buf	7.0		80	1.21	0.08	1.21		TDT	5.2	TDT tubes in wate	TDT	W & F		NCA, 1966
509	Beluga	E	0.017M phosphate buf	7.0		75	6.39	0.81	0.94		TDT	6.0	TDT tubes in wate	TDT	W & F		NCA, 1966
510	Beluga	E	0.017M phosphate buf	7.0		80	0.93	-0.03	0.93		TDT	6.0	TDT tubes in wate	TDT	W & F		NCA, 1966
511	Eklund 17B	B	PY broth	6.8		85	0.49	-0.31	2.60		Survivor curve		screw-cap tube in	counts	PYGS	30°C/8 days	Stringer and Peck, 1996
512	Eklund 17B	B	PY broth	6.8		85	0.09	-1.05	0.42	HS	Survivor curve		screw-cap tube in	counts + courgett	PYGS + courge	30°C/8 days	Stringer and Peck, 1996
513	Eklund 17B	B	PY broth	6.8		85	0.38	-0.42	1.76	HS	Survivor curve		screw-cap tube in	counts + carrot	PYGS + carrot	30°C/8 days	Stringer and Peck, 1996
514	Eklund 17B	B	PY broth	6.8		85	0.39	-0.41	1.81	HS	Survivor curve		screw-cap tube in	counts + mung be	PYGS + mung b	30°C/8 days	Stringer and Peck, 1996
515	Eklund 17B	B	PY broth	6.8		85	0.34	-0.47	1.58	HS	Survivor curve		screw-cap tube in	counts + HEWL	PYGS + potato	30°C/8 days	Stringer and Peck, 1996
516	Eklund 17B	B	PY broth	6.8		85	23.81	1.38	78.99	HR	Survivor curve		screw-cap tube in	counts + HEWL	PYGS + potato	30°C/8 days	Stringer and Peck, 1996
517	Eklund 17B	B	PY broth	6.8		85	0.31	-0.51	1.44	HS	Survivor curve		screw-cap tube in	counts + turnip ju	PYGS + turnip j	30°C/8 days	Stringer and Peck, 1996
518	Eklund 17B	B	PY broth	6.8		85	15.63	1.19	51.86	HR	Survivor curve		screw-cap tube in	counts + turnip ju	PYGS + turnip j	30°C/8 days	Stringer and Peck, 1996
519	Eklund 17B	B	PY broth	6.8		85	0.21	-0.68	0.97	HS	Survivor curve		screw-cap tube in	counts + swede j	PYGS + swede	30°C/8 days	Stringer and Peck, 1996
520	Eklund 17B	B	PY broth	6.8		85	12.58	1.10	41.74	HR	Survivor curve		screw-cap tube in	counts + swede j	PYGS + swede	30°C/8 days	Stringer and Peck, 1996
521	Eklund 17B	B	PY broth	6.8		85	0.19	-0.72	0.88	HS	Survivor curve		screw-cap tube in	counts + swede j	PYGS + swede	30°C/8 days	Stringer and Peck, 1996
522	Eklund 17B	B	PY broth	6.8		85	11.23	1.05	37.26	HR	Survivor curve		screw-cap tube in	counts + swede j	PYGS + swede	30°C/8 days	Stringer and Peck, 1996
523	Eklund 17B	B	PY broth	6.8		85	0.20	-0.70	0.93	HS	Survivor curve		screw-cap tube in	counts + flat bear	PYGS + flat bea	30°C/8 days	Stringer and Peck, 1996
524	Eklund 17B	B	PY broth	6.8		85	10.06	1.00	33.38	HR	Survivor curve		screw-cap tube in	counts + flat bear	PYGS + flat bea	30°C/8 days	Stringer and Peck, 1996
525	Eklund 17B	B	PY broth	6.8		85	0.19	-0.72	0.88	HS	Survivor curve		screw-cap tube in	counts + red cabl	PYGS + red cal	30°C/8 days	Stringer and Peck, 1996
526	Eklund 17B	B	PY broth	6.8		85	7.59	0.88	25.18	HR	Survivor curve		screw-cap tube in	counts + red cabl	PYGS + red cal	30°C/8 days	Stringer and Peck, 1996
527	Eklund 17B	B	PY broth	6.8		85	0.31	-0.51	1.44	HS	Survivor curve		screw-cap tube in	counts + HEWL	PYGS + 10 µg H	30°C/8 days	Stringer and Peck, 1996
528	Eklund 17B	B	PY broth	6.8		85	34.48	1.54	114.39	HR	Survivor curve		screw-cap tube in	counts + HEWL	PYGS + 10 µg H	30°C/8 days	Stringer and Peck, 1996
529	Eklund 17B	B	PY broth	6.8		85	0.32	-0.49	1.49	HS	Survivor curve		screw-cap tube in	counts + HEWL	PYGS + 100 µg	30°C/7 days	IFR unpublished data
530	Eklund 17B	B	PY broth	6.8		85	34.48	1.54	114.39	HR	Survivor curve		screw-cap tube in	counts + HEWL	PYGS + 100 µg	30°C/7 days	IFR unpublished data
531	Eklund 17B	B	PY broth	6.8		85	0.35	-0.46	1.62	HS	Survivor curve		screw-cap tube in	counts + HEWL	PYGS + 1µg HB	30°C/7 days	IFR unpublished data
532	ATCC 9564	E	0.05M phosphate buffe	7.0	1.00	70	10.00	1.00	0.36		Survivor curve		pyrex test tube	counts			Murrell and Scott, 1966
533	ATCC 9564	E			0.90	80	25.84	1.41	25.84		Survivor curve		pyrex test tube	counts			Murrell and Scott, 1966
534	ATCC 9564	E	0.05M phosphate buffe	7.0	1.00	80	0.32	-0.50	0.32		Survivor curve		pyrex test tube	counts			Murrell and Scott, 1966

535	Alaska	E	crabmeat				74	10.05	1.00	1.01		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
536	Alaska	E	crabmeat				77	2.82	0.45	0.81		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
537	Alaska	E	crabmeat				79	1.49	0.17	1.19		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
538	Alaska	E	crabmeat				82	0.71	-0.15	1.63		TDT/Schmidt(1957)	6.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
539	Beluga	E	crabmeat				74	11.22	1.05	1.29		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
540	Beluga	E	crabmeat				77	2.94	0.47	0.91		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
541	Beluga	E	crabmeat				79	1.19	0.08	0.96		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
542	Beluga	E	crabmeat				82	0.84	-0.08	1.83		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
543	Beluga	E	crabmeat				85	0.33	-0.48	1.94		TDT/Schmidt(1957)	6.5	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
544	Crab G21-5	E	crabmeat				74	6.15	0.79	0.85		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
545	Crab G21-5	E	crabmeat				77	1.70	0.23	0.58		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
546	Crab G21-5	E	crabmeat				79	1.09	0.04	0.90		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
547	Crab G21-5	E	crabmeat				82	0.51	-0.29	1.04		TDT/Schmidt(1957)	7.1	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
548	Crab G21-5	E	crabmeat				71	31.88	1.50	0.72		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT	TPGY	26°C/70 days	Lynt *et al.*, 1983
549	190	F	0.067M phosphate buf	7.0			74	9.07	0.96	0.67		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
550	190	F	0.067M phosphate buf	7.0			77	1.66	0.22	0.41		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
551	190	F	0.067M phosphate buf	7.0			79	1.03	0.01	0.80		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
552	190	F	0.067M phosphate buf	7.0			82	0.25	-0.60	0.64		TDT/Schmidt(1957)	5.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
553	202	F	0.067M phosphate buf	7.0			71	42.41	1.63	0.89		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
554	202	F	0.067M phosphate buf	7.0			74	12.68	1.10	0.90		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
555	202	F	0.067M phosphate buf	7.0			77	4.29	0.63	1.02		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
556	202	F	0.067M phosphate buf	7.0			79	0.93	-0.03	0.72		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
557	202	F	0.067M phosphate buf	7.0			82	0.33	-0.48	0.86		TDT/Schmidt(1957)	5.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
558	Craig 610	F	0.067M phosphate buf	7.0			77	6.64	0.82	1.99		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
559	Craig 610	F	0.067M phosphate buf	7.0			79	2.12	0.33	1.70		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
560	Craig 610	F	0.067M phosphate buf	7.0			82	0.84	-0.08	1.88		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
561	Craig 610	F	0.067M phosphate buf	7.0			85	0.37	-0.43	2.30		TDT/Schmidt(1957)	6.3	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
562	202	F	crabmeat				77	9.50	0.98	2.90		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
563	202	F	crabmeat				79	3.64	0.56	2.93		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
564	202	F	crabmeat				82	1.20	0.08	2.65		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
565	202	F	crabmeat				85	0.53	-0.28	3.20		TDT/Schmidt(1957)	6.4	TDT tubes in wate	TDT	TPGY	26°C/182 days	Lynt *et al.*, 1983
566	Mixed strains	B	PY broth	6.8			90	0.51	-0.29	10.99	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/7 days	IFR unpublished data
567	Mixed strains	B	PY broth	6.8			90	23.20	1.37	255.36	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/7 days	IFR unpublished data
568	Mixed strains	B	PY broth	6.8			90	0.58	-0.24	12.50	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/7 days	IFR unpublished data
569	Mixed strains	B	PY broth	6.8			90	31.55	1.50	347.27	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/7 days	IFR unpublished data
570	Eklund 17B	B	0.067M phosphate buf	7.0			75	14.81	1.17	1.35		Survivor curve	4.8	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
571	Eklund 17B	B	broccoli puree	5.8			75	10.70	1.03	1.42		Survivor curve	5.7	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
572	Eklund 17B	B	broccoli puree	5.8			75	6.60	0.82	0.48		Survivor curve	4.4	screw-cap tube in	counts	Broccoli agar	30°C/5 days	IFR unpublished data
573	Eklund 17B	B	potato puree	5.9			75	10.07	1.00	0.96		Survivor curve	4.9	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
574	Eklund 17B	B	potato puree	5.9			75	4.34	0.64	0.54		Survivor curve	5.5	screw-cap tube in	counts	Potato agar	30°C/5 days	IFR unpublished data
575	Eklund 17B	B	0.067M phosphate buf	7.0			78	3.03	0.48	1.16		Survivor curve	4.8	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
576	Eklund 17B	B	broccoli puree	5.8			78	3.17	0.50	1.41		Survivor curve	5.7	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
577	Eklund 17B	B	broccoli puree	5.8			78	1.16	0.06	0.41		Survivor curve	4.4	screw-cap tube in	counts	Broccoli agar	30°C/5 days	IFR unpublished data
578	Eklund 17B	B	potato puree	5.9			78	3.17	0.50	1.24		Survivor curve	4.9	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
579	Eklund 17B	B	potato puree	5.9			78	1.07	0.03	0.46		Survivor curve	5.5	screw-cap tube in	counts	Potato agar	30°C/5 days	IFR unpublished data
580	Eklund 17B	B	0.067M phosphate buf	7.0			80	1.26	0.10	1.26		Survivor curve	4.8	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
581	Eklund 17B	B	broccoli puree	5.8			80	1.46	0.16	1.46		Survivor curve	5.7	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
582	Eklund 17B	B	broccoli puree	5.8			80	0.50	-0.30	0.50		Survivor curve	4.4	screw-cap tube in	counts	Broccoli agar	30°C/5 days	IFR unpublished data
583	Eklund 17B	B	potato puree	5.9			80	0.94	-0.03	0.94		Survivor curve	4.9	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data

584	Eklund 17B	B	potato puree	5.9			80	0.47	-0.33	0.47		Survivor curve	5.5	screw-cap tube in	counts	Potato agar	30°C/5 days	IFR unpublished data
585	Kap B2	B	0.067M phosphate buf	7.0			78	2.23	0.35	0.74	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
586	Kap B2	B	0.067M phosphate buf	7.0			78	70.42	1.85	40.30	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
587	Kap B2	B	broccoli puree	5.8			78	3.18	0.50	1.06	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
588	Kap B2	B	broccoli puree	5.8			78	47.17	1.67	33.10	HR	Survivor curve	13.0	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
589	Kap B2	B	broccoli puree	5.8			78	0.93	-0.03	0.34		Survivor curve	4.6	screw-cap tube in	counts	Broccoli agar	30°C/5 days	IFR unpublished data
590	Kap B2	B	potato puree	5.9			78	3.41	0.53	1.24	HS	Survivor curve	4.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
591	Kap B2	B	potato puree	5.9			78	48.78	1.69	30.01	HR	Survivor curve	9.5	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
592	Kap B2	B	potato puree	5.9			78	0.56	-0.25	0.29		Survivor curve	7.1	screw-cap tube in	counts	Potato agar	30°C/5 days	IFR unpublished data
593	Kap B2	B	0.067M phosphate buf	7.0			75	19.23	1.28	1.22	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
594	Kap B2	B	0.067M phosphate buf	7.0			75	192.30	2.28	47.63	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
595	Kap B2	B	broccoli puree	5.8			75	18.73	1.27	1.21	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
596	Kap B2	B	broccoli puree	5.8			75	77.52	1.89	31.97	HR	Survivor curve	13.0	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
597	Kap B2	B	broccoli puree	5.8			75	6.95	0.84	0.57		Survivor curve	4.6	screw-cap tube in	counts	Broccoli agar	30°C/5 days	IFR unpublished data
598	Kap B2	B	potato puree	5.9			75	18.01	1.26	1.45	HS	Survivor curve	4.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
599	Kap B2	B	potato puree	5.9			75	78.12	1.89	23.19	HR	Survivor curve	9.5	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
600	Kap B2	B	potato puree	5.9			75	2.28	0.36	0.45		Survivor curve	7.1	screw-cap tube in	counts	Potato agar	30°C/5 days	IFR unpublished data
601	Kap B2	B	0.067M phosphate buf	7.0			80	1.09	0.04	1.09	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
602	Kap B2	B	0.067M phosphate buf	7.0			80	36.63	1.56	36.63	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
603	Kap B2	B	broccoli puree	5.8			80	1.11	0.05	1.11	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
604	Kap B2	B	broccoli puree	5.8			80	30.67	1.49	30.67	HR	Survivor curve	13.0	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
605	Kap B2	B	broccoli puree	5.8			80	0.46	-0.34	0.46		Survivor curve	4.6	screw-cap tube in	counts	Broccoli agar	30°C/5 days	IFR unpublished data
606	Kap B2	B	potato puree	5.9			80	2.30	0.36	2.30	HS	Survivor curve	4.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
607	Kap B2	B	potato puree	5.9			80	20.58	1.31	20.58	HR	Survivor curve	9.5	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
608	Kap B2	B	0.067M phosphate buf	7.0			82	0.37	-0.43	1.12	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
609	Kap B2	B	0.067M phosphate buf	7.0			82	28.65	1.46	50.07	HR	Survivor curve	8.3	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
610	Kap B2	B	broccoli puree	5.8			82	0.39	-0.41	1.17	HS	Survivor curve	4.2	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
611	Kap B2	B	broccoli puree	5.8			82	22.83	1.36	32.54	HR	Survivor curve	13.0	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
612	Kap B2	B	broccoli puree	5.8			82	0.19	-0.72	0.52		Survivor curve	4.6	screw-cap tube in	counts	Broccoli agar	30°C/5 days	IFR unpublished data
613	Kap B2	B	potato puree	5.9			82	0.52	-0.28	1.42	HS	Survivor curve	4.6	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
614	Kap B2	B	potato puree	5.9			82	15.77	1.20	25.63	HR	Survivor curve	9.5	screw-cap tube in	counts + LYS	PYGS + LYS	30°C/5 days	IFR unpublished data
615	Kap B2	B	potato puree	5.9			82	0.22	-0.66	0.42		Survivor curve	7.1	screw-cap tube in	counts	Potato agar	30°C/5 days	IFR unpublished data
616	Eklund 17B	B	0.067M phosphate buf	7.0			75	1.15	0.06	0.22		Survivor curve		screw-cap tube in	counts	PYGS	30°C/3 days	IFR unpublished data
617	Eklund 17B	B	0.067M phosphate buf	7.0			75	1.24	0.09	0.23		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data
618	Eklund 17B	B	0.067M phosphate buf	7.0			90	0.64	-0.19	13.79	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/3 days	IFR unpublished data
619	Eklund 17B	B	0.067M phosphate buf	7.0			90	34.13	1.53	375.67	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/3 days	IFR unpublished data
620	Eklund 17B	B	0.067M phosphate buf	7.0			90	0.65	-0.19	14.00	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/6 days	IFR unpublished data
621	Eklund 17B	B	0.067M phosphate buf	7.0			90	36.50	1.56	401.75	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/6 days	IFR unpublished data
622	Eklund 17B	B	PY broth	6.8			75	1.48	0.17	0.28		Survivor curve		screw-cap tube in	counts	PYGS	30°C/2 days	IFR unpublished data
623	Eklund 17B	B	PY broth	6.8			75	1.90	0.28	0.36		Survivor curve		screw-cap tube in	counts	PYGS	30°C/21 days	IFR unpublished data
624	Eklund 17B	B	PY broth	6.8			75	1.29	0.11	0.24		Survivor curve		screw-cap tube in	counts	PYGS + 0.1% N	30°C/1 day	IFR unpublished data
625	Eklund 17B	B	PY broth	6.8			75	1.54	0.19	0.29		Survivor curve		screw-cap tube in	counts	PYGS + 1.5% N	30°C/1 day	IFR unpublished data
626	Eklund 17B	B	PY broth	6.8			75	0.63	-0.20	0.12		Survivor curve		screw-cap tube in	counts	PYGS + 3% Na	30°C/1 day	IFR unpublished data
627	Eklund 17B	B	PY broth	6.8			90	0.68	-0.17	14.65	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/3 days	IFR unpublished data
628	Eklund 17B	B	PY broth	6.8			90	15.65	1.19	172.26	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/3 days	IFR unpublished data
629	Eklund 17B	B	PY broth	6.8			90	0.68	-0.17	14.65	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/6 days	IFR unpublished data
630	Eklund 17B	B	PY broth	6.8			90	15.65	1.19	172.26	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/6 days	IFR unpublished data
631	Eklund 17B	B	PY broth	6.8			80	4.31	0.63	4.31		Survivor curve		screw-cap tube in	counts	PYGS	30°C/6 days	IFR unpublished data
632	Eklund 17B	B	PY broth	6.8			90	0.71	-0.15	15.30	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/4 days	IFR unpublished data

633	Eklund 17B	B	PY broth	6.8			90	40.16	1.60	442.04	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/4 days	IFR unpublished data
634	Eklund 17B	B	PY broth	6.8			80	4.01	0.60	4.01		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
635	Eklund 17B	B	PY broth	6.8			80	6.02	0.78	6.02		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
636	Eklund 17B	B	PY broth	6.8			90	0.77	-0.11	16.59	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/4 days	IFR unpublished data
637	Eklund 17B	B	PY broth	6.8			90	19.42	1.29	213.75	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/4 days	IFR unpublished data
638	Eklund 17B	B	PY broth	6.8			80	5.03	0.70	5.03		Survivor curve		screw-cap tube in	counts	PYGS	10°C/7 days	IFR unpublished data
639	Eklund 17B	B	PY broth	6.8			80	2.59	0.41	2.59		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°C/56 days	IFR unpublished data
640	Eklund 17B	B	PY broth	6.8			80	2.68	0.43	2.68		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°C/56 days	IFR unpublished data
641	Eklund 17B	B	PY broth	6.8			80	3.22	0.51	3.22		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°C/56 days	IFR unpublished data
642	Eklund 17B	B	PY broth	6.8			80	1.45	0.16	1.45		Survivor curve		screw-cap tube in	counts	PYGS under 50	10°C/56 days	IFR unpublished data
643	Eklund 17B	B	PY broth	6.8			80	2.66	0.42	2.66		Survivor curve		screw-cap tube in	counts	PYGS under 2%	10°C/56 days	IFR unpublished data
644	Eklund 17B	B	PY broth	6.8			75	4.95	0.69	0.93		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°C/160 days	IFR unpublished data
645	Eklund 17B	B	PY broth	6.8			75	7.51	0.88	1.42		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°C/160 days	IFR unpublished data
646	Eklund 17B	B	PY broth	6.8			75	7.84	0.89	1.48		Survivor curve		screw-cap tube in	counts	PYGS under 50	10°C/160 days	IFR unpublished data
647	Eklund 17B	B	PY broth	6.8			75	8.86	0.95	1.67		Survivor curve		screw-cap tube in	counts	PYGS under 80	10°C/160 days	IFR unpublished data
648	Eklund 17B	B	PY broth	6.8			75	8.97	0.95	1.69		Survivor curve		screw-cap tube in	counts	PYGS under 50	10°C/160 days	IFR unpublished data
649	Eklund 17B	B	PY broth	6.8			90	1.13	0.05	24.35	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
650	Eklund 17B	B	PY broth	6.8			90	45.25	1.66	498.06	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
651	Eklund 17B	B	PY broth	6.8			90	2.15	0.33	46.32	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
652	Eklund 17B	B	PY broth	6.8			90	16.95	1.23	186.57	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
653	Eklund 17B	B	PY broth	6.8			90	1.79	0.25	38.56	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
654	Eklund 17B	B	PY broth	6.8			90	22.52	1.35	247.88	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
655	Eklund 17B	B	PY broth	6.8			90	1.27	0.10	27.36	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 50	10°C/49 days	IFR unpublished data
656	Eklund 17B	B	PY broth	6.8			90	22.52	1.35	247.88	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 50	10°C/49 days	IFR unpublished data
657	Eklund 17B	B	PY broth	6.8			90	2.45	0.39	68.94		Survivor curve		screw-cap tube in	counts + LYS	PYGS under 2%	10°C/49 days	IFR unpublished data
658	Eklund 17B	B	PY broth	6.8			90	0.83	-0.08	17.88	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/140 days	IFR unpublished data
659	Eklund 17B	B	PY broth	6.8			90	26.59	1.42	292.67	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/140 days	IFR unpublished data
660	Eklund 17B	B	PY broth	6.8			90	0.93	-0.03	20.04	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/140 days	IFR unpublished data
661	Eklund 17B	B	PY broth	6.8			90	27.93	1.45	307.42	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/140 days	IFR unpublished data
662	Eklund 17B	B	PY broth	6.8			90	1.05	0.02	22.62	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 80	10°C/140 days	IFR unpublished data
663	Eklund 17B	B	PY broth	6.8			90	26.88	1.43	295.87	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 80	10°C/140 days	IFR unpublished data
664	Eklund 17B	B	PY broth	6.8			90	6.13	0.79	172.48		Survivor curve		screw-cap tube in	counts + LYS	PYGS under 50	10°C/140 days	IFR unpublished data
665	Eklund 17B	B	PY broth	6.8			90	0.99	0.00	21.33	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/7 days	IFR unpublished data
666	Eklund 17B	B	PY broth	6.8			90	16.50	1.22	181.61	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/7 days	IFR unpublished data
667	Eklund 17B	B	PY broth	6.8			90	0.78	-0.11	16.80	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/7 days	IFR unpublished data
668	Eklund 17B	B	PY broth	6.8			90	10.29	1.01	113.26	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	10°C/7 days	IFR unpublished data
669	Eklund 17B	B	PY broth	6.8			80	3.74	0.57	3.74		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data
670	Eklund 17B	B	PY broth	6.8			80	2.01	0.30	2.01		Survivor curve		screw-cap tube in	counts	PYGS	10°C/21 days	IFR unpublished data
671	Eklund 17B	B	0.067M phosphate buf	6.8			80	2.42	0.38	2.42		Survivor curve		screw-cap tube in	counts	PYGS	30°C/8 days	Stringer *et al.* , 1999
672	Eklund 17B	B	0.067M phosphate buf	7.0			80	4.07	0.61	4.07	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/8 days	IFR unpublished data
673	Eklund 17B	B	0.067M phosphate buf	7.0			80	82.64	1.92	82.64	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/8 days	IFR unpublished data
674	Eklund 17B	B	0.067M phosphate buf	7.0			80	3.15	0.50	3.15	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/98 days	IFR unpublished data
675	Eklund 17B	B	0.067M phosphate buf	7.0			80	27.93	1.45	27.93	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/98 days	IFR unpublished data
676	Eklund 17B	B	PY broth	6.8			80	0.97	-0.01	0.97	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/8 days	IFR unpublished data
677	Eklund 17B	B	PY broth	6.8			80	111.11	2.05	111.11	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/8 days	IFR unpublished data
678	Eklund 17B	B	PY broth	6.8			80	2.00	0.30	2.00		Survivor curve		screw-cap tube in	counts	PYGS	10°C/7 days	IFR unpublished data
679	Eklund 17B	B	PY broth	6.8			80	0.80	-0.10	0.80	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
680	Eklund 17B	B	PY broth	6.8			80	0.69	-0.16	0.69	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
681	Eklund 17B	B	PY broth	6.8			80	0.86	-0.07	0.86	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data

682	Eklund 17B	B	PY broth	6.8			80	15.38	1.19	15.38	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS under 10	10°C/49 days	IFR unpublished data
683	Eklund 17B	B	PY broth	6.8			80	2.33	0.37	2.33		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°C/21 days	IFR unpublished data
684	Eklund 17B	B	PY broth	6.8			80	3.98	0.60	3.98		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°/42 days	IFR unpublished data
685	Eklund 17B	B	PY broth	6.8			80	4.00	0.60	4.00		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°/42 days	IFR unpublished data
686	Eklund 17B	B	PY broth	6.8			80	2.11	0.32	2.11		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°/42 days	IFR unpublished data
687	Eklund 17B	B	PY broth	6.8			80	1.33	0.12	1.33		Survivor curve		screw-cap tube in	counts	PYGS under 10	10°/42 days	IFR unpublished data
688	Eklund 17B	B	PY broth	6.8			80	2.23	0.35	2.23		Survivor curve		screw-cap tube in	counts	PYGS	10°C/119 days	IFR unpublished data
689	Eklund 17B	B	PY broth	6.8			80	3.44	0.54	3.44		Survivor curve		screw-cap tube in	counts	PYGS + 0.01M	10°C/119 days	IFR unpublished data
690	Eklund 17B	B	PY broth	6.8			80	3.84	0.58	3.84		Survivor curve		screw-cap tube in	counts	PYGS + 0.01M	10°C/119 days	IFR unpublished data
691	Eklund 17B	B	PY broth	6.8			80	2.91	0.46	2.91		Survivor curve		screw-cap tube in	counts	PYGS + 0.01M	10°C/119 days	IFR unpublished data
692	Eklund 17B	B	PY broth	6.8			75	6.99	0.84	1.32		Survivor curve		screw-cap tube in	counts	PYGS	10°C/147 days	IFR unpublished data
693	Eklund 17B	B	PY broth	6.8			75	4.58	0.66	0.86		Survivor curve		screw-cap tube in	counts	PYGS (pH 5.5)	10°C/147 days	IFR unpublished data
694	Eklund 17B	B	PY broth	6.8			90	0.71	-0.15	15.30	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + Lys	10°C/21 days	IFR unpublished data
695	Eklund 17B	B	PY broth	6.8			90	18.38	1.26	202.31	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + Lys	10°C/21 days	IFR unpublished data
696	Eklund 17B	B	PY broth	6.8			90	2.81	0.45	60.54	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS (pH 6.0)	10°C/21 days	IFR unpublished data
697	Eklund 17B	B	PY broth	6.8			90	10.76	1.03	118.43	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS (pH 6.0)	10°C/21 days	IFR unpublished data
698	Eklund 17B	B	PY broth	6.8			90	4.81	0.68	135.34		Survivor curve		screw-cap tube in	counts + LYS	PYGS (pH 5.6)	10°C/21 days	IFR unpublished data
699	Eklund 17B	B	PY broth	6.8			90	0.71	-0.15	15.30	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + Lys	10°/42 days	IFR unpublished data
700	Eklund 17B	B	PY broth	6.8			90	19.38	1.29	213.31	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + Lys	10°/42 days	IFR unpublished data
701	Eklund 17B	B	PY broth	6.8			90	1.22	0.09	26.28	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS (pH 6.0)	10°/42 days	IFR unpublished data
702	Eklund 17B	B	PY broth	6.8			90	23.92	1.38	263.29	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS (pH 6.0)	10°/42 days	IFR unpublished data
703	Eklund 17B	B	PY broth	6.8			90	2.39	0.38	67.25		Survivor curve		screw-cap tube in	counts + LYS	PYGS (pH 5.6)	10°/42 days	IFR unpublished data
704	Eklund 17B	B	PY broth	6.8			75	4.45	0.65	0.84		Survivor curve		screw-cap tube in	counts	PYGS + 3% Na	10°C/28 days	IFR unpublished data
705	Eklund 17B	B	PY broth	6.8			75	3.56	0.55	0.67		Survivor curve		screw-cap tube in	counts	PYGS + 4% Na	10°C/28 days	IFR unpublished data
706	Eklund 17B	B	PY broth	6.8			75	0.59	-0.23	0.11		Survivor curve		screw-cap tube in	counts	PYGS + 3% Na	10°C/14 days	IFR unpublished data
707	Eklund 17B	B	PY broth	6.8			90	4.29	0.63	92.43	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + 0.1% N	10°C/161 days	IFR unpublished data
708	Eklund 17B	B	PY broth	6.8			90	26.81	1.43	295.10	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + 0.1% N	10°C/161 days	IFR unpublished data
709	Eklund 17B	B	PY broth	6.8			90	0.87	-0.06	18.74	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + 1.5% N	10°C/161 days	IFR unpublished data
710	Eklund 17B	B	PY broth	6.8			90	22.52	1.35	247.88	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + 1.5% N	10°C/161 days	IFR unpublished data
711	Eklund 17B	B	PY broth	6.8			90	1.53	0.18	32.96	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + 3% Na	10°C/161 days	IFR unpublished data
712	Eklund 17B	B	PY broth	6.8			90	26.38	1.42	290.36	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + 3% Na	10°C/161 days	IFR unpublished data
713	Eklund 17B	B	0.067M phosphate buf	7.0			80	0.42	-0.38	0.42		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
714	2129B	B	0.067M phosphate buf	7.0			80	2.47	0.39	2.47		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
715	Kap B2	B	0.067M phosphate buf	7.0			80	2.57	0.41	2.57		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
716	Kap B5	B	0.067M phosphate buf	7.0			80	2.62	0.42	2.62		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
717	Beluga	E	0.067M phosphate buf	7.0			80	0.42	-0.38	0.42		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
718	Foster B96	E	0.067M phosphate buf	7.0			80	0.59	-0.23	0.59		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
719	Eklund 202F	F	0.067M phosphate buf	7.0			80	0.59	-0.23	0.59		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
720	Craig 610	F	0.067M phosphate buf	7.0			80	0.75	-0.12	0.75		Survivor curve		screw-cap tube in	counts	PYGS	30°C/4 days	IFR unpublished data
721	Eklund 17B	B	0.067M phosphate buf	7.0			75	14.81	1.17	1.21		Survivor curve	4.6	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
722	Eklund 17B	B	0.067M phosphate buf	7.0			78	3.03	0.48	1.11		Survivor curve	4.6	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
723	Eklund 17B	B	0.067M phosphate buf	7.0			80	4.64	0.67	4.64		Survivor curve	4.6	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
724	Kap B2	B	0.067M phosphate buf	7.0			80	1.09	0.04	1.09		Survivor curve	4.2	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
725	Kap B2	B	0.067M phosphate buf	7.0			82	0.37	-0.43	1.11		Survivor curve	4.2	screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
726	Eklund 17B	B	0.067M phosphate buf	7.0			85	2.53	0.40	5.61	HS	Survivor curve	14.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
727	Eklund 17B	B	0.067M phosphate buf	7.0			85	51.00	1.71	128.96	HR	Survivor curve	12.4	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
728	Eklund 17B	B	0.067M phosphate buf	7.0			88	1.50	0.18	4.95	HS	Survivor curve	14.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
729	Eklund 17B	B	0.067M phosphate buf	7.0			88	24.00	1.38	96.51	HR	Survivor curve	12.4	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
730	Eklund 17B	B	0.067M phosphate buf	7.0			90	0.40	-0.40	1.96	HS	Survivor curve	14.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980

731	Eklund 17B	B	0.067M phosphate buf	7.0			90	8.30	0.92	53.07	HR	Survivor curve	12.4	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
732	Eklund 17B	B	0.1M citrate buffer	3.5			88	0.99	0.00	9.90	HS	Survivor curve		screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
733	Eklund 17B	B	0.1M citrate buffer	3.5			88	4.90	0.69	26.12	HR	Survivor curve	10.3	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
734	Beluga	E	0.067M phosphate buf	7.0			80	1.24	0.09	1.24	HS	Survivor curve	9.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
735	Beluga	E	0.067M phosphate buf	7.0			80	36.00	1.56	36.00	HR	Survivor curve	11.8	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
736	Beluga	E	0.067M phosphate buf	7.0			83	0.53	-0.28	0.97	HS	Survivor curve	9.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
737	Beluga	E	0.067M phosphate buf	7.0			83	23.60	1.37	38.52	HR	Survivor curve	11.8	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
738	Beluga	E	0.067M phosphate buf	7.0			85	0.30	-0.52	1.01	HS	Survivor curve	9.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
739	Beluga	E	0.067M phosphate buf	7.0			85	10.40	1.02	27.71	HR	Survivor curve	11.8	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
740	Beluga	E	0.067M phosphate buf	7.0			88	0.15	-0.82	0.92	HS	Survivor curve	9.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
741	Beluga	E	0.067M phosphate buf	7.0			88	6.10	0.79	26.52	HR	Survivor curve	11.8	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
742	Eklund 17B	B	0.1M citrate buffer	3.5			78	2.39	0.38	1.11	HS	Survivor curve		screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
743	Eklund 17B	B	0.1M citrate buffer	3.5			78	40.00	1.60	22.90	HR	Survivor curve	10.3	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
744	Eklund 17B	B	0.1M citrate buffer	4.4			78	3.42	0.53	1.59	HS	Survivor curve		screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
745	Eklund 17B	B	0.1M citrate buffer	4.4			78	52.00	1.72	29.77	HR	Survivor curve	10.3	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
746	Eklund 17B	B	0.067M phosphate buf	7.0			78	3.98	0.60	2.67	HS	Survivor curve	14.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
747	Eklund 17B	B	0.067M phosphate buf	7.0			78	103.00	2.01	64.77	HR	Survivor curve	12.4	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
748	Beluga	E	0.1M citrate buffer	3.5			78	1.77	0.25	0.82	HS	Survivor curve		screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
749	Beluga	E	0.1M citrate buffer	3.5			78	19.30	1.29	10.60	HR	Survivor curve		screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
750	Beluga	E	0.1M citrate buffer	4.4			78	2.12	0.33	0.98	HS	Survivor curve		screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
751	Beluga	E	0.1M citrate buffer	4.4			78	30.00	1.48	16.47	HR	Survivor curve		screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
752	Beluga	E	0.067M phosphate buf	7.0			78	1.52	0.18	0.83	HS	Survivor curve	9.5	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
753	Beluga	E	0.067M phosphate buf	7.0			78	38.00	1.58	23.28	HR	Survivor curve	11.8	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
754	Beluga	E	0.1M citrate buffer + 3	3.8			80	5.84	0.77	5.84		Survivor curve	8.4	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
755	Beluga	E	0.1M citrate buffer + 3	3.8			83	3.01	0.48	5.98		Survivor curve	8.4	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
756	Beluga	E	0.1M citrate buffer + 3	3.8			85	1.48	0.17	5.84		Survivor curve	8.4	screw-cap tube in	counts + LYS	TPG + egg yolk	25°C/3-4 days	Smelt, 1980
757	Eklund 17B	B	0.067M phosphate buf	7.0			90	0.65	-0.19	14.03	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
758	Eklund 17B	B	0.067M phosphate buf	7.0			90	18.28	1.26	201.22	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
759	ATCC 9564	E	0.067M phosphate buf	7.0			90	0.77	-0.11	16.58	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
760	ATCC 9564	E	0.067M phosphate buf	7.0			90	11.44	1.06	125.94	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
761	ATCC 9564	E	0.067M phosphate buf	7.0			75	0.90	-0.05	0.17		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data
762	Hobbs FT50	B	0.067M phosphate buf	7.0			90	0.83	-0.08	17.81	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
763	Hobbs FT50	B	0.067M phosphate buf	7.0			90	28.17	1.45	310.05	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
764	Hobbs FT50	B	0.067M phosphate buf	7.0			75	1.25	0.10	0.24		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data
765	Beluga	E	0.067M phosphate buf	7.0			75	1.63	0.21	0.31		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data
766	Beluga	E	0.067M phosphate buf	7.0			90	0.78	-0.11	16.88	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
767	Beluga	E	0.067M phosphate buf	7.0			90	14.18	1.15	156.13	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
768	Foster B96	E	0.067M phosphate buf	7.0			75	1.56	0.19	0.29		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data
769	Foster B96	E	0.067M phosphate buf	7.0			90	2.11	0.32	45.46	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
770	Foster B96	E	0.067M phosphate buf	7.0			90	28.41	1.45	312.71	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
771	Craig 610	F	0.067M phosphate buf	7.0			75	1.55	0.19	0.26		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data
772	Craig 610	F	0.067M phosphate buf	7.0			90	0.60	-0.22	12.93	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
773	Craig 610	F	0.067M phosphate buf	7.0			90	24.87	1.40	273.74	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
774	Eklund 202F	F	0.067M phosphate buf	7.0			90	0.65	-0.19	13.92	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
775	Eklund 202F	F	0.067M phosphate buf	7.0			90	21.46	1.33	236.20	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
776	CBW25	B	0.067M phosphate buf	7.0			90	1.05	0.02	22.72	HS	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
777	CBW25	B	0.067M phosphate buf	7.0			90	28.25	1.45	310.93	HR	Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/2-3 days	IFR unpublished data
778	CBW25	B	0.067M phosphate buf	7.0			75	3.06	0.49	0.58		Survivor curve		screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data
779	Eklund 202F	F	0.067M phosphate buf	7.0			75	4.91	0.69	0.84		Survivor curve		screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data

780	Eklund 17B	B	0.067M phosphate buf	7.0			75	17.00	1.23	3.20		Survivor curve		screw-cap tube in	counts	PYGS	30°C/5 days	IFR unpublished data	
781	Crab G21-5	E	crabmeat				82	3.00	0.48	6.25		TDT		TDT tubes in wate	TDT	TPGY	26°C/6 days	Solmon *et al.*, 1977	
782	Crab G21-5	E	TPGY broth				82	1.50	0.18	3.13		TDT		TDT tubes in wate	TDT	TPGY		Solmon *et al.*, 1977	
783	Mixed strains: B (Ekl		bechamel sauce	6.49	0.989		80	7.21	0.86	7.21		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
784	Mixed strains: B (Ekl		bechamel sauce	6.49	0.989		85	2.20	0.34	12.27		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
785	Mixed strains: B (Ekl		bechamel sauce	6.49	0.989		92	0.26	-0.59	16.07		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
786	Mixed strains: B (Ekl		bolognaise sauce	5.37	0.987		80	9.27	0.97	9.27		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
787	Mixed strains: B (Ekl		bolognaise sauce	5.37	0.987		85	2.16	0.33	12.04		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
788	Mixed strains: B (Ekl		bolognaise sauce	5.37	0.987		92	0.22	-0.66	13.60		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
789	Mixed strains: B (Ekl		cooked pasta	6.74	0.99		85	2.18	0.34	12.15		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
790	Mixed strains: B (Ekl		cooked pasta	6.74	0.99		88	1.24	0.09	19.38		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
791	Mixed strains: B (Ekl		cooked pasta	6.74	0.99		92	0.52	-0.28	32.14		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
792	Mixed strains: B (Ekl		0.067M phosphate buf	7.0			92	0.06	-1.22	3.71		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
793	Mixed strains: B (Ekl		0.067M phosphate buf	7.0			92	0.47	-0.33	29.05		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
794	Eloise	E	0.067M phosphate buf	7.0			70	15.29	1.18	0.54		Survivor curve		unsealed tubes in	counts	Beef-Infusion ag	33°C/2 days	Graikoski and Kempe, 1964	
795	Detroit	E	0.067M phosphate buf	7.0			65	400.00	2.60	2.68		Survivor curve		unsealed tubes in	counts	Beef-Infusion ag	33°C/2 days	Graikoski and Kempe, 1964	
796	Minneapolis	E	0.067M phosphate buf	7.0			70	14.81	1.17	0.53		Survivor curve		sealed tubes in wa	counts	Beef-Infusion ag	33°C/2 days	Graikoski and Kempe, 1964	
797	Iwanai	E	0.067M phosphate buf	7.0			65	66.67	1.82	0.45		Survivor curve		unsealed tubes in	counts	Beef-Infusion ag	33°C/2 days	Graikoski and Kempe, 1964	
798	E-74	E	0.067M phosphate buf	7.0			85	7.05	0.85	37.40		Survivor curve		unsealed tubes in	counts	Beef-Infusion ag	33°C/2 days	Graikoski and Kempe, 1964	
799	Vancouver He	E	0.067M phosphate buf	7.0			85	6.12	0.79	32.46		Survivor curve		unsealed tubes in	counts	Beef-Infusion ag	33°C/2 days	Graikoski and Kempe, 1964	
800	Not specified	E	distilled water				71	62.90	1.80	1.17			5.2					Mann, 1966	
801	Not specified	E	tuna				71	84.10	1.92	1.02			4.7					Mann, 1966	
802	Saratoga	E	0.067M phosphate buf	7.0			70	33.80	1.53	1.38			7.2	TDT tubes in wate	TDT			Ito et al., 1970	
803	Saratoga	E	0.067M phosphate buf	7.0			73	13.10	1.12	1.31			7.2	TDT tubes in wate	TDT			Ito et al., 1970	
804	Saratoga	E	0.067M phosphate buf	7.0			75	8.20	0.91	1.66			7.2	TDT tubes in wate	TDT			Ito et al., 1970	
805	Saratoga	E	0.067M phosphate buf	7.0			80	1.40	0.15	1.40			7.2	TDT tubes in wate	TDT			Ito et al., 1970	
806	Beluga	E	0.067M phosphate buf	7.0			70	29.30	1.47	1.53			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
807	Beluga	E	0.067M phosphate buf	7.0			73	8.00	0.90	0.96			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
808	Beluga	E	0.067M phosphate buf	7.0			75	5.10	0.71	1.17			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
809	Beluga	E	0.067M phosphate buf	7.0			80	1.40	0.15	1.40			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
810	Alaska	E	0.067M phosphate buf	7.0			70	37.50	1.57	1.96			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
811	Alaska	E	0.067M phosphate buf	7.0			73	11.80	1.07	1.41			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
812	Alaska	E	0.067M phosphate buf	7.0			75	5.30	0.72	1.21			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
813	Alaska	E	0.067M phosphate buf	7.0			80	1.60	0.20	1.60			7.8	TDT tubes in wate	TDT			Ito et al., 1970	
814	1304E	E	0.067M phosphate buf	7.0			70	29.60	1.47	1.85			8.3	TDT tubes in wate	TDT			Ito et al., 1970	
815	1304E	E	0.067M phosphate buf	7.0			73	10.60	1.03	1.44			8.3	TDT tubes in wate	TDT			Ito et al., 1970	
816	1304E	E	0.067M phosphate buf	7.0			75	5.60	0.75	1.40			8.3	TDT tubes in wate	TDT			Ito et al., 1970	
817	1304E	E	0.067M phosphate buf	7.0			80	1.80	0.26	1.80			8.3	TDT tubes in wate	TDT			Ito et al., 1970	
818	Saratoga	E	0.067M phosphate buf	7.0			80	1.17	0.07	1.17			7.1	TDT tubes in wate	TDT			Angelotti, 1970	
819	Beluga	E	0.067M phosphate buf	7.0			80	1.10	0.04	1.10			8.3	TDT tubes in wate	TDT			Angelotti, 1970	
820	Alaska	E	0.067M phosphate buf	7.0			80	1.35	0.13	1.35			7.8	TDT tubes in wate	TDT			Angelotti, 1970	
821	1304E	E	0.067M phosphate buf	7.0			80	1.45	0.16	1.45			8.1	TDT tubes in wate	TDT			Angelotti, 1970	
822	Minnesota	E	crawfish paste				85	8.81	0.94	37.15		TDT/Stumbo(1948)	8.0	TDT tubes in wate	TDT	crawfish paste	30°C/14 days	DePantoja, 1986	
823	Minnesota	E	crawfish paste				90	2.46	0.39	43.75		TDT/Stumbo(1948)	8.0	TDT tubes in wate	TDT	crawfish paste	30°C/14 days	DePantoja, 1986	
824	Mixed strains: B (CD		0.067M phosphate buf	7.0			80	5.45	0.74	5.45		Survivor curve		screw-cap tube in	counts	PYGS	30°C/7 days	IFR unpublished data	
825	Mixed strains: B (CD		0.067M phosphate buf	7.0			80	5.76	0.76	5.76		Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	30°C/7 days	IFR unpublished data	
826	Mixed strains: B (CD		0.067M phosphate buf	7.0			80	5.24	0.72	5.24		Survivor curve		screw-cap tube in	counts	PYGS	25°C/14 days	IFR unpublished data	
827	Mixed strains: B (CD		0.067M phosphate buf	7.0			80	6.51	0.81	6.51		Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	25°C/14 days	IFR unpublished data	
828	Mixed strains: B (CD		0.067M phosphate buf	7.0			80	7.90	0.90	7.90		Survivor curve		screw-cap tube in	counts	PYGS	25°C/21 days	IFR unpublished data	

829	Mixed strains:	B (CD	0.067M phosphate buf	7.0			80	7.90	0.90	7.90		Survivor curve		screw-cap tube in	counts + LYS	PYGS + LYS	25°C/21 days	IFR unpublished data
831	202	F	physiological saline				80	4.90	0.69	4.00		TDT	9.2	sealed ampules in	TDT	CMM	25˚C/35 days	Eklund *et al.*, 1967
832	202	F	physiological saline				90	0.56	-0.25	3.82		TDT	9.2	sealed ampules in	TDT	CMM	25˚C/35 days	Eklund *et al.*, 1967
834	288	F	physiological saline				80	5.60	0.75	4.00		TDT	8.7	sealed ampules in	TDT	CMM	25˚C/35 days	Eklund *et al.*, 1967
835	288	F	physiological saline				90	0.56	-0.25	4.59		TDT	8.7	sealed ampules in	TDT	CMM	25˚C/35 days	Eklund *et al.*, 1967
836	Mixed strains		TPG				80	0.80	-0.10	0.80		Survivor curve			counts	TPG		Membre and McClure, 2006
837	Mixed strains		TPG				80	1.40	0.15	1.40		Survivor curve			counts	TPG		Membre and McClure, 2006
838	Mixed strains		TPG				80	1.70	0.23	1.70		Survivor curve			counts	TPG		Membre and McClure, 2006
839	Mixed strains		TPG				80	1.80	0.26	1.80		Survivor curve			counts	TPG		Membre and McClure, 2006
840	Mixed strains		TPG				80	3.20	0.51	3.20		Survivor curve			counts	TPG		Membre and McClure, 2006
841	Mixed strains		TPG				80	0.20	-0.70	0.20		Survivor curve			counts	TPG		Membre and McClure, 2006
842	Mixed strains		TPG				80	0.30	-0.52	0.30		Survivor curve			counts	TPG		Membre and McClure, 2006
843	Mixed strains		TPG				80	0.30	-0.52	0.30		Survivor curve			counts	TPG		Membre and McClure, 2006
844	Mixed strains		TPG				80	0.30	-0.52	0.30		Survivor curve			counts	TPG		Membre and McClure, 2006
845	Mixed strains		TPG				80	0.30	-0.52	0.30		Survivor curve			counts	TPG		Membre and McClure, 2006
846	Mixed strains		TPG				80	0.60	-0.22	0.60		Survivor curve			counts	TPG		Membre and McClure, 2006
847	Mixed strains		TPG				80	0.60	-0.22	0.60		Survivor curve			counts	TPG		Membre and McClure, 2006
848	Mixed strains		TPG				80	0.60	-0.22	0.60		Survivor curve			counts	TPG		Membre and McClure, 2006
849	Mixed strains		TPG				80	0.60	-0.22	0.60		Survivor curve			counts	TPG		Membre and McClure, 2006
850	Mixed strains		TPG				80	0.70	-0.15	0.70		Survivor curve			counts	TPG		Membre and McClure, 2006
851	Mixed strains		TPG				80	0.70	-0.15	0.70		Survivor curve			counts	TPG		Membre and McClure, 2006
852	Mixed strains		TPG				80	0.80	-0.10	0.80		Survivor curve			counts	TPG		Membre and McClure, 2006
853	Mixed strains		TPG				80	0.90	-0.05	0.90		Survivor curve			counts	TPG		Membre and McClure, 2006
854	Mixed strains		TPG				80	1.20	0.08	1.20		Survivor curve			counts	TPG		Membre and McClure, 2006
855	Mixed strains		TPG				80	1.40	0.15	1.40		Survivor curve			counts	TPG		Membre and McClure, 2006
856	Mixed strains		TPG				80	1.30	0.11	1.30		Survivor curve			counts	TPG		Membre and McClure, 2006
857	Mixed strains		TYD				80	0.20	-0.70	0.20		Survivor curve			counts	TPG		Membre and McClure, 2006
858	Mixed strains		TYD				80	0.20	-0.70	0.20		Survivor curve			counts	TPG		Membre and McClure, 2006
859	Mixed strains		TYD				80	0.60	-0.22	0.60		Survivor curve			counts	TPG		Membre and McClure, 2006
860	Mixed strains		TYD				80	1.00	0.00	1.00		Survivor curve			counts	TPG		Membre and McClure, 2006
861	Mixed strains		TYD				80	5.11	0.71	5.11		Survivor curve			counts	TPG		Membre and McClure, 2006
862	Mixed strains		TYD				80	0.40	-0.40	0.40		Survivor curve			counts	TPG		Membre and McClure, 2006
863	Mixed strains		TYD				80	0.40	-0.40	0.40		Survivor curve			counts	TPG		Membre and McClure, 2006
864	Mixed strains		TYD				80	0.50	-0.30	0.50		Survivor curve			counts	TPG		Membre and McClure, 2006
865	Mixed strains		TYD				80	0.70	-0.16	0.70		Survivor curve			counts	TPG		Membre and McClure, 2006
866	Mixed strains		TYD				80	0.70	-0.16	0.70		Survivor curve			counts	TPG		Membre and McClure, 2006
867	Mixed strains		TYD				80	0.80	-0.10	0.80		Survivor curve			counts	TPG		Membre and McClure, 2006
868	Mixed strains		TYD				80	0.90	-0.05	0.90		Survivor curve			counts	TPG		Membre and McClure, 2006
869	Mixed strains		TYD				80	0.90	-0.05	0.90		Survivor curve			counts	TPG		Membre and McClure, 2006
870	Mixed strains		TYD				80	1.00	0.00	1.00		Survivor curve			counts	TPG		Membre and McClure, 2006
871	Mixed strains		TYD				80	0.30	-0.52	0.30		Survivor curve			counts	TPG		Membre and McClure, 2006
872	Mixed strains		TYD				80	0.40	-0.40	0.40		Survivor curve			counts	TPG		Membre and McClure, 2006
873	Mixed strains		TYD				80	1.00	0.00	1.00		Survivor curve			counts	TPG		Membre and McClure, 2006
874	ATCC 9564	E	fine carot				70	31.41	1.50	1.58		Survivor curve	7.7		counts	TPGY	30˚C/2 days	Duh and Ren, 1995
875	ATCC 9564	E	fine carot				80	1.80	0.26	1.80		Survivor curve	7.7		counts	TPGY	30˚C/2 days	Duh and Ren, 1995
876	ATCC 9564	E	fine carot				90	0.08	-1.10	1.59		Survivor curve	7.7		counts	TPGY	30˚C/2 days	Duh and Ren, 1995
877	Not specified	E					80	1.51	0.18	1.51		TDT		TDT tubes in wate	TDT	W & F		Kralovic, 1973
878	Mixed strains(Eklund		meat medium				75	274.76	2.44	39.04		Survivor curve	5.9	screw-cap tube in	counts	PYGS	25˚C/90 days	Fernandez and Peck, 1997

879	Mixed strains(Eklund	meat medium				80	4.24	0.63	4.24		Survivor curve	5.9	screw-cap tube in	counts	PYGS	25˚C/90 days	Fernandez and Peck, 1997
880	Mixed strains(Eklund	meat medium				85	8.85	0.95	62.29		Survivor curve	5.9	screw-cap tube in	counts	PYGS	25˚C/90 days	Fernandez and Peck, 1997

APPA – Anderson's pork-pea agar, a_w – water activity, CMM – cooked meat medium, *D*-value – decimal reduction time (min), EA – Eugon agar, ESBA – extract-starch-biocarbonate agar, FEM – fortified egg-meat medium, HR – heat resistant fraction, HS – heat sensitive fraction, MAM – Molten agar medium, NVB – Noyes veal-broth medium, PIT – pork-infusion-thioglycollate-starch agar, PYG – peptone-yeast-glucose agar, PYGS – peptone-yeast-glucose-starch medium, RCA – reinforced clostridial agar, RCM – reinforced clostridial medium, SH – sublethal heating, T – temperature (°C), TDT – thermal death time, TPG – tripticase-peptone-glucose agar, TPGY – trypticase-peptone-glucose yeast agar, TPGYT – trypticase-peptone-glucose yeast + 0.1% tripsin, TSA – tryptic-soy agar, TYD – tryptone-yeast dextrose, W & F – Wynne and Foster's pork infusion broth, *z*-value – a change of temperature (°C), for which the *D*-value is reduced/increased by a factor of 10

Appendix 3 Experiment one: Time to visible growth (days) from 10^6 spores $tube^{-1}$ of non-proteolytic *C. botulinum* strains at ten refrigeration temperatures (Stringer and Webb, unpublished data)

Toxin	Strain	4.0°C			5.0°C			5.5°C			6.0°C			6.5°C			7.0°C			8.0°C			9.0°C			10.0°C		
Type B	81-23	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	42	49	39	25	25	25	14	14	14	8	7	7	5	5	5
	81-30	>90	>90	>90	>90	>90	77	46	39	39	25	25	25	18	18	21	14	14	18	8	8	8	7	7	7	5	5	5
	83-01	>90	>90	>90	74	81	42	32	32	39	18	25	21	18	14	18	14	11	14	8	8	8	6	6	6	5	5	5
	86-17	>90	>90	>90	>90	>90	>90	39	28	32	18	18	18	14	14	18	11	11	11	8	8	8	6	6	6	4	4	4
	87-02	>90	>90	>90	>90	>90	>90	>90	35	>90	21	25	25	14	18	18	11	11	11	8	8	7	6	6	6	5	5	5
	90-04	>90	>90	>90	>90	>90	>90	>90	>90	>90	28	32	32	21	18	21	14	14	14	11	11	11	7	8	7	6	6	5
	93-06	>90	>90	>90	32	>90	39	25	25	28	18	18	18	11	11	11	8	8	8	5	6	6	5	5	5	4	4	4
	93-10	>90	>90	>90	42	42	39	25	28	25	14	14	14	11	11	11	11	11	11	6	7	7	6	6	6	5	5	5
	93-11	>90	>90	>90	60	46	39	28	28	25	18	14	14	11	11	11	11	11	11	7	7	7	5	5	5	4	4	4
	02-50	>90	>90	>90	74	49	42	21	25	21	18	18	18	14	14	11	11	11	11	7	7	7	5	6	6	4	4	4
	02-51	>90	>90	>90	>90	>90	>90	>90	>90	56	25	25	25	14	18	14	14	11	11	11	11	11	6	6	6	5	5	5
	05-20	>90	>90	>90	35	>90	>90	25	25	25	18	18	18	14	14	14	11	11	11	11	8	11	6	6	6	5	5	5
	05-25	>90	>90	>90	>90	46	>90	>90	>90	70	21	25	25	14	14	14	11	11	11	7	7	7	6	5	6	5	5	5
	05-29	>90	>90	>90	>90	>90	>90	35	>90	>90	35	32	25	14	18	14	14	11	11	8	8	7	6	6	6	5	5	5
Type E	81-26	>90	>90	>90	>90	74	67	32	35	32	18	18	18	18	18	18	14	11	14	8	8	8	6	6	6	5	5	5
	81-31	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	X	>90	>90	>90	>90	>90	>90	28	25	28	14	14	14	14	11	11
	86-21	>90	>90	>90	25	25	28	18	18	18	14	14	11	11	11	11	11	11	11	6	6	6	5	5	5	4	4	4
	87-01	>90	>90	>90	>90	>90	>90	35	49	49	18	21	21	14	14	14	14	14	14	8	8	8	6	6	7	5	5	5
	93-07	>90	>90	>90	>90	>90	>90	>90	>90	>90	21	35	25	18	18	18	11	11	11	7	7	7	5	6	6	4	4	4
	93-08	>90	>90	>90	28	32	32	14	18	18	14	11	14	11	11	8	8	8	8	7	7	6	5	6	6	4	4	4
	02-06	>90	>90	>90	>90	>90	>90	25	25	25	14	18	18	11	11	14	11	11	11	8	8	8	5	6	6	5	5	5
	02-09	>90	>90	>90	63	56	74	21	25	25	14	14	14	11	11	11	11	11	11	7	7	7	5	5	5	5	5	5
	02-13	>90	>90	>90	32	35	32	14	18	18	11	11	11	11	8	8	8	8	8	7	7	7	5	5	5	4	4	4
	02-14	>90	>90	>90	>90	>90	>90	>90	>90	>90	49	>90	56	18	18	18	11	14	14	7	8	7	6	6	6	4	4	4

	02-15	>90	67	67	21	21	21	18	18	14	11	14	14	11	11	11	8	8	8	6	6	6	5	5	5	4	4	4
	02-21	>90	>90	>90	>90	74	39	28	28	39	11	14	14	11	11	11	8	8	8	6	6	6	5	5	5	4	4	4
	02-22	>90	>90	>90	>90	>90	>90	>90	>90	>90	>90	42	35	18	14	14	11	11	11	6	7	6	6	5	6	4	4	4
	02-24	>90	>90	>90	>90	>90	>90	>90	>90	46	25	25	25	14	14	14	11	11	11	7	6	7	5	6	5	4	4	4
	02-25	>90	>90	>90	>90	>90	>90	32	39	56	14	14	18	11	11	11	8	11	11	7	7	7	5	5	5	4	4	4
	02-26	>90	>90	>90	>90	81	>90	56	39	39	25	21	25	14	14	14	11	11	11	8	8	8	5	5	5	5	5	6
	02-29	>90	>90	>90	28	25	25	21	21	21	14	11	14	11	11	11	11	11	11	6	6	7	5	5	5	4	4	4
	02-32	>90	>90	>90	32	32	32	21	21	21	14	14	14	11	11	11	11	11	11	7	7	7	5	5	5	4	4	4
	02-33	>90	>90	>90	32	32	32	21	21	21	14	14	14	11	11	11	11	11	11	7	7	8	5	5	6	5	5	5
	02-35	>90	>90	>90	42	42	39	21	21	21	14	14	14	11	11	11	8	8	11	7	7	8	5	5	5	5	4	4
	02-47	>90	>90	>90	>90	>90	>90	>90	>90	>90	18	21	18	11	11	11	11	11	11	7	7	6	5	5	5	4	4	4
Type F	86-32	>90	>90	>90	42	>90	>90	35	49	49	21	25	25	18	18	18	14	11	11	7	7	7	5	6	5	5	5	5
	86-33	>90	>90	>90	>90	53	53	28	32	35	18	18	18	14	14	14	11	11	11	6	6	6	5	5	5	4	4	4
	86-34	>90	>90	>90	>90	>90	>90	35	39	39	21	21	21	18	18	18	11	11	11	6	6	6	5	5	5	4	4	4
	06-01	>90	>90	>90	>90	>90	>90	39	53	>90	25	32	28	21	18	18	14	14	14	8	7	8	6	6	6	5	5	5
	06-05	>90	>90	>90	>90	>90	>90	>90	42	42	28	25	39	18	21	28	14	18	18	8	8	8	6	6	6	5	5	5

X – tubes broken

Tests were also carried out at 3.0°C, but growth was not observed in 90 days

Appendix 4 Experiment two: Time to visible growth (days) from different spore concentrations of non-proteolytic *C. botulinum* strains at 6.0°C

Toxin	Type B											Type E									Type F			
Strain/spore concentration	81-23	81-30	83-01	86-17	87-02	87-04	93-06	93-11	02-51	05-20	05-25	81-31	86-21	87-01	02-07	02-09	02-15	02-25	02-26	02-29	86-32	86-33	86-34	06-05
$S = 10^6$ spores tube^{-1}	37	16	12	12	12	16	12	11	20	11	17	40	11	11	11	11	11	11	11	11	12	16	13	17
	59	16	12	12	13	16	12	11	20	11	17		11	11	11	11	11	11	11	11	12	16	14	18
	>60	16	14	13	14	16	13	11	21	11	17	>60	11	11	11	11	11	11	11	11	16	16	14	18
	>60	17	14	13	14	16	13	11	23	11	18	>60	11	11	11	11	11	11	11	11	16	17	14	19
	>60	17	14	13	14	16	13	11	24	11	18	>60	11	11	11	11	11	11	11	11	16	17	16	20
$S = 10^5$ spores tube^{-1}	>60	17	14	14	16	20	16	12	59	12	18	59	11	12	11	11	11	11	11	11	14	17	17	20
	>60	18	14	14	16	20	17	13	>60	12	18		11	12	11	11	11	11	11	11	16	18	18	20
	>60	18	14	14	17	21	18	13	>60	13	19	>60	11	12	11	11	11	11	11	11	18	18	18	20
	>60	20	14	16	23	21	18	13	>60	13	19	>60	11	12	11	11	11	11	11	11	18	18	18	24
	>60	20	16	>60	30	23	19	13	>60	14	19	>60	11	13	11	12	11	11	11	11	19	19	20	24
$S = 10^4$ spores tube^{-1}	>60	20	17	17	21	24	18	14	60	16	20	40	12	13	12	12	12	12	14	11	19	19	19	23
	>60	20	17	17	23	25	19	14	>60	16	23		12	14	12	12	12	12	14	11	19	23	20	24
	>60	23	18	18	24	25	19	16	>60	16	23	>60	12	14	12	12	12	12	14	11	20	23	20	30
	>60	25	19	18	34	32	20	16	>60	18	23	>60	12	16	12	12	12	12	14	11	26	25	21	34
	>60	32	19	18	48	34	23	16	>60	19	24	>60	12	>60	12	12	12	12	14	11	30	31	21	
$S = 10^3$ spores tube^{-1}	>60	25	20	23	28	30	18	16	>60	20	23	>60	13	16	14	13	14	13	16	14	20	20	23	26
	>60	25	20	23	28	32	18	16	>60	20	23	>60	13	16	14	14	14	13	16	14	20	20	23	34
	>60	25	21	24	30	44	19	17	>60	20	25	>60	14	16	14	14	14	13	17	14	23	21	25	>60
	>60	26	21	30	54	44	20	18	>60	25	27	>60	14	16	14	14	14	13	17	14	24	23	25	>60
	>60	27	25	48	>60	>60	20	18	>60	25	37	>60	14	17	14	14	16	14	18	14	28	>60	26	>60
$S = 10^2$ spores tube^{-1}	>60	25	23	30	26	34	23	17	>60	23	25	>60	16	17	16	16	16	16	21	16	27	23	25	>60
	>60	30	23	30	37	37	23	19	>60	25	30	>60	16	18	16	16	16	16	23	16	28	25	27	>60
	>60	30	26	31	37	>60	23	19	>60	27	40	>60	16	18	16	16	16	16	23	16	30	25	31	>60
	>60	31	30	32	48	>60	24	20	>60	30	40	>60	16	18	16	16	16	16	23	16	31	25	>60	>60
	>60	34	37	37	54	>60	25	20	>60	31	44	>60	17	18	16	16	16	16	28	16	32		>60	>60
$S = 10^1$ spores tube^{-1}	>60	31	24	37	28	>60	24	19	>60	25	>60	>60	18	19	17	18	18	17	26	18	23	25	32	>60
	>60	34	26	40	28	>60	27	19	>60	37	>60	>60	18	19	18	18	19	17	30	19	23		>60	>60
	>60	37	30	42	28	>60	28	23	>60	40	>60	>60	18	19	19	18	19	18	30	19	23	>60	>60	>60
	>60	>60	30	48	30	>60	>60	23	>60	48	>60	>60	19	20	20	18	20	18	40	20		>60	>60	>60
	>60	>60	30	54	54	>60	>60	24	>60	51	>60	>60	20	25	>60	19	20	19	42	20	>60	>60	>60	>60
$S = 10^0$ spores tube^{-1}	>60		37	34	28	>60	>60	>60	>60	32	>60	>60	18	20	21	20	19	18	54	20	26	37	>60	>60
	>60	>60	42	40	>60	>60	>60	>60	>60	48	>60	>60	20	21	>60	20	20	19	59	21	>60	>60	>60	>60
	>60	>60	>60	>60	>60	>60	>60	>60	>60	48	>60	>60	21	23	>60	21	20	19	>60	23	>60	>60	>60	>60
	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	21	>60	>60	21	21	19	>60	>60	>60	>60	>60	>60
	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	>60	24	>60	20	>60	>60	>60	>60	>60	>60

Pink colour indicates that tube content turned aerobic and has been excluded from the analysis

Appendix 5 Initial experiment: Carbohydrate utilization test by strains of non-proteolytic *C. botulinum* types B, E and F

Carbohydrate/Strain	81-23	81-26	81-30	81-31*	83-01	86-17*	86-21	86-32	86-33	87-02	93-07	02-25*	05-2*5	06-01*	08-02
Control	6.53	6.10	6.39	6.30	6.33	6.37	6.42	6.59	6.40	6.41	6.29	6.43	6.36	6.41	6.43
Glycerol	5.47	5.57	5.33	5.68	5.41	5.39	6.13	5.84	5.26	5.62	5.53	5.61	5.49	5.89	6.28
Erythritol	6.23	6.31	6.51	6.31	6.40	6.35	6.53	6.61	6.29	6.39	6.27	6.26	6.39	6.38	6.39
D-arabinose	6.41	6.39	6.27	6.27	6.34	6.32	6.36	6.55	6.32	6.33	6.30	6.22	6.35	6.34	6.45
L-arabinose	6.41	6.27	6.41	6.17	6.38	6.31	6.34	6.54	6.34	6.28	6.21	6.25	6.42	6.33	6.28
D-ribose	5.11	5.15	5.14	4.88	5.07	5.06	4.95	5.29	5.38	5.38	5.24	5.13	5.55	5.18	5.15
D-xylose	6.27	6.25	6.37	6.19	6.35	6.24	6.24	6.32	6.33	6.34	6.30	6.18	6.44	6.28	6.31
L-xylose	6.41	6.37	6.52	6.33	6.40	6.38	6.47	6.43	6.41	6.40	6.36	6.27	6.55	6.33	6.50
D-adonitol	6.09	5.88	5.77	6.00	5.83	5.28	5.52	5.35	5.61	5.92	5.61	5.79	5.99	5.60	5.70
Methyl-βD-xylopyranoside	6.58	6.52	6.55	6.48	6.39	6.43	6.47	6.55	6.44	6.47	6.42	6.33	6.50	6.51	6.46
D-galactose	6.46	6.28	6.23	6.02	6.26	6.20	6.22	6.47	6.41	6.52	6.27	6.25	6.34	6.26	6.23
D-glucose	5.20	5.06	4.83	4.90	5.07	4.96	4.83	5.12	5.33	5.30	5.15	5.15	5.41	5.21	5.02
D-fructose	5.20	5.05	5.06	4.81	5.01	4.92	4.94	5.16	5.28	5.38	5.16	5.12	5.34	5.21	4.96
D-mannose	5.08	5.07	5.02	4.94	5.00	5.00	4.92	5.29	5.34	5.36	5.26	5.18	5.83	5.14	5.26
L-sorbose	6.41	6.12	6.39	6.13	6.30	6.29	6.16	6.43	6.40	6.63	6.22	6.17	6.43	6.38	6.18
L-rhamnose	6.55	6.42	6.56	6.27	6.35	6.50	6.35	6.46	6.36	6.45	6.38	6.37	6.53	6.40	6.42
Dulcitol	6.69	6.41	6.58	6.37	6.42	6.49	6.33	6.38	6.40	6.48	6.38	6.43	6.44	6.49	6.37
Inositol	6.01	5.48	6.57	5.92	5.24	5.75	5.38	5.13	5.74	6.08	5.38	5.18	5.82	6.21	5.18
D-mannitol	6.60	6.31	6.48	6.29	6.42	6.36	5.32	6.30	6.40	6.41	6.22	6.30	6.49	6.25	6.34
D-sorbitol	5.48	5.29	5.16	5.03	5.13	5.23	4.89	5.00	5.31	5.32	5.37	5.35	5.40	5.17	5.16
Methyl-αD-mannopyranoside	6.56	6.35	6.41	6.19	6.58	6.40	6.38	6.30	6.40	6.54	6.23	6.25	6.55	6.47	6.54
Methyl-αD-glucopyranoside	6.34	5.84	5.89	5.92	6.22	5.97	6.14	6.20	6.34	5.94	5.81	6.11	6.45	6.17	6.13
N-acetylglucosamine	5.39	5.28	5.00	5.14	5.57	5.16	5.08	5.47	5.45	5.40	5.30	5.31	5.47	5.37	5.39
Amygdalin	6.58	6.09	6.10	6.23	6.42	6.20	6.21	6.38	6.46	6.54	6.31	6.30	6.53	6.28	6.29

Arbutin	6.80	6.19	6.42	6.31	6.16	6.35	6.39	6.42	6.59	6.66	6.38	6.30	6.19	6.38	6.46
Esculin ferric citrate	6.78	6.63	6.55	6.51	6.59	6.59	6.54	6.69	6.74	6.61	6.51	6.55	6.57	6.57	6.68
Salicin	6.62	6.26	6.49	6.35	6.30	6.31	6.43	6.43	6.40	6.44	6.39	6.31	6.61	6.31	6.43
D-cellobiose	6.57	6.21	6.40	6.27	6.31	6.26	6.27	6.31	6.41	6.48	6.25	6.26	6.51	6.35	6.31
D-maltose	6.19	5.12	5.25	4.97	5.09	5.42	4.85	5.24	5.35	5.24	5.21	5.24	5.38	5.21	5.21
D-lactose (bovine origin)	6.35	6.21	6.43	6.19	6.59	6.36	6.42	6.35	6.34	6.38	6.26	6.31	6.59	6.34	6.77
D-melibiose	6.35	6.41	6.60	6.18	6.55	6.41	6.46	6.36	6.40	6.56	6.31	6.26	6.61	6.33	6.89
D-saccharose	5.24	5.09	4.98	4.96	5.18	5.12	5.04	5.27	5.26	5.47	5.29	5.14	6.11	5.30	5.08
D-trehalose	5.22	5.18	5.25	5.28	5.25	5.29	5.13	5.26	5.35	5.42	5.25	5.28	5.57	5.35	5.07
Inulin	6.37	6.33	6.69	6.24	6.48	6.43	6.40	6.38	6.42	6.56	6.28	6.24	6.67	6.39	6.13
D-melezitose	6.57	5.39	6.59	5.05	6.16	6.28	6.46	6.27	6.46	6.46	5.22	5.22	6.57	6.39	5.20
D-raffinose	6.43	6.58	6.44	6.31	6.54	6.45	6.65	6.38	6.48	6.48	6.34	6.38	6.60	6.41	6.33
Amidon (starch)	5.14	6.60	4.90	6.41	4.95	5.13	6.66	5.09	5.43	5.44	6.40	6.16	5.57	5.26	5.18
Glycogen	5.15	6.73	4.99	6.41	4.96	5.02	6.52	5.26	5.31	5.37	6.39	6.40	5.39	5.28	6.51
Xylitol	6.31	6.67	5.28	6.39	5.16	5.06	6.51	6.49	6.31	6.51	5.32	6.44	6.52	6.44	6.39
Gentiobiose	6.41	6.57	6.48	6.35	6.36	6.44	6.54	6.44	6.49	6.63	6.41	6.42	6.54	6.56	6.66
D-turanose	5.13	5.22	5.33	5.29	5.30	5.31	5.03	5.10	5.38	5.64	5.25	5.40	5.41	5.23	5.21
D-lyxose	6.36	6.48	6.60	6.27	6.37	6.47	6.42	6.41	6.44	6.56	6.33	6.31	6.50	6.33	6.43
D-tagatose	6.63	6.61	6.69	6.38	6.55	6.65	6.55	6.54	6.57	6.65	6.48	6.43	6.61	6.43	6.64
D-fucose	6.48	6.67	6.62	6.43	6.57	6.61	6.81	6.50	6.60	6.70	6.51	6.45	6.59	6.46	6.59
L-fucose	6.43	6.61	6.69	6.40	6.51	6.63	6.62	6.42	6.63	6.67	6.57	6.44	6.60	6.50	6.75
D-arabitol	6.54	6.73	6.79	6.53	6.52	6.58	6.58	6.48	6.74	6.69	6.62	6.64	6.64	6.59	6.70
L-arabitol	6.45	6.67	6.87	6.48	6.60	6.67	6.69	6.55	6.57	6.74	6.56	6.53	6.66	6.63	6.82
Potassium gluconate	6.83	6.73	7.57	6.58	7.30	7.20	6.78	6.67	6.70	7.27	6.73	6.63	6.68	6.75	6.74
Potassium 2-ketogluconate	7.08	7.27	7.13	7.18	7.09	7.14	7.17	7.21	7.39	7.23	7.20	7.21	7.21	7.27	7.28
Potassium 5-ketogluconate	6.53	6.76	6.37	6.45	6.52	6.53	6.47	6.68	6.60	6.68	6.56	6.52	6.67	6.60	6.64

The pH is shown at the end of the incubation period

* results present the average of two independent pH measurements

Appendix 6 Expert opinion questioner regarding the concentration of *C. botulinum* spores in batch of milk

Dear Expert -
As part of my project I am exploring different type of data sources that can be used in risk assessments.
I hope you can help me with an example by using expertise to estimate a few parameter values.

My problem centres on spores of *C. botulinum*, in fresh cows' milk. I am considering large batches of milk each with a volume ~10000 litres.

Based on a large number of batches there will be a single mean value for the number of spores per batch.
Can you estimate the largest value of the mean and the smallest value of the mean that are consistent with your belief concerning the number of spores in fresh milk?

Smallest mean = …… (spores per 10000 litres)
Largest mean = …… (spores per 10000 litres)

In a large number of batches each batch will have a different spore load.
Based on your experience what range of spore load would include 95% of all the spore loads in the batches of milk.

The smallest load of the 95% interval = …… (spores per 10000 litres)
The largest load of the 95% interval = …….. (spores per 10000 litres)

This is a very informal exercise.
None of these values will be used in a real safety assessment.
The values you give will only be used anonymously and will never be attributable.

Thank you for your response.