THE SAFETY OF PASTEURISED IN-PACK CHILLED MEAT PRODUCTS WITH RESPECT TO THE FOODBORNE BOTULISM HAZARD

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Abstract

There has been a substantial increase in sales of pasteurised in-pack chilled products over the last decade. It is anticipated that this trend will continue. These foods address consumer demand in being of high quality and requiring little preparation time. The microbiological safety of these foods commonly depends on a combination of a minimal heat treatment, refrigerated storage and a restricted shelf-life. The principal microbiological safety hazard for pasteurised in-pack meat products is foodborne botulism, as presented by non-proteolytic *Clostridium botulinum*. This review provides a summary of research that has contributed to the safe development of these foods without incidence of botulism.

Key words:
Clostridium botulinum*, botulism, pasteurised, sous-vide, meat, chilled*

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Introduction to pasteurised in-pack chilled products

Consumer demand for foods that require minimal preparation time compared with conventional meals, are of high quality, contain low levels of preservatives, and are only minimally processed has led to the development of foods that are pasteurised in-pack. These foods are also known as sous-vide foods, cook-chill ready-to-eat foods, and refrigerated processed foods of extended durability (REPFEDs). These foods are processed at a lower temperature (maximum generally within the range 70°-95°C) than, for example, canned foods. This heat process is intended to minimise loss of sensory and organoleptic quality. After heating, the food is cooled rapidly, and stored at refrigeration temperatures (1° - 8°C). These foods are not sterile, and product shelf-life is dependent on the heat treatment applied and storage temperature. In some circumstances intrinsic properties of the food (e.g. pH, water activity) may also contribute to an extended shelf-life. Typically, the shelf-life can be up to 42 days. Sales of pasteurised in-pack products and other chilled foods are currently increasing at a tremendous rate in many European countries. The European prepared chilled food sector showed a 50% increase in the period 1991-1996, to an estimated € 9,100 million (ECFF, 1998). In the UK alone, sales of prepared chilled foods increased by 45% between 1999 and 2003, to reach an estimated € 7,400 (Chilled Food Association, 2004).

Assessment of microbiological safety hazards associated with pasteurised in-pack chilled products

Pasteurised in-pack products are not sterile, and safety and quality is dependent on a combination of the minimal heat process, storage temperature, shelf-life and perhaps also intrinsic properties of the food (Peck,

1997). The mild heat treatment applied should eliminate cells of vegetative bacteria, but not bacterial or fungal spores. Thus, non-spore-forming pathogens such as *Listeria monocytogenes*, *Yersinia enterocolitica*, diarrheagenic *Escherichia coli*, *Campylobacter jejuni*, and salmonellae should be eliminated. By heating in-pack, post process contamination is prevented. Pasteurised in-pack products are frequently packed under vacuum or an anaerobic atmosphere. This restricts growth of microorganisms that require oxygen for growth, such as molds, but favours growth of microorganisms that can grow in the absence of oxygen. The storage of these foods at refrigeration temperatures prevents growth of mesophilic spore-forming bacteria, although growth may be possible if temperature abuse occurs. Overall, this minimal process favours spore-forming microorganisms that grow in the absence of oxygen at refrigeration temperatures. In particular, concern exists about the potential for growth and neurotoxin production by non-proteolytic *Clostridium botulinum* in the absence of a competing microflora, and the associated foodborne botulism hazard (Peck, 1997; Lund and Peck, 2000). It is essential that pasteurised in-pack products be developed without an associated increase in the incidence of foodborne botulism. Additionally, concern also exists about the hazard presented by psychrotrophic strains of *Bacillus cereus* (Carlin *et al.,* 2000). While food poisoning caused by *B. cereus* is serious, it is generally viewed as a less severe disease than foodborne botulism.

Recommendations and guidelines to ensure the safe production of pasteurised in-pack chilled products with respect to *C. botulinum*

Guidelines and a code of practice have been targeted at ensuring the safe production of these foods by preventing growth and toxin production by non-proteolytic *C. botulinum* (ACMSF, 1992, 1995; ECFF, 1996; Betts, 1996; Gould, 1996; Martens, 1997; Gould, 1999). Growth and toxin production by proteolytic *C. botulinum* is prevented by ensuring storage is below 10°C. Recommendations produced by the UK Advisory Committee on the Microbiological Safety of Food (ACMSF) on procedures to ensure safety with respect to non-proteolytic *C. botulinum* are summarised in Table 1. It is recommended that the heat treatments or combination processes deliver a safety a factor of 10^6 (a 6D [six decimal] process) with regard to spores of non-proteolytic *C. botulinum* (ACMSF, 1992; ECFF, 1996). Possible limitations of these recommendations are described in later sections. It is likely that for a majority of these pasteurised foods, safety will rely on either option (4) or (8), as detailed in Table 1.

Table 1 Recommended procedures to ensure the safety of pasteurised in-pack chilled products with respect to non-proteolytic *C. botulinum* (ACMSF 1992, 1995)

(1) storage at <3.0°C*
(2) storage at ≤5°C and a shelf-life of ≤10 days
(3) storage at 5°-10°C and a shelf-life of ≤5 days
(4) storage at chill temperature** combined with heat treatment of 90°C for 10 min or equivalent lethality (e.g. 70°C for 1675 min, 75°C for 464 min, 80°C for 129 min, 85°C for 36 min (ACMSF, 1992) (note that the European Chilled Food Federation (ECFF, 1996) recommended 80°C for 270 min, 85°C for 52 min)).
(5) storage at chill temperature combined with ≤pH 5.0 throughout the food
(6) storage at chill temperature combined with a salt concentration ≥3.5% throughout the food
(7) storage at chill temperature combined with $\leq a_w$ 0.97 throughout the food
(8) storage at chill temperature combined with a combinations of heat treatment and other preservative factors which can be shown consistently to prevent growth and toxin production by *C. botulinum.*

Notes:
* originally 3.3°C, but growth has now been demonstrated at 3.0°C (Graham *et al*., 1997)
** chill temperature is specified as 8°C in UK

Clostridium botulinum and foodborne botulism

Characteristics of Clostridium botulinum and other neurotoxin producing clostridia

Six physiologically and phylogenetically distinct Gram-positive spore-forming anaerobic bacteria can produce botulinum neurotoxin (Table 2), although the name of *C. botulinum* is retained to emphasise the importance of neurotoxin production (Lund and Peck, 2000). Some strains of *C. baratii* and *C. butyricum* also produce neurotoxin. For each of the six organisms, a non-neurotoxigenic phylogenetically equivalent organism is known (Hatheway, 1992). The different physiology of the six organisms is reflected in the circumstances in which they present a hazard. For example, proteolytic *C. botulinum* and non-proteolytic *C. botulinum* are responsible for most cases of foodborne botulism (Lund and Peck, 2000). Differences in the physiology of these two organisms are summarised in Table 3, and because of these differences they present a hazard in different types of foods. Proteolytic *C. botulinum* is a mesophile, and produces spores of high heat resistance. The canning process for low-acid foods is designed to inactivate spores of this organism, and botulism outbreaks have occurred when the full heat treatment has not been appropriately delivered. Spores of non-proteolytic *C. botulinum* are of moderate heat resistance, but this organism can multiply and form neurotoxin at temperatures as low as 3.0°C. Botulism outbreaks associated non-proteolytic *C. botulinum* have occurred most frequently with processed fish, with for example outbreaks involving vacuum packed smoked fish reported in Sweden and Germany. This organism is the principal concern for the safety of pasteurised in-pack chilled meat products.

Table 2. Characteristics of the six phylogenetically distinct clostridia that can produce the botulinum neurotoxin

Neurotoxigenic organism	Neurotoxins formed	Non-neurotoxigenic equivalent organism	Associated with foodborne botulism
C. botulinum Group I (proteolytic)	A, B, F	*C. sporogenes*	++
C. botulinum Group II (non-proteolytic)	B, E, F	no name given	++
C. botulinum Group III	C, D	*C. novyi*	-
C. botulinum Group IV (*C. argentinense*)	G	*C. subterminale*	-
C. baratii	F	all typical strains	+
C. butyricum	E	all typical strains	+

++ commonly associated, + rarely associated, - not known to be associated

Table 3 Characteristics of the two physiologically distinct clostridia most commonly responsible for foodborne botulism

	Proteolytic *C. botulinum* (*C. botulinum* Group I)	non-proteolytic *C. botulinum* (*C. botulinum* Group II)
neurotoxins formed	A, B, F	B, E, F
minimum growth pH	4.6	5.0
minimum growth temperature	10-12°C	3.0°C
maximum growth NaCl	10%	5%
spore heat resistance ($D_{100°C}$)	>15 min	<0.1 min
foods involved in botulism outbreaks	home canned foods, faulty commercial processing	fermented marine products, dried fish, vacuum packed fish
potential food problems	canned foods	pasteurised in-pack chilled products

Neurotoxins and characteristics of botulism

Foodborne botulism is a severe but rare disease. It is an intoxication resulting from consumption of pre-formed botulinum neurotoxin in food, with as little as 30 ng of neurotoxin sufficient to cause illness and even death. The consumption of as little as 0.1g of food in which a neurotoxin-producing clostridia has grown can result in botulism (Lund and Peck, 2000). There are seven botulinum neurotoxins (A to G), with the type of neurotoxin formed dependent on the producing organism (Table 2). The neurotoxins were originally distinguished on the basis of antigenic response. More recently, however, the amino acid sequence and mode of action of all the neurotoxins have been established (Dodds and Austin 1997; Lund and Peck, 2000). All seven botulinum neurotoxins comprise a heavy and light chain, and are often associated with other proteins (e.g. haemagglutinin, non-toxin non-haemagglutinin). The heavy chains deliver the light chains to the cytosol of the motor neuron, their site of action. The light chains possess zinc endopeptidase activity, and cleave protein components of the acetylcholine-containing synaptic vesicle docking/fusion complex. Each light chain cleaves a specific protein in this complex at a specific site. This cleavage prevents binding of acetylcholine-containing synaptic vesicles, preventing neurotransmitter release and leads to flaccid paralysis of the muscle. Typically symptoms of botulism are neurological and include, blurred vision, dysphagia (difficulty swallowing), generalised weakness, nausea/vomiting, dysphonia (difficulty speaking), dizziness/vertigo, and muscle weakness. Flaccid paralysis of the respiratory muscles can result in death if not treated. In many countries, equine antitoxin is administered in cases of foodborne botulism. Rapid treatment with equine antitoxin and supportive therapy has led to a reduction in the fatality rate to approximately 10% of cases, although full recovery may take many months or even longer. A fatality rate of 10% of cases is very high for a foodborne illness.

Incidence of spores of C. botulinum in foods and the environment.

Spores of proteolytic *C. botulinum* and of non-proteolytic *C. botulinum* are found in soils, sediments and the gastrointestinal tract of animals, and their distribution has been reviewed previously (Dodds, 1992a, 1992b; Dodds and Austin, 1997; Lund and Peck, 2000). Recently, information has been published on the incidence of spores in the environment and in foods in France (Fach *et al*., 2002; Carlin *et al*., 2004). Although often present in low numbers, their ubiquitous nature ensures that raw products cannot be guaranteed free of spores. Foods which are, or can become, anaerobic may allow growth of *C. botulinum* and must therefore be subjected to treatments that destroy spores, or stored under conditions that prevent growth and toxin formation.

Outbreaks of foodborne botulism

The name “botulism” was given to a disease reported in central Europe several hundred years ago that was frequently associated with consumption of blood sausage (“botulism” is derived from the Latin *botulus* meaning sausage). At the end of the nineteenth century, Emile van Ermengem, first isolated a causative organism from home made raw salted ham and the spleen of a man who later died of botulism. In the early part of the twentieth century, many outbreaks of botulism were identified across the world, and were frequently associated with the increased use of canning processes to extend shelf-life. By implementing effective control measures, the incidence of botulism is now lower than a century ago. Foodborne botulism involving commercial processing is uncommon, but on the rare occasions when commercial foods are involved, the medical and economic consequences can be high. It has been estimated that in the USA the cost per case of botulism is approximately $30 million, compared with $10,000-12,000 for each case of illness associated with *Listeria monocytogenes* and *Salmonella* (Setlow and Johnson, 1997). Today, most outbreaks of foodborne botulism are associated with home-prepared foods, where known control measures have not been implemented. For example, in Poland, 1301 outbreaks were reported between 1984-1987, and in Russia, 542 outbreaks were described between January 1998 and September 1999. These high incidences were associated with an increased reliance on the home bottling/canning of foods, reflecting difficult economic conditions. The incidence in Poland has fallen considerably in recent years. Many other countries have lower, but significant rates of foodborne botulism. For example, over the past 20 years, approximately 20-40 cases have been reported annually in Italy, Germany, France, and USA. Examples of recent outbreaks of foodborne botulism are given in Table 4 (see end of the paper). A recent finding has been the association of neurotoxigenic strains of *C. baratii* and *C. butyricum* with foodborne botulism. Outbreaks involving neurotoxigenic *C. butyricum* type E have been reported in China, India and Italy (Anniballi *et al.,* 2002). A suspected outbreak involving neurotoxigenic *C. baratii* type F, was reported in USA in 2001, and was associated with consumption of spaghetti noodles and meat sauce by a 41 year old

woman. A full recovery was eventually made, although 12 weeks were spent on a life support machine (Harvey *et al*., 2002).

Many cases of foodborne botulism have been associated with meat products (Hauschild, 1992; Lucke and Roberts, 1992). In France, Germany and Poland most cases of foodborne botulism are associated with consumption of meat dishes, while in Spain and Italy other foods are more frequently implicated (Lund and Peck, 2000). A large proportion of outbreaks in Germany and France are associated with home or farm salted ham. A majority of meatborne cases in Poland are associated with the home preparation of pork in glass jars (Lucke and Roberts, 1992). Several examples of recent outbreaks of meatborne botulism are included in Table 4. Commercial pasteurised in-pack chilled products have a good safety record with respect to foodborne botulism, although outbreaks involving clam chowder (USA, 1994) and vacuum packed fish (Germany, 1997) have occurred following temperature abuse. It is essential, however, that as new types of food are developed, they are developed safely with respect to the foodborne botulism hazard. Outbreaks involving home prepared vacuum packed fish are also reported (Table 4; see end of the paper).

Control of foodborne botulism hazard presented by proteolytic *C. botulinum* in pasteurised in-pack chilled meat products

Factors influencing growth and toxin formation

Providing that pasteurised in-pack chilled products are stored at an appropriate temperature, growth and toxin production by proteolytic *C. botulinum* is prevented. There is, however, concern that proteolytic *C. botulinum* may grow and form toxin in products that are temperature abused. The effect of individual environmental factors on growth of proteolytic *C. botulinum*, under otherwise optimum conditions, has been established. The minimum temperature at which growth and toxin production occurs is within the range of 10°-12°C. Growth and toxin formation have been described at 12°C in 3-4 weeks (Smelt and Haas, 1978; Peck, 1999), and Ohye and Scott (1953) determined growth rates at 12.5°C. Attempts to establish growth at 10°C or below have been generally unsuccessful (e.g. Ohye and Scott, 1953; Smelt and Hass, 1978; Peck, 1999). Although Tanner and Oglesby (1936) reported growth from vegetative inocula at 10°C, this observation was marred by a temporary rise in the incubator temperature to 20°C, and in the words of the authors "the results of this test may not be very significant". Growth from a spore inoculum was not observed at 10°C in 10 weeks (Tanner and Oglesby, 1936). It is generally accepted that growth is prevented at pH 4.6 or below, that 10% NaCl will prevent growth, and that the minimum water activity permitting growth is 0.93 and 0.96 with glycerol and NaCl, respectively, as the humectants (Hauschild, 1989; Lund and Peck, 2000). The use of other factors to control or prevent growth of proteolytic *C. botulinum* has been reviewed (Hauschild, 1989; Lund and Peck, 2000). The safety of some foods with respect to proteolytic *C. botulinum* may rely on a combination of factors rather than on one single factor. The effect of combinations of pH and NaCl concentration/water activity on time to growth has been determined in laboratory medium (Baird-Parker and Freame, 1967), vacuum-packed potatoes (Dodds, 1989) and in lumpfish caviar (Hauschild and Hilsheimer, 1979). Predictive models that quantify the effect of combinations of environmental conditions on the growth response of proteolytic *C. botulinum* have been developed (Baker and Genigeorgis, 1992; Lund, 1993; Dodds, 1993; Lund and Peck, 2000). These models provide information on interactions between two or more factors, allow predictions to be made for sets of conditions not tested (within the interpolation zone), and are of use in the targeting of challenge testing.

Heat resistance of spores of proteolytic C. botulinum

The relatively high heat resistance of spores of proteolytic *C. botulinum* makes their survival in low acid canned foods a subject of considerable importance. In response to outbreaks of botulism associated with the consumption of inadequately heat-treated foods, studies on the heat resistance of spores of proteolytic *C. botulinum* began in the early part of the twentieth century. From extensive research, it is generally accepted that a heat treatment at 121.1°C for 3 min (or heat treatments of equivalent lethality at other temperatures) will reduce the number of spores of proteolytic *C. botulinum* by a factor of 10^{12} (a 12D process), and this was adopted as the minimum standard for a "botulinum cook" for low acid canned foods (Stumbo *et al*., 1975). The heat resistance of spores of proteolytic *C. botulinum* is generally taken as $D_{121°C}$ = 0.2 min and z = 10°C (Stumbo *et al*., 1975), thus by extrapolation, at 91°C the D-value would be 200 min. The minimal heat treatments applied to pasteurised in-pack and related foods would, therefore, be unlikely to have any significant effect on spores of proteolytic *C. botulinum.*

Combinations of heat treatment, storage temperature and other factors that prevent growth and toxin production from spores of proteolytic C. botulinum

Studies on proteolytic *C. botulinum* have focused on assessing the hazard following temperature abuse. In one study, spores of a mixture of strains of proteolytic *C. botulinum* were inoculated into meat slurry (used as a model food) prepared under N_2, heated at 95°C for 23 min, cooled and subsequently incubated at refrigeration and abuse temperatures (Table 5). The heat treatment had only a marginal effect on time to growth. Growth and toxin production did not occur at 8°C, and at 12°C growth was observed within 21 days and toxin production confirmed (although time to growth was rather variable). At 16°C growth was first observed in 4-7 days, and at 25°C growth was first observed in 2 days (Table 5). These results indicate that if pasteurised in-pack products are abused at 16°C or higher (and possibly even at 12°C), then proteolytic *C. botulinum* is a serious concern.

Evaluation of time to growth and toxin formation in foods

Tests on time to toxin formation have been carried out with pasteurised in-pack pasta products subject to temperature abuse (Table 6). Also, while Del Torre *et al*. (1998) detected toxin formation in salmon-filled tortelli stored at 20°C for 30 days, toxin formation was not detected when this product was stored at 12°C for 50 days. Simpson *et al.* (1995) detected toxin formation after storage at 15°C for 14 days, when a spaghetti and meat-sauce was inoculated with 10^3 spores/g of proteolytic *C. botulinum*. These data emphasise the danger presented by proteolytic *C. botulinum* if these food products are temperature abused.

Table 5 Effect of heat treatment, subsequent incubation temperature and inoculum concentration on time to growth from spores of proteolytic *C. botulinum* types A, B and F in meat slurry (Plowman and Peck, unpublished data)

Incubation temperature	Heat treatment	Incubation time (days) to first observation of growth for each inoculum concentration	
		10^3 spores/tube	10^6 spores/tube
8°C	None	not tested	>60
12°C	None	49	21
	95°C/23min	56	28
16°C	None	6	4
	95°C/23min	7	4
25°C	None	2	2
	95°C/23min	2	2

Table 6 Examples of reported time to toxin formation in pasteurised in-pack chilled products inoculated with a low concentration of spores of proteolytic *C. botulinum* (Glass and Doyle, 1991; Del Torre *et al.,* 1998)

Pasta filling	Product details			Time (days) to toxin formation at specified temperature[a]	
	a_w	pH	atmosphere (CO_2:N_2:O_2)	20°C	30°C
Cheese	0.94	5.7	80:20:0	-[b]	70-NT
Meat	0.95	6.2	0:100:0	-	35-42
ricotta/spinach	0.95	5.9	15:83:2	30-50	-
Salmon	0.95	6.1	15:83:2	15-30	-
Meat	0.95	6.2	15:83:2	30-50	-
Artichoke	0.96	5.6	15:83:2	50-NT	-

[a] - last negative test and first positive test are given (NT = no toxin detected), inoculum spore concentration 1-7 x10^2 spores/g

[b] - not determined

Control of infant botulism hazard presented by proteolytic *C. botulinum* in pasteurised in-pack chilled meat products

Infant botulism is an infection. An immature intestinal flora in infants is unable to prevent colonisation by proteolytic *C. botulinum* (and also neurotoxigenic strains of *C. baratii* and *C. butyricum*), allowing ingested spores to germinate leading to cell multiplication and neurotoxin production. Infants aged between two weeks and six months are most susceptible. Symptoms typically include extended constipation and flaccid paralysis. Infant botulism is rarely fatal. Two sources of spores have been identified, honey and general environmental contamination (e.g. soil, dust). It is estimated that between 10 and 100 spores are sufficient to bring about infection. The first clinical cases of infant botulism were described in USA in 1976, although subsequent investigations revealed earlier cases. Infant botulism has now been reported in twelve European countries, and about half of these were associated with a history of honey consumption (Aureli *et al*., 2002). In view of this association, there are recommendations in several countries that jars of honey should carry a warning indicating that the product is not suitable for infants less than twelve months of age. A disease similar to infant botulism also very rarely affects adults, and occurs when competing bacteria in the normal intestinal flora have been suppressed (e.g. by antibiotic treatment).

It is anticipated that the heat treatment delivered to pasteurised in-pack chilled products will bring about little reduction in numbers of spores of proteolytic *C. botulinum*. While the foodborne hazard is controlled by storage at chilled temperature, the infant botulism hazard is not controlled (since spores will remain in the food, but will be unable to germinate and lead to growth in the food). Thus, there is a concern that these minimally processed foods may represent a risk of infant botulism (and possibly also infectious botulism in susceptible adults). This concern is particularly great for pasteurised in-pack products that are specifically targeted at infants less than twelve months of age.

Control of foodborne botulism hazard presented by non-proteolytic *C. botulinum* in pasteurised in-pack chilled meat products

Factors influencing growth and toxin formation

The minimum temperature at which growth and toxin production have been described is 3.0°C (Graham *et al*., 1997). In this study, vials containing 10 ml of PYGS (peptone, yeast extract, glucose, starch) medium were inoculated at 10^4 spores/ml, and growth was observed at 3.0°C after seven weeks, 3.1°C after six weeks, and 3.2°C after five weeks. The presence of toxin was confirmed. Earlier studies had demonstrated growth and toxin production at 3.3°C within five weeks (Schmidt *et al*., 1961; Eklund *et al*., 1967a, b). Growth and toxin production have not been detected during incubation at 2.1-2.5°C for 90 days (Ohye and Scott, 1957; Schmidt *et al*., 1961; Eklund *et al*., 1967a, b; Graham *et al*., 1997). Maintaining pasteurised in-pack products at a temperature of <3.0°C might be possible in some circumstances (e.g. institutions, catering establishments), but there is doubt as to whether temperatures in this range can always be maintained throughout the distribution chain, particularly in products intended for domestic use. Indeed, regulations require foods in this group to be held at 0°-3°C in Spain, 0°-4°C in France, ≤7°C in Belgium and ≤8°C in the UK (Martens, 1997). Thus, retail products should contain additional hurdles. It is generally recognised that growth and toxin production do not occur below pH 5.0, at a NaCl concentration above 5%, and that the minimum water activity permitting growth is 0.94 and 0.97 with glycerol and NaCl, respectively, as the humectants (Baird-Parker and Freame, 1967; Hauschild, 1989; Graham *et al*., 1996b; Lund and Peck, 2000). The effect of other preservative factors on growth of non-proteolytic *C. botulinum* has been reviewed elsewhere (e.g. Hauschild, 1989; Lund and Peck, 2000). The effect of redox potential and oxygen concentration on growth from unheated spores has been quantified (Lund, 1993). Furthermore, in studies with a model food system (meat slurry), an initial aerobic atmosphere (20% oxygen) did not restrict growth compared to a control atmosphere of nitrogen (Peck, 1999). In these circumstances, although the atmosphere was aerobic, the model food was sufficiently reduced so as to support growth and toxin production by non-proteolytic *C. botulinum*. The use of oxygen as a preservative factor is therefore cautioned, since despite the presence of oxygen, the food itself may be sufficiently reduced and support growth and toxin production by non-proteolytic *C. botulinum* (Snyder, 1996).

In some foods, safety with respect to non-proteolytic *C. botulinum* may be dependent upon more than one preservative factor. For example, although a sub-optimal pH or NaCl concentration might not prevent growth individually, they might in combination. The effect of combinations of pH and NaCl concentration on time to growth at chilled temperatures has been reported (Graham *et al*., 1997). An important development in describing the effect of combinations of preservative factors has been that of predictive models. Predictive models for non-proteolytic *C. botulinum* have been developed in foods and whilst they may predict well for some food groups, they may be of limited use in other types of food. Other models have been developed in laboratory media and may be rather more generic in application. These models provide information on interactions between two or more factors, and can be used to reduce the amount of challenge testing required to ensure product safety. Models have been developed that deliver growth curves, describe the effect of single and multiple factors on the probability of growth, or on time to toxin production at a single inoculum level (Baker *et al*., 1990; Baker and Genigeorgis, 1992; Dodds, 1993; Meng and Genigeorgis, 1993; Lund, 1993; McClure *et al*., 1994; Graham *et al*., 1996b; Whiting and Oriente, 1997; Lund and Peck, 2000; Fernandez *et al*., 2001). Where tested, predictions from these models generally compare well with observed growth and toxin production in independent datasets, giving the user confidence that these models can be used to target, more effectively, challenge testing. Some of these models are freely available through predictive modelling packages [Growth Predictor (www.ifr.ac.uk/Safety/GrowthPredictor/default.html), Pathogen Modeling Program (www.arserrc.gov/mfs/PMP6_Start.htm)]. Published (and in some cases also unpublished) original growth and death curves are compiled and also available free of charge in ComBase (www.combase.cc).

Heat resistance of spores of non-proteolytic C. botulinum

Spores of non-proteolytic *C. botulinum* are of moderate heat resistance, and are considerably less heat resistant than those of proteolytic *C. botulinum*. For example, heating spores for one min in phosphate buffer (pH 7.0) at 85°C, followed by enumeration of survivors on a nutrient medium gave in excess of a 5D kill (Peck *et al*., 1992a). Studies of spore heat resistance by thermal death time methods and by enumeration of survivors have led to estimates of D-values in phosphate buffer, the highest of which have generally been within the range of $D_{82.2°C}$ = 0.5-2.4 min (Lund and Peck, 2000). The measured heat resistance of spores of non-proteolytic *C. botulinum* can be influenced by the menstruum in which the spores are heated and the nutrient medium used for enumeration of survivors. When spores were heated in a model food (meat slurry, pH 6.5), heat treatments at 70°C for >2545 min, 75°C for 1793 min, 80°C for >363 min, 85°C for 36 min, and 90°C for 10 min were necessary to prevent growth from an inoculum of 10^6 spores of non-proteolytic *C. botulinum* (Fernandez and Peck, 1997). Thus, the heat treatments advocated by the ACMSF and the ECFF at 70°-80°C (Table 1) failed to reduce the number of spores of non-proteolytic *C. botulinum* by a factor of 10^6 (a 6D process). The advocated heat treatments were, however, adequate when combined with refrigerated storage and a restricted shelf-life (Fernandez and Peck, 1997).

The heat treatments described in the previous paragraph result in sub-lethal injury to spores of non-proteolytic *C. botulinum*. In particular, the heat treatments inactivate the spore germination system (Peck *et al*., 1992b; Lund and Peck, 1994). A proportion of these heat-damaged spores are permeable to lysozyme, and are able to germinate and give growth in/on media containing lysozyme, giving biphasic survival curves (Sebald and Ionesco, 1972; Alderton *et al*., 1974; Smelt, 1980; Peck *et al*., 1992a, 1992b, 1993; Lund and Peck, 1994; Stringer and Peck; 1996; Peck, 1997). Lysozyme can diffuse through the coat of a proportion (for most strains 0.1 - 1%) of heat-damaged spores, inducing germination by hydrolysing peptidoglycan in the spore cortex (Gould, 1989). Treatment of the heated spores with sodium thioglycollate disrupts the coat of a majority of the spores allowing lysozyme access to the cortex, inducing germination and subsequent growth of vegetative bacteria from a majority of sub-lethally heat-damaged spores. The straight line obtained with thioglycollate-treated spores provides evidence of the mode of action of lysozyme (Peck *et al*., 1992b, 1993; Lund and Peck, 1994; Peck, 1997). Following heat treatment in phosphate buffer, D-values for lysozyme-permeable spores were: $D_{85°C}$ = 100 min, $D_{90°C}$ = 18.7 min, $D_{95°C}$ = 4.4 min (Peck *et al*., 1993). These D-values are approximately two orders of magnitude greater than those obtained when spores were recovered in the absence of lysozyme. Lysozymes from several sources increase the measured heat resistance of spores of non-proteolytic *C. botulinum*, including; hen egg white lysozyme, other type c lysozymes (e.g. from other bird's eggs), other enzymes (chitinase, papain), fruit and vegetable extracts, egg yolk emulsion and horse blood (Peck and Stringer, 1996; Stringer and Peck, 1996; Stringer *et al*., 1999). Lysozyme activity has also been detected in many raw foods, and in most cases the activity is higher than that required to

increase the measured heat resistance of spores of non-proteolytic *C. botulinum* (Peck *et al*., 1992a; Lund and Peck, 1994; Peck and Stringer, 1996). Proposals have also been made to add hen egg white lysozyme and other lytic enzymes to foods as a preservative factor (Nielsen, 1991; Fuglsang, 1995), and enzymes capable of initiating germination may also be produced by other microorganisms (Lund and Peck, 1994). Because of this widespread occurrence of enzymes with lysozyme activity and the relatively high heat stability of lysozyme (Proctor and Cunningham, 1988; Lund and Peck, 1994; Peck and Fernandez, 1995), the effect of lysozyme on sub-lethally heat damaged spores is potentially important in foods that rely on heat-inactivation of spores of non-proteolytic *C. botulinum* for safety. In studies with a model food (meat slurry) with no added lysozyme, heat treatments of 85°C for 36 min, 90°C for 10 min and 95°C for 15 min each prevented an inoculum of 10^6 spores of non-proteolytic *C. botulinum* leading to growth and toxin formation at 25°C in 60 days (Peck *et al*., 1995; Peck and Fernandez, 1995; Graham *et al*., 1996a; Fernandez and Peck, 1997). When hen egg white lysozyme was added to this model food prior to heating (at 625-2400 units/ml), and heat treatments of 85°C for 84 min, 90°C for 34 min or 95°C for 15 min were applied, growth was observed at 25°C after 13, 14 and 32 days, respectively (Peck *et al*., 1995; Fernandez and Peck, 1999). A similar effect may also have been noted with endogenous lysozyme in crabmeat. Enzymes with lysozyme activity have been found in crabmeat, and it is estimated that the concentration is approximately 200 µg/g prior to heating (Lund and Peck, 1994). Heat treatments at 88.9°C for 65 min, 90.6°C for 65 min, 92.2°C for 35 min, or 94.4°C for 15 min were required to prevent growth and toxin formation from 10^6 spores of non-proteolytic *C. botulinum* when the heat treatment was followed by enrichment at 27°C for 150 days (Peterson *et al*., 1997). These heat treatments are substantially greater than those advocated by the ACMSF (1992) and ECFF (1996). The effect of intrinsic properties of foods and other environmental factors on the thermal inactivation of spores of non-proteolytic *C. botulinum* has been studied, and predictive models of thermal inactivation have been produced (Juneja and Eblen, 1995; Juneja *et al*., 1995a, 1995b; Peck and Stringer, 1996; Lindstrom *et al*., 2003).

Combinations of heat treatment, storage temperature and other factors that prevent growth and toxin production from spores of non-proteolytic C. botulinum

It is important to define combinations of preservative factors (e.g. heat treatment and subsequent storage at refrigeration temperatures) that provide an appropriate degree of protection against growth and toxin production by non-proteolytic *C. botulinum*, since the microbiological safety of most pasteurised in-pack products relies on such a process. In studies in which meat slurry was used as a model food, the effect of heat treatment at 65°-95°C combined with subsequent storage at 5°-25°C on time to growth from an inoculum of 10^6 spores of a mixture of strains of non-proteolytic *C. botulinum* was determined (Table 7). Growth was confirmed by the presence of *C. botulinum* neurotoxin. In tests where lysozyme was not added, heat treatments of 70°C for 2545 min, 75°C for 464 min, 80°C for 70 min, 85°C for 23 min, and 90°C for 10 min each prevented growth within 42 days when combined with storage at 8°C (Table 7). The heat treatment at 70°C is greater than that in current recommendations, while the heat treatments at 80°C and 85°C are lower (ACMSF, 1992; ECFF, 1996). A predictive model was developed that described the effect of heat treatment and storage temperature on time to growth (Fernandez and Peck, 1997). The model provided a good description of the data used to generate it, and predictions from the model compared well with observations of time to growth/toxin production in independent experiments (Fernandez and Peck, 1997).

Table 7 Combined effect of lysozyme, heat treatment and subsequent storage temperature on time to visible growth from 10^6 spores of non-proteolytic *C. botulinum* types B, E and F in meat slurry (Peck *et al*., 1995; Peck and Fernandez, 1995; Fernandez and Peck, 1997; Fernandez and Peck, 1999)

Heat treatment	Added lysozyme (units/ml)	Time (days) to growth at specified storage temperature					
		5°C	6°C	8°C	12°C	16°C	25°C
none	0	14	-[a]	7	4	2	1
70°C/529 min	0	35	-	9	7	4	1
70°C/1000 min	0	57	-	21	8	5	2
70°C/1596 min	0	NG[b]	-	35	14	8	3
70°C/2545 min	0	NG	-	50	22	8	3
75°C/285 min	0	NG	-	33	13	10	4
75°C/464 min	0	NG	-	48	38	23	8
75°C/734 min	0	NG	-	NG	18	15	5
75°C/1072 min	0	NG	-	NG	NG	NG	9
80°C/11 min	0	58	-	24	12	7	4
80°C/70 min	0	NG	-	44	19	8	5
80°C/184 min	0	NG	-	NG	37	21	11
80°C/295 min	0	NG	-	NG	NG	NG	33
85°C/23 min	0	NG	-	NG	30	38	15
85°C/36 min	0	NG	-	NG	NG	NG	NG
85°C/52 min	0	NG	-	NG	NG	NG	NG
90°C/10 min	0	NG	-	NG	NG	NG	NG
none	625	-	7	4	2	-	1
65°C/364 min	625	-	11	4	2	-	1
70°C/8 min	625	-	8	6	4	-	1
75°C/27 min	625	-	13	9	5	-	1
70°C/529 min	2400	39	-	9	7	4	1
70°C/1000 min	2400	48	-	17	8	3	2
70°C/1596 min	2400	NG	-	38	16	9	3
70°C/2545 min	2400	NG	-	NG	27	9	4
75°C/285 min	2400	NG	-	33	13	11	4
75°C/464 min	2400	NG	-	NG	23	15	6
75°C/734 min	2400	NG	-	NG	21	11	7
75°C/1072 min	2400	NG	-	NG	NG	NG	10
80°C/11 min	2400	46	-	24	10	8	4
80°C/23 min	625	-	40	23	12	-	3
80°C/184 min	2400	NG	-	NG	NG	19	11
80°C/295 min	2400	NG	-	NG	NG	NG	16
85°C/19 min	625	-	53	53	42	-	6
85°C/23 min	2400	NG	-	NG	23	15	6
85°C/36 min	2400	NG	-	48	26	15	7
85°C/52 min	2400	NG	-	NG	46	23	8
90°C/10 min	2400	NG	-	54	30	18	6
90°C/20 min	480	-	-	NG	51	29	20
95°C/15 min	625	-	NG	NG	NG	-	32

[a] not tested, [b] no growth at day 60

In view of previous comments, it is also appropriate to consider foods that contain lysozyme (Table 7). When relatively gentle heat treatments (e.g. heating at 70°C or 75°C) were applied, a large number of spores survived the heat treatment as measured by recovery in the presence and absence of lysozyme (Fernandez and Peck, 1999). In these circumstances, addition of lysozyme prior to heating had little effect on time to growth in a model food system (Fernandez and Peck, 1999). When more severe heat treatments were applied (e.g. heating at 85°C to 95°C), few or no spores were recovered in the absence of lysozyme, while a substantial number of spores were able to germinate and give growth in the presence of lysozyme (Fernandez and Peck, 1999). Following these heat treatments growth was observed more rapidly and over a wider range of conditions when hen egg white lysozyme was added prior to heating, than when no addition of lysozyme was made (Fernandez and Peck, 1999). From these results combinations of heat treatment and storage temperature required for a specific shelf-life can be estimated for a product suspected to contain lysozyme. These data have been used to develop a predictive model that describes the effect of heating temperature (70°-90°C), heating time (10-2545 min), and storage temperature (5°-25°C) on time to growth with lysozyme added prior to heating (Fernandez and Peck, 1999). Predictions from this model also compared well with the experimental results used to produce it, and with independent reports of time to growth/toxin production (Fernandez and Peck, 1999).

In some pasteurised in-pack products, factors other than heat treatment and subsequent refrigerated storage may also contribute to ensure safety with respect to non-proteolytic *C. botulinum*. The combined effects of heat treatment, pH, NaCl concentration, lysozyme addition, and subsequent storage temperature on time to growth from 10^6 spores of non-proteolytic *C. botulinum* in meat slurry have been described (Figure 1; see the end of the paper). In these tests, meat slurry was prepared at different pH values (pH 5.6-6.5) and different NaCl concentrations (0.6% - 4.9%, salt-on-water). Hen egg white lysozyme was either not added (Figure 1a; see the end of the paper), or was added at 1,200 units/ml prior to heat treatment (Figure 1b; see the end of the paper). Spores from eight strains of non-proteolytic *C. botulinum* types B, E and F were then added in equal numbers to give a final concentration of 10^6 spores per tube of meat slurry. The heat treatment applied were the equivalent of approximately 20 min at each of 80°C, 85°C, 90°C and 95°C (see Graham *et al*., 1996b). After rapid cooling, the tubes were incubated at 5° - 16°C for 90 days. Tubes were observed for growth, and the presence of toxin production confirmed. In the absence of added lysozyme, growth and toxin were observed in tubes that were not heated and tubes heated at 80°C for 20 mins, but not in tubes heated at 85°C or higher (Figure 1a; see the end of the paper). For tubes heated at 80°C for 20 mins, combinations of pH, NaCl concentration and shelf-life were identified that prevented growth from 10^6 spores of non-proteolytic *C. botulinum* (Figure 1a; see the end of the paper). In the presence of added lysozyme, growth and toxin were observed in tubes that were not heated and tubes heated at 80°C for 20 mins, 85°C for 20 mins and 90°C for 20 mins, but not in tubes heated at 95°C for 20 mins (Figure 1b; see the end of the paper). Again combinations of pH, NaCl concentration and shelf-life were identified that prevented growth from 10^6 spores of non-proteolytic *C. botulinum* (Figure 1a; see the end of the paper).

It is important to recognise that the heat treatments advocated by the ACMSF (1992) and ECFF (1996) do not, in themselves deliver a 6D process. For many pasteurised in-pack products, it is necessary to identify a combination process that achieves a 6D process. This will probably include a consideration of storage temperature and shelf-life, and possibly also intrinsic properties of the food or mild preservatives that may be added. It should be noted that in some circumstances, the recommendations of the ACMSF (1992) and ECFF (1996) appear to leave only a small margin of safety, but in other circumstances might lead to unnecessary overprocessing. By adopting effective combination processes it should be possible to avoid potentially dangerous situations and maintain an organoleptically acceptable product. The identification of appropriate combinations of mild preservative factors is an important step forward in the development of rational processes for pasteurised in-pack meat products.

Table 8 Examples of reported time to toxin formation in foods inoculated with a low concentration of spores of non-proteolytic *C. botulinum* (Lund and Peck, 2000)

Food[a]	Inoculum (spores/g)	Time (days) to toxin formation at specified temperature					
		4°C	5°C	8°C	10°C	12°C	16°C
cod[1]	5x10[1]	18	-	8	-	6	-
turkey[2]	10^2	-[b]	-	8	-	5	2
salmon[3]	10^2	21	-	6	-	3	-
catfish[4]	10^2	46	-	6	-	-	3
red snapper[5]	10^3	21	-	9	-	3	3
cooked cauliflower[6]	10^3	-	19	17	13	-	4
cooked mushroom[6]	10^3	-	20	10	10	-	3

[a] references: 1, Post *et al.* (1985); 2, Meng and Genigeorgis (1993); 3, Garcia and Genigeorgis (1987); 4, Reddy *et al*. (1997); 5, Baker *et al.* (1990) Lindroth and Genigeorgis (1986); 6, Carlin and Peck (1996).
[b] tests not carried out at this temperature

6 days at 8°C (Table 8). Other studies have evaluated the safety of pasteurised in-pack chilled meat products. In one study, it was found that the heat treatments applied by industry failed to eliminate small or large numbers of spores of non-proteolytic *C. botulinum* (Table 9). Two of these heat processes (applied to beef cubes and beef liver cubes) followed recommendations made by the ACMSF (1992) and ECFF (1996), but failed to deliver a 6D process. Neurotoxin was formed when two of these products (pork cubes and ground beef) were stored at 8°C, but not at 4°C (Hyytia-Trees *et al*., 2000). During growth, non-proteolytic *C. botulinum* does not produce off odours to the same extent as some other bacteria (e.g. proteolytic clostridia). Comparisons of time to spoilage with time to toxin production by non-proteolytic *C. botulinum* have shown that toxin production often occurs in the absence of spoilage (Peck, 1997).

Process risk modelling for safety of pasteurised in-pack products

A relatively recent development has been that of process risk models for these foods (Barker *et al*., 1999; Carlin *et al.,* 2000; Barker *et al*., 2002, 2004). These process risk models have used a probabilistic modelling approach to assess the risk of foodborne botulism presented by non-proteolytic *C. botulinum* by considering the entire food chain. In process risk modelling, recent developments in mathematics and computing are utilised to assess the magnitude of the risk, and to identify hazard scenarios for each operation in the process. Probabilistic modelling approaches used have included Markov Chain Monte Carlo methods and Bayesian Belief Networks. This information can then be used to identify and prioritise steps to minimize detrimental events and to maximize awareness and process control. This includes the development of a probabilistic framework that accounts for complex combinations of information that relate to a single hazard and which incorporates an intrinsic representation of uncertainties.

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Table 4. Examples of recent outbreaks of foodborne botulism (modified from Peck, 1997; Peck, 2004)

Year Country	Food associated with outbreak	Cases: Total (fatal)	Toxin type: Neurotoxigenic organism
1989 UK	Commercially produced hazelnut yoghurt	27 (1)	Type B: Proteolytic *C. botulinum*
1991 Egypt	Commercially produced uneviscerated salted fish ("faseikh")	>91 (18)	Type E: Non-proteolytic *C. botulinum*
1993 Italy	Commercially produced aubergine in oil	7 (0)	Type B: Proteolytic *C. botulinum*
1993/4 Switzerland	Commercially prepared, dry-cured ham	12/0	Type B: *C. botulinum*[a]
1994 USA	Commercial vacuum packed clam chowder soup	2/0	Type A: Proteolytic *C. botulinum*
1994 USA	Commercial black bean dip	1/0	Type A: Proteolytic *C. botulinum*
1994 USA	Restaurant, potato dip ("skordalia")	30 (0)	Type A: Proteolytic *C. botulinum*
1996 Italy	Commercially produced mascarpone cheese	8 (1)	Type A: Proteolytic *C. botulinum*
1997 Germany	Commercially vacuum packed smoked fish ("Raucherfisch")	2 (0)	Type E: Non-proteolytic *C. botulinum*
1997 Iran	Traditionally made cheese preserved in oil	27 (1)	Type A: Proteolytic *C. botulinum*
1997 Argentina	Home-cured ham	6 (0)	Type E: Non-proteolytic *C. botulinum*
1997 Germany	Home vacuum packed smoked fish ("Lachsforellen")	4 (0)	Type E: Non-proteolytic *C. botulinum*
1998 Croatia	Ham	20 (0)	[b]
1999 Morocco	Commercially produced Mortadella sausage	78 (20)	Type B: *C. botulinum*[a]
2001 USA	Fermented beaver tail and paw	3 (0)	Type E: Non-proteolytic *C. botulinum*
2001 Canada	Fermented salmon roe (two outbreaks)	4 (0)	Type E: Non-proteolytic *C. botulinum*
2001 USA	Commercially produced chilli dish	16 (0)	Type A: Proteolytic *C. botulinum*
2002 South Africa	Commercially produced tinned pilchards	2 (2)	Type A: Proteolytic *C. botulinum*
2002 USA	Muktuk (from Beluga whale)	8 (0)	Type E: Non-proteolytic *C. botulinum*
2003 France	Halal sausage	4 (0)	Type B: *C. botulinum*[a]
2003 Ukraine	Home prepared canned corn	6 (0)	Type B: Proteolytic *C. botulinum*
2003 South Korea	Commercially produced canned sausage	3 (0)	[b]
2003 Norway	Home prepared "rakfisk"	4 (0)	Type B or E: Non-proteolytic *C. botulinum*[c]
2003 Germany	Home prepared dried fish	3 (0)	Type E: Non-proteolytic *C. botulinum*

[a] only toxin identified - unclear whether proteolytic *C. botulinum* type B or non-proteolytic *C. botulinum* type B
[b] toxin reported as present, but type not indicated
[c] likely toxin type and organism

Table 9 Safety evaluation of pasteurised in-pack meat products with respect to non-proteolytic *C. botulinum* (Hyytia-Trees *et al*., 2000)

product	Properties of the pasteurised product				Detection of non-proteolytic *C. botulinum* by PCR and neurotoxin after storage at 8°C			
	NaCl (% wt/vol)	pH	Processing value (min at 82.2°C)	Recommended shelf-life (days)	SL[a]	SL+7d[a]	SL[b]	SL+7d[b]
Pork cubes	0.7	6.0-6.3	22.9	14	-	-	+	++
Pork cubes	0.7	5.8-6.1	153.5	30	+	-	+	-
Beef cubes	0.2	5.8-6.1	496.7	21	-	+	+	+
Beef cubes	0.7	5.6-6.1	186.7	14	-	+	-	-
Pork fillet	2.0	5.8-6.0	10.6	10	+	-	+	-
Roast beef	1.6	5.7-6.1	19.6	30	-	-	-	-
Roast beef	1.9	5.6-5.8	5.5	9	+	-	+	+
Ground beef	0.2	5.5-5.9	0.0	21	-	+	++	++
Beef liver cubes	0.3	6.1-6.3	450.8	21	+	-	-	-
Broiler fillets, mariande	1.4	5.9-6.1	83.9	21	-	-	-	-
Beef, pork, vegetables	1.3	4.8-5.1	2.5	21	-	-	+	+

SL = at recommended shelf-life, SL+7d = at recommended shelf-life plus 7 days
[a] low inoculum of spores (10^2 spores/1.5kg pack), [b] high inoculum of spores (10^5 spores/1.5kg pack)
- PCR test and neurotoxin test both negative, + PCR test positive and neurotoxin test negative, ++ neurotoxin test positive

Figure 1a

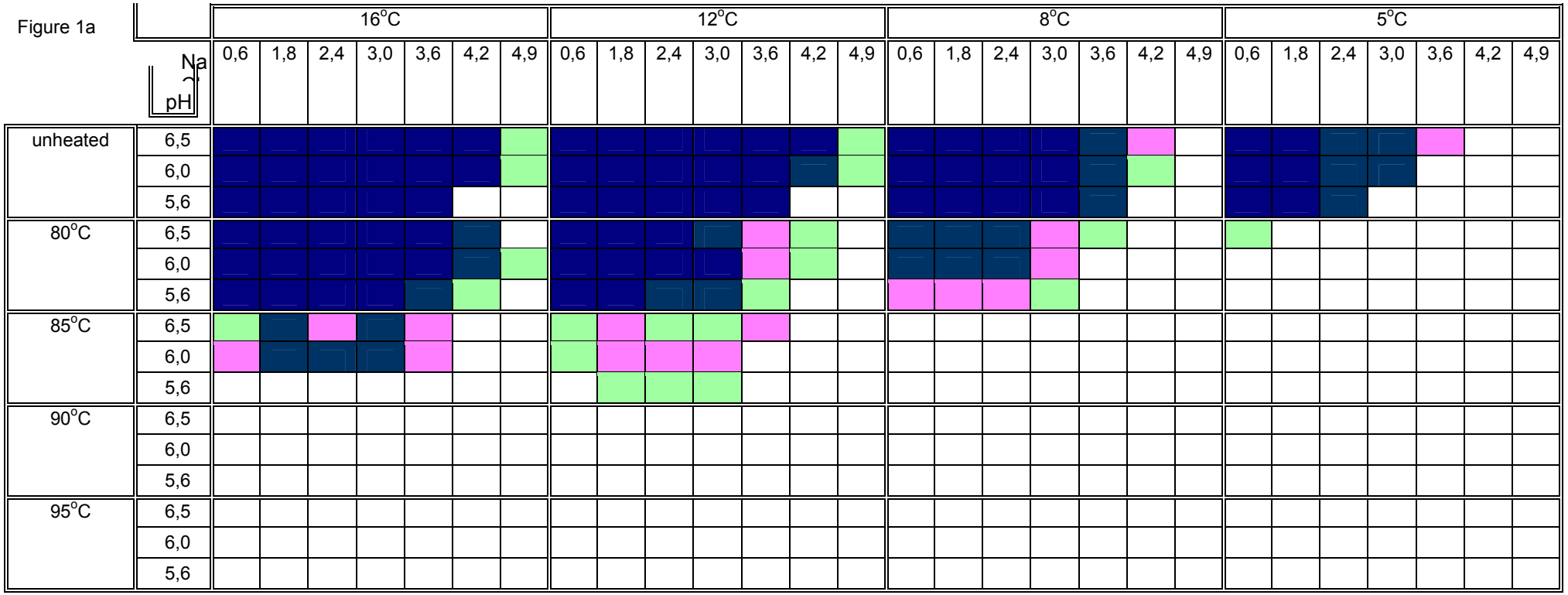
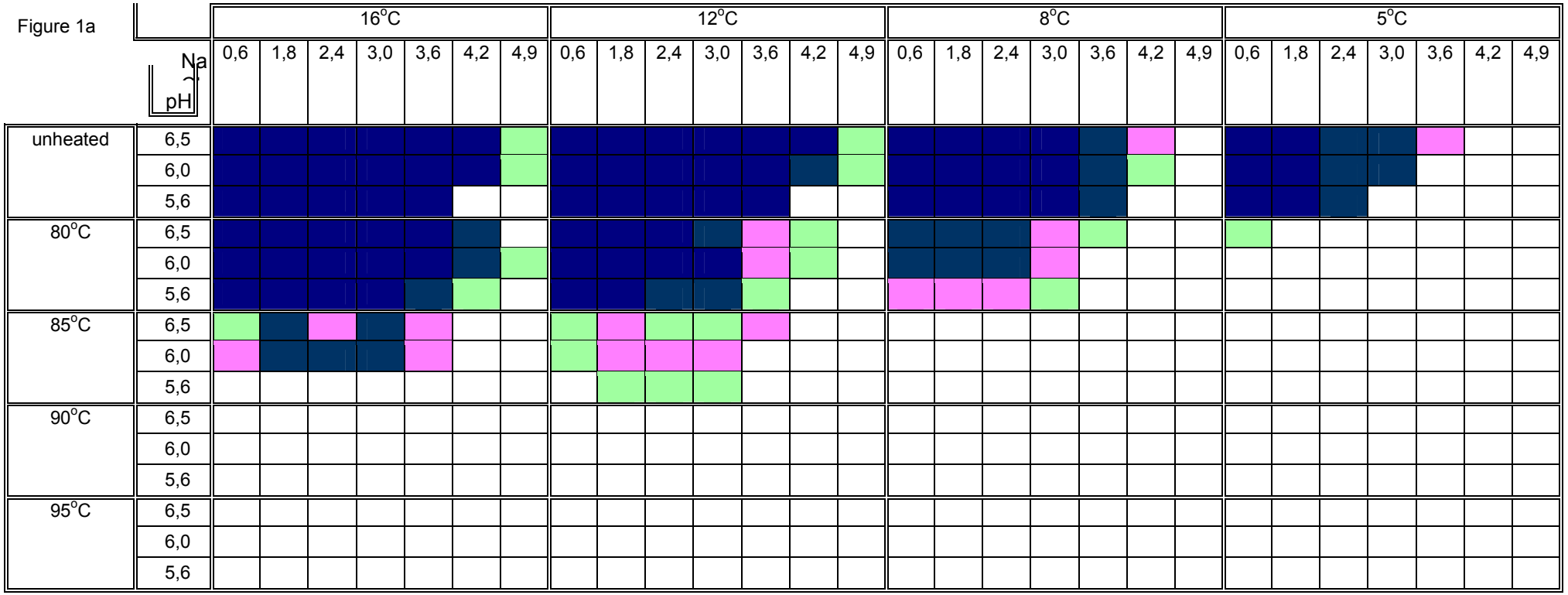


Figure 1 Combined effect of pH, NaCl concentration, lysozyme addition, heat treatment and subsequent storage temperature on time to visible growth from 10^6 spores of non-proteolytic *C. botulinum* types B, E and F in meat slurry (Graham *et al*., 1996a; Peck, Mason and Mitchell, unpublished results). Figure 1a - in absence of added lysozyme.

Figure 1a



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