Clostridium botulinum and the safety of refrigerated processed foods of extended durability

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Refrigerated processed foods of extended durability (e.g. sous-vide foods) rely on a mild heat treatment followed by storage at chill temperature for safety and preservation. The principal microbiological hazard in such foods is growth of non-proteolytic *Clostridium botulinum*. Recent research has identified combinations of mild heat treatment and subsequent refrigerated storage that, when combined with a specified shelf life, provide a defined safety margin with respect to non-proteolytic *C. botulinum*. This article discusses microbiological concerns associated with such minimally processed foods, describes recent studies of factors affecting the survival and growth of non-proteolytic *C. botulinum*, and focuses on the use of combination processes to ensure product safety.

Demand for high-quality foods that are less heavily processed, contain lower levels of preservatives and require minimal preparation time compared with conventional meals has led to the development of refrigerated processed foods of extended durability (REPFEDs). Examples of REPFEDs are sous-vide and cook–chill foods. Sales of REPFEDs are expanding at a tremendous rate in many European countries, the economics of the process are promising, and REPFEDs appear to be a qualitatively better product compared with conventionally cooked foods[1].

REPFEDs are processed at a lower temperature (maximum within the range of 65–95°C) and for longer than, for example, canned foods. The heat process is intended to maximize the sensory and organoleptic qualities of products whose characteristics would be adversely affected by heating at a higher temperature. After heating, products are cooled rapidly, and stored at refrigeration temperatures (1–8°C). Such foods are not sterile, and their shelf life is dependent on the heat treatment applied and the 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 d. REPFEDs are generally prepared in one of three ways: ingredients (which may include both raw and cooked components) are packaged in a heat-stable pouch, a vacuum is applied, the pouch is sealed and the product is then cooked (e.g. sous-vide foods); ingredients are cooked individually and then packaged; or ingredients are cooked, packaged and then heated again.

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Recommendations and guidelines for the safe production of REPFEDs

Novel food processing techniques associated with REPFEDs have created a new niche for the growth of microorganisms. This niche has three important characteristics:

- Many of the foods are packaged under vacuum or an anaerobic atmosphere. This restricts the growth of aerobic bacteria, but favours the growth of anaerobic bacteria.
- The foods receive a mild heat treatment that should eliminate cells of vegetative bacteria, but not bacterial spores.
- The foods are stored at refrigeration temperatures.

This niche favours colonization by microorganisms that produce heat-resistant spores and grow in the absence of oxygen at refrigeration temperatures. In particular, concern exists about the potential for growth and toxin production by *C. botulinum* (Box 1) in the absence of competition from other microorganisms[4,5]. The extended shelf life associated with REPFEDs also provides additional time for toxin production. Several relevant guidelines and codes of practice have been drawn up[6–12]; most are targeted at preventing growth and toxin production by non-proteolytic *C. botulinum*. Recommendations produced in the UK by the Advisory Committee on the Microbiological Safety of Food (ACMSF)[7,8] are summarized in Box 2. Although these recommendations represented an important step forward, some further developments were needed. For example, recommended combinations of chill temperature and heat treatment [as in (4) in Box 2] were based on the recovery of spores at 30°C and not at a chill temperature. Although the recommendations in (5), (6) and (7) are of value, most REPFEDs have a pH value >5, a high water activity and a salt concentration of <3.5%; the principal factors controlling the microbiological safety and quality of such foods are likely to be the heat treatment, storage temperature and shelf life.

In France, regulations for the production of sous-vide foods are targeted at ensuring the overall microbiological quality of the product, and are not aimed at achieving

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Box 1. *Clostridium botulinum* and botulism

Six physiologically and phylogenetically distinct groups of bacteria are capable of producing botulinum neurotoxin. These are *C. botulinum* groups I–IV and some strains of *Clostridium baratii* and *Clostridium butyricum*[2]. These bacteria produce one (or sometimes two) of seven botulinum neurotoxins. The neurotoxins are differentiated as types A–G on the basis of their serological reaction.

There are four types of human botulism: foodborne botulism, wound botulism, infant botulism and intestinal botulism. Botulism is also important in relation to animals and birds. *C. botulinum* group I (proteolytic strains) and *C. botulinum* group II (non-proteolytic strains) are responsible for foodborne botulism. Foodborne botulism is an intoxication involving the consumption of preformed botulinum neurotoxin. As little as 25–50 ng neurotoxin can be lethal. The consumption of as little as 0.1 g of food in which this bacterium has grown and produced the neurotoxin can result in severe illness. Initial symptoms of foodborne botulism may include impaired vision, dry mouth, nausea, vomiting and slight diarrhoea followed by constipation and intestinal pain. They can then progress to muscle weakness and flaccid paralysis, which affects the respiratory muscles and can result in death if not treated.

Over the past 10–20 years, a total of 20–30 cases of foodborne botulism have been reported per year in each of France, Germany, Italy and the USA. In the UK, three outbreaks and 32 cases of foodborne botulism were reported between 1978 and 1996[3]. Although relatively rare, the severity of botulism makes *C. botulinum* an important foodborne pathogen.

specific reductions in numbers of spores of non-proteolytic *C. botulinum*[11,12]. The proposals combine heat treatment with storage at refrigeration temperatures. For products with a shelf life of up to 21 d, a heat treatment that is equivalent to 70°C for 100 min is recommended. To take account of possible risks, the manufacturer is required to check compliance with microbiological criteria after storage at 4°C for 14 d, followed by 8°C for 7 d. For products with a shelf life of up to 42 d, the recommended heat treatment is pasteurization equivalent to heating at 70°C for 1000 min. In this case, compliance is checked after storage at 3°C for 28 d, followed by 8°C for 14 d.

Microbiological safety and spoilage hazards associated with REPFEDs

Spores of *C. botulinum* are found in soils, sediments and the gastrointestinal tract of animals. Despite often being present in low numbers, their ubiquity ensures that raw products cannot be guaranteed to be free of spores. Foods that are, or can become, anaerobic may allow the growth of *C. botulinum* and must therefore be subjected to treatments that destroy spores, or stored under conditions that prevent growth and toxin production. Knowledge of the physiology of these bacteria is required to determine which conditions prevent growth. Of the six physiologically distinct groups of bacteria that are capable of producing botulinum neurotoxin (Box 1), only *C. botulinum* groups I and II are associated with foodborne botulism. These two groups survive and grow under different conditions, and therefore cause problems in different types of foods. Strains of *C. botulinum* group I (proteolytic *C. botulinum*) are unable to multiply at temperatures below 10°C; thus, if storage below this temperature can be assured, these bacteria will not present a hazard in REPFEDs. Strains of *C. botulinum* group II (non-proteolytic *C. botulinum*) can multiply and produce toxin at temperatures as low as 3.0–3.3°C[13–16]. The spores of non-proteolytic *C. botulinum* are moderately heat resistant, and this organism is the principal microbiological hazard in REPFEDs. Non-proteolytic *C. botulinum* produces toxins of types B, E or F, and has been mainly associated with outbreaks of botulism following the consumption of 'fermented' marine products by the northern native populations of Europe, Canada and the USA. During growth, non-proteolytic *C. botulinum* does not produce off-odours to the same extent that, for example, proteolytic clostridia do. Comparisons between time to spoilage and time to toxin production by non-proteolytic *C. botulinum* have shown that toxin production may occur in the absence of spoilage[17,18].

In some circumstances, pathogenic bacteria other than non-proteolytic *C. botulinum* may pose a hazard to the safety of REPFEDs. Proteolytic *C. botulinum* (group I) and *Clostridium perfringens* do not grow below 10°C, and therefore represent a hazard only if foods are stored at temperatures in excess of 10°C. For example, in a challenge test study in which spores of proteolytic *C. botulinum* were inoculated into sous-vide spaghetti and meat sauce, toxin production was detected after storage at 15°C for 14 d[19]. Two outbreaks of foodborne botulism recorded in California, USA, in 1994 were attributed to growth and toxin production by proteolytic *C. botulinum* in clam chowder and in a bean dip that were stored at room temperature instead of at refrigeration temperatures, as intended[20]. The effects of preservative factors on the growth from spores of *C. perfringens* have been studied in sous-vide turkey and beef under conditions of temperature abuse[21,22]. *Bacillus cereus* is capable of slow growth at temperatures below

Box 2. Recommended procedures to ensure the safety of refrigerated processed foods of extended durability (REPFEDs) with respect to non-proteolytic *Clostridium botulinum*

It is recommended that the heat treatments or combination processes reduce the number of viable spores of non-proteolytic *C. botulinum* by a factor of 10^6 (a 6-decimal process)[6,7]. The Advisory Committee on the Microbiological Safety of Food (ACMSF)[7,8] concluded that the safety of REPFEDs with respect to non-proteolytic *C. botulinum* could be ensured by one of the following:

(1) storage at <3.3°C;

(2) storage at ≤5°C and a shelf life of ≤10 d;

(3) storage at 5–10°C and a shelf life of ≤5 d;

(4) storage at chill temperature combined with a 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 (the European Chilled Food Federation recommended alternative equivalent heat treatments, e.g. 80°C for 270 min, 85°C for 52 min[6])];

(5) storage at chill temperature combined with a pH value of ≤5.0 throughout the food;

(6) storage at chill temperature combined with a salt concentration of ≥3.5% throughout the food;

(7) storage at chill temperature combined with a water activity of ≤0.97 throughout the food;

(8) storage at chill temperature combined with combinations of heat treatment and other preservative factors that can be shown consistently to prevent growth and toxin production by *C. botulinum*.

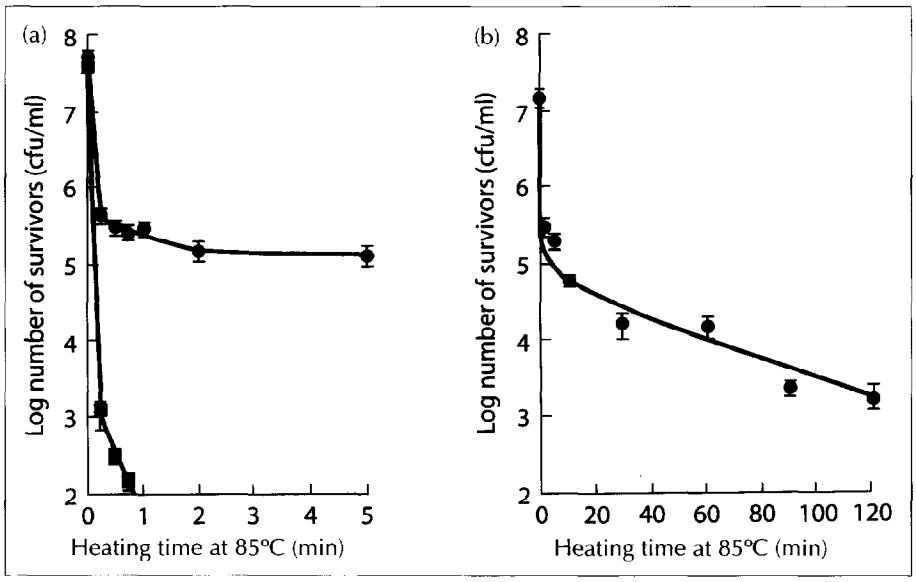

Fig. 1

Determination of the heat resistance of spores of non-proteolytic *Clostridium botulinum* strain 17B by plating on media containing (●) or lacking (■) lysozyme at 625 units (10 μg) per ml and incubation at 30°C. The number of survivors is measured in colony-forming units per ml. Bars represent 95% confidence intervals. (a), Heating at 85°C for up to 5 min. (b), Heating at 85°C for up to 120 min. For further details see Refs 27 and 28.

10°C. However, unlike *C. botulinum*, high numbers of *B. cereus* cells are needed to pose a safety hazard. An initial study indicated that *B. cereus* may represent only a low hazard with respect to the safety of REPFEDs[23]. The mild heat treatments applied to REPFEDs should be sufficient to eliminate vegetative pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Yersinia enterocolitica* and *Salmonella* spp. However, if these products are packaged after heating, recontamination may occur. Some of these pathogens grow at refrigeration temperatures (e.g. *L. monocytogenes* and *Y. enterocolitica*), whereas others survive for long periods (e.g. *E. coli* O157:H7).

Studies of the spoilage of REPFEDs have revealed the potential for growth of clostridia in these foods: non-toxigenic psychrotrophic clostridia have been isolated from spoiled packs of sous-vide-cooked roast beef[24], blown packs of dog rolls[25] and spoiled cans of pasteurized crab meat[26].

Non-proteolytic *C. botulinum* as a potential safety hazard in REPFEDs

Heat resistance of spores of non-proteolytic *C. botulinum*

The measured heat resistance of spores of non-proteolytic *C. botulinum* is dependent on the nutrient medium used to enumerate the survivors. In particular, the addition of lysozyme to the medium increases the number of spores that form colonies and hence increases the measured heat resistance. Heating spores in phosphate buffer (pH 7.0) at 85°C, followed by enumeration of the survivors on a nutrient medium gave in excess of a 5-decimal kill in <1 min (Fig. 1a). The inclusion of hen egg white lysozyme in the recovery medium substantially increased the measured spore heat resistance; the use of this medium indicated that heating spores at 85°C for 5 min and for 120 min gave only a 2.6-decimal kill (Fig. 1a) and a 4.1-decimal kill (Fig. 1b), respectively. There is evidence that the germination system in spores of non-proteolytic *C. botulinum* is destroyed by heating at 85°C and that, in the absence of lysozyme, such heat-damaged spores may remain viable but unable to germinate. The fact that bi-phasic survival curves are observed when spores are recovered in the presence of lysozyme indicates that lysozyme does not increase the measured heat resistance of the entire spore population, just that of the subpopulation of spores in the 'tail' of the survival curve (Fig. 1a and 1b). The spores in this subpopulation possess spore coats that are naturally permeable to lysozyme, which can therefore diffuse from the recovery medium into the heat-damaged spores, inducing germination by hydrolysing peptidoglycan in the spore cortex[29]. The coats of a majority of the spore population are impermeable to lysozyme, and hence lysozyme has no effect on the recovery of these spores. The heat resistance of lysozyme-permeable spores of a representative type B strain has been measured as: $D_{85°C}$ (time at 85°C for a 10-fold reduction in the number of viable spores), 100 min; $D_{90°C}$, 18.7 min; and $D_{95°C}$, 4.4 min[30].

Factors other than hen egg white lysozyme increase the measured heat resistance of spores of non-proteolytic *C. botulinum*. These include other type c lysozymes (e.g. from turkey egg, quail egg, duck egg, human milk)[3], other enzymes (chitinase, papain)[3], egg yolk emulsion[27,31], fruit and vegetable extracts[3,32] and horse blood[31]. Endogenous lysozyme activity has also been detected in many raw foods[3,31]. In most cases the activity is higher than that required to increase the measured heat resistance of spores of non-proteolytic *C. botulinum*[27,31]. The addition of hen egg white lysozyme and other lytic enzymes to foods as a preservative factor has been proposed[33]. This practice might increase the risk of growth and toxin production by non-proteolytic *C. botulinum*. Enzymes that are capable of permeating heat-damaged spores of non-proteolytic *C. botulinum* and initiating germination are also produced by microorganisms, especially sporeformers[31]. Spores of these organisms may survive a mild heat process and lead to growth in food, and thus facilitate growth from spores of non-proteolytic *C. botulinum*.

Some foods are protective to spores during heating, and thus increase the measured spore heat resistance[4,34]. The heat treatments necessary to prevent growth and toxin formation from an inoculum of 10^6 spores of non-proteolytic *C. botulinum* in a model food system (meat slurry without added lysozyme) at 25°C in 60 d were: 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[35,36]. When this model food contained lysozyme (added at 480–625 units/ml before

heating), greater heat treatments were required to prevent growth. Thus, sufficient lysozyme activity remained after heating to induce the germination of heat-damaged spores of non-proteolytic *C. botulinum*. Following heat treatments at 90°C for 20 min and at 95°C for 15 min, growth was observed at 25°C after 20 d and 32 d, respectively[35,37]. Growth was not observed within 60 d without the addition of lysozyme[35]. The heat treatments required at 70–80°C in the absence of added lysozyme and the heat treatments required in the presence of added lysozyme are higher than those in current recommendations for the production of REPFEDs[6,7], and there is a case for considering some revision of these recommendations. Heat treatments at 90°C for 10–20 min or at 95°C for 15 min could result in severe overprocessing of some foods. Although the heat resistance of spores of non-proteolytic *C. botulinum* determined by the enumeration of viable spores at 25°C can be higher than was previously recognized, the combined effects of heat treatment and other controlling factors, particularly refrigerated storage and limited shelf life, may be used to produce a 10^6-fold reduction in the probability of growth of non-proteolytic *C. botulinum*.

The effects of refrigeration temperature and other preservative factors on growth from unheated spores of non-proteolytic *C. botulinum*

Time to toxin production by non-proteolytic *C. botulinum* in foods is influenced by factors such as the initial number of spores present, intrinsic properties of the food (e.g. pH, salt concentration) and other variables (e.g. storage temperature). Many studies have been carried out in which foods have been inoculated with spores of non-proteolytic *C. botulinum* and the time to toxin production established. Examples of the fastest times to toxin formation in fish and meat inoculated with a small number of spores (1–100 spores g^{-1}) of non-proteolytic *C. botulinum* are given in Table 1. Growth and toxin production have also been described in a wide range of cooked vegetables stored at refrigeration and abuse temperatures[41,42]. For example, toxin production from cooked cauliflower inoculated with spores (10^3 spores g^{-1}) of non-proteolytic *C. botulinum* was detected after 21 d at 5°C, after 15 d at 8°C and after 4 d at 16°C[42].

The effects of single preservative factors (e.g. pH, salt concentration, temperature) on the growth of non-proteolytic *C. botulinum* have been established. Generally, growth and toxin production do not occur at a pH value of <5.0, a salt concentration of >5%, or a water activity of <0.97. The minimum temperature at which growth and toxin production occur is often reported as 3.3°C; however, in a recent article, growth and toxin production are described at 3.0–3.2°C[16]. Growth and/or toxin production have been detected at: 4.0°C after 20 d[43], 3.3°C after 31 d[13], 3.2°C after 35 d[16], 3.1°C after 42 d[16], and 3.0°C after 49 d[16], but not at 2.1–2.5°C within 90 d[13–16,44]. Information is currently lacking on growth at 2.5–3.0°C; thus, from a safety point of view, it might be prudent to assume that growth and toxin production occur in this range until it is demonstrated otherwise. Maintenance of a temperature of ≤2.5°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 the case of products that are intended for domestic use. Indeed, current 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[1].

Table 1. Examples of reported time to toxin formation in foods inoculated with a low concentration of spores of non-proteolytic *Clostridium botulinum*

	Time (d) to toxin formation at specified temperature					
Food[a]	4°C	8°C	12°C	16°C	25/30°C	Ref.
Cod	18	8	6	–	1	18
Salmon	21	6	3	–	1	38
Red snapper	NT	12	3	3	1	39
Turkey	–	8	5	2	1	40

[a]Foods inoculated with 1–100 spores of non-proteolytic *C. botulinum* per g
NT, No toxin formed in 60 d (toxin was detected with an inoculum of 10^3 spores g^{-1} in 21 d)
– Indicates tests not carried out at this temperature

In some foods, safety with respect to non-proteolytic *C. botulinum* relies on a combination of preservative factors rather than on a single factor. For example, although the use of a sub-optimal pH value or salt concentration alone might not prevent growth, in combination they might prevent growth in a specified time. The effects of different combinations of pH and salt concentration on time to growth at 3–10°C have been reported recently[16]. The effects of other preservative factors such as lactate[40,45], sorbate[46], nisin and other bacteriocins[47] and modified atmospheres[38,39] have also been considered. Oxygen should be used as a preservative factor only with great caution, because although the atmosphere might be aerobic, the food itself may be sufficiently reduced to support growth and toxin production by non-proteolytic *C. botulinum*[46,48].

An important step in quantifying the response of non-proteolytic *C. botulinum* to combinations of preservative factors has been the development of predictive models. 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 describe the effect of single and multiple factors on the probability of growth, or on the time to toxin production at a single inoculum level[39,40,46,49,50]. A predictive model that generates growth curves, and describes the effects of temperature (4–30°C), pH (5.0–7.3) and salt concentration (0.1–5.0%) on growth from spores of non-proteolytic *C. botulinum* has also been developed[51]. Predictions from these models, where tested, compare well with observed growth and toxin production in challenge test studies with many foods[45,49–51].

Combinations of heat treatment and storage temperature that prevent growth from spores of non-proteolytic *C. botulinum*

Because the microbiological safety of many REPFEDs relies solely on heat treatment and subsequent storage at

Table 2. Combined effects of heat treatment and subsequent storage temperature on the time to visible growth from spores of non-proteolytic *Clostridium botulinum* types B, E and F[a]

Heat treatment	Added lysozyme (units/ml)	Time (d) to growth at specified storage temperature					
		5°C	6°C	8°C	12°C	16°C	25°C
None	0	14	–	7	4	2	1
70°C/105 min	0	14	–	9	6	2	1
70°C/1000 min	0	57	–	21	8	5	2
70°C/2545 min	0	NG	–	50	22	8	3
75°C/464 min	0	NG	–	48	38	23	8
75°C/734 min	0	NG	–	NG	18	15	5
80°C/70 min	0	NG	–	44	19	8	5
80°C/184 min	0	NG	–	NG	37	21	11
85°C/23 min	0	NG	–	NG	30	38	15
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
80°C/23 min	625	–	40	23	12	–	3
85°C/19 min	625	–	53	53	42	–	6
90°C/20 min	625	–	–	NG	51	29	20
95°C/15 min	625	–	NG	NG	NG	–	32

[a]Tubes were inoculated with 10^6 spores of non-proteolytic *C. botulinum*. Tests (including heat treatment and subsequent storage) were conducted in a model food system (meat slurry). When present, hen egg white lysozyme was added at 625 units (10 μg) per ml before heating. For further details see Refs 35–37

– Indicates tests not carried out at this temperature

NG, No growth in 60 d

refrigeration temperatures, it is important that combinations of heat treatment and storage conditions that provide an adequate degree of protection against growth and toxin production by non-proteolytic *C. botulinum* are well defined. The effects of heat treatment and subsequent incubation temperature on growth from spores of non-proteolytic *C. botulinum* in culture media and a model food have been reported[35–37,52]. In the model food studies, the effect of heat treatment at 65–95°C combined with storage at 5–25°C on the time to growth from an inoculum of 10^6 spores of a mixture of strains of non-proteolytic *C. botulinum* was determined (Table 2). Growth was confirmed by the presence of *C. botulinum* neurotoxin. In tests in which 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 d when combined with storage at 8°C (Table 2). The heat treatment at 70°C is greater than that in current recommendations, whereas those at 80°C and 85°C are lower[6,7]. In all, 160 combinations of heat treatment and incubation temperature were tested in this study, and a predictive model was developed that described the effects of heat treatment and storage temperature on the time to growth[36].

In view of previous comments, it is also appropriate to consider foods that contain lysozyme. In these circumstances, growth was observed over a wider range of conditions (Table 2), suggesting that lysozyme activity still remained after even the most severe of these heat treatments. Growth and toxin were detected following heat treatment at 90°C for 20 min when subsequent incubation was at 12°C or higher, and following heat treatment at 95°C for 15 min when incubation was at 25°C but not when incubation was at 6–12°C (Table 2). From these results, combinations of heat treatment and storage temperature that result in a specific shelf life can be estimated for a product that might contain lysozyme.

Regulations produced by the French Ministry of Agriculture for the processing of sous-vide foods include a heat treatment that is equivalent to 70°C for 100 min for foods with a shelf life of 21 d, and require that the food complies with microbiological criteria after storage at 4°C for 14 d, followed by 8°C for 7 d[11,12]. The results presented in Table 2 show that this leaves only a small margin of safety with regard to a process giving an overall 6-decimal reduction of non-proteolytic *C. botulinum* in foods not containing lysozyme. For foods with a shelf life of 42 d, the regulations specify a heat treatment that is equivalent to 70°C for 1000 min, and that the food complies with microbiological criteria after storage at 3°C for 28 d, followed by 8°C for 14 d[11,12]. Although this heat treatment appears to provide a slightly greater margin of safety than that described above, the margin of safety is still small if foods that do not contain lysozyme are maintained at 8°C rather than at a lower temperature (Table 2). Both combination processes would be even less effective if the food contains lysozyme or a similar enzyme.

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

Factors other than heat treatment and subsequent refrigerated storage may also contribute to ensure the safety of REPFEDs with respect to non-proteolytic *C. botulinum*. The combined effects of pH (5.6–6.5), salt concentration (0.6–4.3% in the aqueous phase), lysozyme addition (0 or 1200 units/ml), heat treatment (up to 95°C for 19 min) and storage temperature (5–16°C) on the time to growth in a model food system have been described[53]. The results obtained after heating at 85°C for 18 min are shown in Table 3. The merit of including consideration of pH and/or salt concentration in the combination process is apparent. Lowering the pH and/or increasing the salt concentration extended the time before growth was observed (Table 3). In subsequent studies, heat treatment at 80°C for 20 min was

Table 3. Combined effect of pH, salt concentration, heat treatment and subsequent storage temperature on time to visible growth from spores of non-proteolytic *Clostridium botulinum* types B, E and F[a]

Added lysozyme (units/ml)	pH	Salt (NaCl) concentration (% w/w in aqueous phase)	Time (d) to growth at specified storage temperature			
			5°C	8°C	12°C	16°C
0	6.5	0.6	NG	NG	66	45
0	6.5	2.5	NG	NG	49	29
0	6.5	4.3	NG	NG	NG	NG
0	6.0	0.6	NG	NG	59	40
0	5.6	0.6	NG	NG	NG	NG
0	5.6	2.5	NG	NG	NG	NG
0	5.6	4.3	NG	NG	NG	NG
1200	6.5	0.6	NG	64	24	11
1200	6.5	2.5	NG	43	27	20
1200	6.5	4.3	NG	NG	34	43
1200	6.0	0.6	NG	87	38	21
1200	5.6	0.6	NG	87	38	21
1200	5.6	2.5	NG	NG	31	24
1200	5.6	4.3	NG	NG	NG	NG

[a] Tubes inoculated with 10^6 spores of non-proteolytic *C. botulinum*. Tests (including heat treatment at 85°C for 18 min and subsequent storage) were conducted in a model food system (meat slurry). When present, hen egg white lysozyme was added at 1200 units (25 μg) per ml before heating. For further details see Ref. 53
NG, No growth in 90 d

combined with a slightly reduced pH, a modestly elevated salt concentration and storage at 8°C to prevent growth within 42 d (M.W. Peck *et al.*, unpublished). Following a heat treatment of 80°C for 23 min, growth was observed at 8°C within 23 d when the pH and salt concentration were more conducive for growth (Table 2). By considering intrinsic properties of the food (or by adding mild preservatives), it is possible to reduce the heat treatment given to such minimally processed foods. This is another, important, step forward in the development of rational processes for REPFEDs.

On the basis of experience over many years, some REPFEDs, such as pasteurized crab meat and minimally processed oysters, appear to be safe because they have not been implicated in botulism outbreaks. Yet, spores of non-proteolytic *C. botulinum* have been found in crab meat and may be present in oysters[54–56], and the heat treatments applied are extremely minimal. Blue crab meat is sold as a pasteurized (heated to an internal temperature of 85°C for at least 1 min), refrigerated product with a shelf life of up to 6 months[55]. Oysters receive only a minimal heat treatment (heated to internal temperature of 55°C for 10 min), and with refrigerated storage have a shelf life of 45 d, which can be extended to 60 d by the inclusion of 1.0% salt and 0.13% sorbate[57]. Because the heat treatments given to these products are extremely minimal, safety with respect to non-proteolytic *C. botulinum* seems to depend on a combination of factors, possibly including intrinsic properties of the food. An evaluation of these and similar processes is merited to determine the overall level of safety that these combination processes give, and to determine how they are achieved.

Conclusions and future prospects

The microbiological safety of most REPFEDs is likely to rely on the use of an effective combination of heat treatment, storage temperature and shelf life. In some circumstances, other factors such as intrinsic properties of the food may also contribute to extending shelf life. By adopting effective combination processes, it should be possible to avoid potentially dangerous situations, and maintain an organoleptically acceptable product. Recent research has gone some way to define conditions that could be applied to ensure the safety of REPFEDs with respect to non-proteolytic *C. botulinum*. It is important that this research continues, and that further combination processes are identified that ensure the safety of REPFEDs with respect to non-proteolytic *C. botulinum*. An important step in this process is likely to be the development of additional predictive models. Other important steps are likely to include quantitative risk assessment and the continued implementation of HACCP (hazard analysis and critical control points) procedures. It is hoped that this will lead to the continued safe development of REPFEDs without incidents of botulism. The severity of this illness ensures that the consequences of outbreaks are likely to be significant for consumers and the food industry.

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