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Quantification of factors affecting the probability of development of pathogenic bacteria, in particular *Clostridium botulinum*, in foods

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SUMMARY

The success of a preservation method with respect to *Clostridium botulinum* can be measured by its effect on the probability that a single spore would result in growth and formation of toxin. In canned, low-acid foods, the minimum heat-process is designed to reduce the probability of survival of a single, heat-resistant spore of *C. botulinum* by a factor of 10^{12}. In some foods, safety depends on the combination of inactivation and inhibition of *C. botulinum*. The degree of protection (*Pr*) can be expressed as $Pr = Ds + In$, where *Ds* is the decimal destruction of spores of *C. botulinum* and *In* is the decimal inhibition. A similar approach can be used in the case of other bacteria. $Pr = \log 1/P$, where P = the probability that an individual spore or bacterium will survive and result in growth. P can be estimated as the number of spores or bacteria that survive and initiate growth in a culture medium or food divided by the number of viable spores or bacteria inoculated into the medium or food. The effects of combinations of preservative factors can be measured by their effect on P at a stated temperature for a stated time. In experiments to determine the effects of preservative factors on an anaerobic bacterium such as *C. botulinum* it is essential that oxygen should be eliminated, unless it is controlled as an inhibitory factor. Thus experiments in culture media should be done under strictly anaerobic conditions at a known, low redox potential. The results of experiments to determine the effects of preservative factors on P after a series of incubation times can be modeled by methods similar to those used to model the effect of preservative factors on rate of growth and on lag period. Experiments to determine the effect of preservative factors on the probability of growth from a single spore or bacterium of *C. botulinum* are discussed. A few reports of similar experiments with other bacteria have been published and are described. This approach has the advantage that it takes account of the inoculum level of bacteria.

INTRODUCTION

Preservation factors that are used to prevent or to slow down the growth of foodborne pathogenic bacteria and spoilage microorganisms in foods include storage temperature, humectants, acidulants, preservatives, restriction of oxygen, and increase of carbon dioxide. In order to understand the effects of these factors singly and in combination, studies in culture media are a means of providing a constant, homogeneous environment with controlled amounts of nutrients. Such experiments enable an investigation of the mechanisms of the effects of preservative factors. The equations that are derived from these experiments enable prediction of the effects of different combinations of factors and could indicate useful additive and synergistic effects.

Foods may be limiting in nutrients for some bacteria, they may contain natural inhibitors or protective factors, be physically and chemically heterogeneous and contain a natural or added microflora, that can change during storage. All these properties may modify the effect of preservative factors on bacteria. In order to predict the effect of preservative factors in foods it is important to have an underlying framework of information concerning the effect of these factors in culture media, and then to determine the extent to which predictions based on this framework may have to be modified for particular foods where additional properties influence the situation.

ASSESSMENT OF PRESERVATION METHODS WITH RESPECT TO *Clostridium botulinum* BY THEIR EFFECT ON THE PROBABILITY OF GROWTH FROM SINGLE SPORES OR CELLS

Riemann [32] considered that the success of a preservation method with respect to *C. botulinum* depended on its effect on the probability that one botulinum spore would grow out and give rise to toxin in the preserved product. This principle is used in specification of the heat processing that is used in canning of low-acid foods. In general, the minimum heat process applied is one considered to be sufficient to reduce the probability of survival of heat-resistant spores of *C. botulinum* by a factor of 10^{12}. This concept is based on the following considerations: (i) experimental evidence that the decimal reduction time (D), i.e. the time required to

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kill 90% of the remaining viable spores, remains constant as the total number of viable spores decreases; (ii) the view that a 12-D process provides adequate security; (iii) the assumption that the D-value is not affected by the number of spores per container; (iv) the assessment that a 12-D process for heat-resistant spores of *C. botulinum* is approximately equivalent to heating at 121 °C for 3 min (F_o3) [12]. These rules are applied, in general, in canning processes for low-acid foods.

Canned, cured meats are an exception where a much lower heat treatment (about 0.6 min at 121 °C, 2.4 D) may be used. In these products safety is achieved by both inactivation and inhibition of *C. botulinum* spores by the combined effect of heat, sodium chloride, nitrite and pH. For this type of situation there is a need to quantify the combined effect of these factors. The degree of protection (*Pr*) can be expressed as [7].

$$Pr = Ds + In$$

where *Ds* and *In* are the numbers of 10-fold reductions in survival of and growth from *C. botulinum* spores by heat-inactivation and by inhibition respectively. The thermal process applied to the majority of shelf-stable, canned, cured meats may range from 0.05–0.6 F_o, resulting in *Ds* values of <1–3 log units. For luncheon meats with 5–5.5% brine, 75 or 150 ppm nitrite and 0.6 F_o estimated *In* values were in the range of 4–6 log units [7].

Hauschild [6] estimated the degree of protection as:

$$Pr = \log 1/P$$

where P = the probability that individual spores will survive any heat process, grow out and produce toxin. Thus for a 12-D canning process the protective factor would be 12.

In general, where the effect of preservation conditions on growth of *C. botulinum* is investigated the probability of growth from a single organism (P) in the test conditions is equal to the number of bacteria able to initiate growth in the test conditions (usually estimated by Most Probable Number (MPN) divided by the number inoculated into the test conditions. An example of a study of the effect of a preservative factor on P for growth of *C. botulinum* in a food is a study by Hauschild et al. [8] of the contribution of nitrite to the control of *C. botulinum* in liver sausage. A series of four, 10-fold dilutions of spores of type A and B (proteolytic) *C. botulinum* was inoculated in triplicate into sausages, containing an initial concentration of 0, 50, 100 or 150 ppm nitrite, which were heated at 76–78 °C then incubated at 27 °C. After incubation for the required period of time the sausages were tested for toxin and the probability that a single spore would result in growth and toxin formation in the sausage was estimated as:

$$P = \frac{\text{MPN of spores that initiated growth and gave rise to toxin in sausage}}{\text{the number of viable spores inoculated per sausage } (s) \text{ (measured by plate counts)}}$$

In liver sausage containing 2.1–2.3% NaCl, 50 or 100 ppm input nitrite had little or no effect on the probability of toxin formation in one week, but 150 ppm gave a significant reduction in the risk that one spore would result in formation of toxin (Table 1).

Where foods are challenged with a single level of spores, the MPN of spores resulting in toxin can be calculated as:

$$\text{MPN} = \ln (n/q)$$

where n is the number of food samples analyzed and q is the number of non-toxic samples.

On this basis Hauschild [6] analyzed the results of experiments reported in the literature in which cured meats were challenged with spores of *C. botulinum*, incubated in conditions of temperature abuse and tested for the presence of toxin. In different products the degrees of safety provided by the preservation process, in relation to the risk of growth of *C. botulinum* in conditions of temperature abuse, differed by several log units. For some products, for example, for vacuum-packaged bacon, the P values even in products without nitrite were generally between 10^{-5} and 10^{-7}, whereas for vacuum-packaged turkey roll the safety factor was lower, with P values between 10^{-3} and 10^{-4}.

It should be stressed that the topic being considered is the effect of preservation methods on the probability of growth from a single spore or vegetative bacterium. An assessment of the overall risk of growth of *C. botulinum* in a food would also require information about the likely incidence of *C. botulinum* spores in that food. It is rarely

TABLE 1

The effect of nitrite on the probability of outgrowth and toxigenesis from spores of proteolytic *C. botulinum* (types A and B) in liver sausage (modified from [8])

Nitrite input (ppm)	Expt	Weeks at 27 °C 0.5	1		
		P[a]	P[a]	L[b]	U[b]
0	II	$<2 \times 10^{-6}$	3×10^{-4}	1×10^{-4}	7×10^{-4}
	III		3×10^{-4}	1×10^{-4}	7×10^{-4}
	IV	$<2 \times 10^{-6}$	1×10^{-2}	5×10^{-3}	4×10^{-2}
50	II		8×10^{-5}	3×10^{-5}	3×10^{-4}
	III		8×10^{-3}	3×10^{-3}	3×10^{-2}
	IV		1×10^{-2}	5×10^{-3}	4×10^{-2}
100	II		1×10^{-5}	4×10^{-6}	3×10^{-5}
	III		2×10^{-4}	7×10^{-5}	4×10^{-4}
	IV		2×10^{-2}	8×10^{-3}	6×10^{-2}
150	II		$<2 \times 10^{-6}$		
	III		$<2 \times 10^{-6}$		
	IV		$<2 \times 10^{-6}$		

[a] P = MPN/s where s = no. of challenge spores per sausage, and MPN is derived from the number of sausages that became toxic.
[b] L and U are lower and upper 95% confidence limits of P.

possible to rely on generalizations about such information, and this is not part of the present discussion.

THE EFFECT OF OXYGEN AND REDOX POTENTIAL ON THE PROBABILITY OF GROWTH FROM SINGLE SPORES OR CELLS OF *Clostridium botulinum*

The ability of anaerobic bacteria such as *C. botulinum* to grow in culture media or in foods is influenced strongly by the presence of oxygen. Both the physical exclusion of oxygen and the presence of reducing substances are, in general, considered necessary to establish a low redox potential and allow the initiation of growth of anaerobes from low inocula [9,26].

To assess the effect of storage temperature, pH, acidulants, sodium chloride or other preservative factors on growth of *C. botulinum* it is necessary to specify the conditions of anaerobiosis, in order to understand whether the levels of oxygen and the redox potential contribute to inhibition. In order to determine the maximum ability of *C. botulinum* to survive and multiply in the presence of other inhibitors it is necessary to eliminate oxygen and to establish a low redox potential.

The redox potential of a redox couple is given by

$$E_h = E_o + RT/nF \ln \{[\text{oxidized state}]/[\text{reduced state}]\}$$

where E_h is the electrode potential referred to the standard hydrogen electrode, E_o is the standard electrode potential of the system at 25 °C and pH 7 when the activities of the oxidized and reduced forms are equal, n is the number of electrons transferred for each molecule reduced, the quantities in the square brackets are the activities of the oxidized and reduced forms, R is the gas constant, T is the absolute temperature and F is the Faraday constant [13,16].

Redox potentials measured at a pH other than 7 can be expressed in terms of the equivalent redox potential at pH 7 (E_h7) using the following equation [19]:

$$E_h7 = E_h \text{ at pH } X + 2.303\, RT/nF\,(\text{pH } X - 7)$$

where X is the pH at which measurements were made. At 25 °C, 2.303 RT/nF = 59.1 mV, therefore if n = 1, a pH change of one unit corresponds to a change in E_h of 59.1 mV. Redox potentials are expressed relative to the standard hydrogen electrode, obtained when $[H^+]$ = 1.0 M and pH_2 = 1 atm, the potential of which is defined as zero. Measurement of redox potentials is discussed by Jacob [13] and by Kjaergaard [16].

In a culture medium the redox potential may be the result of the presence of several redox couples, and the controlling factors are often unknown. Measurement of the redox potential has been used, however, in attempts to define the state of reduction of a culture medium when the dissolved oxygen tension is too low to measure with a membrane-covered oxygen electorde, i.e. about 1000-fold lower than the saturation value with air at 1 atm [29].

Oxygen can influence the measured redox potential of a culture medium or a food: (i) by increasing the proportion of the components that are oxidized; and (ii) by interacting directly with the redox electrode. The occurrence of a direct interaction between oxygen and the redox electrode depends on the composition of the solution. In pure water, oxygen does not influence the platinum redox electrode directly because the exchange current for its reduction is too low [2,27]. In salt solutions and culture media with weak redox buffering properties oxygen does directly affect metal electrodes, and may result in mixed potential values due to the effect of both oxygen and other redox systems [15]. The magnitude of the response can depend on both the type of the electrode and the condition of its surface. Standardization of redox electrodes is usually recommended in solutions of a strong redox couple, such as saturated solutions of quinhydrone, at two pH values [16], for example in buffers at pH 4.0 and pH 7.0. At 25 °C the potential of the quinone–hydroquinone (quinhydrone) electrode is given by [18]:

$$E_h, \text{ quinhydrone} = 699 - 59.1 \text{ pH}$$

If it is intended that the redox electrode should measure both redox couples and dissolved oxygen it is necessary also to standardize the response of electrodes to oxygen, for example in aerated phosphate buffer (0.066 M, pH 7, E_h +630 mV at 25 °C) as suggested by Jacob [13], to obtain consistent and meaningful results.

The effect of gases and reducing agents on the redox potential of a culture medium containing peptone, yeast extract, sucrose, starch and thionin is shown in Fig. 1 [20,

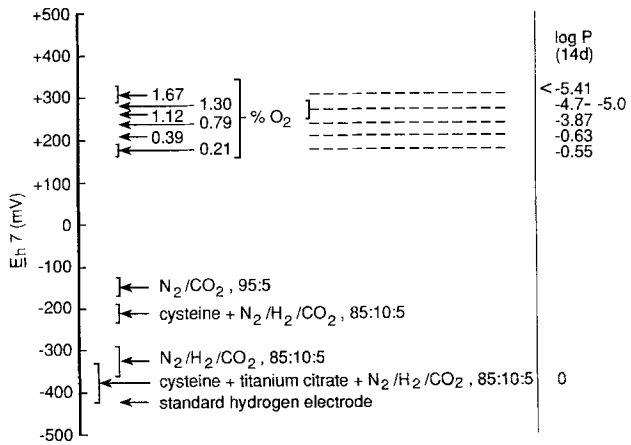

Fig. 1. The effect of gases and reducing agents on the redox potential of a culture medium and on the probability of growth from a single spore of *Clostridium botulinum* type E strain Beluga in 14 days. (Data from 20 and 24). Medium with an $E_h7 < 0$ mV was prepared in strictly anaerobic conditions under $N_2/H_2/CO_2$, 85:10:5 (v/v) or under N_2/CO_2, 95:5 (v/v). Medium with an $E_h7 >$ 0 mV was prepared in strictly anaerobic conditions under N_2/CO_2, 95:5 (v/v) and measured volumes of air were introduced into the final containers of medium before these were sterilized at 121 °C. The concentrations of oxygen in the head-space gas of uninoculated medium were determined by mass-spectrometer.

24]. In various culture media different redox couples will be present and there will be different relationships between concentration of dissolved oxygen and redox potential. It is impossible, therefore, to make simple correlations between dissolved oxygen and redox potential in different media, and the effect of traces of oxygen on anaerobes is likely to depend on the nature of the culture medium. Nevertheless, the relationships between pO_2 and redox potential of culture media reported by Jacob [13] and by Harrison [5] are of the same order as those in Fig. 1.

The relationship between oxygen, redox potential of the medium and probability of growth from a single spore of *C. botulinum* type E is also shown in Fig. 1; more detailed information is given in Table 2. When hydrogen is present in the gas mixture it will influence the growth of *C. botulinum* in several ways [26]: (i) it will act as a reductant, lowering the E_h by reducing constituents of the medium; (ii) it will 'prime' any inoculum that has hydrogenase activity; it is likely that *C. botulinum* forms a hydrogenase, in which case hydrogen may be utilized for production of ATP by reduction of fumarate; (iii) it may protect against oxygen free-radicals.

One of the technical limitations in the type of experiment shown in Table 2 is that germination of clostridial spores, including those of *C. botulinum*, can occur in the presence of oxygen [1]. Germination and emergence of vegetative cells can result in the production of hydrogen, and if high concentrations of spores are present sufficient hydrogen may be formed to result in a marked decrease in the redox potential of the culture medium. In the experiments by Lund and Wyatt [20] and Lund et al. [24] the highest concentrations of spores used were about 10^5 ml^{-1} and germination did not appear to cause significant reduction of the medium, but if more concentrated inocula were used the medium might be reduced during germination, in which case the experiments could not be used to determine the influence of oxygen and redox potential on the probability of growth from a single spore.

In most cases a high redox potential in foods or culture media is caused by the presence of oxygen [9]. There is evidence that inhibition of anaerobes at high redox potential is due primarily to the presence of dissolved oxygen rather than to the redox potential itself, and that high redox potential caused by other oxidizing agents may not be inhibitory. Growth of a butanol-forming clostridium in a culture medium was inhibited by the presence of sufficient oxygen to raise the E_h to +300 mV at pH 7, but in the absence of oxygen the organism grew at an E_h of up to +335 mV when this was caused by the presence of potassium ferricyanide [17]. *Clostridium acetobutylicum* grew at E_h + 370 mV at pH 7 when this was maintained by ferricyanide in the absence of oxygen, but was inhibited at +100 mV when this was produced by aeration, and at −50

TABLE 2

The effect of partial pressure of oxygen on the redox potential of the medium and on the probability (P) for initiation of growth from a single spore of *C. botulinum* type E (modified from [24])

pO_2 (Atm × 10^{-2})	E_h (mV)	Log P for growth in days			
		2	3	5	14
1.67	+294[a] +328[b] (±14)	<−5.41	<−5.41	<−5.41	<−5.41
1.30	+280[a]	<−5.41	−5.36 (−4.52 to −6.80)[c]	−5.01 (−4.30 to −5.95)	−4.69 (−4.08 to −5.39)
1.12	+271[a]	<−5.41	−5.36 (−4.52 to −6.80)	−5.36 (−4.52 to −6.80)	−5.05 (−4.39 to −5.99)
0.79	+250[a]	<−5.41	−4.77 (−4.14 to −5.56)	−4.10 (−3.56 to −4.63)	−3.87 (−3.33 to −4.40)
0.72	+245[a]	−2.63 (−2.03 to −3.30)	−2.22 (−1.65 to −2.84)	−1.14 (−0.49 to −1.80)	−0.97 (−0.32 to −1.62)
0.39	+208[a]	−4.33 (−3.73 to −4.95)	−0.63 (−0.04 to −1.30)	−0.63 (−0.04 to −1.30)	−0.63 (−0.04 to −1.30)
0.21	+170[a] +189 (±12)[b]	−0.55 (+0.06, −1.24)	−0.55 (+0.06, −1.24)	−0.55 (+0.06, −1.24)	−0.55 (+0.06, −1.24)
0	Approx −400[d]	0 (+0.48, −0.48)	0 (+0.48, −0.48)	0 (+0.48, −0.48)	0 (+0.48, −0.48)

Measurements were made in peptone-yeast-sucrose-starch (PYSS) medium containing thionin, 0.002 g L^{-1}, pH 6.8–7.0.

[a]Estimated from a standard curve relating pO_2 to E_h.

[b]Determined by measurement on uninoculated vials of medium.

[c]Figures in parentheses are 95% confidence limits.

[d]Medium at E_h = −400 mV contained cysteine-HCl, 0.5 g L^{-1}, and titanium III citrate, 1.1 mM.

mV when this was maintained by dithiothreitol in the presence of oxygen [28].

In experiments to investigate toxin production by *C. botulinum* type E in fresh herring, Huss and co-workers injected 100 spores of the organism g^{-1} into the muscle of freshly smoked herring [11]. Some samples of the fish were also inoculated with a specific spoilage organism. The fish were then vacuum-packed and stored at 15 °C. All the fish were toxic within 3 days, and the presence of the spoilage organism had little or no effect on toxin production. In fish without the spoilage organism, the E_h in the muscle remained in the range between +200 to +250 mV (Fig. 2), but nevertheless botulinum toxin was formed, indicating that the spores of *C. botulinum* had initiated growth at an E_h higher than +200 mV. In fish inoculated with the spoilage organism the E_h had dropped to about −80 mV after 3 days. There is good evidence that the high E_h in the fish was due to the presence of trimethylamine oxide (TMAO), which controls the E_h regardless of storage atmosphere [10]. Growth of certain spoilage organisms reduces TMAO to trimethylamine, and this change was mainly responsible for the drop in E_h in the fish. In this case a high redox potential in the fish muscle was no guide to the presence of oxygen, and no guide to the risk of growth of *C. botulinum*.

There is evidence that TMAO can act as an electron acceptor for *C. botulinum* type E, and growth of this organism would be expected to lower the E_h in fish muscle by reduction of TMAO and by the formation of hydrogen.

A high redox potential in a culture medium caused by the presence of traces of oxygen has been shown to enhance the inhibitory effect of other factors on growth of *C. botulinum*. In trypticase soy broth pH 7.0 inoculated with approximately 3×10^6 spores of *C. botulinum* type E ml^{-1} and incubated at 30 °C there was no significant difference between the rate of growth at E_h7 −80 mV and that at E_h7 −180 mV [34]. In the presence of 3.5% (w/v) NaCl, however, the lag phase was shorter and growth occurred more rapidly at the lower E_h than at the higher E_h (Fig. 3), and in the presence of 4.0% (w/v) NaCl, growth was detected in the low E_h medium in 48 h at 30 °C but not in the high E_h medium in 200 h. In medium at E_h7 −145 mV or adjusted to −60 mV by allowing access of air, acidic pH and high concentrations of NaCl or of sucrose had a greater inhibitory effect on growth from spores of type A *C. botulinum* at the higher E_h than at the lower E_h [35]. The effect of NaCl on the probability of growth from spores of *C. botulinum* type E was much greater at an E_h7 between +62 and +122 mV, adjusted by the addition of a small amount of air to the culture medium, than at an E_h7 between −426 and −382 mV (Table 3) [20].

THE EFFECT OF PRESERVATIVE FACTORS ON THE PROBABILITY OF GROWTH FROM SINGLE SPORES OR CELLS OF *Clostridium botulinum*

In order to determine the maximum ability of *C. botulinum* to grow in the presence of a range of preservative factors it

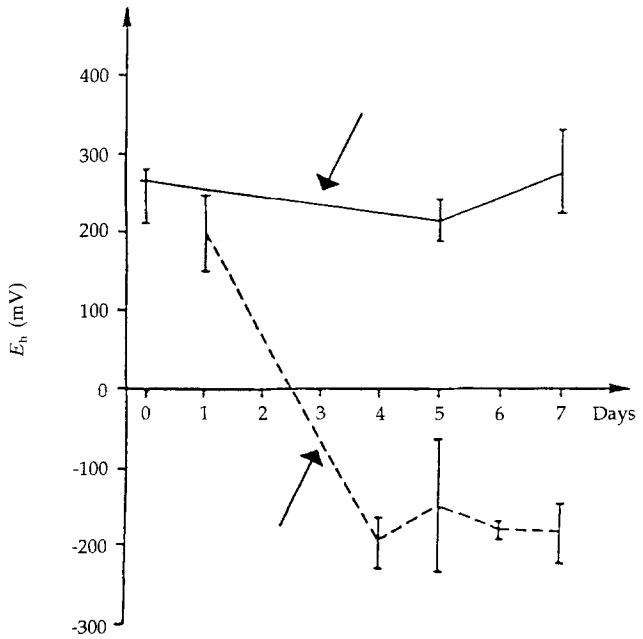

Fig. 2. The redox potential (E_h) in aseptically handled, vacuum-packed smoked herring (—) and vacuum-packed smoked herring inoculated in the loin with 1000 of a 'specific spoilage bacterium' g^{-1} (---) and stored at 15 °C. Similarly handled fish further inoculated with 100 spores of *Clostridium botulinum* type E were toxic three days after packaging (arrow) [11].

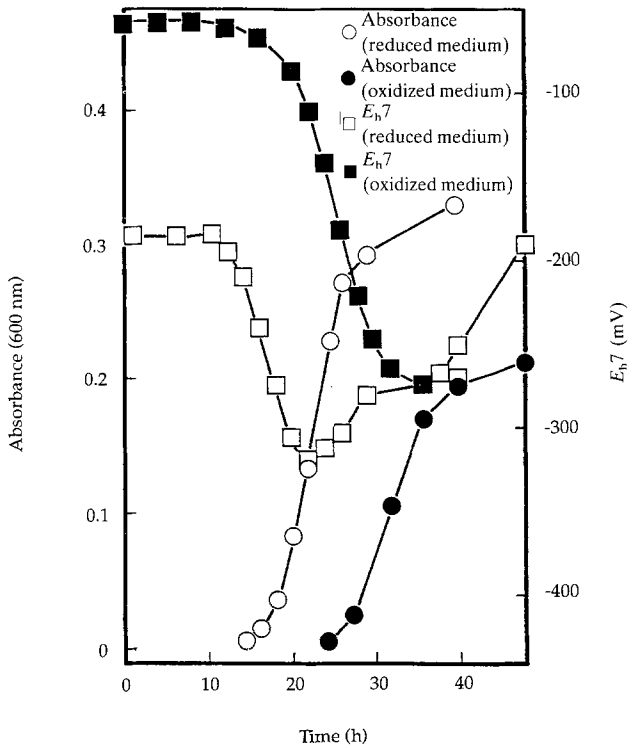

Fig. 3. Growth of *Clostridium botulinum* type E and E_h7 changes in oxidized and pre-reduced anaerobic trypticase soy broth plus 0.05% cysteine and 3% added sodium chloride (giving a total NaCl concentration of 3.5%) [34].

TABLE 3

The combined effect of redox potential, adjusted with air or reducing agents, and NaCl on log P for growth from spores of *C. botulinum* type E, at pH 6.8–7.0 and 20 °C in 5 days (modified from [20])

NaCl (% w/v)	log P	
	E_h −426 to −382 mV	E_h +62 to +122 mV
0.1	0	−1
1.5	–	−2.7
3.25	−1.9	−6.1
3.75	−2.9	–
4.0	−4.2	–

is necessary to eliminate oxygen and establish reducing conditions. In the work of my group we have maintained strictly anaerobic conditions with an E_h7 between −200 mV and −300 mV or lower.

It is generally accepted that growth of *C. botulinum* in foods is inhibited at a pH lower than 4.6, but there have been several reports that growth and formation of toxin can occur at a pH as low as 4.1 in medium that contains a large amount of precipitated protein [33,36,37,38]. It has been suggested that growth in these conditions resulted from the presence of microenvironments in which the pH was above 4.6 [37]. We have been unable to detect growth of *C. botulinum* at pH values below 4.6 in the presence of precipitated protein.

The results of a study of the effect of pH, adjusted with HCl, in medium without precipitated protein, on the probability of growth from a single vegetative bacterium of a strain of type A *C. botulinum* at 30 °C have been reported (Fig. 4) [22]. In these experiments acid pH was much less inhibitory than in the study of the effect on type E spores by Riemann (Fig. 5) [32] whose results showed much less inhibition than reported for type A spores by Montville [25]. The greater resistance to acid pH in the experiments of Lund et al. [22] and of Riemann [32] may be due to the use of liquid media rather than the solid medium used by Montville (Fig. 6) [25], while the fact that the former workers found greater resistance than either of the other two may have been due to the use of vegetative bacteria rather than spores as the inoculum. In the experiments shown in Fig. 4 [22], lowering the pH from 6.8 to 4.6 lowered the log P by a factor of 6. Similar results were obtained with a mixture of type A and proteolytic type B cells.

In a medium adjusted to pH 5.2 with HCl the probability of growth from a single vegetative bacterium of type A *C. botulinum* in three days at 30 °C was 1; in the presence of 50 mmol citric acid L^{-1} at this pH the probability of growth in 14 days was $<10^{-6}$ [4]. The inhibitory effect of ctiric acid was prevented by the addition of metal ions, in particular by Ca^{2+} or Mg^{2+}, suggesting that chelation of

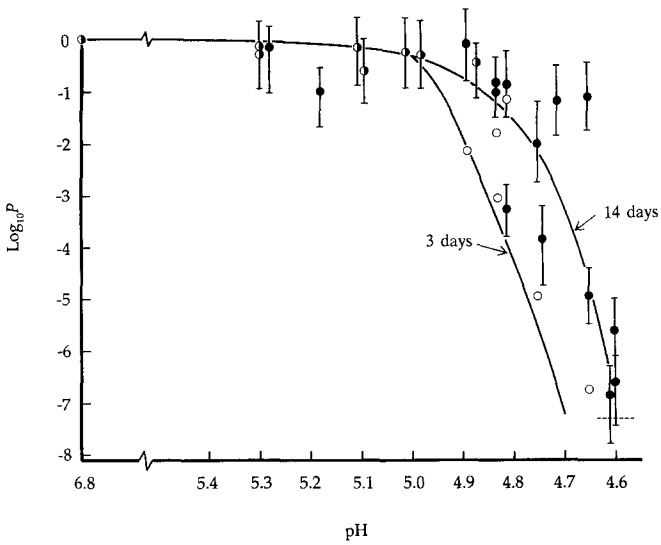

Fig. 4. The effect of pH, adjusted with HCl, on the probability (P) for growth from a single vegetative bacterium of *Clostridium botulinum* type A strain ZK3 in culture medium at 30 °C for 3 days (○) and for 14 days (●). Symbols represent experimental results and continuous lines are predicted from a model. Bars are 95% confidence limits at 14 days, and are omitted, for clarity, from 3-day results [22].

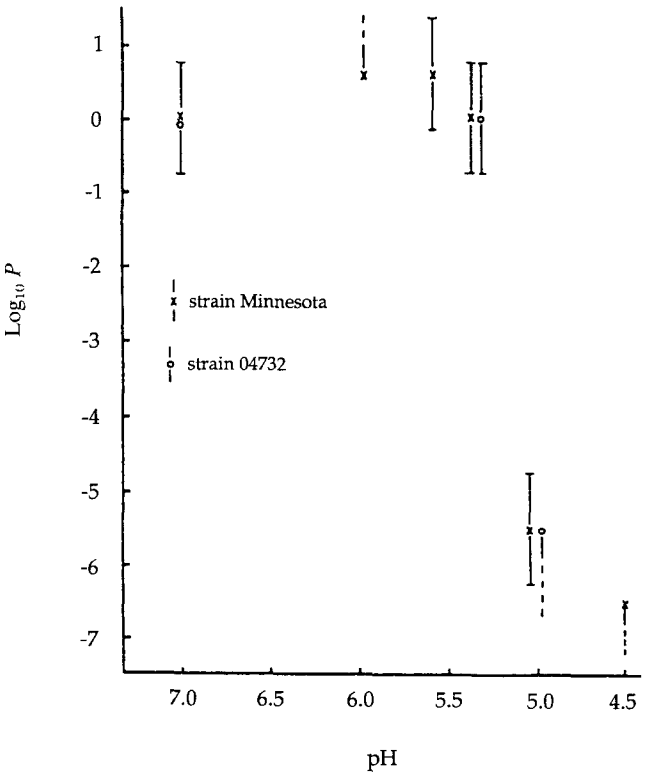

Fig. 5. The effect of pH, adjusted with HCl, on the probability (P) for outgrowth from *Clostridium botulinum* type E spores in brain–heart-infusion medium incubated at 30 °C for months. Bars represent 95% confidence limits. (Modified from [32].)

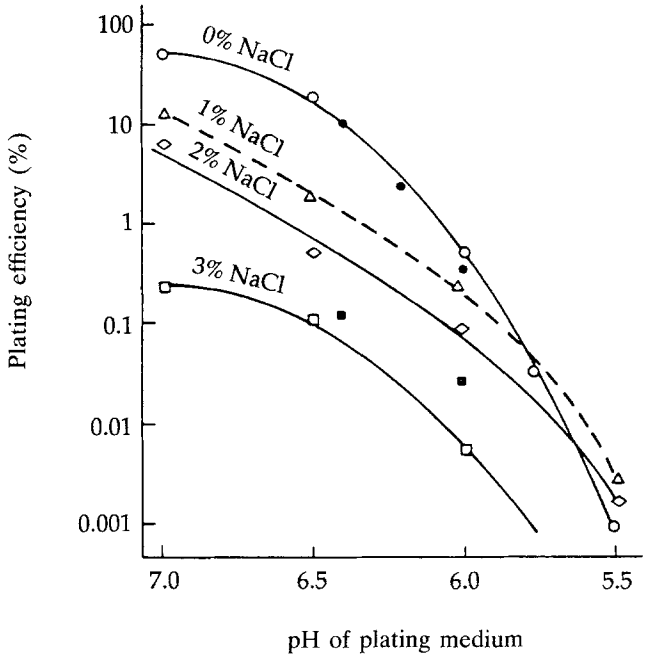

Fig. 6. The effect of pH, adjusted with HCl, and NaCl on plating efficiency of *Clostridium botulinum* type A, strain 62A, spores on botulinum assay medium. ○ = 0%, △ = 1%, ◇ = 2%, □ = 3% NaCl. Closed symbols are from a replicate experiment with a second spore crop [25].

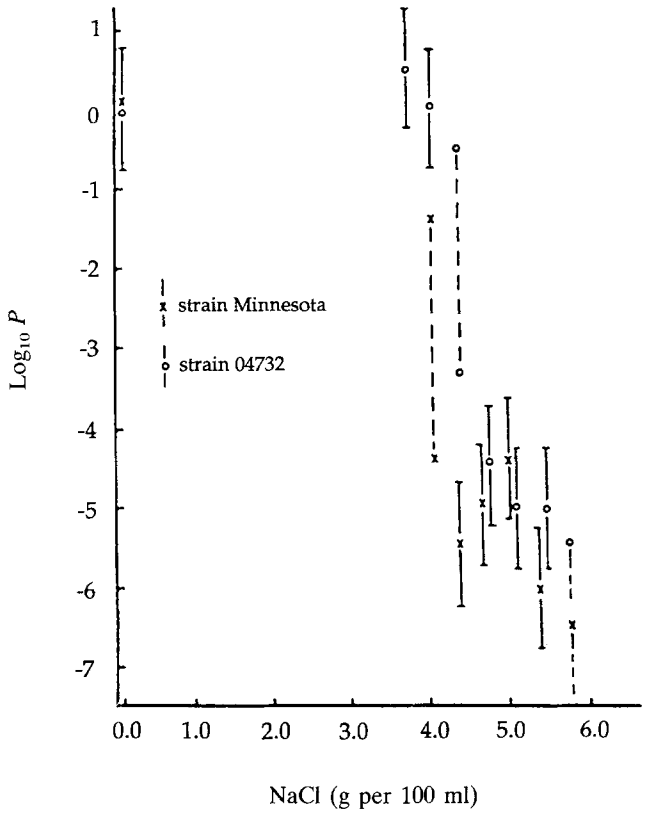

Fig. 7. The effect of salt concentration on the probability (P) for outgrowth from *Clostridium botulinum* type E spores in brain–heart-infusion medium, pH 7.0, incubated at 30 °C for 2 months. Bars represent 95% confidence limits. (Modified from [32].)

metal ions by citrate was responsible for the inhibition. Growth from a spore inoculum was affected similarly.

According to Montville [25], on a medium at pH 7.0 containing 3% NaCl the number of spores of a type A strain able to form colonies in 3 days at 30 °C was over 100-fold lower than that on medium without NaCl (Fig. 6). In contrast, experiments by Riemann [32] showed that for two type E strains at pH 7 incubated for 2 months at 30 °C, NaCl levels lower than 4% had no effect on the probability of growth, but in the presence of 4.5% NaCl log P was reduced by a factor of 5 (Fig. 7). The difference between these reports may be due in part to the use of solid medium and a short incubation time by Montville [25], proteolytic strains (including type A) in general being much more tolerant of high NaCl concentrations than non-proteolytic strains (including type E).

Inhibition of growth from vegetative cells of type A and B (proteolytic) *C. botulinum* by sorbic acid at 30 °C in the pH range 4.9–7.0 was a function of the concentration of undissociated acid [21]. At pH 4.9 a total sorbic acid concentration of 674 mg L^{-1}, equivalent to an undissociated sorbic acid concentration of 283 mg L^{-1} reduced the probability of growth from single vegetative cells in 14 days from between −0.53 and −2.82 to between −6.38 and −7.49 (Table 4). Thus, in foods at a pH of about 5.0 sorbic acid at concentrations used in foods in the UK, usually up to 1000 mg kg^{-1}, could provide a very high safety factor in relation to growth of proteolytic *C. botulinum*.

In studies of the effect of temperature, from 4° to 47 °C, on the probability of growth from spores and vegetative bacteria of proteolytic and non-proteolytic *C. botulinum* log P for growth from single spores or cells of proteolytic strains at 12 °C in 28 days ranged from −3.31 to −6.34, with considerable differences between strains [14]. For non-proteolytic strains log P for growth from single cells or spores at 4 °C in 28 days ranged from 0.76 to −5.92. Regression analyses of the probability of growth of the most capable strains were used to develop equations relating P for initiation of growth of a single spore or cell to temperature and time.

For two type E strains of *C. botulinum* incubated at 30 °C for 2 months log P for growth at pH 5.5 from a spore inoculum was approximately zero, but was about −5 at pH 5.0 (Fig. 5) [32]. At pH 7.0 after incubation at 30 °C for 2 months NaCl concentrations lower than 4% had no effect on the probability of growth, but in the presence of 4.5% NaCl log P was reduced by a factor of 5 (Fig. 7). A combination of pH <5.5 and 3% (w/v) NaCl resulted in a 10^6 reduction in number of spores that resulted in growth in 2 months at 30 °C (Fig. 8). It is clear that lower concentrations of NaCl can contribute to inhibition in combination with other preservative factors.

The combined effects of temperature between 6° and 20 °C, pH, sorbic acid and incubation time on the probability of growth from single vegetative cells of non-proteolytic

TABLE 4

The effect of sorbic acid and pH on the probability (P) of growth from a single vegetative bacterium of *C. botulinum* type A strain ZK3 in a peptone-yeast-glucose-starch (modified PYGS) medium in 14 days at 30 °C [21]

Medium parameter			−log P of growth in 14 days in expt no.		
pH	Total sorbic acid (mg L^{-1})	Undissociated sorbic acid (mg L^{-1})	1	2	3
5.5	0	0	0	0.34	0.15
	1015	156	0.38	1.25	0.28
	1219	188	–	–	1.08
	1421	219	3.23	1.67	3.34
	1828	281	5.53	4.32	5.65
5.1	0	0	0.38	–	0.76
	500	156	0.20	2.20	1.53
	600	188	–	–	1.79
	700	219	1.50	3.56	1.58
	900	282	5.53	5.34	6.08
4.9	0	0	0.53	2.82	1.20
	374	157	0.28	3.41	1.49
	499	189	–	–	1.82
	524	220	4.53	3.48	6.00
	674	283	6.38	6.60	7.49

95% confidence limits of P, ca. ±0.72.

type B strains was investigated by Lund et al. [23] and regression equations were developed to fit the results. At 8 °C and pH 5.3, adjusted with HCl, after 60 days log P was in the range −4 to −6 compared with a value of 0 at 30 °C and pH 7.0 after 2 days (Fig. 9). At 6 °C and pH 5.3 after 60 days log P was <-8. The inhibitory effect of pH was greater at 30 °C and 8 °C than at 16 °C. The effect of sorbic acid was similar to that on proteolytic strains [21]. After 14 days at 30 °C and pH 5.5 in the presence of a total sorbic acid concentration of 1819 mg L^{-1}, equivalent to an undissociated sorbic acid concentration of 280 mg L^{-1} log P was approximately −4. After 60 days at 12 °C and pH 5.5 a total sorbic acid concentration of 1300 mg L^{-1}, equivalent to an undissociated sorbic acid concentration of 220 mg L^{-1}, reduced log P to <-8 (Fig. 10).

In experiments by Lund et al. [21,22,23] and in some of those by Graham and Lund [4] and by Jensen et al. [14] vegetative bacteria were used as in the inoculum. In the majority of experiments to determine the effects of inhibitors on *C. botulinum*, spores are used as the inoculum. This is because it is usually the spores of *C. botulinum* that contaminate foods. In addition, if there is a requirement to investigate toxin formation it is necessary to use spores because these can be freed from toxin before inoculation. Different spore crops of one strain can, however, differ in their properties and particularly in their readiness to germinate in suboptimal conditions. The use of vegetative bacteria may, therefore, allow determination of the most rapid growth possible if germination occurred immediately after inoculation. There is value in having results for both spore and vegetative inocula. An example of the contrast between the time required for growth from an inoculum of vegetative bacteria and that required for growth from spores in adverse conditions is shown by the effect of acid pH [4]. With a vegetative cell inoculum the maximum probability of growth from a single cell at pH 5.2 and 30 °C occurred after three days, whereas from a spore inoculum between 10 and 20 weeks was required for the maximum log P (Table 5).

The information given in these experiments is valuable in that it can demonstrate safety factors of the order of 6 decimal reductions in the probability of growth from single spores or vegetative bacteria of *C. botulinum* and provide equations that relate these factors to the preservative conditions.

In culture media growth of the organism is usually evident as turbidity or changes in the medium, and there is an increasing trend to base experiments on recording of growth where possible rather than on formation of toxin. When studies are made of growth in foods, reliance is often placed on tests for toxin. Riemann [32] reported that he found regularly that a higher number of spores in the inoculum were required for toxin production than for growth. In limited tests in my laboratory we have not seen differences of this order but if this occurs widely it would cause marked

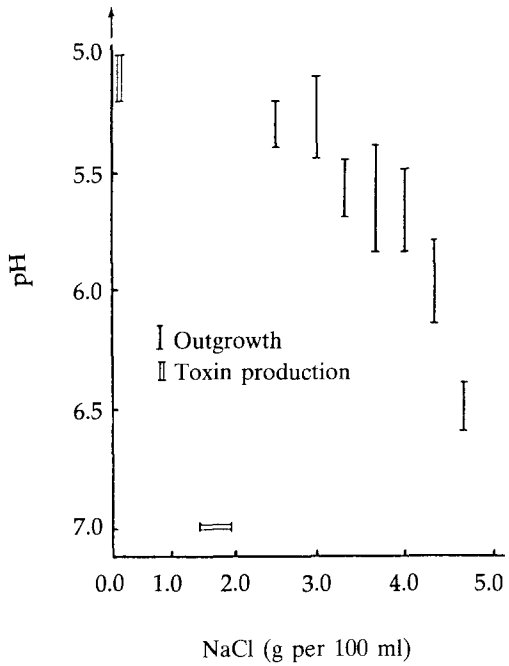

Fig. 8. Combinations of pH and NaCl that caused a reduction of 10^6–10^7-fold in probability (P) for growth from *Clostridium botulinum* type E spores (Minnesota, 04732 and 04735) in brain–heart-infusion medium incubated at 30 °C for 2 months. The 'open' bars indicate levels of NaCl and pH that prevented toxin formation from 10^6 spores of strain Minnesota. (Modified from [32].)

discrepancies between results based on growth and those based on detection of toxin.

THE EFFECT OF PRESERVATIVE FACTORS ON THE PROBABILITY OF GROWTH FROM SINGLE CELLS OR SPORES OF OTHER BACTERIA

A limited number of investigations have been made of the effect of preservative factors on the probability of growth of other bacteria. Studies of the effect of pH and NaCl on the probability of growth of *Staphylococcus aureus* were made by Genigeorgis et al. [3] and multiple regression equations were developed that related the probability of initiation of growth by a single bacterium to the pH and NaCl content of a culture medium. From this the NaCl–pH combinations that would reduce the probability of initiation of growth by a single bacterium by a factor of 10^6 could be calculated. Tests in processed meats showed less inhibition of *S. aureus* than was found in the culture medium [3].

Similar studies of the effect of pH and NaCl on initiation of growth of *Bacillus cereus* were reported by Raevuori and Genigeorgis [30]. The probability of initiation of growth in pasteurized samples of meat and rice adjusted to a range of values of pH and NaCl was also tested. In the food samples less inhibition of *B. cereus* was obtained than in the culture medium.

A study of the effect of temperature, atmosphere and time on the probability of colony formation on blood agar by *Listeria* spp. was reported by Razavilar and Genigeorgis [31]. Of the atmospheres tested, only 100% CO_2 had a marked inhibitory effect, and its effect was greatest at low storage temperature. The log number of bacteria of two strains of *Listeria monocytogenes* needed to initiate colony formation at 4 °C in 14 days in air was 1.11–1.94 and under 100% CO_2 was 4.94–5.87.

We have made some preliminary experiments to determine the effect of pH on the probability of growth of *L. monocytogenes*, and to produce equations describing the results (George and Lund, unpublished). Experiments were done with three strains by making serial 10-fold dilutions and inoculation of each dilution into 16 replicate wells of microplates containing culture medium adjusted to the required pH with HCl. The plates were incubated at 20 °C, examined for visible growth at 24-h intervals and the MPN of the cells that had resulted in growth was calculated and compared with the MPN of cells inoculated. Results for the Scott A strain are shown in Fig. 11. The number of experimental points is limited, but the results show that the

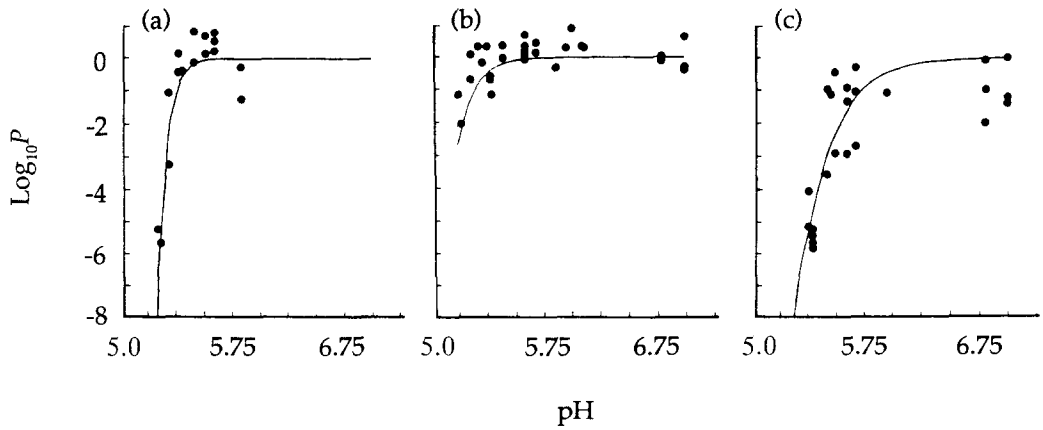

Fig. 9. The effect of pH, adjusted with HCl, on the probability (P) of growth from a single bacterium of non-proteolytic *Clostridium botulinum* type B at (a) 30 °C, (b) 16 °C, and (c) 8 °C after incubation for 60 days [23]. ● = experimental results. Continuous line was calculated from a model. 95% confidence limits for each value of log P are ca. ±0.72.

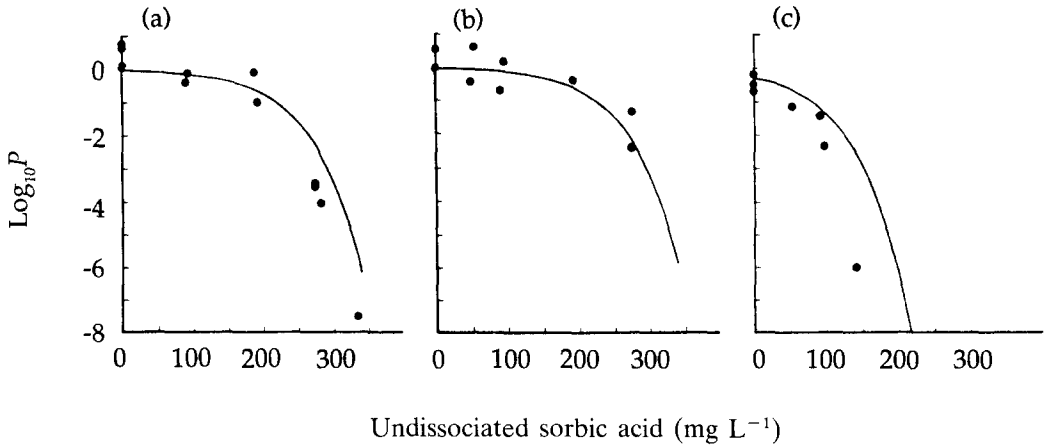

Fig. 10. The effect of undissociated sorbic acid on the probability (P) of growth from a single bacterium of non-proteolytic *Clostridium botulinum* type B at pH 5.5, adjusted with HCl, and incubation temperatures of (a) 30 °C, (b) 20 °C, and (c) 12 °C after incubation for 60 days [23].

TABLE 5

The effect of incubation time on P for growth from a single vegetative bacterium or spore of *C. botulinum* type A, strain ZK3, in culture medium at pH 5.17, adjusted with HCl (modified from [4])

Inoculum	Expt	Log P for growth in (weeks) 0.43	1	4	10	20
Vegetative bacteria	1	0	0	0	0	0
	2	0	0	0	0	0
Spores	1	<−5.84	<−5.84	−3.63	−2.03	−1.59
	2	<−5.84	<−5.84	−2.91	−2.45	−1.03

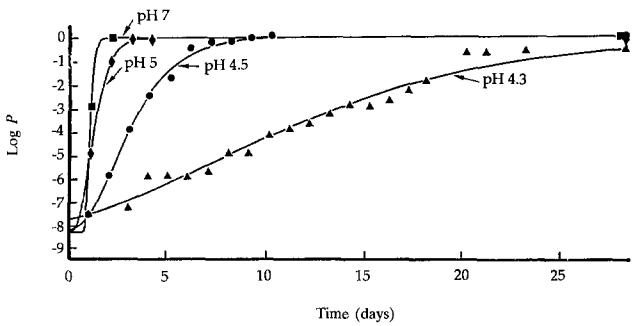

Fig. 11. The effect of pH, adjusted with HCl, and incubation time on the probability of growth from a single cell of *Listeria monocytogenes* strain F4642 (Scott A) at 20 °C. The culture medium was Tryptone Soya broth (Oxoid) plus yeast extract, 3 g L^{-1}, and glucose, 10 g L^{-1}. Serial 10-fold dilutions of a suspension of bacteria were inoculated into 16 replicate wells of medium at each pH, i.e. 10-fold dilutions with 16 replicates per dilution were used to determine the MPN. The MPN of bacteria that had resulted in growth (turbidity) at each pH was recorded daily and used to calculate log P.

relationship between log P and time could be fitted with a Gompertz curve and simple equations were derived that related the parameters B and M of this function to pH.

The Gompertz equation was used as:

$$\log P = A + C\exp\{-\exp[B(t - M)]\}$$

where P is the probability of growth initiation from a single bacterium after time t, A is the log probability as t decreases indefinitely, C is the asymptotic increase in log P as time increases indefinitely, B is the rate of increase of log P at M, and M is the time at which the rate of increase of log P is maximum. For *L. monocytogenes* F 4642 (Scott A) in these experiments $A = -8.2787$, $C = 8.2787$, $B = 10.59 + 2.485$ pH, $M = 774.3 - 323.3$ pH $+ 33.72$ pH^2.

In many experiments to determine log P the 95% confidence limits are wide; these could be reduced by the use of more replicates in MPN counts and of several plates, and therefore a high number of colonies, in counts to determine the number of spores or cells in the inoculum.

Experiments to determine the effect of preservative factors on growth rates of bacteria can give precise information that can be related to the physiology of the microorganism and can be used as a guide to the application of these factors in foods. Determination of effects on the probability of growth from single spores or cells, or on degree of protection, may give less precise results but allows high safety factors to be defined and enables the effect of inoculum size to be determined. There is a need for both types of approach to define the effect of preservative factors on bacteria.

CONCLUSIONS

Growth of *C. botulinum* in foods presents a severe hazard and it is important that the method of preservation of foods should be sufficient to give a high degree of safety against the growth of this bacterium. The heat processing given to low acid foods is, in general, sufficient to give a reduction of 10^{12} in the probability that one heat-resistant spore of a proteolytic

strain of *C. botulinum* will survive, germinate and grow in the food. Where the safety of foods depends on combinations of other preservative factors, with or without heat treatment, it is arguable that the preservation system should reduce the probability that one spore will survive and grow, by a factor of the order of at least 10^6, i.e. provide a degree of protection [7] of 6. Experiments to establish the effect of preservation systems on growth of *C. botulinum* should define and control the anaerobiosis, because this can have a profound effect on results. In order to confirm the effect of a preservation system in a food it is necessary to inoculate the food with at least 10^6 spores or vegetative cells of *C. botulinum* and to demonstrate that these do not initiate growth and toxin formation during the shelf-life of the product, or in abuse conditions that might occur in practice. The development of models that demonstrate the ability of preservation systems to provide degrees of protection of this magnitude requires experiments that can show a change in the degree of protection from 0 to 6–8, i.e. a change in the log probability of growth from 1 to -6 or -8.

In the case of pathogens that present a less severe hazard in foods, the degree of protection required for satisfactory preservation is usually less. Nevertheless pasteurization treatments, for example for liquid whole egg and egg white, are based on the heating required to reduce the number of viable bacteria, in particular salmonellae, by several log cycles. The results of experiments to determine the ability of food-poisoning bacteria to multiply in foods depend on the number of organisms in the inoculum, and studies of the effect of preservation systems on the probability of survival and growth of these bacteria are necessary for the effective design of preservation systems.

A combination of this type of information with that derived from studies of effects on growth rates would provide a valuable assessment of the effect of preservation systems.

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