

Germination, Growth, and Toxin Production of Nonproteolytic *Clostridium botulinum* as Affected by Multiple Barriers

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ABSTRACT: The effect of combinations of pH (6.5, 5.75), NaCl (0.25, 1.75%), and incubation temperatures (7, 13 °C) on spore germination, outgrowth, and time to toxicity of nonproteolytic *Clostridium botulinum* was examined in a broth system. Spores of four toxin type E and nonproteolytic type B were inoculated (10^4 /ml) into Tryptone Peptone Glucose Yeast Extract (TPGY) broth. Cultures were monitored for three weeks, or until toxin was detected. A modified FSIS-amplified ELISA (comparable in sensitivity to the mouse bioassay) was used to screen cultures for neurotoxin. Combinations of the most inhibitory level for each barrier reduced the degree and rate of germination, the lag and growth rate of vegetative cells, and the time to toxicity.

Key words: nonproteolytic *Clostridium botulinum*, germination, neurotoxin, pH, NaCl, temperature

Introduction

NONPROTEOLYTIC *CLOSTRIDIUM BOTULINUM* IS RECOGNIZED as a potential hazard in minimally processed food that is designed for extended refrigerated storage (Conner and others 1989). If spores survive the pasteurization process and the finished product is then stored at temperatures greater than 3.0 °C, there is an unacceptable probability that the surviving spores will germinate and eventually produce neurotoxin (Notermans and others 1990; Brown and others 1991; Graham and others 1997). Instead of relying solely on storage temperature to inhibit *C. botulinum* growth and toxin formation, it would be desirable to incorporate secondary safety barriers into products that act as back-ups to or in conjunction with refrigeration temperature. The individual pH or NaCl concentrations necessary to inhibit toxigenesis may not be organoleptically desirable in many foods. It is also not possible to guarantee storage temperatures less than 3.0 °C after the product is out of the distribution control of the manufacturer. Therefore, combining product pH with NaCl and refrigeration temperature to delay or inhibit *C. botulinum* growth and toxin formation may provide an acceptable alternative. This concept of multiple barriers, or hurdles, to inhibit microbial growth has numerous applications in the food industry (Scott 1989; Leistner and Gorris 1995).

The minimum growth temperature for cells of toxin type E and nonproteolytic B *C. botulinum* at otherwise optimum growth conditions is 3.0 °C (Graham and others 1997). A pH value of 5.0 and NaCl levels of 5.0% and greater are inhibitory to most strains of nonproteolytic spores (Hauschild 1994). Combinations of these properties can act in concert (either additively or synergistically) to slow or inhibit the growth of *C. botulinum*. The minimum inhibitory concentration of one property (for example NaCl) decreases as other antimicrobial factors, such as pH and incubation temperature, are enhanced (Abrahamsson and others 1965; Baird-Parker and Freame 1967; Ohye and Christian 1967; Emodi and Lechowich 1969; Ando and Iida 1970; Graham and others 1996a).

Montville (1984) determined that subpopulations of *C. botulinum* type A spores were capable of producing colonies

at various combinations of pH and NaCl in the enumeration agar. The presence of a small resistant subpopulation may explain the ability of large populations of spores to overcome restrictive environments that completely inhibit low levels of spores (Riemann 1967; Jensen and others 1987).

Germination of clostridial spores can occur under conditions where vegetative growth is not possible. Grecz and Arvay (Arvay 1982) demonstrated germination of type E spores at 2 and 50 °C. The optimum temperature for spore germination was found to be 9 °C while the optimum temperature for vegetative growth was 32.5 °C. In studying the behavior of toxin types A and B spores at near limiting vegetative cell growth temperatures, Smelt and Haas (1978) found that the number of ungerminated (that is, heat resistant) spores decreased over time at 10 °C, but that there was no cell growth or toxin production. Loss of heat resistance over time was used as a measure of germination. At temperatures of 12 °C and greater, germination, growth, and toxin production were possible, depending on the strain. Germination rarely went to completion for a population of spores, even if conditions allowed for rapid outgrowth and toxigenesis.

The objective of this research was to observe the effect of combinations of pH, NaCl, and refrigerated storage temperature on spore germination, outgrowth, and time-to-toxicity for toxin type E and B strains of nonproteolytic *C. botulinum*. Change in total and ungerminated spore numbers over time could provide insight into possible mechanisms of delayed toxin production in the presence of multiple barriers.

Materials and Methods

Organisms

Four *Clostridium botulinum* type E strains (Beluga and O70 from D. Kautter, FDA; Whitefish and Saratoga from V. Scott, NFPA) and four nonproteolytic type B strains (Kapchucka and 17B from D. Kautter, FDA; 2129 and cbw 25B from V. Scott, NFPA) were used. Cultures were checked for purity and toxin type and verified by the mouse bioassay (Solomon and Lilly 1998), using monovalent antitoxin from the Centers for Disease Control and Prevention.

Preparation of spore crops

A modification of the biphasic spore production method of Bruch and others (1968) was used to prepare the inoculum spore crops. The agar phase consisted of 250g ground defatted beef heart; 5g neopeptone (Difco Laboratories Inc., Detroit, Mich., U.S.A.); 5g typtone (Difco); 5g gelatin (Difco); 10g agar (Difco); and 500 ml distilled water. This was mixed in a 2L flask and autoclaved for 30 min at 121 °C. After allowing the agar to cool and solidify, the 8 flasks were allowed to pre-reduce for 24 h at ambient temperature in a Coy Anaerobic Glovebox (Coy Inc., Ann Arbor, Mich., U.S.A.) (gas mix 10% CO₂; 5% H₂; 85% N₂). The liquid phase of the biphasic medium consisted of 50g trypticase (BBL); 5g peptone (Difco); 2g dextrose (Difco); 2g starch (Difco); 0.5g cysteine HCl (Sigma Chemical Co., St Louis, Mo., U.S.A.); and 1L distilled H₂O. The broth was autoclaved for 20 min at 121 °C. After cooling, the broth was placed in the anaerobic chamber and 900 ml was aseptically added to each flask containing the agar phase. The biphasic system was placed at 28 °C and held for an additional 48 h anaerobically to allow the media to pre-reduce and to check for possible contamination.

All 8 strains of nonproteolytic *Clostridium botulinum* were individually inoculated into tubes containing 20 ml of the liquid phase of the biphasic medium and were anaerobically incubated at 28 °C for 48 h. Each 20 ml culture was then added to a flask of biphasic medium and incubated anaerobically at 28 °C for 4 to 7 days until phase contrast microscopic examination of the cultures indicated that >75% of the cells had sporulated.

Spores were harvested by centrifugation at 16,300 x g for 10 min at 4 °C. The spores were then washed by resuspending the pellet in approximately 150 ml sterile distilled water at < 4 °C and centrifuging. This process was repeated at least 5 times for each spore crop. Spores were resuspended in sterile distilled water and stored at 1 °C until needed. Spore crops were enumerated by being heat shocked for 10 min at 60 °C and pour plating with BAM agar (Montville 1981). BAM agar consists of 5.0g yeast extract (Difco), 2.65g nutrient broth (Difco), 2.0g dextrose (Difco), 0.5g sodium thioglycolate (Sigma), 5.0g tryptone (Difco), and 20.0g agar (Difco). Plates were incubated anaerobically at 30 °C for 48 h prior to the counting of colonies.

ELISA

A modification of the FSIS-amplified enzyme-linked immunosorbent assay (ELISA) described by Ransom and others (1993) was used to detect the presence of neurotoxin in growing cultures of nonproteolytic *C. botulinum*. This modification involved the combination of the captured antibodies which were polyclonal goat anti-B and anti-E supplied by Dr. W.H. Lee (FSIS/USDA, Washington, D.C., U.S.A.). Anti-E and anti-B sera were diluted 1:2000 and 1:5000, respectively, in pH 9.6 Carbonate Coating Buffer (CCB) (1.59 g Na₂CO₃, 2.93g NaHCO₃, 1L glass distilled water) and combined 1:1. In addition, polyclonal antisera ((FCM) Rabbit anti-bt A (6/86) (detects hemagglutinin A/B plus neurotoxin A) and (CDS) Rabbit anti-bt E (6/88) (detects neurotoxin E and nontoxic E protein)) were each diluted 1:5000 in PC buffer and then combined 1:1 and used as the sandwich coating. Purified BoNT/B and BoNT/E (from Dr. DasGupta (Food Research Institute, Washington, D.C., U.S.A.) at a concentration of 100 pg/ml (10 pg/well) were used as positive toxin controls. Comparing the ELISA with the mouse bioassay demonstrates that the ELISA was an acceptable alternative for screening large numbers of broth cultures for the presence of neuro-

toxin (Elliott 1997).

Growth experiments

The pH values of TPGY broth were adjusted to 5.75 and 6.5 with 3M HCl. NaCl was added to levels of 0.25 and 1.75% (w/w). Ten ml of the various broth formulations in 16 × 150 mm culture tubes were sterilized at 121 °C for 20 min. All tubes contained an inverted 6 × 50 mm culture tube to aid in detecting gas production. After cooling, all tubes were overlaid with 2 ml of paraffin oil (J.T. Baker, Phillipsburg, N.J.) that had been dry-heat sterilized at 170 °C for 2 h. The tubes were then steamed for 20 min to drive off residual oxygen and cooled to 4 °C prior to inoculation.

Equal concentrations of each of the toxin type B and type E *C. botulinum* spores were combined to give a pooled type B and type E inoculum. In addition to the individual type pooled spores, both toxin types (E/B) were mixed at equal spore concentrations. Spores were heat activated at 60 °C for 10 min immediately prior to inoculation. Twenty ml of chilled inoculum were added to each tube of the TPGY broth (4 °C) through the paraffin oil. Inoculated tubes were incubated at either 7 °C or 13 °C for up to 22 days. Twenty tubes of each pH, salt, spore toxin type and incubation temperature combination were made, allowing up to 10 sets of duplicate tubes to be sampled over the 22 d.

For each sampling time, duplicate tubes were used for total and spore counts. Cells were enumerated by plating on prereduced BAM agar using an Autoplate 3000 (Spiral Biotech, Bethesda, Md., U.S.A.). BAM agar contained 0.002g resazurin (Sigma)/liter as a redox indicator. All manipulations of the tubes and the plating were conducted in a Coy Anaerobic Glovebox. Dilutions were made with prereduced sterile 0.1% peptone (Difco). Plates were incubated at 21 °C for 24 h prior to manual enumeration. It was not possible to use the Laser Bacteria Colony Counter for enumeration due to the transparent nature of the colonies.

Spore counts were made on the same 2 tubes by diluting 1 ml of the broth culture 1:1 with phosphate buffer (pH 7.2) in a 2 ml microfuge tube. The diluted culture was heated at 70 °C for 10 min to destroy any vegetative cells or germinated spores. Spores were enumerated by pour plating with BAM agar and incubating the plates anaerobically at 28 °C for 48 h.

Duplicate samples were refrigerated aerobically at 1 °C for less than 24 h prior to using the ELISA to test for the production of neurotoxin. Enumeration and toxin testing were terminated for any combination of cultural conditions when toxin was detected in at least one of the duplicate samples.

Results and Discussion

EARLY STUDIES SHOWED NO LETHAL EFFECT ON UNGERMENATED toxin type E and B spores heated in phosphate buffer at 70 °C for up to 30 min. However, the combination of exposure to air and heating at 70 °C for 10 min proved to be completely lethal to exponential phase cultures of nonproteolytic *C. botulinum* (data not shown). Loss of spore heat resistance is considered to be the first observable indication of commitment to germination (Foster and Johnstone 1990). It occurs before release of dipicolinic acid, loss of culture turbidity, or phase darkening (Lefebvre and Leblanc 1988).

Results of the growth experiments are presented in Figures 1 to 4. In general, results from mixed toxin types E/B cultures were similar to results observed from type E samples. This reflects the behavior of pooled toxin type E cultures to produce toxin earlier or at the same time and germi-

nate to a lesser degree than the type B cultures under all but 1 cultural combination condition. The 1 condition was with pH 5.75/1.75 % NaCl (Figure 4), in which toxin type B cells grew more rapidly. In this case, the combined E/B growth curve seemed to reflect that of the type B cells.

As expected, decreasing pH, increasing % NaCl, and decreasing temperature individually and in combination had the effect of slowing vegetative growth and increasing the time to toxicity. For vegetative cells expressed as total count, inhibition was evident by increased lag phases and slower growth rates. For sporeformers, length of the lag phase is related to germination rate and degree of germination. Toxin type B spores underwent complete germination under conditions of low salt (Figures 1 and 2). The method used to quantify ungerminated spores only allowed enumeration as low as 20 spores/ml. For the purposes of this study, reduction in ungerminated spores from nearly 104 spores/ml to less than 20/ml is considered complete germination. Presence of 1.75 % NaCl (Figures 3 and 4) prevented complete germination of the toxin type B spores and reduced the degree of germination of the toxin type E spores. Even under the least inhibitory conditions for this study (Figure 1), type E spores did not germinate completely. The discrepancy observed between the total count and the spore count for the type B spores on day zero may be attributed to germination within the first few hours after exposure to germinants in the broth (Figures 1 to 3). Under more stressful conditions, this rapid germination was not observed (Figure 4). Triggering of germination by a germinant has been shown to be pH- and temperature-dependent (Gombas 1984).

Temperature reduction had the effect of reducing the rate

of germination, although in some cases at 7 °C germination proceeded to a greater degree because the experiment was allowed to run longer at the low temperature. Reduction in pH had the effect of both slowing the rate and reducing the degree of germination. This was most evident with the toxin type E spores. A pH value of 5.75 did not prevent complete germination of the type B spores when the NaCl concentration was low. The effect of changing any one environmental factor on germination and outgrowth was more evident at the combined environmental extremes than with the other combinations. For example, the effect of a lower temperature, 7 °C, was more evident at pH 5.75 and 1.75% NaCl.

Complete germination of the spore population does not immediately occur upon exposure to a germinant even if environmental conditions allow for rapid outgrowth. In modeling the germination kinetics of *C. tyrobutyricum*, Mafart (1995) described the decrease in the specific rate of germination over time. Kinetics of spore germination were found to have a significant influence on the lag time before vegetative growth. The distribution of biovariability within a population of germinating *B. cereus* spores was studied by Coote and others (1995) by visually monitoring germination of single spores. They showed that, under ideal conditions, most of the spore population can germinate within a few minutes. However, there is a small segment of the population that may take much longer to initiate germination. The biovariability in germination of individual spores of *C. botulinum* 62A at various temperatures was confirmed by Billon and others (1997).

There is some degree of parallelism between the measurable events of germination: loss of heat resistance, DPA loss,

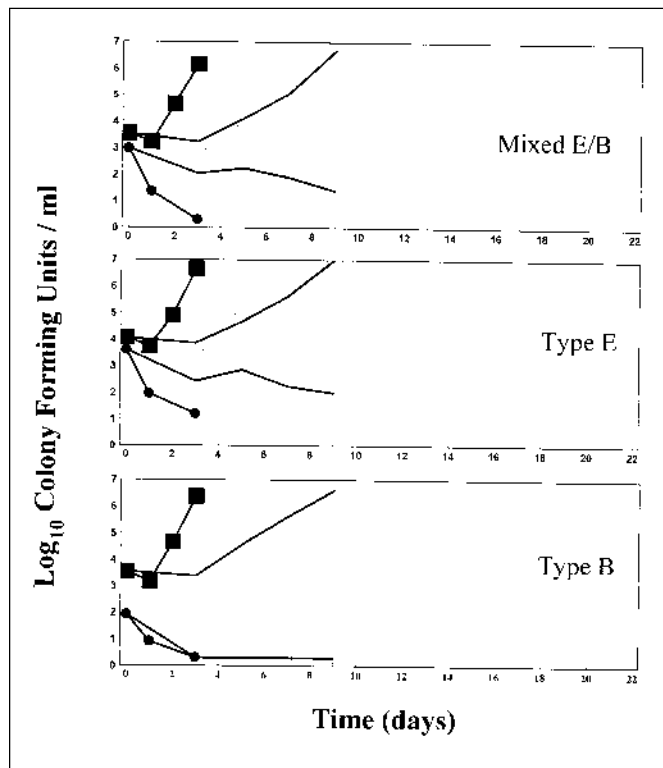


Figure 1—Effect of storage at pH 6.5 and 0.25% NaCl on spores (circles) and total count (squares) of nonproteolytic *C. botulinum* stored at 7 °C (open symbols) and 13 °C (solid symbols)

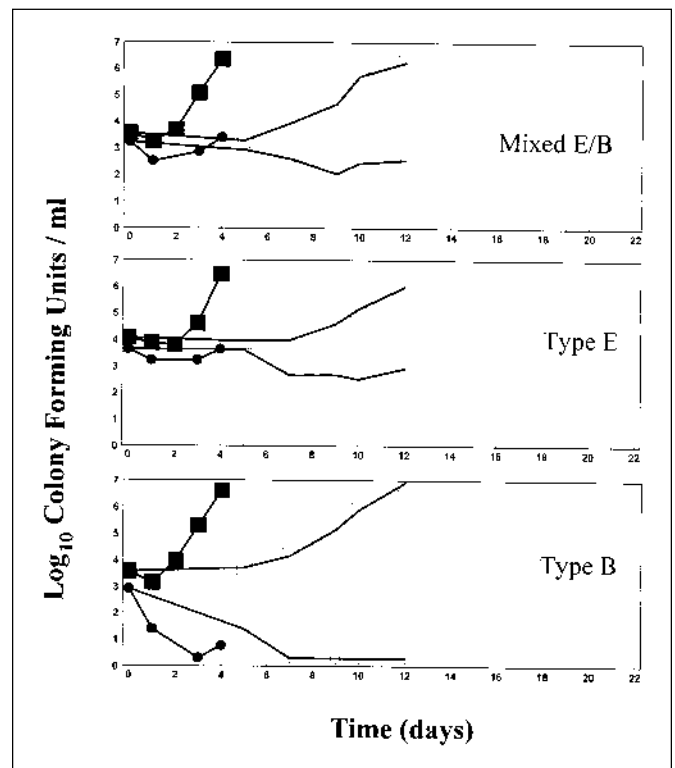


Figure 2—Effect of storage at pH 5.75 and 0.25% NaCl on spores (circles) and total count (squares) of nonproteolytic *C. botulinum* stored at 7 °C (open symbols) and 13 °C (solid symbols)

Table 1—Earliest time to toxicity^a for each pooled nonproteolytic *Clostridium botulinum* spore type and cultural condition

Cultural conditions ^b			Earliest time to toxicity (days)		
Temp.(°C)	pH	NaCl (%)	Spore type ^c		
			Mixed E/B	E	B
13	6.5	0.25	3	3	3
	6.5	1.75	5	5	5
	5.75	0.25	4	4	4
7	5.75	1.75	7	7	7
	6.5	0.25	9	9	9
	6.5	1.75	13	13	15
	5.75	0.25	12	12	12
	5.75	1.75	20	21	21

^aToxin detected with modified FSIS amplified ELISA

^bConditions of anaerobic TYGY broth

^cInitial spore inoculum was 10⁴/ml in 10 ml of broth

and phase darkening. Stressful environmental conditions that affect one of these events could possibly affect the rate of subsequent events, and eventually outgrowth. Outgrowth is characterized by synthesis of new macromolecules and respiration. Inhibition of these metabolic activities even for a short time may be irreversible and can lead to the death of the cell. However, inhibition of germination is caused by interference with either primary spore activation or degradative processes (Vinter 1970). Since germination is the degradative process of changing an activated spore into one ready for outgrowth, asynchronous germination is the rule rather than the exception (Gombas 1984). Such heterogeneity could also explain the ability of only a few spores within a popula-

tion to germinate and grow under stressful conditions, as observed in this experiment and in work done by Montville (1984). Incomplete germination of a population of spores, especially under marginal conditions for growth, may be viewed as a survival strategy for bacteria. Even when conditions for growth are not optimal, a fraction of the spore population (those that do not germinate) will survive to germinate and grow if environmental conditions change.

Enumeration of the total count (vegetative cells and spores) was conducted under strict anaerobic conditions because early experiments have demonstrated that exposure to air was detrimental to nonproteolytic vegetative cells. A comparison of times to toxicity (Table 1) to growth curves (Figures 1 to 4) demonstrates that toxin formation did not occur until the culture grew to at least 10⁵ cells/ml. This contradicts the results of Brown and others (1991). In studies of growth and toxin formation by nonproteolytic *C. botulinum* in sous vide chicken and cod, they observed incidences of toxin formation without concurrent increases in cell numbers. In one case, the number of cells enumerated at the time of toxin production was less than the initial spore inoculum. Aerobic plating of the vegetative cells may have given rise to misleading results in that study.

The time-to-toxicity values in Table 1 are approximated in some cases, because not all of the cultures were tested daily up to the point of toxin detection. The cultures that were not toxin-tested on the day before toxin was evident were the 7 °C/0.25 % NaCl samples. The ability of type E cultures to produce detectable toxin sooner than type B under some conditions may be related to the higher titers of toxin produced at 21 °C by type E strains (Elliott 1997). Time to toxicity

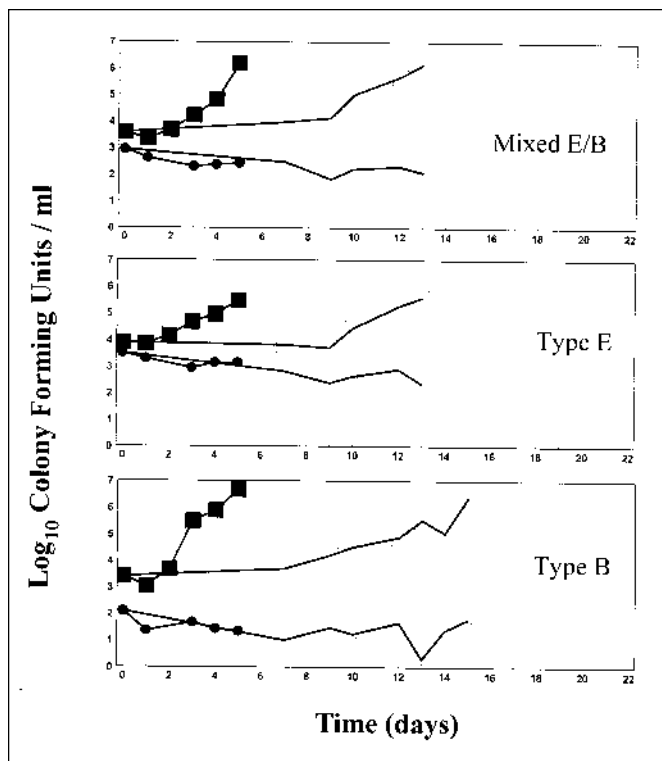


Figure 3—Effect of storage at pH 6.5 and 1.75% NaCl on spores (circles) and total count (squares) of nonproteolytic *C. botulinum* stored at 7 °C (open symbols) and 13 °C (solid symbols)

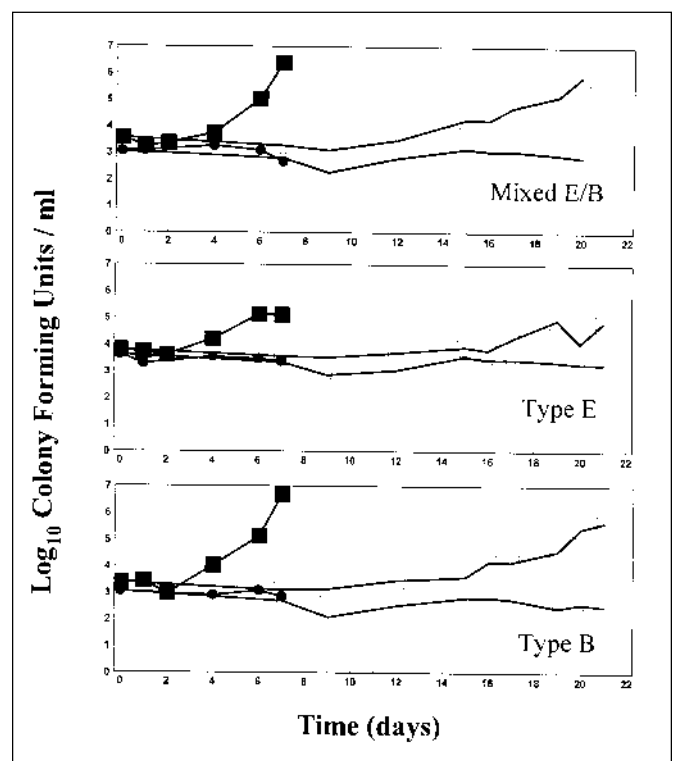


Figure 4—Effect of storage at pH 5.75 and 1.75% NaCl on spores (circles) and total count (squares) of nonproteolytic *C. botulinum* stored at 7 °C (open symbols) and 13 °C (solid symbols)

for the mixed E/B cultures was equivalent to, or in some cases faster than, the individual type cultures.

Toxin was found only in those cultures that had evidence of growth (for example, turbidity and/or gas production). Detection of toxin only after visible growth confirms the findings of Graham and others (1996a). It also indicates that time to turbidity as used by Whiting and Oriente (1997) is a meaningful endpoint in monitoring growth of nonproteolytic *C. botulinum* in developing their predictive model.

The best way to quantify the effect of multiple barriers on microorganisms is through the development of predictive models (Lund 1993). Graham and others (1996b) developed a model to describe the lag time and specific growth rate of nonproteolytic *C. botulinum* as affected by pH, salt, and temperature. Whiting and Oriente (1997) developed a model for nonproteolytic *C. botulinum* type B to predict time to turbidity as a function of spore inoculum, pH, salt, and temperature.

Conclusion

VARIOUS COMBINATIONS OF SUBINHIBITORY LEVELS OF ALL three environmental parameters (pH, NaCl concentration and temperature) slow the rate and degree of spore germination, the rate of vegetative growth, and therefore the time to toxicity. Inhibition of complete germination was observed for increased concentrations of NaCl. This may be a survival mechanism to allow part of the spore population to remain dormant during exposure to borderline stressful conditions that may otherwise not allow vegetative growth.

Since toxin was evident from cultures of both type E and B spores in the entire range of cultural conditions examined, the methods developed in this paper are acceptable to create databases for statistical predictive models where very large numbers of replicates need to be screened for toxin production. Models presented in future publications will be based on combinations of barriers (pH, NaCl, and temperature) similar to those used in these experiments, plus intermediate values, to predict time to toxicity and probability of toxicity by nonproteolytic *C. botulinum*.

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