Journal of Food Protection, Vol. 55, No. 1, Pages 23-27 (January 1992)
Copyright©, International Association of Milk, Food and Environmental Sanitarians

Effect of Headspace Oxygen Concentration on Growth and Toxin Production by Proteolytic Strains of *Clostridium botulinum*

R. C. WHITING* and K. A. NAFTULIN

U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, Pennsylvania 19118

(Received for publication September 11, 1990)

ABSTRACT

A series of experiments was conducted to determine growth and toxin formation by proteolytic strains of *Clostridium botulinum* in broth media that have known pH values (5-7), NaCl concentrations (0-4%), and controlled oxygen-nitrogen atmospheres. Lower pH and higher NaCl levels inhibited growth and toxin production by vegetative cells, but 15% oxygen in the headspace was insufficient for inhibition in all media. When spores were used as inocula and the tubes were gas flushed, outgrowth and toxin production generally occurred only under a 1% or less oxygen atmosphere. Occurrence of growth and toxin was favored by high pH and low NaCl levels and was related to spore inoculum size. Spores were also inoculated into a mixed fermenter with controlled oxygen levels in the headspace. Times to measurable turbidity increased with greater oxygen levels from 36 h at 0.005% O_2 to 109 h at 0.7% O_2; however, growth rates were unaffected by headspace oxygen levels. No toxin was observed with 0.9% O_2, further demonstrating that the critical level of oxygen for germination and growth is approximately 1%.

Clostridium botulinum is characterized as an obligate anaerobe. However, growth and toxin production were demonstrated after outbreaks and in model foods under ostensibly aerobic conditions. For example, sliced luncheon meats, air-packed smoked fish, salted-dried fish, sauteed onions left on the grill, packaged fresh mushrooms, and oriental noodles in oxygen permeable plastic have all supported toxin formation (*9*). Nonproteolytic type E strains in fish-based model substrates developed toxicity in aerobic packaging (*1,14,17,27*).

Early work by Meyer (20) and Dack et al. (*3-5*) indicated proteolytic strains could grow in broth media with headspace oxygen concentrations of 1.7%. Type A spores were reported to grow in the Eh range of -436 to -6 mV (*22*). Spores placed in trypticase soy broth with cysteine and sparged with N_2 (-145 mV) or air (-60 mV) grew equally well at favorable conditions, but growth was delayed with air sparging of broth containing 5-6% NaCl, pH 5.3, or 30% sucrose (*26*).

Predicting the growth of microorganisms in foods requires knowledge of the simultaneous influence of all significant factors. A model for toxin production in fish by nonproteolytic type E botulinum was developed with coefficients for fish type, spore type, type of atmosphere (none containing O_2), temperature, spore inoculum, and aerobic plate count (*1*). Roberts and Jarvis (*23*) modeled the growth of type A spores in pasteurized pork slurries. Their model included storage temperature, NaCl, nitrite, ascorbate, heat treatment, polyphosphate, and high (6.3-6.77) or low (5.54-6.36) pH. Montville (*21*) followed the interaction of pH and NaCl in broth on the culture density of type A vegetative cells. Dodds (*6*) measured the lag time for toxin production by type A and B spores in cooked, vacuum-packed potatoes with controlled a_w and pH. Regression equations for the probability of one spore to produce toxin at a specific a_w-pH were calculated. None of these studies, however, used controlled oxygen levels as a variable.

There is concern that the increasing commercial use of modified atmosphere packaging and precooked foods is increasing the risk of foodborne microbial illnesses including botulism (*11*). A better understanding of the oxygen tolerance of *C. botulinum* as it interacts with pH, salt, and inoculum size is needed. This work examined the growth and toxin formation by proteolytic strains of *C. botulinum* in media that had known pH and NaCl concentrations and were maintained under a controlled oxygen-nitrogen atmosphere headspace. Time for growth and toxin production from both spores and vegetative cells in static broth tubes and from spores in an agitated fermenter was studied.

MATERIALS AND METHODS

C. botulinum

Proteolytic type B strain spores (ATCC 7949) were grown at 35°C for 3 weeks in cooked meat media inside an anaerobic chamber flushed with 5% H_2:10% CO_2:85% N_2 (Coy Laboratory Products, Inc., Ann Arbor, MI). The spore culture was stored

Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Nexus Ex. 1032
Page 1 of 5

inside the chamber. The spore preparations were heat shocked at 80°C for 10 min and enumerated inside the anaerobic chamber by diluting with sterile 0.1% peptone, surface plating on botulinal assay medium (BAM) agar (*12*) with a spiral plater (Spiral Systems, Inc., Bethesda, MD), and incubating at 35°C. BAM consisted of 5.0 g yeast extract, 5.0 g tryptone, 2.65 g nutrient broth, 1.2 g dipotassium phosphate, and 2.0 g dextrose per L (pH 7.3). For studies with vegetative cells, spores were heat shocked and 10 µl inoculated into 20 ml of BAM broth inside the anaerobic chamber. After 48 h at 35°C, 0.1 ml was transferred to each of four tubes containing 20 ml BAM. After 24 h the cultures were washed and resuspended in 20 ml peptone water (total volume).

In the spore germination experiment, spores were heat shocked and diluted with peptone water. Diluted spores (0.10 ml) were then added to BAM media (5.0 ml) to provide a level of 500 spores per ml. For the experiment in the fermenter, three individual strains of type A (69, FDA; 62, FDA; 33, US Army Lab, Natick, MA) and three strains of type B (169, FDA; 999, FDA; ATCC 7949) were individually heat shocked and 0.1 ml inoculated into BAM (100 ml). After incubating inside the anaerobic chamber at 35°C for 3 weeks, the spores were washed, resuspended in water, heat shocked, and enumerated as described above. The individual strains were diluted with water and combined to give an equal strain mixture totaling 2.5 x 10^5 spores per ml. The spore preparation was stored at 6°C for use as inoculum for the fermenter.

Growth of vegetative cells

Culture tubes containing BAM (5.0 ml) with varying pH (pH 5-7 by 0.1 N HCl) and added NaCl (0-4%) were autoclaved and gas equilibrated overnight (ca. 15 h) inside the anaerobic chamber. The loose-capped tubes were inoculated with 0.1 ml vegetative cells (ATCC 7949) to give an average log CFU per ml counts of 3.8. The tubes were placed immediately inside jars fitted with plastic connectors, transferred to an incubator at 20°C, and connected together serially. An oxygen-nitrogen gas mixture with the desired oxygen concentration was then passed through the jars at a constant flow rate of 500 cc/min. The outlet tube of the last jar was submerged in water to create a slight positive pressure in the jars and visually confirm gas flow and absence of leaks. At each sampling time over a 14-d period, the end jar was removed and immediately transferred into the anaerobic chamber where the clostridia in the tubes were enumerated. The tubes were then removed from the chamber and duplicate samples (0.2 ml) placed in a well of a ELISA plate for turbidity measurement at 630 nm. The remainder was stored at 6°C for toxin assay by ELISA (*13*). The amount of toxin from one tube from each run was quantified by mouse bioassay (*8*), and a sample was placed on each ELISA plate as a toxin standard for the other samples on the plate. Two or three replicate tubes were sampled at each time for every pH-NaCl combination. The medium pH and NaCl levels were selected to incorporate the anticipated range of growth using a response surface design. This permitted determination of the variable interactions without the large number of samples required by a full factorial design.

The gas mixtures were formulated by metering and mixing ultrahigh purity nitrogen and custom grade 1%, 10% or 20% oxygen-in-nitrogen (Lindy Division, Union Carbide, Philadelphia, PA). Oxygen levels of the mixtures were assayed with an oxygen analyzer (Systech Instruments, McHenry, IL). The procedure was repeated seven times with oxygen levels of 0.0% (three times), 1.6%, 3.5%, 10.0%, and 15.0%.

To estimate the redox potentials (Eh), uninoculated beakers containing BAM (pH 7.0 and no added NaCl) with the same surface to volume ratio as the culture tubes were autoclaved and equilibrated inside the anaerobic chamber. The beakers were then removed, placed under a flowing nitrogen or 10% oxygen-in-nitrogen, and the platinum and reference electrodes (Radiometer, Denmark) carefully placed 1 cm from the top or bottom of the BAM broth. The Eh was followed until the media were in equilibration with the headspace oxygen. The electrodes were standardized with potassium hydrogen phthalate-quinhydrone (*18*).

Spore outgrowth

The spore inoculation experiment used 5.0 ml BAM with varying pHs and NaCl additions in 15-ml culture tubes. After autoclaving, media were equilibrated inside the anaerobic chamber overnight and inoculated with 0.10 ml ATCC 7949 spores. The tubes were transferred to a plastic glove bag and the bag was flushed three times with the desired oxygen-nitrogen gas mixture. Tubes were then individually flushed for 10 s with the gas mixture and immediately resealed with gas impermeable butyl rubber stoppers (Bellco Glass, Inc., Vineland, NJ). After all tubes were flushed and capped, the tubes were transferred to anaerobic jars which were then flushed for 10 min with 2 L/min of the same gas mixtures and sealed. The jars were incubated at 20°C for up to 90 d. When growth in a tube was evident, the tube was withdrawn and the jar reflushed. The number of days for visible growth was recorded, and the tube was stored at 6°C for confirmation of toxin formation with the ELISA test. The jars were reflushed at 2-week intervals if not previously opened to remove a tube with growth.

Spore outgrowth in a fermenter

Two liters of BAM with 0.5% sodium thioglycolate (pH 7.0 with no added NaCl) was sterilized inside the 6-L bowl of a Techne BR-06 Bioreacter (Techne, Inc., Princeton, NJ). Temperature was maintained at 35.0°C and the broth mixed at 100 rpm. The oxygen-nitrogen gas mixture flowed through a microbial filter into the headspace at 500 cc/min. The oxygen meter was attached to the gas outlet to confirm the oxygen level. The pH was monitored by a submerged electrode interfaced with a microcomputer (Leading Edge D2) with control software by Nomad, Inc. (Livermore, CA). Broth was continuously pumped through a LKB 2138 Uvicord S column densitometer (LKB Instruments, Inc., Gaithersburg, MD) equipped with 408-nm filter and interfaced with the computer. Turbidity and pH values were printed and stored on the microcomputer's hard disk every 30 min. Before inoculation, the fermenter was operated for 24 h to permit detection of any contaminant. The gas mixture was flowing for a minimum of 6 h before inoculation to establish equilibration between headspace and dissolved oxygen. Broth was inoculated with heat-shocked spore mixture (10-ml) containing a total of 2.8 x 10^6 spores. Immediately after inoculating, a sample (4 ml) was withdrawn for Eh measurement. A rubber stopper with Eh electrodes and syringe needle was pressed upon the sample to exclude air and the measurement taken after about 5 min when the reading stabilized. Another sample was transferred to the anaerobic chamber and surface plated on BAM agar plates to confirm inoculum size. Other plates were incubated aerobically to detect contamination by facultative anaerobes. The remaining portion of this sample was mixed with an equal portion of glycerol and stored at -15°C for mouse bioassay. At the end of the fermenter run, another set of samples was taken and analyzed for Eh, bacterial population, Gram stain, catalase activity, possible contamination, and presence of toxin. Preliminary trials were conducted with water and a dissolved oxygen electrode (Associated Bio-engineers and Consultants, Inc., Bethlehem, PA) in the fermenter under identical condition as above to determine the rate of oxygen equilibration after changing from air to nitrogen.

Turbidity data were entered onto an RS/1 table (BBN Software Products Corp., Cambridge, MA) and fitted to the Gompertz equation using a VAX computer and a Gauss-Newton iteration procedure (*2*). Lag times and rate of turbidity increase were calculated from the Gompertz coefficients.

Nexus Ex. 1032
Page 2 of 5

RESULTS AND DISCUSSION

Vegetative cells

The Eh near the bottom of the pH 7 (no added NaCl) medium increased from -117 mV (equilibrated in the reducing atmosphere of the anaerobic chamber) to + 107 mV with flowing nitrogen. Equilibration was 90% complete within 4 h. When 10% oxygen was purged over the medium, the Eh attained +275 mV within 2 h.

With no oxygen in the nitrogen atmosphere above the medium, growth and toxin were consistently observed in media having pH $\geq$ 5.5 and NaCl $\leq$ 3.0 % (Table 1). Growth and toxin were not observed at pH 6.0 with 4.0% NaCl. When the oxygen level was increased to 1.6%, no toxin was detected at the 3.0 and 4.0% NaCl levels and at pH 5.0. Increasing the oxygen levels did not prevent toxin formation in the other five NaCl-pH treatments until 15% flowing headspace oxygen. At that oxygen level, growth and toxin still occurred in two media with pH 6.0 and 7.0 and no added NaCl. For a given medium, the time for measurable toxin formation was only slightly increased by increasing oxygen concentrations.

TABLE 1. *Interaction of oxygen levels with pH and salt content for production of toxin from vegetative cells of* C. botulinum *at 20°C.*

pH	Added NaCl (%)	% oxygen above media				
		0.0	1.6	3.5	10.0	15.0
7.0	0.0	2[a]	2	3	3	3
7.0	4.0	7*	NT	NT		
6.5	1.0	3	2	3	7	NT
6.5	3.0	6	NT	NT		
6.0	0.0	4	2	3	3	7
6.0	2.0	3	3	8	7	NT
6.0	4.0	NT	NT	NT		
5.5	1.0	5	3	6	7	NT
5.5	3.0	5*	NT	NT		
5.0	0.0	7*	NT	NT	NT	NT

[a] Time toxin first detected in days.
* Toxin detected in 2 of 3 runs within 14 d.
NT-No toxin or growth detected within 14 d.
Blank space indicates sample not run.

Douglas and Rigby (*7*) claimed the Eh fell during spore germination and emergence, and Siegel and Metzger (*24,25*) demonstrated that a growing culture in a fermenter can reduce the Eh by 150 mV. We surmise that under favorable pH and NaCl conditions, the vegetative cells were able to maintain the initial Eh in the media and continue to grow, even with subsequently high headspace oxygen concentrations.

The inoculum size affected the time for toxin under 10% oxygen atmosphere in every pH-NaCl medium (Table 2). With 50 CFU per ml inoculum, growth and toxin occurred only at 7.0 with no added NaCl within the 14-d incubation at 20°C. With 5 x 10^5 CFU per ml inoculum, growth and toxin were observed in all media. The amount of toxin produced was not great with this strain (*24*), but it did show an inverse relationship with the time for toxin formation.

TABLE 2. *Effect of inoculum size on the growth and toxin production by vegetative cells grown with a 10% oxygen atmosphere over broth media at 20°C.*

pH	Added NaCl (%)	Inoc. (log)	Time toxin (d)	Toxin (MLD/ml)
7.0	0.0	1.7	9	9
		3.5	3	21
		5.7	1	52
6.5	1.0	1.7	--[a]	0
		3.5	7	22
		5.7	1	128
6.0	0.0	1.7	--	0
		3.5	3	16
		5.7	1	58
6.0	2.0	1.7	--	0
		3.5	7	20
		5.7	4	7
5.5	1.0	1.7	--	0
		3.5	7	30
		5.7	2	110
5.0	0.0	1.7	--	0
		3.5	--	0
		5.7	3	42

[a]-- No growth observed within 14 d.

Spore inocula

When spores were inoculated into media and sealed under varying headspace oxygen concentrations for up to 90 d at 20°C, the oxygen levels that allowed growth and toxin formation were much lower than those observed with vegetative cells (Table 3). At 0.5% or more oxygen, no growth was observed at pH 5.0. The frequency of growth in the other media decreased at 0.5 and 1.0% compared to 0% oxygen. At 2.0% oxygen only one tube had growth but no detectable toxin. Eight tubes, including the tube at 2% oxygen, had growth but no toxin and were confirmed to have gram-positive, catalase-negative rods. It was probable that these tubes were removed for storage at 6°C for the toxin assay without allowing sufficient time for formation of detectable amounts of toxin. Our experience with this ELISA showed it could detect approximately 10 mouse units per ml of toxin.

Fermenter

The previous culture tubes were not agitated, undoubtedly a factor in allowing the continuing growth of the vegetative cells. A series of runs in the fermenter was intended to collaborate the observations of spore growth presented on Table 3 but with mixing and monitored headspace oxygen levels. Trials measuring dissolved oxygen showed a 90% reduction in the first 1 1/2 h after initiating flow of nitrogen. All factors were made optimum for growth including the addition of thioglycolate to the media. The times to turbidity increases were 36 to 60 h with less than 0.4% O_2 and 110 h with 0.5 to 0.7% O_2 (Table

Nexus Ex. 1032
Page 3 of 5

TABLE 3. *Time for growth and toxin production of spores of* C. botulinum *with varying headspace oxygen, pH, and salt levels at 20°C.*

Oxygen (%)	pH	Added NaCl (%)	Ave. time growth (d)[a]	Growth	Toxin
0.0	7.0	0.0	6	10/10[c]	10/10
	7.0	3.0	8	10/10	10/10
	6.0	0.0	8	10/10	10/10
	6.0	1.5	9	10/10	10/10
	5.0	0.0	35	5/10	5/6[d]
	5.0	3.0	--[b]	0/10	0/4
0.5	7.0	0.0	4	5/5	4/5
	7.0	3.0	74	4/5	3/5
	6.0	0.0	89	1/5	1/5
	6.0	1.5	79	1/5	1/3
	5.0	0.0	--	0/5	0/3
	5.0	3.0	--	0/5	0/1
1.0	7.0	0.0	5	3/5	1/5
	7.0	3.0	32	4/5	2/4
	6.0	0.0	34	2/5	1/4
	6.0	1.5	26	1/5	1/3
	5.0	0.0	--	0/5	0/2
	5.0	3.0	--	0/5	0/2
2.0	7.0	0.0	76	1/10	0/8
	7.0	3.0	--	0/10	0/7
	6.0	0.0	--	0/10	0/7
	6.0	1.5	--	0/10	0/7
	5.0	0.0	--	0/10	0/7
	5.0	3.0	--	0/10	0/7

[a] Days to visible growth.
[b] -- No tubes showed turbidity or contained toxin within 90 d.
[c] Numerator is number of positive tubes, denominator total number of tubes tested.
[d] Not all nongrowth tubes were assayed for toxin.

TABLE 4. *Time to turbidity and growth rate of* C. botulinum *in a fermenter with varying headspace oxygen levels at 35°C.*

Oxygen (%)	Time turbidity (h)	Growth rate (Abs/h)
0.005	36	0.70
0.007	45	0.51
0.08	56	0.50
0.15	53	0.28
0.3	60	0.65
0.4	44	0.56
0.5	105	--[a]
0.6	106	0.15
0.7	109	0.35
0.9	>325	NT[b]
1.2	>380	NT

[a] Lag time determined from pH decline.
[b] No increase in turbidity observed.

4). The presence of toxin was confirmed by mouse bioassay in all runs and plate counts showed counts reached 10^7 to 10^8 CFU per ml. At 0.9 and 1.2% oxygen, no turbidity increases indicating vegetative growth were observed after over 325 h incubation and neither run had detectable amounts of toxin. However, plate counts at the end of the runs of 10^5 CFU per ml indicated 2-log cycles of growth had occurred. The absence of toxin may be from insufficient number of cells to produce detectable amounts of toxin, inability of the organism to produce toxin under these conditions, or inactivation of toxin by proteases, denaturation or other chemical reaction (*9*).

Once turbidity began to increase, growth was rapid at all permissive oxygen levels and not consistently affected by the oxygen levels. After maximum growth, the pH declined 0.6 to 1.5 units (average final pH was 6.24) and the Eh declined from an average of +287 to -38 mV (pH corrected). This lowering of redox potential despite continuously flowing oxygen and mixing showed again the capability of *C. botulinum* to create a more favorable environment for itself. These Eh values are higher than the + 144 mV in sterilized milk and tryptone medium with lactose that permitted growth by strain 62A (*15,16*). Nonproteolytic type E strains grew in media with Eh values between + 100 and +250 mV (*14,22*). Lund and colleagues (*18,19*) showed that the number of type E spores necessary to initiate growth must increase exponentially with increasing Eh. Redox potential measurements in a particular complex medium are not specifically related to oxygen concentrations because of the many redox couples involved, some not rapidly reversible or able to react with oxygen (*10*). The controlling factor in growth by anaerobes is the concentration of oxygen and oxygen radicals in the medium and the organism's ability to protect itself from them (*15*).

In summary, this work showed that the critical range of oxygen tolerance of spores of proteolytic strains of *C. botulinum* is approximately 1%. Conditions inside the fermenter of a favorable environment, high spore numbers, and mixing permitted significant growth only at $\leq$0.7% headspace oxygen. However, the number of trials was very limited and the maximum time allowed for growth was 14 d. The spores in static tubes had lower numbers of spores, a more realistic abuse temperature of 20°C, and were incubated for 90 d. They grew under 1.0% oxygen in media having pH values of 6.0 and 7.0. Even though the absolute minimum pH is generally considered to be 4.6 (*9,21*), this work suggested that reducing the pH to 5.0 greatly restricts the ability of the *C. botulinum* to grow. This work also demonstrated the interaction of pH, NaCl, and headspace oxygen in delaying growth and toxin formation. Additional modeling for spore loads realistically expected to be present in foods intended for refrigerated storage is needed to confidently exploit multiple barriers to growth when the food is temperature abused.

Allowing the spores to germinate and multiply before placing them under oxygen atmospheres in unagitated broths demonstrated a great ability by the organism to maintain a suitable environment for continuing growth and toxin production. These quiesence broths could represent a solid food or a liquid during storage and implied that the oxygen surrounding a food is an unreliable barrier to *C. botulinum* growth.

Nexus Ex. 1032
Page 4 of 5

REFERENCES

1. Baker, D. A., and C. Genigeorgis. 1990. Predicting the safe storage of fresh fish under modified atmospheres with respect to *Clostridium botulinum* toxigenesis by modeling length of the lag phase of growth. J. Food Prot. 53:131-140.
2. Buchanan, R. L., H. G. Stahl, and R. C. Whiting. 1989. Effects and interactions of temperature, pH, atmosphere, sodium chloride, and sodium nitrite on the growth of *Listeria monocytogenes*. J. Food Prot. 52:844-851.
3. Dack, G. M., and M. M. Baumgartner. 1928. Effect of reduced oxygen tensions on the growth of *Clostridium botulinum* (type A) in veal infusion broth. J. Infect. Dis. 43:491-494.
4. Dack, G. M., and E. H. Williston. 1929. Effect of oxygen on the viability of young cultures of *C. botulinum*. J. Infect. Dis. 44:26-32.
5. Dack, G. M., W. A. Starin, and M. Werner. 1927. Growth of *C. botulinum* and *C. sporogenes* in veal infusion broth under reduced pressure. J. Infect. Dis. 40:525-532.
6. Dodds, K. L. 1989. Combined effect of water activity and pH on inhibition of toxin production by *Clostridium botulinum* in cooked, vacuum-packed potatoes. Appl. Environ. Microbiol. 55:656-660.
7. Douglas, F., and G. J. Rigby. 1974. The effect of oxygen on the germination and outgrowth of *Clostridium butyricum* spores and changes in the oxidation-reduction potential of cultures. J. Appl. Bacteriol. 37:251-259.
8. Food and Drug Administration. 1978. Bacteriological analytical manual, 5th ed. Association of Official Analytical Chemists, Washington, DC.
9. Hauschild, A. H. W. 1989. *Clostridium botulinum*. pp. 111-189. *In* M. P. Doyle, (ed.). Foodborne bacterial pathogens. Marcel Dekker, New York.
10. Holland, K. T., J. S. Knapp, and J. G. Shoesmith. 1987. Anaerobic Bacteria. Chapman & Hall, New York.
11. Hotchkiss, J. H. 1988. Experimental approaches to determining the safety of food packaged in modified atmospheres. Food Technol. 42 (9):55, 60-62, 64.
12. Huhtanen, C. N. 1975. Some observations on a Perigo-type inhibition of *Clostridium botulinum* in a simplified medium. J. Milk Food Technol. 38:762-763.
13. Huhtanen, C. N., and H. W. Lee. 1990. Factors influencing the ELISA test for botulinal toxin. Institute of Food Technologists' Annual Meeting, Paper # 396.
14. Huss, H. H., I. Schaeffer, A. Pedersen, and A. Jepsen. 1980. Toxin production by *Clostridium botulinum* type E in smoked fish in relation to the measured oxidation reduction potential (Eh), packaging method and the associated microflora. Adv. Fish Sci. Technol. Fishing News Books, Ltd., Farnham, UK. pp. 476-479.
15. Kaufmann, O. W., and R. S. Marshall. 1965. Development of *Clostridium botulinum* type 62A in a trypticase medium autoclaved in the presence of lactose. J. Dairy Sci. 48:670-673.
16. Kaufmann, O. W., and R. S. Marshall. 1965. Factors affecting the development of *Clostridium botulinum* in whole milk. Appl. Microbiol. 13:521-526.
17. Lindroth, S. E., and C. A. Genigeorgis. 1986. Probability of growth and toxin production by nonproteolytic *Clostridium botulinum* in rockfish stored under modified atmospheres. Int. J. Food Microbiol. 3:167-181.
18. Lund, B. M., and G. M. Wyatt. 1984. The effect of redox potential, and its interaction with sodium chloride concentration, on the probability of growth of *Clostridium botulinum* type E from spore inocula. Food Microbiol. 1:49-65 .
19. Lund, B. M., M. R. Knox, and A. P. Sims. 1984. The effect of oxygen and redox potential on growth of *Clostridium botulinum* type E from a spore inoculum. Food Microbiol. 1:277-287.
20. Meyer, K. F. 1929. Maximum oxygen tolerance of *Cl. botulinum* A, B and C, of *Cl. sporogenes* and *Cl. welchii*. J. Infect. Dis. 44:408-411.
21. Montville, T. J. 1983. Interaction of pH and NaCl on culture density of *Clostridium botulinum* 62A. Appl. Environ. Microbiol. 46:961-963.
22. Montville, T. J., and L. K. Conway. 1982. Oxidation-reduction potentials of canned foods and their ability to support *Clostridium botulinum* toxigenesis. J. Food Sci. 47:1879-1882.
23. Roberts, T. A., and B. Jarvis. 1983. Predictive modelling of food safety with particular reference to *Clostridium botulinum* in model cured meat systems. pp. 85-95. *In* T. A. Roberts, and F. A. Skinner, (ed.) Food microbiology: Advances and prospects. Academic Press, New York.
24. Siegel, L. S., and J. F. Metzger. 1979. Toxin production by *Clostridium botulinum* type A under various fermentation conditions. Appl. Environ. Microbiol. 38:606-611.
25. Siegel, L. S., and J. F. Metzger. 1980. Effect of fermentation conditions on toxin production by *Clostridium botulinum* type B. Appl. Environ. Microbiol. 40:1023-1026.
26. Smoot, L. A., and M. D. Pierson. 1979. Effect of oxidation-reduction potential on the outgrowth and chemical inhibition of *Clostridium botulinum* 10755A spores. J. Food Sci. 44:700-704.
27. Stier, R. F., L. Bell, K. A. Ito, B. D. Shafer, L. A. Brown, M. L. Seeger, B. H. Allen, M. N. Porcuna, and P. A. Lerke. 1981. Effect of modified atmosphere storage on *C. botulinum* toxigenesis and the spoilage microflora of salmon fillets. J. Food Sci. 46:1639-1642.

Nexus Ex. 1032
Page 5 of 5