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Clostridium botulinum Growth and Toxin Production in Tomato Juice Containing *Aspergillus gracilis*†

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The ability of spores of one type A and one type B strain of *Clostridium botulinum* to grow and produce toxin in tomato juice was investigated. The type A strain grew at pH 4.9, but not at pH 4.8; the type B strain grew at pH 5.1, but not at pH 5.0. *Aspergillus gracilis* was inoculated along with *C. botulinum* spores into pH 4.2 tomato juice; in a nonhermetic unit, a pH gradient developed under the mycelial mat, resulting in *C. botulinum* growth and toxin production. In a hermetic unit, mold growth was reduced, and no pH gradient was detected; however, *C. botulinum* growth and low levels of toxin production (<10 50% lethal doses per ml) still occurred and were associated with the mycelial mat. The results of tests to find filterable or dialyzable growth factors were negative. It was demonstrated that for toxin production *C. botulinum* and the mold had to occupy the same environment.

Botulism is primarily a hazard in low-acid canned foods. However, an often overlooked fact is that botulism is also a hazard in acid foods (pH <4.6). Home-canned acid foods were implicated in 34 (4.7%) of the 722 reported outbreaks of food-borne botulism from 1899 through 1975 (2-6, 12, 14; K. A. Ito, Annu. Meet. Inst. Food Technol. 37th, Philadelphia, Pa., 1977). Tomato products were implicated in 17 of the 34 outbreaks. This number is too large to be neglected or written off to faulty diagnosis.

Odlaug and Pflug (15) reviewed the problem of botulism in acid foods and indicated that for a botulism hazard to exist in an acid food, there must be a number of contributing conditions. These conditions are: (i) presence of viable *Clostridium botulinum* spores, (ii) presence of other microorganisms due to a failure in delivery and/or post processing contamination, (iii) composition of the food and storage conditions which are particularly conducive to *C. botulinum* growth and toxin production, and (iv) metabiosis.

Metabiosis is defined as the condition where the growth of one organism in a medium makes conditions favorable for the growth of a second organism (9). Tanner et al. (17), de Lagarde and Beerens (8), and Huhtanen et al. (10) were able to detect botulinum toxin in acid foods inoculated with *C. botulinum* spores where molds such as *Penicillium* sp., *Mycoderma* sp., *Trichosphoron* sp., and *Cladosporium* sp. had grown and shifted the pH from <4.6 to >4.6. These are typical cases of metabiosis.

This study was carried out to determine whether *C. botulinum* growth and toxin production would occur in the presence of *Aspergillus* sp. in a specific microenvironment within a food system where the pH was less than 4.6.

(The results are from a Ph.D. thesis submitted by Theron E. Odlaug to the faculty of the Graduate School of the University of Minnesota.)

MATERIALS AND METHODS

***C. botulinum* spores.** Cultures of a *C. botulinum* type A strain, A16037, and a type B strain, B15580, implicated in outbreaks of botulism where home-canned tomato products were the toxin-carrying vehicle (3, 4), were obtained from the Center for Disease Control in Atlanta, Ga. Spore crops were prepared from these strains by using methods described by Odlaug and Pflug (13).

***Aspergillus gracilis* spores.** The strain of mold used in these studies was isolated from a 1-quart (ca. 0.95-liter) jar of spoiled stewed tomatoes. The jar was obtained from I. D. Wolf (Department of Food Science and Nutrition, University of Minnesota). The mold was identified as *A. gracilis* by morphological characteristics, using culture techniques described by Thom and Raper (18).

Conidiospore crops were prepared by using potato dextrose agar (Difco). The procedure was the same as that used by Buchanan et al. (1) for growing conidiospores of *Aspergillus flavus*. A 300-ml flask containing 75 ml of potato dextrose agar was inoculated from the *A. gracilis* stock culture and incubated at 22°C for 21 days.

After incubation the spores were harvested by add-

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ing 45 ml of 0.3% Tween 80 in water solution to the culture flask. The flask was swirled, and the mycelial mat was brushed lightly with a sterile cotton swab to dislodge the conidiospores. The liquid was filtered through two layers of cheesecloth into a centrifuge tube and then centrifuged at 1,200 × *g* for 20 min. The pellet was then resuspended in the Tween solution and stored at 4°C.

Tomato juice. Jars of commercially glass-packed tomato juice were purchased for use throughout the study. The jars were held at 4°C until used. At the time of use, the jars were aseptically opened, and the contents were aseptically poured into a sterile flask. The tomato juice was later dispensed aseptically in test tubes (18 by 150 mm) and milk dilution bottles.

pH measurements. All pH measurements were made with a pH meter (Corning model 610A) and a combination pH electrode (Corning series 500). The instrument was standardized with pH 7.0 and 4.0 buffers before and after sample pH determinations.

Nonhermetic and hermetic experimental units. The ability of *C. botulinum* to grow in the presence of *A. gracilis* in tomato juice was studied in both nonhermetic and hermetic units. Nonhermetic units were units where the cap on milk dilution bottles was loose, allowing free exchange of headspace gas with the outside atmosphere (determined by a vacuum test). Hermetic units were units where the cap was tightened on the bottle and there was no exchange of headspace gas with the outside atmosphere (determined by a vacuum test).

Analysis procedures. Two different procedures were used for analyzing the tomato juice in bottles inoculated with *C. botulinum* and *A. gracilis*.

In the first procedure, the *A. gracilis* mycelial mat was carefully removed from the surface of the tomato juice with a sterile forceps and placed inverted into a petri dish. A combination pH electrode was then placed on the mat, and the pH was measured. Next, three 2-ml samples of the tomato juice were removed from the surface, and the pH of each was measured. After the pH determinations the mold mat and the 2-ml samples were returned to the bottle. The bottle was then shaken, and the pH of the tomato juice was measured (average pH).

A 20-ml amount of the tomato juice was then removed for *C. botulinum* toxin assay by the mouse test (2). Samples of the tomato juice were plated in duplicate by using yeast extract agar as the growth medium to determine the amount of *C. botulinum* present. Nonheated samples and heated samples (80°C for 10 min) were analyzed. All plates were inverted and incubated under a hydrogen-carbon dioxide atmosphere (GasPak) at 32°C for 72 h.

In the second procedure, the mycelial mat was removed as in the first procedure and inverted, and the pH was measured. The mat was then placed into a Pyrex test tube (18 by 150 mm) and macerated with a glass rod, and the pH was measured. Then 10 ml of Butterfield diluent was added to the tube, and the tube was shaken. Samples of this solution were then plated to determine the *C. botulinum* level per mycelial mat, and the solution was assayed for toxin.

Tomato juice was then removed from the bottle in such a manner as to lower the level of tomato juice in the milk dilution bottle 10 mm per sample time (15 ml). For each sample the pH was measured, and the *C. botulinum* population level per milliliter was determined, as in the first procedure. Each sample was checked for the presence of *C. botulinum* toxin.

Description of experiments. (i) Minimum pH for *C. botulinum* growth. This experiment was carried out to determine the minimum pH for growth of the *C. botulinum* spores used in this study. Sterile flasks were aseptically filled with tomato juice, and the pH was adjusted to the desired level with 1.0 N NaOH. For each pH level each of a series of Pyrex tubes (18 by 150 mm) was filled with 20 ml of tomato juice and inoculated with 0.1 ml of the *C. botulinum* spore suspension to yield either 10^1 or 10^3 spores per ml of tomato juice. At each pH level tested, nine tubes were prepared for a particular spore concentration. The tubes were heated at 80°C for 10 min, layered with a 1:1 mineral oil–paraffin mixture, and incubated. Three tubes were incubated at 32°C, three tubes were incubated at 22°C, and three tubes were used to determine the initial pH and the number of spores per milliliter of tomato juice and to check for the possible presence of toxin by the mouse test.

The tubes were observed periodically over a 120-day period. If gas formation was observed in any tube, the substrate was assayed for toxin, and the pH and the number of *C. botulinum* spores per milliliter were determined. If after 120 days no growth had been observed, as evidenced by gas formation, the pH and the *C. botulinum* spores per milliliter of substrate were determined. In some of these tubes the substrate was assayed for toxin.

To determine the number of *C. botulinum* spores per milliliter of substrate in a tube after incubation, 5 ml was pipetted into a test tube. The tubes were heated for 10 min at 80°C to destroy vegetative cells, and then a series of dilutions was prepared and duplicate samples from each dilution were pipetted into petri plates. Yeast extract agar (15) was added to each plate. The plates were inverted and incubated in anaerobic jars under a hydrogen–carbon dioxide atmosphere for 72 h at 32°C.

(ii) *C. botulinum* growth and toxin production in tomato juice containing *A. gracilis*. These experiments were conducted to determine whether *C. botulinum* growth and toxin production would occur in tomato juice (pH 4.2) when *A. gracilis* was also present in the substrate. Milk dilution bottles containing 100 ml of tomato juice (pH 4.2) were inoculated with *A. gracilis* conidiospores and *C. botulinum* type A spores that had been heated at 80°C for 10 min. Nonhermetic and hermetic units were used in these tests. The concentration of spores and incubation time varied depending on the test.

After incubation, analyses were carried out to determine whether *C. botulinum* growth and toxin production had taken place and whether there were any changes in pH in the tomato juice medium.

(iii) *C. botulinum* growth and toxin production in tomato juice containing *A. gracilis* in a two-compartment system. The objective of these experiments was to determine whether the *C. botulinum* spores could produce toxin if they were physically separated from the *A. gracilis* spores. A two-

compartment system was constructed by using dialysis tubing.

The use of dialysis tubing in tomato juice was similar to the method used by Willardsen et al. (20) for containing *Clostridium perfringens* in dialysis tubing within a beef sample. Dialysis tubing with a 4.8-nm pore size and a 22-mm diameter (Arthur H. Thomas Co., Philadelphia, Pa.) was used. The tubing was made from regenerated cellulose. The molecular weight cutoff for this tubing was 12,000. This size of tubing prevented the microorganisms from passing from one side of the tubing to the other. To sterilize the dialysis tubing, it was cut into 8-cm strips and placed in a flask containing a 10% glycerin solution. The dialysis tubing was then autoclaved at 121.1°C for 15 min. After sterilization the dialysis tubing was rinsed with sterile distilled water.

Four experimental test conditions were evaluated: *C. botulinum* and *A. gracilis* outside; *C. botulinum* outside and *A. gracilis* inside; *A. gracilis* outside and *C. botulinum* inside; and both organisms inside the dialysis tubing. The test conditions are further described in Table 1.

To prepare the system, one end of the tubing was tied closed to make a pouch, and 10 ml of tomato juice containing the appropriate inoculum was pipetted into the pouch. The open end was then tied closed, and the pouch was held with a sterile forceps and rinsed with approximately 1 liter of sterile distilled water. The pouch was then placed into a milk dilution bottle with a hermetic seal containing 100 ml of tomato juice plus the appropriate inoculum.

The bottles with dialysis tubing were incubated at 32°C for 25 days, after which time the pH values of the outside and inside substrates were measured, and the substrates were analyzed for *C. botulinum* and the presence of toxin.

(iv) Growth of *C. botulinum* in filtered *A. gracilis* spent medium. The objective of these experiments was to determine whether there were any filterable growth factors produced by *A. gracilis* that would allow *C. botulinum* growth and toxin production in a medium where the pH was less than 4.6. Three milk dilution bottles with hermetic seals containing 100 ml of pH 4.2 tomato juice were inoculated with 0.1 ml of an *A. gracilis* spore suspension so that there were about 10^3 conidiospores per ml of tomato juice. Each bottle was incubated at 32°C for 25 days.

After incubation the contents of each bottle were centrifuged at 1,200 × *g* and 4°C for 20 min. The pH was then determined. In all three bottles the pH of the centrifuged spent medium was ≤4.3. After centrifugation, half of the supernatant (spent medium) was removed and adjusted to pH 7.0 with 1.0 N NaOH. The pH 7.0 medium and the pH ≤4.3 medium were then filtered through a 0.45-μm membrane filter and pipetted in 20-ml amounts into tubes (18 by 150 mm).

TABLE 1. *Two-compartment system experiments*[a]

Test condition	Material in tomato juice outside the dialysis tubing	Material in tomato juice inside the dialysis tubing
A	*C. botulinum* spores	None
	A. gracilis spores	None
B	*C. botulinum* spores	*A. gracilis* spores
C	*A. gracilis* spores	*C. botulinum* spores
D	None	*C. botulinum* spores
	None	*A. gracilis* spores

[a] There were approximately 10^3 spores of each organism per ml of tomato juice (pH 4.2).

The two sets of tubes from each bottle were inoculated with 0.1 ml of the *C. botulinum* type A spore suspension so that there were about 10^3 spores per ml of spent medium. The tubes were layered with a 1:1 paraffin-mineral oil mixture and incubated at 32°C for 120 days or until growth as evidenced by gas formation was observed.

The same procedure described above was duplicated with tomato juice that had not been inoculated with the *A. gracilis* conidiospores.

At the end of the 120-day period or when growth as evidenced by gas formation was observed, the contents of each tube were assayed for *C. botulinum* toxin.

(v) Quantification and stability of *C. botulinum* toxin in tomato juice. Three tests were conducted to determine the titer of *C. botulinum* toxin in tomato juice in hermetic units after incubation at 32°C for 25 days. Initially there were 10^3 *C. botulinum* spores per ml and 10^3 *A. gracilis* conidiospores per ml. Tests were also conducted to determine the stability of the toxin in tomato juice.

After incubation the contents of the bottle were centrifuged at 1,200 × *g* for 20 min and filtered through a 0.45-μm membrane filter. The tomato juice supernatant was split into two equal portions and stored at 4 and 22°C. At approximately 5-day intervals 5 ml was removed and tested for *C. botulinum* toxin by the mouse test. Five mice were injected at three different dilutions (10^0, 10^{-1}, and 10^{-2}). The diluent was gelatin-phosphate (2). The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (7).

RESULTS

(i) Effect of pH on *C. botulinum* growth and toxin production. The results of tests to measure the effect of pH on the growth and toxin production of *C. botulinum* type A spores in tomato juice are shown in Table 2. After 120 days of incubation, regardless of the incubation temperature or spore level, there was no growth or toxin production at or below pH values of 4.8. There was no growth at pH values from 4.8 to 5.1 in any tubes containing approximately 10 spores per ml.

In those tubes with 10^3 spores per ml there was growth and toxin production at pH 5.0 and incubation temperatures of 22 and 32°C. At pH 4.9 there was no growth or toxin production in 120 days at 22°C, but at 32°C two of three tubes were positive for toxin within 17 days; the tube that was negative at the end of the experiment (120 days) had a pH of 4.8.

The results of tests to measure the effects of pH on growth and toxin production of *C. botulinum* type B spores in tomato juice are shown in Table 3. Four pH levels were evaluated; the

TABLE 2. *Effect of pH on growth and toxin production of C. botulinum type A (A16037) spores in tomato juice*

Initial pH	Initial no. of spores/ml (mean)	Incubation temp (°C)	Conditions after incubation				Length of incubation (days)
			Toxin	Gas	No. of spores/ml (mean)	pH (mean)	
5.1	14	22	−,−,−[a]	−,−,−[a]	18	5.1	120
	14	32	−,−,−	−,−,−	20	5.1	120
	1.7×10^2	22	+,+,+	+,+,+	5.9×10^2	5.1	10
	1.7×10^3	32	+,+,+	+,+,+	7.0×10^2	5.0	10
5.0	16	22	−,−,−	−,−,−	16	5.0	120
	16	32	−,−,−	−,−,−	17	5.0	120
	1.7×10^2	22	+,+,+	+,+,+	2.0×10^3	5.1	16
	1.7×10^3	32	+,+,+	+,+,+	1.8×10^3	5.0	18
4.9	17	22	−,−,−	−,−,−	17	4.9	120
	17	32	−,−,−	−,−,−	19	4.9	120
	1.7×10^3	22	−,−,−	−,−,−	1.8×10^3	4.9	120
	1.7×10^2	32	+,+	+,+	2.1×10^3	4.9	17
	1.7×10^3	32	−	−	1.8×10^3	4.8	120
4.8	1.9×10^3	32	−,−,−	−,−,−	1.9×10^3	4.8	120
	1.9×10^2	22	−,−,−	−,−,−	1.9×10^3	4.8	120
4.7	19	22	NT[b]	−,−,−	18	4.7	120
	19	32	NT	−,−,−	14	4.7	120
	1.7×10^3	22	NT	−,−,−	1.7×10^3	4.7	120
	1.7×10^3	32	NT	−,−,−	1.7×10^3	4.7	120

[a] For each combination of initial pH, initial number of spores, and incubation temperature, the results from three individual tubes are given.
[b] NT, Not tested.

TABLE 3. *Effect of pH on growth and toxin production of C. botulinum type B (B15580) spores in tomato juice*

Initial pH	Initial no. of spores/ml (mean)	Incubation temp (°C)	Conditions after incubation				Length of incubation (days)
			Toxin	Gas	No. of spores/ml (mean)	pH (mean)	
5.2	9.0	22	−,−,−[a]	−,−,−[a]	10	5.2	120
	9.0	32	−,−,−	−,−,−	8.0	5.2	120
	1.1×10^3	22	+,+,+	+,+,+	4.9×10^3	5.2	40
	1.1×10^3	32	+,+,+	+,+,+	7.2×10^3	5.2	40
5.1	10	22	−,−,−	−,−,−	7.0	5.1	120
	10	32	−,−,−	−,−,−	8.0	5.1	120
	1.1×10^3	22	−,−,−	−,−,−	9.9×10^2	5.1	120
	1.1×10^3	32	−,−,−	−,−,−	1.0×10^3	5.1	120
5.0	13	22	NT[b]	−,−,−	9.0	5.0	120
	11	32	NT	−,−,−	6.0	5.0	120
	1.1×10^3	22	NT	−,−,−	7.9×10^2	5.0	120
	1.1×10^3	32	NT	−,−,−	7.9×10^2	5.0	120
4.9	10	22	NT	−,−,−	7.0	4.9	120
	12	32	NT	−,−,−	4.0	4.9	120
	1.1×10^3	22	NT	−,−,−	6.8×10^2	4.9	120
	1.1×10^3	32	NT	−,−,−	7.4×10^2	4.9	120

[a] For each combination of initial pH, initial number of spores, and incubation temperature, the results from three individual tubes are given.
[b] NT, Not tested.

only positive result was at the highest level, pH 5.2, where growth and toxin were found in those tubes with 10^3 *C. botulinum* spores per ml.

(ii) *C. botulinum* growth and toxin production in tomato juice containing *A. gracilis*. Table 4 shows the results of a test where *C. botulinum* and *A. gracilis* were inoculated together into tomato juice in four nonhermetic units. The entire contents of the bottle were analyzed after 25 days of incubation, when gas bubbles were observed below the mycelial mat in all of the units. The growth of *A. gracilis*

changed the pH at the surface from an initial pH level of 4.6 to a pH of >6.2 in all cases. There was *C. botulinum* growth and toxin production in all of the experimental units.

Table 5 shows the results of two tests where *C. botulinum* and *A. gracilis* were inoculated together into tomato juice in a nonhermetic unit and where successive layers of tomato juice in the unit were analyzed. A pH gradient was observed in each unit, the pH decreasing with distance from the mycelial mat. The increase in the *C. botulinum* population over the initial number of spores per milliliter was greatest at the surface. The counts decreased with distance from the mycelial mat.

Table 6 shows the results of the test where *C. botulinum* and *A. gracilis* were inoculated together into tomato juice in hermetic units. A thin mycelial mat was visible on the surface of the tomato juice in each experimental unit after 3 to 5 days of incubation. There was no measurable increase in the *C. botulinum* population, and no toxin was detected after 10 days of incubation. After 15 days of incubation there was an increase in the *C. botulinum* population, but no toxin was detected. After 20 days of incubation one unit had toxin production, and the other unit had no significant change in the *C. botulinum* population or toxin production. At both 25 and 30 days *C. botulinum* growth and toxin production were detected in the tomato juice. The maximum pH measured in this test was 4.35.

TABLE 4. *C. botulinum type A growth and toxin production in tomato juice containing A. gracilis in a nonhermetic unit: analysis of the entire contents*[a]

Bottle no.	Surface pH	Avg pH	Toxin	Amt of *C. botulinum* (CFU/ml) in:	
				NHS[b]	HS[b]
1	6.8	6.8	+	1.0×10^5	2.9×10^4
2	7.1	6.7	+	2.1×10^6	1.9×10^3
3	6.7	6.5	+	8.1×10^5	7.1×10^3
4	6.3	5.4	+	1.1×10^5	1.2×10^3

[a] Initial conditions in the tomato juice: pH, 4.2; *C. botulinum*, 2.5×10^3 spores per ml; *A. gracilis*, 10^3 spores per ml. Incubation was at 32°C for 25 days.

[b] NHS, Nonheated sample; HS, heated sample (80°C for 10 min).

(iii) *C. botulinum* and *A. gracilis* in tomato juice in a two-compartment system. The results of two tests are summarized in Tables 7 and 8. When *C. botulinum* and *A. gracilis* were together, either inside or outside the dialysis tubing, there was *C. botulinum* growth and toxin production. Table 7 shows the results of the two tests where *C. botulinum* and *A. gracilis* were outside the dialysis tubing and no microorganisms were inside. Samples taken throughout the bottle were analyzed. No pH gradient was detected. The growth of *C. botulinum* appeared to be confined to the mycelial mat and the 10 mm of tomato juice below the mycelial mat. At the other levels of tomato juice analyzed there appeared to be no increase in the *C. botulinum* population over the original number of *C. botulinum* spores present. However,

TABLE 5. *C. botulinum type A growth and toxin production in tomato juice containing A. gracilis in a nonhermetic unit analysis of successive layers*[a]

Distance from mycelial mat (mm)	pH	Toxin	Amt of *C. botulinum* (CFU/ml) in:	
			NHS[b]	HS[b]
Test 1				
Mycelial mat	6.5, 6.5[c]	+,+[c]	1.6×10^6, 2.0×10^{6c}	6.0×10^3, 4.2×10^{3c}
0–10	5.3, 5.4	+,+	1.7×10^6, 2.3×10^6	1.6×10^3, 1.4×10^3
10–20	5.1, 5.0	+,+	1.3×10^6, 1.9×10^6	9.4×10^2, 2.0×10^3
20–30	5.0, 5.0	+,+	8.6×10^5, 7.1×10^5	8.7×10^2, 1.5×10^3
30–40	4.7, 4.9	+,+	1.0×10^5, 1.3×10^6	1.1×10^3, 3.4×10^3
40–50	4.6, 4.8	+,+	6.1×10^5, 7.9×10^5	2.4×10^3, 2.2×10^3
Test 2				
Mycelial mat	6.6, 6.7	+,+	1.7×10^6, 9.0×10^5	6.8×10^3, 5.2×10^3
0–10	5.4, 5.4	+,+	2.2×10^6, 1.4×10^6	1.1×10^3, 1.0×10^3
10–20	5.1, 5.1	+,+	1.9×10^6, 1.1×10^6	7.8×10^2, 1.9×10^3
20–30	4.9, 5.0	+,+	8.4×10^5, 3.1×10^5	1.6×10^3, 2.0×10^3
30–40	4.8, 5.1	+,+	6.4×10^5, 2.1×10^5	1.6×10^3, 1.2×10^3
40–50	4.8, 4.8	+,+	4.2×10^5, 7.4×10^5	2.6×10^3, 2.8×10^3

[a] Initial conditions in the tomato juice: pH, 4.2; *C. botulinum*, 2.5×10^3 spores per ml; *A. gracilis*, 10^3 spores per ml. Incubation was at 32°C for 25 days.

[b] NHS, Nonheated sample; HS, heated sample (80°C for 10 min).

[c] For each distance the results from two individual bottles are given. For mycelial mats the amount of *C. botulinum* is given as CFU/mat.

TABLE 6. *C. botulinum type A growth and toxin production in tomato juice containing A. gracilis in a hermetic unit: analysis of the entire contents*[a]

Bottle no.	No. of days at 32°C	Surface pH	Avg pH	Toxin	Amt of *C. botulinum* (CFU/ml) in:	
					NHS[b]	HS[b]
1	5	4.20	4.20	−	2.5×10^3	2.5×10^3
2	5	4.20	4.20	−	2.4×10^3	2.5×10^3
3	10	4.20	4.20	−	2.2×10^3	2.4×10^3
4	10	4.20	4.20	−	2.3×10^3	2.5×10^3
5	15	4.20–4.30	4.20	−	3.1×10^4 (est)[c]	1.7×10^3
6	15	4.20–4.25	4.25	−	3.1×10^4 (est)	2.1×10^3
7	20	4.20–4.25	4.20	−	2.6×10^3	2.4×10^3
8	20	4.25–4.30	4.20	+	3.1×10^4 (est)	7.1×10^3
9	25	4.20–4.35	4.25	+	2.7×10^4	3.1×10^3
10	25	4.25–4.30	4.25	+	1.6×10^4	6.7×10^3
11	30	4.25–4.30	4.25	+	1.9×10^4	5.0×10^3
12	30	4.25–4.30	4.25	+	3.7×10^4	2.7×10^3

[a] Initial conditions in the tomato juice: pH, 4.2; *C. botulinum*, 2.5×10^3 spores per ml; *Aspergillus*, 10^3 spores per ml. Incubation was at 32°C.

[b] NHS, Nonheated sample; HS, heated sample (80°C for 10 min).

[c] est, Estimated values.

TABLE 7. *Two-compartment system experiments: C. botulinum type A growth and toxin production in tomato juice containing A. gracilis in a hermetic unit*[a]

Distance from mycelial mat (mm)	pH	Toxin	Amt of *C. botulinum* (CFU/ml) in:	
			NHS[b]	HS[b]
Test 1				
Mycelial mat	4.2, 4.25[c]	+,+[c]	1.3×10^4, 1.8×10^4[c]	9.8×10^2, 1.4×10^3[c]
0–10	4.2, 4.2	+,+	1.3×10^5, 1.2×10^5	4.0×10^3, 7.7×10^3
10–20	4.2, 4.2	+,+	2.1×10^4, 3.0×10^4	2.4×10^3, 2.1×10^3
20–30	4.2, 4.2	+,+	2.2×10^4, 2.4×10^4	3.0×10^3, 2.5×10^3
30–40	4.2, 4.2	−,−	2.5×10^3, 2.9×10^3	2.7×10^3, 2.4×10^3
40–50	4.2, 4.2	−,−	2.5×10^3, 2.4×10^3	2.6×10^3, 2.7×10^3
Test 2				
Mycelial mat	4.3, 4.25	+,+	9.8×10^3, 4.7×10^4	1.8×10^3, 2.1×10^3
0–10	4.2, 4.2	+,+	7.8×10^5, 9.2×10^4	1.2×10^3, 5.1×10^3
10–20	4.2, 4.2	+,+	8.4×10^3, 5.4×10^3	2.2×10^3, 2.6×10^3
20–30	4.2, 4.2	−,+	2.9×10^3, 3.1×10^3	3.0×10^3, 2.7×10^3
30–40	4.2, 4.2	−,−	2.5×10^3, 2.6×10^3	2.8×10^3, 2.8×10^3
40–50	4.2, 4.2	−,−	2.7×10^3, 2.5×10^3	2.5×10^3, 2.7×10^3

[a] Table 1, test condition A. Initial conditions in the tomato juice: pH, 4.2; *C. botulinum*, 2.5×10^3 spores per ml; *A. gracilis*, 10^3 spores per ml. Incubation was at 32°C for 25 days.

[b] NHS, Nonheated sample; HS, heated sample (80°C for 10 min).

[c] For each distance the results from two individual bottles are given. For mycelial mats the amount of *C. botulinum* is given as CFU/mat.

toxin was found to a depth of 30 mm, indicating some diffusion.

Table 8 shows the results of the two tests where both organisms were inside the dialysis tubing. Small increases in *C. botulinum* and toxin production were detected. No pH value >4.2 was measured. No toxin was detected in the tomato juice surrounding the dialysis tubing.

Also shown in Table 8 are the results of the two tests where the two organisms were physically separated by dialysis tubing. There was no *C. botulinum* growth or toxin production inside or outside.

(iv) *C. botulinum* growth in filtered *A. gracilis* spent medium. The results of those tests where *C. botulinum* spores were inoculated into *A. gracilis* spent medium are summarized in Table 9. *C. botulinum* did not grow and produce toxin in the *A. gracilis* filtered tomato juice spent medium or in the tomato juice filtered medium at a pH of less than 4.6, but when the pH was adjusted to 7.0, there was growth of *C. botulinum* and toxin production. At pH 7.0 all tubes were positive for growth and toxin production after 4 days of incubation.

These results indicated that under the conditions employed in these tests no filterable growth factor from the mold capable of allowing

TABLE 8. *Two-compartment system experiments: C. botulinum type A growth and toxin production in tomato juice containing A. gracilis in a hermetic unit*

Test condition[a]	Tomato juice outside the dialysis tubing				Tomato juice inside the dialysis tubing				
	Surface pH	*Aspergillus* growth	Toxin	Amt of *C. botulinum* (CFU/ml) in: NHS[b]	Amt of *C. botulinum* (CFU/ml) in: HS[b]	Surface pH	*Aspergillus* growth	Toxin	Amt of *C. botulinum* (CFU/ml) in: NHS[b]	Amt of *C. botulinum* (CFU/ml) in: HS[b]
Test 1										
B	4.2, 4.2[c]	−, −[c]	−, −[c]	2.6×10^3, 2.8×10^3	2.2×10^3, 2.4×10^3	4.2, 4.2[c]	+, +[c]	−, −[c]	0, 0[c]	0, 0[c]
C	4.2, 4.2	+, +	−, −	0, 0	0, 0	4.2, 4.2	−, −	−, −	1.9×10^3, 2.4×10^3	2.2×10^3, 1.6×10^3
D	4.2, 4.2	−, −	−, −	0, 0	0, 0	4.2, 4.3	+, +	+, +	7.6×10^3, 4.8×10^3	2.0×10^3, 8.3×10^2
Test 2										
B	4.2, 4.2	−, −	−, −	2.5×10^3, 2.4×10^3	2.2×10^3, 2.6×10^3	4.2, 4.2	+, +	−, −	0, 0	0, 0
C	4.3, 4.2	+, +	−, −	0, 0	0, 0	4.2, 4.2	−, −	−, −	2.7×10^3, 2.1×10^3	2.0×10^3, 2.5×10^3
D	4.2, 4.2	−, −	−, −	0, 0	0, 0	4.2, 4.2	+, +	+, +	3.7×10^3, 6.1×10^3	2.4×10^3, 1.5×10^3

[a] Table 1, test conditions B, C, and D. Initial conditions in the tomato juice: pH, 4.2; *C. botulinum*, 2.5×10^3 spores per ml; *A. gracilis*, 10^3 spores per ml. Incubation was at 32°C for 25 days.
[b] NHS, Nonheated sample; HS, heated sample (80°C for 10 min).
[c] For each test condition the results from two individual bottles are given.

C. botulinum to grow and produce toxin in a medium at a pH of <4.6 could be demonstrated.

(v) *C. botulinum* toxin stability in tomato juice. Table 10 shows the results of tests to determine the *C. botulinum* toxin titer in tomato juice in a hermetic unit after incubation at 32°C for 25 days in the presence of *A. gracilis*. The titer for three tests ranged from 4.7 to 8.4 LD_{50}/ml of tomato juice; the mean was 6.5 LD_{50}/ml of tomato juice. As Table 6 shows, the corresponding population level for this amount of toxin is approximately 10^4 *C. botulinum* colony-forming units (CFU) per ml.

Also in Table 10 are the results of studies to determine the stability of the preformed toxin in tomato juice supernatant when stored at 4 and 22°C. The *C. botulinum* toxin formed in tomato juice kept its activity for 30 days when stored at 4°C. Two of the three replicate test samples retained toxin activity for 20 days at 22°C.

DISCUSSION

Limiting pH for *C. botulinum* growth. The tubes of tomato juice with *C. botulinum* spores were observed for 120 days. Townsend et al. (19) did not find any *C. botulinum* toxin production in foods with pH levels greater than 4.6 after 75 days of incubation at 30°C. Ito et al. (11), in studying the limiting pH for *C. botulinum* growth in cucumber puree, did not find any positive tubes after 18 days of incubation at 30°C. Huhtanen et al. (10) studied the limiting pH for *C. botulinum* growth in tomato juice and did not find any positive tubes after 30 days of incubation at 35°C. In this study no positive tubes were observed after 40 days of incubation at 32°C.

It is interesting that none of the tubes containing about 10 *C. botulinum* spores per ml of tomato juice were positive for growth and toxin production. Ito et al. (11) were able to get growth in cucumber puree at pH 5.0 with a *C. botulinum* inoculum of 10^6 spores per ml but not with 10^2 spores per ml. Ito (Annu. Meet. Inst. Food Technol. 37th, Philadelphia, Pa., 1977) suggested that it may be more desirable to use the larger inocula in pH inhibition studies of *C. botulinum* spores because of this phenomenon. It may be that only a fraction of the spore population is capable of germination and outgrowth at more acid pH values and that, when the inoculum is larger, the probability of germination and outgrowth in an experimental unit increases.

***C. botulinum* and *A. gracilis* in tomato juice.** It was observed during preliminary experiments that the amount of growth of *A. gracilis* in tomato juice could be controlled by the degree

TABLE 9. *C. botulinum A16037 spores in filtered Aspergillus spent tomato juice medium and in filtered tomato juice*

Filtered substrate	Tube no.	pH	No. of *C. botulinum* spores/ml	No. of days at 32°C	Gas	Toxin
Aspergillus spent tomato juice medium	1	4.25	10^3	120	−	−
	2	4.30	10^3	120	−	−
	3	4.25	10^3	120	−	−
Aspergillus spent tomato juice medium	4	7.0	10^3	3	+	+
	5	7.0	10^3	4	+	+
	6	7.0	10^3	4	+	+
Tomato juice	7	4.2	10^3	120	−	−
	8	4.2	10^3	120	−	−
	9	4.2	10^3	120	−	−
Tomato juice	10	7.0	10^3	4	+	+
	11	7.0	10^3	4	+	+
	12	7.0	10^3	4	+	+

of oxygen exclusion from the milk dilution bottles. When the closure was nonhermetic, a thick mycelial mat formed on the surface after 3 to 5 days of incubation, and the mycelial mat appeared to reach a maximum size after 8 to 10 days of incubation. When there was a hermetic seal, a thin mycelial mat formed after 3 to 5 days of incubation. The mycelial mat was very thin compared with the thick mycelial mat that formed in the nonhermetic unit. Even after 60 days of incubation, the mycelial mat in the hermetic unit did not increase in thickness. However, by loosening the cap, which allowed oxygen to enter, the mycelial mat increased in thickness within 3 to 5 days. This visual qualitative difference in the thickness of the mycelial mat was demonstrated repeatedly by adjusting the tightness of the cap at the time of inoculation with the mold.

The *Aspergillus* organism used in this study was capable of growing in the tomato juice and raising the pH above 4.6 to where *C. botulinum* growth and toxin production occurred. The change in pH from 4.2 to approximately pH 6.5 at the surface occurred only when the unit was nonhermetic. Apparently oxygen required by this organism for growth was not limited under these conditions.

Huhtanen et al. (10) were able to demonstrate a pH gradient in tomato juice when *Cladosporium* sp. was present. Their results were similar to the results reported in Table 5 for *A. gracilis* in tomato juice. Huhtanen et al. indicated that a heavy mold mat was present after 3 days of incubation. This seems to indicate that oxygen was not limiting in the cultures used by Huhtanen et al. In this study with *A. gracilis* in tomato juice in a nonhermetic unit, a heavy mycelial mat developed after 3 to 5 days of incubation, whereas in a hermetic unit the growth of the mycelial mat was reduced.

TABLE 10. *C. botulinum toxin titer and stability when formed in tomato juice containing A. gracilis in a hermetic unit*[a]

Initial toxin titer in the unit (LD_{50}/ml)	Storage temp (°C)	Length of toxin stability (days)					
		5	10	16	20	25	30
6.3	4	+	+	+	+	+	+
	22	+	+	+	+	+	+
8.4	4	+	+	+	+	+	+
	22	+	+	+	+	+	+
4.7	4	+	+	+	+	+	+
	22	+	+	+	+	−	−

[a] Initial conditions in the tomato juice: pH, 4.2; *C. botulinum*, 2.5×10^3 spores per ml; *A. gracilis*, 10^3 spores per ml. Incubation was at 32°C for 25 days.

When *A. gracilis* and *C. botulinum* were inoculated into tomato juice in a hermetic unit, *C. botulinum* growth and toxin production were demonstrated even though the pH measured both at the surface and throughout the tomato juice was less than 4.6. The major increase in the *C. botulinum* population was associated with the mycelial mat and near the surface. The titer of the *C. botulinum* toxin formed in tomato juice in the hermetic unit with *Aspergillus* present was very low, <10 LD_{50}/ml of tomato juice. It has been reported that under optimum conditions *C. botulinum* can produce as much as 2×10^6 LD_{50}/ml of culture medium (16).

The results presented in this study suggest that the growth of the *Aspergillus* organism on the surface of tomato juice creates a microenvironment within or adjacent to the mycelial mat or directly below the mycelial mat where the pH was probably greater than 4.6 and where *C. botulinum* spores can germinate, reproduce, and produce toxin. The low toxin titers found under these conditions and the small increases in population suggest that the volume of the microen-

vironment favorable to *C. botulinum* growth and toxin production may be very small.

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