**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% 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 (2-5, 12, 14; K. A. Ito, Amnu. 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.

Odiaug 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 past processing contamination, (iii) composition of the food and storage conditions which are particularly conducive to *C. botulinum* growth and toxin production, and (iv) metabolism.

Metabolism 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 Beeren (8), and Huhntan et al. (10) were able to detect botulinum toxin in acid foods inoculated with *C. botulinum* spores where molds such as *Penicillium* sp., *Mycodermia* sp., *Trichosporon* sp., and *Cladosporium* sp. had grown and shifted the pH from <4.6 to >4.6. These are typical cases of metabolism.

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, A169057, 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 32°C for 21 days. After incubation the spores were harvested by add-
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 cheese cloth into a centrifuge tube and then centrifuged at 1,200 x 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 instruments were 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. graciles* 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 on the bottle remained in a tight position, preventing free 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. graciles*.

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 (45°C for 10 min) were analyzed. All plates were inverted and incubated under a hydrogen-carbon dioxide atmosphere (GaPlak) at 32°C for 72 h.

In the second procedure, the mycelial mat was removed as was standardized with pH 7.0 and 4.0 buffers before and after sample pH determinations. After incubation, analyzed, 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 ml 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 10^4 or 10^6 spores per ml of tomato juice. At each pH level tested, nine tubes were prepared for a pressor spore concentration.

The tubes were heated at 60°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. (ii) **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, analyzed, 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.

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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 22°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, analyzed, 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 ml 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. The tubes were heated at 60°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 were determined. In some of these tubes the substrate was assayed for toxin.

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compartment system was constructed by using dialysis tubing.

The use of dialysis tubing in tomato juice was similar to the method used by Willardson 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% glycerol solution. The dialysis tubing was then autoclaved at 121.1°C for 16 min. After sterilization, the dialysis tubing was rinsed with sterile distilled water.

Four experimental test conditions were evaluated: C. botulinum and A. gravis 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 sterile forceps and rinsed with approximately 1 liter of sterile distilled water. The pouch was then placed into a milk dialysis 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 dialysis 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 10³ spores 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 x 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 into 20-ml amounts into tubes (18 by 150 mm).

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 10³ 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 35 days. Initially there were 10³ C. botulinum spores per ml and 10³ 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 x 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 32°C. At approximately 6-day intervals 0 ml was removed and tested for C. botulinum toxin by the mouse test. Five mice were injected at three different dilutions (10³, 10², and 10¹). The diluent was peptone-phosphate (2). The 50% lethal dose (LD₅₀) 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 10³ spores per ml.

In those tubes with 10³ 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 these 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 1. Two-compartment system experiments

<table>
<thead>
<tr>
<th>Test condition</th>
<th>Material in tomato juice outside the dialysis tubing</th>
<th>Material in tomato juice inside the dialysis tubing</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C. botulinum spores None A. gracilis spores A. gracilis spores</td>
<td>A. gracilis spores C. botulinum spores</td>
</tr>
<tr>
<td>B</td>
<td>C. botulinum spores None A. gracilis spores A. gracilis spores</td>
<td>A. gracilis spores C. botulinum spores</td>
</tr>
<tr>
<td>C</td>
<td>None A. gracilis spores C. botulinum spores A. gracilis spores</td>
<td>None A. gracilis spores</td>
</tr>
<tr>
<td>D</td>
<td>None A. gracilis spores C. botulinum spores A. gracilis spores</td>
<td>None A. gracilis spores</td>
</tr>
</tbody>
</table>

*There were approximately 10³ spores of each organism per ml of tomato juice (pH 4.2).
only positive result was at the highest level, pH 5.2, where growth and toxin were found in those tubes with 10⁶ 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 >8.2 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. gravis 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 range 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. gravis 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.

(iii) C. botulinum and A. gravis 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. gravis 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. gravis 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 6. C. botulinum type A growth and toxin production in tomato juice containing A. gracilis in a hermetic unit: analysis of the entire contentsa

<table>
<thead>
<tr>
<th>Bottle no.</th>
<th>No. of days at 32°C</th>
<th>Surface pH</th>
<th>Avg pH</th>
<th>Toxin</th>
<th>Amt of C. botulinum (CFU/ml) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NISb</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>4.20</td>
<td>4.20</td>
<td>−</td>
<td>2.5 × 10^5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>4.20</td>
<td>4.20</td>
<td>−</td>
<td>2.4 × 10^5</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>4.20</td>
<td>4.20</td>
<td>−</td>
<td>2.2 × 10^5</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>4.20</td>
<td>4.20</td>
<td>−</td>
<td>2.3 × 10^5</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>4.20-4.30</td>
<td>4.20</td>
<td>−</td>
<td>3.1 × 10^6 (est)</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>4.20-4.25</td>
<td>4.25</td>
<td>−</td>
<td>3.1 × 10^6 (est)</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>4.20-4.25</td>
<td>4.20</td>
<td>−</td>
<td>2.6 × 10^5</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>4.25-4.30</td>
<td>4.20</td>
<td>+</td>
<td>3.1 × 10^6 (est)</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>4.20-4.35</td>
<td>4.25</td>
<td>+</td>
<td>2.7 × 10^5</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>4.25-4.30</td>
<td>4.25</td>
<td>+</td>
<td>1.6 × 10^5</td>
</tr>
<tr>
<td>11</td>
<td>30</td>
<td>4.25-4.30</td>
<td>4.25</td>
<td>+</td>
<td>1.9 × 10^5</td>
</tr>
<tr>
<td>12</td>
<td>30</td>
<td>4.25-4.30</td>
<td>4.25</td>
<td>+</td>
<td>3.7 × 10^5</td>
</tr>
</tbody>
</table>

a Initial conditions in the tomato juice: pH, 4.2; C. botulinum, 2.5 × 10^3 spores per ml; Aspergillus, 10^6 spores per ml. Incubation was at 32°C.

b NIS, 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

<table>
<thead>
<tr>
<th>Distance from mycelial mat (mm)</th>
<th>pH</th>
<th>Toxin</th>
<th>Amt of C. botulinum (CFU/ml) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NISb</td>
</tr>
<tr>
<td>Test 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycelial mat</td>
<td>4.2, 4.25</td>
<td>+, +</td>
<td>1.3 × 10^6, 1.8 × 10^6</td>
</tr>
<tr>
<td>0-10</td>
<td>4.2, 4.2</td>
<td>+,+</td>
<td>1.3 × 10^6, 1.2 × 10^6</td>
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<tr>
<td>10-20</td>
<td>4.2, 4.2</td>
<td>+,+</td>
<td>2.1 × 10^6, 3.0 × 10^6</td>
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<tr>
<td>20-30</td>
<td>4.2, 4.2</td>
<td>+,+</td>
<td>2.2 × 10^6, 2.4 × 10^6</td>
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<tr>
<td>30-40</td>
<td>4.2, 4.2</td>
<td>-,-</td>
<td>2.5 × 10^6, 2.9 × 10^6</td>
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<tr>
<td>40-50</td>
<td>4.2, 4.2</td>
<td>-,-</td>
<td>2.5 × 10^6, 2.4 × 10^6</td>
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<tr>
<td>Test 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycelial mat</td>
<td>4.3, 4.25</td>
<td>+,+</td>
<td>9.8 × 10^6, 4.7 × 10^6</td>
</tr>
<tr>
<td>0-10</td>
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<td>+,+</td>
<td>7.8 × 10^6, 9.2 × 10^6</td>
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<td>20-30</td>
<td>4.2, 4.2</td>
<td>-,-</td>
<td>2.9 × 10^6, 3.1 × 10^6</td>
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<tr>
<td>30-40</td>
<td>4.2, 4.2</td>
<td>-,-</td>
<td>2.5 × 10^6, 2.6 × 10^6</td>
</tr>
<tr>
<td>40-50</td>
<td>4.2, 4.2</td>
<td>-,-</td>
<td>2.7 × 10^6, 2.5 × 10^6</td>
</tr>
</tbody>
</table>

a Table I test condition A. Initial conditions in the tomato juice: pH, 4.2; C. botulinum, 2.5 × 10^6 spores per ml; A. gracilis, 10^6 spores per ml. Incubation was at 32°C for 25 days.

b NIS, 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/matt.
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 LD₅₀/ml of tomato juice; the mean was 6.5 LD₅₀/ml of tomato juice. As Table 6 shows, the corresponding population level for this amount of toxin is approximately 10⁴ 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. (10) 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 10⁶ spores per ml but not with 10⁴ spores per ml. Ito (Annu. Meet. Inst. Food Technol. 37th, Philadelphia, Pa., 1977) suggested that it may be more desirable to use the larger inoculum 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
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 9. C. botulinum A16037 spores in filtered Aspergillus spent tomato juice medium and in filtered tomato juice**

<table>
<thead>
<tr>
<th>Filtered substrate</th>
<th>Tube no.</th>
<th>pH</th>
<th>No. of C. botulinum spores/ml</th>
<th>No. of days at 22°C</th>
<th>Gas</th>
<th>Toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus spent tomato juice medium</td>
<td>1</td>
<td>4.25</td>
<td>10³</td>
<td>10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.30</td>
<td>10³</td>
<td>12</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.25</td>
<td>10³</td>
<td>12</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Aspergillus spent tomato juice medium</td>
<td>4</td>
<td>7.0</td>
<td>10³</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.0</td>
<td>10³</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7.0</td>
<td>10³</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>7</td>
<td>4.2</td>
<td>10³</td>
<td>12</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.2</td>
<td>10³</td>
<td>12</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4.2</td>
<td>10³</td>
<td>12</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>10</td>
<td>7.0</td>
<td>10³</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>7.0</td>
<td>10³</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7.0</td>
<td>10³</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Initial toxin titer in the unit (LD₅₀/ ml)</th>
<th>Storage temp (°C)</th>
<th>Length of toxin stability (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>8.4</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>4.7</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30</td>
</tr>
</tbody>
</table>

*Initial conditions in the tomato juice: pH, 4.2; C. botulinum, 2.5 × 10⁶ spores per ml; A. gracilis, 10⁵ 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 LD₅₀/ml of tomato juice. It has been reported that under optimum conditions C. botulinum can produce as much as 2 × 10⁷ LD₅₀/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-
environment favorable to \textit{C. botulinum} growth and toxin production may be very small.

\textbf{ACKNOWLEDGMENTS}

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\textbf{LITERATURE CITED}


