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Thermal Destruction of *Clostridium botulinum* Spores Suspended in Tomato Juice in Aluminum Thermal Death Time Tubes[1]

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The heat destruction characteristics of *Clostridium botulinum* spores suspended in tomato juice and phosphate buffer were determined by the survivor curve method with aluminum thermal death time tubes. Two type A strains of *C. botulinum* and a type B strain were evaluated. Strains A16037 and B15580 were implicated in outbreaks of botulism involving home-canned tomato products. Strain A16037 had a higher heat resistance than either 62A or B15580. The mean thermal resistance (*D*-values) for A16037 in tomato juice (pH 4.2) were: 115.6°C, 0.4 min; 110.0°C, 1.6 min; and 104.4°C, 6.0 min. The mean *D*-values for A16037 in Sorensen 0.067 M phosphate buffer (pH 7) were: 115.6°C, 1.3 min; 110.0°C, 4.4 min; and 104.4°C, 17.6 min. At each test temperature, the *D*-values were approximately three times higher in buffer than in tomato juice. The *z*-value for *C. botulinum* A16037 spores in tomato juice was 9.4°C, and in buffer the *z*-value was 9.9°C. The use of aluminum thermal death time tubes in a miniature retort system makes it possible to determine survivor curves for *C. botulinum* spores at 121.1°C. This is possible because the lag correction factor for the aluminum tubes is only about 0.2 min, making possible heating times as short as 0.5 min.

The problem of botulism associated with tomato products is of concern to both consumers and government agencies (34). A fact often overlooked is that home-canned acid foods were implicated in 34 (4.7%) of the 722 *Clostridium botulinum* intoxications from 1899 through 1975 (9–11, 29). Tomato products were implicated in 17 of the 34 outbreaks. This number is too large to be neglected or written off to faulty diagnosis. Therefore, *C. botulinum* in acid foods is a potential public health hazard.

There was a reported outbreak of botulism involving commercially processed tomato catsup in 1915 (15, 29). Since this time there have been no reported outbreaks associated with commercially canned acid foods (9–11). Considering that over 775 billion cans of commercially canned foods have been consumed since 1930 (27), the problem of *C. botulinum* intoxications involving acid foods is a problem of home-canned and not of commercially canned foods.

Based on data in the literature, it would appear to be impossible for *C. botulinum* to grow and produce toxin below a pH of 4.6 (6, 12, 19–21, 25, 26, 40, 41). However, tomato products can support the growth of *C. botulinum* and toxin production if the pH is greater than 4.7 (18, 34).

The heat processes used to preserve acid foods are not severe enough to destroy the spores of *C. botulinum*, because the acidity of the product is relied upon to prevent the germination and growth of this organism (14, 34, 41). Little is known about the heat resistance of *C. botulinum* in tomato products. This investigation of the heat resistance of *C. botulinum* spores in a tomato substrate was stimulated by the recent cases of botulism associated with home-canned tomato products (7, 8).

(This work was taken in part from a thesis submitted by Theron E. Odlaug to the faculty of the graduate school of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.)

MATERIALS AND METHODS

Spores. Cultures of a *C. botulinum* type A strain, A16037, and a type B strain, B15580, were obtained from the Center for Disease Control in Atlanta, Ga. These strains were implicated in outbreaks of botulism in which home-canned tomato products were the toxin-carrying vehicle (7, 8). The classical 62A strain was obtained from the American Type Cul-

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ture Collection. Spore crops were prepared using beef heart infusion medium as described by Wheaton and Pratt (43). After maximum sporulation at 32°C, estimated by microscopic examination, the spores were separated from the solid portion of the infusion by passing the mixture through two layers of cheesecloth. The spores were separated from the broth by centrifugation at 1,200 × *g* for 20 min. The spore crop was cleaned by sonic treatment using a S-12 Branson sonifier for 3 min at 20 kHz followed by repeated washings with distilled water and centrifugation. Cleanliness was estimated by the loss of stainability by crystal violet. The washed spores were suspended in distilled water and stored at 4°C.

Subculture medium. Yeast extract agar (3) was used for subculturing the heat-treated spores as well as for enumeration of the initial spore concentration (yeast extract [BBL], 10.0 g; soluble starch, 1.0 g; K_2HPO_4, 2.0 g; agar, 15 g; distilled water, 1,000 ml; pH 7.2). The medium was sterilized for 15 min at 121°C in 300-ml amounts in 500-ml Erlenmeyer flasks. Immediately before pouring into the plates, the following additions were aseptically made to each flask: 3.75 ml of a 10% sodium thioglycolate solution, 3.75 ml of a 40% dextrose solution, and 7.5 ml of a 4% sodium bicarbonate solution. Dextrose was sterilized by filtration, and the other two compounds were sterilized by heat.

Tomato juice (pH 4.2). Twelve 1-quart (ca. 1-liter) jars of commercially glass-packed tomato juice were purchased to be used throughout this study. The jars of tomato juice were held at 4°C from the time of purchase until used. Before each heating test, each jar was removed from the refrigerator, washed, dried, flamed on the top and sides, and opened with a disk cutter (Difco) under a laminar-flow hood. The entire contents of the jar were poured into a sterile flask.

Sorensen 0.067 *M* phosphate buffer. Sorensen 0.067 M phosphate buffer (pH 7.0) was prepared by mixing 61.1 ml of a stock solution of 0.067 M disodium phosphate (Na_2HPO_4) with 38.9 ml of a stock solution of 0.067 M potassium acid phosphate (KH_2PO_4). The prepared buffer was autoclaved for 15 min at 121°C.

Aluminum tubes. Aluminum tubes were used for holding the spores during the heat destruction tests. The overall length of the tubes was 152 mm with a 6-mm outside diameter. The inside diameter of the tube was 3 mm. The tube was threaded at both ends; the ends were closed by a plastic cap screw and "O" ring; the volume of the reservoir was approximately 1 ml. A sketch of the aluminum thermal death time tube is shown in Fig. 1.

Inoculation of test substrate. The heat resistance of the spores was determined in tomato juice and buffer. Before a heating test, 29.7 ml of the test substrate was added to a sterile Pyrex test tube (25 by 150 mm) and 0.3 ml of spore suspension was added. The contents were then mixed and distributed into the aluminum thermal death time tubes.

Filling of aluminum tubes. One milliliter of the inoculated substrate was deposited into each of the aluminum tubes with a hypodermic syringe. The tubes were hermetically closed with a plastic cap screw and "O" ring. The filled tubes were held in an ice-water bath until heating. At least three replicate units were evaluated at each heating time. A minimum of four heating times were utilized in each survivor curve determination.

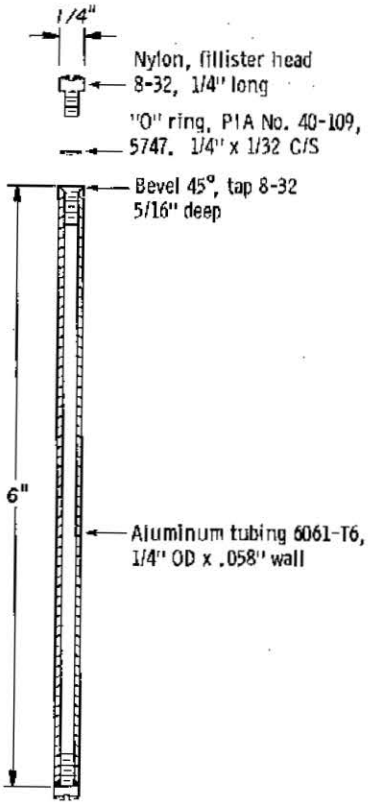

FIG. 1. *Aluminum thermal death time tube.*

Heating of aluminum tubes. The substrate in the aluminum tubes was mixed on a Vortex mixer for 30 s immediately before heating. The tubes were then placed in a metal rack in the miniature retort. Immediately after heating, the tubes were placed into an ice-water bath. The rods remained in the ice bath until the procedure for spore recovery was initiated.

Procedures to recover and plate samples from aluminum tubes. After heating, the substrate in the tubes was again mixed on a Vortex mixer for 30 s. The cap screw on one end of the tube was removed, and the contents were removed with a hypodermic syringe. The substrate was deposited into a sterile premarked petri plate in a single large droplet. Portions of this droplet were either plated directly in duplicate or were added to dilution blanks and plated in duplicate. Twenty milliliters of yeast extract agar was poured into each petri plate and mixed with the portion of the spore suspension. After the agar had solidified, the plates were inverted, placed in an aerobic jar under a GasPak hydrogen-carbon dioxide atmosphere, and incubated at 30°C. All plates were incubated for 9 days, when colony counts were at a maximum.

Analysis of colony count data. The semilogarithmic model was used as the basis for correlating

the heat destruction data. The general equation is: $\log N = -U/D + \log N_0$, where N = number of surviving organisms after treatment (U), N_0 = initial number of organisms, U = sterilizing value at test temperature, and D = time for a 90% reduction in the microbial population. In using this model we recognize, as discussed by Pflug and Bearman (32), that all microbial heat destruction data will not fit the semilogarithmic model exactly but that it is the most convenient and usable model available today.

In survivor curve experiments to evaluate the effect of heat stress on microbial spores, all conditions except heating time were held as constant as possible. The resulting data are the number of colony-forming units for each test heating time and for the unheated control. The data were analyzed with the aid of a digital computer. In the analysis procedure the survivor data for the unheated controls (N_0) were separated from the survivor data at the several heating times. The survivor data for the several test heating times (not including the unheated controls) were correlated using the simple linear regression of the logarithm of the survivors versus sterilizing value (13). The slope of the regression line was used to estimate the thermal resistance parameter (D). The zero-time intercept of the regression line (Y_0) was calculated. The Y_0 and N_0 values were used to calculate the intercept ratio (IR): $IR = \log Y_0/\log N_0$.

The statistical variation of these parameters and the point-to-point D-value of the data were calculated by the computer. A survivor curve graph was also prepared by the computer.

The z-values were determined by a linear regression analysis of the logarithm of the D-values versus temperature.

Lag correction factors for aluminum tubes. Temperatures of the sample in the tube were measured during the heating and cooling tests with 30-gauge thermocouples that could be located at the midpoint, 77 mm from the end, or 25 mm above the bottom or below the top of the tube. The thermocouple lead wires passed through a 2-mm hole in the plastic screw that was sealed with epoxy resin.

The cavity in the tube was filled with 1.0 ml of the liquid sample. Water, a convection heating material, and mushroom puree (prepared from canned mushrooms), a conduction heating material, were used in these tests.

These tests were carried out at 121°C in a miniature retort. At zero time the tubes were placed in the miniature retort, heated for 1 min, and then cooled. The tubes were kept vertical in the retort.

Analysis of heat penetration data. The time-temperature data for each test were plotted on semilogarithmic paper according to the method of Ball (4). The temperature response parameter, f_h, and lag factor, j_h, were calculated. Lag correction factors were calculated using the methods of Ball (4, 5) and Stumbo (39) using a j_c of 1.2 and T_1 (retort temperature)-T_2 (cooling temperature) of 82.2°C (180°F).

RESULTS

Lag correction factors. The mean f_h and j_h values for water and mushroom puree heated in aluminum tubes are shown in Table 1. The temperature response parameter (f_h) of 0.09 min for the top portion of the water-filled tube indicates that heating was slightly faster at the top than in the middle and bottom, where the f_h was 0.12 min. Heating of the mushroom puree was somewhat slower, as expected: the f_h was 0.14 min at the middle and bottom and 0.13 min at the top. These small f_h values indicate the rapid temperature response of the 1-ml samples in the aluminum tubes. In Table 2 are shown the lag correction factors for water and mushroom puree in aluminum tubes calculated for two temperatures. The difference in the lag correction factor between the two test substrates was small. At 121.1°C and z = 10°C, the lag correction factor for water was 0.18 min, and for mushroom puree it was 0.23 min. The difference is 0.05 min or 3.0 s. Since this difference is small and the majority of heating times are greater than 1 min, a lag correction factor of 0.2 min was used to correct the heating times in all tests.

Survivor curve tests. The heat resistance of strains 62A and B15580 at 110.0°C is shown in Table 3; the heat resistance of A16037 at 104.4, 110, and 115.6°C is given in Table 4. The D(110°C)-value for the type B strain in tomato

TABLE 1. *Summary of aluminum tube heating experiments*

Test substrate	Thermocouple junction position in tube[a]	f_h (min)	j_h (min)
Water	Top[b]	0.09	1.1
	Middle	0.12	1.2
	Bottom[c]	0.12	1.2
Mushroom puree	Top[b]	0.13	1.2
	Middle	0.14	1.1
	Bottom[c]	0.14	1.2

[a] Six experiments for each condition.
[b] Twenty-five millimeters below the top.
[c] Twenty-five millimeters above the bottom.

TABLE 2. *Lag correction factors (t_l) for aluminum tubes*

Substrate	z-value (°C)	t_l (min) for T_1[a]	
		104.4°C	121.1°C
Convection heating (water)	7.8	0.18	0.19
	10	0.17	0.18
	12.2	0.16	0.17
Conduction heating (mushroom puree)	7.8	0.23	0.24
	10	0.22	0.23
	12.2	0.20	0.22

[a] T_1, Retort temperature.

juice was 0.7 min; for the 62A strain, it was 1.0 min; and for the A16037 strain, it was 1.6 min.

The D(110°C)-value for the 62A strain in buffer was 2.8 min; for the type B strain, it was 1.4 min; and for the A16037 strain, it was 4.4 min.

Because of its higher heat resistance, tests were carried out with the A16027 strain of *C. botulinum* to determine its z-value. The results of the survivor curve tests at three temperatures for type A16037 spores heated in tomato juice (pH 4.2) are shown in Table 4, and results

TABLE 3. *Summary of survivor curve tests for Clostridium botulinum 62A and B15580 at 110.0°C*

Test no.	Spore strain	D-value (min)	95% CI[a] (min)	IR[b]
Tomato juice				
T07010A	62A	0.98	0.84–1.17	0.86
T07026A	62A	0.92	0.75–1.14	0.89
Mean		0.95		0.88
T07010B	B15580	0.87	0.77–1.01	0.72
T07026B	B15580	0.61	0.53–0.71	0.79
Mean		0.74		0.76
Buffer				
T06302B	62A	2.72	2.50–3.00	0.91
T07012A	62A	2.89	2.55–3.34	0.87
Mean		2.80		0.89
T06355A	B15580	1.37	1.28–1.47	0.98
T07004A	B15580	1.34	1.23–1.48	1.01
Mean		1.36		1.00

[a] CI, Confidence interval.
[b] IR, Intercept ratio.

TABLE 4. *Summary of survivor curve tests for Clostridium botulinum type A spores (A16037) suspended in tomato juice (pH 4.2)*

Test no.	Test temp (°C)	D-value (min)	95% CI[a] (min)	Width of 95% CI (min)	IR[b]
T06167B	115.6	0.40	0.36–0.45	0.09	0.99
T06181B	115.6	0.38	0.35–0.40	0.05	0.99
T06195B	115.6	0.39	0.36–0.42	0.05	0.99
Mean		0.39			0.99
T06134B	110.0	1.59	1.43–1.79	0.36	0.92
T06169B	110.0	1.56	1.36–1.83	0.47	0.94
T07010C	110.0	1.50	1.27–1.71	0.44	0.97
Mean		1.55			0.94
T06138B	104.4	6.02	5.58–6.53	0.95	1.04
T06149B	104.4	6.09	5.74–6.49	0.75	1.05
T06153D	104.4	5.89	5.51–6.32	0.81	1.02
Mean		6.00			1.04

[a] CI, Confidence interval.
[b] IR, Intercept ratio.

TABLE 5. *Summary of survivor curve tests for Clostridium botulinum type A spores (A16037) suspended in Sorensen 0.067 M phosphate buffer (pH 7.0)*

Test no.	Test temp (°C)	D-value (min)	95% CI[a] (min)	Width of 95% CI (min)	IR[b]
T06111B	115.6	1.35	1.18–1.57	0.39	0.96
T06153B	115.6	1.27	1.14–1.44	0.30	0.96
Mean		1.31			0.96
T06194B	110.0	4.29	3.83–4.88	1.05	0.94
T06212A	110.0	4.47	3.88–5.23	1.35	0.98
Mean		4.38			0.96
T06205A	104.4	16.19	15.26–17.25	1.99	1.00
T06217A	104.4	19.10	17.69–20.77	3.08	0.98
Mean		17.64			0.99

[a] CI, Confidence interval.
[b] IR, Intercept ratio.

in Sorensen 0.067 M phosphate buffer (pH 7.0) are given in Table 5.

The A16037 spores had a higher heat resistance in buffer compared with the tomato juice substrate. The D-values for spores in tomato juice (Table 4) were 30% of the D-values for spores in buffer (Table 5) at 115.6°C, 35% at 110.0°C, and 34% at 104.4°C. These results indicate that the relative differences between D-values for spores in tomato juice compared with buffer were similar at the three test temperatures.

The shape of the survivor curves for the *C. botulinum* type A16037 spores, as indicated by the intercept ratio, did not differ greatly with test temperature and test substrate. Typical survivor curves for each test temperature for tomato juice and buffer are shown in Fig. 2. The rapid destruction of spores in tomato juice compared with spores in buffer is evident when the survivor curves at each test temperature are compared.

The D-value data in Tables 4 and 5 for the type A16037 spores were used in calculating the z-values shown in Table 6. The z-value was 9.4°C (17.0°F) for the spores suspended in tomato juice and 9.9°C (17.8°F) for the spores suspended in Sorensen 0.067 M phosphate buffer.

DISCUSSION

Aluminum thermal death time tubes. In selecting an inoculum-substrate container to be used in the *C. botulinum* thermal resistance tests, we wanted a unit with these attributes: unbreakable (for safety reasons), reusable, easily sealed and opened, small lag correction fac-

tor so tests could be carried out at 121°C, relatively inexpensive, and easily used in a miniature retort heating system. The result was the development of the aluminum thermal death time tube. We believe that this unit, used with a miniature retort heating system, is equal to or better than other methods currently available for carrying out survivor curve tests with *C. botulinum* spores (24, 33, 35).

Heat resistance tests. There have been a number of published studies on the resistance of *C. botulinum* spores (1, 2, 16, 22, 23, 31, 37, 38, 42, 44). In only one of these studies, that of Xezones and Hutchings (44), was there an extensive investigation of the effect of pH on the *D*-value of *C. botulinum* spores suspended in food. We found that the results of Xezones and Hutchings were more informative when the logarithm of the *D*-value data at each temperature was plotted versus pH. The resulting graph for spores heated in the spaghetti-tomato sauce-cheese substrate at the four temperatures is shown in Fig. 3. The results were similar for *C. botulinum* spores heated in macaroni

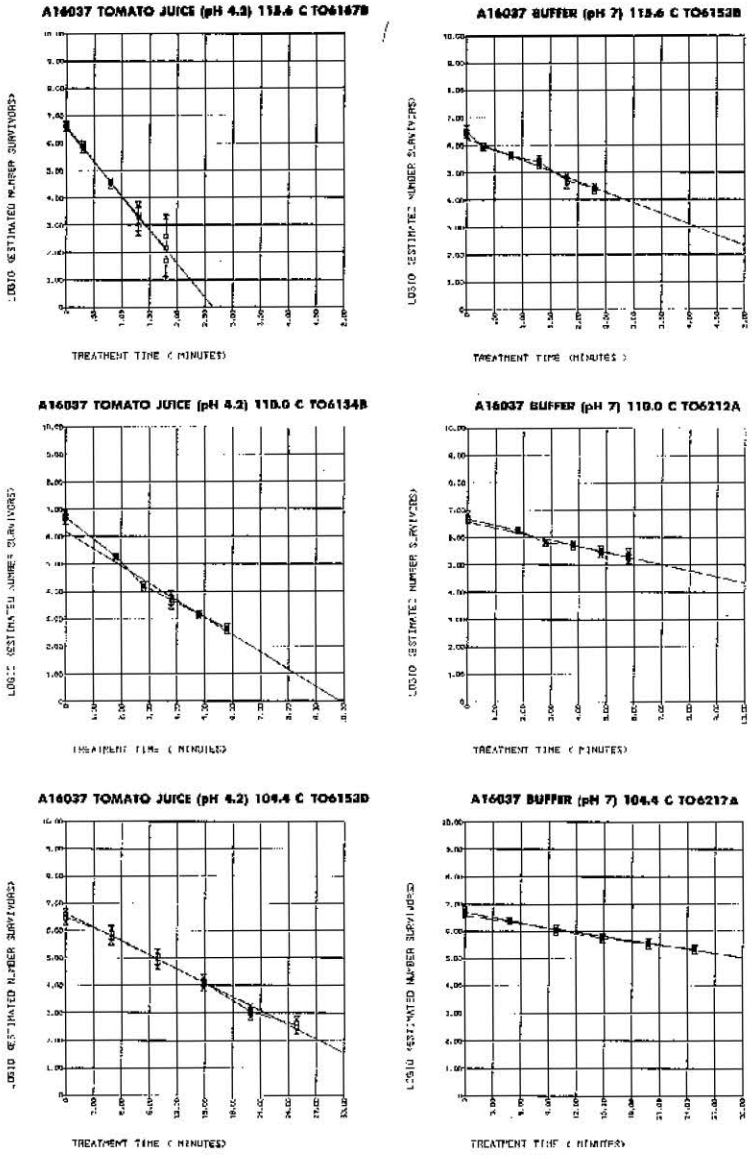

FIG. 2. *Survivor curves for Clostridium botulinum A16037 spores.*

TABLE 6. *z-value results for Clostridium botulinum type A spores (A16037)*

Substrate	z-value (°C)	95% CI[a] (°C)	Width of 95% CI (min)
Tomato juice (pH 4.2)	9.43	9.28–9.60	0.32
Sorensen 0.067 M PO_4 buffer (pH 7.0)	9.93	9.12–10.89	1.77

[a] CI, Confidence interval.

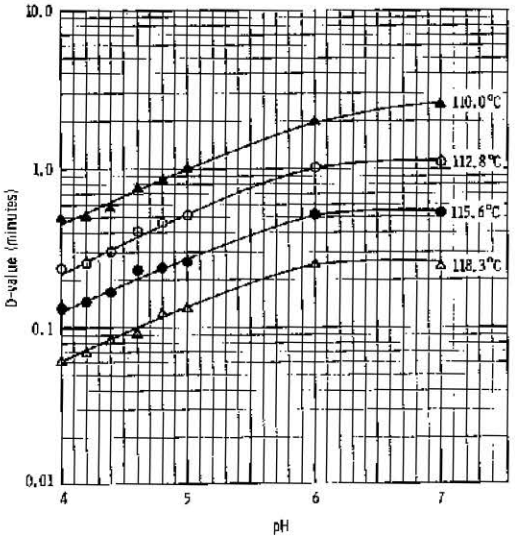

FIG. 3. *D-values at four temperatures for spores of Clostridium botulinum suspended in a spaghetti-tomato sauce-cheese substrate as a function of pH, from the data of Xezones and Hutchings (44).*

creole and Spanish rice. The effect of pH on the *D*-value of *C. botulinum* spores was essentially the same regardless of the test temperature.

The analysis of the data of Xezones and Hutchings (44) indicates that for *C. botulinum* spores heated in a spaghetti-tomato sauce-cheese, macaroni creole, or a Spanish rice substrate, the *D*-values at the four test temperatures were 3.5 to 5 times higher when the substrate pH was 7.0 compared with when the substrate pH was 4.2. The results in this study were similar to the results of Xezones and Hutchings in that, at pH 7.0 in buffer, the resistance of the spores was higher than at pH 4.2 in tomato juice, and the relative difference between *D*-values was similar regardless of the test temperature.

Lower heat resistance at lower acid pH values has been suggested by Grecz et al. (17) to be explained partly by the dissociation constants of Ca-dipicolinic acid and by Alderton et al. (1, 2) to be a function of the chemical form of the spore (resistant calcium form and sensitive hydrogen form).

The type A strain (A16037) used in this study was implicated in an outbreak of botulism involving a jar of home-canned tomato juice that contained type A toxin (8). This organism has a higher heat resistance in buffer and tomato juice than strain 62A. The extrapolated *D*(100°C)-value for A16037 spores was, in buffer, 47 min and in tomato juice, 18 min. The heat processes currently used for canning tomato products (pH < 4.6) are not designed to destroy *C. botulinum* spores because they will not grow and produce toxin where the pH is below 4.6. If the pH of the product is raised above 4.6 surviving *C. botulinum* spores may germinate, grow, and produce toxin. Under certain specific and rather unique conditions, *C. botulinum* can grow with another microorganism within a system where the pH is less than 4.6 (8, 18, 28, 36).

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