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Heat Resistance of *Clostridium botulinum* Type B Spores Grown from Isolates from Commercially Canned Mushrooms[1]

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ABSTRACT

The heat resistance of ten *Clostridium botulinum* type B spore crops was determined in mushroom puree and 0.067M Sorenson phosphate buffer (pH 7). The spore crops were grown from *Clostridium botulinum* isolates obtained from commercially canned mushrooms. The D-values for all of the *C. botulinum* spore crops were overall slightly higher in the buffer than in mushroom puree. The mean D(110.0 C)-value for the ten spore crops in buffer was 1.17 min and for the spores in mushroom puree the mean D(110.0 C)-value was 0.78 min. The mean D(115.6 C)-value in buffer for the ten spore crops was 0.24 min compared to a mean D(115.6 C)-value of 0.19 min for spores in mushroom puree. The *C. botulinum* type B spores tested in this study had a heat resistance that was less than the classical heat resistance for *C. botulinum* spores.

During 1973 and 1974, the Food and Drug Administration (FDA) conducted a survey of all domestic and imported canned mushrooms held in warehouses in the United States. As a result of this survey canned mushrooms from nine commercial canners were found to contain *Clostridium botulinum* (*1*). The cans were from seven U.S. producers and two foreign producers.

The objective of this study was to determine the wet-heat resistance of the *Clostridium botulinum* type B spore crops produced from the isolates obtained from commercially canned mushrooms. Heat resistance tests were done at 110 C (230 F) and 115.6 C (240 F) with the spores suspended in two different substrates: mushroom puree and 0.067 M Sorensen phosphate buffer (pH 7.0).

The heat resistance of these *Clostridium botulinum* spore crops can be used as an aid in ascertaining if the recent detection of botulinum toxin in commercially canned mushrooms (*1*) was due to the failure to deliver a minimum botulinum cook or to post-processing contamination.

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MATERIALS AND METHODS

Spores

Ten cultures of *Clostridium botulinum* type B isolated from commercially canned mushrooms were used to produce 10 spore crops.

The cultures were grown in Stumbo's beef heart casein medium (*6*) in 100-ml bottles, incubated at 30 C for 15 to 20 days followed by refrigeration for 7 to 30 days. The spore crops were harvested by filtration through sterile cheese cloth layered with glass wool and centrifuged. All spore crops were washed five times with sterile distilled water and were not treated with lysozyme. The spores were suspended in sterile distilled water, stored at 4 C, and counted by the 3-tube MPN procedure using trypticase-peptone-glucose-yeast extract broth. The spore codes and the codes for the producers of the cans of mushrooms that were the source of the isolates are shown in Table 1.

TABLE 1. Clostridium botulinum *type B isolates.*

Univ. of Minn. spore code	FDA strain designation	Company
SKAF	002-642G-1A	A
SKBF	083-383G-2	B
SKCF	083-384G-2	B
SKDF	083-424G-7	B
SKEF	039-612G-10	C
SKFF	011-273G-E	D
SKGF	083-384G-4	B
SKHF	038-390G-6	B
SKIF	083-390G-12	B
SKJF	038-424G-4	B

Substrate preparation

Sorensen 0.067M phosphate buffer, pH 7 (*2*). Two-ml quantities of the buffer in 18 × 150 mm screw-capped glass tubes were autoclaved and stored at 4 C and used within 48 h of preparation. On the day of a test, tubes containing buffer were removed from the refrigerator and allowed to reach room temperature. Five min before heating .05 ml of the appropriate spore crop was added to the buffer in each of the glass test tubes using an Eppendorf pipette. Inoculation of the spores was done in a laminar flow hood. The buffer and spore inoculum were mixed and tubes were loaded into the assigned retort and were heated at the predetermined temperature for the designated time.

Mushroom puree. A single case of 4-oz. cans of commercially produced mushrooms stems and pieces, stored at 4 C was used throughout this study. Opening of the cans and other aseptic manipulating were carried out in a laminar flow hood. Before a heating test the content of one can was removed aseptically and blended (Waring Aseptic Dispersall, Model A5-1) with 1.0 ml of spore crop for

1 min at low speed. The puree was kept in the blender container at less than 20 C for the duration of the test period, which never exceeded 1 h. The pH of the puree ranged from 6.3 to 6.5.

To facilitate removal of specified amounts of inoculated puree from the blender container and the subsequent transfer to glass test tubes, a 10-ml syringe was fitted with a 130 mm × 4-mm (ID) stainless steel tube. The free end of the stainless steel tube was placed into a needle valve with an "O"-ring packing located at the base of the blender container and the syringe was filled with the puree. The puree was then delivered in 2.0 ± 0.1-ml amounts to the bottom of each of five test tubes.

The inoculated mushroom puree was added to glass tubes 5 min before heating. The tubes were then loaded into the assigned retort and heated at the predetermined temperature for the designated time.

Heat resistance testing

Heat resistance testing was done using the multiple replicate fraction-negative method (3) with screw-cap test tubes as the test unit. To do each fraction-negative test, six 18 × 150-mm screw-cap test tubes containing approximately 10^7 spores in the appropriate substrate were heated for five or six heating times. The tubes were heated in miniature retorts at 110 C (230 F) and 115.6 C (240 F). At the end of the heating period tubes were quickly transferred to 80-C water bath and held in this water bath for 2 min.

Twenty ml of pork infusion agar (6) at 80 C was added to each tube followed by 5 ml of a 1:1 paraffin-mineral oil mixture. The tubes were incubated at 30 C for 60 days. Visually observed gas formation and/or colony formation were used as indication of survival.

The Spearman-Karber (SK) method was used for D-value calculation (5). At the shortest heating time all the units must be positive and at the longest heating time all the units must be negative. At the intermediate times various fractions of the replicate tubes would be negative. To obtain data usable by the SK method a preliminary test with widely spaced heating times was first used. Subsequent tests were done until data usable by the SK method were obtained.

Determining the initial numbers of spores

The initial number of spores (N_0) for the fraction-negative tests was determined by serial dilutions and pour plate method using pork infusion agar (PIA). For tests with the buffer substrate, serial dilutions of 1-ml aliquots from three unheated tubes were made with Butterfield's phosphate buffer, pH 7.2. The N_0 in the mushroom puree was determined by serial dilutions of three 1-ml aliquots removed from the puree in the blender container.

Two aliquots (0.1 and 1.0 ml) from each dilution bottle were plated in duplicate and 20 ml of PIA was added to the plates. The plates were inverted, placed in anaerobic jars under a hydrogen-carbon dioxide atmosphere (GasPak) and incubated at 30 C for 48 h.

RESULTS AND DISCUSSION

The D-value results for each of the 10 spore crops heated at 110 and 115.6 C in 0.067 M Sorensen phosphate buffer (pH 7.0) and in mushroom puree are presented in Table 2. The response of the 10 spore crops to the four test conditions was variable.

The D-value data in Table 2 were statistically analyzed; there was no significant correlation between the D-values for spores heated in buffer and in mushroom puree at either 110 or 115.6 C, or between the D-values for the spores heated in mushroom puree at 110 and 115.6 C. The most significant correlation was between the values for the spores heated in buffer at 110 and 115.6 C. The results of the statistical analysis were basically the same when the D-values were analyzed by either of several methods: (*a*) the product moment correlation on the D-value, or on the logarithm of the D-value, (*b*) Spearman's rank correlation, or (*c*) Kendall's Tau.

Considering the correlation between the D-values for the spores in buffer at 110 and 115.6 C, heating *C. botulinum* spores in mushroom puree produced results that were quite variable. The variation may have been due to an interaction between the spores and the mushroom puree during heating or during incubation.

When heated in buffer, the several spore crops showed a range of heat resistance levels at 110 and 115.6 C, which suggests that there were several *C. botulinum* type B variants in the group.

For the purpose of evaluating mushroom sterilization processes, the results of tests of all 10 of the spore crops were examined. The D(110 C)-values for the spores heated in mushroom puree ranged from 0.49 to 0.99 min and in buffer from 1.02 to 1.38 min. The D(115.6 C)-value for the spores heated in mushroom puree ranged

TABLE 2. *D-value results for the ten* Clostridium botulinum *type B spore crops heated at 110.0and 115.6 C in mushroom puree and 0.067M phosphate buffer.*

Spore suspension code	110.0 C Tests				115.6 C Tests			
	Mushroom puree		0.067M phosphate buffer, pH 7		Mushroom puree		0.067M phosphate buffer (pH 7)	
	D-Value (min)	95% C.I.[a] (min)	D-Value (min)	95% C.I. (min)	D-Value (min)	95% C.I.[a] (min)	D-Value (min)	95% C.I.[a] (min)
SKAF	.99	0.84 - 1.14	1.13	1.01 - 1.25	.14	0.09 - 0.20	.22	0.19 - 0.26
			1.08	0.89 - 1.26	.12	0.09 - 0.16	.22	0.19 - 0.26
SKBF	.87	0.69 - 1.04	1.10	0.98 - 1.21	.18	0.15 - 0.22	.19	0.14 - 0.23
			1.02	0.86 - 1.18	.19	0.15 - 0.22	.22	0.18 - 0.25
SKCF			1.06	0.91 - 1.21	.14	0.08 - 0.20		
SKDF	.68	0.50 - 0.86	1.26	1.13 - 1.40	.15	0.11 - 0.20	.24[b]	—
			1.23	1.10 - 1.37	.18	0.15 - 0.22	.22	0.16 - 0.28
SKEF	.49	0.30 - 0.69	1.10	0.95 - 1.25			.11	0.05 - 0.17
SKFF	.78	0.64 - 0.91			.30	0.24 - 0.36	.35	0.30 - 0.39
SKGF	.89	0.77 - 1.01	1.22	1.04 - 1.39	.14	0.10 - 0.18	.18	0.13 - 0.22
SKHF	.96	0.87 - 1.05			.39	0.33 - 0.44	.27	0.21 - 0.34
SKIF	.79	0.70 - 0.88	1.28	1.14 - 1.42	.12	0.09 - 0.15	.32	0.25 - 0.39
SKJF	.58	0.44 - 0.70	1.38	1.27 - 1.50	.19	0.16 - 0.23	.29	0.21 - 0.37
Mean	.78		1.17		.19		.24	
Range	.49 - .99		1.02 - 1.38		.12 - .39		.11 - .35	

[a]95% confidence for the D-value.
[b]Data not sufficient to calculate variance of the D-value.

from 0.12 to 0.39 min and in buffer from 0.11 to 0.35 min.

There were marked differences between D-values for spores heated in buffer compared to spores heated in mushroom puree. At 110 C the mean D-value for spores heated in mushroom puree was 0.78 min and in buffer 1.17 min. At 115.6 C the mean D-value for the spores heated in mushroom puree was 0.19 min and in buffer 0.24 min.

The mean width of the D-value confidence interval (CI) expressed as a percent of the D-value was 39% for mushroom puree at 110 C, 25% for buffer at 110 C, 49% for mushroom puree at 115.6 C, and 48% for buffer at 115.6 C. The D-values for the spores heated in buffer at 110 C showed the smallest mean width of the CI. For the spores heated in buffer the difference between the mean D-value CI at 110 and 115.6 C was quite large, 25 versus 48%. The effect of normal variation of time and temperature, that is the same regardless of test time and temperature, probably produced a correspondingly greater percent error in the results at 115.6 C because of shorter heating times. The wider mean CI for the spores heated in mushroom puree at 110 C compared to buffer at 110 C suggests that the mushroom puree contributes to variability.

By extrapolation equivalent D(121.1 C)-values were calculated from D(110.0 C)-values and D(115.6 C)-values for the 10 spore crops tested using a z of 10 C. These values are presented in Table 3. The mean D(121.1 C)-values for spores heated in the mushroom puree were .05 min and .06 min calculated from D(110.0 C) and D(115.6 C)-values, respectively. The mean D(121.1 C)-values for spores heated in the 0.067 M phospahte buffer were .07 min and .09 min calculated from D(110.0 C) and D(115.6 C) values, respectively.

TABLE 3. *Extrapolated D(121.1 C)-Values for the ten* Clostridium botulinum *type B spore crops calculated from D(110.0 C)-values and D(115.6 C)-values using a z-value of 10 C.*

	Calculated from D(110.0 C)-value		Calculated from D(115.6 C)-value	
	Mushroom puree	0.067M phosphate buffer, pH7	Mushroom puree	0.067M phosphate buffer, pH 7
Spore code	D(121.1 C) min	D(121.1 C) min	D(121.1 C) min	D(121.1 C) min
SKAF	.08	.09	.04	.06
		.08	.03	.06
SKBF	.07	.08	.05	.05
		.09	.05	.06
SKCF		.08	.04	
SKDF	.05	.10	.05	.07
		.10	.03	.06
SKEF	.04	.09		.03
SKFF	.06		.08	.10
SKGF	.07	.09	.04	.05
SKHF	.07		.11	.08
SKIF	.06	.10	.03	.09
SKJF	.04	.11	.05	.08
Mean	.06	.09	.05	.07
Range	.04 - .08	.08 - .11	.03 - .11	.03 - .10

The spore crops tested in this study were not very heat resistant compared to the classical D(121.1 C)-value (calculated from the data of Esty and Meyer by Schmidt) of 0.2 min (*4*). The spores in this study heated in a mushroom puree or in buffer had D(121.1 C)-values that were 15 to 55% of the classical value. The mean D(121.1 C)-value for spores heated in mushroom puree was about 25% of the classical resistance value and for the buffer was about 40% of the classical value. The largest equivalent D(121.1 C)-value was 0.11 min for strain SKHF heated in mushroom puree and for strain SKJF heated in 0.067 M phosphate buffer.

The isolates for these spore crops were obtained from canned mushrooms that had been processed in canning plants with deficiencies in equipment (inaccurate or broken thermometers, faulty piping, etc.) and in operating procedures (poor fill control, inadequate venting, etc.) (*1*). If we assume that the initial number of *C. botulinum* spores per can was 100 and that cans containing toxin were found at the rate of one can per 100,000 can-lot; the F_0-value that would produce these conditions if the spores had D(121.1 C)-values of 0.03 to 0.11 min would be 0.21 to 0.77 min. An acceptable F_0 for public health safety for low-acid canned is 3.0 min. Since all of the *C. botulinum* spore crops tested had significant heat resistance the possibility is very high that the spores survived the heat processes and that they did not leak into the container after processing.

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