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Growth Potential of *Clostridium botulinum* in Fresh Mushrooms Packaged in Semipermeable Plastic Film

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Fresh mushrooms (*Agaricus bisporus*) were inoculated in the stem, gill, or cap with *Clostridium botulinum* spores. They were placed with uninoculated mushrooms in paper board trays, which were then covered and sealed in a polyvinyl chloride stretch film to simulate prepackaged mushrooms available at retail stores. When incubated at 20 C, botulinum toxin could be detected as early as day 3 or 4, when the mushrooms still appeared edible. Mushrooms inoculated in the stem with 1,000 type A spores frequently became botulinogenic; higher spore levels were needed if gills or caps were inoculation sites. Type B spores were less apt to produce toxic mushrooms. Respiration of the fresh mushrooms used up O_2 more rapidly than could enter through the semipermeable wrapping film, so that the equilibrium O_2 concentration became low enough for growth of *C. botulinum*. Inoculated mushrooms did not become botulinogenic when held at 4 C.

The recent concern about the potential botulism hazard associated with commercially canned mushrooms originated with the finding in 1973 of *Clostridium botulinum* toxin in the product of one company (11). In the ensuing intensive investigation, botulinum toxin was found in canned mushrooms processed by at least six domestic and two foreign companies (D. A. Kautter, personal communication). Strict adherence to approved processing methods should prevent a recurrence of this problem.

Aside from use in canned form, commercially grown mushrooms are also sold retail as fresh produce, prepackaged in flexible plastic films of restricted gas permeability. Use of fresh mushrooms has been increasing in the United States; in 1974 about 37% of the mushrooms were sold as fresh produce and 63% were sold in canned form. In Europe the proportion is 65% fresh and 35% canned (4).

Development of botulinum toxin in canned mushrooms indicates that harvested mushrooms can be contaminated naturally with *C. botulinum*. The study to be reported here considers the possibility of this organism growing and producing its toxin in prepackaged fresh mushrooms. Toxin production seemed possible if (i) fresh mushrooms are similar in chemical composition to canned mushrooms so that they satisfy the nutritional requirements for growth of *C. botulinum*, (ii) fresh mushrooms respire and use O_2 more rapidly than the rate at which the gas can enter the package through the semipermeable wrapping film, and (iii) the resulting equilibrium is a low O_2 tension within the packages of mushrooms.

The inoculated pack approach was used. Fresh mushrooms were inoculated with *C. botulinum* spores and repackaged together with uninoculated ones in polyvinyl chloride wrapping film, and the inoculated specimens were tested for botulinum toxin after incubation.

MATERIALS AND METHODS

Mushrooms. Fresh mushrooms (*Agaricus bisporus*) were purchased at a supermarket that receives daily shipment of this produce. Tests were started on the day mushrooms were purchased.

Wrapping film. The wrapping film was one used commercially for packaging fresh mushrooms. It is a polyvinyl chloride film (0.75 mil thick) called PWSH (produce wrap, shrink, hand). Transmission of O_2 is 800, and of CO_2 it is 6,000 $cm^3/100\ in^2$ (645 cm^2) per 24 h at 77 F (25 C); the moisture vapor transmission rate is 20 $g/100\ in^2$ per 24 h at 100 F (37.8 C) and 90% relative humidity (manufacturer's specifications).

Spores. *C. botulinum* strains were obtained from K. Ito, National Canners Association Western Regional Research Laboratory, Berkeley, Calif., and the type of toxin produced is indicated by a letter in the strain designation. Spore suspensions were prepared by repeatedly washing the spore crops, which had been grown in polypeptone-Trypticase broth, with sterile water (3). Stock spore suspensions contained few vegetative cells and had about 10^9 spores/ml. Spores used in the tests were from stock suspensions that had been stored about 1 year at 4 C.

Viable spores were enumerated as the average of triplicate five-tube, most-probable-number tests (1).

A dilution of the stock spore suspension was heated at 80 C for 10 min. Serial decimal dilutions of this suspension in distilled water were prepared, and appropriate ones were cultured in cooked meat medium (Difco Laboratories, Detroit, Mich.). Incubation was at 37 C for at least 2 weeks. Although contamination was not a problem, cultures developing from the highest dilution giving growth were routinely tested for the proper type of botulinum toxin.

Inoculation of mushrooms. Separate mushrooms were inoculated by one of three methods. Caps were inoculated by smearing 0.1 ml of spore inoculum over the surface; in some instances, six superficial punctures into the cap were made through the deposited spore film with a 26-gauge hypodermic needle. Gills were inoculated by depositing 0.1 ml of spores into the underside of the caps in about 10 droplets. Stems were inoculated with 20 μl of inoculum distributed in 10 well-separated injections. Mushrooms of about 12 g were used.

Packaging of mushrooms. Mushrooms were packaged at 0.5 lb (227 g) per paper board tray of approximately 5.25 by 4.5 and 2.5 inches (ca. 13 by 11 by 6 cm) in height. Two inoculated mushrooms were placed in a box and surrounded by uninoculated specimens. The box was then covered in a single layer of polyvinyl chloride film, except for overlapping on the bottom. The overlapping film ends were sealed with a hot-air gun, and the open sides were closed by folding the film that extended beyond the box across the bottom. Sealing these ends to the previously made bottom seal resulted in mushroom packages comparable to those obtainable at retail stores.

Each variation of test conditions was tested with a separate package of mushrooms.

Incubation. Repackaged mushrooms were incubated at 20 C. The important exceptions were controls for the several packages of mushrooms being tested together in one experiment. These controls were held at 4 C for the longest incubation time being used in the particular trial (usually 6 days). The controls, made each time a spore suspension was tested, were mushrooms inoculated in the stems with 10^5 or 10^6 spores.

Analysis of headspace gas. Mushrooms were repackaged in the laboratory and held at room temperature. Gas in the packages was sampled into a Hamilton gas syringe after inserting the needle through the covering film. O_2 and CO_2 analyses were made with an FM720 gas chromatograph (F and M Scientific Corp., Avondale, Pa.), using a hot wire detector and 6-foot (ca. 183-cm) copper columns, respectively, of molecular sieve 5A and silica gel, both 30 to 60 mesh. Samples were 300 μl, carrier gas was helium at 50 to 60 ml/min, temperature of detection and injection ports was 250 C, and the oven temperature was 70 to 75 C isothermal. N_2 was calculated as total gas minus the sum of O_2 and CO_2. O_2 concentration was then obtained as percentage of total O_2, CO_2, and N_2.

Test for toxin. The two spore-inoculated mushrooms in a box were recovered, and most of the uninoculated part was discarded by removing, as appropriate, the cap or stem. The two saved portions were weighed and ground together in a mortar with an equal amount (wt/vol) of pH 6.4, 0.2% phosphate buffer containing 0.1% gelatin. The extract, obtained as the supernatant fluid resulting from centrifugation of the homogenate, was injected intraperitoneally into two mice (0.5 ml/mouse) for the preliminary toxicity test.

Survival of mice for 4 days indicated the absence of botulinum toxin. Toxic extracts nearly always killed animals within 48 h and were retested to confirm the lethal agent as botulinum toxin. Three pairs of mice were used; one pair was challenged with the extract, each animal of the second pair was given 1 IU of type A antitoxin (0.5 ml/animal, intraperitoneally) before injecting the extract, and the third pair was similarly protected with type B antitoxin before being challenged with the sample. Death of the unprotected and one other pair confirmed the presence of botulinum toxin, with the type of antitoxin that provided protection identifying the toxin type in the extract.

Difficulty was seldom encountered. A few extracts did kill in the preliminary test but not when the samples, which had been stored at 4 C, were retested in the confirmatory test. Such samples were considered negative for botulinum toxin. The toxin type demonstrated in the tests was always consistent with the one expected of the spore inoculum.

Quantitation of toxin. After botulinum toxin had been identified, the extract was diluted serially in twofold increments with the phosphate-gelatin buffer. The selected dilutions were injected intraperitoneally into separate groups of six mice. Deaths within 4 days were tabulated, and the mean lethal dose per gram of mushroom was calculated by the Reed and Muench procedure (10).

RESULTS

The first efforts were directed toward determining the O_2 concentration that develops inside boxes that contain fresh mushrooms and are sealed in the wrapping film. Headspace gas samples were obtained 2 h after repackaging; in some instances, the puncture hole made for sampling was sealed with Scotch tape immediately as the needle was withdrawn, so that the package could be retested later.

A wide range of values was obtained (Table 1), but the trend was the rapid disappearance of O_2, so that in 2 h the headspace gas had less than 2% oxygen. Packages with high (>10%) O_2 concentration probably were incompletely sealed with the wrapping film, since they still had these high levels when retested at 7 days.

An exploratory experiment showed that mushrooms inoculated in the stem with 10^5 type A spores were botulinogenic after 6 days at 20 C. A series of tests was then done with differing spore levels of several *C. botulinum*

TABLE 1. *Oxygen concentration (as percentage of total O_2, CO_2, and N_2) in headspace of packages of mushrooms sealed in polyvinyl chloride film and held at room temperature*

Holding time	No. of packages	Oxygen (%) in headspace	
		Range	Mean
2 h	16	1.0–1.9	1.4
	4	2.0–3.9	2.6
	4	4.0–19.0	12.1
7 days	5	1.0–1.9	1.4
	2	2.0–3.9	2.7
	3	4.0–19.0	15.7

strains, with results that are consolidated in Table 2. Larger or smaller spore loads may have been tested, but only the smallest inoculum resulting in detectable toxin is cited. For test conditions not resulting in toxin formation only the highest tested spore level is given.

Although the tabulated data show that fresh mushrooms can become botulinogenic, the data are meant to illustrate trends only. This limitation is placed because of some difficulty in duplicating results. However, the results presented have been obtained frequently enough so that they, or closely similar data, can be expected in at least one of several trials. Considering the nature of botulism and the strong overall indication that botulinum toxin can be formed in fresh mushrooms, any condition that often, but not necessarily always, gives rise to toxin formation is considered important. The probable major factor contributing to the difficulty in consistently duplicating results is suggested by the high O_2 values sometimes found in the headspace analyses (Table 1). Greater reproducibility of results was obtained during the latter part of the work, presumably because packaging improved with experience.

Type B strains seemed less able to grow and produce toxin in mushrooms than type A strains. To obtain toxic mushrooms generally required spore inocula significantly greater than those needed for type A strains. Because of this difference, subsequent work was done with type A spores.

Repackaged mushrooms incubated for 6 days were often spoiled to the extent that they would not be accepted as food by most people. A study was done to determine whether or not toxin could be formed before this state of spoilage was reached. Inoculated packs were prepared, and the toxin titer in the inoculated mushrooms was determined at daily intervals of incubation. The appearance of the mushrooms at these times was recorded (Table 3).

The shortest incubation time at which toxin was detected was 3 days; when titrated, titers of toxin per gram of mushroom were 140 mouse mean lethal doses or lower. At the time when toxin was first found, mushrooms had not yet deteriorated to the degree that they would necessarily be discarded as being unfit for eating. Toxin titers increased with incubation time to as high as 10,000 mean lethal doses/g. This latter value is an exceptional one; generally the maximum was in the several hundred mean lethal doses per gram range.

Experiments considered so far used spore suspensions heated (10 min, 80 C) on the day they were inoculated into mushrooms to inactivate residual toxin persisting in the stock spore suspensions. That significant toxicity was not being introduced into mushrooms with the inocula was shown by the absence of toxin in control mushrooms held at 4 C and by survival of mice challenged with 0.5 ml of the working spore suspension.

Heating of the spore inocula could affect results by activating spores (8) to facilitate their germination in fresh mushrooms. This possibility was tested with a spore suspension that had never been heated and with two others held 30 or more days at 4 C after being heated, so that the spores should have reverted to their unactivated state. The complication of toxin in the spore inocula was avoided, because diluting stocks of about 10^9 spores/ml to the working 5×10^5/ml or lower level reduced toxicity beyond the detectable concentration. The control packages verified this assumption.

The results (Table 4) showed that mushrooms inoculated with heated and unheated spores of strain 56A became toxic at essentially the same time. Results with the other two strains, when compared to data obtained previously with spores heated on the day they were inoculated into mushrooms (Table 2), also indicated that *C. botulinum* spores need not be heat activated to germinate and grow in fresh mushrooms.

DISCUSSION

The possible practical significance of the data depends on mushrooms being acceptable as food when botulinum toxin can be detected in them. In our experiments, mushrooms that were experimentally inoculated with *C. botulinum* spores could be botulinogenic by day 3 or 4, when they still might be considered edible.

During most of the work the spoilage rates of repackaged mushrooms were generally as indicated in Table 1, but significantly different rates were observed with some lots. Mushrooms

TABLE 2. *Formation of botulinum toxin in fresh mushrooms inoculated with C. botulinum spores and repackaged with polyvinyl chloride wrapping film*[a]

Strain	Spore inoculum per mushroom	Site[b]	Toxin type found
69A	10^4	Cap (P), gill	A
	2×10^3	Stem	A
19A	5×10^4	Cap (P, NP)	A
	10^3	Gill, stem	A
62A	10^5	Gill	A
	10^3	Stem	A
90A[c], 11A[c]	10^4	Cap (P, NP), gill, stem	A
213B	10^6	Cap (P)	B
	10^5	Gill	0
	2×10^5	Stem	0
169B	10^6	Cap (P, NP)	0
	10^6	Gill	B
	10^4	Stem	B
113B	10^6	Gill, stem	0
185B[c], 13983B[c]	10^6	Gill	0
	10^6	Stem	B

[a] Incubation was at 20 C for 6 days. Spore suspensions were heated for 10 min at 80 C on the day of use.
[b] For cap, P and NP are, respectively, punctures or no puncture in cap through deposited spore layer.
[c] Single trial with indicated inoculum.

TABLE 3. *Botulinum toxin titers and appearance of mushrooms inoculated with C. botulinum spores and held at 20 C*[a]

Spore inoculum		Days	LD_{50}/mushroom		Appearance[b]
Strain	Spores/mushroom		Stem	Gill	
19A	5×10^4	1	0	0	Good
		2	0	0	Good
		3	140	0	Good
		4	50	34	Fair
		5	600	50	Poor
		6	10,000	100	Poor
11A	10^4	1	0		Good
		2	0		Good
		3	50		Fair
		4	970		Fair
		5	3,200		Poor
90A	10^4	2	0		Good
		3	0		Good
		4	80		Fair
		5	640		Poor

[a] The inoculum was dropped onto the gill or injected into the gill. LD_{50}, Mean lethal dose.
[b] Subjective assessment by two individuals. Good, Acceptable as food; fair, might be used, especially by selecting out the better ones; poor, unlikely to be eaten.

used during December through March, in particular, deteriorated more slowly, so that they appeared good at 4 days and were fair even after 6 days, although toxin had developed by day 4 of incubation. Variable elapsed times between harvest of the product and purchase at the store could be important in determining the time to spoilage.

C. botulinum has been found on fresh mushrooms (2), but the test method was not devised to determine on which part of the mushroom the organisms might be concentrated. Less than 100 *C. botulinum* per 100 g of mushroom were generally enumerated, but the possibility of higher contamination levels cannot be eliminated at the present time. In the inoculated pack experiments reported here, the lowest inoculum level giving rise to toxic mushrooms with some frequency was 10^3 type A spores per mushroom stem. However, there were rare in-

TABLE 4. *Production of botulinum toxin in fresh mushrooms inoculated with C. botulinum spores not heated on the day of injection*

Strain	Spore inoculum		Botulinal toxin identified	
	Spores/mushroom	Site	Type	Time tested[a]
56A[b]	5×10^4	Stem	A	At 4 but not 3 days
	5×10^4	Gill	A	At 4 but not 3 days
62A[c]	10^3	Stem	A	At 6 days only
19A[c]	10^3	Stem	A	At 6 days only

[a] Mushrooms were rated good (56A) and fair (62A, 19A) when toxin was found.
[b] Spore suspensions include those heated (80 C for 10 min) immediately before use and those never heated; results are the same for both.
[c] Spores were heated 30 days before being used to inject mushrooms.

stances, not included in Table 3, of toxin being formed in mushrooms inoculated with 5×10^2 spores.

Compared to type A, higher type B spore inocula were generally needed to produce botulinogenic mushrooms. This difference was unexpected in that type B toxin was the type found consistently in the investigation of the commercially canned mushrooms.

The most important reason for packaging with semipermeable films is to minimize the spoilage rate of the fresh mushrooms. Extension of shelf life when mushrooms are prepackaged is, among other reasons, due to lowered O_2 and increased CO_2 concentrations resulting from the equilibrium between respiration by the mushrooms and the exchange of gases through the packaging film (7). The equilibrium headspace O_2 concentration of 1 to 2% in most of our test packages is consistent with those found by Nichols and Hammond (7), who showed also that the several polyvinyl chloride films they studied resulted in essentially similar equilibrium O_2 concentrations when incubation was at 18 C.

Although reducing substances of mushrooms could contribute to lowering the oxidation-reduction potential to levels compatible for growth of anaerobes (6), the primary reason for toxin production in the packaged, fresh mushrooms is the low O_2 concentration that develops inside the packages. Apparently, *C. botulinum* has O_2 tolerance, enabling it to grow in 1 to 2% O_2, as can some other so-called anaerobes (5).

Packaging with a film that gives a higher equilibrium O_2 concentration as well as acceptable shelf life would avoid the potential botulism hazard indicated by the inoculated pack study of packaged mushrooms held at room temperature. An alternative control method based on the same considerations is suggested by results obtained in preliminary experiments (unpublished data) with mushrooms inoculated in the stem with 10^5 type A spores and then incubated in packages for 6 days at 20 C. These mushrooms did not become botulinogenic if a single perforation of 1/8 inch (3.175 mm) in diameter had been made in the overwrapping film.

In agreement with 10 to 12 C being the generally accepted lowest temperature range at which proteolytic *C. botulinum* types A and B can grow (9), inoculated mushrooms held at 4 C never became toxic. Refrigeration is, therefore, a means to prevent formation of type A and B toxin by proteolytic *C. botulinum*. This temperature alone might not prevent type E *C. botulinum* growth (9), but the higher equilibrium O_2 concentration expected at temperatures closer to 4 C than 18 C (7) could be sufficient to inhibit formation of toxin by this type of *C. botulinum*.

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