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The Relationship Between Botulinal Toxin Production and Spoilage of Fresh Tomatoes Held at 13 and 23°C Under Passively Modified and Controlled Atmospheres and Air

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ABSTRACT

The formation of botulinal toxin relative to spoilage of fresh whole tomatoes was investigated at 13 and 23°C under passively modified (MA) and controlled atmospheres (CA) and air. Tomatoes were subsurface inoculated with a composite of type A and proteolytic and nonproteolytic type B strains of *Clostridium botulinum* spores. Some were also inoculated with *Alternaria* mold spores. MA (1.0-2.9% O_2) was passively established by a combination of product respiration and package permeability. CA was established by placing tomatoes in continuously flushed (1% O_2 20% CO_2, balance N_2) Plexiglass plastic containers. Tomatoes were tested for botulinum toxin by the mouse assay at the time when they first became inedible based on predefined stages of decay rather than specific storage times in order to determine the relationship between spoilage and botulinal toxigenesis. All tomatoes became inedible according to the established criteria within 17 to 46 d depending on the storage temperature and atmosphere. Botulinum toxin was not detected in the 24 composite samples of inedible tomatoes (representing 99 tomatoes) which were tested at the time they first became inedible. Toxin was detected in four of five additional composite samples (representing 10 tomatoes) which were held 2 to 9 d beyond the time they were first determined to be inedible. These data indicate that MA-packaged tomatoes can become toxic but only after becoming severely spoiled beyond the point of being organoleptically acceptable. The risk of botulism from consumption of extended shelf life whole tomatoes appears to be insignificant.

Modified atmosphere packaging (MAP), whether active or passive, can extend the time in which respiring fruits and vegetables remain organoleptically acceptable (*9*). Extension of shelf life through MAP has several positive implications for product quality and marketing and several MAP products have been introduced into retail commerce (*13*).

Unpackaged fresh fruits and vegetables have seldom been implicated in outbreaks of botulism. This is probably due to a combination of factors including the inability of *Clostridium botulinum* spores to germinate and produce toxin at low pH, the presence of O_2, and the inherent rapid spoilage of most unpackaged produce. Packaged unprocessed or minimally processed vegetables have in a few instances been implicated in botulism. Most recently, shredded cabbage that was thought to have been packaged in a modified atmosphere (MA) was implicated as the cause of botulism in people who had eaten coleslaw made from the cabbage. Laboratory experiments confirmed that *C. botulinum* type A spores would grow and produce toxin in cabbage stored in flexible film bags containing 70% CO_2 and 30% N_2 while still remaining edible in the judgment of the investigators (*14*).

Produce with a pH $\leq$4.6 such as tomatoes have not, to our knowledge, been implicated in botulism. However, a pH of $\leq$4.6 is not sufficient to absolutely preclude toxigenesis by *C. botulinum* under all circumstances. *C. botulinum* types A and B have been shown to produce toxin in an optimal medium at a pH of 4.2 (*18*), and under strict reducing atmospheres toxin has been produced at pH values as low as 4.07 in chemically defined media (*19*). However, toxin production has not been demonstrated in acidified foods at such low pH values.

Our concern was that technologies which significantly extend shelf life of even high acid fruits or vegetables could allow sufficient time for pathogen growth in products which would not, with conventional handling, support toxigenesis before becoming organoleptically unacceptable. The major safety consideration in MAP produce is whether or not absolute organoleptic spoilage (i.e., the produce becomes inedible) occurs before or after the food becomes pathogenic.

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The objective of this study was to determine if whole fresh tomatoes packaged under modified or controlled atmospheres and held at temperatures typical of commercial practice would support toxigenesis by *C. botulinum* types A and B prior to becoming organoleptically unacceptable. We first developed an "edibility" rating system as an objective measurement of tomato quality. We then conservatively applied this system to an inoculated sample storage study. We also wanted to know the effect of mold infection on botulinal toxin formation. The results of a separate preliminary study have been reported elsewhere (*6*).

MATERIALS AND METHODS

Experimental design

Inoculated tomatoes were stored at two temperatures (13 and 23°C) and three atmospheric conditions (passively modified atmosphere, controlled atmosphere, and air) for a total of six environments. Each tomato was inoculated in one of the following ways: *C. botulinum* spores only; *C. botulinum* spores and *Alternaria* spores; *Alternaria* spores only, and sterile water only. Tomatoes were sampled at various predefined stages of decay rather than specific time points in order to determine the relationship between spoilage and botulinal toxigenesis.

Tomatoes

The BHN 22 variety of tomatoes used in this study was grown in Mexico, harvested at the breaker stage as defined in the U.S. Grade Standards (*17*), and imported to San Diego, CA. Tomatoes were then shipped overnight to the laboratory (Gainesville, FL) where they were inspected, and ca. 45% selected for use based on the following criteria: weight (170 to 230 g), color (light red to red as defined in ref. *17*), and overall high quality.

Tomatoes were washed gently by hand for 1 min in batches of 50 in pH 6.0 (adjusted with 0.01 M HCl) warm tap water containing 500 mg/L sodium lauryl sulfate and 150 mg/L sodium hypochlorite followed by rinsing for 1 min in tap water containing 150 mg/L sodium hypochlorite (pH 6.0) before inoculation. Rinsed tomatoes were air dried overnight after being covered with absorbent toweling with no tomato-to-tomato contact.

Botulinum and Alternaria *cultures*

The composite inoculum of *C. botulinum* spores consisted of approximately equal numbers of 4 type A strains (33A, 36A, 62A, and 69A), 4 proteolytic type B strains (AccB, 53B, 169B, and 213B), and 2 nonproteolytic type B strains (17B and 2B). Individual washed spore crops were prepared by the biphasic method (*1*) and were stored at 4°C prior to use.

A single species of *Alternaria* was isolated as the predominant mold from tomatoes that had been allowed to spoil at 25°C in air. The purified *Alternaria* mold was propagated on YM agar (Difco Laboratories, Detroit, MI) at 25°C. After 10 d incubation, *Alternaria* spores were harvested from the surface of the agar plates, transferred to sterile 0.85% saline, and the conidial density estimated using a hemacytometer (*2*). This suspension was further diluted with sterile 0.85% saline to 2.5 x 10^4 spores per ml.

Inoculations

Each tomato was inoculated in one of five ways: sterile water only, *Alternaria* spores only, *C. botulinum* spores only, and two types of *Alternaria* and *C. botulinum* coinoculations. Tomatoes stored in MA and in air were inoculated on the same day, tomatoes from another batch to be stored in controlled atmosphere (CA) were inoculated about 1 month later. The 10 individual suspensions of *C. botulinum* spores of known concentration were diluted and pooled to yield a suspension containing 5.0 x 10^5 spores per ml. The pooled suspension was heat shocked at 60°C for 13 min and then cooled in an ice bath. Ten- and 100-fold dilutions were made to yield 5.0 x 10^4 and 5.0 x 10^3 spores per ml suspensions. The three suspensions were used to deliver (in a volume of 0.02 ml) target inocula levels of 10,000 (high), 1000 (medium), and 100 (low) spores at each tomato inoculation site. Three self-adhesive differently colored paper dots were applied at equidistant points along the equator of each tomato to identify the points of inoculation. High, medium, and low inoculations were made by injecting 0.02 ml of each spore concentration at a separate point on each tomato just below the tomato skin surface, and within 2 to 3 mm of the edge of the appropriate dot. Tomatoes injected only with 0.02 ml sterile water or only with 500 *Alternaria* spores at one site on the equator served as negative controls. A fourth group of tomatoes were inoculated with both *C. botulinum* and *Alternaria* spores as described above, except that the *Alternaria* inoculations were made by making three 6 mm long x 6 mm deep surface cuts and flushing each by syringe with a 0.02-ml suspension containing 2.5 x 10^4 mold spores per ml (500 spores). Cuts were made on the tomato's equator within 2 cm of a botulinal spore inoculation site. All injections were made with 0.5-cc sterile insulin syringes fitted with 28 gauge, 0.5-inch needles (Becton Dickinson, Rutherford, NJ).

Ten additional tomatoes were inoculated at three sites with the high target level (10,000 spores) of *C. botulinum* and at one site with 500 *Alternaria* spores. The inoculations were as described above, except that the *C. botulinum* spores were injected near the stem scar, on the equator, and near the blossom scar of each tomato. The *Alternaria* spores were inoculated about 2 cm from the equatorial botulinal spore inoculation site. These tomatoes were stored at 23°C in the MA and served as positive controls based on a smaller preliminary study which indicated that this procedure would yield toxin (*7*).

Three replicate inoculated tomatoes from each inoculation time were reserved for zero time enumeration of *C. botulinum* spores. Actual spore level was estimated by aseptically removing and weighing three wedges (ca. 10 g each) from each tomato encompassing each inoculation site. Each wedge was then diluted 1:10 in sterile deionized water, homogenized, and analyzed by the five-tube, most probable number (MPN) technique using trypticase-peptone-glucose-yeast extract broth (TPGY) without added trypsin. TPGY tubes were incubated (5 d, 35°C) before determination of the presumptive MPN per gram on the basis of turbidity in the lower half of the tube. Turbid tubes from the highest dilutions were randomly selected and confirmed using phase microscopy and testing supernatants for botulinal toxin. The confirmed MPN per gram was used to determine the number of spores at each inoculation site.

Packaging and storage

All samples were stored at 12.3 ± 0.2 or 23.0 ± 0.2°C (i.e., ambient). All packaging materials were supplied by the Dow Chemical Company.

A passively MA was established in the headspace of sealed packages by a combination of package permeability and tomato respiration. Immediately after inoculation, two tomatoes were placed stem-scar down into PVC trays with a packet containing 15 g of NaCl to reduce relative humidity. A rigid PVC insert was placed on an inner lip of each tray just above the tomatoes and a sheet of EVA film heat-sealed over the tray. Tomatoes stored in air were packaged the same as passively MA samples except that 32-mm dia. holes were punched in both ends of the packages to allow gas exchange. CA packaged tomatoes were packaged the same as air stored samples except a CA was established by

placing punctured packages in Plexiglass plastic containers that were continuously flushed (1 L/min) with premixed compressed gas (1% O_2, 20% CO_2, balance N_2).

Headspace gas samples were taken just prior to opening with a 10-cc syringe and analyzed with a Mocon® PG-100 CO_2 analyzer and an Anatop® OXY 211 O_2 analyzer. Samples of gas from the Plexiglass container headspace were tested twice daily (once daily on weekends).

Spoilage evaluation and sample collection

Changes in tomato quality were assessed by rating eight parameters on a scale of 1 to 9 (Table 1). Tomatoes assigned an average rating from 9 to 5 were considered edible, 4 to 2 partially edible, and inedible if any one factor had rating of 1 as defined in Table 1. In practice, several quality factors (Table 1) deteriorated to a score of 1 simultaneously. The odor of each package upon opening was noted and characterized.

Sample collection was based on quality rating and not on a predetermined time schedule. The objective of this protocol was to assay samples of edible, partially edible, and inedible tomatoes at intervals that, in the aggregate, represented the product life span (time from initial high quality to first becoming inedible) under each storage condition. Only MA tomatoes which had $\leq 6\%$ O_2 were tested for toxin so that any packages which had leaks would not be tested. Storage times for tomatoes ranged from 18 to 54 d, depending on storage conditions and the type of inocula.

For each sample set (tomatoes of the same inocula type and storage environment), composite samples were tested for botulinal toxin beginning with tomatoes that had been stored for the longest time (i.e., inedible) and working towards the tomatoes that had been stored for the shortest time (i.e., edible). Samples from the 10,000 spore inoculation sites were tested first, with lower target level spore inoculations available for testing only if samples with higher inocula levels proved positive. When two composite samples from a given time point were negative, no further testing of that sample set was undertaken. The assumption was that if two consecutive samples were negative, younger samples and/or samples inoculated with fewer spores would also be negative.

Toxin assay

Tomatoes to be tested for toxin were removed from the package (after testing the headspace and noting product odor) and transferred to a sanitized plastic cutting board. Tomato wedges (ca. 30 g) encompassing the *C. botulinum* inoculation sites were aseptically removed, placed in separate Stomacher® 400 bags, and hand massaged for approximately 1 min. The pH of the resulting homogenate was measured using an Orion Ross™ combination pH probe. The pH probe was briefly immersed in a 0.1 N NaOH to inactivate any botulinal toxin that might have been present, and then rinsed with deionized water.

The sample was weighed, mixed with an equal amount of sterile gel phosphate buffer (pH 6.2), homogenized (1 min in a TekMar™ Stomacher® 400), and placed in a sterile S/P® diSPO® specimen container. Samples were then stored at -20°C until tested for toxin. Frozen samples were held at room temperature for 1 h and then thawed at 4-7°C for 18 to 24 h. Thawed samples were gently mixed in the sample cup and 8 ml of sample pooled into a 50-ml centrifuge tube along with one to five other samples. The composite was centrifuged at 12,000 rpm for 15 min at 4-10°C and the supernatant used to make three samples for toxin testing (untreated, trypsin treated, and boiled supernatant) as described in the Bacteriological Analytical Manual (5). Pairs of

TABLE 1. *Tomato quality rating guide.*

Rating	9	7	5	3	1
Overeall Quality	Excellent	Good	Fair	Poor	Inedible
% Edible	100	100	75	50	0
Saleability	Saleable	Saleable	Unsaleable	Unsaleable	Unsaleable
General description	No signs of decay. No postharvest blemishes.	Minor signs of decay visible to trained eye, but of little significance to consumer.	Decay evident, but not serious. Decay restricted to areas easily cut away by consumer.	Serious decay. Consumer unlikely to eat any of tomato, but could salvage at least 50% if necessary.	Massive decay. Consumer reluctant to touch tomato.
Mold	None	Fine growh. Visible only to trained eye.	Growth readily detected by consumer, but restricted to no more than two spots.	Lush growth, but limited to 5% of surface area.	Lush growth covers more than 10% of surface area.
Shrivel	None	Visible only to trained eye.	Readily detected by consumer, but resricted to no more than two spots.	Large area (size of half dollar), or several small areas.	Massive shrivel. Dominates view of tomato.
Bruising	None	Visible only to trained eye.	Readily detected by consumer, but resricted to shoulder area or other areas easily cut away. Moderate discoloration.	Serious bruising. Evidenced by heavy discoloration (darkening), or water-logged appearance.	Massive bruising.
Internal decay	None	None	Minor. Restricted to no more than two areas near tomato surface -- easily cut away.	Serious. Evidenced by heavy discoloration or water-logged appearance.	Massive. Evidenced by tissue rupture and leakage of juice.

white mice (ICR strain) were injected intraperitoneally with 0.5-ml samples and observed for up to 96 h for symptoms of botulism and deaths. A sample was considered positive for toxin if at least one of two mice died. Composite trypsin treated and boiled supernatants from positive samples were not lethal.

RESULTS AND DISCUSSION

Mean headspace gas concentrations for passively modified samples stored at 13°C were 2.7% O_2 and 4.9% CO_2 for tomatoes inoculated with *C. botulinum* spores only, and 1.5% O_2 and 8.4% CO_2 for tomatoes inoculated with *C. botulinum* and *Alternaria* spores. At 23°C, headspace gas concentrations were 2.9% O_2 and 5.9% CO_2 and 1.0% O_2 and 11.8% CO_2 for tomatoes inoculated with *C. botulinum* spores only and with *C. botulinum* and *Alternaria* spores, respectively. Mean headspace gas concentrations for CA storage at 13°C were 1.6% O_2 and 19.4% CO_2, and 1.4% O_2 and 19.8% CO_2 for CA storage at 23°C.

Table 2 summarizes the results for samples tested on the day in which they were rated inedible (i.e., one or more quality scores of 1, Table 1). All of the 24 composite samples (representing 99 tomatoes) tested negative for botulinal toxin. Since toxin was not detected in the inedible samples, none of the edible or partially edible (as defined in the Methods section) samples were tested. The average pH for 37/99 samples was ≥4.60 at the end of the storage period. Samples coinoculated with *Alternaria* spores had an average pH of 4.60, while those inoculated with *C. botulinum* only were slightly lower at pH 4.56. Samples incubated in air had an average pH of 4.71, while those in MA and CA had pHs of 4.53 and 4.49, respectively. The odor of most samples at the time of analysis was described as "sour" or "sweet/sour".

Four of five positive control samples were toxic after 26 d of storage under MA at 23°C (Table 3). This was 8 d longer than similarly stored tomatoes tested as soon as they became inedible (Table 2) and 2 to 9 d after the positive control samples were given quality ratings of 1 (i.e., 2 to 9 d after becoming inedible). This additional incubation time yielded tomatoes that were grossly decayed beyond the point of edibility as defined by the quality rating system (Table 1). The combination of a very active microbial population in these samples and variations in packaging led to variability in the gas composition in the packages (Table 3). The appearance of the toxic tomatoes on the day of testing was so unappetizing that we considered the probability of human consumption of tomatoes at this level of deterioration to be remote. Headspace O_2 concentrations for the toxic samples were 1.1-1.6%. The pH of 15/30 tomato wedges comprising the five composite samples was ≥4.60. The odor of the toxic samples was characterized as "strong sour" or "sweet/alcohol" when they were removed from the packages. This suggested that anaerobic fermentation had occurred.

Tomatoes injected with only sterile water or mold spores were not tested for toxin because trypsin-treated and boiled supernatants from positive samples tested negative indicating that the deaths were due to botulinum toxin and not due to the tomatoes themselves nor due to products of mold growth.

TABLE 2. *Results of botulinal toxin tests and mean pH measurements for tomatoes inoculated with* Alternaria *and/or* C. botulinum *and stored under different atmospheres and temperatures.*

No. of *C. botulinum* spores inoculated per site	No. of *Alternaria* spores inoculated per site	Storage temp. °C	Storage atm.	Range of days in storage	Mean pH of samples	No. of toxic/no. tested samples
4100	0	13	air	42-46	4.49	0/5
4100	500	13	air	24-33	4.59	0/8
4100	0	23	air	38-42	4.79	0/7
4100	500	23	air	24-27	4.97	0/6
4100	0	13	MA	42-46	4.63	0/10
4100	500	13	MA	27-28	4.54	0/8
4100	0	23	MA	41-46	4.51	0/5
4100	500	23	MA	17-18	4.44	0/9
12000	0	13	CA	21-40	4.45	0/6
12000	500	13	CA	18-37	4.43	0/11
12000	0	23	CA	13-31	4.46	0/10
12000	500	23	CA	27-31	4.61	0/14

It is widely accepted that the minimum pH at which *C. botulinum* will germinate and grow in foods to produce toxin is 4.7 (*11,16*). In order to provide a margin of safety, the U.S. Food and Drug Administration has prescribed that canned foods with a pH >4.6 must be retorted to inactivate *C. botulinum*. The pH of virtually all tomato cultivars is normally below 4.6 (*3,5*). Individual tomatoes (typically overripe or supporting mold growth) may have pH values greater than 4.6 (*4,8,12*). For all tomatoes evaluated in this study (all storage conditions and inocula types), 4 of 360 (1.1%) wedges from edible tomatoes had pH values greater than 4.6, 49 of 495 (9.9%) of wedges from partially edible tomatoes had pH values greater than 4.6, and 173 of 435 (40%) of wedges from inedible tomatoes had pH values greater than 4.6. Within the subset of inedible tomatoes, 98 of 162 (60.5%) of samples from air storage, 45 of 147 (30.6%) of MA samples, and 30 of 126 (23.8%) of CA samples had pH values greater than 4.6. These data indicate that senescence and decay of tomatoes can raise the pH. It has been demonstrated that molds which are known to produce basic amines enhance the production of toxin, probably by increasing localized pH. For example, *C. botulinum* can produce toxin in tomato juice when there is overgrowth by *Aspergillus gracilis* (*10*). The higher pH of the air-stored tomatoes probably reflects a greater degree of mold growth in this atmosphere compared to the reduced O_2 MA and CA atmospheres.

Several of the positive control samples (Table 3) had pHs <4.6 yet were toxic. Toxigenesis at pHs below 4.6 has been reported previously (*19*). Our pH measurements were

TABLE 3. *Results of botulinal toxin tests, package headspace analyses, composition, and pH measurements of positive control samples (tomatoes inoculated with* C. botulinum *and* Alternaria *and stored under modified atmospheres for 26 d at 23°C).*

Composite sample number	$\%0_2$	$C0_2$	Hold time (d) (a)	pH at inoculation site: Stem	Equator	Blossom	Aroma	Result of toxin test on composite sample
1a	1.6	21.6	9	4.87	4.85	4.73	strong sour	Positive for toxin
1b	1.6	21.6	9	4.72	4.47	4.58	strong sour	Positive for toxin
2a	1.1	12.1	9	4.43	4.45	4.42	strong sour	Positive for toxin
2b	1.1	12.1	9	4.64	4.45	4.32	strong sour	Positive for toxin
3a	1.3	16.6	6	4.82	5.60	4.83	sweet/alcohol	Positive for toxin
3b	1.3	16.6	2	4.42	4.46	4.48	sweet/alcohol	Positive for toxin
4a	6.9	6.8	0	4.63	5.40	4.59	sour	Negative for toxin
4b	6.9	6.8	2	6.20	5.30	4.63	sour	Negative for toxin
5a	1.1	4.1	9	4.60	4.70	4.78	strong sour	Positive for toxin
5b	1.1	4.1	9	4.51	4.44	4.46	strong sour	Positive for toxin

(a) Elapsed time between the day each tomato was assigned a quality rating of 1 (indicating inedibility) and the day the tomatoes were removed from storage for analysis.

made on homogenized samples which may not accurately reflect the pH at the site of mold growth.

Clostridium botulinum is an anaerobe with a low tolerance for O_2. However, as Sperber (*15*) has pointed out, even though *C. botulinum* is an anaerobe, it still may grow in the presence of low amounts of O_2. It is not the composition of the headspace gas that affects growth but rather the Eh of the food. The Eh of many foods stored in atmospheres containing 1-5% O_2 may still be low enough to allow toxin production. This is likely the case in our work. All toxic tomatoes were stored in atmospheres containing at least 1% O_2. The presence of low amounts of O_2 is not sufficient to preclude the formation of toxin, even in high acid products such as tomatoes.

CONCLUSION

The failure of *C. botulinum* to grow and produce toxin before extreme spoilage indicates that tomatoes stored under the modified and controlled atmospheres used in this study do not represent a significant botulism risk. Product abuse in distribution, at retail, or by the consumer would likely accelerate spoilage and therefore represents a fail-safe response with respect to botulism risk. Product abuse that destroys the integrity of the MA package may also be considered fail-safe, since the package headspace becomes aerobic and fresh, unpackaged tomatoes have not been documented to represent a botulism risk. Although the tomatoes themselves supported botulinum toxigenesis, this only occurred several days after the tomatoes passed the conservatively defined end point of becoming inedible. With the exception of this extreme spoilage, no other single factor including initial pH, time, temperature, atmosphere, inoculum level, or mold was sufficient for toxigenesis nor a predictor of toxigenesis.

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