Nonproteolytic *Clostridium botulinum* Toxigenesis in Cooked Turkey Stored under Modified Atmospheres

KATHLEEN A. LAWLOR,* MERLE D. PIERSON, CAMERON R. HACKNEY, JAMES R. CLAUS, AND JOSEPH E. MARCY

Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0418, USA

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ABSTRACT

The ability of nonproteolytic *Clostridium botulinum* type B spores to grow and produce toxin in cooked, uncured turkey packaged under modified atmospheres was investigated at refrigeration and mild to moderate abuse temperatures. Cook-in-bag turkey breast was carved into small chunks, surface-inoculated with a mixture of nonproteolytic *C. botulinum* type B spores, packaged in O₂-impermeable bags under two modified atmospheres (100% N₂ and 30% CO₂:70% N₂), and stored at 4, 10, and 15°C. Samples were analyzed for botulinal toxin and indigenous microorganisms, as well as subjected to sensory evaluation, on days 0, 7, 14, 28, 42, and 60. Given sufficient incubation time, nonproteolytic *C. botulinum* type B grew and produced toxin in all temperature and modified atmosphere treatment combinations. At moderate temperature abuse (15°C), toxin was detected by day 7, independent of packaging atmosphere. At mild temperature abuse (10°C), toxin was detected by day 14, also independent of packaging atmosphere. At refrigeration temperature (4°C), toxin was detected by day 14 in product packaged under 100% N₂ and by day 28 in product packaged under 30% CO₂:70% N₂. Reduced storage temperature significantly delayed toxin production and extended the period of sensory acceptability of cooked turkey, but even strict refrigeration did not prevent growth and toxigenesis by nonproteolytic *C. botulinum*. At all three storage temperatures, toxin detection preceded or coincided with development of sensory characteristics of spoilage, demonstrating the potential for consumption of toxic product when spoilage-signaling sensory cues are absent.

Changing lifestyles and growing consumer demand for freshness, nutrition, and convenience have prompted the marketing of an ever-increasing number and variety of cooked, ready-to-eat, and microwave-ready food products. Many of these items receive only minimal thermal processing, contain no added preservatives, and depend upon refrigeration (4°C or less) as the primary, if not sole, barrier to growth of pathogenic and spoilage microorganisms during product distribution and storage. Because refrigeration temperatures employed by retailers and consumers can often be as high as 15.5°C (10, 27), simply chilling these foods cannot guarantee their safety or stability. Additional antimicrobial barriers are needed to ensure wholesomeness and quality throughout their expected storage life.

Modified atmosphere packaging (MAP) has become a popular means of extending the shelf-life of refrigerated precooked foods, especially meat and poultry products. By replacing the air in the package headspace with CO₂, N₂, or a mixture of gases, MAP can delay the food’s inherent biochemical and microbiological spoilage mechanisms, thereby imparting a preservative effect without the use of chemical additives (considered by many consumers as undesirable). However, by suppressing the growth of normally occurring aerobic spoilage organisms, the combination of O₂ exclusion and CO₂ augmentation used in MAP can encourage proliferation of anaerobic pathogens before evidence of spoilage occurs, thus raising questions about the safety of products packaged and stored under modified atmospheres (8). Of particular concern to processed poultry manufacturers is the potential for growth of psychrotrophic (nonproteolytic) strains of *Clostridium botulinum* in uncured, ready-to-eat products. These strains are capable of producing toxin at temperatures as low as 3.3°C (6, 13, 24–26) without the accompanying signs of putrefactive spoilage used by many consumers to judge whether or not a product is fit for human consumption. Although the incidence of botulinial spores in processed meat and poultry products is quite low (less than 1 spore/kg) (7), the ubiquitous nature of the organism suggests that environmental contamination is, at some point, inevitable. Thus, in the absence of traditional curing agents or back-up preservative systems to prevent toxigenesis during prolonged storage or under conditions of temperature abuse, these products have the potential to become vehicles for the transmission of foodborne botulism.

While many investigators have studied the ability of proteolytic (putrefactive) strains of *C. botulinum* to grow and produce toxin in processed poultry products, few have reported on the behavior of nonproteolytic strains of the organism in such products (15, 16, 20, 21). Where data are available, they have generally been obtained under conditions of moderate to severe temperature abuse (15 to 30°C), using products formulated with multiple combinations of...
antibotulinal agents (e.g., salt, nitrite, sorbate, phosphate, organic acids), exposed to substantial spore challenges (10^3 to 10^6/g), and stored under vacuum. Very few studies have evaluated the behavior of nonproteolytic _C. botulinum_ in products formulated without high levels of traditional preservatives and under conditions more commonly encountered in commercial practice (i.e., low numbers of contaminating spores, and storage under refrigeration [≤4°C] or mild temperature abuse [8 to 12°C]) (15, 16). We are not aware of reports addressing the behavior of nonproteolytic _C. botulinum_ in uncured poultry products packaged and stored under modified atmospheres, where the presence of CO₂ (a known botulinal spore germinant (1, 29)) might actually promote the growth of this anaerobic pathogen. The purpose of the present study was to assess the potential for toxin production in cooked, uncured, low-salt turkey products inoculated with low levels of nonproteolytic _C. botulinum_ spores, packaged under modified atmospheres, and stored for extended periods of time under refrigeration and mild to moderate temperature abuse.

**MATERIALS AND METHODS**

**Spore preparation and maintenance.** Nonproteolytic _C. botulinum_ strains 2B, 17B, and 25765B were obtained from the culture collection of the Department of Food Science and Technology at Virginia Polytechnic Institute and State University, Blacksburg, Va. Spore suspensions of individual strains were prepared at 30°C in Fernbach flasks using a biphasic culture system (2, 3). The liquid phase contained sterile distilled water; the solid phase contained (wt/vol): 5% tryptosate (Difco Laboratories, Detroit, Mich.), 0.5% peptone (Difco), 0.4% glucose (BBL, Cockeysville, Md.), 0.2% sodium thiglycollate (BBL), and 3% agar (BBL). Spores of each strain were harvested by centrifugation at 2,520 × g (4°C/20 min; Sorvall RC-5B refrigerated superspeed centrifuge, DuPont Instruments, Wilmington, Del.), washed in sterile distilled water, centrifuged, and the resultant pellet enzymatically cleaned at 45°C for 2 h in a pH 8.1 K_2 HPO_4 (Fisher Scientific Company, Pittsburgh, Pa.) buffer containing 100 µg/ml lysozyme (Sigma Chemical Company, St. Louis, Mo.) and 50 µg/ml trypsin 1:250 (Difco). The enzyme-treated suspension was centrifuged, washed an additional six times in sterile distilled water, and the final pellet suspended in sterile distilled water. The cleaned spore suspension (~10^9 spores/ml) was subdivided into 2-ml aliquots and stored at −20°C until needed (up to 1 year). Viable spore counts for each strain were determined in duplicate at the time of use by the trypticase-peptone-glucose-yeast extract roll tube method (9).

**Product preparation.** Boneless uncured turkey breasts were vacuum-sealed in ~3.2-kg quantities and cooked for 6 h to an internal temperature of 72.2°C by a commercial processor, using a three-stage (60°C → 71°C → 82°C) high-humidity oven. Immediately following the final process chill step (0°C core temperature), the cook-in-bag breasts were packed in ice-filled coolers (3 ± 1°C) and transported (~2.5 h) to the Department of Food Science and Technology at Virginia Polytechnic Institute and State University. Upon arrival, they were transferred to a cold room (0.5 ± 1.5°C) for cutting and packing. Breasts were aseptically removed from their vacuum cooking bags and carved into chunks ranging in size from 1.9 cm × 1.3 cm × 1.3 cm to 3.8 cm × 1.9 cm × 1.3 cm. Chunks were commingled and distributed in 25-g aliquots into 22.5-cm × 15.5-cm high-barrier (coextruded ethyl-vinyl alcohol) bags (O₂ transmission rate: 3–6 cc/m²/24 h at 40°F and 0% relative humidity; CO₂ transmission rate: 9–16 cc/m²/24 h at 40°F and 0% relative humidity; H₂O vapor transmission rate: 0.5–0.6 g/100 in²/24 h at 100°F and 100% relative humidity [Cryovac Division, Sealed Air, Inc., Duncan, S.C.]), color-coded by treatment variable. To accommodate workflow, product bags (pouches) were held on ice in the cold room until inoculation (~2 h).

Six random product samples were collected throughout the packaging operation for subsequent zero-time physical and microbiological analyses. Proximate analysis results were provided by the supplier.

**Product inoculation and MAP.** A portion of the product samples was reserved prior to inoculation, to serve as a negative, uninoculated control for all treatments evaluated in this study. Each individual product pouch was treated, sealed, inspected, and placed on ice before advancing to the next pouch. The inoculum volume was previously determined, so as not to alter product water activity (a_w). The inoculating pipettor was calibrated before use, and separate, sterile disposable pipet tips were used for each inoculation.

A stock spore suspension (containing approximately equal numbers of each of the three nonproteolytic _C. botulinum_ test strains) was prepared in sterile distilled water immediately prior to inoculation and serially diluted in sterile distilled water to obtain the spore concentration necessary to achieve a target inoculum level of 100 to 500 spores/g of product. This working suspension (inoculum) was heat-shocked at 60°C/10 min (TE45 thermostatic circulating water bath, Neslab Instruments, Inc., Portsmouth, N.H.), cooled, and held in an ice/water bath under constant agitation during inoculation and packaging operations. Actual inoculum spore counts were determined by plating in duplicate onto trypticase-peptone-glucose-yeast extract roll tubes (9) at the start and finish of all inoculation procedures.

Ice-filled product coolers were transferred as needed from the cold room to the laboratory for inoculation. Individual product pouches were removed from the cooler and a total inoculum volume of 200 µl was aseptically dispensed dropwise over the top surface of the product chunks in the pouch (Eppendorf model 4710 adjustable pipettor, Brinkmann Instruments, Inc., Westbury, N.Y.). To ensure uniformity in sample treatment, negative product controls received 200 µl of sterile distilled water in place of the inoculum.

After inoculation, the pouch was gently shaken to distribute its contents evenly. The designated modified atmosphere (100% N₂ or 30% CO₂:70% N₂) was applied via a two-stage vacuum and gas-filling process with impulse sealing (Multivac A300 vacuum sealer, Multivac, Kansas City, Mo.; Smith 180 SCFH proportional tri-gas blinder, Smith Equipment, Watertown, S.D.). The pouch was visually inspected for seal integrity and “pillow pack” dimensions (~15 by 12.5 by 3.5 cm) and briefly held in an ice-filled cooler until all replicate pouches of the same treatment variable were modified atmosphere-packaged (~20 min). The MAP pouches were then removed from the cooler and placed in random, preassigned locations in a preassigned incubator.

**Product incubation and sampling.** MAP product was incubated for up to 60 days at 4, 10, and 15°C in separate Precision Scientific model 815 low-temperature incubators (Fisher Scientific). Incubator temperature was monitored daily using a mercury thermometer (Fisher Scientific) partially submerged in anhydrous glycerol (J. T. Baker Chemical Co., Philadelphia, Pa.), and some variation (+1 to 1.5°C) was observed. At predetermined intervals (0, 7, 14, 28, 42, and 60 days), triplicate samples of each inocu-
lated and uninoculated treatment variable were evaluated for gas production (swelling), headspace gas composition, aroma/appearance, aerobic plate count (APC), modified psychrotrophic plate count (mPSY) (11), lactic acid bacteria (LAB) count, and botulinal toxin production. Any treatment variable testing positive for botulinal toxin on 2 consecutive sampling days was discontinued from the study. Product pH, water activity (aw), and botulinal spore counts were determined on day 0 only.

Analyses of pH, aw, and headspace gases. Product pH and aw were determined on day 0 using six random turkey breast samples collected during the pouch-filling operation. Intact champs (approximately 1.9 cm × 1.3 cm × 1.3 cm) were used for aw measurement (Decagon CX-1 water activity system, Decagon Devices, Inc., Pullman, Wash.). Homogenized samples (25 g turkey + 25 g distilled water, blended for 2 min; Stomacher 400 lab blender, Tekmar Co., Cincinnati, Ohio) were used for pH measurement (Accumet model 610A pH meter with combination electrode, Fisher Scientific).

Headspace gas composition (percent CO2, O2, N2) was determined at every sampling time on triplicate intact packages from each treatment variable (inoculated and uninoculated), using a Fisher Hamilton model 29 gas partitioner (Fisher Scientific), equipped with DEHS/Chromosorb P and molecular sieve 13X columns, a thermal conductivity detector, and a Hewlett-Packard model 3396A reporting integrator (Hewlett-Packard Co., Avondale, Pa.). Helium flow rate was 40 ml/min and cell temperature was 70°C. An Airco BOC blood gas mixture (5.13% CO2:14.92% O2; 79.95% N2; The BOC Group, Inc., Murray Hill, N.J.) was used to standardize the partitioner. Headspace samples (1 cc) were withdrawn through a 1.3-cm-thick foam septum and collected in a 1-cc Gastight #1001 glass syringe (Hamilton Co., Reno, Nev.).

Sensory evaluation. Following headspace analysis, random samples from each treatment condition were assigned random three-digit codes and presented to a three-member untrained sensory panel (immunized with pentavalent botulinal toxoid) for observations related to product acceptability, based collectively on aroma and visual appearance. Sample packages were opened immediately before evaluation, and panelists were asked to judge sensory acceptability using the following five-point category scale: definitely would use, probably would use, might use/might not use, probably would not use, definitely would not use. Sensory unacceptability was determined as that point at which panelists reported they probably would not use or definitely would not use the product.

Microbiological analyses. Nonproteolytic C. botulinum spore counts in triplicate inoculated and uninoculated samples of each treatment variable were determined in duplicate on day 0 only (to verify inoculum levels), using the trypticase-peptone-glucose-yeast extract roll tube method (9).

Following sensory evaluation, each product pouch was briefly kneaded with a cold cylindrical roller to produce a paste suitable for microbiological sampling. At all sampling times, triplicate inoculated and uninoculated samples of each treatment variable were analyzed for APC, mPSY, and LAB. Eleven-gram aliquots of the sample pastes were blended with 99 ml 0.1% peptone diluent (Difco) (2 min; Stomacher 400 lab blender), serially diluted as necessary, and pour-plated in duplicate using standard methods agar (Difco) containing 0.005% (wt/vol) 2,3,5-triphenyltetrazolium chloride (Fisher Scientific) for APC and mPSY, and all-purpose Tween (APT) agar (Difco) containing 0.0032% (wt/vol) bromcesol purple (BCP) dye (Fisher Scientific) (APT+BCP) for LAB. APT+BCP plates were overlaid with approximately 10 ml of APT+BPC prior to incubation. APC plates were incubated at 35°C/48 h; mPSY and APT+BPC plates were incubated at 20°C/72 h. Lenticular, subsurface yellow (acid-producing) colonies on APT+BPC (purple background) were Gram-stained and tested for catalase reaction. Acid-producing, catalase-negative, gram-positive nonspore-forming rods/cocci were presumptively identified as LAB (28).

Mouse bioassay for botulinal toxin. At each scheduled sampling time, triplicate inoculated and uninoculated samples of each treatment variable were analyzed for the presence of botulinal toxin using the mouse bioassay, according to Food and Drug Administration Bacteriological Analytical Manual procedures (12). Three extracts of each test sample were prepared in gelatin phosphate buffer (pH 6.2): (i) untreated, (ii) trypsin-treated (1.8 ml extract + 0.2 ml saturated aqueous trypsin; incubated at 35 to 37°C/1 h), and (iii) heat-treated (1.5 ml extract heated at 100°C/10 min, then cooled). For each sample extract preparation, duplicate male ICR mice (18 to 20 g; Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were each injected intraperitoneally with 0.5 ml of the extract and observed for 72 h for clinical signs/symptoms of, and death resulting from, botulism.

Statistical analysis. Treatments were arranged as a 6 × 2 factorial in a randomized complete block design. All treatments were performed in triplicate, and the entire study was repeated twice. Bacterial counts (CFU/g) were transformed into base-10 logarithms, and sensory ratings were converted from a word scale into numerical values ranging from 5 (definitely would use) to 1 (definitely would not use). Analysis of variance was used to determine if significant (P < 0.05) differences existed between treatments, using the general linear models procedure of SAS Institute (SAS), Cary, N.C. (23). Means were separated using the least significant difference method (SAS), employing a significance level of 0.05. Analysis of botulinal toxin production was performed using a chi-square (2 × k, contingency) test (22, 23), wherein toxic samples were scored as 1 and nontoxic samples were scored as 0.

RESULTS AND DISCUSSION

Product composition. The product used in this study contained 15.2% protein, 79.4% moisture, 0.8% fat, 1.5% sucrose, 1.8% salt, and 0.4% phosphate. Initial product pH was 6.20 and water activity (aw) was 0.951.

Changes in headspace CO2 levels. Mean headspace CO2 levels increased by up to 2% (P < 0.05) in inoculated and uninoculated product during the first week of storage at 15°C (regardless of packaging atmosphere), and there was approximately a 3-log increase in lactic acid bacteria counts (data not shown). There was no further CO2 accumulation by week 2 (the point at which the 15°C treatment variable was terminated), consistent with a leveling-off of lactic populations. Package headspace composition remained unchanged (P > 0.05) over time in the inoculated and uninoculated product stored at 10 and 4°C (regardless of packaging atmosphere), consistent with the failure of low levels of background LAB to proliferate at these temperatures.

Changes in indigenous microorganisms. No indigenous C. botulinum spores were detected in uninoculated turkey at any time during the study. APC of all product samples were ≤10 CFU/g at time zero. Storage temperature
significantly affected ($P < 0.05$) mean APC of uninoculated turkey, producing overall population increases of 4 to 5 logs at $15^\circ C$, but only 1.5 logs or less at $10^\circ C$ or $4^\circ C$ (Fig. 1). In *C. botulinum*-inoculated turkey, no significant differences ($P > 0.05$) in final aerobic population levels were observed between storage temperatures. Packaging atmosphere had no significant effect ($P > 0.05$) on APC of uninoculated product but did significantly affect ($P < 0.05$) aerobic counts of *C. botulinum*-inoculated product. Total aerobic populations of inoculated turkey packaged under $30\%$ CO$_2:70\%$ N$_2$ were 1 to 2.5 logs lower than in inoculated turkey packaged under $100\%$ N$_2$, an effect that became more pronounced as storage temperature decreased. Although competition by *C. botulinum* and other anaerobes may account for a portion of this APC decline, the increased solubility of CO$_2$ at lower temperatures (19) and well-documented inhibitory effect of CO$_2$ against aerobic spoilage organisms (5) is the more likely cause.

Modified psychrotrophic plate counts (mPSY) of all product samples were <100 CFU/g at time zero. Overall levels of LAB in uninoculated turkey packaged under $100\%$ N$_2$ or $30\%$ CO$_2:70\%$ N$_2$ increased approximately 3 logs within the first week of storage at $15^\circ C$, but this was the only significant effect ($P < 0.05$) observed (data not shown). There were no overall changes ($P > 0.05$) in LAB populations in uninoculated product at $10$ or $4^\circ C$ (regardless of packaging atmosphere) or in inoculated product under any treatment condition. This ex-
Table 1. Time to toxin detection and sensory unacceptability in cooked turkey inoculated with a mixture of nonproteolytic type B C. botulinum spores,™ packaged under modified atmospheres, and stored at refrigeration and abuse temperatures

<table>
<thead>
<tr>
<th>Storage temperature (°C)</th>
<th>Modified atmosphere</th>
<th>Days to toxin detection</th>
<th>Days to sensory unacceptability&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Days to toxin detection</th>
<th>Days to sensory unacceptability</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>100% N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>7 (3/3)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7</td>
<td>7 (3/3)</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>30% CO&lt;sub&gt;2&lt;/sub&gt;:70% N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>14 (1/3)</td>
<td>14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14 (2/3)</td>
<td>14&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>100% N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>28 (1/3)</td>
<td>60</td>
<td>60</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>30% CO&lt;sub&gt;2&lt;/sub&gt;:70% N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>28 (1/3)</td>
<td>60</td>
<td>60</td>
<td>42</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean inoculum size: 54 spores/g in trial 1; 43 spores/g in trial 2.

<sup>b</sup> Time to sensory unacceptability, as determined by evaluation of aroma and appearance.

<sup>c</sup> Number of toxic samples/number of samples tested.

<sup>d</sup> Although mean sensory rating was unacceptable, at least one of the three panelists would consume this product.

It is interesting to note that, in both trials, time to sensory unacceptability did not always coincide with product toxicity. This resulted in many instances where product that was actually toxic was rated as acceptable, and therefore considered suitable for consumption. In some cases, sensory panelists did not uniformly regard deteriorative changes in aroma/appearance as necessarily constituting unacceptability. Consequently, product that was assigned a collective sensory panel rating of unacceptable was rated as acceptable by at least one panel member.

The results of this study demonstrate that MAP and refrigerated storage are not sufficient barriers to prevent nonproteolytic C. botulinum growth and toxigenesis in ready-to-eat poultry products formulated without intrinsic antitoxigenic safety factors. Aroma and visual appearance cannot be relied upon to provide consumer protection, because sensory unacceptability does not always coincide with toxigenesis. Background contaminants (such as LAB or psychrophilts) cannot be relied upon to out-compete or inhibit C. botulinum, because both types and numbers of spores, a packaged under modified atmospheres, and stored at refrigeration and abuse temperatures.
indigenous microorganisms can vary considerably from one product lot to another. In order to ensure the safety of refrigerated, precooked poultry products throughout their expected storage life, additional antibotulinum barriers are necessary. Intervention strategies employing lactate and other organic acid salts (14–16), microbially derived bactericidal/bacteriostatic proteins or glycoproteins (bacteriocins) (17), or competitive inhibition and antagonism by lactic starter cultures (18) could provide viable alternatives to the use of traditional preservatives, and would be consistent with consumers’ demands for natural ingredients and processes. Lactic acid and its lactate salts are already Food and Drug Administration-approved and commercially available for use in muscle food products. Nisin, the bacteriocin produced by Lactobacillus lactis subsp. lactis, is Food and Drug Administration-approved for use in processed cheese products. Nisin, egg white lysozyme, and other anti- toxinogenic compounds or organisms in food systems and, ultimately, their safety and stability in commercial practice. Stricter control of refrigeration (<3°C) throughout the retail, food service, and consumer handling chain would also serve to enhance the inhibitory effects of these and other antibotulinum agents (18, 19, 21), and thus reduce the risk of precooked poultry products becoming vehicles for the transmission of foodborne botulism.

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REFERENCES