Growth and Production of Toxin of *Clostridium botulinum* Type E in Rainbow Trout under Various Storage Conditions

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**ABSTRACT**

Rainbow trout (*Oncorhynchus mykiss*) were inoculated with 3 to 4 log₉ spores per g of fish of a mixed pool of four strains of *Clostridium botulinum* type E (Beluga, Minnesota, G21-5, and 070). The trout were vacuum-skin packaged with either oxygen-barrier or oxygen-permeable films. Trout packaged with oxygen-permeable film were stored at 4°C for 21 days, while trout packaged with oxygen-barrier film were stored either at 4°C for 21 days or at 10°C for 15 days. Storage at 10°C was used to simulate commercial temperature abuse. *Clostridium botulinum* outgrowth was determined by a most-probable-number (MPN) method using (tryptone peptone yeast extract glucose trypsin) anaerobic broth. Toxin production was evaluated using a mouse bioassay. Psychrotrophic and anaerobic populations increased with time regardless of packaging type. After 6 days at 10°C, botulinum toxin was detected in the packaged trout; however, the fish was noticeably spoiled before that time. No botulinum toxin was detected in trout packaged with either barrier or permeable films and stored at 4°C for 21 days, although the product was considered spoiled by day 12.

Key words: *Clostridium botulinum*, vacuum-skin packaging, rainbow trout

Packaging methods that provide the consumer with high quality and convenient fish products are desirable in the marketplace (4). In addition, the attractiveness of the package may enhance product sales. Vacuum packaging provides the means to meet these consumer demands. There continues to be concern about the use of vacuum packaging with fresh, refrigerated fish products because of the possible increased risk from the growth and toxin production of *Clostridium botulinum* type E (10). *Clostridium botulinum* type E spores are naturally present in aquatic environments and are capable of growth at temperatures as low as 3.3 to 4°C. It is possible that fish contaminated with *C. botulinum* type E which is vacuum packaged and held under refrigeration temperatures above 4°C may support the production of botulinum toxin. The hypothesis is that in this environment the microorganism may have a competitive advantage over the normal psychrotrophic bacteria that are not able to grow under the reduced oxygen environment inside the package. If this occurs, then some investigators believe that the noticeable spoilage characteristics associated with fish items spoiled by the typical aerobic psychrotrophic bacteria may not be noticeable and a consumer may inadvertently ingest fish containing botulinum toxin (10).

Growth of *C. botulinum* and toxin production in vacuum-packed fish products can be prevented by maintaining the products at a temperature below 3°C during distribution and storage. In some instances, with current commercial practices this may not be done (13). Thus it is important to determine if the production of botulinum toxin in packaged fish at abusive, refrigerated storage temperatures precedes spoilage. If the product is inedible due to spoilage it may not pose a realistic health threat, since the consumer would reject the product. The objective of the present study was to investigate the potential for *Clostridium botulinum* outgrowth and toxin production in vacuum-skin-packaged rainbow trout at two temperatures, 4°C and 10°C, with the natural microflora present. From the data obtained, a decision can be made on whether the risk of toxin production results from the packaging type used or the storage temperature used.

**MATERIALS AND METHODS**

**Spore preparation**

Four strains of *C. botulinum* type E (Beluga, Minnesota, G21-5, and 070) were obtained from the Food and Drug Administration, Washington, D.C., and were maintained at room temperature in reinforced clostridial broth (Oxoid Ltd., Basingstoke, Hants., England). Prior to inoculation of the rainbow trout, spores of each strain were prepared and enumerated by the method of Lindroth and Genigeorgis (17).

**Experimental design**

Rainbow trout (*Oncorhynchus mykiss*) were harvested from the University of Georgia Aquaculture Ponds in Cohutta, GA. Rainbow trout were headed, gutted, washed, and kept on ice for use the following day. Dressed trout were inoculated with a mixed pool of four strains of *C. botulinum* type E spores by dipping them into a Butterfield’s phosphate buffer solution (19) containing 6 to 7 log₁₀ *C. botulinum* spores per ml. After 30 min, the rainbow trout were placed on a sterile screen and the excess liquid was allowed to drain over a 30-min period. The dipping procedure allowed for a target spore load on the rainbow trout of 3 to 4 log₁₀ spores per g of fish. Control samples were dipped in the same way, except the solution did not contain *C. botulinum* spores. Oxygen-barrier...
(oxygen transmission rate: 3 to 6 cm$^3$/m$^2$/24 h/atm at 4°C, 0% RH) or oxygen-permeable (oxygen transmission rate: 930 cm$^3$/m$^2$/24 h/atm at 23°C, 75% RH) films (Trigon Intact skin packaging film), designed for the RM331 Mark III Mini Intact Machine (Trigon Packaging Corp., Redmond, WA) were used to vacuum-skin-package the rainbow trout. Packages were prepared film-to-film with a sealing temperature of 120°C for 20 s. Rainbow trout packaged with oxygen-permeable film were stored at 4°C for 21 days. Trout packaged with oxygen-barrier film were stored at either 4°C for 21 days or 10°C for 15 days. During the 4°C storage of trout vacuum-skin packaged with the oxygen permeable film, samples were analyzed at 3-day intervals. Rainbow trout samples vacuum-skin packaged with the oxygen-barrier film and stored at 4°C were analyzed on storage days 0 and 21. Samples held at 10°C were analyzed on days 0, 1, 2, 4, 6, 10, and 15. For each storage temperature, three replications of both inoculated and control samples were performed.

**Most-probable-number (MPN) procedure for** C. **botulinum**

The 5-tube MPN method using (tryptone peptone yeast extract glucose trypsin) enrichment broth developed by Lilly et al. was used (16). Trypsin (0.25 ml of 0.5% trypsin (Difco 1.250) per ml of extract) was added to the broth to inactivate the bacteriocins produced by nontoxigenic organisms and to aid in isolating C. botulinum type E from mixed cultures (16). Rainbow trout samples ranging from 10 to 15 g were homogenized with a stomacher (Stomacher Lab Blender 400, Tekmar Co., Cincinnati, OH; model #S10-400) and serial dilutions were made with Butterfield’s phosphate buffer (19). Dilutions (1ml) were transferred to the TPYGT broth tubes (9 ml in 16 x 150 mm tubes), and the tubes were incubated anaerobically at 30°C for 48 hours. Calculations to determine MPN counts were made using tables found in the Compendium of Methods for the Microbiological Examination of Foods (18).

**Psychrotrophic enumeration**

Psychrotrophic populations in rainbow trout samples were determined on the initial packaging day and at the intervals stated previously. The serial dilutions made for the MPN enumeration were plated onto plate count agar (Difco Laboratories, Detroit, MI) plates which were incubated aerobically at 4°C for 10 days prior to counting the colonies which developed.

**Toxin analysis**

The procedure to assay botulinum toxin followed the Centers for Disease Control protocol with a slight modification (7). The overnight suspension prepared with equal parts of homogenized rainbow trout and equal parts gelatin phosphate buffer was centrifuged (Sorvall model GLC-l; Newtown, CT) at 3,000 rpm for 30 min. The resulting supernatant was retained and analyzed as outlined in the CDC protocol using male Swiss ICR mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN).

**Spoilage endpoint**

Stored rainbow trout were considered spoiled when the psychrotrophic populations exceeded approximately 6 log$_{10}$ CFU/g and there was an obvious presence of noticeable off-odors. Psychrotrophic populations of this size on food of aquatic origin are often considered a possible indicator of spoiled product (15). Since the product was potentially contaminated with C. botulinum toxin, spoilage detection by organoleptic means other than aroma was not possible for safety reasons.

| TABLE 1. Presence of Clostridium botulinum type E toxin in vacuum-skin-packaged rainbow trout held at 4°C and inoculated with C. botulinum type E spores or left uninoculated (control). Packaging material was an oxygen-permeable film. |
|---|---|---|
| Day | Control, Oxygen-Permeable Film | Inoculated, Oxygen-Permeable Film |
| 0 | 0/3$^a$ | 0/3$^b$ |
| 3 | 0/3 | 0/3 |
| 6 | 0/3 | 0/3 |
| 9 | 0/3 | 0/3 |
| 12 | 0/3 | 0/3 |
| 15 | 0/3 | 0/3 |
| 18 | 0/3 | 0/3 |
| 21 | 0/3 | 0/3 |

$^a$ Number of positive toxin-containing trout samples.  
$^b$ Total number of trout samples tested.

**RESULTS**

Botulinum toxin was not detected in any of the oxygen-permeable film packages during the 21 days of storage (Table 1). The psychrotrophic population increased by 5.4 log cycles and the anaerobic population increased by 3.2 log cycles on rainbow trout vacuum-skin packaged in oxygen-permeable film during storage at 4°C (Fig. 1 and 2). Although the initial size of the psychrotrophic population on the trout was low, the presence of mucus typically present on trout was noticed. The products appeared spoiled between 12 and 15 days of storage based on psychrotrophic populations and the presence of off-odor.

Rainbow trout samples packaged in oxygen-barrier film did not produce botulinum toxin during 21 days of storage. After 21 days of 4°C storage, the psychrotrophic population of the inoculated samples increased by 5.5 log cycles and the anaerobic population increased by 3.4 log cycles. The data for the samples packaged in the oxygen-
The anaerobic population increased by a similar magnitude (5.1 log cycles) on rainbow trout packaged with oxygen-barrier film and held at 10°C. The anaerobic population increased by approximately 4.0 log cycles but spoilage occurred by day 3 of storage (Fig. 3 and 4). Toxin was produced by day 6 on rainbow trout inoculated with C. botulinum and stored at 10°C (Table 2). Inoculated samples were unacceptable for consumption by day 3 of storage based on psychrotrophic populations and off-odor characteristics. The off-odor did not dissipate or fade away after the samples were removed from the packaging film.

**Figure 2.** Enumeration of anaerobic populations in vacuum-skin-packaged rainbow trout samples inoculated with Clostridium botulinum type E spores (+) and uninoculated controls (Q) stored at 4°C. Packaging material was an oxygen-permeable film.

**TABLE 2.** Presence of Clostridium botulinum type E toxin in vacuum-skin-packaged rainbow trout held at 10°C and inoculated with C. botulinum type E spores or left uninoculated (control). Packaging material was an oxygen-barrier film.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control, Oxygen-Barrier Film</th>
<th>Inoculated, Oxygen-Barrier Film</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>1</td>
<td>0/3</td>
<td>0/3</td>
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<tr>
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<td>3/3</td>
</tr>
<tr>
<td>15</td>
<td>0/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>

* Number of positive toxin-containing trout samples.
* Total number of trout samples tested.

**DISCUSSION**

In this experimental design the normal microflora present on the rainbow trout was not reduced or eliminated by treating the trout samples with either 70% ethanol dippings or autoclaving. Many previous investigations have used such treatments to modify the product so that the background microflora was not present to compete with C. botulinum after it had been introduced. With no competitive microflora present, production of botulinum toxin might precede detectable spoilage. By not altering the background microflora, a more realistic interaction between natural background microflora and the C. botulinum spores can occur in vacuum-skin-packaged fish products. In addition to having the normal background microflora present on the trout, variables studied in this investigation were vacuum packaging and temperature effects.

The findings of this study, which pointed out that detectable spoilage preceded botulinum toxin production, are supported by several investigators who have found that...
"generally" spoilage was apparent before 
Clostridium botulinum
toxigenesis occurred in vacuum-packaged raw fish products held below 10°C (3, 8, 11, 12). However, other investigators have speculated that toxin production by 
Clostridium botulinum may precede organoleptic spoilage in fish samples that have been vacuum packaged (2, 8, 9, 10, 11, 14, 17). The results possibly differ because samples were prepared differently, interfering with the natural background flora on the fish and thus influencing the findings. Based on these contrasting results, it can be concluded that spoilage should not be used as the sole indicator of toxigenesis in vacuum-packaged fish products (2).

This inoculated-pack study indicates that temperature appeared to have a greater impact on the amount of spoilage and toxin production in vacuum-packaged rainbow trout than the packaging type used. These results indicate that the shelf life of vacuum-skin-packaged trout can be extended without the risk of 
Clostridium botulinum toxigenesis if the temperature is maintained at or below 4°C. This study confirmed the results obtained by several investigators, that abusive time and temperature storage conditions of fish products more significantly affected toxigenesis than the type of packaging material used (1, 2, 5, 6, 12).

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