Challenge Studies with *Clostridium botulinum* in a Sous-Vide Spaghetti and Meat-Sauce Product

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ABSTRACT

Challenge studies were carried out to evaluate the safety of a reformulated sous-vide processed spaghetti and meat-sauce product (a0 0.992-0.972, pH 4.5-6) inoculated with *Clostridium botulinum* types A and B spores. Following processing at 75°C for 36 min (equivalent to a 13 D process for *Streptococcus faecium*), samples were stored at 15°C and analyzed at selected time intervals for toxin production and visible signs of spoilage. Toxin was detected in samples of pH 5.5 after 14-21 days and in products of pH 5.25 after 35 days. Toxin was not detected in any samples of pH < 5.25 within 42 days storage at 15°C. All products of pH 5.75 and 6 were visibly spoiled, i.e., swollen due to CO2 production, prior to toxigenesis. However, for products of pH 5.5 and 5.25, toxigenesis preceded spoilage. Subsequent studies were done to test the effect of additional salt (1-3% w/w) on the safety of a product of pH 5.5 at mild temperature-abuse storage conditions (i.e., 15°C). Toxin production was not delayed in samples containing < 1.5% added salt, while > 1.5% salt (w/w) prevented toxin production throughout the 42-day storage period at 15°C. Microwave heating of products for 5-10 min at full or half power in a domestic microwave (800 watts) inactivated the preformed toxin in all samples.

Key words: Sous-vide, spores, *C. botulinum*

Over the past few years, there has been a tremendous increase in minimally processed vacuum-packed (sous-vide) refrigerated foods on the market as a result of consumer demands for foods that offer greater convenience and time savings in preparation. Experts predict that these minimally processed refrigerated foods will replace about 25% of the canned and frozen foods in supermarkets within the next 10 years, due to their fresher taste and the fact that they are preservative-free (9).

“Sous-vide” means “under vacuum”; it describes a processing technique whereby freshly prepared foods are vacuum sealed in individual packages and subsequently pasteurized at time/temperature combinations sufficient to destroy all vegetative pathogens (20, 26). Under good manufacturing practices, sous-vide processed products have a shelf life of 20 to 30 days when stored at refrigeration temperatures (25, 32). However, regulatory authorities recognize that minimally processed foods may be a potential public-health risk due to the growth of food-borne pathogens which may survive the heat process (2, 4).

The heat treatment applied in sous-vide processing results primarily in the destruction of vegetative cells, while spores, if present, will often survive the mild heat-processing conditions. Of most concern in such commercially processed foods is the survival and growth of *C. botulinum*. Sous-vide products contain sufficient nutrients, low salt levels, high pH, and an oxygen-free environment conducive to the growth of and toxin production by *C. botulinum*.

The *C. botulinum* species is very heterogeneous, and two main groups causing human botulism are recognized: viz., group I, the proteolytic types A, B, and F; and group II, the non-proteolytic types B, E, and F. The proteolytic strains have a minimum growth temperature of 10°C, are more acid tolerant and produce very heat-resistant spores (14, 30). Therefore, if sous-vide foods are stored at temperatures higher than 10°C, there is a risk of growth of *C. botulinum* with concomitant production of toxin. Also, if the heat treatment is insufficient to destroy group II spores, even proper refrigeration may not be sufficient, as these strains may grow at 4°C.

The purpose of this study was to determine the risk of survival of, and toxin production by, proteolytic strains of types A and B spores in a sous-vide spaghetti and meat-sauce product at mild temperature-abuse storage conditions. The effects of additional barriers (salt and pH) to control the growth of *C. botulinum* in the minimally processed product were also studied, as well as the effect of microwave heating on the preformed botulinum toxin.

MATERIALS AND METHODS

Production of spores

The strains of *C. botulinum* used were proteolytic types A6, 62A, 17A, 317121A, CK2-A, MRB, 1B, 13983-IIB, 368B, and 426B, obtained from the Bureau of Microbial Hazards, Health Protection Branch (HPB), Health and Welfare, Ottawa. Spore suspensions of the individual strains were prepared by growing the strains in trypticase peptone glucose yeast-extract (TPGY) broth at 35°C for 10 days according to methods outlined by Health and Welfare, Canada, Ottawa (16). Spores were harvested with sterile distilled water, centrifuged at 17,500 x g for 20 min at 2 to 5°C, resuspended in gelatin-phosphate buffer (pH 6.6) and heat-shocked at 80°C for 10 min. The number of spores/ml in each suspension was

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enumerated on Wynne’s agar (Difco, Detroit, Michigan) as outlined by Hauschild and Hilsheimer (15). For inoculation, spores were pooled from each strain into a gelatin-phosphate buffer to give a final concentration of 10^9/ml spores.

**Product inoculation and packaging**

The spaghetti and meat-sauce product was prepared by adding 200 g of previously cooked spaghetti to 175 g of commercially produced meat sauce. The spaghetti with meat sauce was weighed directly into a pre-tared stomacher bag and inoculated with 0.5 ml of prepared spore inoculum to give a final inoculum of 10^3 spores/g of sample. The contents of the bags were then thoroughly massaged by hand to mix the sample. The mixture was then carefully transferred into a thermoformed oriented polypropylene tray and vacuum packaged with a top film of Wallo peel (polyester/polyamide/EVOH, O2 transmission rate, 12 cc/m²/atm at 23°C) using a vacuum skin/thermoforming packaging system (Trigon Model # RM331 MMIS Inc., Ontario, Canada).

**Effect of pH and salt**

The effects of pH and salt on the growth of, and toxin production by, *C. botulinum* were investigated in reformulated products. Samples were adjusted to various pH levels (5.0, 5.25, 5.5, 5.75, and 6.0) by adding appropriate amounts of 1.0 N NaOH to the meat sauce. The products were then inoculated with *C. botulinum* types A and B spores, packaged, heat processed, stored at 15°C and assayed for toxin.

The pH of each sample was measured in duplicate by inserting the probe of a calibrated Acumet pH meter (Fisher Scientific, Montreal) directly into the samples.

The effect of salt was studied at pH 5.5 only. Appropriate amounts of salt were added to the meat sauce to give final salt concentrations of 0.5, 1, 1.5, 2, 2.5, and 3%, w/w. The a_w of the final products was measured using a Decagon water-activity meter (model CX-II, Hotpack Refrigerated Bath Circulator, Canada Inc., Waterloo, Ontario). The salt concentration was measured in the reformulated products using a Horiba compact salt meter C-121 (Horiba Instruments Inc., Irvine, CA) previously calibrated with 1% and 5% standard NaCl solutions.

**Thermal processing and sampling of product**

The prepared samples were processed at time/temperature conditions previously determined by Simpson et al. (29) to achieve a 13 D (decimal) reduction in *S. faecium*, i.e., heating in a water bath maintained at 75°C for 36 min. Products were then cooled rapidly to 4°C, and stored at 15°C for up to 42 days. Triplicate samples were withdrawn and analyzed for toxin after the first 14 days and subsequently at 7-day intervals until the end of the storage period.

**Toxin assay**

At each sampling time, 20 g of sample from triplicate packages in each treatment were weighed into stomacher bags with 20 g of gelatin-phosphate buffer, homogenized in the stomacher for 1 min, and centrifuged at 17,500 x g for 20 min. The supernatant was then filter-sterilized and 0.5 ml samples were injected intraperitoneally in duplicate into mice (20–25 g) and the animals were observed for 3 days for symptoms of botulism (pinched waist, laboured breathing and/or death).

To confirm the presence of *C. botulinum* toxin, randomly selected samples that killed mice were retested with antitoxin. For neutralization and toxin typing, 0.4 ml of botulinum antiserum A and B were added to 1.4 ml of a 1:1 dilution of the supernatant, and left at room temperature for 45 min, and then injected into mice and monitored as described previously for symptoms of botulism.

The time until toxin production was defined as the earliest time at which toxin was detected in any given specific treatment. For example, if for a specific treatment one or two out of three samples were toxic on day 14 and then the same fractions were again toxic after 21 days in another set of replicates, the reported time until toxin production was recorded as day 14.

**Sensory evaluation**

Samples were visually examined for swelling due to gas production, as well as changes in color and odor. For unswollen packages, the presence of toxin was used to indicate spoilage.

**Headspace gas analysis**

Samples which were found to be swollen were analyzed for headspace-gas composition by withdrawing gas samples using an 0.5 ml gas-tight syringe (Precision Sampling Corp., Baton Rouge, FL) through silicone seals attached to the outside of each package. Headspace gas was analyzed with a Varian gas chromatograph (Model 3300, Varian Canada Inc.), fitted with a thermal conductivity detector and using Porapak Q with molecular sieve 5A (80–100 mesh) columns in series. Helium was used as the carrier gas at a flow rate of 20 ml min⁻¹. The column oven was set at 80°C. The detector and detector were set at 100°C. Peaks were recorded and analyzed with a Hewlett-Packard integrator (model 3930A, Hewlett-Packard Co., Avondale, PA).

**Calculation of probability**

The number of toxic samples was converted to the most probable number (MPN) of spores able to initiate growth and toxin production in each set of conditions. The MPN was calculated from the Halvorson-Ziegler equation (11) as: ln (n/q), where n is the number of samples analyzed and q the number of nontoxic samples as described by Hauschild (13).

The probability of toxigenesis from a single spore was then defined as P(%) = (MPN x 100)/10^w', where w' is the number of samples analyzed and 10^w' the number of spores/g.

When no samples were toxic at 15°C, P(%) was taken as 10⁻⁴ (8). The P(%) values calculated were plotted against storage time, a_w, or pH. Using the predictive equations and taking log P for the first day toxin production was detected in a sample and equating this to the actual observed day, predicted time of toxin production was calculated for the various values of a_w and pH.

**Statistical analyses**

The effects of the experimental factors (pH, a_w) on P(%) and on the time until first toxin production (lag time) were further investigated by a simple regression technique which was used to select the best model for predicting P(%) and lag time. All statistical calculations were performed using the general linear models procedure of the STAT graphics.
TABLE 1. Time of botulinum toxin production in formulated sous-vide spaghetti and meat-sauce product stored at 15°C as influenced by pH and water activity (aw).

<table>
<thead>
<tr>
<th>Barrier Used In Sample</th>
<th>Days to Toxin Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Effect of pH</td>
<td></td>
</tr>
<tr>
<td>Control (pH 4.4)</td>
<td>&gt;42</td>
</tr>
<tr>
<td>pH 5</td>
<td>&gt;42</td>
</tr>
<tr>
<td>pH 5.25</td>
<td>35</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>21</td>
</tr>
<tr>
<td>pH 5.75</td>
<td>21</td>
</tr>
<tr>
<td>pH 6</td>
<td>14</td>
</tr>
<tr>
<td>B. Effect of salt or aw</td>
<td></td>
</tr>
<tr>
<td>[NaCl]</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>0.992</td>
</tr>
<tr>
<td>1.0%</td>
<td>0.986</td>
</tr>
<tr>
<td>1.5%</td>
<td>0.983</td>
</tr>
<tr>
<td>2.0%</td>
<td>0.976</td>
</tr>
<tr>
<td>2.5%</td>
<td>0.975</td>
</tr>
<tr>
<td>3.0%</td>
<td>0.972</td>
</tr>
</tbody>
</table>

1 pH of all samples was 5.5; initial salt content of commercial product determined as 0.5%.

Figure 1. Effect of pH on the observed probability of toxigenesis by one C. botulinum spore in sous-vide spaghetti and meat-sauce product incubated at 15°C for up to 42 days.

Figure 2. Effect of aw on the observed probability of toxigenesis by one C. botulinum spore in sous-vide spaghetti and meat-sauce product (pH 5.5) incubated at 15°C for up to 42 days.

Microwave heating

Samples in which toxin was detected were individually microwave heated in their original containers (opened, but contained in another bag to prevent moisture loss) at either full or half power for 5 or 10 min, using a conventional domestic microwave oven (Kenmore brand, Sears Canada) with 800 watts power. Immediately after microwave heating, the temperature of each product was measured at five spots using a calibrated digital thermometer. Products were then re-examined for the presence of toxin as described previously.

RESULTS AND DISCUSSION

This work extends previous studies on the microbiological changes that occurred in a sous-vide spaghetti and meat-sauce product which indicated the survival of spores and heat resistant lactic acid bacteria (29). The products used in this study were similar in formulation to previously prepared products and the commercially produced product in terms of their approximate composition, water activity, and pH. However, it was necessary to raise the initial pH of the product used in this study from 4.4 to 5.5, i.e., the average pH of the samples used in earlier thermal processing/storage studies. The variations in the pH values were attributed to changes in the production/formulation of the commercially produced meat sauce, e.g., different batches of tomatoes.

A storage temperature of 15°C was used to represent mild temperature abuse, since surveys of retail cases in supermarket and domestic refrigerators indicated that 20% exceeded temperatures of 10°C (7,12,36).

The inoculum level of C. botulinum used in this study was substantially higher than contamination levels which have been found in either fresh or processed meat products. Several authors have shown that the concentration of spores range from 0.00004 to 0.00167/g in raw and processed meat products (1,10,1418,33). Therefore, the inoculum level and the storage conditions used in this study represent the worst case scenario for this kind of sous-vide product.

It is evident that the probability of toxigenesis increased with storage time, but decreased as either the aw (increased salt concentration) or pH was decreased (Table 1, Figs. 1, 2). The effect of pH on the growth of and toxin production by

TABLE 2. Predicted time until toxin production and actual time observed during storage at 15°C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Model No.</th>
<th>Days Until Toxin Production Predicted</th>
<th>Days Until Toxin Production Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.0</td>
<td>1</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>pH 5.75</td>
<td>1</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>1</td>
<td>21.3</td>
<td>21</td>
</tr>
<tr>
<td>pH 5.25</td>
<td>1</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>aw (0.992)</td>
<td>2</td>
<td>25.5</td>
<td>21</td>
</tr>
<tr>
<td>aw (0.986)</td>
<td>2</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>aw (0.983)</td>
<td>2</td>
<td>51</td>
<td>42</td>
</tr>
</tbody>
</table>

a $l/log P = 0.107779 - 0.0784 pH (r^2 = 0.91)$
b $l/log P = 4.78373 - 5.12605088 aw (r^2 = 0.95)$
C. botulinum is shown in Table 1, and the corresponding observed probability of toxigenesis by one C. botulinum spore is shown in Fig. 1. Toxin was not detected in any control samples (pH 4.4 or pH 5.0) at day 42 of storage at 15°C while toxin was detected in samples at pH 6 as early as day 14 for the same storage temperature. For products reformulated to pH 5.5 or 5.75, toxin was detected at day 21, while in the products at pH 5.25, toxin was not detected until day 35 at 15°C.

Products reformulated to pH 6 and 5.75 were visibly spoiled, i.e., swollen, at days 14 and 21 respectively, when toxin production was detected. Swelling was due to headspace CO₂ production: this was similar to spoilage profiles of lactic acid bacteria (LAB) in previous shelf-life studies of the product (unpublished data), although gas produced by C. botulinum could also contribute to the swelling. However, of most concern was that there was no swelling or discoloration in samples of pH 5.25 and 5.5, when toxin was detected i.e., toxigenesis preceded spoilage.

The minimum pH requirement for growth of proteolytic C. botulinum is in the range 4.6–4.8, although for many strains it may be well over 5.0 (14, 24, 34). The results of this study confirm the effect of pH in controlling toxin production by proteolytic C. botulinum types A and B spores in minimally processed sous-vide products with pH < 5.0.

However, products with a pH > 5.0 could obviously pose a public-health hazard, and additional barriers, e.g., aₗ reduction, are necessary in medium-acid products (pH 5–6) to prevent the growth of and toxin production by C. botulinum, particularly at mild temperature-abuse conditions.

To test the effect of an additional barrier, salt concentration, on toxin production by C. botulinum, the spaghetti and meat-sauce product was reformulated to contain an additional 1–3% salt (Table 2). A pH of 5.5 was chosen since (1) toxigenesis preceded spoilage in these products, and (2) the average pH of the commercially produced sous-vide pasta/meat-sauce products was 5.3 ± 0.2.

The effects of decreasing aₗ through variation in salt concentration on toxin production in products reformulated to pH 5.5 (original salt content 0.5%) is shown in Table 1 and Fig. 2. Addition of salt to the reformulated product decreased its aₗ from 0.992 (control) to 0.972 for products containing an additional 3% salt. This additional salt and reduction in aₗ had a significant effect on toxin production by C. botulinum. In control samples (pH 5.5, no added salt), toxin was detected at day 21, while in product containing an additional 1% salt, toxin was not detected until day 28 when samples were stored at 15°C. However, at salt concentrations greater than 1.5%, toxin was not detected in any samples throughout the 42-day storage period at 15°C. The results of this study confirm the importance of salt (NaCl) in controlling food-borne C. botulinum. The decrease in water activity obtained by adding salt and the delay in toxin production at higher salt levels is consistent with previous observations (3, 5, 31). These authors reported minimum water activities of 0.94–0.96 for C. botulinum types A and B spores in NaCl-containing foods media. Although a lower minimum water activity was observed for growth of and toxin production by C. botulinum in NaCl-adjusted substrates, the inhibitory effect of lower salt concentrations, and hence slightly higher aₗ values, in this study can be attributed to the combined synergistic effect of reduced aₗ, pH, and storage temperature (3, 21, 22, 28).

Mathematical modeling has been used in several studies to assess the safety of food products with respect to C. botulinum (8, 17, 19). In this study, a logarithmic transformation of the probability data resulted in a better fit. The relationship between the time until first toxin production (lag time) and the variables (salt, aₗ and pH) were determined in a similar manner. Only terms which were statistically significant by analysis of variance were included in the models. The resulting models and the predicted time until toxin production, as well as the actual time observed, are shown in Tables 2. A good correlation was observed between predicted and observed times until toxin production. These findings are consistent with observations made by previous investigators that mathematical models can be used to quantify the risk of C. botulinum growth in foods affected by different food and environmental parameters (6, 8, 13, 17, 19).

Several methods have been examined for inactivating botulinum toxin in foods without destroying nutrients or the organoleptic qualities of the products. These include heating and freezing (27). The consumer appeal of many ready-to-eat convenience foods, such as sous-vide products, is that these products can be reheated quickly by microwave energy. However, there is a paucity of data on the effect of microwave energy on the stability of preformed toxin in sous-vide products, especially products which appear organoleptically acceptable to the consumer. The effect of microwave-heating toxic sous-vide products in a domestic microwave (800 watts power) for either 5 or 10 min at both full power and half power was investigated. No attempt was made to measure the initial or final concentration of toxin prior to or after microwave heating. However, it was evident that microwave energy denatured preformed toxin in the sous-vide spaghetti and meat-sauce product. No toxin was detected after microwave-heating toxic samples of pH 5.25, 5.5, 5.75, and 6.0 at either full or half power for either 5 or 10 min. The internal temperature of the microwave-heated products measured by inserting a digital thermometer directly into the products after microwave heating showed that product temperature ranged from 95–100°C in products heated at full power for 5 and 10 min respectively and from 85–99°C for products heated at half power for the same time periods. These results confirm previous studies which showed that C. botulinum types A and B toxins can be inactivated at 60–85°C for time periods up to 20 min (35). However, the complete inactivation of toxin after microwave heating for 5 min is contrary to the observations of Notermans, Dufrenne, and Lund (23) who reported that complete inactivation of preformed toxin in products required heating in a domestic microwave for at least 10 min. The differences in these results may be attributed to the lower power of the microwave, i.e., 700 watts versus 800 watts used in the two studies. It may also relate to differences in the level of inoculum. In their study, Notermans, Dufrenne, and Lund (23) used levels of 10⁶ spores/g sample as compared to 10⁵/g used in this study. If the latter is the case, then it may be inferred that higher spore levels probably result in higher toxin production, and hence the degree of inactivation achieved would depend on the duration of the heating time.

In conclusion, the study indicates that both pH and salt exert antimicrobial/antibotulinal effects, resulting in inhibition of growth and toxin production by C. botulinum types A and B spores in sous-vide spaghetti and meat sauce. Since a worst-case
scenario was presented in this study, it would be expected that if lower levels of *Clostridium botulinum* spores are present in the raw ingredients used in formulation of sous-vide products, a reduction in pH or aₙ (through the addition of salt) could be used either alone, or in combination, to inhibit the growth of clostridial spores. Although microwave heating inactivated preformed toxins in the product, it will not be judicious to recommend it as a safety factor to reduce the botulism risk in minimally processed, microwave-heated food products. This can best be achieved through proper refrigerated storage of products and/or product reformulation to suitable aw and pH levels to ensure the public-health safety of products subjected to temperature abuse at any stage of their storage and distribution.

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