Predictive Model Describing the Effect of Prolonged Heating at 70 to 80°C and Incubation at Refrigeration Temperatures on Growth and Toxigenesis by Nonproteolytic *Clostridium botulinum*

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**ABSTRACT**

There is growing interest in the food industry in the use of long heat treatments in the range of 70 to 90°C to produce minimally processed foods that have an extended shelf life at refrigeration temperatures. The risk of growth and toxin production by nonproteolytic *Clostridium botulinum* in these foods is of concern. The effect of heat treatments at 70, 75, 80, 85, and 90°C combined with refrigerated storage for 90 days on growth from 10⁶ spores of nonproteolytic *C. botulinum* (types B, E, and F) in an anaerobic meat medium was studied. The following heat treatments prevented growth and toxin production during 90 days provided that the storage temperature was no higher than 12°C: 75°C for ≥1,072 min, 80°C for ≥230 min, 85°C for ≥36 min, and 90°C for ≥10 min. Following heating at 70°C for 2,545 min and storage at 12°C, growth was first observed after 22 days. A factorial experimental design allowed a predictive model to be developed that described the incubation time required before the first sample showed growth as a function of heating temperature (70 to 80°C), period of heat treatment (up to 2,545 min), and incubation temperature (5 to 25°C). Predictions from the model provided a valid description of the data used to generate the model, and agreed with observations made previously.

Key words: Predictive model, *Clostridium botulinum*, heat treatment, REPFEDs

In the last few years the chilled-food market has experienced a tremendous growth, with a wide variety of products available to the consumer through retail and catering outlets. New packaging and processing techniques have been designed to maintain sensory and microbiological quality for extended periods. One group of chilled products, refrigerated processed foods of extended durability (REPFEDs), such as *sous vide* foods, receive a mild heat treatment and are then stored at a refrigeration temperature (15, 16). They are often packaged under vacuum or modified low-oxygen atmospheres to extend their shelf life. This packaging restricts the growth of aerobic spoilage organisms while creating conditions favoring growth of anaerobic microorganisms.

*Clostridium botulinum* produces an extremely powerful neurotoxin, and the consumption of foods in which this pathogen has grown can lead to severe illness or death. Unlike proteolytic strains, nonproteolytic strains of *C. botulinum* (types B, E, and F) are capable of growth and toxin production at temperatures as low as 3.3°C (8, 9) and, although spores are readily inactivated when heated at 100°C, they may survive milder heat treatments (16). Nonproteolytic strains of *C. botulinum*, therefore, represent a potential hazard for these minimally processed foods. It has been recommended, in Europe, that for chilled foods with a risk of growth of nonproteolytic *C. botulinum* (e.g., foods with a high pH or a low salt concentration) the heat treatment applied should give a 10⁶ reduction of viable spores of nonproteolytic *C. botulinum*, and that this would be achieved by a heat treatment at 90°C for 10 min or an equivalent treatment (1, 10).

The food industry has shown considerable interest in the use of equivalent heat treatments (i.e., longer heat treatments at lower heating temperatures) rather than 90°C for 10 min. A longer heat treatment at a lower heating temperature may maintain sensory and organoleptic quality in products where their characteristics would be adversely affected by heating at a higher temperature for a shorter time. Temperatures of 70 to 90°C are considered to form the practical range of most cooking regimes. Several committees and advisory bodies have written guidelines recommending appropriate heating times at temperatures ranging from 70 to 90°C. The different guidelines are based on different experimental data, and there is some variation in the recommendations made. The European Chilled Food Federation (ECFF) (10) recommended that the heating periods necessary to produce a 10⁶ reduction of viable spores of nonproteolytic *C. botulinum* at 80, 85, and 90°C were 270.3, 51.8, and 10.0 min respectively. A z value of 7°C was used...
The Advisory Committee on the Microbiological Safety of Food (ACMSF) (1) provided guidelines for temperatures between 70 and 90°C. The recommended heating periods to produce a 10^6 reduction in spores of nonproteolytic C. botulinum at 70, 75, 80, 85, and 90°C were 1675, 464, 129, 36, and 10 min respectively. A z value of 9°C was used (1). The French Ministry of Agriculture published recommendations to regulate the extension of shelf life of ready-to-eat meals (17, 18). For products with a shelf life of up to 21 days, a heat treatment equivalent to 100 min at 70°C was recommended. To take possible risks into account the manufacturer was required to verify that these products complied with microbiological criteria after storage at 4°C for 14 days followed by 8°C for 7 days. For products with a shelf life of up to 42 days followed by 8°C for 14 days.

The measured heat resistance of spores of nonproteolytic C. botulinum is influenced by factors such as strain, heating menstruum, and particularly the presence of lysozyme during recovery (15, 16). Generally, $D_{32.2^\circ C}$ has been reported to be less than 2 min when spores have been recovered in the absence of lysozyme (15). When spores were recovered in the presence of lysozyme, their measured heat resistance was considerably greater (20). Sufficient lysozyme activity also remained following heating at 90°C for 19.8 min to increase the measured heat resistance of spores of nonproteolytic C. botulinum (21).

Mathematical models have been published to describe the effect of environmental factors on growth from unheated spores of nonproteolytic C. botulinum. Baker and Genigeorgis (2) published a model to predict the lag time of nonproteolytic C. botulinum in various fish homogenates as a function of incubation temperature (4 to 30°C), modified atmosphere (70% CO₂/30% N₂ or 100% CO₂) and inoculum (10⁻² to 10⁴ spores/sample). This model provided a good description of growth from unheated spores of nonproteolytic C. botulinum in foods (3), and incubation temperature was the most important factor affecting time to toxin production (2, 4). Graham et al. (11) produced a model that predicts the combined effect of pH (5.0 to 7.3), NaCl concentration (0.1 to 5%) and temperature (4 to 30°C) on growth from unheated spores of nonproteolytic C. botulinum. Predictions from this model compared well with observations of growth in foods in many studies (11). The time required for growth from heated spores is likely to differ to that from unheated spores as a proportion of heated spores may be inactivated and others may be sublethally injured, affecting their ability to germinate and grow at low temperatures.

The combined effect of heat treatment and storage temperature on growth from spores of nonproteolytic C. botulinum has been tested in a model food. Treatments equivalent to heating at 65°C for 364 min, 70°C for 8 min, and 75°C for 27 min had little effect on growth and toxin formation compared with unheated spores. For example, growth at 25°C was observed after 1 day (22). After treatments equivalent to heating at 80°C for 23.3 min, 85°C for 19.0 min, and 90°C for 19.8 min, growth at 25°C was observed after 3 and 8 days, but not after 90 days, respectively (21) (Peck, Fairbairn and Lund, unpublished data).

Although models have been developed to predict growth from unheated spores of nonproteolytic C. botulinum at refrigeration temperatures, models have not been developed that describe the combined effect of heat treatment and incubation temperature on growth and toxin production from heated spores of nonproteolytic C. botulinum. The aim of this work was to investigate the effect of long periods of heat treatment and incubation temperature on growth and toxin production and to use this information to produce a mathematical model that describes growth and toxin production by nonproteolytic C. botulinum as a function of heat treatment and incubation temperature.

**MATERIALS AND METHODS**

**Bacterial cultures and spores**

Cultures of nonproteolytic C. botulinum types B (Eklund 2B, Hobbs FTS0), E (Beluga, Foster B96), and F (Eklund 202F, Craig 610) were originally obtained from J. S. Crowther, Unilever Research, Colworth, UK. Type B strain Eklund 17B (NCIB 10642) and type E strain Hazen 36208 (NCIB 10660) were from the National Collection of Industrial Bacteria, UK. All were maintained as previously described (19). It was confirmed that the concentration of toxin formed in peptone–yeast extract–glucose–starch medium (PYGS) (14) was greater than 100 mouse lethal doses per ml (22) and that all the strains multiplied rapidly at 8°C.

Spores were produced on a two-phase medium and washed as described previously (19). Tests were made to confirm that the spores had a heat resistance similar to that described previously (20).

**Preparation of meat medium**

An anaerobic meat medium was prepared containing raw ground beef (Brooks, Norwich, U.K.), 500 g; glucose, 10 g; NaCl, 10 g; soluble starch, 10 g; and glass-distilled water to 1000 g. The mixture was steamed in a boiling-water bath for 20 min to cook the meat, and then ground in a food processor for 4 periods of 20 s to produce very small particles. The meat slurry was boiled for another 30 min, with evaporation minimized by use of a trap. The flask was weighed at the end of boiling to estimate any loss of water, and deoxygenated glass-distilled water (prepared by strict anaerobic technique) was added when necessary to give the required weight. The medium was cooled to 50°C with constant stirring in a stream of oxygen-free nitrogen for 1 h, and the pH was adjusted from approximately 5.8 to 7.1 with 5 M KOH. A total of approximately 6 ml of 5 M KOH was added per liter of medium. The medium was continuously stirred and maintained at 50°C to keep the fat molten, while 20-ml volumes were dispensed by strict anaerobic technique into anaerobic culture tubes (18 by 150 mm) (Bellico, Vineland, NJ), which were capped with gray butyl rubber septa (Jencons Scientific, Leighton Buzzard, UK) and sealed with aluminum caps (Pierce and Warriner, Chester, UK). This procedure ensured an even distribution of fat throughout the tubes. The
medium was sterilized by autoclaving at 121°C for 15 min, stored at 1°C, and used within 3 weeks of preparation. The pH of the medium after autoclaving was 6.5 ± 0.1, and the average final fat content was 8.8% (±0.1%, wt/wt). The water activity of the meat medium was measured using a CX-2 water activity system (Decagon Devices, Pullman, WA); the average value was 0.99.

**Preparation of spore suspension and inoculation of meat medium**

A suspension of spores (approximately 5 × 10^6/ml) containing an equal number of spores of each of the eight strains of nonproteolytic *C. botulinum* (3 type B strains, 3 type E strains, and 2 type F strains) was made in saline (0.85%, wt/vol) prepared under N₂. Using a syringe, 0.2-ml volumes of suspension were added to sealed tubes of medium that had been prewarmed at 45°C for 10 min to melt and mix the fat. The tubes were then shaken to disperse the added spores and cooled immediately in an ice-water bath. Five replicate tubes were used for each combination of heat treatment and incubation temperature. The inoculated tubes were heat treated within 1 h of the addition of spores.

**Measurement of the heat treatments applied**

In order to monitor the temperature during thermal treatments, eight thermocouples (copper/copper nickel thermocouple probes (type T) in a 1.6-mm diameter stainless-steel probe, R.S. Components, Corby, UK) were sealed singly into tubes of uninoculated meat medium. Each probe had been calibrated previously against certified precision mercury-in-glass thermometers in a range of 65° to 95°C. The temperature was recorded to the nearest 0.1°C. Temperatures were recorded by a Data Acquisition Unit (Series 410, Anvile Instruments, Camberley, UK), with the response of the probes monitored every 6 s for the shorter heat treatments, and every 30 s for heat treatments longer than 600 min. The heat treatment applied was assessed by placing the temperature-monitoring tubes (those with thermocouples) towards the center of the rack containing tubes to be heat treated; this placement ensured that they received the lowest heat treatment of all tubes. An appropriate heat treatment was applied by immersing the rack of tubes rapidly in a large water bath (W38 bath; Grant Instruments, Cambridge, UK) set at the desired temperature, and the temperature of the bath was monitored by a precision mercury-in-glass thermometer. When the core temperature of the meat medium was within 0.1°C of that required, timing was started. After an appropriate period of time at the desired temperature, the rack of tubes was removed and plunged into a deep ice bath. The tubes were then shaken vigorously to effect a rapid cooling. When the core temperature of the meat medium had fallen below 10°C in all monitored tubes, the tubes were dried and transferred to an appropriate incubator.

**Incubation of tubes after heat treatment**

After heat treatment and cooling, five replicate tubes of meat medium previously inoculated with spores of *C. botulinum* were incubated in low-temperature incubators (Astell-Hearson, model MK III), the temperatures of which were monitored with platinum resistance thermometers connected to a data logger (Anvile Instruments) and recorded at intervals of 30 min. The target incubation temperatures were 5, 8, 12, 16, and 25°C for all the heat treatments applied. The temperature of the incubators was measured with a probe sealed into a tube of distilled water instead of a tube with meat slurry. The platinum resistance thermometers (British Standard Grade II) were calibrated to an accuracy of ±0.1°C over the range of use. At the end of each experiment, the data were analyzed to determine the mean temperature and temperature variation.

**Enumeration of survivors**

Serial dilutions (10-fold) of heat-treated spores were prepared in dilution PYGS broth (14) using strict anaerobic technique. Appropriate dilutions were inoculated (200 μl volumes) into five replicate vials of PYGS broth (10 ml) using disposable, sterile 1-ml syringes and 26-gauge needles. Inoculated vials were incubated at 30°C and examined for growth at 3-day intervals for 2 weeks. Growth was assessed by visible turbidity and production of gas. From the number of vials that showed growth the most probable number (MPN) of viable spores in the original sample was calculated (13). Enumeration of unheated controls was also carried out to establish the initial number of viable spores per tube of meat slurry. The probability that a single spore would initiate growth and toxigenesis (P) was calculated as the MPN of spores that resulted in growth per the MPN of spores inoculated. The value log(1/P) represents the expected number in logarithmic units (log,10) of spores required for one spore to result in growth (7).

**Determination of growth and toxin production**

All the tubes were examined at least every 2 or 3 days for signs of growth. Growth of *C. botulinum* in meat medium was indicated by obvious formation of gas. In some circumstances a few gas bubbles or minor cracks in the meat medium occurred as a result of the manipulations; this was not taken to indicate growth. At the end of the experiment, samples of medium to be tested for toxin were centrifuged (15,000 × g, 10°C, 15 min). The supernatant was stored at 1°C until it was tested for toxin. Tests for the presence of toxin were made using an enzyme-linked immunoassay (ELISA) procedure (6) modified from a method originally described by Potter et al. (23). For each heat treatment, samples of the lowest incubation temperature that showed growth and the highest incubation temperature that did not show growth were tested for toxin. In most of the sets of conditions in which some, but not all, of the 5 replicate vials showed visible signs of growth and gave positive results in the ELISA, samples from each of the 5 replicate vials were tested for toxin by intraperitoneal injection into mice, as described previously (22).

**Modeling**

For each measured temperature (Tm), the lethal rate (L) with reference to the target temperature (Tt) was calculated from the formula L = 10[(Tm−Tt)/z], where z is the change in temperature in degrees centigrade resulting in a 10-fold change in decimal reduction time. A z value of 6°C was used as this value gave the best fit when times to growth were compared taking into account heat treatment and incubation temperature. The lethality of the heat treatment (K), in terms of the equivalent time (min) at the target temperature (Tt), was calculated from the equation, K = Σ(L. Δt), where Δt is the time (min) between temperature measurements. The same procedure was used to calculate K_{T50}, the lethality of the heat treatment in terms of equivalent time (min) at 75°C. Here, the target temperature used in the formula was 75°C.

The incubation times required before the first observation of growth (y) were modeled as a function of the equivalent heating time at 75°C (K_{T50}) and incubation temperature (T). Data in the temperature range 70 to 80°C were used; heat treatments at 85 and 90°C were not included in the model, as growth was observed only at the shortest heating times. The equivalent heating time at 75°C (K_{T50}) was used in the model rather than heating time and heating temperature as there were only three values for heating tempera-
ture. A biquadratic response surface was used, which was represented by a polynomial of the form $\ln(y) = c_1 + c_2 \cdot K_{75^\circ C} + c_3 \cdot T + c_4 \cdot K_{75^\circ C} \cdot T + c_5 \cdot K_{75^\circ C}^2 + c_6 \cdot T^2$, where $\ln(y)$ is the natural logarithm of the dependent variable of the model, time to the first tube showing growth, and $c_1$ through $c_6$ are the coefficients to be estimated.

**RESULTS**

**Heat treatments applied**

When the temperature approached the target values, there was a relatively slow rate of increase in temperature (Fig. 1). Therefore, for the shorter time heat treatments, the come-up period contributed significantly to the lethality of the heat treatment (Fig. 1, Table 1). The cool-down period was between 4 and 10 min in most of the treatments and the decrease in temperature was relatively fast (Fig. 1, Table 1). The lethal effect of the total heat treatment is expressed as the equivalent time at the target temperature and the equivalent time at $75^\circ C$ (Table 1).

**Calculation of the number of spores that survived each heat treatment**

The number of viable spores decreased as the heating time increased (Table 2). The detection limit was 10 viable spores per tube. The decrease that resulted from heat treatments at $70^\circ C$ was by less than a factor of $10^6$ (1,000-fold) in all cases. For heat treatments at $75^\circ C$, reductions in excess of $10^{13}$ were achieved. The decrease that resulted from heat treatments at 80, 85, and $90^\circ C$ was greater than a factor of $10^6$ except for one heat treatment at each of 80 and $85^\circ C$ (Table 2).

**Growth of nonproteolytic C. botulinum in meat medium after heat treatment and incubation at chill temperature**

The actual mean incubation temperatures were 25.0, 15.8, 11.5, 7.8, and 4.9°C for the heat treatments tested in experiment 1, and 25.3, 15.9, 11.8, 7.9, and 4.7°C for the heat treatments tested in experiment 2 (see Table 1). At no time did the measured incubation temperature exceed the target incubation temperature by more than $1^\circ C$.

The effect of heat treatment and subsequent incubation temperature on time to growth from an inoculum of $10^9$ spores of nonproteolytic C. botulinum is shown in Table 3. Growth of C. botulinum was recorded when formation of gas was evident. Following heating at $70^\circ C$ for 2,545 min, growth at $8^\circ C$ was observed in 50 days. Heat treatments of $75^\circ C$ for 1,072 min, $80^\circ C$ for 230 min, $85^\circ C$ for 23 min, and $90^\circ C$ for 10 min, all prevented growth at $8^\circ C$ for 90 days.

![FIGURE 1. Changes in temperature of meat medium during heat treatment of spores of nonproteolytic C. botulinum in meat medium. The temperature in reference tubes was measured at intervals of 6 s, and the mean temperature is shown. The three heat treatments shown are $70^\circ C$ for 529 min, $75^\circ C$ for 285 min, and $80^\circ C$ for 184 min.](image-url)
TABLE 2. Effect of heat treatments on the survival of spores of nonproteolytic \textit{C. botulinum}

<table>
<thead>
<tr>
<th>Heat treatment\textsuperscript{a}</th>
<th>Temperature (°C)</th>
<th>Heating time at target temp (min)</th>
<th>MPN viable spores per tube</th>
<th>( P )</th>
<th>( \log 1/P \textsuperscript{c} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated</td>
<td>0</td>
<td>( 6.0 \times 10^5 )</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>70</td>
<td>104.9</td>
<td>( 4.0 \times 10^4 )</td>
<td>6.7 \times 10^{-2}</td>
<td>1.17</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td>529.1</td>
<td>( 5.4 \times 10^4 )</td>
<td>9.1 \times 10^{-2}</td>
<td>1.04</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td>998.9</td>
<td>( 6.8 \times 10^2 )</td>
<td>1.1 \times 10^{-3}</td>
<td>0.29</td>
<td>2.95</td>
</tr>
<tr>
<td></td>
<td>1596.3</td>
<td>( 1.4 \times 10^3 )</td>
<td>2.4 \times 10^{-3}</td>
<td>2.62</td>
<td>2.62</td>
</tr>
<tr>
<td></td>
<td>2065.9</td>
<td>( 6.3 \times 10^3 )</td>
<td>1.1 \times 10^{-2}</td>
<td>1.97</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>2544.5</td>
<td>( 2.6 \times 10^3 )</td>
<td>4.4 \times 10^{-3}</td>
<td>2.36</td>
<td>2.36</td>
</tr>
<tr>
<td>75</td>
<td>284.6</td>
<td>( 2.0 \times 10^2 )</td>
<td>3.4 \times 10^{-4}</td>
<td>3.47</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>463.1</td>
<td>( 8.0 \times 10^1 )</td>
<td>1.3 \times 10^{-4}</td>
<td>3.87</td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>1071.5</td>
<td>( 1.8 \times 10^1 )</td>
<td>3.0 \times 10^{-5}</td>
<td>4.52</td>
<td>4.52</td>
</tr>
<tr>
<td>80</td>
<td>11.4</td>
<td>( 1.3 \times 10^3 )</td>
<td>2.2 \times 10^{-3}</td>
<td>2.66</td>
<td>2.66</td>
</tr>
<tr>
<td>85</td>
<td>35.7</td>
<td>( 5.9 \times 10^1 )</td>
<td>1.0 \times 10^{-4}</td>
<td>4.00</td>
<td>4.00</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All other heat treatments resulted in an MPN per tube of \(<10\) spores.
\textsuperscript{b} \( P \), probability of growth and toxin formation at 30°C from a single spore.
\textsuperscript{c} \( \log 1/P \), log number of spores required for one spore to grow and produce toxin at 30°C.

(0°C). This model provides a valid description of the data used to generate it. This equation gives predictions for a range of conditions in which the model was generated.

TABLE 3. Effect of heat treatment and subsequent incubation temperature on time to growth of nonproteolytic \textit{C. botulinum}

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Time (days) to growth at incubation temperature (°C):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated</td>
<td>( 60 )</td>
<td>( 7 )</td>
<td>( 1 )</td>
</tr>
<tr>
<td>( 70 )</td>
<td>( 14 )</td>
<td>( 9 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 80 )</td>
<td>( 14 )</td>
<td>( 11 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 90 )</td>
<td>( 14 )</td>
<td>( 13 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 100 )</td>
<td>( 14 )</td>
<td>( 15 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 110 )</td>
<td>( 14 )</td>
<td>( 17 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 120 )</td>
<td>( 14 )</td>
<td>( 19 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 130 )</td>
<td>( 14 )</td>
<td>( 21 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 140 )</td>
<td>( 14 )</td>
<td>( 23 )</td>
<td>( 2 )</td>
</tr>
<tr>
<td>( 150 )</td>
<td>( 14 )</td>
<td>( 25 )</td>
<td>( 2 )</td>
</tr>
</tbody>
</table>

\textsuperscript{a} First number is time (days) before the first tube showed growth; number in parenthesis is time when all tubes showed growth.
\textsuperscript{b} NG, no growth or toxin at day 90.
\textsuperscript{c} First number is time (days) before the first tube showed growth, second number is the number of tubes (of 5) that showed growth and toxin at day 90.

Toxin formation by nonproteolytic \textit{C. botulinum}

Tubes were tested for toxin at the end of the experiment. Using the ELISA technique, toxin was detected in at least one tube in each treatment where growth was recorded, and toxin was not detected in any tubes in which growth had not been detected by gas production. In the samples that were tested for toxin in the mouse test as well as by the ELISA, the results of the two tests were in agreement in all cases.

Modeling

The second-order polynomial derived from 63 data points was \( \ln(y) = 4.574 + 0.002 \cdot K_{75°C} - 0.263 \cdot T + 0.00004 \cdot K_{75°C} \cdot T - 0.0000007 \cdot K_{75°C}^2 + 0.00364 \cdot T^2 \), where \( \ln(y) \) is the natural log of time (in days) to the first observation of growth, \( K_{75°C} \) is the equivalent heating time (min) at 75°C (a transformation factor of \( z = 6°C \) can be used), and \( T \) is the incubation temperature (°C). This model should only be used to obtain predictions within the boundaries where growth was observed. These are an equivalent heat treatment at 75°C for 15 to 150 min followed by an incubation temperature of 5 to \(<8°C\), heating at 75°C for 15 to 1,250 min followed by incubation at 8 to \(<25°C\), and heating at 75°C for 15 to 2,500 min followed by incubation at 25°C. The equivalent heat treatments may be converted from within the range of 70 to 80°C, as this is the range in which the model was generated.

The percentage of variance accounted for using the \( r^2 \) statistic was 87% and the value of the residual mean square error (RMSE) for \( \ln(y) \) was 0.39. The RMSE provides a measure of the goodness of fit of the model to the data (5). Time to growth as fitted by the second-order polynomial compared well with the observed (observed) time to growth (Fig. 2). Thus, the model provides a valid description of the data used to generate it.

This equation gives predictions for a range of condi-
DISCUSSION

This study has determined the effect of heat treatments in the range 70 to 90°C with subsequent incubation at refrigeration temperatures on growth from 10⁶ spores of nonproteolytic C. botulinum in a model food product. A model has been developed that predicts the time to growth of this pathogen as a function of heat treatment and subsequent incubation temperature with an inoculum level of 10⁶ spores. The initial number of 10⁶ spores per tube was chosen because the heat treatments recommended by the ECFF (10) and ACMSF (1) were established to reduce the number of viable spores of nonproteolytic strains of C. botulinum by a factor of 10⁶ (6D process); the rationale for this criterion has been discussed previously (22).

The French Ministry of Agriculture made two recommendations for the safe production of refrigerated ready-to-eat meals with an extended shelf life (17, 18). For products with a shelf life of up to 21 days, a heat treatment of 100 min at 70°C (or the equivalent) was recommended followed by a check of compliance, to take into account possible risks, by storage at 4°C for 14 days followed by 8°C for 7 days. In this study, following a heat treatment of 105 min at 70°C, growth was observed at 5°C in 14 days and at 8°C in 9 days, suggesting that this French recommendation may leave only a small margin of safety with regard to a 6D process for nonproteolytic C. botulinum. For products with a shelf life of up to 42 days, a heat treatment of 1,000 min at 70°C (or the equivalent) was recommended followed by a check of compliance by storage at 3°C for 28 days and 8°C for 14 days. In the present work, following a heat treatment of 1,000 min at 70°C, growth was observed at 8°C in 21 days, suggesting a wider margin of safety than given by the other French recommendation. In other studies it has been observed that incubation of spores of nonproteolytic C. botulinum at temperatures lower than 3°C (where spores can germinate but not result in vegetative growth) did not reduce the lag time from spores (Rodrigo, Fernandez, and Peck, unpublished data). A shelf life of 42 days based on a heat treatment of 1,000 min at 70°C followed by an incubation at 3°C for 28 days and then by 14 days at 8°C should therefore ensure a 6D process in relation to nonproteolytic C. botulinum.

In the UK, the ACMSF stated that heat treatments at 70°C for 1,675 min, 75°C for 464 min, and 80°C for 129 min were equivalent to 90°C for 10 min, and would give a 10⁶ reduction of spores of nonproteolytic C. botulinum (1). The ECFF recommended that heat treatment at 80°C for 270 min would also give a 10⁶ reduction (10). In this study, none of these heat treatments alone gave the desired kill, as tested by the ability of the spores to germinate and result in growth at 25°C. The heat treatments at 70 and 75°C recommended by the ACMSF (1) were particularly ineffective. Indeed to achieve a 10⁶ reduction of spores of nonproteolytic C. botulinum it would be necessary to heat at 70°C for more than 2,545 min, at 75°C for 1,793 min, and at 80°C for more than 363 min, or to combine heat treatment with storage at refrigeration temperatures to give the required degree of protection against C. botulinum. Appropriate shelf lives can be derived from the data presented here. Heat treatments recommended by the ACMSF and ECFF to give a 10⁶ reduction of spores of nonproteolytic C. botulinum of 36 min at 85°C (1), 52 min at 85°C (10) and 10 min at 90°C (1, 10) all delivered this kill in the tests reported here when incubation was at 25°C for 90 days. Growth and toxin production were detected in one of 20 tubes heated at 90°C for 10 min and subsequently incubated at 16 or 25°C. If one viable spore remained in the tube following heating, the heat process indicated a 10⁶ reduction in the number of viable spores (i.e., from 1.2 × 10⁷ spores in 20 tubes to 1 spore in 20 tubes).

Observations of time to growth made in this study following heating at 85 and 90°C are consistent with those made previously. For example, Graham et al. (12) heated 10⁶ spores of nonproteolytic C. botulinum in a meat medium at
85°C for 18 min, and observed growth in 29 days at 16°C and in 49 days at 12°C, but not in 90 days at an incubation temperature of 8°C; in this study following heating at 85°C for 23 min growth was observed in 38 days at 16°C, 30 days at 12°C, and 75 days at 8°C. A failure to observe growth following heating at 90°C for more than 15 min in this study is consistent with a previous report of no growth in 93 days in 10 tubes that were heated at 90°C for 19.8 min and subsequently incubated at 16°C or 25°C (21).

Predictions from the model were compared with observations in previous studies where time to growth from 10⁶ spores of nonproteolytic C. botulinum was determined (Table 4). Predictions from the model were marginally faster than those observed by Peck, Fairnburn, and Lund (unpublished data). This difference may be due to the larger number of strains used in the previous work. Slightly faster growth from unheated spores at refrigeration temperatures was also noted compared to data from the studies reported here. In a further study, when 10⁶ spores of a mixture of eight strains of nonproteolytic C. botulinum were heated in a meat medium for 20 min at 80°C and incubated at 8, 12, and 16°C, growth was observed in 21, 11, and 6 days respectively (Mitchell, Mason and Peck, unpublished data). Predictions from the model for these conditions were 20, 10, and 5 days, respectively (Table 4). Therefore, predictions from the model compare well with data obtained in other studies without lysozyme. This indicates that the model can be used to predict shelf life with respect to providing a safety factor of 10⁶ in relation to spores of nonproteolytic C. botulinum of a food given a mild heat treatment (at 70 to 80°C) and stored at a refrigeration temperature. In other studies time to growth and the range of conditions supporting growth were considerably affected by inclusion of hen egg white lysozyme (HEWL) in the meat medium prior to heating (12, 21).

For example, following heating at 90°C for 19.8 min and subsequent incubation at 12°C, growth was observed in 31 days with HEWL included at 50 μg/ml prior to heating, but not in 93 days when no addition was made (21). Therefore, this model should only be used for those food products that are known not to contain lysozyme. An alternative model based on heating a food containing lysozyme has been developed (Fernandez and Peck, unpublished data). The occurrence of lysozyme in foods has been reviewed recently (16).

This model fulfills the requirement of the food industry for a rapid and reliable method of determining the effect of heat treatment and storage temperature on the ability of a food to support growth and toxin production by nonproteolytic C. botulinum. The model will therefore help assure the safety of a range of food commodities and formulations and contribute to effective targeting of the more expensive and time-consuming challenge testing of foods, encouraging the safe development of new minimally processed, refrigerated foods (especially REPFEDs). This will also be of practical benefit in the development of a comprehensive hazard analysis critical control point (HACCP) quality-assurance program to define adequate heat treatment, storage conditions, and shelf life for the products with respect to providing a safety factor of 10⁶ in relation to spores of nonproteolytic C. botulinum. The model does not take into account the hazard posed by proteolytic strains of C. botulinum, and this hazard must be considered at incubation temperatures in excess of 10°C.

This model was developed using a food prepared at optimal pH and NaCl concentration, and therefore represents a worst-case scenario, excepting the presence of lysozyme. Further models need to be developed that describe the effect of pH, NaCl, and other preservative factors on growth from heat-treated spores of C. botulinum that are subsequently incubated at refrigerated temperatures.

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