

**Demonstration of the safe shelf-life of fresh meat with
respect to non-proteolytic *Clostridium botulinum***

**Project Report
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SUMMARY

The aim of this study was to consider the safety of a shelf-life of 21-28 days at 3-5°C, or 10-15 days at 8-10°C for fresh red meats with respect to growth of, and toxin formation by, *C. botulinum*. This included carrying out a literature review on growth and neurotoxin production by non-proteolytic *C. botulinum* in fresh meat, the use of Combase to predict the growth of non-proteolytic *C. botulinum* and a challenge test to determine the conditions under which neurotoxin can occur in fresh meats.

Combase predictions indicate that meats with pH values of 5.5, 5.75, 6.0 and 6.5 would have to be stored below 8, 7, 6 and 5°C respectively to prevent growth within 10 days. Only meat with a pH of 5.5 stored at 4°C was predicted not to support growth within 28 days. These predictions do not support assignment of a shelf-life of 21 days at 5°C, or more than 10 days at 8-10°C.

There is very little information in the literature on growth and toxin production by non-proteolytic *C. botulinum* in fresh red meat. Although the data is limited, and some is of questionable quality, what has been published shows growth can occur in fresh within 28 days at 5°C.

In a challenge test, samples of raw beef, pork and lamb, supplied by EBLEX and BPEX, were inoculated with spores of a mixture of strains of non-proteolytic *Clostridium botulinum* types B and E at a concentration of 10⁶ spores per 100g pack. After vacuum packing, the samples were incubated at 5, 8 or 10°C and samples were taken for analysis of toxin formation after selected times of up to 63 days. A majority of the packs showed spoilage as judged by visible gas formation and off colour.

Non-proteolytic *C. botulinum* was able to grow and produce high concentrations of toxin within 10 days at 8 or 10°C in a sterile meat based culture medium but toxin was not detected in any of the samples at 5°C. Toxin production in the raw meat samples was very sporadic but was observed in 28 days in lamb at 5°C and within 28 days in beef, pork and lamb at 8°C. At a nominal temperature of 10°C toxin was first observed after 10 days in pork and lamb and 28 days in beef. In view of the observation of toxin formation at 10 days at 10°C (the first day on which toxin was tested for), a shelf-life of more than 10 days at 8-10°C would appear to leave only a small margin of safety to allow for possible time and/or temperature abuse by consumers. Thus, these data do not support assignment of a shelf-life of more than 10 days at 8-10°C. When raw meat was stored at 5°C, toxin was detected after 28 days, but not at day 24 or earlier. A commonly used approach to food safety is that the product should be demonstrated to be safe for 150% of its shelf-life at the test (or even an abuse) temperature. The demonstration of no toxin formation within 24 days at 5°C may indicate that a shelf-life of 16 days at 5°C is justified.

In conclusion, the data available on growth of non-proteolytic *C. botulinum* in fresh meat is limited and confused. It suggests that the probability of growth and toxin formation in refrigerated raw meat may be low but it can occur. At this time there does not appear to be sufficient scientific evidence to support a shelf-life of greater than 10 days at 8°C in fresh red meat. The position at 5°C is less clear. The challenge test work indicates that a 16 day shelf-life might be justified, while ComBase predicts an increase in viable count of 0.5 log unit at pH = 6.0,

and 2.0 log unit if pH = 6.5 after 16 days at 5°C. Neither of these increases in viable count are considered acceptable. Literature data indicate growth and toxin formation in raw meat in 21-27 days at 4°C. A shelf-life of 21-28 days at 5°C does not appear to be justified.

1.0 INTRODUCTION

In 1993, Lucke and Roberts reported that fresh meat has an excellent safety record with respect to foodborne botulism, and that no associated outbreaks could be identified (Lucke and Roberts, 1993). A review of the present literature also fails to identify any outbreaks of foodborne botulism associated with fresh meat. The only exception to this is fresh “fermented” meat, such as beaver or seal, consumed by the native peoples of Canada and Alaska (Stringer and Peck, 2008; Peck, 2010). However these fermented meats products have little in common with fresh vacuum-packed meat that is produced and sold in the UK. Thus, in view of the very large quantities of vacuum-packed fresh meat produced in the UK per annum, the present practice would seem to leave a large margin of safety, although the actual safety margin or controlling factors are presently unknown. However, it is important, that as new techniques such as vacuum-packing are used, it needs to be demonstrated that any extended shelf-life remains safe with respect to foodborne botulism.

FSA guidelines (<http://www.food.gov.uk/multimedia/pdfs/publication/vacpacguide.pdf>) on the shelf life of vacuum packed and modified atmosphere packed foods in relation to *C. botulinum* specify a number of specified “controlling factors” that can be used with such products. In respect to the safety of fresh meat, three are of particular interest:

Storage below 3°C

Storage at $\leq 8^{\circ}\text{C}$ for a maximum of 10 days

A combination of heat and preservative factors which can be shown consistently to prevent growth and toxin production by non-proteolytic *C. botulinum*

Using storage temperature as the sole controlling factor may present difficulties for operators who rely on vacuum packed storage for maturation or stock control of fresh meat. Many SMEs do not have chillers capable of operating continuously at $<3^{\circ}\text{C}$. Furthermore the guidelines may also reduce the benefit of investing in innovative retail packaging strategies. There is therefore a need to meet the third “controlling factor” described above, that is to identify combinations of factors that can be used to prevent growth and toxin formation by *C. botulinum* in fresh vacuum-packed meat. It is most likely that this will be a combination of storage temperature and time, since fresh meat is not heat treated and the pH and water activity of fresh meat cannot be changed. Suitable controlling factors can be determined by using predictive microbiological models or by inoculated challenge test studies.

It is important to note that the FSA guidelines were based on best evidence and designed for use with all vacuum packed and modified atmosphere packed foods, and it is well known that different foods present a different risk. For example, the ACMSF (1992) ranked food categories as high, medium or low risk. In this categorisation, fresh meat was considered low risk.

Foodborne botulism is a severe neuromuscular disease caused by consumption of preformed botulinum neurotoxin. The botulinum neurotoxin is the most potent substance known, and consumption of as little as 30-100ng of neurotoxin can be fatal. In view of the severity of foodborne botulism, the species known as *Clostridium botulinum* is defined solely on the basis of an ability to form botulinum neurotoxin, rather than any phylogenetic relationship. In consequence, *C. botulinum* is a heterogeneous species that comprises four phylogenetically and physiologically distinct bacteria, known as *C. botulinum* Groups I to IV, with the distinction between *C. botulinum* Groups I to IV strong enough to merit four different species. Group I

(proteolytic) *C. botulinum* and Group II (non-proteolytic) *C. botulinum* are responsible for virtually all cases of foodborne botulism. These two organisms differ physiology (Table 1), and present different hazards in foods.

	Group I Proteolytic <i>C. botulinum</i>	Group II Non-proteolytic <i>C. botulinum</i>
Neurotoxins formed	A, B, F	B, E, F
Non-neurotoxicogenic equivalent clostridia	<i>C. sporogenes</i>	no species name given
Minimum growth temperature	10-12°C	2.5-3.0°C
Optimum growth temperature	37°C	25°C
Minimum growth pH	4.6	5.0
Maximum NaCl conc. allowing growth	10%	5%
Minimum water activity for growth, humectant: NaCl/glycerol	0.94/0.93	0.97/0.94
Spore heat resistance	D _{121°C} = 0.21 min	D _{82.2°C} = 2.4

Table 1. Characteristics of group I (proteolytic) and Group II (non-proteolytic) *C. botulinum*

Proteolytic *C. botulinum* is mesophilic. Growth and neurotoxin formation have been reported in 3-4 weeks at 12°C, but not at 10°C or below (Peck 2006). Proteolytic *C. botulinum* has the ability to form neurotoxin in grossly temperature abused (25°C) vacuum-packed beef (Lucke and Roberts 1993) but is not a hazard in correctly stored chilled foods where the temperature remains below 10°C. Non-proteolytic *C. botulinum* is a psychrotrophic bacterium with an optimum growth temperature of 25°C (Peck 2006). Growth and neurotoxin formation reported at 3.0°C to 3.3°C in five to seven weeks, but not at 2.1°C-2.5°C in 12 weeks (Peck and Stringer 2005) These strains form a single neurotoxin of type B, E, or F.

Fresh meat would appear to be a good substrate for microbial growth and the spoilage of chilled vacuum packed meat by various cold-tolerant clostridia is well described (Adam, 2010). It would therefore seem possible that non-proteolytic *C. botulinum* could grow and produce toxin in fresh vacuum packed meat stored at 3°C or above. Growth and neurotoxin by non-proteolytic *C. botulinum* in vacuum-packed lamb chunks has been investigated by Moorhead and Bell (1999, 2000). Toxin could be formed in lamb chunks spiked with spores of non-proteolytic *C. botulinum* and stored at 2°C, 4°C and higher temperatures. In view of the generally accepted minimum growth temperature of 3°C, the report of neurotoxin formation at 2°C is unexpected. Other than these studies, the potential for growth and neurotoxin formation by non-proteolytic *C. botulinum* in vacuum-packed fresh meat does not appear to have been greatly tested.

The purpose of this study is to determine the potential for non-proteolytic *C. botulinum* to grow and form neurotoxin in vacuum-packed meat.

2.0 LITERATURE REVIEW: Growth and neurotoxin production by non-proteolytic *C. botulinum* on fresh meats

2.1 Search plan

Data was collected in January 2011 from a manual search of the IFR *C. botulinum* reference collection and online searches of a number of databases. The search terms used were:

botulinum in any field AND (**meat** or **pork** or **lamb** or **beef**) in any field.

As there was a very low hit rate the search was extended to include poultry, chicken or turkey meat in addition to red meat.

The databases searched were

Web of Knowledge
CABI
AGRICOLA
FTSA Direct
PUBMED (Medline)
Scirus
Google scholar

Potential references were screened manually and useful data was summarised below. The lack of data precluded the generation of a summarising spreadsheet.

2.2 Results

Vacuum and modified atmosphere packaging is widely used in the meat industry to both extend product shelf-life and improve handling, storage and distribution of the product. Despite being common practice, there is a surprising lack of published studies on the growth of non-proteolytic *C. botulinum* in these products. Limited data is available on growth of non-proteolytic *C. botulinum* in processed meats and very little has been published on fresh meat.

Hyytiä-Trees *et al.* (2000) conducted challenge studies on 16 different sous-vide food products. Although most of the products were heat treated before incubation, their tests did include unheated ground beef. Ground beef was surface inoculated with 0.1 or 196 spores g⁻¹ of a mixture of five strains of non-proteolytic *C. botulinum* (31-2570 E, 4062 E, C-60 E, 706 B and FT 10 F). Samples (1500g) were vacuum-packed and stored at 4° and 8°C for 21 and 28 days. Growth of *C. botulinum* in the product was detected at the appropriate storage time using a quantitative PCR and the presence of toxin was determined by mouse bioassay. Neither growth nor toxin were detected in 21 days at 4° or 8°C in the samples inoculated at 100 cfu kg⁻¹. Growth of type E strains was detected after 28 days at 8°C but not 4°C. In meat inoculated at the higher level of 10^{5.3} cfu kg⁻¹, growth of type E strains was detected after 21 days at 4° and 8°C and toxin was detected in the 8°C sample.

Moorhead and Bell (1999) surface inoculated lamb chumps (anterior end of leg comprising gluteus, obliquus and adductor muscles) with 5×10^6 spores (10^4 cm^{-2}) of a mixture of four botulinum toxin producing organisms, proteolytic *C. botulinum* type A, non-proteolytic *C. botulinum* type B (ATCC 25765 and 17B which are the same strain), non-proteolytic type E and a type E toxin producing strain of *C. butyricum*. Vacuum packed samples were heat shrunk for 2-3s at 80-85°C and then stored at -1.5, 0, 2, 4, 6, 8, 10, 12 and 15°C for up to 84days. The incubators used were set with weekly defrost cycles such that the meat surface temperature increased on average to 3.1°C above the set temperature but returned to the set temperature within 2.5h. Samples were removed when packs appeared blown and then tested for the presence of toxin by mouse assay. The results are summarised in Table 2.

	Time to blown pack and confirmed toxin presence (days)								
Incubation temperature (°C)	-1.5	0	2	4	6	8	10	12	15
Sample A	>84	>84	55	27	17	11	6	3	3
Sample B	>84	>84	55	27	20	11	7	3	3

Table 2. Time to blown pack in duplicate samples of vacuum-packed lamb chumps. Toxin was detected in all blown packs (Moorhead and Bell 1999)

In view of the generally accepted minimum growth temperature for non-proteolytic *C. botulinum* of 3°C, the report of neurotoxin formation at 2°C is unexpected. The temperature in the packs did go above 2.0°C but the authors state this was for short periods: They report that for growth in 55 days at a nominal temperature of 2.0°C, the temperature was between 2.5° and 3.3°C for 11h 45min and between 3.3° and 5.1°C (the maximum temperature) for 7h. Growth would not be expected in these short periods above 3°C based on data available from broth systems. Toxin was detected by mouse assay but there is no mention of toxin neutralisation by antibodies. Toxin neutralisation would have aided confirmation that the toxic effect was specifically due to botulinum toxin rather than any non-specific toxic effect, although the authors do state that only samples inoculated with *C. botulinum* became toxic. Lack of neutralisation also means it is not possible to say which of the *C. botulinum* species was thought to be responsible for the toxin production.

Moorhead and Bell (2000) conducted a second study to compare the effects of vacuum packing and modified atmosphere packing under carbon dioxide on the growth of *C. botulinum* in refrigerated lamb. Lamb chumps were prepared as described above but either vacuum packed or packed in oxygen impermeable bags with 1.5L carbon dioxide per kg meat. Packs were incubated at 2° or 4°C for up to 84 days. Duplicate packs were tested for toxin by mouse assay at periods of between 21 and 84 days. The results are shown in Table 3. All samples tested were found to be toxic in mice. The results again suggest botulinum toxin can be produced in lamb in less than three weeks at 4°C and less than six weeks at 2°C. However, it would have been instructive to see some negative samples to confirm the positive results were not caused by non-specific toxicity to mice.

Storage time (days)	Storage conditions			
	4°C		2°C	
	Vacuum	CO ₂	Vacuum	CO ₂
21	++	++		
28	++	++		
35	++	++		
48			++	++
55			++	++
63			++	++
84	++	++	++	++

Table 3. Time to botulinum toxin detection in duplicate samples of lamb chumps either vacuum-packed or packed under carbon dioxide (Moorhead and Bell 2000) ++ = both duplicate packs positive. No symbol = not tested

Warnecke *et al.* (1967) tested the ability of a *C. botulinum* type E strain to grow in ground beef. Samples (250g) were inoculated with approximately 10^5 cells per gram, vacuum packed in oxygen impermeable bags and stored at 3, 13 or 23°C (also stated as 41, 55 and 72°F equivalent of 5, 13 and 22°C). Samples were evaluated regularly for changes in visual appearance and gas formation and when excessive gas formation was observed, samples were frozen for toxin testing. Toxin was detected by injecting 0.5ml of meat sample in water (50/50) into four mice. Samples that caused mortality were also tested after being mixed with type E antiserum. The results table in this manuscript shows that 10 of the 14 mice injected with extracts from beef stored at 41°F (5 or 3°C) died. However, it is difficult to interpret this data as the manuscript does not state incubation times or which samples were positive. It also states that “all samples of ground beef including controls produced mortality in mice” but when frozen and re-injected into mice at a later date no mortality was noted from samples that had been incubated at 41 or 55°F. Pivnick and Bird (1965) had previously reported a problem in challenge testing vacuum packed meats where inoculated or non-inoculated samples became toxic for mice but this toxicity was not necessarily due to botulinum toxin as activity was not suppressed by the presence of antitoxins. They also observed that such non-specific activity disappeared on dilution at 1:5 or 1:10 or after freezing at -20°C. Such data makes the Warnecke *et al.* (1967) study difficult to interpret.

An abstract of a thesis by Schocken-Iturrino (1981) reported that *C. botulinum* grew in nearly all inoculated test samples of vacuum packed meat products stored at >3°C. However, details of the foods tested, *C. botulinum* strains used and incubation times could not be found as this thesis could not be obtained and e-mails to the author have so far failed to elicit any response.

Data on the growth of *C. botulinum* at temperatures of 10°C and below was recently reviewed (Peck, 2006; Peck *et al.* 2006; Peck *et al.* 2008). Part of this study included collection of

challenge test data. This included data on cooked meat products and raw and cooked fish (shown in Appendix 9). In Appendix 9 the time to the first observation of growth or toxin and the last time when all replicate samples were negative was noted. Where the time between the last negative sampling and the first positive sample is four days or less, a Time to Toxin (TTT) has been calculated using $TTT = \sqrt{(\text{time to last negative sampling} \times \text{time to first positive sample})}$, whether data is for toxin formation (T) or growth (G). The data for fish and meat samples are shown in Figure 1. Data where the time between last negative sampling and first positive sample was greater than four days was not included in the graph as these time to toxin results could not be plotted accurately. For example, a food is inoculated with *C. botulinum*, incubated at 10°C for 28 days, and then tested and found to contain botulinum toxin. The toxin could have been formed any time from day 1 to day 28 so any estimate of TTT would have a large error associated with it.

The data in Appendix 9 shows many foods that were negative for botulinum toxin after 28 days, however, these data also illustrate that non-proteolytic *C. botulinum* can grow in many foods in refrigeration conditions. Growth was observed in less than 21 days at 5°C in several foods including cooked pork with stuffing, pasteurised liver pate chubs and sous-vide beef with gravy. Growth at 10°C or below in less than 15 days was observed in sous-vide rack of lamb, sous-vide beef homogenate, cooked minced beef, bone in ham, jellied ox tongue, lamb hot pot and lamb curry. These data show that meat can be a good substrate for growth by non-proteolytic *C. botulinum* and that this organism is a potential hazard in cooked foods assigned shelf-lives of longer than 10 days. However, the results are not directly applicable to fresh meat as the conditions for growth in fresh and cooked meats may differ.

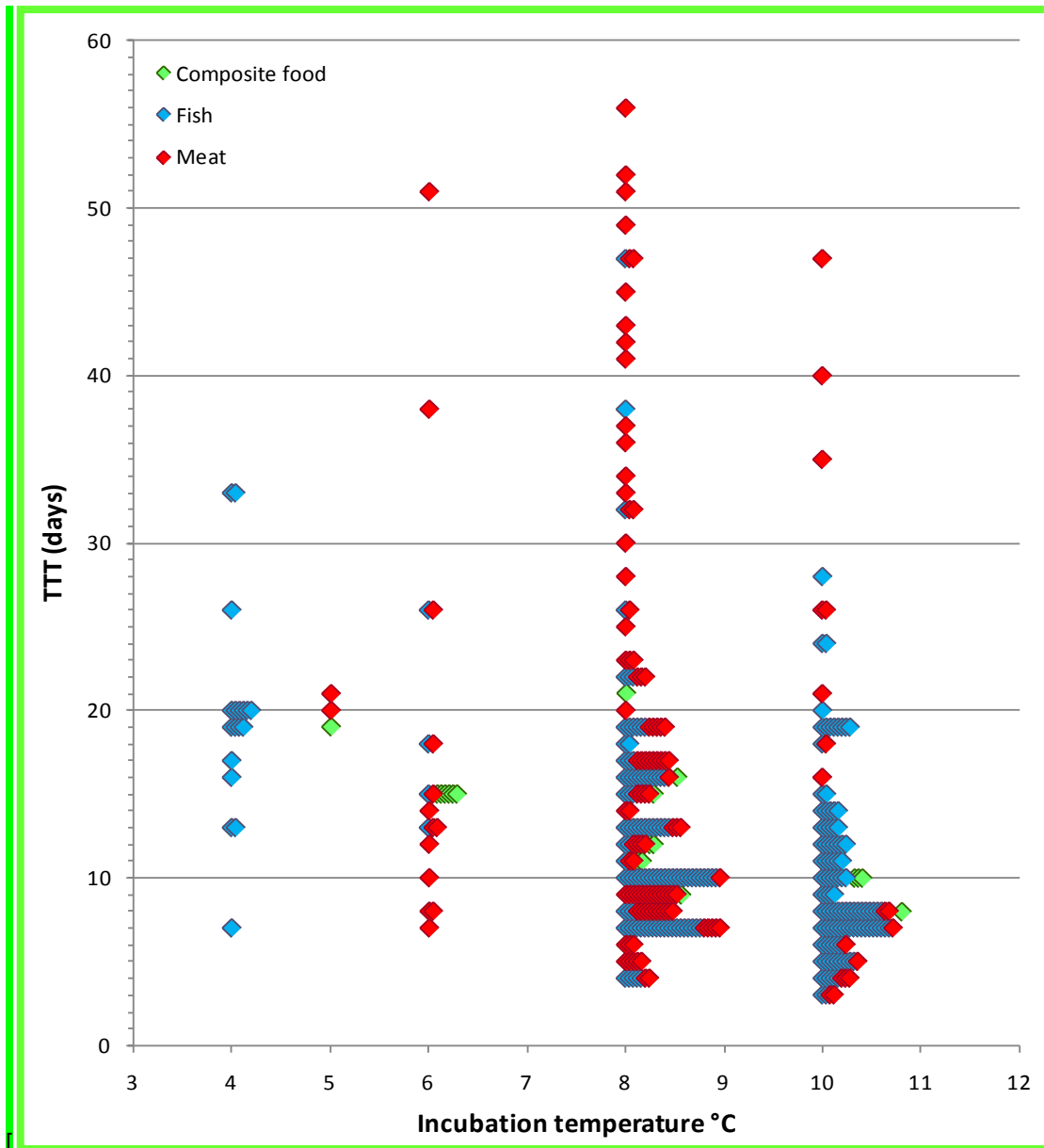


Figure 1. Reported times to toxin formation (TTT) by non-proteolytic *C. botulinum* in foods at refrigeration temperatures adapted from (Peck *et al.* 2006). Observations made in meat (mostly processed), fish or composite meals at 4°, 5°, 6°, 8° and 10°C are shown. Where there is more than one observation at each temperature/time, successive observations are plotted to the right to give an indication of the number of data at each point.

3.0 COMBASE GROWTH PREDICTIONS

Combase (www.combase.cc) consists of two parts. The first part is a database that displays measured microbial responses (growth or survival) as a function of environmental conditions (e.g. temperature, pH, water activity) in laboratory media or specific food. The second part is a modelling toolbox that can be used to generate predictions of the growth or survival of pathogens as a function of environmental conditions. These predictions are based on some of the data recorded in the database and modelled with classic predictive modelling techniques.

3.1 Measured growth data

Most of the data on growth of non-proteolytic *C. botulinum* at chilled temperatures in the Combase database has been measured in culture media: There are 159 data sets (growth curve or growth rate) for non-proteolytic *C. botulinum* in culture medium but only 16 data sets in meats, seafoods or dairy products. There are five data sets for growth in meat at 12°C or below. In each case the data only shows a growth rate based on the time for a 3 log increase in concentration. The results are summarised in table 4. The data is listed under “Other or unknown type of meat” rather than a named meat so these studies may have been conducted in a culture medium such as Robertson’s cooked meat rather than fresh meat. The recorded doubling times for these samples are between 12 and 44 hours at 10°C which corresponds to 5 and 18 days respectively for a 3 log increase in cell numbers. Similarly the data suggests a 3 log increase would take between 10 and 18 days at 5°C. However, it is difficult to apply these data to growth in fresh meat as they do not include any information on lag and the growth matrix is not clear.

Temperature (°C)	pH	NaCl (%)	Maximum Rate (log ₁₀ (CFU/h)	Doubling Time (h):
5.0	6.4	2.6	0.0123	24.50
5.0	6.4	1.8	0.0071	42.60
10	6.4	2.6	0.0071	42.50
10	6.4	2.1	0.0068	44.21
10	6.4	1.8	0.0243	12.40

Table 4. Growth rates for a mixture of strains of type B, E and F non-proteolytic *C. botulinum* in “Other or unknown type of meat” recorded in Combase database.

3.2 Predicted growth data

Combase predictor was used to make predictions of growth of non-proteolytic *C. botulinum* in fresh meat. Predictions are based on the main environmental factors effecting growth; temperature, pH and water activity or salt. Typical salt concentrations were calculated for raw beef, pork and lamb from the sodium content listed in composition data from McCance and Widdowson's The Composition of Foods integrated dataset 2002. All meats had calculated NaCl concentrations between 0.1 and 0.25%. Most samples were around 0.2% NaCl and this figure was used for all the predictions. Meat ultimate pH values were provided by Dr. K. Matthews (EBLEX). Beef samples from different plants had pH values of: 5.58 (SD 0.11), 5.51 (SD 0.15),

5.48 (SD 0.04), 5.71 (SD 0.1) and 5.48 (0.12). Lamb samples had pH values of: 5.51 (SD 0.04) and 5.56 (SD 0.08). For beef and lamb it was decided to base predictions on a typical pH of 5.5 with a worst case pH of 6.0. Pork pH values were obtained from Homer and Matthews (1998). They found the mean ultimate pH of pig carcasses was 5.54 in the summer and 5.75 in the winter and none of the carcasses had a pH greater than 6.5. For pork, predictions were based on pH values of 5.5 and 5.75 with a worst case pH of 6.5.

Predictions were made for samples with a salt content of 0.2% and pH values of pH 5.5, 5.75, 6.0 and 6.5 for temperatures at 1°C intervals between 4 and 10°C. Four degrees centigrade is the lower limit of the Combase non-proteolytic *C. botulinum* model as below this temperature growth is very inconsistent. The predictions are shown in Figures 2, 3, 4, and 5. The predictions indicate that meats with pH values of 5.5, 5.75, 6.0 and 6.5 would have to be stored below 8, 7, 6 and 5°C respectively to prevent growth within 10 days. Only meat with a pH of 5.5 stored at 4°C was predicted not to support growth within 28days.

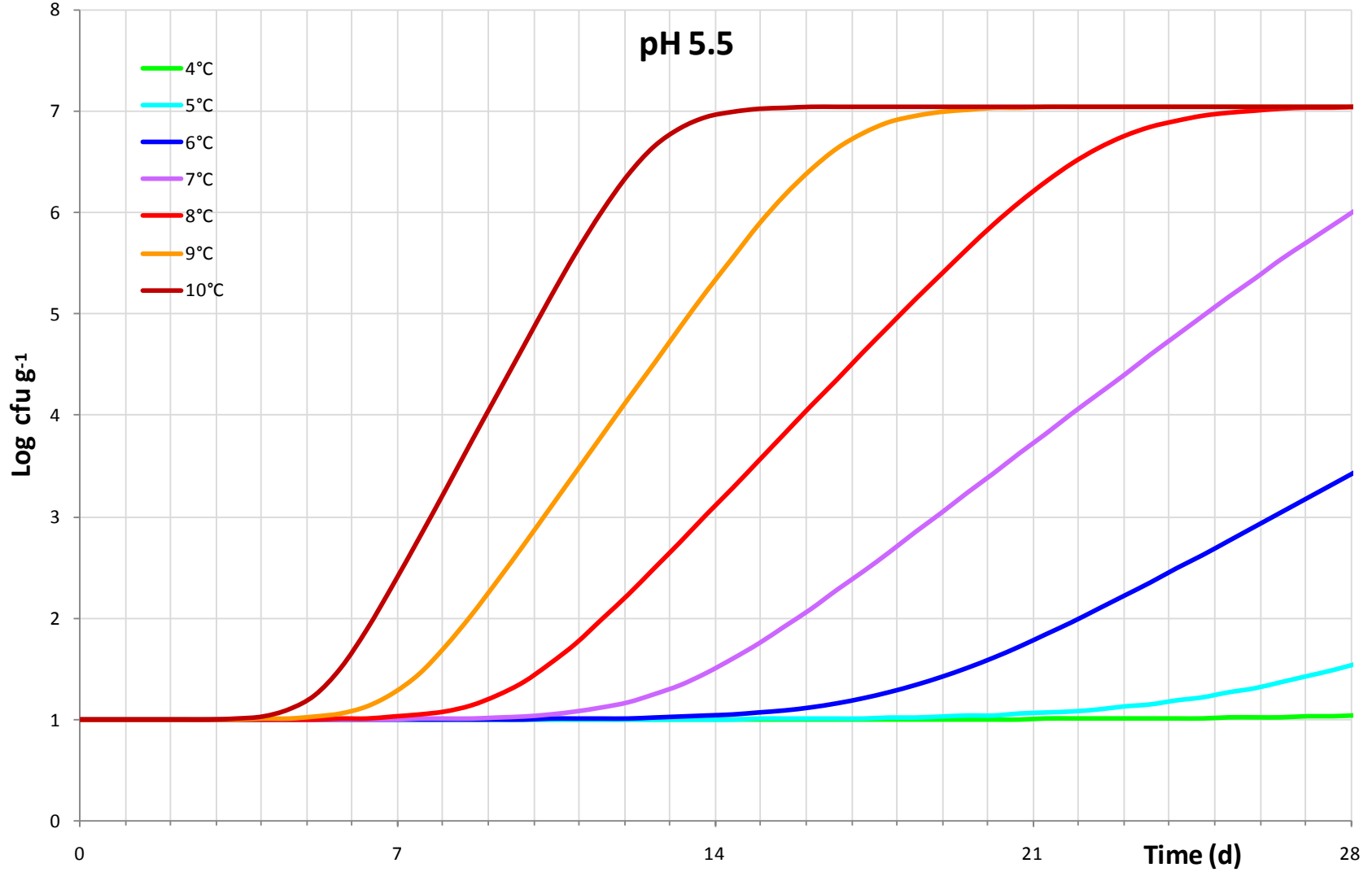


Figure 2 Predicted growth of non-proteolytic *C. botulinum* at refrigeration temperatures in conditions with a pH value of 5.5, a typical ultimate pH for beef, lamb and summer pork, and a salt content of 0.2%.

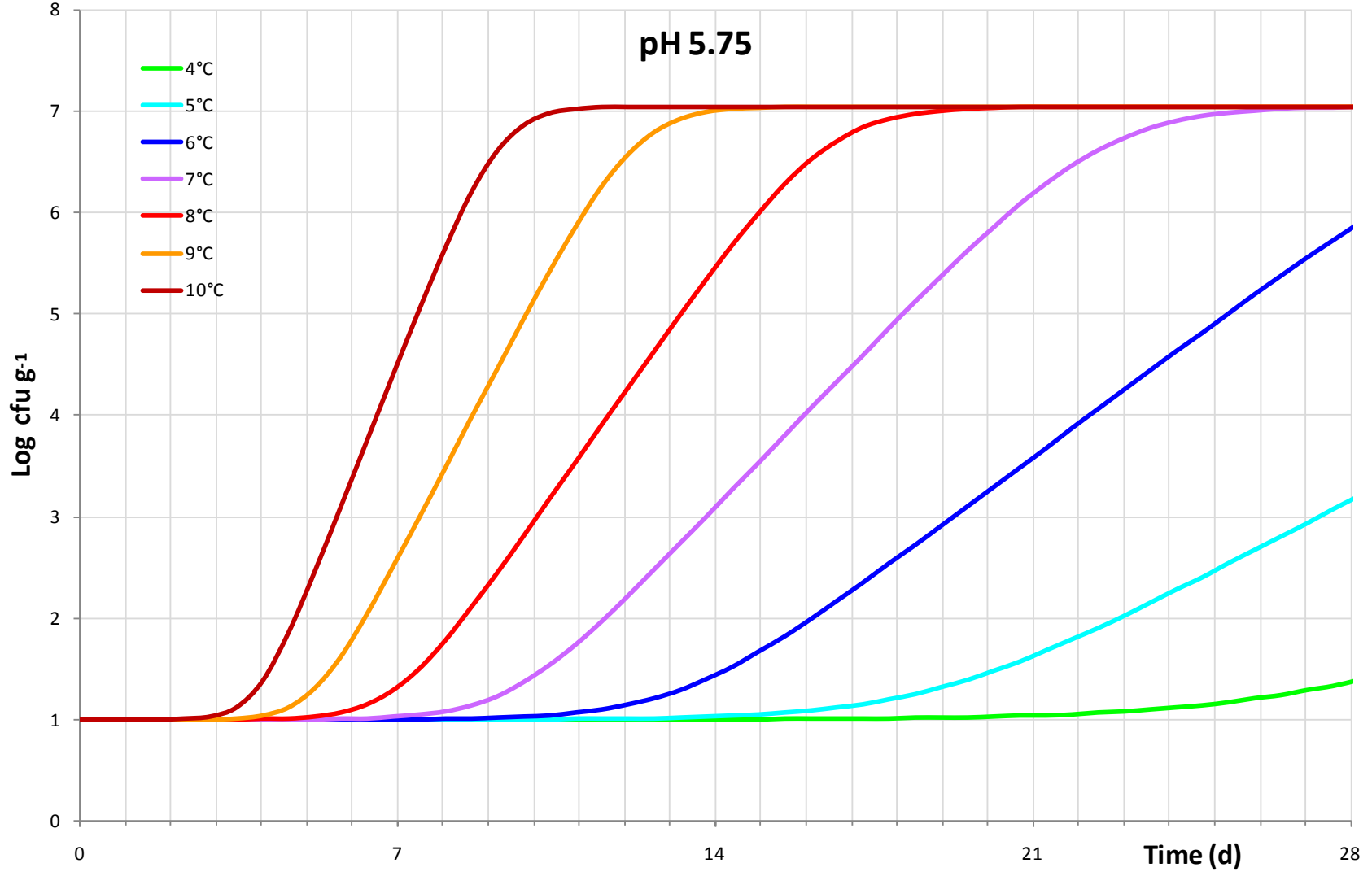


Figure 3 Predicted growth of non-proteolytic *C. botulinum* at refrigeration temperatures in conditions with a pH value of 5.75, a typical ultimate pH for pork in winter, and a salt content of 0.2%.

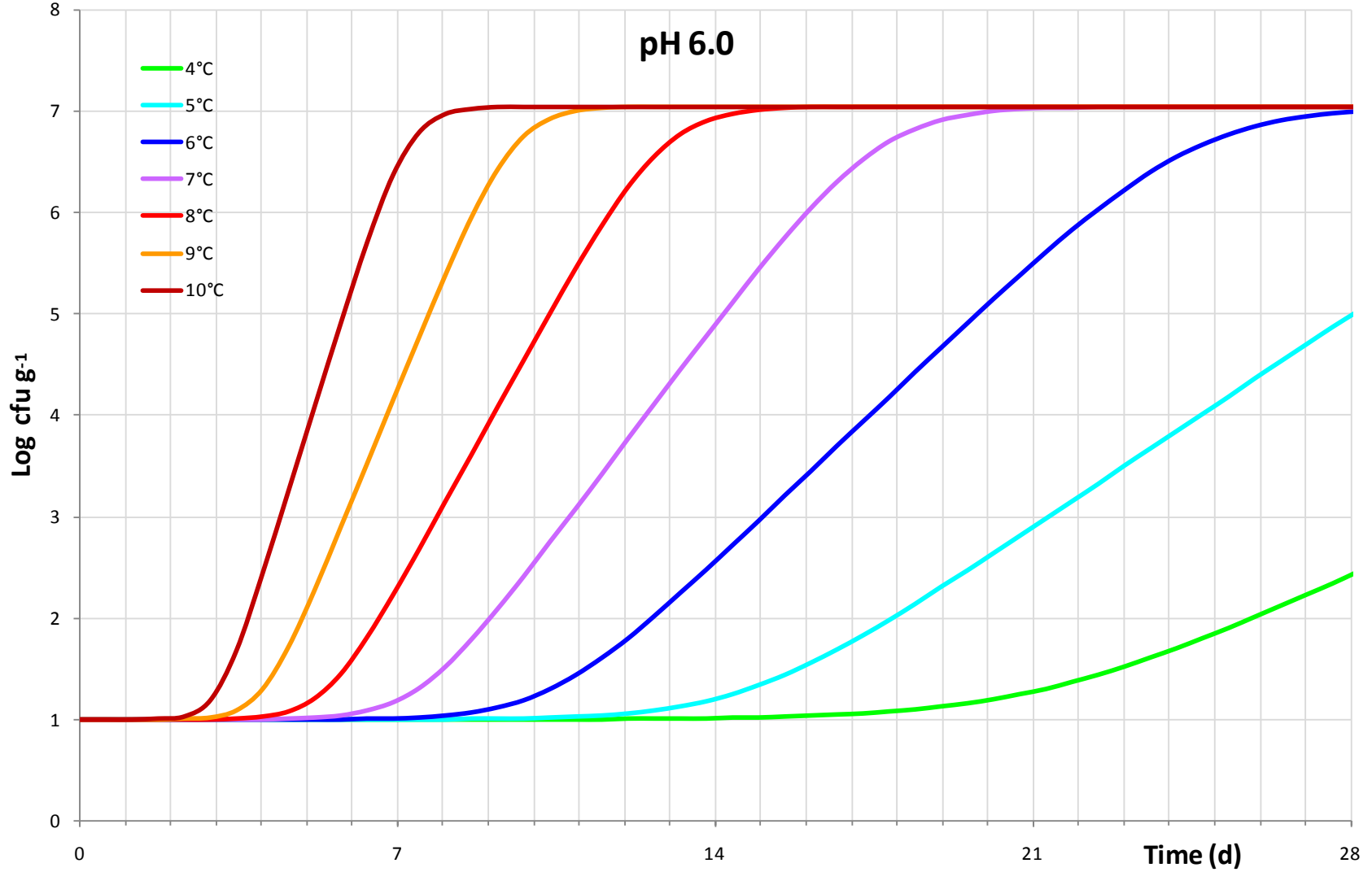


Figure 4 Predicted growth of non-proteolytic *C. botulinum* at refrigeration temperatures in conditions with a pH value of 6.0, a worst case ultimate pH for beef and lamb, and a salt content of 0.2%.

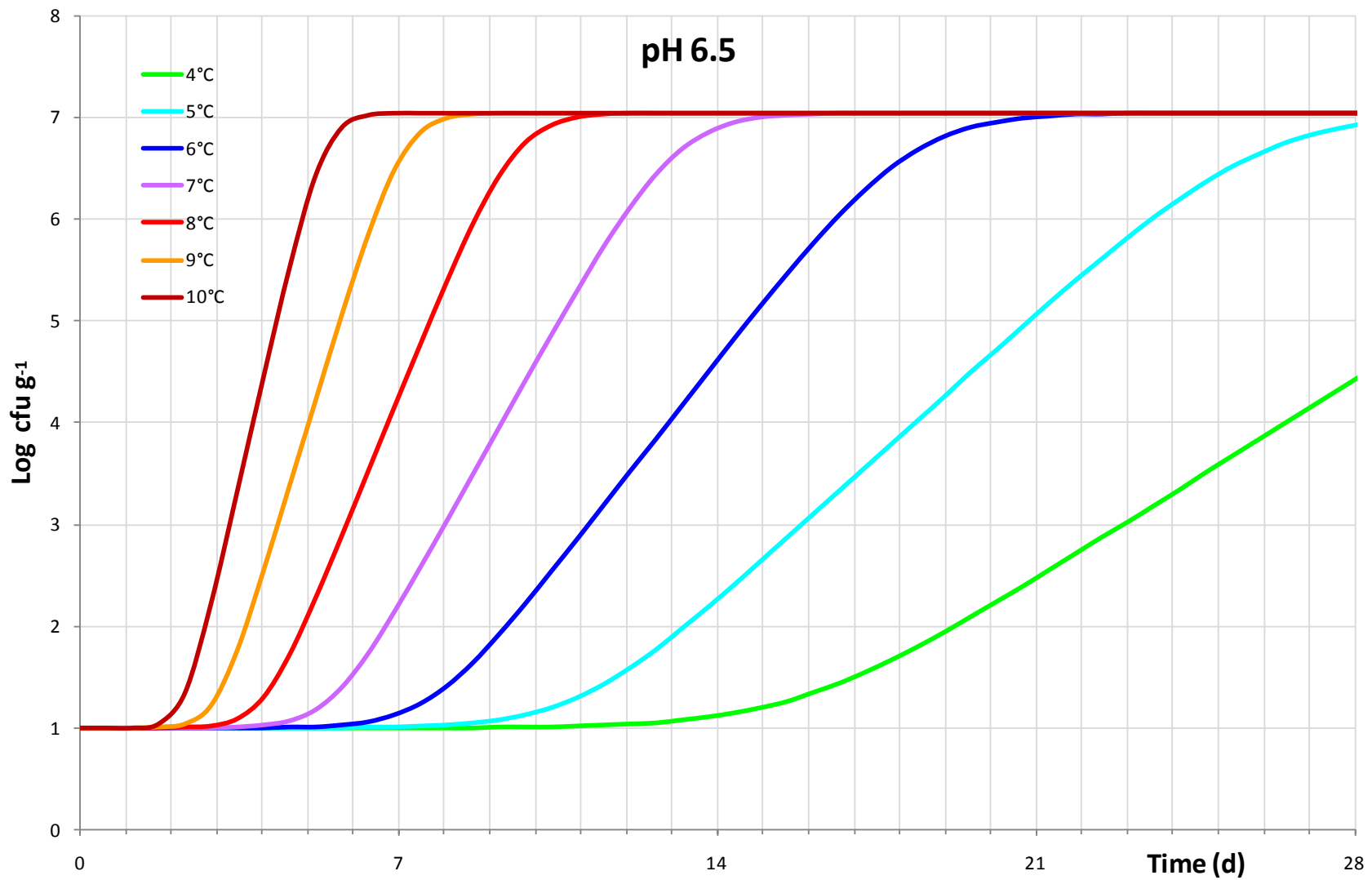


Figure 5 Predicted growth of non-proteolytic *C. botulinum* at refrigeration temperatures in conditions with a pH value of 6.5, a worst case ultimate pH for pork, and a salt content of 0.2%.

4.0 CHALLENGE TESTS IN VACUUM PACKED MEAT

4.1 Introduction

Beef, pork and lamb samples of different muscles taken from different abattoirs were inoculated with spores of non-proteolytic *C. botulinum* and incubated at temperatures up to 10°C for up to 63 days to assess the potential for these meats to support the growth of and toxin formation by this organism. The ability to grow on irradiated meat was also tested to assess the effect of the initial level of microbial flora on growth. Growth in Robertson's cooked meat medium (CMM), a sterile culture medium, pH 7.3, consisting of particles of defatted beef heart in a nutrient broth, was used as a control to test the ability of non-proteolytic *C. botulinum* to grow and produce toxin at the incubation temperatures.

4.2 Methods

Samples

Challenge tests were conducted using three types of beef, two of pork and two of lamb (Table 5). Meat samples arrived as approximately 100g single steak type portions each packed in an unsealed gas impermeable plastic bag (approx 15x18cm). The sample weights, treatments and replicate code numbers are shown in Appendix 1. All samples except those for irradiation arrived under refrigeration the day after cutting and on arrival were transferred to a 2°C refrigerator and processed the same day. Irradiated samples were frozen and on arrival, repacked in dry ice and sent to be irradiated at 25 to 40 kGy. After irradiation the samples were returned still frozen then thawed overnight at 2°C before inoculation.

Sample	Description	Date cut	Date inoculated
Beef 1	longissimus - sirloin from a multi species plant, delivered and cut at old MLC demonstration shop to replicate "dirty" plant cutting room. Temp >7°C.	13/09/2010	14/09/2010
Beef 2	Vastus - thick flank from multi species plant, cut and prepared on site to replicate "clean" plant cutting room. Temp <7°C.	13/09/2010	14/09/2010
Beef 3	Gluteus medius. - main rump muscle from multi species plant, cut and prepared on site to replicate "clean" plant (as 2)	13/09/2010	14/09/2010
Pork 4	longissimus - loin from multi species plant, cut and prepared on site to replicate "clean" plant (as 2)	04/10/2010	05/10/2010
Pork 5	longissimus - loin from multi species plant, cut and prepared on site to replicate "clean" plant (as2)	04/10/2010	05/10/2010
Lamb 6	A mix of chump (gluteus medius) thick flank (vastus) topside (semimembranosus) silverside (gluteobiceps) loin (longissimus) selected from a single species lamb plant. Delivered and prepared as sample 1	11/10/2010	12/10/2010
Lamb 7	As 6 but cut under strict hygiene conditions in chiller area. room temperature <7°C.	11/10/2010	12/10/2010
Beef 8	as sample 1, but irradiated prior to testing	13/09/2010	19/10/2010
Pork 9	as sample 4, but irradiated prior to testing	11/10/2010	19/10/2010
Lamb 10	as sample 6, but irradiated prior to testing	11/10/2010	19/10/2010
CMM			28/09/2011

Table 5 Meat samples used in the challenge study.

jars under a headspace of 10% CO₂ : 90% H₂ for 48h at 30°C. The actual spore concentration was 1.067×10^7 spores ml⁻¹.

Inoculation

Meat samples were surface inoculated with 100µl of spore suspension (10⁶ spores) per 100g pack distributed between six separate sites. Packs were flattened by hand (no significant massaging), vacuum packed and then placed in ice until all the packs of one treatment had been prepared. The time required for inoculation and vacuum packaging when samples were not on ice was around 55s per pack. Sealed packs were placed into a gas jars and flushed with anaerobic gas mix (5%CO₂ : 10%H₂ : 85%H₂). This was to prevent any oxygen ingress into the pack during storage.

CMM (10ml in glass, rubber stoppered bottles) was boiled to removed any dissolved oxygen, cooled to the appropriate temperature and then inoculated with 100µl of spore suspension.

Incubation

Processed packs and bottles of CMM were incubated at the appropriate temperature. The temperature of the incubators was monitored using a Labguard system set to trigger a warning if the temperature was $\pm 1^\circ\text{C}$ from the set point.

At the appropriate sampling times, packs were removed from the chilled incubators, a brief description of their visual appearance was made and then they were frozen at -20°C until required for toxin extraction.

Toxin extraction

Food samples were removed from storage at -20°C, defrosted at 2°C overnight, and then blended for two minutes with 100ml 1% gelatine phosphate buffer in a Stomacher. Negative control samples were prepared from uninoculated non-incubated meat samples. An aliquots of the homogenised sample was transferred to a sterile 15ml centrifuge tube and frozen at -20°C until required for toxin testing.

Toxin measurement

All samples were tested for the presence of type B and type E botulinum neurotoxins using a sandwich type ELISA. The ELISA method is described in Appendix 2. Samples were defrosted overnight at 2°C, diluted 1 in 2 in casein buffer and clarified by centrifugation at 3000g. Each test plate consisted of duplicate test samples, a standard curve made from 1 in 2 dilutions of a toxoid standard and negative and positive control samples. Negative controls were extracts made from samples that had not been inoculated and had been frozen on day of receipt. For the pork, lamb and beef samples, the standard curves were prepared in the appropriate negative control meat extract. For the CMM and irradiated meat samples, standard curves were prepared in PBST. The positive control was a diluted PYGS culture supernatant of either a type B or a type E *C. botulinum* culture.

The presence or absence of toxin was determined from the toxoid standard curve. Samples were classified as positive if both duplicates gave an absorbance >1 or an absorbance greater than 2ng toxoid per well for CMM or beef and 6ng per well for pork or lamb. Two ng type E toxoid was equivalent to approximately 0.06 ng type E toxin complex.

4.3 Results

Visual examination of packs for spoilage

A summary of the visual observations made on sample packs after storage for is shown in Appendix 3. All the samples lost their tight vacuum packed appearance within 10 days. Pork samples were judged unacceptable after 17 days at 8 or 10°C or 24 days at 5°C due to the production of a thick, creamy exudate. The beef samples were judged to look acceptable until day 56 and the lamb samples throughout the experiment. The pattern of spoilage in the irradiated meat appeared to be different to the non-irradiated meat with a great deal more gas production.

Temperature data

The average incubation temperatures are shown in Table 7

Nominal temperature	Average temperature (°C)	Standard Deviation (°C)	Time > 1°C above set temp (h)	Time > 2°C above set temp (h)
2°C	2.2	0.19	1.3	0.5
5°C	5.2	0.66	17.8	12
8°C	7.8	0.27	0	0
10°C	9.5	2.10	157	64.5

Table 7 Summary of incubation temperatures.

The 10°C incubator broke down during the trial and, although the samples were moved to a new incubator before they warmed up, the performance of the replacement incubator was more variable primarily cycling from 8 to 12°C. The maximum temperature during the trial was 12.5°C. Although this variability in the 10°C incubator temperature was greater than planned, it is probably more representative of temperature abuse conditions in a domestic refrigerator. Incubation should be considered to have occurred at 8 to 12°C (and is henceforth called nominal 10°C).

Toxin detection

The ELISA results for all samples are shown in Appendices A4 to A8 and the results are summarised in Table 8 and Table 9. The results in the CMM confirm non-proteolytic *C. botulinum* could grow quickly and produce high levels of toxin within 10 days at 8 or 10°C. Toxin was not detected in the samples at 5°C.

Toxin production in the meat samples was very sporadic with only pork 4 showing high toxin concentrations and reproducible toxin production in all three replicate samples. Toxin formation was detected in pork and lamb at 10 days at 10°C (earlier samples were not tested), and in lamb at 5°C in 28 days (but not 24 days).

Toxin was not detected in any of the irradiated meat samples.

	Matrix	Lot	0	Storage conditions – inoculated packs								Uninoculated
				2-5°C								2-5°C
				17d	21d	24d	28d	35d	42d	56d	63d	42
Type B toxin	Beef	1	---	---	---	---	---	---	---	---	---	---
	Beef	2	---	---	---	---	---	---	---	---	---	-+-
	Beef	3	---	---	---	---	---	---	---	---	---	---
	Pork	4	---	---	---	---	---	---	---	---	---	---
	Pork	5	---	---	---	---	---	---	---	---	---	---
	Lamb	6	---	---	---	---	---	---	---	---	---	---
	Lamb	7	---	---	---	---	---	---	---	---	---	---
	Irr-beef	8	---	---	---	---	---	---	---	---	---	---
	Irr-pork	9	---	---	---	---	---	---	---	---	---	---
	Irr-lamb	10	---	---	---	---	---	---	---	---	---	---
	CMM	-		---	---	---	---	---	---	---	---	+++
Type E toxin	Beef	1	---	---	---	---	---	---	---	---	---	---
	Beef	2	---	---	---	---	---	---	---	---	---	---
	Beef	3	---	---	---	---	---	---	---	---	---	---
	Pork	4	---	---	---	---	---	---	---	---	---	---
	Pork	5	---	---	---	---	---	---	---	---	---	---
	Lamb	6	---	---	---	---	---+	---	---	+++	---	-+-
	Lamb	7	---	---	---	---	-+-	---	---	---	---	---
	Irr-beef	8	---	---	---	---	---	---	---	---	---	---
	Irr-pork	9	---	---	---	---	---	---	---	---	---	---
	Irr-lamb	10	---	---	---	---	---	---	---	---	---	---
	CMM	-		---	---	---	---	---	---	---	---	---

Table 9 Summary of toxin test for samples incubated at 2-5°C. Each box represents one test condition and shows the results for triplicate samples.

4.4 Discussion

The results in the CMM show that non-proteolytic *C. botulinum* could grow quickly at 10°C and produce high concentrations of toxin within 10 days. The type E strains could produce toxin within 10 days at 8°C whereas type B toxin was not detected for the 28 day observation period of this experiment. For the spore cocktail used in this experiment it appears that at least one of the type E strains was able to grow more rapidly under these conditions. Neither type B nor type E toxin was detected in the CMM incubated at 5°C. Although most (but not necessarily all) strains of non-proteolytic *C. botulinum* would be expected to grow at 5°C in ideal conditions, CMM is not a fully anaerobic medium: the meat is under broth in a head space of air. Anaerobes can grow in the reducing conditions at the bottom of the CMM medium with growth causing further reduction of the redox potential allowing further growth. Failure to grow at 5°C is likely to be a combination of an increased solubility of oxygen at lower temperatures combined with the increase lag time, reduced growth rate and decreased probability of growth of non-proteolytic *C. botulinum* below 8°C. These results illustrate the critical nature of temperature control in preventing growth of *C. botulinum* with a difference of only 3°C making the difference between growth within 10 days and no growth within 42 days.

The limit of detection for toxin used in this study was lower for beef or CMM, than for pork or lamb. Meat that had not been inoculated and was frozen on day 0 was used to make negative control samples which were used to assess the background reading for each plate. Toxin can normally be detected when the absorbance measured in a well is significantly greater than that of the negative control. For CMM and beef, a detection level of 0.06ng neurotoxin complex per well was used as this value was slightly greater than the control plus three standard deviations so could be distinguished from negative samples. For both lamb and the pork samples it was observed that the absorbance in all the incubated samples was slightly higher than that in the unincubated controls. This is unlikely to be as a result of low level toxin production in all samples as the measured absorbance did not vary with incubation temperature or time. It is more likely to be the result of the unincubated sample not being truly representative of the absorbance of incubated samples which would have been associated with much higher bacterial spoilage. The detection limit of the lamb and pork ELISAs was increased to 0.18ng neurotoxin complex per well so that the increased background did not result in false positive values.

Growth was not observed within 21 days at 8°C but was observed within 10 days in samples nominally incubated at 10°C. This suggests there is a potential for *C. botulinum* growth in vacuum packed meats assigned a shelf-life of 10-15 days if that product is subjected to vary mild temperature abuse. Toxin production was related to the meat samples with more of pork lot 4 able to support growth than pork lot 5 and more samples of lamb lot 6 becoming toxic than lamb lot 7. This shows that the probability of growth and toxin by *C. botulinum* production in red meats depends on the intrinsic properties of individual samples. Toxin was detected in two of the uninoculated samples, beef lot 1 and lamb lot 6. Both these lots were cut at the at old MLC demonstration shop.

Toxin detection at 5°C was very poor. No growth was observed in 24 days or less and only 5 toxic samples were detected during the trial. Storing meat at 5°C for 21 days would appear to be close to the boundary of where growth could occur. It is normally recommended that growth

should not occur within 1.5-times the length of the required shelf-life to allow a safety margin in case of product abuse. Two lamb samples were toxic after 28 days but not at day 24. This would suggest the shelf-life at 5°C should not be longer than 16 days.

Toxin was not detected in any of the irradiated meat samples during this trial. The irradiation dose used in this study was relatively high and would have been expected to reduce the number of either non-proteolytic or proteolytic *C. botulinum* spores by a factor of 10^6 . Visual examination seemed to show growth in the irradiated samples with some bags blowing and a creamy exudate being formed. It should also be noted that the irradiated samples had previously been frozen whereas the other samples were inoculated from chilled. This could have affected the amount of exudate. If growth was occurring without toxin production, either a psychrotrophic spoilage clostridium was able to survive the irradiation and grow in the meat, or the *C. botulinum* were growing in the irradiated meat without producing toxin, or something in the meat destroyed any of the toxin protein produced. Subsequent studies were carried out to investigate this phenomenon further, but failed to identify organisms other than *C. botulinum* in meat extracts and toxin spiked into the irradiated meat extracts was stable for 1 week at 10°C. The paradox between apparent growth and failure to detect toxin in the irradiated samples warrants further investigation.

It has been suggested that non-proteolytic *C. botulinum* doesn't grow in fresh meat as it is a poor competitor of the natural flora. Using irradiation to reduce the background flora did not result in an increase in observed toxin production by non-proteolytic *C. botulinum*. Also, in samples that had not been irradiated, toxin production was only observed after products appeared spoiled suggesting *C. botulinum* was able to form toxin despite the presence of high levels of spoilage bacteria. These results indicate that competition is not the explanation for why *C. botulinum* grows relatively poorly in raw meat.

5.0 CONCLUSIONS

The aim of this work was to consider the safety with respect to growth of and neurotoxin formation by *C. botulinum* of a shelf-life of 21-28 days at 3-5°C, or 10-15 days at 8-10°C for fresh red meats. Since a protection factor of 10^6 is recommended when assessing the safety of chilled foods (FSA, 2008), and it is usually recommended that a product can be demonstrated as being safe for one and a half times the expected shelf-life, this means we would need to show no growth from 10^6 spores of non-proteolytic *C. botulinum* within 42 days at 5°C or 21 days or 10°C.

The current FSA guidelines on the shelf life of vacuum packed and modified atmosphere packed foods (FSA, 2008) specify a number of “controlling factors” that can be used to prevent the growth and toxin production by non-proteolytic *C. botulinum* in chill foods with an assigned shelf-life of more than 10 days. They are chill temperatures (less than or equal to 8°C) and:

- a heat treatment of 90°C for 10 minutes or equivalent lethality
- a pH of 5.0 or less throughout the food
- a salt level of 3.5% or more (aqueous) throughout the food
- an a_w of 0.97 or lower throughout the food
- storage below 3°C
- a combination of heat and preservation factors which has been shown to consistently prevent growth and toxin production by *C. botulinum*

As fresh vacuum-packed meat is not heat treated and the pH and water activity cannot be changed, the factors preventing growth during storage are likely to be combinations of storage temperature and time along with the intrinsic properties of the meat. Suitable controlling factors can be determined by using predictive microbiological models or by inoculated challenge test studies.

Using ComBase to predict the time to growth in substrates of the same pH and salt content as fresh red meat clearly shows there is potential for growth and toxin production by non-proteolytic *C. botulinum* in less than 21 days at 5°C or 10 days at 8°C. While these predictions are useful, it should be remembered that these models are based on the primary controlling factors of temperature, pH and water activity and have been constructed to be fail-safe. They do not include other potential inhibitory factors such as the competitive flora. Fresh meat could only be considered safe if it could be shown there was a specific and predictable factor associated with it that would make growth less likely in meat than in fully anaerobic sterile media.

Information in the literature on challenge test studies using non-proteolytic *C. botulinum* in fresh red meat is very limited. Although data are limited, and some is of questionable quality, what has been published shows growth can apparently occur in fresh within 21 days at 4°C, and within 11 days at 8°C.

The data gathered in the challenge test in this report suggests that, although the conditions tested might be close to the limits of growth for non-proteolytic *C. botulinum*, growth in some meat samples could occur within 10 days at a nominal temperature of 10°C and 28 days at 5°C.

None of the available challenge test data show that it would be safe to extend the shelf-life of fresh red meat beyond 10 days at 8°C. In view of the observation of toxin formation in 10 days at 10°C (the first day that toxin was tested for), a shelf-life of more than 10 days at 8°C would appear to leave only a small margin of safety to allow for potential time and/or temperature abuse by consumers. However, this proposed way forward would seem to be in conflict with the observed production and sale of large quantities of vacuum packed red meats without incidence of botulism. From this information it has been suggested that the risk of botulism is actually quite low (Peck *et al.* 2006). Possible additional controlling factors to account for this discrepancy include low contamination levels, the presence of an inhibitory background microflora, spoilage preceding toxin formation or food being heated before consumption to inactivate any toxin that has been formed.

The lack of data on growth of non-proteolytic *C. botulinum* may relate to an historic belief that, although non-proteolytic *C. botulinum* is a hazard in fish and aquatic mammals, it is of less relevance in meat. Lucke *et al.* (1981) stated that “it is generally believed that they are of little relevance to meat products for two reasons: i) with few exceptions, all psychrotrophic *C. botulinum* strains hitherto have been isolated from samples of aquatic origin, not from meat or meat products; ii) psychrotrophic strains of *C. botulinum* are more sensitive to heat and curing salts than their mesophilic, proteolytic counterparts. Therefore even less likely to develop in a meat product.” These two statements are not applicable to fresh meat. The greater sensitivity of non-proteolytic *C. botulinum* to heat treatments is of no consequence in fresh meat. Also, non-proteolytic *C. botulinum* has been isolated from raw meats (Lucke *et al.*, 1981, Simunovic *et al.* 1985). Indeed, the first strain of *C. botulinum* isolated, which was associated with an outbreak of botulism from ham in 1895, is thought to have been a non-proteolytic *C. botulinum* type B strain from the description of the physiological attributes. Furthermore, the name “botulism” is derived from the Latin word “botulus” meaning sausage, reflecting a historic association between food poisoning and sausages.

Several bacteria have been reported as being able to inhibit toxin production by non proteolytic *C. botulinum* (Valenzeula *et al.* 1967; Crandall *et al.* 1994; Rodgers *et al.* 2004). However, the fact that toxin is produced in foods such as raw fish or cooked meats that are not sterile, and even the meat samples in this report shows that *C. botulinum* can grow in the presence of at least some types of spoilage flora. Unless a specific inhibitory strain had been added as a preservative, natural flora would not seem to meet the criteria of a factor that can be “shown to consistently prevent growth and toxin production by *C. botulinum*.”

The data on growth of *C. botulinum* in chilled meat is confused. The data generated in this study suggests that the probability of growth below 5°C is low but it can occasionally occur within 28 days and predicting when it will occur is difficult. We are not able at this time to provide scientific evidence to support the hypothesis that the shelf-life of fresh meat could be safely extended to 21-28 day at 5°C or to more than 10days at 8° to 10°C.

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APPENDICES

Appendix A1. List of samples, code numbers, weights, treatments and toxin results

See file Appendix 1 list of samples.xls

Appendix A2. Protocol for *C. botulinum* toxin ELISA

Principle

The 'sandwich' ELISA uses Metabionics Inc antibodies as the capture antibody, and the same antibodies biotinylated as the detector antibody. The assay signal is produced with a commercial horseradish peroxidase-streptavidin conjugate and TMB substrate. The protocol is based partly on Ferreira *et al.* (2003) Journal of AOAC International. **86**(2): 314-331 and advice from Mike Goodnough, Metabionics Inc.

Coating Plates

Coat Nunc Maxisorp microwell plates with 100 μ l per well of anti-B or anti-E capture antibody at a concentration of 1 μ g ml⁻¹ in carbonate-bicarbonate buffer (pH 9.6).

Carbonate-bicarbonate buffer (pH 9.6): dissolve 1.59 g l⁻¹ of NaCO₃ and 2.93 g l⁻¹ of NaHCO₃ in dH₂O. Check pH is 9.6 \pm 0.1.

Incubate plates overnight at 4°C, discard the antibody solution and tamp dry on paper towel.

Blocking Plates

Block unreacted sites by adding 300 μ l of casein assay buffer (pH 7.8) to each well.

Casein assay buffer: is 1% (w/v) vitamin-free casein (Sigma, C3400) pH 7.8.

Incubate plates for 90 min in a humidified box at 37°C.

Empty plates by inversion, tamp dry on paper towel and dry inverted at room temperature for 2 h.

Seal plates in vacuum-packed bags and store at -20°C until use.

Detector antibody

Biotinylate anti-B or anti-E antibodies using Pierce EZ-Link Micro Sulfo-NHS-LC-Biotin kit (21935). Dissolve biotinylated antibodies in 50% glycerol and store at -20°C.

Wash buffer

The wash buffer is phosphate-buffered saline-tween (PBST). Dissolve the contents of one sachet of PBST powder (Sigma) in 1000 ml of ultrapure water.

Enzyme conjugate:

Streptavidin-Horseradish Peroxidase (Prozyme 'high signal', Europa Bioproducts PZCJ30H).

Substrate

Super sensitive Tetramethylbenzidine (TMB) (Sigma).

Assay

1. Remove plates from freezer and defrost casein buffer.
2. Dilute samples 1 in 2 in casein assay buffer. Add 100 μl per well of sample to the antibody-coated plates. Include all necessary blanks.
3. Dilute purified toxoid standards to an appropriate concentration in casein assay buffer. Add to 100 μl per well to antibody-coated plates.
4. Incubate plates in a humidified box for 2 h at 37°C.
5. Wash x5, 300 μl per well of PBST and tamp dry on paper towel.
6. Add 100 μl per well of 1 $\mu\text{g ml}^{-1}$ biotinylated detector antibody in casein assay
7. Incubate plates in a humidified box for 1 h at 37°C.
8. Wash x5, 300 μl per well of PBST and tamp dry on paper towel.
9. Add 100 μl of streptavidin-horseradish peroxidase enzyme conjugate diluted 1:5000 in PBST to each well.
10. Incubate plates in a humidified box for 1 h at 37°C.
11. Wash x 5, 300 μl per well of TBST and tamp dry on paper towel.
12. Add 100 μl of TMB substrate to each well. Monitor colour development in blank wells and stop the reaction when required by adding 50 μl of 0.3M H_2SO_4 per well.
13. Read absorbance at 450nm.

Appendix A3. Visual examination of packs for spoilage

A summary of the visual observations made on sample packs after storage for is shown in file Appendix 3 visual results.xls

Appendix A4. ELISA results for Cooked Meat Medium

The Cooked Meat Medium ELISA measurements are shown in file Appendix 4 CMM ELISA data.xls

Appendix A5. ELISA results for beef

The beef ELISA measurements are shown in file Appendix 5 Beef ELISA data.xls

Appendix A6. ELISA results for pork

The pork ELISA measurements are shown in file Appendix 6 Pork ELISA data.xls

Appendix A7. ELISA results for lamb

The beef ELISA measurements are shown in file Appendix 7 Lamb ELISA data.xls

Appendix A8. ELISA results irradiated meat samples

The irradiated meat ELISA measurements are shown in file Appendix 5 Irr ELISA data.xls

Appendix A9. Summary of time to toxin data for processed meats and meat containing foods.

A summary of time to toxin data for processed meats and meat containing foods is shown in file Appendix 9 Growth below 10 data