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Predicted and observed growth and toxigenesis by *Clostridium botulinum* type E in vacuum-packaged fishery product challenge tests

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Abstract

The observed growth and toxigenesis by *Clostridium botulinum* type E in vacuum-packaged unprocessed, raw pickled and cold-smoked rainbow trout stored at slightly abusive temperatures were compared to predictions generated by two currently available predictive microbiological programs, Food MicroModel and Pathogen Modelling Program. In unprocessed fish there was only a 2 log increase in type E cell count at the time the toxicity first occurred after 2 weeks storage at 8°C. Neither growth or toxin production was observed in raw pickled fish with a NaCl concentration of 6.7% (w/v) during 6 weeks storage at 6°C. In cold-smoked fish with a NaCl level of 3.2% (w/v) toxic samples were detected after 3 and 4 weeks storage at 8°C and 4°C, respectively, without any increase in type E count. Both models were hampered by limitations to controlling environmental factors set by the programs which also had an adverse effect on the reliability of predictions. Most predictions generated by the models were inconsistent with the results observed in the challenge studies. In certain situations, the models seemed to be 'fail-safe', in that, the growth rate predicted from the model was faster or a predicted time to toxicity shorter than that which actually occurred in the food. In other situations, the predictions showed the product to be safe when it was not. The results demonstrate the need for further development and rigorous validation of the models before they are accepted for wider use by inspecting officials and the food industry.

Keywords: *Clostridium botulinum*; Predictive modelling; Fishery products

1. Introduction

Novel foods that are not shelf stable are being manufactured in increasing numbers. Many of these foods rely primarily upon maintenance of proper refrigeration to prevent spoilage and ensure microbiological safety. Traditionally, the risk of growth of pathogenic microorganisms and possible toxin production in foods has been determined through the use of inoculated pack studies. Now, however, there

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are too many products, alternate ingredients, and process variations to conduct a complete laboratory evaluation of each possible contingency and potential foodborne pathogen for each product. Therefore, predictive food microbiology, the modelling of microbial populations, particularly those of foodborne pathogens, has become an active field of research. Whiting and Buchanan (1994) recently proposed a three-level model classification scheme comprised of primary, secondary and tertiary models. Primary level models describe the change in microbial numbers over time and secondary level models indicate how the features of primary models change with respect to one or more environmental factors, such as pH, temperature and a_w. Tertiary level models are personal computer software packages using the pertinent information of primary and secondary level models to generate the desired graphs, predictions and comparisons. Use of these predictive models in the food industry has been warranted by rapid and cost effective provision of useful information for making decisions in many situations. These include product development, prediction of safety and shelf life of products, identifying critical control points in quality control, and planning laboratory tests.

Although relatively rare, the severity of botulism makes *Clostridium botulinum* an important foodborne pathogen. World wide, ~450 botulism outbreaks with 930 incidents are recorded annually, 12% of which are caused by psychrotrophic serotype E (Hatheway, 1995). Outbreaks caused by type E are usually associated with fish and in northern temperate regions, which include Canada, Alaska, Russia and Japan. Common sources are traditionally prepared fermented fish, whale and seal foods of ethnic groups such as North American Eskimos and the Jewish Community. Although a majority of the outbreaks reported have been caused by home-preserved fish, there have been recent increases in the number of type E botulism cases associated with commercially manufactured vacuum-packaged fishery products (Anonymous, 1991; Öberg, 1994; Korkeala et al., 1998). The variety of vacuum-packaged lightly processed novel types of fishery products with long shelf lives has expanded rapidly. These products are developed and released into the market without accurate knowledge about their microbiological safety with respect to *C. botulinum* (Hyytiä et al., 1997; Korkeala et al., 1998). Therefore, it is to the benefit of industry, inspecting officials and consumers to develop mathematical microbiological growth models which could be used to predict how changes in formulations or storage conditions may affect microbial growth. However, current models cannot be used with confidence until their validation in various foods is tested by comparing the predictions to data obtained from inoculated pack studies (Whiting and Buchanan, 1994).

The purpose of the present study was to investigate two currently available modelling software programs to evaluate their ability to determine the safety of different types of vacuum-packaged fishery products with respect to *C. botulinum* type E. By using quantitative PCR, we were able to study the association between *C. botulinum* growth and toxin production at different growth conditions. The study consisted of three inoculated pack studies performed at slightly abusive refrigerated storage temperatures using vacuum-packaged rainbow trout which were either (I) unprocessed, (II) raw pickled or (III) cold-smoked. Raw pickled and cold-smoked products are commercially available.

2. Materials and methods

2.1. Fish

In all inoculation studies rainbow trout (*Oncorhyncus mykiss*) were obtained from a local fish manufacturing plant where they were beheaded and filleted. The fillets had an average weight of 600–900 g.

2.2. Brining

In studies II and III (Table 1) the rainbow trout fillets were brined at a local fish manufacturing plant using the injection method. The pressure used in the brine injection machinery (Fomaco 44/176, Fomaco Food Machinery Company A/S, Køge, Denmark) was 1.6 bar and the concentration of the brine was 21% (w/w). In inoculation study II the fillets were dry-salted overnight at 3°C after curing by the injection method. The waterphase NaCl concentrations of the final products were 6.7±0.9% and 3.2±0.2% (mean±S.D.) in inoculation studies II and

Table 1
Details of *Clostridium botulinum* type E strains and inocula used in challenge studies I, II, and III

Study	Inoculum (cfu/g)[a]	Strain	Origin	Source
I	A: 9.6×10^{-1}	Beluga E	Fermented white whale flippers, Alaska, USA	Dolman/Lindroth[b]
		92 E	Marine environment, Pacific Coast, USA	Eklund/Lindroth[b]
		C-60 E	Dried mutton, Faeroes, Denmark	SSI[c]
	B: 1.2	K-44 E	Rainbow trout, Pellinki, Finland	Our own isolate
		K-45 E	Rainbow trout, Pellinki, Finland	Our own isolate
		K-52 E	Rainbow trout, Jurmo, Finland	Our own isolate
II	2.1×10^{2}	Beluga E	Fermented white whale flippers, Alaska, USA	Dolman/Lindroth[b]
		211 E	Pickled herring, Vancouver, Canada	Dolman/Lindroth[b]
		KA-2 E	Seola Creek strain, USA	Riemann/Lindroth[b]
III	A: 1.4×10^{-1}	Beluga E	Fermented white whale flippers, Alaska, USA	Dolman/Lindroth[c]
	and	211 E	Pickled herring, Vancouver, Canada	Dolman/Lindroth[c]
	B: 4.1	C-94 E	Sealmeat, Greenland, Denmark	SSI[c]

[a]An equal number of spores of each strain were used.
[b]Strains were collected from various sources by Seppo Lindroth (University of California, Davis). The preceding name denotes the person who presumably first isolated the strain.
[c]SSI: Statens Serum Institut, Copenhagen, Denmark.

III, respectively. Before inoculation the fillets were sliced into portions weighing 200±10 g each.

2.3. Sample inoculation

Details of the strains and inocula of *Clostridium botulinum* type E used in studies I, II and III are presented in Table 1. Spore suspensions of individual strains were prepared according to the method of the Food and Agricultural Organization (1991). Before inoculation, the suspensions were enumerated according to Doyle (1991) and surface inoculation of the fish fillets was performed with non-heat-shocked spores as previously described (Hyytiä et al., 1997).

2.4. Processing and storage conditions

The raw pickling process used in study II was performed by slicing and vacuum-packaging fish fillets that had been ripened overnight in dry-salt. In study II, dill and other spices typical of raw pickled fish products were not used in order to minimize uncontrolled factors in the samples. In study III, the inoculated fillets were cold-smoked for 20 h in an electronically controlled, electrically heated smokehouse equipped with an external smoke generator (Vemag; Kerres GmbH, Sulzbach/Murr, Germany) at 18–21°C. In all studies the inoculated fillets were vacuum-packaged (Multivac A 300/16 1986; Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) in polyamide/polyethylene films (Wipak Oy; Nastola, Finland) with an oxygen permeability of 31 $cm^3/m^2/24$ h (23°C, 50% RH) and a water vapour permeability of 1.6 $g/m^2/24$ h (38°C, 90% RH) either immediately after inoculation and/or processing. The samples were stored at 8°C for 5 weeks, at 6°C for 6 weeks and at 4°C or 8°C for 6 weeks in studies I, II and III, respectively. In Finland, a shelf life of 3–4 weeks is usually established for products corresponding to those used in studies II and III.

2.5. Sampling procedures

In inoculation study I, *C. botulinum* type E counts, botulinum toxin, pH and reduction potential (Eh) were analyzed immediately after inoculation and thereafter once a week for 5 weeks from five parallel samples for both inocula for a total of 60 samples. Water activity a_w was determined immediately after inoculation as a composite of five parallel samples for both inocula. In study II, both type E counts and botulinum toxin were analyzed immediately after inoculation and then once a week for 6 weeks from four parallel samples for a total of 28 samples. Four samples were stored for an extended period of 26 weeks and were analyzed for type E count and toxigenesis. NaCl concentration and pH were de-

termined at the time of inoculation from ten and four parallel uninoculated samples, respectively. In study III, type E counts were determined immediately after processing and then weekly for 6 weeks from two parallel samples for each inoculum level and storage temperature for a total of 56 samples. Botulinum toxin was analyzed after 3, 4, 5, and 6 weeks storage from three parallel samples for each inoculum and storage temperature for a total of 48 samples. NaCl concentration and pH were determined at the time of inoculation from five parallel uninoculated samples.

2.6. PCR-detection and quantification

Samples were examined for the presence of *C. botulinum* type E using a quantitative PCR analysis, which was based on a three-tube most probable number (MPN) procedure (Hielm et al., 1996). Briefly, logarithmic dilutions of samples were inoculated into tubes of tryptone-peptone-glucose-yeast extract (Difco, Detroit, MI) broth and incubated anaerobically at 26°C for 3 days. Washed and boiled cells from the overnight cultures were used as a template for PCR. DynaZyme™ DNA polymerase (cloned from *Thermus brochianus*; Finnzymes, Espoo, Finland) and a 96-well PTC-100 thermal cycler (MJ Research, Watertown, MA) were employed. The size of the amplified PCR products was determined by agarose gel electrophoresis with comparison to standard DNA fragments (DNA molecular weight marker VI; Boehringer Mannheim, Mannheim, Germany). Results were reported as the mean counts of parallel samples.

2.7. Toxin analysis

The procedure for the assay of botulinum toxin followed the Nordic Committee on Food Analysis (1991) protocol, with minor modifications. Comminuted fish samples (20–25 g) were homogenised with gelatin phosphate buffer in a ratio 1:2 (w/v) for 2 min in a Stomacher 400 Lab Blender (Seward Medical, London, UK). The homogenate was centrifuged at 10 000–15 000 × *g* for 15 min at 6°C (Sigma 3K 30; Sigma Laborzentrifugen GmbH, Osterode/Harz, Germany). Trypsin activated extracts (1.0 ml) were injected intraperitoneally into two white mice (20–25 g). The mice were observed for botulinal symptoms for 7 days. If death with typical symptoms was recorded for one or both of the mice, the injection was repeated with a heat-treated sample. The bioassays were approved by the Committee on Animal Experimentation of the Faculty of Veterinary Medicine.

2.8. NaCl, pH, reduction potential (Eh) and water activity (a_w) determinations

The waterphase NaCl concentrations were determined according to the method of the Nordic Committee on Food Analysis (1974). Results represent the mean concentrations of parallel samples. pH was determined from homogenates of minced fish and distilled water in a ratio 1:1 (w/v) using a digital Microprocessor pH 537 measuring device (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). The same device was used for the Eh measurements by placing the electrode into the fish flesh immediately after opening the vacuum-package. Results presented were the mean values of parallel samples of each sampling time. A_w was analyzed using minced samples in a Durotherm measuring apparatus (Lufft GmbH, Fellbach, Germany).

2.9. Predictive microbiological models

Two predictive microbiological modelling programs, Food MicroModel version 2.0 (Leatherhead Food Research Association, Leatherhead, Surrey, UK) and Pathogen Modelling Program version 5.0 (USDA Eastern Regional Research Center, Wyndmoor, PA) were used for the generation of growth and time-to-toxicity predictions.

The growth model for non-proteolytic *C. botulinum* types B, E and F by the Food MicroModel program uses temperature, pH, a_w or waterphase NaCl concentration and initial number of organisms as controlling factors. The model does not provide predictions for the lag time of toxin production. The predictions for growth in studies I, II and III were determined by using the controlling factors present in each study with the exception of the initial number of organisms and waterphase NaCl concentration which were set by the limitations of the program. The input values of the controlling factors used for the predictions were in inoculation study I: temperature = 8°C, pH = 6.5 (inoculum A) or 6.4 (inoculum B), $a_w = 0.985$ (inoculum A) or 0.992

(inoculum B), initial number of organisms = 1 log cfu/g (minimum value); in inoculation study II: temperature = 6°C, pH = 6.1, NaCl (w/v) = 4.5% (maximum value), initial number of organisms = 2.3 log cfu/g; and in inoculation study III: temperature = 4°C or 8°C, pH = 5.9, NaCl (w/v) = 3.2%, initial number of organisms = 1 log cfu/g (minimum value).

The lag time predictive model for non-proteolytic *C. botulinum* toxin production in vacuum-packaged raw fish by the Pathogen Modelling Program uses temperature, aerobic plate count (APC) and initial number of organisms as controlling factors and was used in study I. The input values of the controlling factors were: temperature = 8°C, APC = −2 log cfu/g (minimum) or 3 log cfu/g (maximum), initial number of organisms = 2.3 log cfu/sample (inoculum A) or 2.4 log cfu/sample (inoculum B). The result was reported as the time to detectable toxin production. The time-to-turbidity predictive model for non-proteolytic *C. botulinum* type B uses temperature, pH, waterphase NaCl level and initial number of organisms in the food as controlling factors and was used in studies II and III. Limits were set by the program for waterphase NaCl concentration and storage temperature. The input values of the controlling factors in inoculation study II: temperature = 6°C, pH = 6.1, NaCl (w/v) = 4.0% (maximum value), initial number of organisms = 4.6 log cfu/sample; and in inoculation study III: temperature = 5°C (minimum value) or 8°C, pH = 5.9, NaCl (w/v) = 3.2%, initial number of organisms = 1.4 log cfu/sample (inoculum A) or 2.9 log cfu/sample (inoculum B). The result was reported as the time (τ) when the probability of growth reached half of the maximum probability of growth over the entire storage period (inoculation study III) or the lower 95% confidence limit of the tau value (inoculation study II).

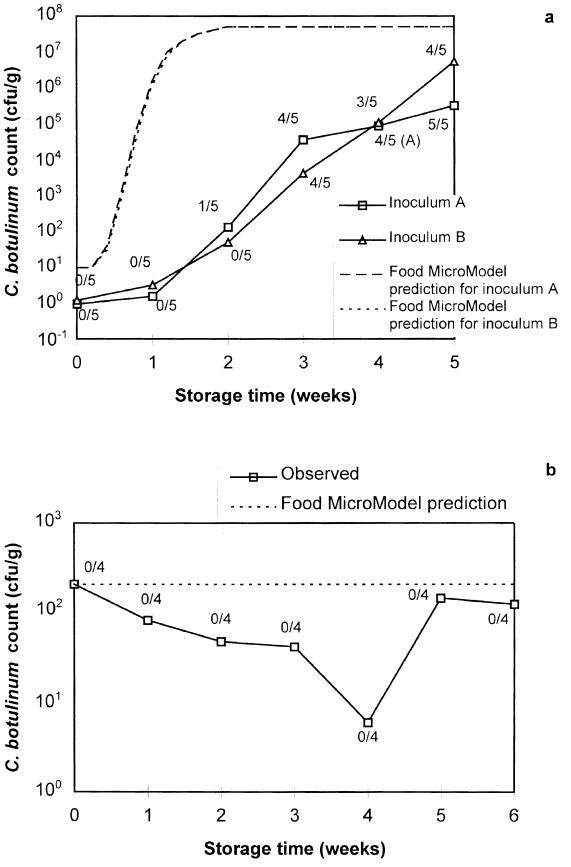

Fig. 1. Predicted growth for *Clostridium botulinum* type E (a) with two low level inocula of A and B in vacuum-packaged unprocessed rainbow trout stored at 8°C for 5 weeks and (b) with a high level inoculum in vacuum-packaged raw pickled rainbow trout stored at 6°C for 6 weeks with observed growth and toxin production. Ratios indicate the number of toxic samples over the number of samples analyzed.

3. Results

3.1. Inoculation study I

Fig. 1a shows the predicted growth for non-proteolytic *C. botulinum* by the Food MicroModel at growth conditions present in inoculation study I and the observed growth of *C. botulinum* type E together with toxin analysis results. The time for detectable toxin production predicted by the Pathogen Modelling Program varied from 5 to 8 days depending on the level of APC used as the controlling factor (minimum 1×10^{-2} cfu/g, maximum 1×10^{3} cfu/g). There was approximately a 2 log increase in *C. botulinum* type E count when the first samples became toxic following 2 weeks storage at 8°C. Samples with inoculum B showed slightly slower growth and lower toxigenesis than samples containing inoculum A. The changes in pH and Eh during 5 weeks of storage at 8°C are presented in Table 2.

Table 2
Clostridium botulinum type E count, toxin production, pH, reduction potential (Eh) and water activity (a_w) in vacuum-packaged unprocessed rainbow trout stored at 8°C for 5 weeks

Inoculum	Storage time (weeks)	*C. botulinum* type E count (cfu/g)	Toxigenesis	pH	Eh (mV)	a_w
A	0	9.6×10^{-1} (2.0)[a]	0/5[b]	6.45 (0.08)[a]	+ 27 (3)[a]	0.985
	1	1.5 (2.1)	0/5	6.53 (0.05)	− 224 (281)	ND[c]
	2	1.3×10^{2} (1.0×10^{2})	1/5	6.24 (0.10)	− 450 (43)	ND
	3	3.6×10^{4} (3.0×10^{4})	4/5	6.60 (0.07)	− 359 (34)	ND
	4	8.6×10^{4} (1.6×10^{5})	4/5	6.74 (0.12)	− 362 (14)	ND
	5	3.2×10^{5} (5.5×10^{5})	5/5	6.95 (0.07)	− 357 (12)	ND
B	0	1.2 (1.5)	0/5	6.41 (0.05)	+ 17 (6)	0.992
	1	3.2 (0.8)	0/5	6.58 (0.08)	− 369 (156)	ND
	2	4.9×10^{1} (3.8×10^{1})	0/5	6.21 (0.10)	− 442 (27)	ND
	3	4.1×10^{3} (2.6×10^{3})	4/5	6.40 (0.08)	− 365 (15)	ND
	4	1.1×10^{5} (2.0×10^{5})	3/5	6.77 (0.08)	− 368 (10)	ND
	5	5.4×10^{6} (1.2×10^{7})	4/5	6.87 (0.08)	− 366 (14)	ND

[a]Standard deviation.
[b]Number of toxic samples/number of samples analyzed.
[c]ND, not determined.

3.2. Inoculation study II

According to the prediction by the Food MicroModel there would be no growth in raw pickled rainbow trout during 6 weeks of storage at 6°C (Fig. 1b). The lower confidence limit of τ was predicted to be 8 days by the Pathogen Modelling Program. The observed *C. botulinum* type E count declined during the first 4 weeks of storage and showed approximately a 2 log increase thereafter, but did not return to the initial inoculation level. No toxin production was detected throughout the storage period. The four samples which were stored for 26 weeks at 6°C were all positive for toxin, with no increase observed in the *C. botulinum* count (50±100 cfu/g; mean±S.D.).

3.3. Inoculation study III

Fig. 2a and b presents the growth of non-proteolytic *C. botulinum* in cold-smoked rainbow trout as predicted by the Food MicroModel and the observed growth at 4°C and 8°C, respectively. A τ of >90 days was predicted by the Pathogen Modelling Program at both storage temperatures. However, toxic samples were observed after 3 weeks storage at 8°C and 4 weeks at 4°C. No increase in *C. botulinum* type E counts was detected at either temperature during 6 weeks of storage even though the Food MicroModel predicted exponential growth after 2 weeks at 8°C.

4. Discussion

Direct measurement of *C. botulinum* growth in foods is difficult, therefore information is scarce concerning the correlation between increase in cell count and toxin production. The quantitative PCR-detection method used in this study allowed for the enumeration of *C. botulinum* type E and the plotting of growth curves. In inoculation study 1, toxigenesis only occurred after a 2–3 log increase in cell count. The observed lag time of 14 days for toxin production at 8°C agrees well with the results of Baker and Genigeorgis (1990) who reported type E toxigenesis after 15 days at 8°C in vacuum-packaged fresh salmon homogenate with an inoculation of 1 cfu/g. The moderately slow growth of type E may have been due to the inhibiting influence of competing microflora. The use of non-heat-shocked spores and surface inoculation instead of deep inoculation may also have retarded the growth. The slightly slower growth and toxigenesis observed with inoculum B may be attributable to strain dependent differences. Inoculum A contained laboratory maintained strains isolated from various sources, while inoculum B consisted of strains isolated recently

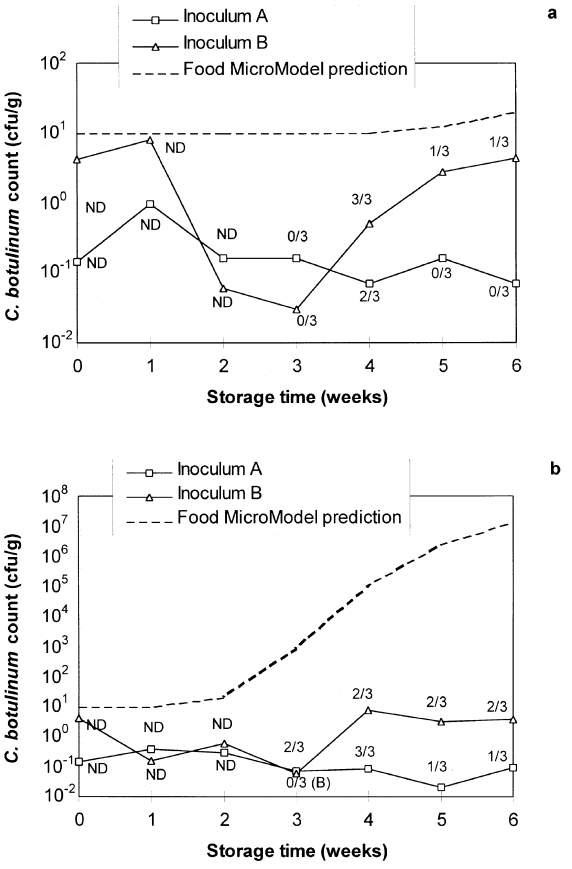

Fig. 2. Predicted growth for low (A) and medium (B) level inocula of *Clostridium botulinum* type E in vacuum-packaged cold-smoked rainbow trout stored at 4°C (a) and 8°C (b) for 6 weeks with observed growth and toxin production. Ratios indicate the number of toxic samples over the number of samples analyzed. ND, not determined.

from rainbow trout. An initial decrease in pH after the first week of storage followed by a constant increase during the remainder of the storage time were observed. The decrease may be attributable to the dissolution of CO_2 into the aqueous environment of the fish flesh and the subsequent increase to the production of volatile basic compounds, such as ammonia, by fish spoilage bacteria (Reddy et al., 1997). Strong reducing conditions prevailed in the unprocessed fish during the entire storage time due to vacuum-packaging and activity of fish spoilage bacteria (Huss, 1979).

That no growth was observed during inoculation study II was expected due to the high NaCl concentration of the product. The maximum NaCl level in brine for the growth of non-proteolytic *C. botulinum* is 5% (Dodds, 1993). When placed in an adverse environment microbial populations decline over time (Whiting and Buchanan, 1994). Interestingly, toxicity occurred when storage time was grossly extended. Although growth and toxigenesis were highly unlikely, in theory, due to the inhibitory NaCl level, growth may have been possible in small pockets of microenvironments created by the uneven distribution of NaCl. The high S.D. of the mean NaCl concentration of raw pickled fish reflected the difficulties that are encountered in the salting of fish fillets which results in significant package to package variation in the NaCl level. In our experience, NaCl concentrations may vary within a single raw pickled fillet by as much as 1.5%. Simultaneous destruction of spores at one location in a sample and germination followed by growth at another location may explain why no increase in *C. botulinum* type E count was detected despite the presence of toxin production in studies II and III. In study III, the combination of NaCl and cold-smoking together with a decrease in storage temperature from 8°C to 4°C extended the lag time for toxin production to 4 weeks as compared to 2 weeks observed in study I. This is in accordance with observations of Garcia et al. (1987) who reported toxigenesis after 21 days at 4°C in vacuum-packaged unprocessed salmon inoculated with a pool of 13 strains of non-proteolytic types B, E and F at level 2 cfu/g.

The generation of predictions for *C. botulinum* type E growth and lag times for toxin production in the three inoculation studies highlighted some limitations associated with the currently used predictive modelling programs. In the Food MicroModel the smallest possible initial number of organisms in food, 10 cfu/g, was unrealistic when considering the natural contamination level of *C. botulinum* type E in fish and fishery products. The highest spore load of any *C. botulinum* serotype found in any food was the type E count of 5.3 cfu/g in Danish farmed trout (Huss et al., 1974). In a recent type E prevalence study conducted by our group in Finland, the highest count detected was 2.7 cfu/g in fresh whitefish (Hyytiä et al., 1998). The vacuum-packaged fishery products, however, had a significantly lower contamination level of 6.0×10^{-2} cfu/g (Hyytiä et al., 1998). Baker and Genigeorgis (1990) recommended an inoculum level of 100 cfu/g for *C. botulinum*

challenge studies in order to create an adequate margin of safety, but they also stated that increased spore loads have the greatest effect on shortening the time to toxin detection at refrigerated temperatures < 8°C. In studies I and III we attempted to simulate as accurately as possible the natural contamination levels present in fish. In study II, a higher inoculation level was used since no growth was expected due to the high NaCl concentration of the product.

Another limitation of the Food MicroModel was that it did not give lag time predictions for toxin production. If a product is considered safe until the growth of *C. botulinum* begins then the predicted and observed results in studies I and III were inconsistent. Based on the prediction, safe storage time for vacuum-packaged unprocessed rainbow trout at 8°C was less than 2 days. However, the first toxic samples did not occur until after 2 weeks of storage and by that time the predicted growth was already in late exponential phase. Many predictive models are designed to be 'fail-safe' which means that the growth rate predicted from the model will be faster or a predicted time-to-toxicity will be shorter than that which actually occurs in the food (Whiting and Buchanan, 1994). In study III, on the other hand, no growth was predicted to occur in vacuum-packaged cold-smoked rainbow trout during 6 weeks storage at 4°C. However, the first toxic samples were observed after 4 weeks. Predictions from all models are most accurate when all experimental factors are close to the midpoint of their overall range. As any factor changes, moving towards its limits, there will be greater variation in the predictions (Whiting and Buchanan, 1994). The confidence limits associated with predictions are markedly increased with increasing response times (McMeekin et al., 1997). In study III both the low storage temperature of 4°C and the moderately high NaCl level of 3.2% contributed to long response time, and thus to an inaccurate prediction.

The main problem associated with the Pathogen Modelling Program was that no appropriate model for *C. botulinum* type E growth and toxin production in fishery products was included. The fish model was applicable only to time-to-toxicity predictions in unprocessed fish. It is worth speculating whether the time for detectable toxin production gained from the fish model would have been more accurate if the program had allowed for higher APCs than the maximum level of 1×10^3 cfu/g. This level is ~ 2 log units too low for most fresh fish (Ikawa and Genigeorgis, 1987; Reddy et al., 1996, 1997). Garcia and Genigeorgis (1987) demonstrated increased lag times for *C. botulinum* when the level of initial microbial population (APC) of the fish was increased. The model for the non-proteolytic type B growth and toxin production was applicable to fishery products, but the predictions of this model cannot be regarded as reliable due to the limitations for temperature and NaCl concentration set by the program. The environmental factors in studies II and III were simply out of the ranges of the model. Additionally, the level of inocula in study III was so low that the model was not valid.

Deviations from predictions, such as those detected in the present study, do not necessarily imply that the model is defective but more likely that knowledge of some food ecosystems is incomplete and factors other than those used in the model development have an effect on microbial behaviour (McMeekin et al., 1997). Most models are developed in broths under constant conditions and do not account for changing environmental variables, such as fluctuating temperatures during distribution and storage, or internal package to package variation in intrinsic factors, such as level and distribution of NaCl. Additionally, models do not incorporate the effects of physiologic status of microbes, the activities of many commonly used antimicrobials, such as sorbates and bacteriocins, or the impacts of microbial competition or time of spoilage (Whiting and Buchanan, 1994; Schaffner and Labuza, 1997). Therefore, the true applicability of currently available models to real life situations is questionable. Extensive research is still needed to further develop a reliable predictive model for *C. botulinum* growth, toxigenesis and nonthermal death with special emphasis on the range of the environmental factors, which should include values expected to be of interest to the model user.

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