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Safety Evaluation of Sous Vide-Processed Products with Respect to Nonproteolytic *Clostridium botulinum* by Use of Challenge Studies and Predictive Microbiological Models

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Sixteen different types of sous vide-processed products were evaluated for safety with respect to nonproteolytic group II *Clostridium botulinum* by using challenge tests with low (2.0-log-CFU/kg) and high (5.3-log-CFU/kg) inocula and two currently available predictive microbiological models, Food MicroModel (FMM) and Pathogen Modeling Program (PMP). After thermal processing, the products were stored at 4 and 8°C and examined for the presence of botulinal spores and neurotoxin on the sell-by date and 7 days after the sell-by date. Most of the thermal processes were found to be inadequate for eliminating spores, even in low-inoculum samples. Only 2 of the 16 products were found to be negative for botulinal spores and neurotoxin at both sampling times. Two products at the high inoculum level showed toxigenesis during storage at 8°C, one of them at the sell-by date. The predictions generated by both the FMM thermal death model and the FMM and PMP growth models were found to be inconsistent with the observed results in a majority of the challenges. The inaccurate predictions were caused by the limited number and range of the controlling factors in the models. Based on this study, it was concluded that the safety of sous vide products needs to be carefully evaluated product by product. Time-temperature combinations used in thermal treatments should be reevaluated to increase the efficiency of processing, and the use of additional antibotulinal hurdles, such as biopreservatives, should be assessed.

The term "sous vide" means "under vacuum" and describes a processing technique whereby freshly prepared foods are vacuum sealed in individual packages and then pasteurized at time-temperature combinations sufficient to destroy vegetative pathogens but mild enough to maximize the sensory characteristics of the product (39, 40). After cooking, the products are chilled, stored refrigerated, and reheated before consumption. Sous vide foods are mainly used in mass catering and restaurants (30). Compared with traditional cooking methods, sous vide has many advantages (40, 42). Economic benefits include better use of labor and equipment through centralized production and extended shelf life due to vacuum packaging, which by excluding oxygen inhibits oxidative processes and growth of spoilage organisms. The shelf life of a sous vide product can be as long as 42 days (42). In addition, the reduced need for preservatives and flavor enhancers, better preservation of vitamins, and retention of most of the original food juices all contribute to higher quality of sous vide foods over conventional meals.

Concerns associated with sous vide processing involve the microbiological safety of the products (40). The psychrotrophic food-borne pathogens and particularly nonproteolytic group II *Clostridium botulinum* bacteria are of concern due to the methods of preparing, distributing, and storing these products. Mild heat treatments in combination with vacuum packaging may actually select for *C. botulinum* and increase the potential for botulism. Sous vide products are generally formulated with little or no preservatives and frequently do not possess any intrinsic inhibitory barriers (pH, a_w, or NaCl) that either alone or in combination would inhibit growth. Therefore, strict adherence to refrigerated storage below 3.3°C must be maintained to ensure the safety of sous vide products with respect to nonproteolytic *C. botulinum* (1). However, the temperature control in chill chains is often inadequate, and temperature abuse is common throughout distribution and retail markets and by consumers (8, 16, 27).

Recent research has identified combinations of mild heat treatment and subsequent refrigerated storage that, when combined with a specified shelf life, provide a defined safety margin with respect to nonproteolytic *C. botulinum* (10, 15, 46). Based on these research results, the Advisory Committee on the Microbiological Safety of Food (1) recommended certain procedures to ensure the safety of refrigerated processed foods of extended durability. According to these recommendations, heat treatments or combination processes should reduce the number of nonproteolytic *C. botulinum* bacteria by a factor of 10^6 (a 6-decimal [6-D] process). However, the capability of a combination process to consistently prevent growth and toxin production by *C. botulinum* in a particular product must be reliably demonstrated.

There are two main approaches that can be used to evaluate the stability and the safety of a product with respect to food-borne pathogens. Traditionally, the effect of thermal processing on pathogenic microorganisms, as well as the risk of their growth and possible toxin production in foods, has been determined through the use of inoculated pack studies. Now, however, there are too many products, alternate ingredients, and process variations to conduct a complete laboratory evaluation of each possible contingency and potential food-borne pathogen for each product. Therefore, predictive food microbiology, the modeling of microbial populations, particularly those of food-borne pathogens, has become an active field of

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TABLE 1. Details of the 16 sous vide-processed products included in the study

Product no.	Ingredient(s)[a]	Size (g)	*P* value[b] (min)	NaCl (% [wt/vol])	pH[c]
1	Pork cubes	1,500	22.9	0.7	6.0–6.3
2	Pork cubes	1,500	153.5	0.7	5.8–6.1
3	Beef cubes	1,500	496.7	0.2	5.8–6.1
4	Beef cubes	1,500	186.7	0.7	5.6–6.1
5	Pork fillet	1,300	10.6	2.0	5.8–6.0
6	Beef roast	1,400	19.6	1.6	5.7–6.1
7	Beef roast	1,500	5.5	1.9	5.6–5.8
8	Ground beef	1,500	0.0	0.2	5.5–5.9
9	Beef liver cubes	1,500	450.8	0.3	6.1–6.3
10	Broiler fillets, marinade	1,000	83.9	1.4	5.9–6.1
11	Rice, vegetables, pork, seafood	1,500	102.0	1.9	5.8–6.0
12	Rice, water, milk	1,500	ND[d] (high)	1.1	6.1–6.7
13	Beef, pork, water, vegetables	1,500	2.5	1.3	4.8–5.1
14	Beef, vegetables, water	1,500	349.2	1.3	4.7–5.3
15	Water, potatoes, beef, vegetables	1,500	370.1	1.0	5.3–5.8
16	Pork, vegetables, water	1,500	118.9	1.0	4.9–5.3

[a] Main ingredients in diminishing order.
[b] *P*, pasteurization value based on the measured temperatures in the product during the processing.
[c] The pH range measured from the samples during the study.
[d] ND, not determined. The measurement did not completely succeed due to the high liquid consistency of the product.

research. Predictive models are equations which can use the information from a large database to predict inactivation or growth of microorganisms under defined conditions. 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 (47).

The present study was performed to evaluate the safety of 16 different types of sous vide-processed products with respect to nonproteolytic *C. botulinum*. The efficiency of thermal processes to inactivate botulinal spores and the subsequent effect of mildly abusive storage temperatures on *C. botulinum* outgrowth and toxigenesis were studied by using inoculated pack studies and two currently available predictive microbiological programs.

MATERIALS AND METHODS

Products. Sixteen sous vide-processed products of various types were evaluated for safety with respect to nonproteolytic *C. botulinum*. The details of the products are described in Table 1. The ingredients of each product were obtained from local processors and were transported to the laboratory in refrigerated vacuum packages at 2°C.

Product inoculation and vacuum packaging. A mixture of five nonproteolytic *C. botulinum* strains was used in the inoculum: three type E strains (31-2570 E, 4062 E, and C-60 E), one type B strain (706 B), and one type F strain (FT 10 F). The strains were of North American and European origin and were isolated from seafood and meat products between the 1960s and the 1980s. The spore suspensions of individual strains were prepared according to the method recommended by the Food and Agricultural Organization (12), and the concentration of each spore suspension was determined as described by Doyle (9). The spore mixture used for inoculation contained an equivalent number of non-heat-shocked spores of each strain diluted in sterile distilled water; 5 ml of dilution contained the required spore load for one sample. Low (2.0-log-CFU/kg) and high (5.3-log-CFU/kg) levels of inoculum were used. The inoculation was performed in vacuum pouches by spraying the spore mixture evenly on the surfaces of solid products or by thoroughly mixing the spore mixture into liquid products. The vacuum pouches (250 by 500 mm) were prepared from nylon-polyethene multiple-layer laminate films (Wipak Oy, Nastola, Finland) with an oxygen permeability of 17 $cm^3/m^2/24$ h/atm (23°C, 50 to 70% rH) and a water vapor permeability of 1.3 $g/m^2/24$ h (23°C). Samples were vacuum packaged (Multivac A 300/16 1986; Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) immediately after inoculation.

Thermal processing. The samples were cooked in a process autoclave (Stock Pilot Rotor 900 G; Hermann Stock Maschinenfabrik GmbH, Neumünster, Germany) by using either full water immersion or water spray circulation cooking, depending on the product. Uninoculated samples were used to probe (Ellab; Ellab A/S, Roedovre, Denmark) measure the core temperature during the process. The temperatures measured at the coolest part of the product as a function of processing time were used to calculate the inactivation ratio (*P*/*t*) for each processing time by using the formula $P/t = 10^{(T - T_{ref})/z}$ (5), where *P* is the pasteurization value (minutes) and *t* is the processing time (minutes) at the actual temperature, *T* (degrees Celsius). T_{ref} is the reference temperature related to pasteurization process. *z* value is the temperature change (degrees Celsius) necessary to change the decimal reduction value (*D*) by a factor of 10. Of the nonproteolytic serotypes, type B has the most heat-resistant spores. Accordingly, the T_{ref} and *z* values used in the present study, 82.2 and 16.5°C ($D = 32.3$ min), respectively, were those for type B (4). *P* values for each product (Table 1) were calculated by integrating the inactivation ratio curve over processing time. After the thermal process, the samples were cooled to 13 to 40°C in the autoclave with cold water either by spraying or by full immersion depending on the method used in the heat process. The final cooling to 2°C was accomplished in cold storage.

Storage conditions and sampling procedures. After processing, samples of each product were stored at 4 and 8°C. The samples were analyzed for the presence of *C. botulinum* type B, E, and F cells and botulinum neurotoxin after the shelf life typically recommended for a corresponding commercially available product and 7 days after that. The analyses were performed with three parallel samples for each storage temperature and inoculum level. pH was analyzed for four parallel inoculated samples immediately after processing and at both sampling times after storage.

Microbiological quality (aerobic plate count [APC], number of sulfite-reducing clostridia, lactic acid bacteria, and yeasts and molds) of selected products (no. 1, 3, 4, 5, 6, 7, 8, 10, and 11) was determined by using uninoculated samples in parallel with sensory evaluation. All analyses were performed on single samples in duplicate. The samples stored at 8°C were examined immediately after processing, in the middle of the recommended shelf life, after the recommended shelf life, and after the safe-storage time predicted by the Pathogen Modeling Program (PMP).

Detection of *C. botulinum*. Twenty grams of each sample was examined for the presence of *C. botulinum* type B, E, and F cells by PCR analysis as described by Hielm et al. (21), with some modifications. The quantification was based on a 1-dilution-level most-probable-number (MPN) series (11). Briefly, 20 tubes containing 10 ml of tryptone-peptone-glucose-yeast extract (Difco, Detroit, Mich.) broth with 625 IU of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml were each inoculated with 1 g of thoroughly homogenized sample. Enrichment cultures were incubated at 26°C in an anaerobic cabinet with an internal atmosphere of 85% N_2–10% CO_2–5% H_2 (MK III; Don Whitley Scientific Ltd., Shipley, United Kingdom) for 3 days. Washed and boiled cells from overnight (18-h) cultures were used as a template for PCR. DynaZyme DNA polymerase (Finnzymes, Espoo, Finland) and a 96-well PTC-100 thermal cycler (MJ Research, Watertown, Mass.) were used. The sizes of the amplified PCR products were determined by agarose gel electrophoresis with comparison to standard DNA fragments (DNA molecular weight marker VI; Boehringer Mannheim GmbH, Mannheim, Germany).

Toxin analysis. The procedure for the assay of botulinum toxin followed the Nordic Committee on Food Analysis protocol (35), with modifications described by Hyytiä et al. (24).

pH analysis. pHs of homogenates of minced sample and distilled water in a ratio of 1:1 (wt/vol) were determined with a digital Microprocessor pH 537

TABLE 2. Detection of nonproteolytic *C. botulinum* types B, E, and F by quantitative PCR analysis in sous vide-processed products with a low inoculum level (2.0 log CFU/kg) after storage at 4 and 8°C until sell-by date and 7 days after sell-by date

Product no. (recommended shelf life in days)	No. of PCR-positive samples/no. of samples analyzed[a]			
	Sell-by date		1 wk after sell-by date	
	4°C	8°C	4°C	8°C
1 (14)	0/3	0/3	0/3	0/3
2 (30)	3/3 (1.19 ± 0.61; E, B)	2/3 (0.83; E)	0/3	0/3
3 (21)	0/3	0/3	0/3	1/3 (0.41; E, B)
4 (14)	0/3	0/3	0/3	1/3 (1.16; B)
5 (10)	3/3 (0.41; E, F)	2/3 (0.60; E)	0/3	0/3
6 (30)	0/3	0/3	0/3	0/3
7 (9)	1/3 (0.41; E)	1/3 (0.41; E)	0/3	0/3
8 (21)	0/3	0/3	0/3	1/3 (0.74; E)
9 (21)	2/3 (0.83; E)	1/3 (0.41; E)	0/3	0/3
10 (21)	0/3	0/3	1/3 (0.41; E)	0/3
11 (21)	1/3 (0.41; E)	0/3	1/3 (0.90; E, B)	1/3 (0.90; B)
12 (21)	0/3	0/3	2/3 (0.41; E)	2/3 (0.72; B, E)
13 (21)	0/3	0/3	0/3	0/3
14 (30)	0/3	0/3	0/3	0/3
15 (21)	3/3 (0.74; E)	1/3 (0.74; F)	0/3	0/3
16 (21)	0/3	0/3	0/3	0/3

[a] Data in parentheses are mean MPN log CFU per kilogram in PCR-positive samples (standard deviation of the cell counts is reported if more than two parallel samples were positive) and the serotype(s) detected, in diminishing order.

measuring device (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany).

Determination of microbiological and sensory quality. The APC, lactic acid bacteria, yeasts and molds, and sulfite-reducing clostridia were determined by the methods of the Nordic Committee on Food Analysis (34, 36–38) by using plate count agar (Difco), MRS agar (Oxoid, Basingstoke, United Kingdom), YGC agar (Difco), and SFP agar base (Difco), respectively.

A trained laboratory panel of 10 judges evaluated the appearance and the aroma of the uninoculated samples heated to a service temperature typical of the product by using a five-point structured category scale (32). Each evaluation contained a marked reference sample that was obtained from a fresh production batch. In addition to a marked reference sample, in each session a fresh reference sample was hidden among the stored samples. A score of 2 on the category scale indicated that there was a "just-detectable" deterioration in sensory quality compared to that of the marked reference, a score of 3 indicated a "clearly detectable but not unacceptable" deterioration, and a score of 5 indicated that the judge had considered the sample unacceptable for human consumption. All samples were evaluated twice, and means of scores were calculated over replicates for each sample.

Predictive microbiological models. Food MicroModel (FMM), version 2.5 (Leatherhead Food Research Association, Leatherhead, Surrey, United Kingdom), was used to generate predictions for the lethal effect of each thermal process on nonproteolytic *C. botulinum* spores. FMM and PMP, version 5.0 (U. S. Department of Agriculture Eastern Regional Research Center, Wyndmoore, Pa.), were used to generate predictions for the safe-storage times after processing with the assumption that all or one part of the spores survived the thermal process.

The thermal death model for nonproteolytic *C. botulinum* type B by the FMM has temperature (80 to 95°C), water-phase NaCl level (0 to 5%), and pH (4.0 to 7.4) as controlling factors. The model predicts the estimated minimum decrease in the number of *C. botulinum* spores. The growth model for nonproteolytic *C. botulinum* types B, E, and F by the FMM has temperature (4 to 30°C), pH (5.1 to 7.5), and water-phase NaCl concentration (0 to 4.5%) as controlling factors. The minimum value for the initial number of organisms is 10 CFU/g. The estimate of the safe-storage time was based on the calculated lag time for growth. The time-to-turbidity predictive model for nonproteolytic *C. botulinum* type B by the PMP uses temperature (5 to 28°C), pH (5.0 to 7.0), water-phase NaCl level (0 to 4.0%), and initial number of organisms in the food (1 to 10^5 CFU/product unit) as controlling factors. The safe-storage time was reported as the lower 95% confidence limit of the tau, which is the time when the probability of growth reached half of the maximum probability of growth over the entire storage period.

The highest pH level measured from the samples of each product during the study and the water-phase NaCl level analyzed by the raw-material suppliers (Table 1) were used as input values in all predictions. The thermal death predictions were based on the lowest temperature recorded at the center of the product, and heating and cooling times were taken into account. Since initial number of organisms is not a controlling factor in the FMM growth model, 5.3 log CFU/kg was used as an input value in growth predictions. PMP time-to-turbidity predictions were generated by using the FMM thermal death model predictions as to the level of surviving spores in each product as an input value for initial number of organisms. In both growth models, 5°C was used as the lower storage temperature to enable the comparison of models.

RESULTS

During storage at 4 and 8°C, a distinct difference between low- and high-inoculum samples was observed, in that substantially fewer samples with a low inoculum were positive for *C. botulinum* in PCR analysis and no toxigenesis was observed (Tables 2 and 3). Storage temperature did not appear to have a considerable effect on the number of PCR-positive samples or on the number of *C. botulinum* bacteria in positive samples at either inoculum level. However, toxigenesis was detected only in samples stored at 8°C (products 1 and 8). Eighty-seven percent of the PCR-positive samples contained serotype E. The prevalence of serotypes B and F in positive samples was 15 and 9%, respectively.

The microbiological quality of the products studied remained unchanged during the storage period at 8°C with APCs being mainly <1.0 log CFU/g. The highest counts (3.0 log CFU/g) were detected in products 5 and 11. The numbers of sulfite-reducing clostridia, lactic acid bacteria, and yeasts and molds were below detectable levels (1.0 log CFU/g) in all products studied. The sensory quality remained good with 4 of the 126 evaluated samples having a mean score of 2 or below, indicating that difference from the fresh reference sample was just detectable in most cases, and all scores being below 3, showing no unacceptable deterioration in examined samples.

Two distinct groups could be discerned among the 16 products subjected to challenge testing. A high-risk group consisted of four products (no. 1, 7, 8, and 13) that had high numbers of *C. botulinum* bacteria in PCR analysis with one or both inoculum levels at both sampling times and/or that showed toxigenesis (Tables 2 and 3). Two products (no. 14 and 16) were designated safe since they were negative for *C. botulinum* cells and botulinum neurotoxin at both sampling times in all different treatment groups. Differences in *P* value, NaCl concentration, and pH were observed between high-risk and safe products (Table 4). The remaining 10 products presented increased

TABLE 3. Detection of nonproteolytic *C. botulinum* types B, E, and F by quantitative PCR analysis in sous vide-processed products with a high inoculum level (5.3 log CFU/kg) after storage at 4 and 8°C until sell-by date and 7 days after sell-by date

Product no. (recommended shelf life in days)	No. of PCR-positive samples/no. of samples analyzed[a]			
	Sell-by date		1 wk after sell-by date	
	4°C	8°C	4°C	8°C
1 (14)	1/3 (0.74; F)	3/3 (1.91 ± 2.01; E)	1/3 (2.18; E)	3/3 (1.93 ± 1.75; E)[b]
2 (30)	3/3 (0.80 ± 0.16; E, B)	3/3 (0.55 ± 0.23; E)	0/3	0/3
3 (21)	0/3	2/3 (1.01; E)	1/3 (0.41; B)	2/3 (0.60; E)
4 (14)	0/3	0/3	0/3	0/3
5 (10)	2/3 (0.41; E, F)	2/3 (0.41; E)	0/3	0/3
6 (30)	0/3	0/3	1/3 (0.41; E)	0/3
7 (9)	2/3 (0.41; E)	1/3 (0.41; E)	3/3 (1.70 ± 1.43; E)	2/3 (1.50; E)
8 (21)	3/3 (1.36 ± 0.79; E)	3/3 (0.97 ± 0.79; E)[b]	0/3	0/3[b]
9 (21)	1/3 (0.90; E, B)	0/3	0/3	0/3
10 (21)	0/3	0/3	0/3	0/3
11 (21)	0/3	0/3	0/3	1/3 (0.41; E)
12 (21)	0/3	0/3	2/3 (1.19; E)	3/3 (0.94 ± 091; E)
13 (21)	2/3 (1.54; E)	1/3 (0.41; E)	3/3 (1.41 ± 1.58; E)	1/3 (1.04; E)
14 (30)	0/3	0/3	0/3	0/3
15 (21)	0/2	0/3	0/3	0/3
16 (21)	0/3	0/3	0/3	0/3

[a] Data in parentheses are mean MPN log CFU per kilogram in PCR-positive samples (standard deviation of the cell counts is reported if more than two parallel samples were positive) and the serotype(s) detected, in diminishing order.
[b] One sample contained botulinum neurotoxin.

botulism risk by being positive in PCR analysis on one or several occasions.

According to the thermal death predictions by the FMM, 5 of the 16 products studied (no. 3, 9, 12, 14, and 15) had a thermal process that appeared to meet the Advisory Committee on the Microbiological Safety of Food requirement of a 6.0-log-unit reduction and would be adequate to eliminate the spores in the high-inoculum samples (Table 5). In seven products (no. 3, 9, 11, 12, 14, 15, and 16), the predicted reduction in spore numbers appeared to be adequate to achieve the 2.0- to 2.3-log-unit reduction required to eliminate the spores in low-inoculum samples. The thermal death predictions agreed well with the measured P values but not with the observed PCR results. The safe-storage times predicted by the FMM were in general substantially shorter than those predicted by PMP (Table 5). According to the FMM prediction, only three products (no. 13, 14, and 16) appeared to be safe within the limits of their recommended shelf life regardless of the storage temperature. According to the PMP, all products were safe at 5°C with the low inoculum level, but only 6 (no. 3, 9, 12, 14, 15, and 16) of the 16 products were safe within the limits of their recommended shelf life if storage temperature and initial number of surviving spores were increased.

TABLE 4. Physical and chemical features of high-risk and safe sous vide products

Physical or chemical feature	High risk[a] (products 1, 7, 8, and 13)	Safe[b] (products 14 and 16)
P value[c]	7.7 (0.0–22.9)	234.1 (118.9–349.2)
NaCl (% [wt/vol])[d]	1.0 (0.2–1.9)	1.2 (1.0–1.3)
pH[d]	5.8 (5.1–6.3)	5.3

[a] Samples strongly positive for *C. botulinum* and/or botulinum neurotoxin at both sampling times.
[b] Samples negative for *C. botulinum* and botulinum neurotoxin at both sampling times.
[c] Means of the lowest values measured for each product and the range.
[d] Means of the highest values measured for each product and the range.

DISCUSSION

Due to the ubiquitous spread of *C. botulinum* in nature (17, 19, 20), contamination of raw ingredients of food products by botulinal spores is possible and even probable. However, the number of spores in different foods has been generally reported to be low (17, 18, 23, 26). The challenge tests of this study were designed to simulate as closely as possible the natural contamination level in foods (low-level inoculum) and to present a worst-case scenario to obtain an adequate margin of safety (high-level inoculum). The results gained from the inoculation studies question the current recommendations for safe processing set out by the Advisory Committee on the Microbiological Safety of Food. Based on the calculated P values, the thermal processes of five products (no. 3, 9, 12, 14, and 15) appeared to be adequate to achieve the 6-D reduction in botulinal spores (criterion: the ratio of P value to processing time required for 6-D reduction being ≥ 1) which is recommended to ensure the safety of refrigerated processed foods of extended durability with respect to nonproteolytic *C. botulinum* (1, 3). However, only one of these products (no. 14) was determined to be safe in challenge tests. The results of the inoculated pack studies revealed that the majority of thermal processes were inadequate to eliminate the spores even with the low inoculum level. The presence of botulinal spores in nonsterile low-acid vacuum-packaged foods must be considered a serious risk due to the high probability of temperature abuse and mishandling of these types of products (8, 16, 27). The botulism risk of sous vide products is additionally increased by the absence of spoilage flora and by the long sensory shelf life which allows toxigenesis before sensory spoilage occurs. However, to our knowledge, sous vide products have not been implicated as a cause of a botulism outbreak so far.

The comparison of high-risk products with safe products pointed out the factors contributing to the increased botulism risk. A low P value seemed to be the most significant single factor increasing the botulism risk in the products. A considerable overlap was observed in NaCl content and pH values between different risk groups, though high-risk products

TABLE 5. Thermal inactivation of nonproteolytic *C. botulinum* spores based on the measured temperatures during the processing of 16 sous vide products as predicted by FMM and safe-storage times as predicted by FMM and PMP

Product no. (recommended shelf life in days)	Predicted reduction in amt of spores (log units)	Lag time for growth by FMM (days)[a]		Time to turbidity by PMP (days) for initial no. of nonproteolytic *C. botulinum* bacteria before thermal processing (log CFU/kg)			
				5°C		8°C	
		5°C	8°C	2.0	5.3	2.0	5.3
1 (14)	NE[b]	7	3	32	<4.4[c]	18	<2.4[c]
2 (30)	1.4	9	3	>42[d]	13	23	7
3 (21)	>12.0	9	3	>90	>90	>90	>90
4 (14)	1.6	9	3	>42[d]	15	22	9
5 (10)	NE	13	5	>38[d]	<4.0[c]	9	<2.3[c]
6 (30)	NE	10	4	>36[d]	<4.0[c]	21	<2.2[c]
7 (9)	NE	19	8	>42[d]	<4.4[c]	25	<2.5[c]
8 (21)	NE	12	5	42	<6.2[c]	24	<3.5[c]
9 (21)	>12.0	7	3	>90	>90	>90	>90
10 (21)	0.5	9	4	>41[d]	48	24	3
11 (21)	2.2	13	5	>90	22	>90	13
12 (21)	>12.0	7	3	>90	>90	>90	>90
13 (21)	NE	>90	60	>52[d]	<7.3[c]	44	<4.5[c]
14 (30)	6.1	>90	>69	>90	>90	>90	>90
15 (21)	>12.0	21	15	>90	>90	>90	>90
16 (21)	3.8	>90	>65	>90	>50[d]	>90	43

[a] The initial number of nonproteolytic *C. botulinum* bacteria before thermal processing was 5.3 log CFU/kg.
[b] NE, no effect; processing temperature was below the limits of the model.
[c] The initial number of organisms was outside the limits of the model.
[d] The result was extrapolated from the probability curve generated by the model.

tended to have slightly lower NaCl concentrations and higher pHs than safe products. The growth and inactivation of microorganisms can be substantially affected by food type (42). The main ingredient of the four high-risk products was meat, while both of the two safe products contained a large amount of vegetables. It has been observed that the high fat content in meat-based foods can increase the heat resistance of microorganisms (2, 22). Moreover, meat contains high amounts of osmoprotectants (e.g., proline, betaine, and carnitine), essential amino acids, and lytic enzymes (lysozyme) which either increase the heat resistance of botulinal spores or facilitate the germination and growth of the heat-injured survivors (42). On the other hand, certain vegetables have been reported to suppress the growth of nonproteolytic *C. botulinum* due to their inherent antibotulinal characteristics such as low pH, inadequate nutrient contents, or antimicrobial compounds (6, 28).

Overall, the results of the PCR analyses indicated that very little growth occurred during the storage. Toxin production by nonproteolytic *C. botulinum* has been shown to occasionally occur with very weak growth or no detectable growth (6, 14, 24, 25). It is also possible that all true *C. botulinum*-positive samples were not detected due to the large size of the samples and the low inoculum levels. Despite thorough homogenization at the time of inoculation and sampling, the spores were probably unevenly distributed. Only a single 20-g aliquot from each 1,000- to 1,500-g sample was examined for the presence of botulinal spores, with the detection limit of the PCR method used being 0.4 log CFU/kg. Some products, on the other hand, were positive for botulinal spores at the first sampling but negative in the second. This correlates with the observation that the number of microorganisms is known to decline over time when placed in an adverse environment (31). None of the intrinsic factors (NaCl and pH) of the products alone was inhibitory to the growth of *C. botulinum*. However, when sublethal levels of NaCl and pH were combined with low storage temperature, the conditions may not have allowed for the survival of heat-injured spores (29).

To our knowledge, the predictive models used in the present study have not been previously validated in sous vide products with respect to nonproteolytic *C. botulinum*. The predictions by the FMM thermal death model were found to be unreliable in the 16 sous vide products studied. The model appeared to give high values for the logarithmic reduction of spores, since spores were observed even in those products with a low inoculum level which were predicted to have 6-D reduction in spore numbers. The FMM growth model predictions cannot be directly compared with the data obtained from the present challenge tests, since the model predicts lag time for growth and the observed results do not give evidence as to when growth began. The PMP time-to-turbidity model appeared to generate long safe-storage times for low-inoculum samples, since spores and/or slight growth was detected in most products. However, with the high inoculum level the model predicted considerably shorter safe-storage times for most products, including those that were considered to exhibit high risk. The failure of both growth models to predict safe-storage times for different types of vacuum-packaged fishery products with respect to *C. botulinum* type E and *Listeria monocytogenes* has been recently reported (7, 25). The poor agreement of predicted and observed results in the above-mentioned studies and in this study was partly due to the limited number of controlling factors in the models. For example, the level and nature of the natural bacterial flora in the products and the product formulation have an effect on growth by food-borne pathogens. The models have been developed in broths under constant conditions and do not account for different changing variables in food products and characteristics of different bacterial strains that affect microbial behavior (41, 47). Additionally, in many cases the levels of the controlling factors in the products studied were simply out of range or operated near the outer limits of those set by the models, which contributed to inaccurate predictions. With these types of products, models should not be used. Instead, safety evaluation should be done by inoculation studies.

The results of the present study indicate that the safety of sous vide products with respect to nonproteolytic *C. botulinum* has to be carefully evaluated product by product. An increase in processing time and temperature would seem a logical solution in view of the difference in the *P* values observed between high-risk and safe products. However, the degree of benefit gained from increased thermal processing is obviously greatly dependent on the type of product. Additionally, adverse effects on sensory and nutritional qualities by increased thermal treatment are the opposite of the original idea of sous vide processing. Another alternative to improve safety would be to add additional hurdles to products. Biopreservatives, such as nisin and organic acids, are known to have an antibotulinal effect (33, 43, 45). However, even a slight change in formulation or processing conditions warrants a safety evaluation by challenge tests since the predictive models available to date appear to frequently provide misleading predictions. Furthermore, use of time-temperature indicators in individual product packages would record the storage history of a product (40, 44) and might lead to enhanced temperature control in chill chains. Additionally, for evaluators to be able to make confident risk assessments and to avoid being unnecessarily overcautious, additional data on the prevalence and numbers of spores of psychrotrophic *C. botulinum* in different categories of sous vide-processed foods is needed (13).

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REFERENCES

1. **Advisory Committee on the Microbiological Safety of Food.** 1992. Report on vacuum packaging and associated processes. Her Majesty's Stationery Office, London, United Kingdom.
2. **Ben Embarek, P. K., and H. H. Huss.** 1993. Heat resistance of *Listeria monocytogenes* in vacuum-packaged pasteurized fish fillets. Int. J. Food Microbiol. **20:**85–95.
3. **Betts, G. D. (ed.).** 1996. Code of practice for the manufacture of vacuum and modified atmosphere packed chilled foods with particular regard to the risk of botulism. Campden & Chorleywood Food Research Association, Chipping Campden, United Kingdom.
4. **Brown, H.** 1990. Evaluation of shelf life for chilled foods. Technical manual no. 28. The Campden Food and Drink Research Association, Chipping Campden, United Kingdom.
5. **Brown, K. L.** 1993. Principles of heat preservation, p. 33. *In* J. A. G. Rees and J. Bettison (ed.), Processing and packaging of heat preserved foods. Blackie Academic & Professional, London, United Kingdom.
6. **Carlin, F., and M. W. Peck.** 1995. Growth and toxin production by nonproteolytic and proteolytic *Clostridium botulinum* in cooked vegetables. Lett. Appl. Microbiol. **20:**152–156.
7. **Dalgaard, P., and L. V. Jørgensen.** 1998. Predicted and observed growth of *Listeria monocytogenes* in seafood challenge tests and in naturally contaminated cold-smoked salmon. Int. J. Food Microbiol. **40:**105–115.
8. **Daniels, R. W.** 1991. Applying HACCP to new-generation refrigerated foods at retail and beyond. Food Technol. **45**(6)**:**122, 124.
9. **Doyle, M. P.** 1991. Evaluating the potential risk from extended-shelf-life refrigerated foods by *Clostridium botulinum* inoculation studies. Food Technol. **45**(4)**:**154–156.
10. **Fernández, P. S., and M. W. Peck.** 1997. 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*. J. Food Prot. **60:**1064–1071.
11. **Finnish Standardization Association.** 1979. MPN technique in microbiological examination of water. Standard no. 4447. Finnish Standardization Association, Helsinki, Finland.
12. **Food and Agricultural Organization.** 1991. Manual of food quality control 12. Food and Agricultural Organization of the United Nations, Rome, Italy.
13. **Gould, G. W.** 1999. Sous vide foods: conclusions of an ECFF Botulinum Working Party. Food Control **10:**47–51.
14. **Graham, A. F., D. R. Mason, F. J. Maxwell, and M. W. Peck.** 1997. Effect of pH and NaCl on growth from spores of non-proteolytic *Clostridium botulinum* at chill temperature. Lett. Appl. Microbiol. **24:**95–100.
15. **Graham, A. F., D. R. Mason, and M. W. Peck.** 1996. Inhibitory effect of combinations of heat treatment, pH, and sodium chloride on growth from spores of nonproteolytic *Clostridium botulinum* at refrigeration temperature. Appl. Environ. Microbiol. **62:**2664–2668.
16. **Harris, R. D.** 1989. Kraft builds safety into next generation refrigerated foods. Food Process. **50:**111–112, 114.
17. **Hauschild, A. H. W.** 1989. *Clostridium botulinum*, p. 111–189. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, New York, N.Y.
18. **Hauschild, A. H. W.** 1992. Epidemiology of human foodborne botulism, p. 68–104. *In* A. H. W. Hauschild and K. L. Dodds (ed.), *Clostridium botulinum*—ecology and control in foods. Marcel Dekker, New York, N.Y.
19. **Hielm, S., J. Björkroth, E. Hyytiä, and H. Korkeala.** 1998. Prevalence of *Clostridium botulinum* in Finnish trout farms: pulsed-field gel electrophoresis typing reveals extensive genetic diversity among type E isolates. Appl. Environ. Microbiol. **64:**4161–4167.
20. **Hielm, S., E. Hyytiä, A.-B. Andersin, and H. Korkeala.** 1998. A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and Baltic Sea sediment samples. J. Appl. Microbiol. **84:**133–137.
21. **Hielm, S., E. Hyytiä, J. Ridell, and H. Korkeala.** 1996. Detection of *Clostridium botulinum* in fish and environmental samples using polymerase chain reaction. Int. J. Food Microbiol. **31:**357–365.
22. **Hobbs, G.** 1976. *Clostridium botulinum* and its importance in fishery products. Adv. Food. Res. **22:**135–185.
23. **Huss, H. H., A. Pedersen, and D. C. Cann.** 1974. The incidence of *Clostridium botulinum* in Danish trout farms. I. Distribution in fish and their environment. J. Food Technol. **9:**445–450.
24. **Hyytiä, E., S. Eerola, S. Hielm, and H. Korkeala.** 1997. Sodium nitrite and potassium nitrate in control of nonproteolytic *Clostridium botulinum* outgrowth and toxigenesis in vacuum-packed cold-smoked rainbow trout. Int. J. Food Microbiol. **37:**63–72.
25. **Hyytiä, E., S. Hielm, M. Mokkila, A. Kinnunen, and H. Korkeala.** 1999. Predicted and observed growth and toxigenesis by *Clostridium botulinum* type E in vacuum-packaged fishery product challenge tests. Int. J. Food Microbiol. **47:**161–169.
26. **Hyytiä, E., S. Hielm, and H. Korkeala.** 1998. Prevalence of *Clostridium botulinum* type E in Finnish fish and fishery products. Epidemiol. Infect. **120:**245–250.
27. **Kalish, F.** 1991. Extending the HACCP concept to product distribution. Food Technol. **45**(6)**:**119–120.
28. **Larson, A. E., E. A. Johnson, C. R. Barmore, and M. D. Hughes.** 1997. Evaluation of the botulism hazard from vegetables in modified atmosphere packaging. J. Food Prot. **60:**1208–1214.
29. **Lund, B. M., and M. W. Peck.** 1994. Heat resistance and recovery of spores of non-proteolytic *Clostridium botulinum* in relation to refrigerated, processed foods with an extended shelf-life. J. Appl. Bacteriol. Symp. Suppl. **76:**115S–128S.
30. **Martens, T.** 1996. The 'sous vide' process, p. 335–345. *In* S. A. Taylor, A. Raimundo, M. Severini, and F. J. M. Smulders (ed.), Meat quality and meat packaging. European Consortium for Continuing Education in Advanced Meat Science and Technology, Utrecht, The Netherlands.
31. **McMeekin, T. A., J. Brown, K. Krist, D. Miles, K. Neumeyer, D. S. Nichols, J. Olley, K. Presser, D. A. Ratkowsky, T. Ross, M. Salter, and S. Soontranon.** 1997. Quantitative microbiology: a basis for food safety. Emerg. Infect. Dis. **3:**541–549.
32. **Meilgaard, M., G. V. Civille, and B. T. Carr.** 1987. Sensory evaluation techniques. CRC Press, Inc., Boca Raton, Fla.
33. **Meng, J., and C. A. Genigeorgis.** 1994. Delaying toxigenesis of *Clostridium botulinum* by sodium lactate in sous-vide products. Lett. Appl. Microbiol. **19:**20–23.
34. **Nordic Committee on Food Analysis.** 1986. Aerobic micro-organisms. Enumeration at 30°C in meat and meat products. Method no. 86, 2nd ed. Nordic Committee on Food Analysis, Espoo, Finland.
35. **Nordic Committee on Food Analysis.** 1991. Botulinum toxin. Detection in foods, blood and other test materials. Method no. 79, 2nd ed. Nordic Committee on Food Analysis, Espoo, Finland.
36. **Nordic Committee on Food Analysis.** 1991. Lactic acid bacteria. Determination in meat and meat products. Method no. 140. Nordic Committee on Food Analysis, Espoo, Finland.
37. **Nordic Committee on Food Analysis.** 1993. Yeasts. Detection in foods. Method no. 149. Nordic Committee on Food Analysis, Espoo, Finland.
38. **Nordic Committee on Food Analysis.** 1994. Sulphite-reducing Clostridia. Determination in foods. Method no. 56, 3rd ed. Nordic Committee on Food Analysis, Espoo, Finland.
39. **Peck, M. W.** 1997. *Clostridium botulinum* and the safety of refrigerated processed foods of extended durability. Trends Food Sci. Technol. **8:**186–192.
40. **Rhodehamel, E. J.** 1992. FDA's concerns with sous vide processing. Food Technol. **46**(12)**:**73–76.
41. **Schaffner, D. W., and T. P. Labuza.** 1997. Predictive microbiology: where are we, and where are we going? Food Technol. **51**(4)**:**95–99.

42. **Schellekens, M.** 1996. New research issues in sous vide cooking. Trends Food Sci. Technol. **7:**256–262.
43. **Scott, V. N., and S. L. Taylor.** 1981. Effect of nisin on the outgrowth of *Clostridium botulinum* spores. J. Food Sci. **46:**117–120.
44. **Skinner, G. E., and J. W. Larkin.** 1998. Conservative prediction of time to *Clostridium botulinum* toxin formation for use with time-temperature indicators to ensure the safety of foods. J. Food Prot. **61:**1154–1160.
45. **Sofos, J. N., F. F. Busta, and C. E. Allen.** 1979. Botulism control by nitrite and sorbate in cured meats: a review. J. Food Prot. **42:**739–770.
46. **Stringer, S. C., D. A. Fairbairn, and M. W. Peck.** 1997. Combining heat treatment and subsequent incubation temperature to prevent growth from spores of non-proteolytic *Clostridium botulinum*. J. Appl. Microbiol. **82:**128–136.
47. **Whiting, R. C., and R. L. Buchanan.** 1994. Microbial modeling. Food Technol. **48**(6)**:**113–120.