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http://hdl.handle.net/1975/766

Inhibition of Growth of Nonproteolytic *Clostridium botulinum* Type B in Sous Vide Cooked Meat Products Is Achieved by Using Thermal Processing but Not Nisin

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ABSTRACT

The safety of refrigerated processed foods of extended durability (REPFEDs) with respect to nonproteolytic *Clostridium botulinum* is under continuous evaluation. In the present study, mild ($P_{85.0}^{7.0}$ values 0 to 2 min [P, pasteurization value; z-value 7.0°C; reference temperature 85.0°C]) and increased ($P_{85.0}^{7.0}$ values 67 to 515 min) heat treatments were evaluated in relation to survival of nonproteolytic *C. botulinum* type B spores in sous vide processed ground beef and pork cubes. The use of two concentrations of nisin in inhibition of growth and toxin production by nonproteolytic *C. botulinum* in the same products was also evaluated. A total of 96 samples were heat processed and analyzed for *C. botulinum* by BoNT/B gene-specific polmerase chain reaction and for botulinum toxin by a mouse bioassay after storage of 14 to 28 days at 4 and 8°C. Predictably, after mild processing all samples of both products showed botulinal growth, and one ground beef sample became toxic at 8°C. The increased heat processing, equivalent to 67 min at 85°C, resulted in growth but not toxin production of *C. botulinum* in one ground beef sample in 21 days at 8°C; in the pork cube samples no growth was detected. The increased heating of both products resulted in higher sensory quality than the milder heat treatment. Nisin did not inhibit the growth of nonproteolytic *C. botulinum* in either product; growth was detected in both products at 4 and 8°C, and ground beef became toxic with all nisin levels within 21 to 28 days at 8°C. Aerobic and lactic acid bacterial counts were reduced by the addition of nisin at 4°C. The study demonstrates that the mild processing temperatures commonly employed in sous vide technology do not eliminate nonproteolytic *C. botulinum* type B spores. The intensity of each heat treatment needs to be carefully evaluated individually for each product to ensure product safety in relation to nonproteolytic *C. botulinum*.

Refrigerated processed foods of extended durability (REPFEDs) are a large group of novel foods including sous vide processed food products. In sous vide technology fresh or freshly prepared foods are vacuum-packed, pasteurized in water autoclaves, and rapidly cooled to chilled temperatures. Typical of sous vide foods is their enhanced sensory and nutritional quality. Hermetic sealing prevents moisture loss, and vacuum packaging and chilled storage allow for extended shelf lives of up to 42 days *(33)*. The sensory quality of these products is due to the minimal processing of raw materials, combined with mild to moderate pasteurization and limited use of salt and other preservatives *(31, 33)*. The microbiological quality and safety of sous vide products relies mainly upon refrigerated storage. The pasteurization temperatures applied in sous vide technology are typically 65 to 95°C. These temperatures are sufficiently high to destroy most vegetative bacterial cells but mainly too low to destroy bacterial spores. Of most concern in these products is the survival, outgrowth, and toxin production by *Clostridium botulinum* (11, 28). The growth of *C. botulinum* is supported by the absence of oxygen and of a competing microflora, in combination with a high water

activity. Moreover, nonproteolytic *C. botulinum* has been reported to grow and produce toxin at a temperature as low as 3.0°C *(12)*.

Outlines for the safe processing of REPFEDs in relation to nonproteolytic *C. botulinum* have been proposed by the Advisory Committee on the Microbiological Safety of Foods in 1992 *(1)* and the European Chilled Food Federation (ECFF) in 1996 *(7)*. If the product shelf life exceeds 10 days, a heat treatment of 90°C for 10 min or of equivalent lethality followed by chilled storage should be achieved *(1, 7)*. Such a heat treatment is expected to result in a 6-log reduction in the initial spore count (a $6D$ process), thus ensuring the safety of these food products without the use of other controlling factors. According to the proposed equivalent criteria set by the ECFF *(7)*, corresponding to the 90°C for 10 min process are 52 min at 85°C or 270 min at 80°C; the Advisory Committee on the Microbiological Safety of Foods, on the other hand, reports effective heat treatments to be 36 min at 85°C, 129 min at 80°C, 464 min at 75°C, or 1,675 min at 70°C *(1)*. This is because ECFF applied a z-value of 7°C, whereas the Advisory Committee on the Microbiological Safety of Foods based their calculations on a z-value of 9°C. In the light of the wide variety in equivalence criteria the pasteurization processes employed in the manufacturing of REPFEDs

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should be carefully evaluated and their safety ensured. High processing temperatures are widely considered to reduce the sensory quality of these foods, and much milder heat treatments are thus often used *(14)*. Additional safety factors such as chilled storage at a temperature below 3°C, water activity <0.97, pH <5, a restricted shelf life, or a combination of these factors are strongly recommended *(1, 7)*. If milder heat treatments are used, evidence must be provided that the safety of the food product with respect to nonproteolytic *C. botulinum* is ensured by other preservation factors *(1, 4, 7)*. Due to the increasing number of new products and alternate ingredients, challenge testing by inoculated pack studies has increasingly been replaced by predictive modelling, which is more economical and less time consuming. Some of the predictions gained from these models, however, have been shown to be inconsistent with the results of inoculated pack studies *(16)*.

Some preservatives have been shown to inhibit the growth of psychrotrophic bacteria in sous vide foods. Sodium lactate delayed toxin production by nonproteolytic *C. botulinum* in beef, chicken, and salmon *(21)*. Natural antimicrobials such as nisin have been shown to possess anticlostridial activity and to extend the shelf life of dairy products at levels of 5 to 500 IU/g *(2, 5, 20, 27, 34, 35, 37)*. The Joint Food and Agricultural Organization-World Health Organization Expert Committee accepted nisin as a food additive in 1969, but in many countries its use is permitted chiefly in dairy products. The potential of nisin as an additional preservative hurdle in sous vide processed foods needs further evaluation.

In a previous safety evaluation of 16 sous vide food products, we demonstrated the necessity of challenge testing by inoculated pack studies and showed that most heat treatments employed in manufacturing of these products were insufficient to destroy the nonproteolytic *C. botulinum* *(17)*. In the same study, only two products were regarded as safe, i.e., were found to show neither growth nor toxin production by nonproteolytic *C. botulinum* during storage at slightly abused temperatures of 4 and 8°C. All other products yielded growth at one or more sampling times and storage temperatures, and two meat products were toxic at the end of the storage period. The heat treatments employed in the manufacturing of these two products were estimated to result in a less than 1-log reduction in the botulinal spore count as compared to the references set by ECFF *(7)*.

The aim of the present challenge study was to evaluate the lethality of heat treatments calculated to correspond to a 6D process *(7)* with respect to nonproteolytic *C. botulinum* type B in sous vide processed ground beef and pork cubes (inoculation study I). The effect of increased heating on the sensory quality of the two products was also studied. Another aim was to study the effect of two concentrations of nisin on the growth and toxin production by nonproteolytic *C. botulinum* type B in the same products (inoculation study II), treated by a mild pasteurization process.

MATERIALS AND METHODS

Sous vide products. The sous vide products were ground beef and pork cubes. The diameter of each pork cube was approximately 2 cm. The ingredients of both products were obtained from local processors and were transported to the laboratory in vacuum packages at 2°C. The ingredients were lightly browned on an industrial processing line covered with teflon surface without the use of fat or other additional ingredients. The mean pH of the ground beef was 6.0 and that of the pork cubes 6.2. The NaCl concentration (wt/vol) was determined by the supplier by Mohr's method *(18)* and was 0.2% in the ground beef and 0.7% in the pork cubes. The final size of each sample was 1.5 kg.

Inoculation with *C. botulinum* strains and packaging. The inoculum consisted of a mixture of nonproteolytic type B *C. botulinum* strains (Eklund 2B, Eklund 17B, and Hatheway 706B in study I; Eklund 2B and Eklund 17B in study II). The spore suspensions of each strain were prepared according to the instructions of the Food and Agricultural Organization *(10)* and enumerated by the most probable number (MPN) method as described by Doyle *(6)*. The inocula consisted of equivalent numbers of non-heat-shocked spores of each strain diluted in sterile distilled water, with a final inoculation level of log 5.3 CFU/kg in an inoculation volume of 5 ml. The samples were placed in vacuum pouches, and the inoculum was sprayed evenly on the surfaces of pork cubes or thoroughly mixed into the ground beef. Vacuum pouches (250 by 500 mm) were made from nylon-polyethene multiple layer laminate films (Wipak Oy, Nastola, Finland) with an oxygen permeability of 17 cm³/m²/24 h/atm (23°C, 50 to 70% relative humidity) and a water vapor permeability of 1.3 g/m²/24 h (23°C). All samples were vacuum packaged (Multivac A 300/16 1986; Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) immediately after bacterial inoculation and the addition of nisin if applicable. Uninoculated samples were prepared for temperature measurements, microbiological analysis, and sensory evaluation.

Nisin. A solution containing 150,000 IU/ml of nisin (Nisaplin 1,000,000 IU/kg; Aplin & Barrett Ltd., Beaminster, Dorset, UK) was prepared in 0.02 M HCl and stored at 4°C for 24 h. Nisin (250 IU/g or 500 IU/g) was added to the products immediately before inoculation with *C. botulinum* spores. Products prepared without nisin served as controls.

Thermal processing. All samples were cooked in a process autoclave (Stock Pilot Rotor 900 G; Hermann Stock Maschinenfabrik GmbH, Neumünster, Germany), using water spray circulation cooking for the ground beef and full water immersion for the pork cubes. After thermal processing the samples were cooled in the autoclave for 1 h, using cold water (5°C) either by spraying or by full immersion according to the method used in the heat process. Final cooling to 2°C was accomplished in a cold store with the same temperature.

The core temperature of the products throughout cooking was measured with a temperature probe (Ellab; Ellab A/S, Roedovre, Denmark) in two uninoculated samples, and the data were used to calculate the inactivation ratio (P/t) for each processing time. The equation used was $P/t = 10^{(T-T_{ref})/z}$ *(3)*, where P is the pasteurization value (min) and t is the processing time (min) at the actual temperature T (°C). T_{ref} is the reference temperature related to the pasteurization process. The z-value is the change in temperature (°C) required to yield a 10-fold change in the thermal processing time ($D_{T_{ref}}$). The T_{ref}, z, and $D_{85°C}$ values used in the present study, 85°C, 7°C, and 8.7 min, respectively, were those recommended by the ECFF *(7)*. Pasteurization values ($P_{85.0}^{7.0}$, where 7.0 denotes the z-value and 85.0 denotes the reference temperature) for each product during each heat process were calculated by integrating the inactivation ratio curve over the processing time. The $P_{85.0}^{7.0}$-value describes the lethality of a heat process

FIGURE 1. *Temperature curves of mild* ($P_{85.0}^{70}$*-values 0 to 2 min) and increased* ($P_{85.0}^{70}$*-values 67 to 515 min) heat treatments employed for sous vide processed ground beef and pork cubes.* —◆—, *Mild heat treatment, ground beef;* —■—, *increased heat treatment, ground beef;* —▲—, *mild heat treatment, pork cubes;* —×—, *increased heat treatment, pork cubes.*
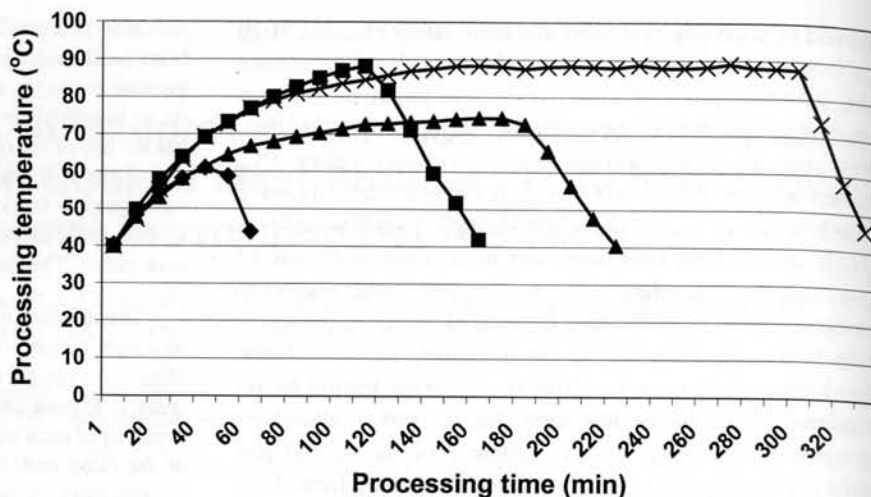
as the equivalent time (min) at the reference temperature (85°C) *(3)*.

In study I, mild heat treatments ($<1D$) were compared to the increased heat treatments ($>6D$) recommended by the ECFF *(7)*. During mild heat treatment, the product core temperatures reached 62.3 and 74.7°C in the ground beef and pork cubes, respectively (Fig. 1). The calculated $P_{85.0}^{70}$-value was 0 min for the ground beef and 2 min for the pork cubes. During the increased heat process, the product core temperature in ground beef reached 88.3°C, whereas the maximum core temperature in the pork cubes was 89.9°C. The corresponding $P_{85.0}^{70}$-value for the ground beef was 67 min and that for the pork cubes 515 min (Fig. 1). Thus, the increased heat treatments employed for both products exceeded the recommended reference heat treatment, the $P_{85.0}^{70}$-value of which is 52 min *(7)*. In study II, the above-mentioned mild heat processes were used for both products, in order to detect the possible effect of nisin.

Storage conditions and sampling procedures. The processed samples were stored at 4 and 8°C. The presence of *C. botulinum* and botulinum toxin were analyzed from three parallel samples stored at both temperatures for 21 days (ground beef) or 14 days (pork cubes) after processing in both inoculation studies, as well as 7 days later in study II. In study II, the microbiological quality of the samples was determined at the same time as the samples were analyzed for *C. botulinum*. In inoculation study I, sensory evaluation of uninoculated samples was carried out 1 day after processing and at the end of the storage period. In both studies the pH was measured (Microprocessor pH 537; Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) from each

sample 1 day after processing. Twenty-five grams of each sample was homogenized with distilled water (vol/vol 1:1) for the measurement.

Detection of *C. botulinum* and microbiological analysis. All samples were examined for the presence of *C. botulinum* type B by polymerase chain reaction (PCR) analysis *(15)* based on the detection of the botulinum neurotoxin type B gene. This method allows detection of both spores and vegetative cells. In brief, 20 1-g samples from each sample were each transferred into 10 ml of Trypticase-peptone-glucose-yeast extract broth containing 625 IU/ml of lysozyme *(19)* and incubated anaerobically at 26°C for 72 h. The cells from 1-ml samples of overnight cultures were washed and heated and used as a template in the PCR reaction. A 96-well PTC-100 thermal cycler (MJ Research, Watertown, Mass.) and DynaZyme DNA polymerase (Finnzymes, Espoo, Finland) were employed. The size of the amplified PCR products was determined in agarose gels by comparison with standard DNA fragments (DNA molecular weight marker VI; Boehringer Mannheim, Mannheim, Germany). Quantification was obtained by one-dilution MPN technique using a 20-tube standard *(36)*. If all 20 tubes were positive in the PCR analysis, the estimated quantity of *C. botulinum* present in the sample was determined as greater than the MPN estimate for a sample yielding 19 positive tubes doubled, corresponding to a cell count of log MPN >2.5 CFU/kg. A cell count of log 2.5 CFU/kg was used in calculating the mean cell counts (Tables 1 and 2). Similarly, when all tubes were negative in the PCR analysis, the estimated cell count was determined as less than half of the MPN estimate for a sample yielding one positive tube, corresponding to log MPN <0.1 CFU/kg. A cell

TABLE 1. *PCR detection and toxigenesis of nonproteolytic* C. botulinum *type B in two sous vide processed meat products cooked by mild ($P_{85.0}^{70}$ values 0 to 2 min) and increased ($P_{85.0}^{70}$ values 67 to 515 min) heat treatments in inoculation study I*

Product	Storage time (days)	Storage temperature (°C)	Mild		Increased	
			PCR[a]	Toxigenesis[a]	PCR	Toxigenesis
Ground beef	21	4	3/3 (1.9; 1.6–2.2)[b]	0/3	0/3 (0.1)	0/3
		8	3/3 (2.5)	3/3	1/3 (0.2; <0.1–0.4)	0/3
Pork cubes	14	4	3/3 (1.7; 1.5–1.8)	0/3	0/3 (0.1)	0/3
		8	3/3 (2.4; 2.2–>2.5)	0/3	0/3 (0.1)	0/3

[a] Number of positive samples/number of all samples analyzed.
[b] Mean log MPN CFU/kg; minimum and maximum values for cell count.

TABLE 2. *PCR detection and toxigenesis of nonproteolytic* C. botulinum *type B in two sous vide processed products treated by mild heat treatment (*$P^{70}_{85.0}$ *values 0 to 2 min) and two concentrations of nisin in inoculation study II*

			Nisin level (IU/g)					
			0		250		500	
Product	Storage time (days)	Storage temperature (°C)	PCR[a]	Toxigenesis[a]	PCR	Toxigenesis	PCR	Toxigenesis
Ground beef	21	4	3/3 (1.3; 0.9–1.6)[b]	0/3	3/3 (1.7; 1.6–1.8)	0/3	3/3 (1.6; 1.3–1.7)	0/3
		8	3/3 (1.9; 1.5–2.2)	1/3	2/3 (1.2; <0.1–2.2)	0/3	3/3 (1.4; 0.4–2.1)	0/3
	28	4	2/3 (0.8; <0.1–1.6)	0/3	3/3 (1.1; 0.7–1.7)	0/3	3/3 (1.3; 1.0–1.6)	0/3
		8	3/3 (1.8; 1.3–>2.5)	3/3	3/3 (2.1; 1.9–>2.5)	3/3	3/3 (2.2; 1.9–>2.5)	3/3
Pork cubes	14	4	1/3 (0.4; <0.1–0.9)	0/3	0/3 (0.1)	0/3	2/3 (0.9; <0.1–1.4)	0/3
		8	2/3 (0.9; <0.1–1.8)	1/3	1/3 (0.6; <0.1–1.5)	0/3	1/3 (0.4; <0.1–0.9)	0/3
	21	4	3/3 (0.9; 0.4–1.5)	0/3	3/3 (0.8; 0.4–1.2)	0/3	1/3 (0.3; <0.1–0.7)	0/3
		8	2/3 (1.0; <0.1–1.9)	0/3	3/3 (1.0; 0.7–1.3)	0/3	3/3 (1.2)	0/3

[a] Number of positive samples/number of all samples analyzed.
[b] Mean log MPN CFU/kg; minimum and maximum values for cell count.

count of log 0.1 CFU/kg was used in calculating the mean cell counts (Tables 1 and 2).

The presence of botulinum neurotoxin in the samples was analyzed by the mouse bioassay, according to the Nordic Committee on Food Analysis *(23)*. The results from the PCR and toxin assays were analyzed by using the chi-square test and Fisher's exact test. In both studies, three parallel samples per each heat treatment, storage temperature, storage time, and nisin level, if applicable, were analyzed.

In study II, the microbiological quality of both products, including the aerobic plate count, number of sulfite-reducing clostridia, lactic acid bacteria, and yeast and molds was determined according to the Nordic Committee on Food Analysis *(22, 24–26)*. A total of three parallel samples were analyzed for each nisin level, storage temperature, and storage time.

Sensory evaluation. In study I, the sensory quality of uninoculated samples treated by mild or increased heat processes was evaluated by a trained 10-member panel using a quantitative profiling method. The evaluation was performed 1 day after processing and at the end of the storage period at 4°C. Prior to evaluation,

FIGURE 2. *Effect of mild (*$P^{70}_{85.0}$*-values 0 to 2 min) and increased (*$P^{70}_{85.0}$*-values 67 to 515 min) heat processing on sensory quality of sous vide processed ground beef stored at 4°C for 21 days.* —●—, *Mild heat treatment;* —■—, *increased heat treatment;* ***P < 0.001; *P < 0.05.

the samples were heated in ceramic containers in an oven so that the core temperature of each sample reached 70°C. Immediately after heating, when the product temperature was approximately 65°C, the evaluation was performed in duplicate by using separate sample packages. The sensory profile applied to describe the sensory quality of these meat products comprised the qualities of brownness, grayness, aroma, flavor (0 = weak, 10 = strong), tenderness (0 = tough, 10 = tender), and juiciness (0 = dry, 10 = juicy). The intensities of the attributes were rated on 10-unit scales using a computerized sensory data collection system (Panel 5, version 4.1; Legolas, Espoo, Finland). The means of attributes were calculated and the statistical significance of the differences between samples treated by mild and increased heating was tested with a three-way analysis of variance, taking into account the heat treatment, storage time, and duplicate. Because storage time had no effect on sensory quality, the results are presented in the form of figures showing only the effect of heat treatment at the end of the storage time (Figs. 2 and 3).

RESULTS

Inoculation study I. After the mild heat treatment, *C. botulinum* was detected by PCR technique in all samples of both products (Table 1). In addition, all ground beef samples stored at 8°C were toxic. The mean *C. botulinum* count in all samples varied between log MPN 1.7 and 2.5 CFU/kg. After the increased heat processing, one sample of ground beef still contained *C. botulinum*, with an MPN estimate for the cell count of log 0.4 CFU/kg; in the pork cube samples *C. botulinum* was not detected. Toxigenesis was not observed in either product after the increased heat processing.

Ground beef samples with increased heating had a browner and less gray appearance, they were more tender and juicier, and had a more intense aroma and flavor than the samples processed by the mild heat treatment, at both sampling times (Fig. 2). Similar differences were observed in the pork cubes: tenderness and juiciness were found to be intensified by the increased heat processing (Fig. 3). Storage time did not affect the sensory quality of either product.
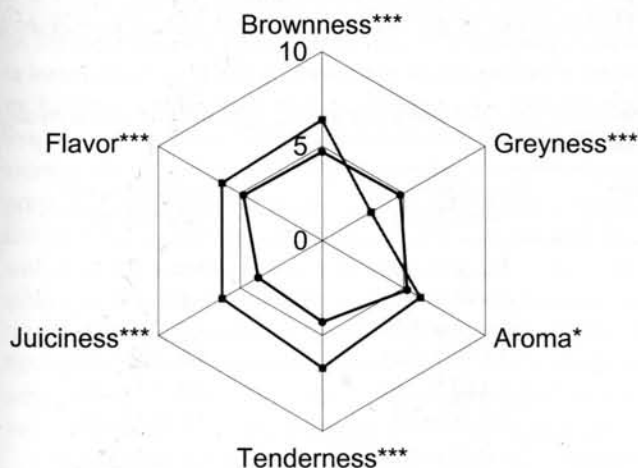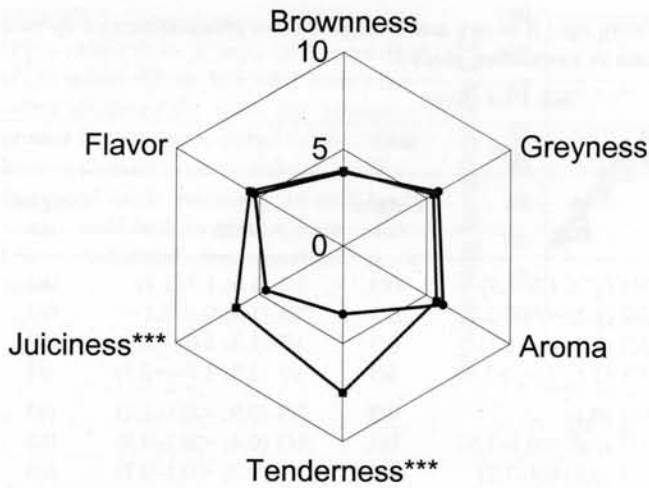
FIGURE 3. Effect of mild ($P_{85.0}^{7.0}$-values 0 to 2 min) and increased ($P_{85.0}^{7.0}$-values 67 to 515 min) heat processing on sensory quality of sous vide processed pork cubes stored at 4°C for 14 days. —●—, Mild heat treatment; —■—, increased heat treatment; ***P < 0.001.

Inoculation study II. Neither of the nisin concentrations inhibited the growth of nonproteolytic C. botulinum in either of the two products (Table 2). In the case of the ground beef, 23 nisin-treated samples (96%) and 11 (92%) control samples without nisin contained C. botulinum, regardless of storage time, temperature, or nisin concentration. All ground beef samples stored at 8°C for 28 days and one sample stored at 8°C for 21 days were toxic. The mean C. botulinum count in the ground beef varied between log MPN 0.8 and 2.2 CFU/kg. The individual cell count in samples containing botulinum toxin varied between log 1.3 and >2.5 CFU/kg.

In comparison to the ground beef, fewer samples of pork cubes were positive for C. botulinum by the PCR and toxin assay at both nisin concentrations and at both storage temperatures (Table 2). Fourteen (58%) nisin-treated samples and eight (67%) control samples were positive for C. botulinum type B. One control sample stored at 8°C for 14 days was toxic. The mean botulinal count varied between

log MPN 0.1 and 1.2 CFU/kg. The individual botulinum count in the toxic sample was log 1.8 CFU/kg.

By the end of the storage period, the mean aerobic plate count reached log 5.6 CFU/g in the ground beef and log 1.0 CFU/g in the pork cubes (Table 3). Lactic acid bacterial counts reached log 5.4 CFU/g in the beef and log 0.6 CFU/g in the pork. The number of sulfite-reducing clostridia and yeast and molds in both products were below the detection limit (<log 1 CFU/g) throughout the storage period. At 4°C the mean log counts of aerobic and lactic acid bacteria in ground beef were log 2.9 to 4.3 CFU/g higher in the control samples than in the nisin-treated samples. At 8°C the difference was minor. The microbiological quality of pork cubes did not change during storage at either temperature.

DISCUSSION

The mild processing used in both inoculation studies was clearly insufficient to destroy nonproteolytic C. botulinum type B spores. A total of 68 (81%) samples processed by the mild heat treatments in studies I and II contained C. botulinum, and toxin production occurred in 14 samples (17%) (Tables 1 and 2). The increased heat treatments used in the present study were expected to result in complete destruction of the inoculated spore count, which was log 5.3 spores/kg. However, after the increased heat treatment of ground beef with a $P_{85.0}^{7.0}$-value of 67 min and estimated lethality of 7.7D, C. botulinum was still detected in one sample at 8°C, because growth was observed in 1 tube out of 20, corresponding to an MPN estimate of log 0.4 CFU/kg. The calculated reduction achieved by this heat treatment was thus only 4.9D. This indicates that not all of the recommended pasteurization processes (7) ensure a 6-log reduction in the spore count in ground beef. This is in agreement with earlier studies (9, 30). Additional controlling factors are needed to ensure the product safety. In these ground beef samples, the growth of nonproteolytic C. botulinum may be allowed by the pH and the low NaCl concentration. Such a product must be stored uninterruptedly below 3°C. However, due to the likelihood that this storage temperature

TABLE 3. Aerobic plate counts and lactic acid bacterial counts determined in sous vide processed ground beef and pork cubes treated by mild heat treatment ($P_{85.0}^{7.0}$ values 0 to 2 min) and two concentrations of nisin

Product (storage time)	Nisin concentration (IU/g)	Storage temperature (°C)	Aerobic plate count (log CFU/g)	Lactic acid bacteria (log CFU/g)
Ground beef (24 days)	0	4	5.6 (4.4–6.7)	5.5 (4.3–6.8)
		8	5.4 (4.1–6.7)	5.4 (4.1–6.8)
	250	4	2.9 (2.6–3.2)	2.7 (2.3–3.1)
		8	3.1 (0.7–5.4)	3.0 (0.5–5.5)
	500	4	1.3 (0.5–2.6)	2.0 (1.7–2.3)
		8	4.6 (1.3–7.9)	4.8 (1.8–7.8)
Pork cubes (14 days)	0	4	1.0 (0.5–2.0)	0.6 (0.5–0.6)
		8	0.5	0.5
	250	4	0.5	0.5
		8	0.5	0.6 (0.5–0.6)
	500	4	0.8 (0.7–1.7)	0.6 (0.5–0.6)
		8	0.5	0.5

will be exceeded at the retail and consumer level, the shelf life of such product should be restricted. Following the pasteurization process with a $P_{85.0}^{7.0}$-value of 515 min, *C. botulinum* was not detected in any pork cube sample, and none of these samples contained botulinum toxin. This was expected, because the estimated lethality of this process greatly exceeded the $6D$ concept.

According to a previous study *(8)*, heating log 6 spores of nonproteolytic *C. botulinum* at 85°C for 23 min resulted in growth and toxigenesis at 8°C in the absence of lysozyme within 87 days *(8)*. In the presence of lysozyme, after heating the same number of nonproteolytic spores at 85°C for 18.1 *(13)*, 19 *(29)*, and 57.8 min *(9)*, growth and toxin production at 8°C were observed after incubation of 64, 53, and 75 days, respectively. Moreover, heating at 85°C for 19 min yielded growth at 12°C in 42 days *(29)*. This heat treatment combined with storage below 12°C and a shelf life of not more than 28 days was considered to reduce the risk of growth from spores of nonproteolytic *C. botulinum* by a factor of log 6 *(29)*. In the present study, after heating log 5.3 spores at 85°C for 67 min, nonproteolytic *C. botulinum* type B was detected at 8°C within 21 days, although toxin production was not observed. Whether the positive result in the PCR analysis was due to spores or vegetative cells is not known. However, in the light of previous studies *(8, 13, 29)*, if the shelf life of this product is extended or if the product is stored at an abused temperature, it is highly likely that toxin production will occur. The present result was obtained in samples corresponding to industrial sous vide products in relation to their size, packaging, and processing, whereas in the earlier experiments the spores were heated in test tubes containing meat medium *(8, 13, 29)*. It is essential that the heat inactivation of spores from nonproteolytic *C. botulinum* is also evaluated under the true conditions prevailing for an actual product. This necessitates challenge testing and supports the earlier observation *(16)* as to the importance of inoculated pack studies, performed in parallel with predictive models that are mainly based on extensive in vitro test series.

In study II, nisin did not appear to inhibit the growth or toxin production by nonproteolytic *C. botulinum* type B in either product (Table 2). Nisin has been reported to show its highest stability and heat resistance in the presence of low fat and protein, under acidic conditions, and at low storage temperatures *(32, 35)*. According to previous reports, it is ineffective at a pH >7 *(35)*. In the two products investigated in the present study the pH was 6.0 and 6.2, and the protein content was relatively high. It is thus possible that nisin had decreased activity in both products, as no inhibition of botulinal growth was observed. Whether the value of nisin in ensuring the safety of meat products with regard to nonproteolytic *C. botulinum* could be improved by acid treatment warrants further investigations.

In accordance with a previous report on nonproteolytic *C. botulinum* types B, E, and F in sous vide products *(17)*, the storage temperature had a marked effect on the botulinum toxin production: a total of 13 ground beef samples from both studies and 1 pork cube sample in study II showed toxigenesis after insufficient heat treatment and storage at a slightly abused temperature of 8°C, whereas none of the samples became toxic after storage at 4°C (Tables 1 and 2). More emphasis should thus be put on the control of storage temperature of sous vide products with long shelf lives.

In study II, high numbers of aerobic and lactic acid bacteria were detected in ground beef (Table 3). This indicates that the mild heat treatment employed for ground beef was not only insufficient to destroy spores of *C. botulinum* but also too mild to eliminate the vegetative bacteria. In the pork cubes the number of aerobic and lactic acid bacteria remained under the detection limit until the end of storage. The addition of nisin at concentrations of 250 and 500 IU/g appeared to reduce the mean number of aerobic and lactic acid bacteria by a factor of log 3 to 4 in ground beef at 4°C. The same influence could be observed at 8°C, but the reduction in the aerobic and lactic acid bacterial counts was less obvious.

Sensory evaluation in study I revealed marked differences in both products and throughout the storage period between samples processed by the mild and increased heat treatments (Figs. 2 and 3). Aroma, flavor, tenderness, juiciness, and brownness were considered more intense in the samples processed by the increased heat treatment. All these changes can be regarded as beneficial in meat quality. This finding is encouraging, with regard to intensifying the heat treatments employed for sous vide meat products in order to improve product safety. However, as sous vide products are often used as ingredients in meals that are further processed, more information is required to see whether the aforementioned changes affect the sensory quality of the end product.

This study showed that the mild processing temperatures commonly employed in sous vide technology do not eliminate nonproteolytic *C. botulinum* type B spores in meat products. Limited intrinsic preservation and even slightly abused storage temperatures allow outgrowth and toxin production by nonproteolytic *C. botulinum*. It is therefore essential to intensify heat treatments in order to ensure the product safety in relation to nonproteolytic *C. botulinum*. This study also demonstrates the necessity of challenge testing whenever a new product, or an old product with changes in product formulation or manufacturing procedures, is to be launched on the market. The applicability of the recommended heat treatments *(1, 7)* set for the processing of REPFEDs should be evaluated individually for each product to determine their actual lethality with regard to nonproteolytic *C. botulinum* spores in different types of products.

ACKNOWLEDGMENTS

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