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Inhibitory Effect of Combinations of Heat Treatment, pH, and Sodium Chloride on Growth from Spores of Nonproteolytic *Clostridium botulinum* at Refrigeration Temperature

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Nonproteolytic strains of *Clostridium botulinum* will grow at refrigeration temperatures and thus pose a potential hazard in minimally processed foods. Spores of types B, E, and F strains were used to inoculate an anaerobic meat medium. The effects of various combinations of pH, NaCl concentration, addition of lysozyme, heat treatment (85 to 95°C), and incubation temperature (5 to 16°C) on time until growth were determined. No growth occurred after spores were heated at 95°C, but lysozyme improved recovery from spores heated at 85 and 90°C.

Botulism is caused by consumption of the neurotoxin produced by the anaerobe *Clostridium botulinum*. If this bacterium grows in food, it will likely produce sufficient toxin to cause severe illness or death. Refrigerated processed foods of extended durability (14, 15) are preserved by mild heat treatment followed by refrigerated storage. Nonproteolytic strains of *C. botulinum* (but not proteolytic strains) are capable of growth at temperatures as low as 3.3°C (7, 8, 23) and present a potential hazard in these foods, particularly if they are stored under vacuum or in a modified atmosphere (15, 16).

The United Kingdom Advisory Committee on the Microbiological Safety of Food (ACMSF) has recommended that for low-acid, refrigerated, processed foods with a shelf life of more than 10 days, safety may be ensured by heat treatment, low pH, low water activity, high NaCl concentration, or a combination of these factors. Heat treatment at 90°C for 10 min was deemed adequate to reduce the risk of growth of nonproteolytic *C. botulinum* by a factor of 10^6 (1). However, lysozyme, which is present in many foods (15), has been shown to improve recovery from heated spores (17, 18), and there are reports of growth from spores of nonproteolytic *C. botulinum* in lysozyme-containing media that had been given a heat treatment in excess of 90°C for 10 min (19, 20). Thus, in some circumstances it might be difficult to ensure a 10^6 reduction in spore numbers for nonproteolytic *C. botulinum* without using very high temperatures or long heating times, either of which would adversely affect many foods. The way to ensure safety must be to identify treatments that will permit milder heat treatments to be combined with other preservative factors, thus maintaining the nutritional and organoleptic qualities of the food.

The aim of this study was to identify combinations of heat treatment, pH, NaCl concentration, and incubation temperature that reduced the risk of growth from spores of nonproteolytic *C. botulinum*, in a strictly anaerobic meat medium, by a factor of at least 10^6.

Preparation and inoculation of meat medium. An anaerobic meat medium was prepared as described by Peck et al. (20) except that it was boiled and dispensed in 20-ml volumes under a flow of nitrogen instead of under a mixture of H_2-CO_2-N_2. NaCl was added at a concentration of 0.5, 2.0, or 3.5% (wt/wt). The dry weight of the medium was 18.4% (mean of nine determinations; dried at 100°C overnight). The concentration of NaCl added was thus calculated to be equivalent to 0.6, 2.5, or 4.3% (wt/wt), respectively, in the aqueous phase. Before the medium was dispensed, the pH was adjusted with 1 M HCl or KOH to give a pH of 5.6, 6.0, or 6.5 after autoclaving. The pH was determined after autoclaving and again after 3 months for at least two tubes per batch, and it was within 0.07 U of the target pH in all cases. A filter-sterilized solution of hen egg white lysozyme was added to half of the tubes to a final lysozyme concentration of 25 μg/ml (1,200 U/ml) as described previously (20).

Spores of eight strains of nonproteolytic *C. botulinum* (type B strains 2B, 17B, and FT50; type E strains Beluga, Hazen 36208, and Foster B96; and type F strains 202F and Craig 610B) were produced as described previously (17). A suspension of spores was then prepared (5×10^6/ml) comprising approximately equal numbers of each strain. Spore suspension (0.2 ml) was added by syringe to the tubes of medium, which had been prewarmed to 45°C to melt the fat and allow the spores to be dispersed by shaking. This gave an inoculum of approximately 10^6 spores per 20-ml volume of meat medium. Five replicate tubes were inoculated for each combination of heat treatment, NaCl concentration, pH, lysozyme, and recovery temperature.

Heat treatment and incubation. Tubes were either unheated, heated at 85°C for approximately 18 min (equivalent to 90°C for 4.2 min, assuming a *z* value of 8°C [20]), heated at 90°C for approximately 20 min, or heated at 95°C for 19 min (equivalent to 90°C for 80 min, assuming a *z* value of 8°C). Tubes were heated by submersion in a large water bath (W38; Grant Instruments, Cambridge, United Kingdom), and the temperature was monitored with eight microthermistors (Grant Instruments) sealed into uninoculated tubes as described previously (20). The rate of increase in temperature became very slow as the temperature of the meat medium approached the target temperature. As the temperature was close to the maximum for a large part of the come-up time (i.e., the time required for the temperature at the center of the medium to come within 0.1°C of the target temperature), this period contributed significantly to the lethality of the treatments. Cool-down time, on the other hand, was very rapid (4 to

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TABLE 1. Heat treatment of spores of nonproteolytic *C. botulinum* in a meat medium

Expt no.	Heating temp (°C)	Come-up time[a] (min)	Time at target temp[b] (min)	Heat lethality (equivalent time [min] at target temp)[c]
1	85.0	13.5	12.3	18.1
	90.0	14.4	13.9	20.2
	95.0	21.0	9.5	19.0
2	85.0	14.3	11.3	17.5
	90.0	16.3	11.9	19.3
3	85.0	12.6	13.0	17.8
	90.0	13.9	14.4	20.3

[a] Time required for the temperature at the center of the meat medium to come within 0.1°C of the target temperature.
[b] Length of time that the temperature at the center of the meat medium was within 0.1°C of the target temperature.
[c] Calculated by the method of Peck et al. (20).

5 min). The heating times (averaged for the eight microthermistors) and the total lethality (expressed as the equivalent time at the target temperature, thus taking into account the killing that occurs during the whole period of heat treatment) are shown in Table 1. After heat treatment and cooling, tubes of meat medium were incubated in low-temperature incubators (Astell-Hearson) at 5, 8, 12, or 16°C to simulate both refrigerated storage and mild abuse. The temperatures of the incubators were recorded at 15-min intervals as described previously (12). The average temperatures over the 3-month incubation period were within 0.2°C of the target temperature, with a standard deviation of 0.5°C or less. Tubes were examined for signs of gas formation daily for the first 14 days and three times per week thereafter for at least 90 days.

Growth from spores of nonproteolytic *C. botulinum* after heating and subsequent incubation at 5 to 16°C. Lysozyme had no significant effect on growth from unheated spores (Table 2), presumably because the germination system that lysozyme replaces after heat treatment was undamaged. Growth was prevented by a combination of 5°C-4.3% NaCl or 12°C-pH 5.6-4.3% NaCl. Revised ACMSF recommendations (2) stated that foods stored between 5 and 10°C should have a maximum shelf life of 5 days or less, not 10 days as proposed originally (1). The merit of this change is supported by our observation of growth from unheated spores in 5 days at 8°C (Table 2). Toxin production has also been detected following growth from nonproteolytic *C. botulinum* spores in less than 10 days at 8°C in whiting and cod (21), salmon (10), rockfish (13), and turkey breasts (11). Growth in less than 10 days at 8°C has also been indicated by a predictive model (12). Growth from unheated spores occurred in 12 days at 5°C (Table 2), which is similar to the 11 days required for toxin production at 5°C in vacuum-packed herring (3). Thus, although the results presented here are most relevant to meat-based products, they are compatible with reports of growth in fish and poultry. The ACMSF also stated that at temperatures below 10°C, 3.5% NaCl will prevent growth of nonproteolytic *C. botulinum* (1). Yet in the experiments reported here, 4.3% NaCl, although inhibitory, did not prevent growth under all conditions. This is compatible with an earlier report of growth of nonproteolytic *C. botulinum* type E in laboratory media in the presence of 3.5 to 4.3% NaCl at 7.2°C (9) and with model predictions (12).

Lysozyme, when added prior to heating, substantially improved growth from spores heated at 85°C for the equivalent of approximately 18 min (Table 3). This is seen both in the time

TABLE 2. Effect of lysozyme, pH, NaCl concentration, and recovery temperature on growth from unheated spores of nonproteolytic *C. botulinum* types B, E, and F

Expt no.	Added lysozyme (μg/ml)	pH	% NaCl[a]	No. of days required for growth in meat medium incubated at[b]:			
				16°C	12°C	8°C	5°C
1	0	6.5	0.6	2, 2, 2, 2, 2	2, 2, 2, 2, 2	5, 5, 5, 6, 6	12, 12, 12, 12, 12
2	0	6.5	0.6	2, 2, 2, 2, 2	3, 3, 3, 3, 3	5, 5, 6, 6, 6	13, 14, 14, 14, 18
1	0	6.5	2.5	2, 2, 2, 2, 2	4, 4, 4, 4, 4	8, 8, 8, 8, 9	24, 27, 27, 27, 27
1	0	6.5	4.3	6, 7, 8, 8, 9	12, 17, 22, 31, >104[c]	64, 66, 73, 78, >104	>104
2	0	6.0	0.6	2, 2, 2, 2, 2	3, 3, 3, 3, 3	6, 6, 6, 6, 9	13, 14, 14, 14, 14
2	0	5.6	0.6	2, 2, 2, 2, 2	4, 4, 4, 4, 4	9, 9, 9, 9, 9	14, 18, 18, 18, 18
3	0	5.6	0.6	3, 3, 3, 3, 3	5, 5, 5, 5, 5	8, 8, 8, 8, 8	10, 11, 11, 13, 14
3	0	5.6	2.5	5, 5, 5, 5, 5	6, 6, 6, 6, 6	14, 17, 17, 17, 17	31, 35, 35, 45, 47
3	0	5.6	4.3	>95	>95	>95	>95
1	25	6.5	0.6	2, 2, 2, 2, 2	2, 2, 2, 2, 2	5, 5, 5, 6, 6	12, 12, 12, 12, 12
2	25	6.5	0.6	2, 2, 2, 2, 2	3, 3, 3, 3, 3	5, 5, 5, 5, 6	13, 14, 14, 18, 18
1	25	6.5	2.5	2, 2, 2, 2, 2	4, 4, 4, 4, 4	7, 7, 8, 8, 8	24, 24, 24, 24, 27
1	25	6.5	4.3	6, 7, 8, 8, 12	11, 11, 11, 20, >104	34, 36, 49, 49, >104	>104
2	25	6.0	0.6	2, 2, 2, 2, 2	3, 3, 3, 3, 3	6, 6, 6, 6, 6	13, 13, 14, 18, 18
2	25	5.6	0.6	2, 2, 2, 2, 2	3, 4, 4, 4, 4	9, 9, 9, 9, 9	18, 18, 18, 18, 18
3	25	5.6	0.6	3, 3, 3, 3, 3	5, 5, 5, 5, 5	7, 7, 7, 7, 7	10, 10, 11, 11, 13
3	25	5.6	2.5	5, 5, 5, 5, 5	5, 5, 5, 6, 6	13, 13, 13, 14, 17	26, 26, 26, 31, 31
3	25	5.6	4.3	75, 95, 95, >95	>95	>95	>95

[a] Percent (wt/wt) NaCl in the aqueous phase of the medium.
[b] For each set of conditions, five replicate tubes were inoculated with 10^6 spores.
[c] >104, no growth in one or more tubes by the end of the incubation period (104 days).

TABLE 3. Effect of lysozyme, pH, NaCl concentration, and recovery temperature on growth from spores of nonproteolytic *C. botulinum* types B, E, and F heated at 85°C[a]

Expt no.	Heating time (min)[b]	Added lysozyme (μg/ml)[c]	pH	% NaCl[d]	No. of days required for growth in meat medium incubated at[e]:			
					16°C	12°C	8°C	5°C
1	18.1	0	6.5	0.6	55, 55, 71, 71, >104[f]	76, 104, >104	>104	>104
2	17.5	0	6.5	0.6	45, 52, >91	66, >91	>91	>91
1	18.1	0	6.5	2.5	29, 41, 59, >104	49, 62, 87, >104	>104	>104
1	18.1	0	6.5	4.3	>104	>104	>104	>104
2	17.5	0	6.0	0.6	40, 63, 66, 66, 80	59, >91	>91	>91
2	17.5	0	5.6	0.6	>91	>91	>91	>91
3	17.8	0	5.6	0.6	>95	>95	>95	>95
3	17.8	0	5.6	2.5	>95	82, >95	>95	>95
3	17.8	0	5.6	4.3	>95	>95	>95	>95
1	18.1	25	6.5	0.6	11, 12, 17, 20, 29	24, 27, 29, 29, 31	64, 71, 76, 83, 85	104, >104
2	17.5	25	6.5	0.6	28, 31, 31, 31, 33	35, 40, 47, 49, 56	75, 75, 84, 87, 87	>91
1	18.1	25	6.5	2.5	20, 20, 20, 22, 24	27, 31, 31, 31, 34	43, 43, 45, 59, 62	104, 104, 104, >104
1	18.1	25	6.5	4.3	43, 64, >104	34, 83, >104	>104	>104
2	17.5	25	6.0	0.6	21, 28, 28, 38, 42	38, 54, 59, 61, 68	87, >91	>91
2	17.5	25	5.6	0.6	21, 28, 28, 28, 28	38, 40, 42, 54, 54	87, >91	>91
3	17.8	25	5.6	0.6	24, 24, 26, 33, 33	17, 26, 42, 42, 56	>95	>95
3	17.8	25	5.6	2.5	24, 24, 28, 31, 40	31, 33, 47, 59, 59	95, >95	>95
3	17.8	25	5.6	4.3	>95	>95	>95	>95

[a] Heat treatment and recovery were performed in meat medium.
[b] Heating time refers to total lethality of treatment. See Table 1 for details.
[c] Lysozyme was added to the meat medium prior to heat treatment.
[d] Percent (wt/wt) NaCl in the aqueous phase of the medium.
[e] For each set of conditions, five replicate tubes were inoculated with 10^6 spores.
[f] >104, no growth in one or more tubes by the end of the incubation period (104 days).

required for growth and in the range of conditions under which growth was observed. The amounts of time required for growth at 12 and 8°C after heating at 85°C in the presence of lysozyme (24 to 56 days and 64 to 87 days, respectively) (Table 3) were similar to those reported previously after a similar heat treatment but with only 10 μg of lysozyme added per ml prior to heating (20). The relative lack of inhibition at pH 5.6 in the presence of lysozyme (Table 3) may be due to lysozyme being more heat resistant at pH 5.6 than at pH 6.5 (5) so that more spores survived the heat treatment, thus offsetting the inhibitory effect of lowering the pH.

Heat treatment at 90°C for the equivalent of approximately 20 min was sufficient to prevent growth if lysozyme was not added (Table 4). In the presence of lysozyme, this heat treatment did not prevent growth under conditions of temperature abuse, with growth first being observed after 29 days at 16°C, after 49 days at 12°C, and after 80 days at 8°C (Table 4). Growth was prevented provided that the 90°C heat treatment was combined with 4.3% NaCl, incubation at 5°C, or the combination pH 6.0-12°C or 2.5% NaCl-pH 5.6-16°C. In the presence of lysozyme, times to first observation of growth were similar to those reported after a similar heat treatment with 25 μg of lysozyme per ml (19).

Heating spores at 95°C for the equivalent of 19 min prevented growth under all the conditions tested in experiment 1 (results not shown), and this heat treatment was not used in any further experiments. In previous work, however, after heating spores at 95°C for the equivalent of 15 min, growth was reported in meat medium plus lysozyme at 25°C (20); thus, heating spores at 95°C may not be sufficient to prevent growth under all conditions.

The lysozyme concentration used in the experiments reported here, 25 μg/ml of meat medium, is comparable to concentrations in foods. These vary from 2 to 28 μg/g in vegetables and from 10 to 200 μg/g in fish and seafood, with much higher concentrations in eggs (14, 15, 22). The heat resistance of lysozyme is strongly dependent on the heating medium and is unknown in most foods. Data presented here indicated that after heating in meat medium at 90°C for 20 min, sufficient lysozyme activity remained to increase the measured heat resistance of spores of nonproteolytic *C. botulinum*. Similar observations have been made previously when lysozyme at 5 μg/ml was heated in meat medium at 90°C for 20 min (19) or in water at 121°C for 15 min (24). Growth of nonproteolytic type B strains has been observed in salmon after being heated at 88.9°C for 46 min, suggesting that native lysozyme in the salmon had survived the heat treatment (6).

Toxin production. Toxin determinations were performed on at least three samples for each condition tested. Samples were frozen at the end of the 90-day incubation time, then thawed and centrifuged (18,000 × *g*, 15 min, 10°C), and the supernatant fluids were tested for toxin by an enzyme-linked immunosorbent assay (ELISA) procedure (4). Selected samples that showed growth under the most extreme conditions were also tested for toxin by the mouse bioassay (20). On no occasion was toxin detected under conditions which did not result in visible growth, while toxin was always detected under conditions in which growth was recorded (results not shown). The mouse bioassay was used to test for toxin in 65 samples near the limits of growth, and this confirmed the observations of growth and the ELISA toxin test results.

Conclusion. We have shown that in the presence of hen egg white lysozyme at 25 μg/ml (1,200 U/ml) in a meat medium, heat treatment at 90°C for 20 min does not ensure a reduction

TABLE 4. Effect of lysozyme, pH, NaCl concentration, and recovery temperature on growth from spores of nonproteolytic *C. botulinum* types B, E, and F heated at 90°C[a]

Expt no.	Heating time (min)[b]	Added lysozyme (μg/ml)[c]	pH	% NaCl[d]	No. of days required for growth in meat medium incubated at[e]:			
					16°C	12°C	8°C	5°C
1	20.2	0	6.5	0.6	>104[f]	>104	>104	>104
2	19.3	0	6.5	0.6	>91	>91	>91	>91
1	20.2	0	6.5	2.5	>104	>104	>104	>104
1	20.2	0	6.5	4.3	>104	>104	>104	>104
2	19.3	0	6.0	0.6	>91	>91	>91	>91
2	19.3	0	5.6	0.6	>91	>91	>91	>91
3	20.3	0	5.6	0.6	>95	>95	>95	>95
3	20.3	0	5.6	2.5	>95	>95	>95	>95
3	20.3	0	5.6	4.3	>95	>95	>95	>95
1	20.2	25	6.5	0.6	29, 31, 34, 34, 34	49, 52, 59, 59, 66	87, 104, 104, 104, >104	>104
2	19.3	25	6.5	0.6	49, 52, 56, 61, 66	59, 68, 70, 70, 70	>91	>91
1	20.2	25	6.5	2.5	34, 34, 45, 55, 104	62, 62, 69, 71, 87	80, 104, >104	>104
1	20.2	25	6.5	4.3	>104	>104	>104	>104
2	19.3	25	6.0	0.6	49, 59, >91	>91	>91	>91
2	19.3	25	5.6	0.6	38, 40, >91	>91	>91	>91
3	20.3	25	5.6	0.6	31, >95	>95	>95	>95
3	20.3	25	5.6	2.5	>95	>95	>95	>95
3	20.3	25	5.6	4.3	>95	>95	>95	>95

[a] Heat treatment and recovery were performed in meat medium.
[b] Heating time refers to total lethality of treatment. See Table 1 for details.
[c] Lysozyme was added to the meat medium prior to heat treatment.
[d] Percent (wt/wt) NaCl in the aqueous phase of the medium.
[e] For each set of conditions, five replicate tubes were inoculated with 10^6 spores.
[f] >104, no growth in one or more tubes by the end of the incubation period (104 days).

of spores of nonproteolytic *C. botulinum* by a factor of 10^6 when the incubation period is 3 months. In order to help formulate a meat-based food that is safe with respect to this organism at 5 to 16°C, suitable combinations of treatments can be identified from the work described here. If foods are likely to be maintained at temperatures above 10°C, however, growth of and toxin production by proteolytic strains of *C. botulinum* must also be prevented.

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