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Growth of and Toxin Production by Nonproteolytic *Clostridium botulinum* in Cooked Puréed Vegetables at Refrigeration Temperatures

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Seven strains of nonproteolytic *Clostridium botulinum* (types B, E, and F) were each inoculated into a range of anaerobic cooked puréed vegetables. After incubation at 10°C for 15 to 60 days, all seven strains formed toxin in mushrooms, five did so in broccoli, four did so in cauliflower, three did so in asparagus, and one did so in kale. Growth kinetics of nonproteolytic *C. botulinum* type B in cooked mushrooms, cauliflower, and potatoes were determined at 16, 10, 8, and 5°C. Growth and toxin production occurred in cooked cauliflower and mushrooms at all temperatures and in potatoes at 16 and 8°C. The *C. botulinum* neurotoxin was detected within 3 to 5 days at 16°C, 11 to 13 days at 10°C, 10 to 34 days at 8°C, and 17 to 20 days at 5°C.

Six physiologically and phylogenetically distinct groups of clostridia are capable of producing the botulinum neurotoxin (18). *Clostridium botulinum* groups I and II are responsible for human foodborne botulism. *C. botulinum* group II (nonproteolytic) strains are psychrotrophic, produce toxins of types B, E, or F, and are capable of growth and toxin production at 3.3°C (18). Thus, nonproteolytic strains of *C. botulinum* pose a hazard in products that rely on a mild heat treatment and refrigeration for preservation, e.g., sous-vide products and other refrigerated processed foods of extended durability (REPFEDs) (26, 27). Vegetables are frequent ingredients of REPFEDs, and cooked vegetables are considered a high risk with regard to nonproteolytic psychrotrophic *C. botulinum* (2). A wide range of cooked vegetables incubated at 30°C supported growth and toxin production by a mixture of strains of nonproteolytic *C. botulinum* (8). Nevertheless, there is little information on the growth of nonproteolytic *C. botulinum* in cooked vegetables at refrigeration temperatures and under conditions of mild temperature abuse. In contrast, growth of and toxin production by nonproteolytic *C. botulinum* in laboratory media (16, 17, 22, 25, 28), in meat and poultry (1, 14, 29, 30), and in fish and seafood (6, 7, 12, 13, 19–21, 23, 24, 36, 39, 40) have been extensively studied.

The aim of this work was (i) to determine the ability of each of seven strains of nonproteolytic *C. botulinum* to grow and produce toxin in a range of cooked vegetables at mild abuse and refrigeration temperatures and (ii) to study the effect of temperature ranging from 5 to 16°C on the kinetics of growth of and toxin production by nonproteolytic *C. botulinum* type B in selected vegetables.

Growth of and toxin production by different strains of nonproteolytic *C. botulinum* in a range of vegetables. Cooked purées were prepared from fresh vegetables, distributed anaerobically in 10-ml volumes, and sterilized as described previously (8). The strains used were nonproteolytic *C. botulinum* type B (Eklund 2B, Eklund 17B, and Hobbs FT50), type E (Beluga and Hazen 36208), and type F (Eklund 202F and Craig 610). The origin and maintenance of the strains and the preparation of spore suspensions were as described previously (8, 34). Suspensions of spores of each strain were diluted in sterile glass-distilled water to give a concentration of 10^5 viable spores per ml. A 100-μl sample of each spore suspension was inoculated individually into two to four replicate vials containing 10 ml of cooked puréed vegetables to give a final concentration of 10^3 spores per ml. In some cases, tests were performed with different batches of cooked vegetables. Vials were incubated for 15 to 60 days at 30 and 10°C. Vials were observed daily for production of gas bubbles for the first 15 days of incubation and then every 3 to 4 days. On the final day of incubation, samples were tested for toxin by an enzyme-linked immunosorbent assay (ELISA) method (8, 37), and in some cases, the mouse test was also used (35).

At 30°C, growth and/or toxin production by all strains was detected in mushrooms, potatoes, and cauliflower, as well as by five strains in broccoli, four strains in asparagus, and two strains in kale (data not shown). Cooked bean sprouts supported the growth of strain 17B, the only strain tested. At 10°C, visible growth (gas production) was detected in mushrooms within 7 days of incubation with six of the inoculated strains, and production of toxin was confirmed (Table 1). The presence of the botulinum neurotoxin was demonstrated in inoculated vials of mushrooms, cauliflower, broccoli, asparagus, and kale with an ELISA and the mouse test (Table 1). In the ELISA, most positive samples gave an A_{492} substantially greater than the threshold for a positive ELISA as calculated by Potter et al. (37), indicating the production of a significant amount of toxin.

This work has confirmed the ability of nonproteolytic *C. botulinum* to grow and produce toxin in a range of cooked vegetables, such as mushrooms, cauliflower, potatoes, broccoli, asparagus, bean sprouts, and kale at 30°C (8). Some vegetables, such as mushrooms and cauliflower, supported growth of all strains tested, while fewer strains grew on other vegetables (e.g., kale). Growth and toxin production have now also been demonstrated at refrigeration temperatures. Five vegetables that supported growth at 30°C supported the growth of at least one strain at 10°C. This article is the first report of toxin production at 10°C by nonproteolytic *C. botulinum* in asparagus, broccoli, cauliflower, kale, and mushrooms; toxin production has also been previously reported in potatoes (5, 31) and peas (15).

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TABLE 1. Growth of and toxin production by strains of nonproteolytic *C. botulinum* in vials of cooked puréed vegetables incubated at 10°C[a]

Vegetable or medium	pH	Total incubation time (days)	Result for nonproteolytic *C. botulinum* test strain													
			Hobbs FT50		Eklund 17B		Eklund 2B		Beluga		Hazen 36208		Eklund 202F		Craig 610	
			TVG[b]	Toxin[c]	TVG	Toxin	TVG	Toxin	TVG	Toxin	TVG	Toxin	TVG	Toxin	TVG	Toxin
PYGS	6.86	15	5–9	+	6–10	+	6–9	+	5	+	6	+	5	+	5	+
Mushroom	6.41	15[d]	5–7	++	6–7	++	7–8	++	6–12	++	GNO	++	5	++	5–6	++
Cauliflower	5.47	60	GNO	+*	GNO	++	GNO	−	GNO	−	GNO	−	GNO	++	23	+*
Broccoli	5.49	60	19–23	++	GNO	++	GNO	++	GNO	−	GNO	−	GNO	++	GNO	++
Asparagus	5.33	60	8	+	GNO	+	GNO	−	GNO	−	GNO	−	GNO	−	GNO	++
Kale	5.27	58	GNO	++	GNO	−	GNO	−	GNO	−	GNO	−	GNO	−	GNO	−

[a] Growth and toxin production were determined with vials containing 10 ml of cooked puréed vegetables under anaerobic conditions. Each vial was inoculated with ca. 10^3 spores of the indicated test strain per ml.

[b] TVG, time to visible growth (days); GNO, growth not observed.

[c] +, toxin detected in the ELISA; ++, toxin detected in the ELISA and in the mouse test; +*, toxin detected in the ELISA but not in the mouse test; −, toxin not detected in the ELISA. Note that there was no toxin in cooked potatoes with any of the strains.

[d] Total incubation time was 60 days for strain Hazen 36208.

Effect of temperature on the growth of nonproteolytic *C. botulinum* type B in cooked puréed potatoes, mushrooms, and cauliflower. The effect of temperature on growth curves of nonproteolytic *C. botulinum* type B was tested in cooked vegetables prepared as described above, except that they were cooled under a headspace of nitrogen and 100-ml volumes were distributed into 150-ml bottles in an anaerobic cabinet (Don Whitley Scientific, Leeds, United Kingdom) filled with oxygen-free H_2-CO_2-N_2 (10:5:85 [vol/vol]) before sterilization at 121°C for 15 min. The pHs of the cooked vegetables were 5.71 in potatoes, 6.29 in mushrooms, and 5.56 in cauliflower (each was the average of duplicate measurements). The water activity of cooked puréed vegetables was measured with a Decagon Aqualab CX2 dew point water activity meter (GBX SARL, Romans-sur-Isère, France) and was 0.99 for each vegetable purée (each was the average of four replicate samples). A suspension was prepared which contained equal numbers of spores of six strains of nonproteolytic *C. botulinum* type B (Eklund 2B, Eklund 17B, Hobbs FT50, Colworth 151, 2129B, and 4672U-1) and had a final concentration of 10^6 spores per ml. A 100-μl sample of this suspension was used to inoculate each of the 100-ml vials of cooked puréed potatoes, mushrooms, and cauliflower to give a final concentration of 10^3 spores per ml. Test vials (one vial per temperature and per vegetable, precooled before inoculation) were then transferred to low-temperature incubators. Temperatures were recorded every 15 min throughout the incubation period (17). Target temperatures were 16, 10, 8, and 5°C. For more than 98.8% of the time, the temperatures were lower than the target temperature + 1.0°C. Viable counts were determined at inoculation and then at appropriate intervals during incubation. Inoculated vials were observed for production of gas bubbles at each sampling time. A 1-ml sample was removed to determine the viable count and replaced by a volume of oxygen-free N_2-H_2 (90:10 [vol/vol]). Counts were made by preparing 10-fold dilutions in PYGS (25) under a flow of oxygen-free N_2-H_2 (90:10 [vol/vol]) and spreading 100-μl samples on duplicate plates of VL blood agar (17). The spread plates were transferred within 10 min to anaerobic jars under a headspace of H_2-CO_2 (90:10 [vol/vol]) and incubated for 48 h at 30°C. At each sampling time, a further sample was removed, diluted 10-fold, and kept frozen at −18°C for up to 8 weeks prior to analysis for toxin.

Significant growth of nonproteolytic *C. botulinum* type B occurred in cooked puréed potatoes at 16 and 8°C and in cooked mushrooms and cooked cauliflower at 16, 10, 8, and 5°C. No growth was detected in cooked potatoes at 10 and 5°C after 27 and 38 days of incubation, respectively. Curves were fitted to the growth data with the Baranyi model (4) to derive doubling time, lag time, and time to a 1,000-fold increase. Fitted growth curves are shown for mushrooms (Fig. 1). At each incubation temperature, time to a 1,000-fold increase was more rapid in mushrooms than in cauliflower, and that in cauliflower was more rapid than that in potatoes (Table 2). Time to toxin was taken as the first sampling time at which an A_{492} higher than 0.5 was obtained when 10-fold-diluted samples were tested in the ELISA. This is probably equivalent to more than 50 50% mouse lethal doses of type B toxin per ml (37). Similar results were obtained when time to visible growth and time to toxin were measured (Table 2) and when individual strains were tested (Table 1). Toxin was detected within 3 to 5 days at 16°C, 11 to 13 days at 10°C, 10 to 34 days at 8°C,

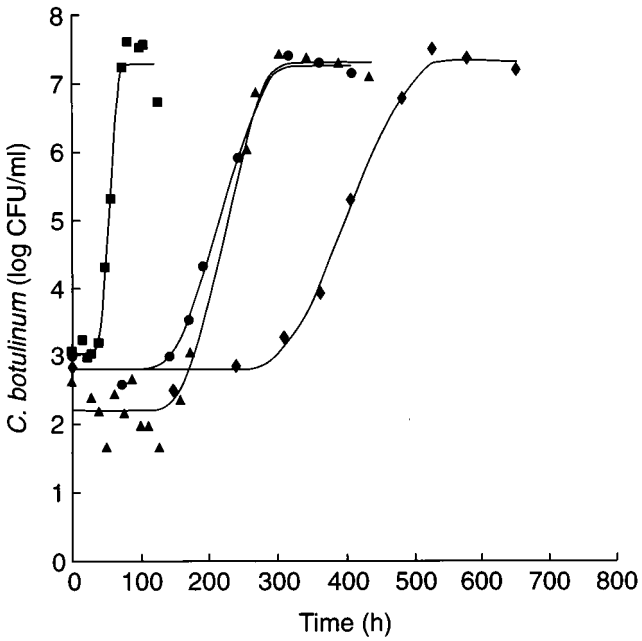

FIG. 1. Effects of incubation temperature on the growth from spores of a mixture of strains of nonproteolytic *C. botulinum* type B in cooked puréed mushrooms at pH 6.29. Points are actual counts at 16°C (■), 10°C (▲), 8°C (●), and 5°C (◆). The lines show the fitting of the counts to the Baranyi equation.

TABLE 2. Growth of and toxin production by nonproteolytic *C. botulinum* type B in vials of cooked puréed vegetables at temperatures between 5 and 16°C

Vegetable	Target temp (°C)[a]	Calculated time[b]			RMSE[c]	Observed time		Toxin in the mouse test
		Doubling (h)	Lag (h)	To 1,000-fold increase (days)		To visible growth (days)	To toxin (days)[d]	
Potato	16	2.6	83	4.7	0.63	4.5	5.3	NT[e]
Mushroom	16	2.1	38	2.6	0.22	2.9	2.9	NT
Cauliflower	16	3.0	52	3.4	0.16	3.9	3.9	NT
Mushroom	10	7.0	161	9.6	0.63	10.5	10.5	+
Cauliflower	10	12.6	235	15.0	0.43	13.1	13.1	+
Potato	8	10.3	628	30.4	0.29	31.0	33.9	+
Mushroom	8	8.9	146	9.8	0.37	7.1	10.2	+
Cauliflower	8	11.3	288	16.8	0.39	14.9	17.1	+
Mushroom	5	12.4	304	17.8	0.43	20.0	20.0	+
Cauliflower	5	8.5	383	19.6	0.62	21.0	19.0	+

[a] There was no growth in cooked potatoes at 10 and 5°C.
[b] Growth parameters (doubling time, lag time, and time to a 1,000-fold increase) were estimated with the Baranyi equation (4).
[c] RMSE, root mean square error of fit of curve to data.
[d] First incubation time with $A_{492} > 0.5$ of ELISA reading in 10-fold-diluted samples. The A_{492} of diluted samples on inoculation was <0.1.
[e] NT, not tested.

and 17 to 20 days at 5°C. The presence of botulinum neurotoxin in samples was confirmed with the mouse test (Table 2).

The doubling times of nonproteolytic *C. botulinum* type B in cooked vegetables at 16, 10, 8, and 5°C were generally shorter than those observed in laboratory medium under optimum conditions (16, 17, 32, 33). When exact incubation temperature, pH, and NaCl concentration were input into a predictive model for nonproteolytic *C. botulinum* developed in this laboratory (17) and available through Food MicroModel (Food MicroModel, Ltd., Leatherhead, Surrey, United Kingdom), the predicted doubling times, lag times, and time to a 1,000-fold increase in culture medium were shorter (often marginally) than those observed in cooked vegetables.

The earliest times to toxin detection in other foods have generally ranged from 1 to 4 days at 30°C, from 6 to 20 days at 8 to 10°C, and from 14 to 30 days at 4 to 6°C (1, 3, 6, 7, 9–14, 19–21, 23, 24, 30, 36, 38, 41). The earliest time to toxin detection in vegetables is within this range, albeit toward the longer end. From these findings, it can be concluded that the potential for growth of and toxin production by nonproteolytic *C. botulinum* is as high in some cooked vegetables as in fish, meat, or poultry.

Conclusion. This work has shown that many vegetables supported growth and toxin production by nonproteolytic *C. botulinum* at refrigeration temperatures and that the time to toxin production is within the range reported for other food groups (e.g., meat, fish, and poultry). Consequently, recommendations and guidelines proposed to control the growth of nonproteolytic *C. botulinum* and ensure the safety of REPFEDs (2, 26) apply to REPFEDs made of or containing vegetables. Recommendations include a short shelf life for products that rely only on refrigeration temperature for preservation, with additional preservative factors (heat treatment, lowered pH, and/or lowered water activity) necessary for products with an extended shelf life.

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