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Effect of Sodium Nitrite on Toxin Production by *Clostridium botulinum* in bacon

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Pork bellies were formulated to 0, 30, 60, 120, 170, or 340 μg of nitrite per g of meat and inoculated with *Clostridium botulinum* via pickle or after processing and slicing. Processed bacon was stored at 7 or 27 C and assayed for nitrite, nitrate, and botulinal toxin at different intervals. Nitrite levels declined during processing and storage. The rate of decrease was more rapid at 27 than at 7 C. Although not added to the system, nitrate was detected in samples during processing and storage at 7 and 27 C. The amount of nitrate found was related to formulated nitrite levels. No toxin was found in samples incubated at 7 C throughout the 84-day test period. At 27 C, via pickle, inoculated samples with low inoculum (210 *C. botulinum* per g before processing and 52 per g after processing) became toxic if formulated with 120 μg of nitrite per g of meat or less. Toxin was not detected in bacon formulated with 170 or 340 μg of nitrite per g of meat under these same conditions. Toxin was detected at all formulated nitrite levels in bacon inoculated via the pickle with 19,000 *C. botulinum* per g (4,300 per g after processing) and in samples inoculated after slicing. However, increased levels of formulated nitrite decreased the probability of botulinal toxin formation in bacon inoculated by both methods.

Recent studies on canned, perishable, cured meat and wieners showed that increased nitrite levels decreased the probability of botulinal toxin formation (1, 4). However, the impact of nitrite upon toxigenesis differed somewhat between the two products. This difference is probably due, in part, to differences in formulation and processing employed in the manufacture of the two products. Bacon is formulated and processed differently from either canned, perishable, cured meat or wieners. Thus, results from the foregoing studies would not necessarily apply to bacon.

This study was conducted as one of a series undertaken cooperatively by the American Meat Institute, the Food and Drug Administration, and the United States Department of Agriculture to determine the minimal level of sodium nitrite required in bacon for consumer acceptance and botulinal protection. The initial studies on bacon were designed to investigate three distinct aspects and were conducted concurrently. Project I investigated the effect of nitrite level on product manufacture, chemical changes, product acceptance, off-flavor, growth of microbial spoilage organisms, and color. Project II examined the roles of nitrite level, cooking method, and ascorbates on the formation of *N*-nitrosopyrrolidine. Preliminary results from the first two projects have been reported (3). When completed, the first two projects will be reported as a separate paper. Project III, reported herein, investigated the degree to which nitrite retards or prevents growth of *Clostridium botulinum* in bacon. Additional research is now underway at the Food Research Institute, University of Wisconsin, to investigate the effect, if any, that high levels of sodium ascorbate have on the inhibition of botulinal toxin by sodium nitrite in bacon.

MATERIALS AND METHODS

Experimental design. The experimental variables are listed in Table 1. The design for the uninoculated portion of the experiment was a full replicate of a 6 × 6 × 2 (nitrite level × storage time × incubation temperature) factorial with one package for each treatment combination. A similar 6 × 6 × 2 (nitrite level × storage time × inoculum level) design was used for inoculated samples. There were five packages

TABLE 1. *Experimental design*

Factor	Variables
Nitrite level	0, 30, 60, 120, 170, and 340 μg/g of meat
Method of inoculation	Via pickle, after slicing
Desired inoculum level	0, 100, and 10,000 spores/g of meat
Storage time	
Uninoculated samples	Before smoking, after smoking; 7, 14, 28, and 56 days
Inoculated samples	After smoking; 7, 14, 28, 56, and 84 days
Holding temperature	7 C, 27 C

for each treatment combination for samples inoculated via the pickle solution and two replicates for samples inoculated with a sand-spore mixture after slicing.

Inoculum. Spores of five type A (77A, 62A, 12885A, 33A, and 62A) and five type B (ATCC7949, 41B, 40B, 53B, and Lamanna B) strains of *C. botulinum* were used. Tubes of peptone colloid medium (Difco) modified by the addition of 0.1% glucose were inoculated with a heat-shocked spore suspension of the individual strains. After 16 h of incubation at 37 C, fresh tubes of the medium were inoculated, incubated for 4 h, and again transferred. After 4 h of incubation, these cultures were inoculated into the sporulation medium of Schmidt and Nank (5) consisting of 5% Trypticase (BBL), 0.5% peptone (Difco), and 0.05% sodium thioglycolate. The final cultures were incubated for 7 days at 37 C. Spores were harvested by centrifugation, washed several times, and suspended in sterile, distilled water. Samples of each suspension were heat-shocked (80 C for 15 min), and spore counts were determined by a three-tube most probable number procedure in modified peptone colloid medium (2). A single suspension was prepared containing equal numbers of spores of each strain. A portion of this spore mixture was heat-shocked, diluted, and added to the pickle solutions for inoculation of the bacon. A second portion of the heat-shocked spore suspension was mixed with sterile sand and dried under vacuum over phosphorus pentoxide at room temperature for inoculation of bacon after slicing.

Preparation and inoculation of bacon. Raw, frozen pork bellies, curing ingredients, and water used for preparing pickle were from the same stocks used in the project I research and were supplied by Armour and Co. (Oak Brook, Ill.). Two bellies at each nitrite and spore inoculum level were pumped with curing pickle to an 11% gain and drained to an approximate 10% gain. The pickle contained sodium chloride (13.3%), sucrose (3.1%), tripolyphosphate (2.6%), sodium isoascorbate (0.23%), and the various concentrations of sodium nitrite and water. These concentrations of ingredients in the pickle were 10 times the levels desired in bacon. The drained bellies were smoked and processed to an internal temperature of 53 C over an 8.5-h period. Smoke from hardwood shavings was added during the initial 2.5 h. The processed bellies were held at −2.2 C for 36 h and sliced. The two bellies for each nitrite and spore inoculum level were divided into thirds. One slice from each third of the two bellies was placed in a Curpolene 200 (Curwood, Inc., New London, Wis.) pouch (six slices total, weighing 125 to 150 g), and the package was sealed under vacuum. The packages were stored at 7 or 27 C.

Uninoculated bacon was prepared in the same manner except that spores were not added to the pickle. This bacon was handled and stored in the same manner as the inoculated bacon and was used for all chemical analyses. A portion of this bacon was inoculated with the dried sand-spore mixture after slicing.

Toxin assay and determination of spore levels. The samples were randomized and labeled so that each package was designated for analysis at a specific time. However, samples which swelled prior to this time were removed from incubation and analyzed. The packages were weighed, and the entire contents of each package were blended for 1 min with an equal weight of gelatin phosphate buffer. The slurry was centrifuged, and 0.5 ml of the supernatant was injected into each of five mice (three unprotected and two protected with type AB botulinal antitoxin from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada). Death of the unprotected mice within 4 days and survival of the protected 2 mice were considered proof of the presence of botulinal toxin.

Inoculum levels before smoking were determined by removing three plug samples (about 6.3 cm^2/sample) from one of the two bellies at each nitrite level. The plug samples from each belly were composited for determination of inoculum level.

Viable counts were determined on nonheat-shocked samples of bacon before and after processing and inoculated pickle solutions by the three-tube most probable number procedure. The pickle solutions were filtered through 0.45-μm pore size filters. After rinsing the filters several times to minimize contamination by pickle ingredients, the filters were cut up, placed in phosphate buffer (pH 7.2), and agitated to dislodge the organisms, and counts per ml of pickle were determined. All bacon samples were blended as for toxin assay, and the slurry was diluted for determination of counts.

Chemical analyses. A composite of nine plugs bored randomly from the bacon bellies was used for chemical analysis of bacon before and after heat processing. Nitrite and nitrate concentrations were determined as previously reported (1).

RESULTS

Samples of finished bacon at the various nitrite levels were analyzed for sodium chloride, moisture, fat, protein, and water activity. The range and average for these values, plus calculated brine concentrations, are shown in Table 2.

Concentrations of nitrite found before smok-

TABLE 2. *Composition of finished bacon*

Determination	Sodium chloride (%)	Moisture (%)	Brine (%)	Water activity	Protein (%)	Fat (%)
Range	1.3–1.5	23.0–32.9	4.33–6.12	0.955–0.960	7.7–10.1	52.7–65.1
Average	1.47	28.1	4.9	0.958	9.25	57.9

TABLE 3. *Effect of processing, storage time, and temperature on depletion of added nitrite*

Added nitrite (μg/g of meat)	Nitrite values (μg/g of meat)										
	Before processing	After processing	No. of days stored at 7 C					No. of days stored at 27 C				
			7	14	28	54	84	7	14	28	54	84
0	0	0	0	0	0	0	0	0	0	0	ND[a]	ND
30	26	10	8	6	6	6	1	3	2	1	ND	ND
60	54	19	12	9	7	10	2	1	0	1	ND	ND
120	96	46	20	22	10	4	4	6	4	1	2	2
170	220	95	48	49	26	14	9	14	6	0	1	1
340	380	160	104	93	51	42	26	56	24	3	1	1

[a] ND, Not determined, for samples were putrid and nitrite levels were very low at 28 days.

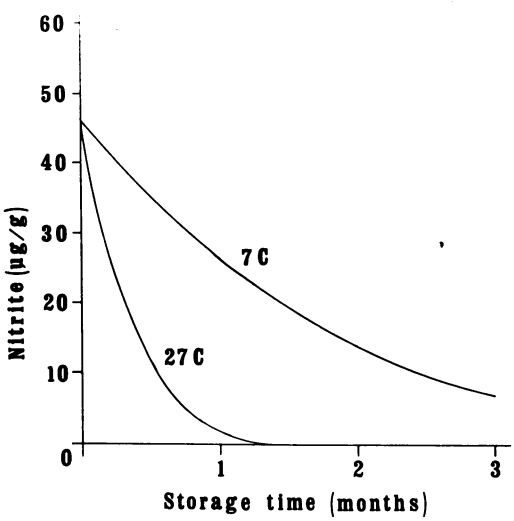

FIG. 1. *Comparison of predicted residual nitrite levels in bacon stored at 7 and 27 C with an initial formulation to 170 μg of nitrite per g of bacon.*

ing corresponded to formulated levels (Table 3). However, approximately 50 to 80% of the nitrite was lost during processing (i.e., heating to 53 C followed by holding for 36 h at −2.2 C). A further time-temperature-dependent reduction in nitrite occurred during storage. For example, Fig. 1 shows a best fitting (least squares) line fitted to predicted residual nitrite values for product formulated to 170 μg of nitrite per g and held at 27 and 7 C. There was a geometric decline in nitrite levels with time at both temperatures. However, the rate of decrease was more rapid at 27 than at 7 C.

Nitrate was not added to the system; however, analysis showed the presence of nitrate during processing and storage at 27 and 7 C (Table 4). The amount of nitrate found was related to formulated nitrite levels.

The range and logarithmic average counts of *C. botulinum* in the inoculating pickle, pumped bellies, and the finished bacon are shown in Table 5. These values represent counts across all nitrite levels. Although there was considerable variation, the counts were independent of the nitrite levels. Thus, exposure to nitrite, particularly the high levels in the pickle, had no effect on the inoculum. Three samples of bacon were analyzed per inoculum level after inoculating bacon with the sand-spore mixtures. The logarithmic averages were 40 and 3,400 per g with ranges of 18 to 86 and 1,800 to 4,600 per g for the low and high inoculum levels, respectively. Sixty samples of bacon inoculated via the pickle and 24 samples inoculated with the sand-spore mixtures were tested after packaging and without incubation. All were nontoxic, demonstrating that toxin was not carried into the product by the inocula.

Table 6 shows the number of confirmed botulinal toxic samples inoculated via pickle and held at 27 C. Increased levels of nitrite decreased the rate of toxin production and the total number of samples ultimately containing toxin. The level of nitrite necessary to inhibit toxin production was dependent upon inoculum level. At the low inoculum level no toxin was confirmed in samples formulated with 170 or 340 μg of nitrite per g throughout the storage period. At the high inoculum level, toxin was

TABLE 4. *Effect of processing, storage time, and temperature on nitrate concentrations*

Added nitrite (μg/g of meat)	Nitrate values (μg/g of meat)										
	Before processing	After processing	No. of days stored at 7 C					No. of days stored at 27 C				
			7	14	28	54	84	7	14	28	54	84
0	0	19	36	15	16	35	18	0	10	10	ND[a]	ND
30	0	29	63	26	22	35	10	0	17	18	ND	ND
60	0	37	49	49	22	36	32	5	0	19	ND	ND
120	18	48	58	130	25	64	62	58	31	21	26	16
170	35	70	102	135	34	103	67	73	23	35	26	33
340	35	80	63	149	60	137	82	112	217	51	32	16

[a] ND, Not determined.

TABLE 5. *Clostridium botulinum counts in pickle and bacon when inoculated via pickle*

Inoculum	Log_{10} avg	Range
Low inoculum		
Pickle	1,200[a]	930-2,400
Bacon		
Before processing	210[b]	150-680
After processing	52[b]	18-220
High inoculum		
Pickle	330,000[a]	93,000-1,000,000
Bacon		
Before processing	19,000[b]	4,300-43,000
After processing	4,300[b]	720-8,600

[a] Each value is an average (per milliliter) of six different pickle samples.

[b] Each value is an average per gram) of 10 bacon samples.

detected in bacon at all nitrite levels, but with decreasing frequency as the formulated level increased. All botulinogenic samples were proteolyzed. Samples stored for up to 84 days at 7 C were nontoxic and showed no evidence of proteolysis.

Table 7 shows the numbers of botulinal toxic samples after holding bacon at 27 C when inoculated by the sand-spore mixture after slicing. As was true for samples inoculated before processing, increased nitrite levels in the bacon reduced the rate of toxin development and eventual number of toxic samples. Relatively few samples were confirmed to be toxic at 170 or 340 μg of nitrite per g; however, there was at least one toxic sample at all nitrite levels studied. The total number of botulinogenic samples at the low inoculum, zero nitrite level was unexpectedly low.

TABLE 6. *Effect of sodium nitrite on toxin production by C. botulinum in bacon stored at 27 C; inoculated via pickle*

Inoculum level	Added nitrite (μg/g of meat)	No. of toxic samples after 7 to 84 days					Total no. of toxic samples
		7[a]	14	28	56	84	
Low (52/g)	0	4	5	15	—[b]	—	24[c]
	30	4	5	14	—	—	23
	60	0	3	5	4	2	14
	120	0	0	2	3	3	8
	170	0	0	0	0	0	0
	340	0	0	0	0	0	0
High (4,300/g)	0	5	5	15	—	—	25
	30	1	4	8	4	3	20
	60	0	2	2	5	4	13
	120	0	2	2	3	5	12
	170	0	1	2	5	5	13
	340	0	2	0	0	1	3

[a] Numbers across represent days of incubation.

[b] No samples remained for testing.

[c] Each nitrite level initially contained 25 test samples.

TABLE 7. *Effect of sodium nitrite on toxin production by C. botulinum in bacon inoculated with sand-spore mixture and stored at 27 C after slicing*

Inoculum level	Added nitrite (μg/g of meat)	No. of toxic samples after 7 to 84 days					Total no. of toxic samples
		7[a]	14	28	56	84	
Low (40 spores/g)	0	0	1	0	1	0	2[b]
	30	0	1	2	2	2	7
	60	0	2	1	2	2	7
	120	0	0	0	0	1	1
	170	0	0	0	0	1	1
	340	0	0	0	0	1	1
High (3,400 spores/g)	0	2	0	1	2	1	6
	30	2	2	2	2	2	10
	60	0	2	2	2	2	8
	120	0	0	1	1	1	3
	170	0	0	1	0	0	1
	340	0	0	1	0	0	1

[a] Numbers across represent days of incubation.
[b] Each nitrite level initially contained 10 test samples.

DISCUSSION

The results show that nitrite retards toxin production by *C. botulinum* in bacon stored at 27 C. Increased nitrite levels resulted in decreased toxin formation. The nitrite was shown to be effective with two methods of inoculation, simulating contamination of the bacon before and after processing.

Levels of *C. botulinum* in the bacon after smoking were lower than before the smoking process (Table 5). This approximate fourfold decrease in counts at both inoculum levels may be attributed to loss of inoculum during drainage of pickle from the bacon during processing and germination of spores with subsequent death during the 8.5 h of slow heating.

Viable counts of *C. botulinum* were similar in the bacon inoculated by the two methods. The bacon inoculated via the pickle contained 52 and 4,300 *C. botulinum* per g after processing for the low and high inoculum levels, respectively. This closely compares with the counts of 40 and 3,400 per g for the bacon inoculated with the sand-spore mixture. On this basis, a comparison of toxicity results can be made between bacon inoculated before and after processing.

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