

# Comparison of Organic Acid Salts for *Clostridium botulinum* Control in an Uncured Turkey Product

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## ABSTRACT

Health concerns have led consumers toward purchasing nitrite-free, low salt meat and poultry products. Lacking these barriers to control growth of bacterial pathogens, such products carry heightened risks for botulism, especially if temperature abused. To address this threat, five organic acid salts were evaluated as potential antibotulinal agents. Ground turkey breast was formulated with 1.4% NaCl, 0.3% sodium pyrophosphate, 0-6% organic acid salts, 10% ice, and 500 spores per g of a 6-strain mixture of proteolytic *Clostridium botulinum*. Vacuum-packaged product (10 g) was heated in 75°C water for 20 min, cooled, and incubated for up to 18 d at 28°C. Botulinal neurotoxin was detected by mouse bioassay at 2 d in samples which lacked any of the test compounds. Samples containing 2% acid salt developed neurotoxin, which was detected at 2, 2, 4, 5, and 5 d for pyruvate, citrate, lactate, acetate, and propionate, respectively. With 6% acid salt additions, samples remained neurotoxin free until 7 d with pyruvate, 18 d with citrate, and >18 d for the remaining compounds. Monocarboxylic acid salts exhibited antibotulinal activity related to their dissociation constants ( $pK_a$ ). Citrate did not fit this pattern, however, suggesting a different mechanism of action. This study reveals that a variety of organic acid salts possess activity that can be used alone or possibly in combination to enhance the safety of nitrite-free turkey products.

Demand for safe, extended shelf-life refrigerated foods has sparked a search for new and novel agents to control the growth of foodborne bacterial pathogens. Organic acids and their salts have been shown to inhibit a variety of these hazardous organisms, including: *Salmonella* (3), *Listeria monocytogenes* (23), *Yersinia enterocolitica* (9), *Staphylococcus aureus* (12), and *Aeromonas hydrophila* (15). Many of the organic acids and their salts are generally recognized as safe by the U.S. Food & Drug Administration (USFDA), and their wide consumer acceptance makes them attractive as potential compounds to protect foods from pathogen growth.

Little is known, however, about the efficacy and mechanism of action of organic acids or their salts on *Clostridium botulinum*. In one study, Maas et al. (10) demonstrated the efficacy of 2.5-3.5% sodium lactate in inhibiting *C. botuli-*

*num* neurotoxin production for up to 10 d at 27°C in a cook-in-bag turkey product. *C. botulinum* is an organism of central importance to the maintenance of food safety because germination and outgrowth of the thermotolerant spores yields a potent neurotoxin, which can lead to foodborne intoxication if ingested.

Recent outbreaks of foodborne botulism involving commercial products which were temperature abused occurred principally in foods which lacked additional protection such as decreased pH, reduced water activity, or the addition of antimicrobial agents. Salts of organic acids have the potential to provide a recommended second barrier to control growth of *C. botulinum*. Therefore, in this study we evaluated five organic acid salts to assess their antibotulinal activity in an uncured turkey product.

## MATERIALS AND METHODS

### Spore cultures

Six proteolytic strains of *C. botulinum* strains, three type A (62A, 69, 33) and three type B (169, FDA 999, C11) were heat shocked (80°C for 10 min), then sporulated in anaerobic botulinal assay medium broth at 35°C as described by Huhtanen (7). After approximately 2 weeks, spores were harvested by centrifugation, washed with sterile distilled H<sub>2</sub>O, and enumerated by plating and incubating at 35°C for 48 h on botulinal assay medium agar plates inside a flexible anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). Each strain was tested for homogeneity and diagnostic biochemical markers, as described previously (11). Equal numbers of the six strains were pooled and stored at 4°C in sterile distilled water. The mixture was tested for viability prior to each experiment and periodically for toxin type following conventional methods (19).

### Product formulation

Ten pounds (ca. 4.53 kg) of turkey breast meat were obtained from a local distributor, deboned, and double ground with 3/8 in. (ca. 9.5 mm) then 3/16 in. (ca. 4.8 mm) grinder plates using a laboratory grinder (Hobart Corp., Troy, OH). Seven 500-g batches were prepared and each received 1.4% NaCl (7 g, wt/wt) and 0.3% sodium pyrophosphate (1.5 g, wt/wt; Fisher Scientific Company, Fair Lawn, NJ). In addition, 0-6% (0-30 g, wt/vol) of one of the following acid salts in sterile distilled H<sub>2</sub>O (50 ml total volume) were added to five of the batches: sodium acetate (J. T. Baker Chemical Co., Phillipsburg, NJ), sodium lactate (Fisher Scientific Co., Fair Lawn, NJ), sodium pyruvate (Sigma Chemical

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Corp., St. Louis, MO), sodium citrate (J. T. Baker) and sodium propionate (Sigma). All compounds were monocarboxylic acid salts except citrate, which has three carboxyl groups. As needed, the pH levels of the turkey formulations were modified from an initial pH of 5.7-5.8 to a target level of 6.0 using a buffer solution of the specific organic acid salt. The pH levels of the acid salts were adjusted using HCl or NaOH, by using a pH meter equipped with a combination electrode (Model PHM82, Radiometer A/S, Copenhagen). Food mixtures were blended with a Hobart Model N-50 mixer for 1 min at the slowest speed. One of the two remaining samples was used as a positive control (inoculated with spores) and the other a negative control (uninoculated).

#### Turkey product inoculation and incubation

Each 500-g formulation was placed into heat sealable plastic bags, weighed, and 500 spores per g were inoculated into all but the negative control by adding an average of 5 ml of a  $7.6 \times 10^4$  spores per ml suspension of the spore mixture. Bags were heat sealed and mixed well to ensure equal spore distribution. After mixing,  $10 \pm 0.1$ -g samples were weighed into filter stomacher bags (Tekmar, Cincinnati, OH). Stomacher bags were folded and placed into high oxygen barrier bags (All-Vak #13,  $O_2$  permeation = 1.0 cc/100 in<sup>2</sup>/24 h at 25°C, International Kenfield Distributing Co., Rosemont, IL), vacuum sealed, heated in 75°C H<sub>2</sub>O for 20 min, cooled in crushed ice, and incubated at 28°C for 0-18 d. At the end of each incubation period, samples were frozen at -18°C until tested for neurotoxin; in no case was this longer than 3 d. Three samples per treatment were tested at each designated sampling time.

#### Neurotoxin bioassay

Twenty milliliters of 0.2% gelatin phosphate (pH 6.2) was added to the turkey formulations in each stomacher bag and the contents were then macerated with a Model 400 Stomacher (A. J. Seward, London) for 2 min. Filtrates were transferred into sterile tubes and centrifuged at  $1500 \times g$  for 5 min. Each supernatant fluid was tested for botulinum neurotoxin by injecting intraperitoneally 0.5 ml into each of two 15- to 20-g Swiss-Weber albino mice (West Jersey Biological Co., Wenonah, NJ). Mice were acclimated for 24 h prior to challenge, then observed for 96 h postinjection for typical botulinal symptoms (19). Selected samples were neutralized with 300 µl of types A, B, and polyvalent antitoxins (Centers for Disease Control and Prevention, Atlanta,

GA), following FDA procedures (19), to confirm clinical observations.

#### Sensory evaluation

Samples were evaluated by at least one experienced investigator for evidence of off-odors (pungent, putrefied) and textural changes (mushiness, exudate, friability). All investigators were immunized against botulinum neurotoxin.

## RESULTS

The mouse bioassay (Table 1) demonstrated that all of the compounds tested delayed neurotoxin production. Positive controls were toxic at 2 d, while no neurotoxin was found in the negative controls during the 18-d testing period. Samples became toxic at 2, 2, 4, 5, and 5 d for those treated with 2% pyruvate (0.18 M), citrate (0.07 M), lactate (0.22 M), acetate (0.24 M), and propionate (0.21 M), respectively. When concentrations were increased to 6%, time to toxicity increased to 7 d for pyruvate (0.54 M), to 18 d for citrate (0.20 M), and to >18 d for propionate (0.62 M), acetate (0.72 M), and lactate (0.66 M). Neutralization experiments with antitoxin confirmed the presence of botulinal neurotoxin. They also demonstrated that only type A neurotoxin was synthesized, as was observed previously when mixed strains were used (11).

The effect of modulating the pH level of the turkey formulations on delay of neurotoxin was investigated for acetate. Two percent acetate (0.24 M), which was buffered to appropriate pH levels, was added to each of the formulations to yield final pH levels of 5.5, 6.0, or 6.5. Formulations were processed, and samples were stored and assayed as described above. Mouse bioassay results are shown in Table 2. No toxin was detected in the uninoculated controls (pH 6.0). Samples receiving 2% acetate and having formulation pH levels of 5.5, 6.0, and 6.5 became toxic at 7, 6, and 5 d, respectively. Complimentary mineral acid or base controls, which were adjusted to identical pH levels of 5.5, 6.0, and 6.5 were toxic at 2, 2, and 3 d, respectively.

TABLE 1. Detection of *C. botulinum* neurotoxin in inoculated uncured turkey formulated with two levels of different organic acid salts and incubated at 28°C.

Sample	Days											
	0	2	3	4	5	6	7	8	9	11	14	18
+ Control	-	4/6 <sup>a</sup>	6/6	6/6								
-	-	-	-	-	-	-	-	-	-	-	-	-
2% Propionate	-	-	-	-	1/6	5/6						
6%	-	-	-	-	-	-	-	-	-	-	-	-
2% Citrate	-	1/6	3/6									
6%	-	-	-	-	-	-	-	-	-	-	-	2/6
2% Acetate	-	-	-	-	6/6	6/6						
6%	-	-	-	-	-	-	-	-	-	-	-	-
2% Lactate	-	-	-	5/6	6/6							
6%	-	-	-	-	-	-	-	-	-	-	-	-
2% Pyruvate	-	2/6	5/6									
6%	-	-	-	-	-	-	2/6	5/6				

<sup>a</sup> Positive samples/total samples. A sample was considered positive when duplicate mice demonstrated botulism symptoms. Two trials were performed, with each trial including three replicate samples.

- = Negative for neurotoxin after 72 h challenge.

Sensory evaluation for off-odors and texture (Table 3) generally followed a similar temporal pattern as the bioassay data. Lactate, pyruvate, and citrate treated samples became toxic prior to detection of off-odors or soft texture, however. This is significant because of the implication that sensory indicators are unreliable to alert consumers to the danger associated with these toxin-bearing products. Similar observations for garlic-in-oil were reported by Solomon and Kautter (17).

### DISCUSSION

In the present study, five organic acid salts were evaluated for antibotulinal activity. When compared on a molar basis, citrate proved to be most effective (Tables 1, 4), i.e., 0.2 M citrate (6%) delayed toxigenesis far longer than did any of the other acid salts at this corresponding molar concentration. Propionate, acetate, and lactate were intermediate in delaying toxin, in that order; pyruvate was the least effective of the compounds tested.

The efficacy of the monocarboxylic acid salts was related to the combined effect of concentration and to the

$pK_a$ , especially the level of the compound remaining in the undissociated form (listed in Table 4). The relationship between ionization level and antibotulinal activity is demonstrated graphically in Fig. 1 which shows a linear relationship between the square root transformation of the undissociated fraction of the monocarboxylic acid salts and the delay in toxin detection. This correlation is borne out further in Table 2, which shows that lowering the pH level of acetate, with a concomitant shift toward the undissociated form, yielded greater delays in toxin synthesis. The data presented here also demonstrated that a specific acetate effect occurred, because all acetate samples remained toxin free longer than did the complimentary mineral acid controls.

All of the monocarboxylic acid salts used in this study are present in food naturally or approved as intentional food additives. Propionate is present in Swiss cheese at concentrations of up to 1% (1). The antibacterial mode of action has been ascribed to disruption of amino acid metabolism (4,6,22). Acetic acid occurs naturally in vinegar at a level of 4% and is used in curing muscle foods and in condiments (2). Pyruvate occurs naturally in all living

TABLE 2. pH Effects on neurotoxin development in inoculated uncured turkey formulated with two levels of different organic acid salts and incubated at 28°C.

Sample	pH	Time to neurotoxin detection									
		0	1	2	3	4	5	6	7	8	
Negative control <sup>1</sup>	5.7	-	-	-	-	-	-	-	-	-	-
	5.5 <sup>2</sup>	-	-	2/3 <sup>3</sup>	3/3	-	-	-	-	-	-
	6.0	-	-	3/3	2/3	-	-	-	-	-	-
	6.5	-	-	-	3/3	3/3	-	-	-	-	-
Acetate	5.5	-	-	-	-	-	-	-	-	3/3	2/3
	6.0	-	-	-	-	-	-	2/3	3/3	-	-
	6.5	-	-	-	-	-	1/3	2/3	-	-	-

<sup>1</sup> No spores inoculated.

<sup>2</sup> pH Level adjusted with 1 N HCl or NaOH.

<sup>3</sup> Positive samples/total samples. A sample was considered positive when duplicate mice demonstrated botulism symptoms.

- = Negative for neurotoxin after 72 h challenge.

TABLE 3. Sensory evaluation of inoculated uncured turkey formulated with two levels of different organic acid salts and incubated at 28°C.

Sample	Days											
	0	2	3	4	5	6	7	8	9	11	14	18
+ Control	-	-	+	+	-	-	-	-	-	-	-	-
-	-	-	-	-	-	+	+	-	-	-	-	-
2% Propionate	-	-	-	-	-	-	-	-	-	-	-	-
6%	-	-	-	-	+	+	-	-	-	-	-	-
2% Citrate	-	-	-	-	-	-	-	-	-	-	-	-
6%	-	-	-	-	-	-	-	-	-	-	-	-
2% Acetate	-	-	-	+	+	-	-	-	-	-	-	-
6%	-	-	-	-	-	-	-	-	-	-	-	-
2% Lactate	-	-	-	-	+	+	-	-	-	-	-	-
6%	-	-	-	-	-	-	-	-	-	-	-	-
2% Pyruvate	-	-	-	-	+	+	-	-	-	-	-	+
6%	-	-	-	-	-	-	-	-	-	-	-	-

+ = Samples with pungent odor.

- = No pungent odor detected.

TABLE 4. Concentration and dissociation of acid salts used in inoculated uncured turkey.

Acid salt	%	Molarity	pK <sub>a</sub>	pH	Dissociation ratio [HA]/[A <sup>-</sup> ]	[COOH]	[COOH] + [COO <sup>-</sup> ]
Propionate	2	0.21	4.87	6.0	0.077	0.016	0.21
	6	0.62				0.048	0.62
Citrate	2	0.07	1 = 3.13	6.0	1 = 0.0013	0.056	0.21
	6	0.20	2 = 4.76 3 = 6.40		2 = 0.059 3 = 2.5	0.16	0.60
Acetate	2	0.24	4.75	6.1	0.046	0.011	0.24
	6	0.72		6.0	0.056	0.042	0.72
Lactate	2	0.22	3.86	5.8	0.012	0.0026	0.22
	6	0.66				0.0079	0.66
Pyruvate	2	0.18	2.50	5.9	0.0004	0.0001	0.18
	6	0.54		5.8	0.0005	0.0003	0.54

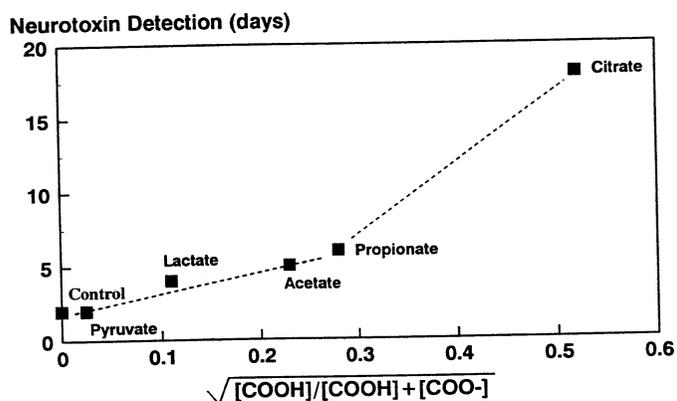


Figure 1. Relationship between neurotoxin detection time and dissociation of 0.2 M acid salts at pH 6.

things and is used commercially as a flavoring agent. Lactate is found in fermented foods as a product of glycolysis and has been shown previously to delay *C. botulinum* toxigenesis. Maas et al. (10) showed that sodium lactate delayed *C. botulinum* toxin production in a cook-in-bag turkey product by a direct lactate ion effect. The authors proposed that either lactate inhibited anaerobic energy generation directly or by lactate flux across cell membranes. The current work supports their observation that lactate delays neurotoxin development.

Inhibitory actions of monocarboxylic salt acids may involve several mechanisms, including: a pH lowering effect on the intracellular pH; alterations in cell membrane permeability, with a disruption of substrate transport; or depletion of the level of reducing agents available for electron transport systems (1). The implication for low acid foods, such as muscle foods, is that salts of monocarboxylic organic acids having higher pK<sub>a</sub> are more effective in delaying *C. botulinum* toxigenesis.

The antibotulinal activity of citrate, a hydroxy tricarboxylic acid, does not follow the pattern exhibited by the monocarboxylic acid salts. Citrate was the most effective compound tested on a molar basis. This effect cannot be explained only by the level of citric acid remaining in the undissociated form. Fig. 1 shows a comparison among the carboxylate dissociation levels from citrate and the other acids. The ratio of the unionized and total carboxylate concentration (at the experimental pH value) indicates a linear relationship among the monocarboxylate acid salts, but it deviates for citrate. This suggests a different mechanism of action.

Several studies demonstrated that the antibotulinal mechanism of citrate inhibition is by chelation of metals, which would be favored when multiple carboxylic acid moieties are ionized (8,16). Furthermore, Graham and Lund (5) demonstrated in a culture medium the action of citrate in delaying *C. botulinum* neurotoxin production and presented data showing that the combined effect of cation chelation and subsequent mineral deprivation was the mechanism of action. Nottermans et al. (13) reported that a mixture of citric and ascorbic acids inhibited toxigenesis by type B strains in vacuum-packaged potatoes.

Alternatively, using a model system at 26°C, Tsang et al. (18) found that at a pH level below 5.0, acetic acid was a more effective inhibitor of *C. botulinum* type E spores than citric acid. In addition, Wong et al. (21) found that in acidified media citric acid was less effective than HCl in preventing the germination of *C. botulinum* type A spores.

Thus, since many pathogens grow at or near ideal refrigeration temperatures (14), organic acid salts may serve as secondary barriers to control bacterial growth in refrigerated foods, particularly those that are temperature abused. These organic acid salts occur widely in nature and are used currently by the food industry (20). They are attractive alternatives to other compounds because they satisfy consumers' demand for natural antimicrobial agents. In conclu-

sion, this study reveals that a variety of acid salts possess activity that can be used alone or possibly in combination to enhance the safety of meat and poultry products.

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