

Antibotulinal Properties of Selected Aromatic and Aliphatic Ketones

BOBBY L. BOWLES* and ARTHUR J. MILLER

ABSTRACT

Several aromatic and aliphatic ketones were tested for inhibitory activity against *Clostridium botulinum* spores and cells. Six-tenths mM 3-heptanone, 3-hexanone, or benzophenone delayed spore germination in botulinal assay medium (BAM) broth at 32°C. Sporicidal activity was observed for 1,250 mM 2,3-pentanedione, while 2-octanone, 3-octanone, or benzophenone were effective at 2,500 mM. In general, higher concentrations were required to inhibit vegetative cells than to prevent spore germination. Maximum activity against vegetative cells was observed at 25 mM acetanisole (4'-methoxyacetophenone), 2,3-butanedione, 2,3-pentanedione, 2-pentanone, or benzophenone, and inhibition was independent of pH. Five-tenths mM acetanisole inhibited dipicolinic acid release, 100 mM reduced 20 min 80°C thermal resistance, and 5.0 mM delayed toxigenesis in BAM broth at 32°C. Furthermore, inhibitory activity of acetanisole was comparable to that observed in BAM broth when tested in commercially prepared chicken and beef broths. The spectrum of antibotulinal activity was dependent upon carbon chain length, carbonyl position, number of carbonyls, and aromaticity. The inhibitions observed suggest that aliphatic and aromatic ketones might have potential as novel antimicrobial agents.

Aromatic and aliphatic ketones are reactive carbonyl compounds that are recognized for their characteristic aromas, flavors, and therapeutic properties. A number of naturally occurring and synthetic ketones are used in perfumes, cosmetics, incense, as food flavoring agents, and are important in a variety of medical and biological materials (13,16). They are characterized by one or more carbonyls attached to a hydrocarbon structure. The reactive properties of these compounds are due to the carbonyl moiety, and the type and location of other functional groups.

A number of ketones are currently approved as food additives. Several ketones and other carbonyl-containing compounds have been demonstrated to have antimicrobial activity (2,4,6,7,14,15). Little information exists, however, on their inhibitory activity against foodborne pathogens, and in particular, against sporeforming bacteria such as *Clostridium botulinum* (2,7,9). Therefore, it was the aim of this study to

determine the activity of several ketones which are approved as food additives, against *C. botulinum* spores and cells.

MATERIALS AND METHODS

Cultures

A spore mixture containing 3 type A (33, 62A, 69) and 3 type B (999, 169, ATCC, 7949) proteolytic *C. botulinum* strains was used throughout the study. Individual strain spore suspensions were prepared by culturing in botulinal assay medium (8) without thioglycollate (BAM) for 21 d at 32°C in a flexible anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). Anaerobiosis was maintained using a gas mixture consisting of 10% CO₂, 5% H₂, and 85% N₂ by periodic gas-exchange flushes and palladium catalyzed O₂ removal. Spore crops were harvested by three successive centrifugations at 17,310 × g for 10 min at 5°C with sterile distilled H₂O washes. Spore pellets were suspended in sterile distilled H₂O, heat shocked (80°C/10 min), and stored at 5°C prior to use. *C. botulinum* confirmation was based on Gram reaction, cellular morphology, neurotoxin production by mouse bioassay, lipase, catalase, and oxidase activities (5). Each spore crop was quantified and the 6-strain spore mixture prepared by combining equal concentrations of the individual strains to create a final concentration of 4.72 × 10⁵ spores per ml. Viability and germination rate were tested initially on individual strains, and monthly on spore mixtures (5). Spore suspensions were quantified prior to storage and immediately before antimicrobial testing.

Aromatic and aliphatic ketones

Acetanisole (4'-methoxyacetophenone) (99%), 2,3-butanedione (99%), 2-butanone (99.5%), 2-heptanone (98%), 3-heptanone (98%), 4-heptanone (98%), 3-hexanone (98%), 2,3-pentanedione (97%), 2-pentanone (97%), 2-octanone (98%), 3-octanone (99%), 2-undecanone (99%), benzophenone (99%), and acetoin (99%) were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Structures and some properties of the ketones tested are listed in Table 1. Stock solutions of the ketones tested were prepared (wt/vol or vol/vol) in 95% reagent grade ethanol according to their respective normal states at 25°C, and purities. Each ketone stock solution was stored at 5°C or at room temperature in an appropriate container, according to their respective reactivity. Final ethanol concentrations (47.5-0.02%) of treatments for antimicrobial testing were below those reported to be sporostatic or sporicidal (11,12).

Determination of spore minimal inhibitory concentrations (MIC)

A modified version of the Association of Official Analytical Chemists method of analysis for sporicidal activity was employed

TABLE 1. Aromatic and aliphatic ketones tested.

Compounds	Structure	NS ^a	FW ^b	BP ^c
2-Butanone	$\text{H}_3\text{C}-\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$	L	72.11	80
2,3-Butanedione	$\text{H}_3\text{C}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$	L	86.09	88
Acetoin	$\begin{array}{c} \text{OH} \quad \text{O} \\ \quad \parallel \\ \text{H}_3\text{C}-\text{C}-\text{C}-\text{CH}_3 \\ \\ \text{H} \end{array}$	S	88.11	148
2-Pentanone	$\text{H}_3\text{C}-(\text{CH}_2)_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$	L	86.13	100 -101
2,3-Pentanedione	$\text{H}_3\text{C}-\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$	L	100.12	110 -112
3-Hexanone	$\text{H}_3\text{C}-(\text{CH}_2)_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\text{CH}_3$	L	100.16	123
2-Heptanone	$\text{H}_3\text{C}-(\text{CH}_2)_4-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\text{CH}_3$	L	114.19	149 -150
3-Heptanone	$\text{H}_3\text{C}-(\text{CH}_2)_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\text{CH}_3$	L	114.19	146 -149
4-Heptanone	$\text{H}_3\text{C}-(\text{CH}_2)_2-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_2-\text{CH}_3$	L	114.19	145
2-Octanone	$\text{H}_3\text{C}-(\text{CH}_2)_5-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$	L	128.22	173
3-Octanone	$\text{H}_3\text{C}-(\text{CH}_2)_4-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_2-\text{CH}_3$	L	128.22	167 -168
2-Undecanone	$\text{H}_3\text{C}-(\text{CH}_2)_8-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$	L	170.30	231 -232
Acetanisoole	$\text{H}_3\text{C}-\text{O}-\text{C}_6\text{H}_4-\text{C}-\text{CH}_3$	S	150.18	152 -154 /26mm
Benzophenone	$\text{C}_6\text{H}_5-\text{C}-\text{C}_6\text{H}_5$	S	182.22	49 -51

Structural and physical properties obtained from Aldrich Chemical Company, Inc., Milwaukee, WI.

^a NS = normal state (liquid or solid) at 25°C.

^b FW = formula weight.

^c BP = boiling point.

to determine the effect of ketones on *C. botulinum* spores (1). Test compounds were serially diluted (2,500, 1,250, 625, 312.5, 156.25,...0.15 mM) in BAM broth and inoculated with 0.1 ml of a 4.72×10^5 CFU/ml heat-shocked (80°C/10 min) activated spore suspension. After anaerobic exposure for 6 h at 32°C, each test

concentration culture was subcultured (0.1 ml) to five replicate tubes of thioglycollate broth prior to (sporidical activity) and after heat-shock (80°C/10 min) treatment (sporostatic or antigerminative activity) to destroy germinated spores. Thioglycollate broth tubes were incubated aerobically for 48 h at 32°C and examined for

turbidity. Lack of growth in ≥ 4 thioglycollate broth tubes inoculated with nonheat- or heat-treated test cultures was interpreted, respectively, as sporicidal or sporostatic (i.e., antigerminative).

Determination of vegetative minimal inhibitory concentrations

The inhibitory effect of aromatic and aliphatic ketones on *C. botulinum* cells was tested at 4 concentrations (25, 125, 250, and 500 mM) and at 3 pH levels (6.0, 7.0, 8.0) in BAM agar. Test compounds were added to BAM agar (100 ml) after autoclaving to yield the desired concentration range at each of three pH values. Treatments were conducted in triplicate for each compound and concentration and included 0 mM controls. The agar plates were surface streak, inoculated with a 24-h culture of *C. botulinum*, and incubated anaerobically for 48 h at 32°C. The growth response of the organism in the presence of ketones and the effect of pH on inhibitory activity were compared with those of unsupplemented BAM controls.

Dipicolinic acid (DPA) release

DPA release was estimated using the colorimetric assay of Janssen et al. (9). Acetanisoole was added to 9.9-ml BAM broth tubes to create a concentration series of 0.5, 1, 2, 3, 4, 5, 50, 100, 200, and 300 mM. Test media were inoculated with 0.1 ml of a heat-shocked (80°C/10 min) spore suspension (4.72×10^5 CFU/ml) and incubated 9 h anaerobically at 32°C. Nine-hour cultures were centrifuged at $1,500 \times g$ for 10 min and the supernatant fluid retained for colorimetric analysis. One milliliter of a freshly prepared 0.5 M acetate buffered chromogenic reagent was added to 4.0 ml of culture supernatant fluid. Optical density was measured at 440 nm using a Shimadzu UV-VIS Model 160 spectrophotometer (Kyoto, Japan), and DPA content was calculated from a standard curve of known DPA concentrations (0-160 $\mu\text{g/ml}$).

Effect of ketones on spore thermal resistance

C. botulinum spores (8.20×10^6 CFU/ml) were aerobically exposed to 100 mM of acetanisoole in 5.0-ml glass vials containing BAM broth for 30 min at 25°C, and the exposure media transferred to an 80°C Exacal high temperature water bath (NesLab Instruments Inc., Newington, NH) for 5-20 min. A Keithley Metrabyte datalogger Model DDL 4100 (Taunton, MA) was used to monitor temperature and equilibration time. An equilibration time of 97 s was required for acclimation of treatment samples to 80°C. Samples were removed, cooled in an ice bath, plated in duplicate onto BAM agar plates using a Spiral Systems Model D plating instrument (Cincinnati, OH), and incubated anaerobically at 32°C for 48 h. Plates were enumerated using Spiral Systems Model 500A, then converted into bacterial counts with Spiral Biotech CASBA™ II BEN software (Bethesda, MD). Spore thermal resistance was evaluated by comparing the population densities of acetanisoole-treated and untreated BAM samples. A 50-min exposure control (25°C) of nonheat-treated spores with acetanisoole was included to confirm spore viability in the absence of thermal treatment.

Inhibitory activity of acetanisoole in chicken and beef broths

Commercially prepared canned chicken and beef broths were dispensed in 9.9-ml portions to sterile test tubes and acetanisoole added to yield concentrations of 2.0, 3.0, 4.0, and 5.0 mM. The broths were inoculated with 0.1 ml of a heat-shocked (80°C/10 min) 4.72×10^5 CFU/ml spore suspension, incubated anaerobically at 25°C, and examined at 24-h intervals for turbidity. The commercial chicken and beef broths contained, respectively, 4.0 g protein, carbohydrate, and fat (chicken or beef); each at a final concentration of 1% (wt/vol).

Effect of acetanisoole on toxigenesis

One-tenth milliliter of a heat-shocked (80°C/10 min) spore suspension (4.72×10^5 CFU/ml) was added to BAM broth tubes

(5.0 ml) that were supplemented with 5, 10, 25, 50, or 100 mM of acetanisoole. Tubes were incubated anaerobically for 48 h at 32°C, then centrifuged ($1500 \times g/10$ min) to remove cellular debris. A 72-h bioassay was conducted on duplicate Swiss-Webber 15- to 20-g mice of either sex by intraperitoneal injection (0.5 ml) of undiluted culture supernatant fluid. Polyvalent antiserum controls were included on some samples to confirm clinical symptoms as botulism (5).

Structure/activity comparisons

The ketones were ranked according to their sporostatic, sporicidal, and vegetative cell MICs using Lotus 1-2-3 (Lotus Development Corporation, Cambridge, MA). Comparisons were based on a variety of chemical and physical properties and included: (i) Carbon chain length; (ii) number of carbonyls; (iii) boiling point; (iv) formula weight; and (v) the aromatic or aliphatic nature of the R-group.

RESULTS

The aromatic and aliphatic ketones tested varied in their ability to delay 6 h germination of *C. botulinum* spores in BAM broth (Table 2). 3-Heptanone, 3-hexanone, and benzophenone were most active; 0.6 mM delayed the events associated with termination of the cryptobiotic state of *C. botulinum* spores. 2-Heptanone, 4-heptanone, 2-octanone, and 2-undecanone were antigerminative at 4.88 mM, while 2-butanone and acetoin (78.12 mM) were the least active of the compounds tested. Sporicidal activity was observed with 1,250 mM 2,3-pentanedione, and with 2,500 mM of 2-octanone, 3-octanone, or benzophenone.

The MICs of aromatic and aliphatic ketones against *C. botulinum* vegetative cells were higher than those observed for spores, and in some instances inhibition was pH dependent (Table 3). Twenty-five millimolars acetanisoole, 2,3-butanedione, 2,3-pentanedione, 2-pentanone, or benzophenone were most active against cells at all pH values tested. At pH 6.0, 25 mM of 2-heptanone, 3-heptanone, 4-heptanone, 3-hexanone, 2-undecanone, and acetoin were equally effective against *C. botulinum* cells. Higher pH values, however,

TABLE 2. Inhibitory activity of ketones against *C. botulinum* spores at 32°C in BAM broth.

Test compound	Sporostatic MIC ^a	Sporicidal MIC
	(mM)	(mM)
Acetanisoole	39.06	>2500 ^b
2,3-Butanedione	9.78	>2500
2-Butanone	78.12	>2500
2-Heptanone	4.88	>2500
3-Heptanone	0.6	>2500
4-Heptanone	4.88	>2500
3-Hexanone	0.6	>2500
2,3-Pentanedione	19.53	1250
2-Pentanone	39.06	>2500
2-Octanone	4.88	2500
3-Octanone	19.53	2500
2-Undecanone	4.88	>2500
Benzophenone	0.6	2500
Acetoin	78.12	>2500

^a Minimal inhibitory concentration.

^b Indicates that concentrations below this level did not inhibit spore germination.

reduced the inhibitory activity of these compounds. MICs of 2-heptanone and acetoin were 4 times higher at pH 7.0, while those of 3- and 4-heptanone, and 3-hexanone were 10 times greater. Aliphatic ketones ≥ 8 carbons were least active against vegetative cells, and their inhibitions were pH dependent.

The spectrum of activity of ketones against *C. botulinum* spores and vegetative cells was tested further using acetanisole. The parameters tested included: (i) Release of dipicolonic acid, (ii) thermal resistance, and (iii) 48 h toxigenesis. Acetanisol, an aromatic ketone, inhibited DPA release, lowered thermal resistance, and delayed toxigenesis. Increasing acetanisole concentrations from 0.1 to 100 mM reduced DPA release exponentially, and at ≥ 150 mM, no DPA was released (Fig. 1). Prior incubation with 100 mM acetanisole reduced by 5 \log_{10} spore populations incubated for 20 min at 80°C in BAM broth (Fig. 2). A 2.5 \log_{10} linear decline was observed during the first 15 min of incubation, while the population dropped another 2.5 \log_{10} during the last 5 min of treatment. Spore concentrations of unsupplemented heated controls and samples treated with 100 mM acetanisole for 50 min at 25°C were constant for the duration of the test. Five millimolars acetanisole delayed 48 h toxin production (Table 4) by *C. botulinum* in BAM broth at 32°C and delayed germination in chicken and beef broths for 8 d at 25°C (Table 5).

TABLE 3. Inhibitory activity of ketones in BAM agar against *C. botulinum* cells at 32°C.

Test compound	48 h MIC ^a (mM) at pH value		
	6.0	7.0	8.0
Acetanisol	25	25	25
2,3-Butanedione	25	25	25
2-Butanone	25	25	125
2-Heptanone	25	125	250
3-Heptanone	25	250	500
4-Heptanone	25	250	500
3-Hexanone	25	250	>500 ^b
2,3-Pentanedione	25	25	25
2-Pentanone	25	25	25
2-Octanone	25	>500	>500
3-Octanone	25	>500	>500
2-Undecanone	25	500	>500
Benzophenone	25	25	25
Acetoin	25	125	500

^a Minimal inhibitory concentration.

^b No inhibition observed at the highest concentration tested.

DISCUSSION

Although several ketones were shown previously to retard bacterial growth, their approved use in foods remains limited to flavor enhancement (10,13,14). In the present study, we demonstrated further their potential antibacterial activity. The activity of ketones was dependent upon hydrocarbon length, location and number of carbonyl groups (i.e., accessibility, steric hinderance, positive charge), aromatic or aliphatic nature of the R-group, and pH.

Six-tenths millimolar 3-hexanone, 3-heptanone, or benzophenone was most effective in delaying germination. Antigerminative activity of aliphatic ketones decreased with increasing chain length, and their activities were dependent upon carbonyl location within the hydrocarbon structure. Aliphatic ketones of ≥ 4 carbons (acetoin, and 2-butanone)

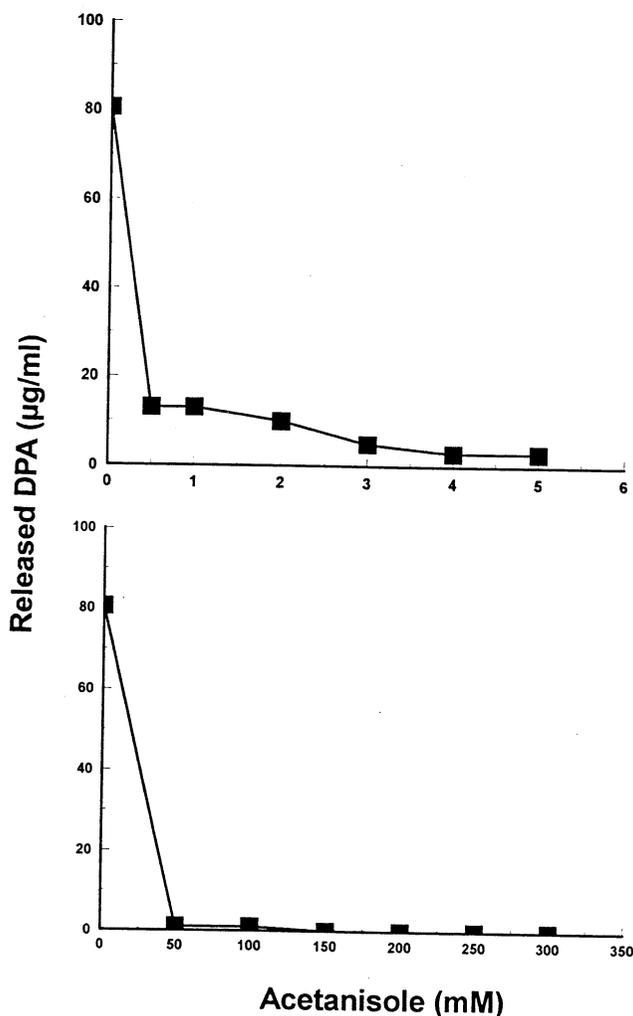


Figure 1. The effect of acetanisole on dipicolonic acid (DPA) release from *C. botulinum* spores in BAM broth after 9 h anaerobic incubation at 32°C.

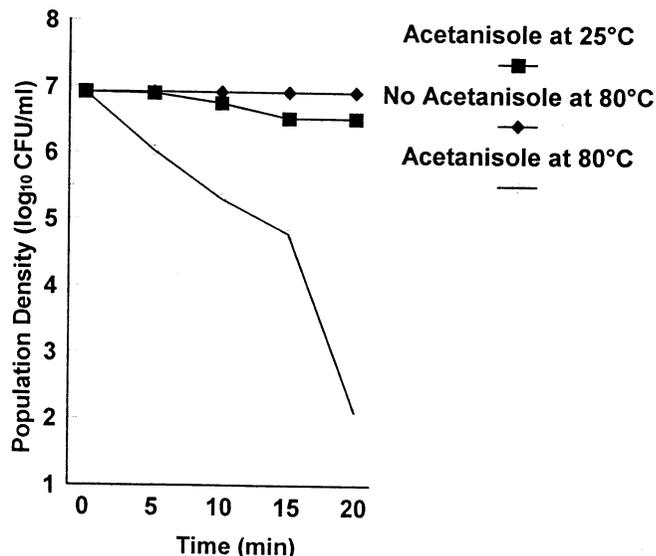


Figure 2. Effect of acetanisole (100 mM) on thermal resistance of *C. botulinum* spores at 80°C in BAM broth. The heat-treated sample was an unsupplemented control.

were the least inhibitory. Location of the carbonyl group at the number 3-carbon position maximized antigerminative activity of heptanone, while the 2-position was most effective for octanone. Sporocidal activity of the ketones tested occurred at much higher concentrations than those observed as antigerminative, and activity was independent of R-group chain length or aromaticity. The most effective compound (2,3-pentanedione, 1,250 mM) was a 5-carbon aliphatic dicarbonyl, while MICs two times higher were required for two 8-carbon aliphatics (2- and 3-octanone) and an aromatic ketone (benzophenone).

Aliphatic and aromatic ketones were less active against *C. botulinum* cells than spores. At pH 7.0, two of the six most active compounds were aromatic, 2 dicarbonyls, and 2 aliphatic. In general, the inhibitory properties of aromatic and dicarbonyl ketones against cells were independent of pH, while those of the aliphatics were pH dependent. Lower molecular weight compounds were more effective than ketones of ≥ 6 carbons and retained activity at pH values ≥ 7.0 . The inhibitory activities of eight carbon aliphatics were enhanced when the carbonyl group was located at the number 2-carbon position and addition of a hydroxyl group to 2-butanone (i.e., acetoin) antagonized vegetative cell inhibition.

To further define the potential antitoxigenic activities of ketones, acetanisole (4'-methoxyacetophenone), an aromatic ketone, was tested for activity against DPA release, thermal resistance, and neurotoxicity. The compound reduced DPA

TABLE 4. The effect of acetanisole on 48 h toxigenesis of *C. botulinum* in BAM broth at 32°C.

Acetanisole (mM)	Final pH	Neurotoxin ^a
0 ^b	6.90	+
5	6.83	-
10	6.82	-
25	6.81	-
50	6.80	-
100	6.80	-
Control ^c	6.90	-

^a Test results of a 72-h bioassay.

^b Unsupplemented BAM control.

^c Unsupplemented BAM broth treated with polyvalent antiserum prior to bioassay.

+ = Positive botulin bioassay.

- = Negative botulin bioassay.

TABLE 5. The effect of acetanisole on germination of *C. botulinum* spores in commercially prepared broths.

Incubation time (d)	Antigerminative MIC ^a (mM) at 25°C	
	Chicken broth	Beef broth
2	2.0	2.0
4	4.0	2.0
6	5.0	4.0
8	5.0	5.0

^a Minimal inhibitory concentration. Acetanisole was tested at 2.0, 3.0, 4.0, and 5.0 mM. Growth was assessed by comparing test cultures with unsupplemented (inoculated and uninoculated) commercial broth controls.

release and thermal resistance and delayed 48 h neurotoxin production. Several resistance properties (i.e., thermal, radiation) of bacterial spores have been attributed to the chemical nature of spore coats and DPA content. DPA release occurs during the initial phase of germination, and its inhibition may be due to the surface active properties of ketones and other carbonyl-containing compounds (16). Antigerminative properties of acetanisole observed in a bacteriological medium were retained when tested in commercially prepared canned chicken and beef broths. Retention of sporostasis in commercial canned broths demonstrates the presumptive efficacy of ketones as antimicrobials in food products.

The antitoxigenic activity of aliphatic ketones was generally less than those found in a previous investigation on aldehydes (submitted for publication). Ketones were less active than aldehydes against *C. botulinum* spores. The activity of ketones against vegetative cells, however, was greater than those observed for aldehydes, and in several instances independent of pH. The results obtained in this study confirm the importance of chain length and aromaticity to the inhibitory properties of carbonyl-containing compounds against *C. botulinum* spores and cells. Differences in the antitoxigenic properties of ketones and aldehydes may be attributed to the steric hindrance in ketones that results from the attachment of two alkyl or aryl groups to the carbonyl atoms, and the tendency of two electron-repelling alkyl or aryl groups to reduce the positive charge of the carbonyl carbon atom (16). Furthermore, the resonance nature of an aromatic ketone reduces the positive charge of the carbonyl carbon atom and are thus less active than aliphatics (16).

These data suggest that aliphatic and aromatic ketones have potential as antitoxigenic agents in foods and are indicative of the activity of carbonyl-containing compounds against spores and cells of *C. botulinum*. Inhibitory activity was shown to be dependent upon a number of structural characteristics, such as: (i) carbon chain length; (ii) location and number of carbonyl groups; and (iii) aromaticity. Additional analyses are needed to test other structurally similar compounds and to conduct challenge studies in foods over extended periods in the presence of these reactive compounds.

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