

Modulation of thermal resistance of proteolytic *Clostridium botulinum* spore by aromatic flavor carbonyls

*Phenylacetaldehyde (PH), benzaldehyde (BE), cinnamaldehyde (CI) and piperonal (PI) were tested, singly and in various combinations, to determine their effect on thermal resistance at 90°C of proteolytic Clostridium botulinum spores. Spore thermal resistance was modulated to varying degrees by the flavor compounds tested. Population densities of ethanol and unsupplemented controls, however, were unchanged for the duration of the test. At 100 mM concentrations, 90°C thermal death decimal reduction times ($D_{90^\circ\text{C}}$) of 15.5, 10.7, 9.3 and 11.6 min were observed, respectively, for PH, BE, CI and PI. Inhibitions comparable with those of single aldehydes were observed when various combinations of the aromatic aldehydes were tested. At a total carbonyl conc. of 100 mM, $D_{90^\circ\text{C}}$ of 36, 14, 26, 14, 15 and 22 min were required to achieve a decimal reduction in population density for PH+BE, PH+CI, PH+PI, BE+CI, BE+PI and CI+PI supplemented treatments, respectively. Similar botulinal thermal resistance modulations occurred in the presence of three- and four-aldehyde combinations. These data indicate that certain approved aromatic flavor compounds have potential as food additive controls against proteolytic *C. botulinum* spores. Furthermore, the observed additive and synergistic combinational effects indicate that organoleptic restrictions or food substrate incompatibilities may be reduced.*

Introduction

Bacterial spores are, in general, highly resistant structures that remain viable in extreme environments (Holland et al. 1969, Sussman and Halvorson 1966). Relatively small quantities of a chemical agent may prevent

germination or act as a germinant (Vinter 1970), however, and sporostatic concentrations of a chemical agent are usually significantly less than those required to inhibit vegetative cell growth (Cook and Pierson 1983, Ram et al. 1979, Smith and Dawes 1989). *Clostridium botulinum* and other sporeforming pathogenic or spoilage bacteria are usually important food industry concerns or public health hazards after germination, outgrowth and vegetative cell proliferation, and heat treatment remains the major destruction method (Hauschild 1989). Furthermore, *Desulfotomaculum* and *Sporolactobacillus* spp. with chemical, thermal and

irradiation resistances comparable with, and in some instances greater than, those of *Bacillus* and *Clostridium* spp. (Botha and Holzapfel 1988, 1987, Doores 1983, Doores and Westhoff 1981) are now recognized as potential food spoilage sporeforming bacteria (Donnelly and Busta 1981, Doores 1983, Nakayama et al. 1984). The list of compounds that can inhibit or lower the resistance of bacterial spores is short and limited in application (Cook and Pierson 1983). Thus, chemical or physical approaches to reducing the thermal resistances of foodborne sporeforming pathogenic or spoilage bacteria are desired.

Bacterial spore thermal resistance has been attributed to their structural composition and to the physical state and location of specific components (Gombas 1983). For example, alpha/beta small acid soluble proteins (Setlow and Setlow 1993), the 'glassy state' configuration of vital spore protoplast macromolecules and supermolecular assemblies (Sapru and Labuza 1993), mineralization (Beaman and Gerhardt 1986), and dipicolinic acid/calcium content and DPA-chelate stabilities (Mallidis and Scholefield 1987) are important determinants in spore thermal resistance.

C. botulinum is an anaerobic, Gram-positive, sporulating bacillus that synthesizes a potent neurotoxin. The disease induced by the neurotoxin is of international concern with distinct seasonal and strain regional differences (Hauschild 1989, Sugiyama 1990). While the incidence of botulism is rare, outbreak potential remains, and *C. botulinum* growth and toxin production has been demonstrated in several new packaging technologies (Bean and Griffin 1990, Conner et al. 1989, Hauschild 1989). *C. botulinum* spores are the important initial contaminants, and control measures are primarily directed against spore transition phases (e.g., activation, germination and/or outgrowth) to ensure botulism-safe foods.

Although the anti-bacterial activity of various plant constituents, such as aldehydes and ketones, is well-documented (Ismail and Pierson 1990, LaBaw and Desrosier 1954, Raccach 1994), few attempts have been made to assess their inhibitory effect on endosporu-

lating foodborne bacterial pathogens. A variety of naturally occurring aromatic and aliphatic aldehydes and ketones were recently demonstrated to have anti-botulinal properties, and several aromatics reduced proteolytic *C. botulinum* thermal resistance (Bowles and Miller 1993a, b). However, little is known about the inactivation kinetics of spore populations heated in the presence of single or various combinations of aromatic aldehydes approved as food additives. As such, the potential application of aromatic aldehydes as thermal processing enhancing food additives was assessed by determining the thermal resistance at 90°C of proteolytic *C. botulinum* spores in the presence of single or combined concentrations of phenylacetaldehyde (PH), benzaldehyde (BE), cinnamaldehyde (CI) or piperonal (PI).

Materials and Methods

Cultures

A spore suspension of proteolytic *C. botulinum* 169B was prepared as previously described (Bowles and Miller 1993a, b). *C. botulinum* confirmation was based on Gram-reaction, cellular morphology, neurotoxin production by mouse bioassay, lipase, catalase, and oxidase activities (Centers for Disease Control 1974). All analyses, unless otherwise indicated, were conducted using a heat-shock (10 min at 80°C) activated $7.0 \log_{10}$ cfu ml⁻¹ *C. botulinum* 169B spore suspension.

Test compounds

BE (99%), CI (99%), PI (99%) and PH (90%) were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI, USA. Some properties of the aldehydes tested are listed in Table 1. Stock solutions were prepared (wt/vol or vol/vol) in 95% reagent grade ethanol according to their respective normal states at 25°C and purities. Final ethanol concentrations of treatments for antimicrobial testing were below those reported to be sporestatic or sporicidal (Koransky et al. 1978).

Activity against proteolytic *C. botulinum* spore 90°C thermal resistance

Single aldehyde treatments. *C. botulinum* spores were exposed for 30 min at 25°C to 50 or 100 mM of PH, BE, CI or PI in 17.0 ml glass vials containing cooked meat medium (CMM, pH 7.2±0.2) (DIFCO, Detroit, MI, USA). The vials were then transferred to a 90°C Exacal high temperature water-bath (NesLab Instruments Inc., Newington, NH, USA) for 0–30 min. A Keithley Metrabyte datalogger model DDL 4100 (Taunton, MA, USA) was used to monitor temperature and equilibration time. Three samples were removed at 5 min intervals, cooled, and 0.1 ml of each replicate diluted, respectively, into 9.9 ml 0.1% peptone-H₂O (pH 7.2; DIFCO, Detroit, MI, USA). The diluted samples were plated in triplicate onto brain–heart infusion agar (DIFCO, Detroit, MI, USA) using a Spiral Systems Model D plating instrument (Cincinnati, OH, USA) and incubated 48 h at 32°C in Model IV SHEL LAB Bactron anaerobic/environmental chamber (Sheldon Manufacturing, Inc., Cornelius, OR, USA). Surviving populations were enumerated, and bacterial counts converted into thermal death

values using Lotus 1-2-3 Release 4.01 (Lotus Development Corporation, Cambridge, MA, USA) regression analysis. Only survival curves with values in the straight portion, with a correlation coefficient (r^2)>0.90 were used. The heat resistance data were analyzed by analysis of variance to determine if there were statistically significant differences among the treatments. A triplicate set of 60 min exposure controls (25°C) of non-heat-treated spores, with 50 or 100 mM of the respective aldehydes or with and without added ethanol, was included to confirm viability in the absence of thermal treatment.

Combined aldehyde treatments. *C. botulinum* spores were exposed for 30 min at 25°C to combinations of two, three, or four aldehydes (100 or 50 mM total carbonyl conc.) of PH, BE, CI, or PI in 17.0 ml glass vials containing CMM. The aldehyde combinations tested were PH+BE, PH+CI, PH+PI, BE+CI, BE+PI, CI+PI, PH+BE+CI, PH+CI+PI, BE+CI+PI, and PH+BE+CI+PI. The treatments were thermally processed and thermal death values determined as previously described. Non-heat-treated controls (25°C)

Table 1. Flavor compounds

Description	Compound			
	Phenylacetaldehyde	Benzaldehyde	Cinnamaldehyde	Piperonal
Normal state (25°C)	Liquid	Liquid	Liquid	Solid
Formula weight	120.15	106.20	132.16	150.13
Boiling point (°F)	195	178–197	250–252	264
Regulatory status	synthetic flavor 21CFR 172.515	GRAS 21CFR 182.60	GRAS 21CFR 182.60	GRAS 21CFR 182.60
Botanical source	Synthetic	<i>Prunus amygdalus</i> var. <i>dulcis</i>	<i>Cinnamomum zeylanicum</i>	<i>Piper nigrum</i>
Solubilities	Slightly in H ₂ O; EtOH; Et ₂ O	350 pt. H ₂ O; sol. in EtOH, Et ₂ O, oils	700 pt. H ₂ O; 7 vol. 60% EtOH; Et ₂ O; chloroform	500 pt. H ₂ O; EtOH; Et ₂ O
Sensory character	Sweetly flora, lilac & hyacinth	Almond	Sweetly spicy, cinnamon	Pepper, aromatic, spicy, pungent
Toxicity data & CODEN	orl-rat LD50: 1550 mg/kg FCTXAV 17,377-79	orl-rat LD50: 1300 mg/kg FCTXAV 2,327,64	orl-rat LD50: 2220 mg/kg FCTXAV 2,327,64	orl-rat LD50: 2700 mg/kg TXAPA9 6,378,64

Structural and physical properties obtained from Aldrich Chemical Company, Inc., Milwaukee. Merck Chemical Index (11th edn.) (Merck & Co. Inc., Rahway, NJ, USA), and the AVI Source Book of Flavors (Westport, CT, USA).

were included for each of the aldehyde combinations tested, to confirm viability in the absence of thermal treatment.

Activity of aldehyde-combination treatments in beef broth. Commercially prepared beef broth (initial pH 5.63) was dispensed into 17.0 ml glass vials, and three or four aldehyde-combinations added to achieve a total carbonyl concentration of 50 or 100 mM. The aldehyde combinations tested were PH+BE+CI, PH+CI+PI, BE+CI+PI, and PH+BE+CI+PI. The treatments were thermally processed and thermal death values determined as previously described.

Results and Discussion

In general, the aromatic aldehydes PH, BE, CI and PI were found to be effective agents for reducing the time and temperature required for thermal inactivation of proteolytic *C. botulinum* 169B spores. Population densities of ethanol and unsupplemented controls were not changed during the course of the test. When subjected to a non-lethal heat treatment at 90°C in the presence of 100 mM PH (pH 6.2), BE (pH 6.2), CI (pH 6.4) or PI (pH 7.0), thermal resistance of proteolytic *C. botulinum* spores was reduced (Fig. 1). One-hundred millimolar CI ($D_{90^\circ\text{C}}=9.3$ min) was the most active carbonyl tested, with 10.7, 11.6, 15.5 min required to achieve a decimal reduction in population density for BE, PI and PH supplemented treatments, respectively. However, activity of the compounds against spore thermal resistance was reduced when tested at 50% lower concentration (data not shown). At 50 mM, decimal reduction times for PH (pH 6.5), BE (pH 6.6), CI (pH 6.6) and PI (pH 7.0) supplemented treatments, were increased to 28, 73, 31 and 163 min, respectively; however, the values were lower than the controls (with and without added ethanol).

The antagonistic effect of the aromatic flavor carbonyls on proteolytic *C. botulinum* thermal resistance was retained, and in some instances enhanced when two-, three- or four-aldehyde-combinations were tested in cooked

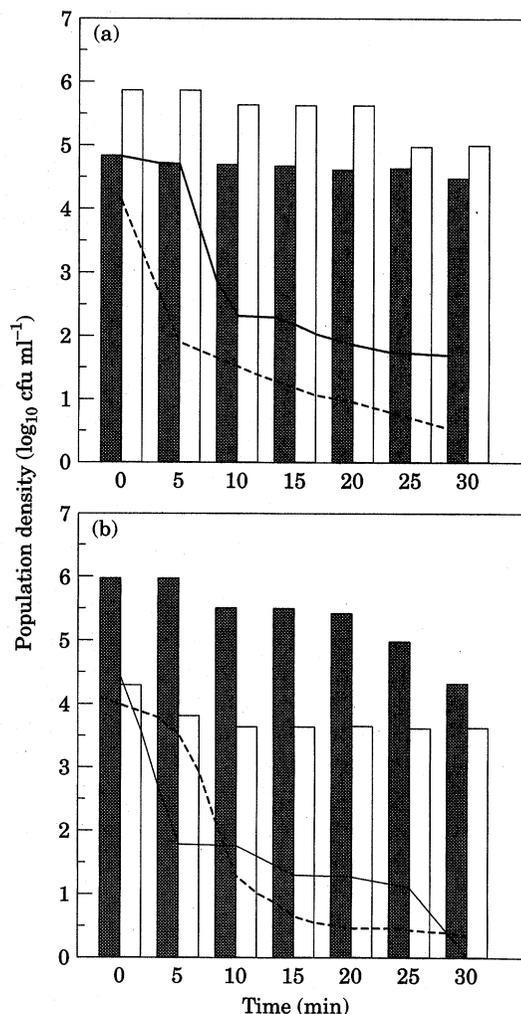


Figure 1. Effect of selected aromatic flavor compounds (100 mM) on thermal resistance at 90°C of spores of proteolytic *Clostridium botulinum* in cooked meat medium. (a) (▣) 100 mM Phenylacetaldehyde+25°C pH 6.17; (---) 100 mM Phenylacetaldehyde+90°C $D_{90}=15.51$; (□) 100 mM Benzaldehyde+25°C pH 6.24; (—) 100 mM Benzaldehyde+90°C $D_{90}=10.71$. (b) (▣) 100 mM Cinnamaldehyde+25°C pH 6.39; (—) 100 mM Cinnamaldehyde+90°C $D_{90}=9.37$; (□) 100 mM Piperonal+25°C pH 7.04; (---) 100 mM Piperonal+90°C $D_{90}=11.58$.

meat medium (Table 2). At a total carbonyl concentration of 100 mM, 50 mM combinations of BE and CI or PH and CI were the most active two-aldehyde combinations, while a 50 mM combination of PH and BE was the least effective. Although 50 mM PI ($D_{90^\circ\text{C}}=163$)

was previously shown to be the least effective single carbonyl treatment, its inhibitory activity was enhanced when combined with the other aromatic aldehydes. Similar inhibitions were observed when two-aldehyde combinations were tested at a total carbonyl concentration of 50 mM. Twenty-five millimolar combinations of PH and BE, CI or PI, reduced *C. botulinum* spore thermal resist-

ance. In all instances, moreover, thermal death times were lower than those observed for 50 mM quantities of single carbonyls. Of the 25 mM aldehyde combinations tested (50 mM final carbonyl conc.), BE+PI was most effective, followed in order of increasing decimal reduction times by PH+CI, BE+CI, PH+PI, PH+BE and CI+PI. Reduced proteolytic *C. botulinum* thermal resistance was

Table 2. Effect of various carbonyl combinations on thermal resistance at 90°C of spores of proteolytic *Clostridium botulinum*^a in cooked meat medium

Carbonyl combination	Conc. (mM) ^c	Final pH	Time (min) to 90°C	Log ₁₀ cfu ml ⁻¹ Time 0 ^d	D _{90°C} (min)
PH+BE	50	6.47	3.50	4.61	22
	100	6.06	5.55	3.01	36
PH+CI	50	6.20	3.55	4.92	14
	100	5.85	6.00	3.95	14
PH+PI	50	6.67	3.55	5.34	17
	100	6.44	5.30	2.77	26
BE+CI	50	6.57	3.30	5.18	19
	100	6.26	4.00	1.97	14
BE+PI	50	6.78	3.40	4.97	11
	100	6.53	4.45	5.62	15
CI+PI	50	6.83	3.35	4.78	24
	100	6.61	5.50	3.85	22
PH+BE+CI	50	6.28	4.00	3.86	26
	100	6.44	4.40	3.74	20
PH+CI+PI	50	6.28	5.35	3.62	24
	100	6.21	4.30	2.41	14
PH+BE+CI+PI	50	6.45	4.50	3.64	31
	100	6.12	4.00	2.99	42

^a7.0 Log₁₀ cfu ml⁻¹ was the initial inoculum for each treatment.

^bPH, phenylacetaldehyde; BE, benzaldehyde; CI, cinnamaldehyde; PI, piperonal.

^cAppropriate amounts of each compound added to achieve a 50 mM or 100 mM total carbonyl concentration.

^dPopulation density at temperature equilibrium.

Table 3. Proteolytic *Clostridium botulinum* spore^a thermal resistance in commercial beef broth supplemented with various aldehyde combinations

Carbonyl combination	Conc. (mM) ^c	Final pH	Time (min) to 90°C	Log ₁₀ cfu ml ⁻¹ Time 0 ^d	D _{90°C} (min)
PH+BE+CI	50 ^a	5.39	4.25	3.86	9
	100 ^b	5.18	3.40	3.74	38
PH+CI+PI	50	5.54	4.00	3.62	16
	100	5.26	3.30	2.41	64
BE+CI+PI	50	5.48	4.00	4.05	21
	100	5.26	3.50	3.86	22
PH+BE+CI+PI	50	5.51	3.50	3.64	19
	100	5.30	4.50	2.99	31

^a7.0 Log₁₀ cfu/ml was the initial inoculum for each treatment.

^bPH=phenylacetaldehyde; BE=benzaldehyde; CI=cinnamaldehyde; PI=piperonal.

^cAppropriate amounts of each compound added to achieve a 50 mM or 100 mM total carbonyl concentration.

^dPopulation density at temperature equilibrium.

also observed when three-aldehyde combinations were tested. At a total aldehyde concentration of 100 mM, D_{90} times of 14, 20 and 70 min were observed for 33.33 mM combinations of PH+CI+PI (pH 6.21), and PH+BE+CI (pH 6.44), respectively. When all four aromatic aldehydes were tested in combination, activity was retained. Approximately 25% less time was required at 50 mM than at 100 mM total carbonyl concentration to achieve a \log_{10} cfu ml^{-1} population reduction, however, proteolytic *C. botulinum* thermal resistance modifications appeared to be independent of these factors. We hypothesize that the consistently observed reduced activity of three- or four-aldehyde combinations of >50 mM final carbonyl concentration may be attributed to concentration dependent interactions between individual aldehydes, or to increased competitive binding of less inhibitory aldehydes to spore coat reactive sites.

The effect of the aromatic aldehydes on 90°C proteolytic *C. botulinum* thermal resistance was retained when tested in a commercial beef broth (Table 3). Proteolytic *C. botulinum* thermal resistance at 90°C was most reduced when heated in the presence of a three-aldehyde combination (50 mM total carbonyl conc.) that consisted of 12.5 mM combinations of PH+BE+CI, with 9 min required to achieve a decimal reduction in population density. The thermal death values of 50 mM total-carbonyl treatments were consistently less than those observed for 100 mM three- or four-aldehyde combinations.

These data indicate that carbonyl concentration and aromaticity are equally important factors in proteolytic *C. botulinum* thermal resistance modification. At lower concentrations, comparable inhibitions can be achieved when highly active aldehydes are combined with less active carbonyls. The anti-botulinal activity of the aldehyde-combinations tested appear to be concentration and substrate dependent; 50 mM total carbonyl concentrations were in several instances significantly more effective than 100 mM. Although various acclimation times and pH values were observed for the aromatic aldehyde supplemented treatments, modulation of proteolytic *C. botulinum* thermal resist-

ance appeared to be independent of these factors. Various synthetic plant auxins that are also naturally occurring, were shown to reduce the thermal resistance of a flat sour sporeforming bacillus (LaBaw and Desrosier 1954), and thermal inactivation of *Bacillus cereus*, *B. subtilis* and *B. coagulans* spores was enhanced in a 75%-butter/25%-water emulsion (Fuchs and Clausen 1976). Diacetyl (2,3-butandione), an α -dicarbonyl, is a naturally occurring butter component.

Conclusions

Several approved flavor compounds were shown to reduce the thermal resistance of proteolytic *C. botulinum* 169B spores. Although optimal activity was observed at 100 mM, individual carbonyl concentrations of 33.33, 25 or 12.5 mM were as effective when two-, three- and four-aldehyde-combinations were tested, and in some instances, activity was enhanced. These data indicate that phenylacetaldehyde, benzaldehyde, cinnamaldehyde or piperonal may be employed to decrease the risk of botulism in food, and that combinations may be used to enhance activity, or to reduce/alleviate objectionable sensory characters or food substrate restrictions.

References

- Beaman, T. C. and Gerhardt, P. (1986) Heat resistance of bacterial spores correlated with protoplast hydration, mineralization, and thermal adaptation. *Appl. Environ. Microbiol.* **52**, 1242–1246.
- Bean, N. H. and Griffin, P. M. (1990) Foodborne disease outbreaks in the United States, 1973–1987: pathogens, vehicles, and trends. *J. Food Protect.* **53**, 804–817.
- Botha, S. J. and Holzappel, W. H. (1988) Resistance of vegetative cells and endospores of *Sporolactobacillus* to gamma-irradiation. *Int. J. Food Microbiol.* **7**, 169–172.
- Botha, S. J. and Holzappel, W. H. (1987) Resistance of *Sporolactobacillus* to potassium sorbate and sodium nitrate. *Int. J. Food Microbiol.* **5**, 331–336.
- Bowles, B. L. and Miller, A. J. (1993a) Antibotulinal properties of aromatic and aliphatic aldehydes. *J. Food Protect.* **56**, 788–794.

- Bowles, B. L. and Miller, A. J. (1993b) Antibotulin properties of aromatic and aliphatic ketones. *J. Food Protect.* **56**, 795–800.
- Centers for Disease Control. (1974) Detection of *Clostridium botulinum* and botulin toxin. In *Laboratory Methods in Anaerobic Bacteriology, CDC Laboratory Manual*. U.S. Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, pp. 41–44, Atlanta, Georgia.
- Conner, D. E., Scott, V. N. and Bernard, D. T. (1989) Potential *Clostridium botulinum* hazards associated with extended shelf-life refrigerated foods: a review. *J. Food Safety* **10**, 131–153.
- Cook, F. K. and Pierson, M. D. (1983) Inhibition of bacterial spores by antimicrobials. *Food Technol.* **37** (11), 115–126.
- Donnelly, L. S. and Busta, F. F. (1981) Anaerobic sporeforming microorganisms in dairy products. *J. Dairy Sci.* **64** (1), 161–166.
- Doores, S. (1983) Bacterial spore resistance—species of emerging importance. **37** (11), 127–134.
- Doores, S. and Westhoff, D. (1981) Heat resistance of *Sporolactobacillus inulinus*. *J. Food Sci.* **46** (3), 810–812.
- Fuchs, A. and Clausen, M. (1976) Heat resistance of bacterial spores in dry butterfat, in butter/water emulsion and in phosphate buffer. *Milchwirtsch. Forsch.* **5**, 91–97.
- Gombas, D. E. (1983) Bacterial spore resistance to heat. *Food Technol.* **37** (11), 105–110.
- Hauschild, A. H. W. (1989) *Clostridium botulinum*. In *Foodborne Bacterial Pathogens* (E. Doyle, M. P.), pp. 112–189, Marcel Dekker, Inc., New York.
- Holland, D., Barker, A. N. and Wolf, J. (1969) Factors affecting germination of clostridia. In *Spores IV*. (Ed. Campbell, L. L.), pp. 317–325. Bethesda, Maryland, American Society for Microbiology.
- Ismail, A. A. and Pierson, M. D. (1990) Inhibition of germination, outgrowth, and vegetative growth of *Clostridium botulinum* by spice oils. *J. Food Protect.* **53**, 755–758.
- Koransky, J. R., Allen, S. D. and Dowell, V. R., Jr. (1978) Use of ethanol for selective isolation of sporeforming microorganisms. *Appl. Environ. Microbiol.* **35**, 762–765.
- LaBaw, G. D. and Desrosier, N. W. (1954) The effect of synthetic plant auxins on the heat resistance of spores. *Food Res.* **19**, 98–105.
- Mallidis, C. G. and Scholefield, J. (1987) Relation of the resistance of bacterial spores to chemical composition and structure i. relation to core components. *J. Appl. Bacteriol.* **62**, 65–69.
- Nakayama, A., Kadota, H. and Sonobe, J. (1984) Classification and identification of the bacteria causing obligate-anaerobic flat sour spoilage. *J. Food Hygiene Soc. Jap.* **24** (4), 297–309.
- Raccach, M. (1994) The antimicrobial activity of phenolic antioxidants in foods: a review. *J. Food Safety* **6**, 141–171.
- Ram, B. P., Rana, R. S. and Gollakota, K. G. (1979) Inhibition of germination of *Bacillus cereus* T spores by phenylglyoxal. *Folia Microbiol.* **24**, 228–233.
- Sapru, V. and Labuza, T. P. (1993) Glassy state of bacterial spores predicted by polymer glass-transition theory. *J. Food Sci.* **58** (2), 445–448.
- Setlow, B. and Setlow P. (1993) Binding of small acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. *Appl. Environ. Microbiol.* **59** (10), 3418–3423.
- Smith, K. T. and Dawes, J. W. (1989) The preferential inhibition of *Bacillus subtilis* spores outgrowth by chloroquine. *Arch. Microbiol.* **152**, 251–257.
- Sugiyama, H. (1990) Botulism. In *Foodborne Diseases* (Ed. Cliver, D. C.), pp. 108–125. San Diego, Academic Press.
- Sussman, A. and Halvorson, H. O. (1966) *Spores. Their Dormancy and Germination*. pp. 116–125, 150–185, and 216–270. New York, Harper and Row.
- Vinter, V. (1970) Germination and outgrowth: effect of inhibitors. *J. Appl. Bacteriol.* **33**, 50–58.