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Inhibition of Bacterial Spore Growth by Fatty Acids and Their Sodium Salts

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ABSTRACT

The antimicrobial activity of 11 fatty acids and their salts was tested on spores of *Clostridium botulinum* 62A, *Clostridium sporogenes* PA3679, and *Bacillus cereus* F4165/75.

Linolenic acid was the most inhibitory fatty acid and lauric acid was the most inhibitory of the saturated fatty acids. Minimum inhibitory concentrations ranged from 50-150 μg/ml for lauric acid, ≥150 μg/ml for myristic acid, 30-100 μg/ml for linoleic acid, and 10-75 μg/ml for linolenic acid depending on the strain. Caprylic, capric, palmitic, stearic, arachidic, and erucic acids showed only partial inhibition (44 to 90%) at concentrations as high as 150 μg/ml.

Addition of 0.2-0.3% (wt/vol) starch neutralized the inhibitory effect of palmitic, linoleic, and linolenic acids but had no effect on lauric acid even when increased to 1%.

Lauric, linoleic, and linolenic acids were shown to inhibit spore germination as measured by loss of spore heat resistance.

The antimicrobial properties of fatty acids and fatty acid salts or esters have been known for many decades. Several investigations have demonstrated the inhibitory effects of these compounds on vegetative bacteria, yeasts, molds, viruses, and tumor cells. The results of these investigations have been compiled by Bayliss (*3*), Kodicek (*13*), Nieman (*14*), and Kabara (*11*). Other researchers demonstrated that compounds such as starch, serum albumin, cholesterol, lecithin, Ca^{2+}, and Mg^{2+} can antagonize the antimicrobial properties of fatty acids (*7,8*).

Comparatively few studies have addressed the effect of fatty acids on bacterial spores, and the information available is rather scant and often inconclusive or controversial. Blocher and Busta (*4*) identified the need for systematic research in this area. Kabara (*12*) reported some possibilities for the use of some fatty acids or their esters as a component of a food preservative system comprising food grade phenolics and chelators.

The present investigation addresses the effect of different fatty acids and their salts on spores of two foodborne pathogens, *Clostridium botulinum* 62A and *Bacillus cereus* F4165/75, and one spoilage bacterium, *Clostridium sporogenes* PA3679.

MATERIALS AND METHODS

Bacterial strains

Spores of *C. botulinum* 62A, *C. sporogenes* PA3679, and *B. cereus* F4165/75 were obtained from the culture collection of the Department of Food Science and Nutrition, University of Minnesota, St. Paul.

Preparation of spore suspensions

The spore suspension of *C. botulinum* 62A was prepared according to the method of Schmidt and Nank (*18*) and Christiansen et al. (*5*). *C. sporogenes* PA3679 spores were prepared on pork infusion medium containing 5 g of glucose per L using a biphasic culture technique (*1*). *B. cereus* F4165/75 spores were prepared on fortified nutrient agar according to the method of Johnson et al. (*10*).

Each spore suspension was cleaned by repeated washings in sterile distilled water and by differential centrifugations. After each centrifugation, spore suspensions were observed for stratification. Strata were separated into individual centrifuge bottles, and wet mounts were observed using a phase contrast microscope (Olympus EHT, Tokyo, Japan). Strata containing predominantly vegetative cells were discarded. PA3679 spore suspension required an enzymatic treatment using trypsin and lysozyme as described by Foegeding an Busta (*6*). Final pellets containing 90% spores for PA3679 and 98% spores for 62A and F4165/75 strains were stored in sterile distilled water at 4 ± 2°C.

Effect of free fatty acids and fatty acid salts on bacterial spores

Eleven free fatty acids, (caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), linolenic ($C_{18:3}$), arachidic ($C_{20:0}$), and erucic acid ($C_{22:1}$), were obtained from Nu Chek Prep, Inc. (Elysian, MN) with a purity >99%. Unsaturated fatty acids were flushed with nitrogen and sealed under high vacuum before shipment. Each fatty acid was dissolved in 95% ethanol solution to a final concentration of 100 mg/ml. Sodium salts of fatty acids were also prepared by mixing a portion of the fatty acid with the appropriate amount of 0.1 N NaOH. All fatty acid and salt solutions were filter sterilized at low temperature (6-10°C) using 0.45 μm Millipore filter.

Portions of the sterile fatty acid or salt solutions were aseptically added to 500-ml Erlenmeyer flasks containing 150 ml of sterile culture medium and thoroughly mixed. The contents of each flask was then equally dispensed into either petri plates previously inoculated with 100-300 heat-activated spores of *B. cereus* F4165/75 or test tubes (20 x 200 mm) inoculated with 250-300 heat-activated spores of *C. botulinum* 62A or *C.*

sporogenes PA3679. Heat activation was for 15 min at 70°C for F4165/75, 15 min at 80°C for 62A, and 9 min at 100°C for PA3679. *Bacillus* spores were enumerated on Trypticase soy agar (TSA, BBL) (*10*) and clostridial spores were enumerated on supplemental yeast extract agar (Difco) (*15,16*). Anaerobiosis was achieved for clostridia using the modified Lee tube technique described by Ababouch and Busta (*2*). Upon solidification, the plates were incubated at 30°C for 48 h and the modified Lee tubes at 32°C for 48 h.

The maximal amount of ethanol that was carried into the culture medium (1%) did not inhibit colony formation from spores. Levels of 5% ethanol were shown to have no effect (data not shown).

Three to five concentrations were tested for each fatty acid or salt solution. These concentrations ranged from 10 to 100 μg/ml in increments of 10 to 20 μg/ml for strong inhibitors and from 50 to 150 μg/ml (up to 500 μg/ml for some salts) in increments of 50 μg/ml for weak inhibitors. The control contained no fatty acid or salt.

Antagonism of fatty acid inhibition by starch

The effect of soluble starch (Fisher) on eight fatty acids (caprylic, capric, lauric, palmitic, stearic, and arachidic acids at 150 μg/ml each, linoleic and linolenic acids at 100 μg/ml each) was tested using spores of *C. botulinum* 62A and *C. sporogenes* PA3679. The procedure described before was adopted except that starch was mixed into the culture medium at 0.2, 0.3, 0.4, or 1% (wt/vol). Controls consisted of inoculated culture medium containing no fatty acid and no starch, or no starch.

Effect of fatty acids on spore germination

The effect of lauric acid (50 μg/ml), linoleic acid (30 μg/ml), and linolenic acid (10 μg/ml) on the germination of *B. cereus* F4165/75 spores in Trypticase soy broth (TSB, BBL) was investigated. Germination was measured by loss of heat resistance (*10*) in triplicate trials for each fatty acid. Spores of *B. cereus* F4165/75 in distilled water were heat activated at 70°C for 15 min prior to the inoculation of TSB, previously equilibrated at 30°C and containing the fatty acid. The initial population in TSB was ca. 10^6 spores per ml. Three- milliliter samples were taken every 15 min for 2 h; 1 ml was immediately diluted and plated and the remaining 2 ml were heat shocked for 15 min at 70°C prior to plating. Enumerations of total populations (unheated sample) and spore populations (heated sample) were performed on TSA supplemented with 0.3% (wt/vol) starch. Care was exercised to ensure that the amount of fatty acid that was carried from TSB into TSA was insignificant (<1 μg/ml) and similarly present in corresponding (timewise) TSA plates inoculated from the control or from the inhibitor-containing TSB. All plates were incubated for 20-24 h at 30°C. The percentage of germinated spores at a given sampling time was calculated as follows:

$$\% \text{ germination} = \left[1 - \left(\frac{\text{spore population}}{\text{total population}}\right)\right] \times 100$$

Experimental design

A completely randomized design was used to study the effect of free fatty acids and fatty acid salts on bacterial spores. Ten petri plates or 10 modified Lee tubes were used to enumerate viable spores for each strain and each level of each inhibitor, except for the starch experiments where five instead of 10 petri plates were used. Two to three trials were performed for each strain and inhibitor. For each experiment, data from each fatty acid treatment level were geometrically averaged, and analysis of variance was conducted on the geometric means (*19*) at a level of significance $\alpha = 0.05$.

RESULTS

Figures 1 and 2 represent the effect of free fatty acids on spores of *C. sporogenes* PA3679 and *C. botulinum* 62A, respectively. Table 1 presents the minimum inhibitory concentrations of the different free fatty acids and fatty acid salts tested. Minimum inhibitory concentration is defined as the lowest concentration tested that showed total inhibition of colony formation within 48 h of incubation. The ability of PA3679 and 62A spores to form colonies was affected differently by the fatty acids tested. Lauric acid was the most inhibitory saturated fatty acid. Short-chain (number of carbons n < 12) saturated acids were less active than myristic acid and less (at 50 and 100 μg/ml) or equally (at 150 μg/ml) active as palmitic acid in inhibiting PA3679 spores ($\alpha = 0.05$). Inhibitory activity was observed for all acids against 62A spores except for arachidic acid which was the least inhibitory.

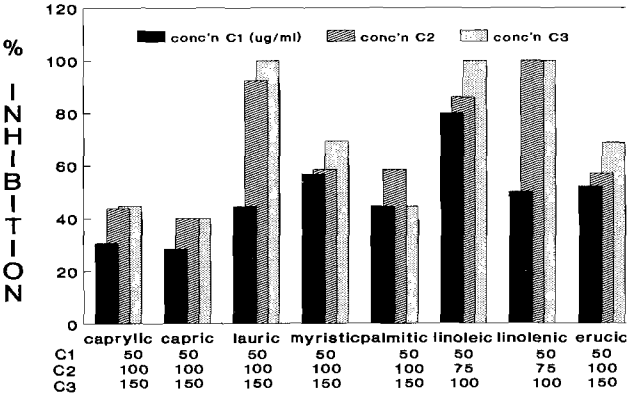


Figure 1. *Inhibition of* C. sporogenes *PA3679 spore growth by fatty acids.*

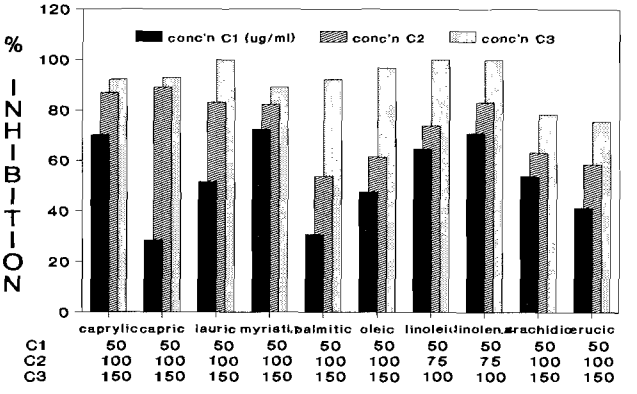


Figure 2. *Inhibition of* C. botulinum *62A spore growth by fatty acids.*

These data show also that among unsaturated fatty acids, linolenic acid showed the highest inhibitory activity and was equally or more active than lauric acid. *C. botulinum* 62A spores were more sensitive than *C. sporogenes* PA3679 spores to short-chain saturated fatty acids, whereas linoleic and linolenic acids were more active against PA3679 spores as compared to 62A spores.

Figures 3 and 4 represent the effect of fatty acid salts on spores of *C. botulinum* 62A and *B. cereus* F4165/75, respectively. These data, along with those in Table 1, confirm previous findings with free fatty acids. Sodium

Table 1. *Minimum inhibitory concentration*[a] *(in μg/ml) of fatty acids and their salts on bacterial spores of* C. sporogenes *PA3679,* C. botulinum *62A,* and B. cereus *F4165/75.*

Fatty Acid	Free Fatty Acids		Fatty Acid Salts	
	PA 3679	62 A	62A	F 4165/75
Caprylic $C_{8:0}$	>>150	>150	ND[b]	>>150
Capric $C_{10:0}$	>>150	>150	>>200	ND
Lauric $C_{12:0}$	150	150	150	50
Myristic $C_{14:0}$	>150	>150	>150	200
Palmitic $C_{16:0}$	>>150	>150	>>150	>>250
Stearic $C_{18:0}$	ND	ND	>150	>250
Oleic $C_{18:1}$	ND	150	>150	>150
Linoleic $C_{18:2}$	100	100	50	30
Linolenic $C_{18:3}$	75	100	40	10
Arachidic $C_{20:0}$	ND	>150	>>300	>>500
Erucic $C_{22:1}$	>150	>150	>>300	>>500

[a] Defined as the lowest concentration of fatty acid or salt tested that showed total inhibition of colony formation on solid culture medium after 48 h of incubation.

[b] ND - not determined.

>> Less than 50% inhibition of colony formation was observed at the concentration indicated in the table, except for sodium palmitate which demonstrated 63% inhibition on 62A spores.

> 75 to 93% inhibition of colony formation was observed at the concentration indicated in the table.

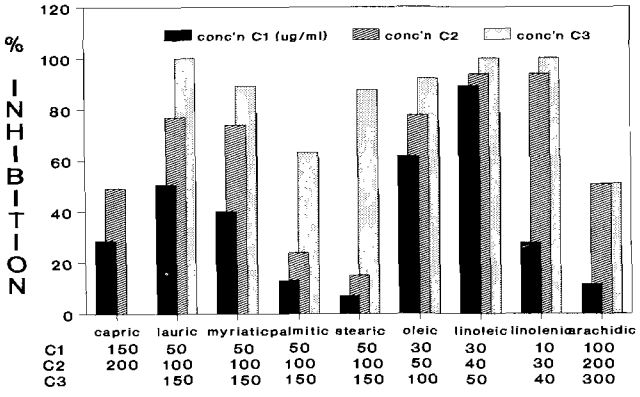


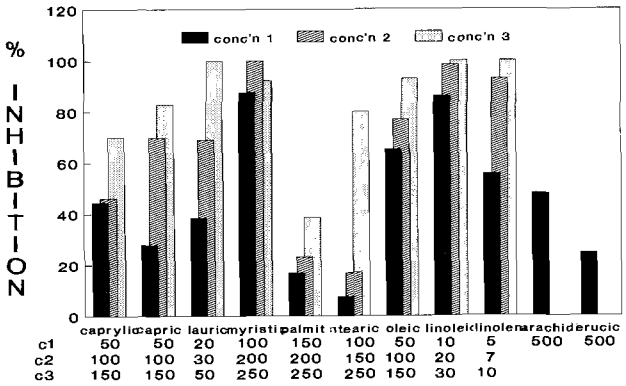
Figure 3. *Inhibition of* C. botulinum *62A spore growth by fatty acid salts. C_{20} exerted 55% inhibition at 300 μg/ml. $C_{22:1}$ exerted very little (<5%) inhibition up to 500 μg/ml.*

laurate was the most inhibitory sodium salt of saturated fatty acids, and sodium linolenate was the most active salt tested. The inhibitory activity of sodium salts of C_{18} fatty acids increased as the unsaturation increased.

Sodium salts of long-chain unsaturated fatty acids were more inhibitory against 62A spores than the corresponding free fatty acids, whereas the opposite was observed for palmitic and myristic acids ($\alpha = 0.05$). This may indicate the importance of solubility for unsaturated fatty acids and of lipophilicity for long-chain saturated acids as discussed later (*20*).

Spores of *B. cereus* were more sensitive than spores of *C. botulinum* to the action of sodium laurate, linoleate, and linolenate ($\alpha = 0.05$).

Addition of starch to the culture medium affected differently the inhibitory action of fatty acids on spores of *C. botulinum* 62A (Table 2). Similar results were obtained



Fiqure 4. *Inhibition of* B. cereus *F4165/75 spore growth by fatty acid salts. C_{20} exerted 48% inhibition at 500 μg/ml. $C_{22:1}$ exerted ≤25% inhibition up to 500 μg/ml.*

with *C. sporogenes* spores (data not shown). The antagonistic concentrations of starch were 0.2% (wt/vol) for palmitic acid and 0.3% for linoleic and linolenic acids. Addition of 1% starch partially affected the antibacterial activity of caprylic, capric, stearic, and arachidic acids and had no neutralizing effect on inhibition by lauric acid.

Figure 5 represents the effect of lauric, linoleic, and linolenic acids on *B. cereus* spore gemination measured as a loss of heat resistance. In the absence of free fatty acids, *B. cereus* F4165/75 spores germinated rapidly in TSB reaching 99% germination in 45 min, but the extent of germination was insignificant (<5%) in TSB supplemented with the fatty acids.

DISCUSSION

Free fatty acids and their salts or esters have been shown to inhibit a variety of microorganisms (*11*). The present investigation confirms that free fatty acids can also exert inhibitory activity against bacterial spores. Early studies (*7*) demonstrated the inhibitory effect of sodium oleate, sodium linoleate, and sodium linolenate on spores of different species of *Clostridium* and *Bacillus* but failed to demonstrate any effect for sodium laurate, and data from replicate experiments were often not comparable. Roth and Halvorson (*17*) reported that these compounds per se were not inhibitory and ascribed their antimicrobial activity to autoxidation by-products such as peroxides. Tonge (*21*) reported that oxidized C_{18} unsaturated fatty acids were only marginally more bacteriostatic than the unoxidized forms. Kabara (*11*) ascribed these and other early contradictory findings to the impurity of the fatty acids tested. In the present investigation, the fatty acids tested had a purity ≥99%, and care was exercised to prevent oxidation of unsaturated fatty acids.

Our findings indicate that free fatty acids such as lauric, linoleic, and linolenic or their salts may provide a good alternative to the use of other food preservatives such as nitrates or even sorbates, which showed toxic reactions in humans at high concentration (*12*). Further research is needed to assess the relevance of these findings to food systems.

Galbraith et al. (*8,9*) speculated that a fatty acid must be in solution yet remain sufficiently lipophilic to absorb

Table 2. *Antagonism of fatty acid inhibition of spores of* C. botulinum *62A by starch.*

Fatty Acid (μg/ml)	Controls: No starch No fatty acid	Controls: No starch With fatty acid	Starch Levels in % (w/v): 0.2	0.3	0.4	1	Minimum antagonistic starch level in % (w/v)
Caprylic (150)	2.32[a]	1.17	-	-	1.50	1.36	>1[c]
Capric (150)	2.32	1.02	-	-	1.26	1.89	>1
Lauric (150)	2.32	0.30	-	-	0.62	0.60	>1
Palmitic (150)	2.32[b]	1.10	2.28	2.29	-	-	0.2
Stearic (150)	2.32	1.30	-	-	2.10	2.14	>1
Linoleic (100)	2.32	0.30	2.03	2.23	-	-	0.3
Linolenic (100)	2.32	0.20	2.18	2.29	-	-	0.3
Arachidic (150)	2.32	1.61	-	-	-	2.15	>1

[a] Each value represents the geometric mean of five replicate colony counts on supplemental yeast agar (*15,16*).
[b] Underlined values are not significantly different according to the "honestly significant difference test" of Neuwman and Keuls (*19*).
[c] Minimal antagonistic concentrations represent the lowest concentration of starch (in w/v) that reversed completely the inhibitory effect of the tested fatty acid.

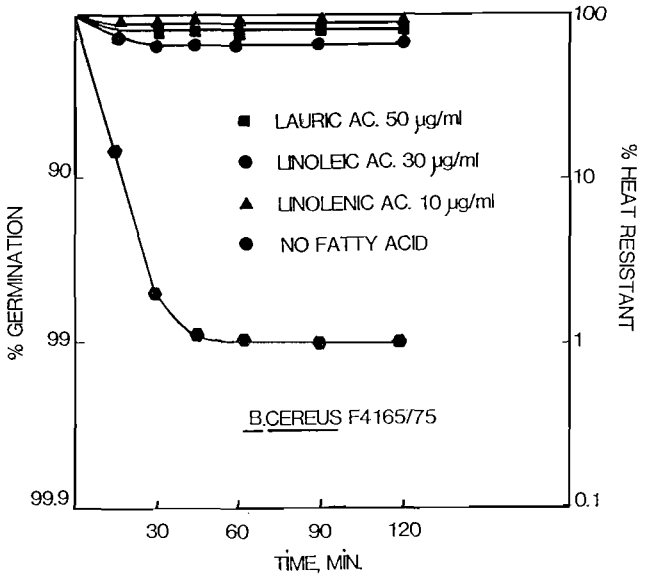


Figure 5. *Inhibition of* B. cereus *F4165/75 spore germination, as measured by loss of heat resistance, by lauric, linoleic, or linolenic acids.*

onto the cell surface and thus inhibit nutrient uptake into vegetative bacteria. Of the saturated fatty acids, the inversely related properties of water solubility and lipophilic activity would be optimum in lauric acid (*8*). The decreased solubility of longer chain saturated fatty acids would not be compensated by the increase in lipophilic activity. The C_{18} unsaturated fatty acids differ from the long-chain saturated acids in behaving as liquids in solution instead of as hydrated solids and thus combine increased solubility with high lipophilic activity (*8*). In addition, the increase in the number of double bonds tends to increase steric effect and the surface area requirements of fatty acids molecules. Thus, fewer molecules of unsaturated acids would be required to occupy a given area on the bacterium wall and absorb onto sensitive sites.

In the present investigation, fatty acids were shown to inhibit spore germination, possibly by binding to spore envelopes and thus inhibiting the binding of germinants to germination sites. The binding of fatty acids to spore envelopes could be governed by the physicochemical properties of the fatty acids, namely lipophilicity and solubility. These properties are greater for lauric acid and C_{18} unsaturated fatty acids as stated earlier (*8*). This could explain the high inhibitory activity of lauric, linoleic, and linolenic acids.

Alternatively, fatty acids may inhibit spore germination by blocking one or more post-triggering steps, i.e., connecting reactions, in the germination process. This could be by uncoupling of energy metabolism or oxidative phosphorylation. Such roles have been demonstrated for fatty acids in relation to the inhibition of vegetative cell growth (*9*).

Furthermore, these findings do not preclude the possibility that other steps in the spore cycle, namely, outgrowth or vegetative cell multiplication or both, are inhibited by the fatty acids.

Starch has been added to several culture media in order to prevent the inhibitory action of fatty acids (*17*). Our data show that starch is only antagonistic to palmitic, linoleic, and linolenic acids but not to lauric acid. Other compounds antagonistic to fatty acids inhibition should be identified and used to neutralize the inhibitory effects if other fatty acids are suspected of being present in culture media and interfering with the cultivation procedure. Serum albumin, cholesterol, lecithin, and Ca^{2+} and Mg^{2+} cations were shown to antagonize the inhibitory action of various fatty acids (*8*). The relevance of these findings in relation to bacterial spores is yet to be investigated.

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