ANTIBIOTICS IN FOOD PROCESSING

Additives Accelerating Death of Spores by Moist Heat

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Three distinct views of the possible role of antibiotics in food preservation are compared: prevention or retardation of the outgrowth of viable spoilage organisms, killing of vegetative cells including germinating spores, and synergizing effect on the thermal death of spores of heat-resistant spoilage bacteria. Whether subtilin may act through distinct mechanisms in exhibiting various types of activity is considered. The behavior of other antibiotics and the implications for screening of antibiotics to be used with heat processing in food preservation are discussed.

IN THE PRESERVATION OF FOOD FROM MICROBIOLOGICAL DETERIORATION, there are two basic principles. The goal may be to kill all spoilage microorganisms and prevent recontamination. This objective usually resolves itself into killing heat-resistant spores by relatively severe heat treatments, although methods based on irradiation by electrons have attracted much interest recently. The alternative is to avoid outgrowth of the spoilage organisms. By outgrowth is meant a degree of microbiological activity that perceptibly lowers the quality of the product. It frequently is not necessary to prevent all metabolic activity. Indeed, some reproduction of certain of the contaminating microorganisms may be tolerable or desirable, as in pickling or cheese making.

Prevention of outgrowth is the older principle and it remains the method of practice for most foodstuffs. It is probable that storage of dry seeds of wild cereals enabled the human race to develop urban civilizations (*22*). Ancient processes other than drying involve concentration to sirups or self-limiting fermentations. In more recent times the use of cold storage and of chemical preservatives has become important, as has the rapid distribution of fresh produce over long distances.

Prevention of Food Spoilage

Even a temporary delay in onset of spoilage may have practical significance. Such delays may sometimes be obtained by scrupulous care to avoid contamination and by the use of processes designed to reduce the initial level of contamination, as by pasteurization in the distribution of milk. It is likely that antibiotics or other chemical inhibitors will prove useful in specific applications such as have been described for meat (*25*), fish (*24*), and fresh vegetables. For example, it has been reported (*21*) that dipping fresh spinach in dilute streptomycin solution, even though followed by thorough rinsing, suffices to confer 1 or 2 days' additional shelf life by protecting against the bacterial slime that sometimes appears in fresh spinach that has been packaged in cellophane.

Another possible application involves the use of subtilin and Terramycin to repress the food-poisoning micrococci and other spoilage organisms in custard pastry fillings. Food poisoning toxins from strains of *Micrococcus* and *Salmonella* may be formed without obvious spoilage and, although rarely fatal, they are sufficiently unpleasant to make desirable an economical treatment ensuring their absence. The experiments that have been reported by Godkin and Cathcart (*9*) indicate that not only are inoculations of food-poisoning bacteria controlled but also the development of the normal heat-resistant flora is delayed for at least 3 days at 37° C.

Although most fermented foods depend for their stability on the formation of lactic or other acid or ethyl alcohol, other microbially produced chemicals may be involved in the stability of cheese, according to Hirsch (*10*). An enhanced preservative action against spoilage by *Clostridium sporogenes* and *Clostridium butylicum* has been achieved by the addition of milk cultured with a special strain of *Streptococcus lactis* so that it contained a relatively high concentration of the antibiotic nisin (*14*). Botulism has rarely been traced to cheese, a food that *a priori* would seem especially favorable to the development of *C. botulinum* (*10*). Whether nisin or other antibiotics retard the development of botulinum toxin in cheese remains unreported.

The situation is much different for another great class of foods, the low-acid or neutral foods such as vegetables and meats, which are sterilized by severe heating and protected from subsequent recontamination by canning or bottling. Long-maintained preservation is desired rather than a moderate delay in the onset of spoilage. Inasmuch as the products lack an intrinsic resistance to spoilage, it is necessary that all but an extremely small fraction of the cans be free of viable spoilage organisms. A direct empirical observation of the effectiveness of a treatment is rarely practical. As a consequence, test systems must be devised that will permit the calculation of processing schedules that will yield a specified low probability of survival of viable spores of a particular test organism added in a specified large number to the particular foodstuff under consideration. The basic data for such calculations are of the sort that are illustrated in Figure 1. (Two independent relationships are given in the same figure; they may be differentiated by the solid and dashed lines.) Parameter D refers to the elapsed time required for a 90% reduction in number of surviving spores at constant temperature; parameter z refers to the increase in temperature required for 90% reduction in time required for an arbitrary extent of killing.

The linear relationship of the logarithm of the number of survivors to time at constant temperature—that is, the constancy of D—holds surprisingly well for homogeneous populations of spores.

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Table I. Factors Influencing Death of Spores at Higher Temperatures (8)

Factors determined at time of heating

- Water, salt, and sugar concn. and pH of menstruum
- Vegetable juices *vs.* phosphate buffers in menstruum
- Physical make-up of menstruum (oil stratification)
- Numbers and clumping of spores
- Temperature

Predetermined factors

- Bacterial strain
- Conditions of sporulation
- Treatment of spores subsequent to sporulation; storage conditions

The linear relationship of the logarithm of the time required for a specified reduction in number of survivors to the temperature—that is, the constancy of *z*—also has been found to hold throughout the investigated range of processing temperatures. The effectiveness of a processing schedule is determined by an integration of the lethal effect of the particular temperature at a particular location within the can on the particular number of survivors at that location at a particular time over the whole contents of the can and over the entire period of heating, holding, and cooling. Ingenious techniques and approximations have been devised to achieve this purpose (*23*), but it suffices here to consider parameter *D*. (The *z* value is relatively invariable.) Before discussing the factors that affect *D*, however, it is necessary to consider what test organisms are to be used as a basis for process calculations.

Experience has demonstrated the suitability of most nonacid foods for the development of *C. botulinum* toxin, so that the overriding consideration in the processing of such foods has been the provision of an extremely wide margin of safety with respect to this bacterium. Tests are made with strains of *C. botulinum* especially selected for high heat resistance and with other bacteria that have caused nontoxic spoilage under field conditions. If any process were to make possible a substantial reduction in the severity of the heat treatment it would be necessary to study its effect not only on those organisms recognized at present as troublemakers but also on those others which might become troublesome if the new process were not also effective against them.

Test organisms other than *C. botulinum* include a nontoxic *Clostridium* sp. (P.A. 3679) with much greater resistance to heat, a strain of *Bacillus coagulans* (*B. thermoacidurans*) with extraordinary resistance to heat in acid media, and various thermophiles which are still more resistant than P.A. 3679 (*23*). Among the latter is a non-gas-forming strain of *B. circulans* (*B. stearothermophilus* F.S. 1518). Complete sterility with respect to thermophiles frequently is foregone, for they will not cause spoilage unless the products are stored at high temperatures, whereas the processing necessary for their complete elimination would severely damage many products.

A great many factors are recognized to influence thermal death of spores; the principal ones cited by Esty in his 1928 review (*8*) are listed in Table I. Heat sterilization is much less effective for dry than for moist spores. This fact may account for the resistance of spores under dry heat, in heavy sirups, in solutions stratified with oil, and in dry clumps. Spores may be severalfold more resistant in certain vegetable media than in others, although pH and other important factors are controlled, so that natural but yet unidentified constituents of vegetable extracts may influence thermal death.

The major category that must now be added to this 1928 list comprises those organic compounds that are active in low concentrations, such as certain constituents of essential oils, antibiotics, and other compounds. Some of the published observations are listed in Table II. It appears that a variety of substances will accelerate the death of spores in the presence of water at higher temperatures.

Survey of 67 Antibiotics

It was desired to determine to what extent the prevention of outgrowth of spores by the presence of an antibiotic during incubation would be useful in selecting antibiotics capable of accelerating the death of spores heated severely in the presence of moisture. Preliminary results obtained for 67 antibiotics that were readily available show a poor correlation of the two tests. The antibiotics that gave positive results are listed in Table III. The test bacterium was P.A. 3679; less comprehensive comparisons with *C. botulinum* are in substantial agreement.

Inhibition end points for the outgrowth of spores were determined by an adaptation of the gradient-plate method to anaerobic conditions, as was described briefly by Andersen *et al.* (*3*). The antibiotic agar wedge in the bottom o the plate was covered by a thin uniform layer containing approximately 50,000 spores. The antibiotic-free agar wedge was poured on top and the plate completed by adding a flat glass disk and thioglycollate agar. Nigericin was suspended in 0.1% Tween 20 preparatory

Figure 1. Parameters D and z used for calculation of heat processing schedules (arbitrary example)

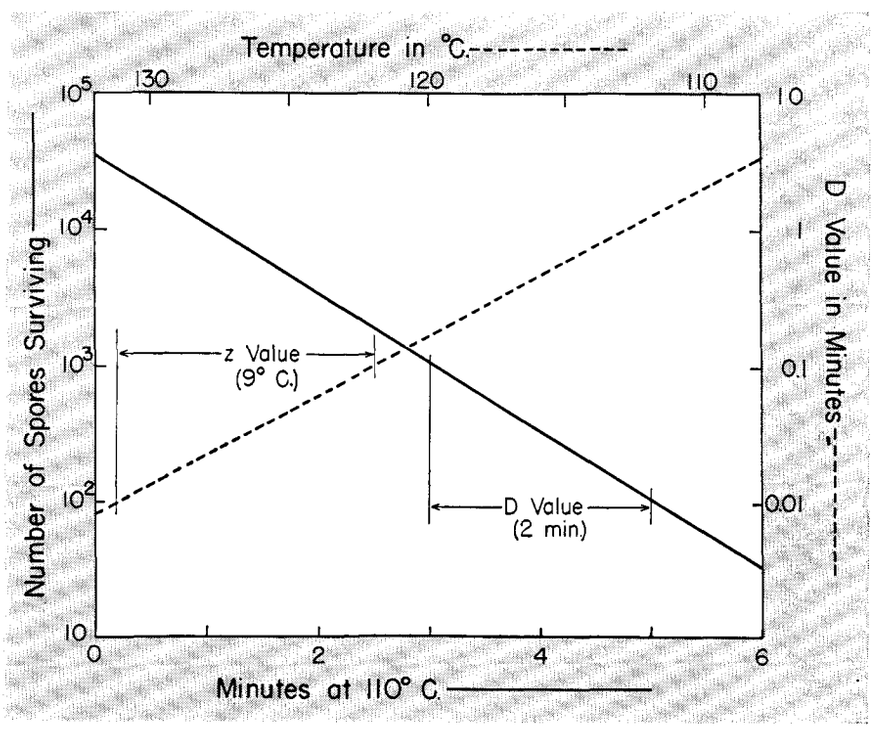

Table II. Agents Accelerating Death of Spores at Higher Temperatures

Agent and Ref.	Concn., P.P.M.	Menstruum	Temp., ° C.	Spores of	Reduction in D, %
Subtilin (*13*)	14	Pea puree	110–127	*C. botulinum* 62A	57
			116–132	*C.* sp. (P.A. 3679)	53
Allyl isothiocyanate (*11*)	50	Tomato juice	88–99	*B. coagulans* (ATCC 8038)	25
Garlic oil (*5*)	30–125	Tomato juice	100	*B. coagulans* (ATCC 8038)	35–40
Cinnamon oil (*5*)	30–125	Tomato juice	100	*B. coagulans* (ATCC 8038)	40
Clove oil (*4, 5*)	420–1000	Tomato juice	96–104	*B. coagulans* (ATCC 8038)	25–35
Black pepper (*4*)	10,000	Tomato juice	96–104	*B. coagulans* (ATCC 8038)	20
Msc. vegetable extracts (*12*)	10,000	Phosphate buffer, pH 7	94	Flat sour 787	~90
Lupulone (*12*)	100	Phosphate buffer, pH 7	94	Flat sour 787	~90
Indoleacetic acid (*12*)	174	Phosphate buffer, pH 7	94	Flat sour 787	~40
Vitamin K_5 (*19*)	25–200	Phosphate buffer, pH 7	115	*C.* sp. (P. A. 3679)	25–75
Ascorbic acid (*19*)	100	Phosphate buffer, pH 7	115	*C.* sp. (P. A. 3679)	−50

Table III. Antibiotics (of 67 Tested) Found Active Against *Clostridium* sp. (P.A. 3679)[a]

	Concn. Affecting Outgrowth of Spores in Agar, P.P.M.			
	Complete inhibition	50% reduction in colony size	50% reduction in colony numbers	Reduction in D Value, %
Nigericin	0.2	0.08	0.18	−13
Oxytetracycline	0.2	0.14	0.18	−6
Erythromycin	0.25	0.2	0.2	10
Penicillin G	0.3	0.2	0.2	10
Chlorotetracycline	0.7	0.4	0.5	−6
Subtilin[b]	0.6	0.6	<0.03	53(*13*), 54[c]
Subtilin methyl ester[b]	1.5	1.5	~0.05	35,51
Rhodomycetin	1.5	1.5	0.3	15
Chloramphenicol	1.7	1.1	1.1	10
Achromycin	3.5	3	3	−3
Thiolutin	6	3	3	3
Cinnamycin	9	8	8	7,11
Tyrothricin	13	4	4	<4
Laterosporin A	17	17	2.5	10
Methylol gramicidin	20	11	2	8
Polypeptin	25	25	2	0
Nisin	~40	~40	0.2	57,55
Catenulin	>40	>40	>40	15
Hexahydrolupulone	>40	>40	>40	17
Usnic acid	>25	>25	>25	−>20, −16

[a] Antibiotics included in this table reduced the outgrowth of spores in agar plates at 20 p.p.m., altered the D value by 15% at 14 p.p.m., or did both.
[b] Concentrations calculated in terms of 100% potency subtilin, although materials of 70 and 90% potency were used, respectively, in tests of subtilin and esters. The latter contained 12.9 equivalents of methoxyl per 10,000 grams. A product with 5.1 eq. gave essentially the same end points for spore outgrowth.
[c] Tested at 7 p.p.m.

to the outgrowth test. Tyrothricin, methylol gramicidin, and rhodomycetin were dissolved in propylene glycol and dispersed in water or 0.1% Tween 20. This resulted in maximum concentrations of 0.01% Tween 20 and 0.2% propylene glycol in the anaerobic plates, which did not affect spore outgrowth.

The effects on *D* value were determined by the procedure described by LeBlanc *et al.* (*13*) for subtilin. All antibiotics were tested at 14 p.p.m. and 120.1° C.; all tests were incubated for 90 days. Replicate values in Table III refer to experiments performed at different times. The *D* values for 18 controls run with the same spore suspension but at different times averaged 1.55 ± 0.105 minutes (standard deviation). A combination of subtilin and oxytetracycline was no more effective than subtilin alone.

The increased resistance conferred by 14 p.p.m. of usnic acid (indicated by negative reductions in *D* values in Table III) is noteworthy. A similar effect with a moderate concentration of ascorbic acid on spores of this bacterium was reported earlier (Table II). The findings suggest that other chemicals capable of increasing the thermal resistance of spores may occur in some foodstuffs. The existence of substances with this property also may provide a useful tool for investigation of the nature of death of spores under the influence of "moist heat."

Of the antibiotics that appear to accelerate the thermal death of spores, subtilin and nisin are polypeptides produced by strains of *Bacillus subtilis* and *Streptococcus lactis*, respectively. Both contain the thio ether amino acids, lanthionine and a β-methyllanthionine.

Forty-seven antibiotics were without effect either on outgrowth prevention (at 20 p.p.m. after 3 days) or on *D* value (less than 15% change with 14 p.p.m.). Twenty-three were unidentified concentrates. The others were:

Actinomycin	Fumagillin
Actithiazic acid	Grifolin
Aspergillic acid	Helixins A and B
Bacitracin	Licheniformin A5
Burdock antibiotic	Neomycin
Circulin sulfate	Pleocidin
Citrinin	Polymixin D
Clavicin	Streptomycin
Comirin	Streptothricin
Dihydroquercitin	Subtenolin
Dihydrotomatidine	Tomatodine
Endomycin	Tomatine

Prospects for Use of Antibiotics in Canned Foods

The significance of the effect on *D* values lies in part in the possibility that the use of such an agent might allow substantial reductions in the severity of heat-processing schedules for certain foods and also in the possibility that more substantial margins of safety may be established for other foods that occasionally give trouble with thermophiles or other non-toxin-producing bacteria. The existing schedules represent a difficult compromise between quality and stability, particularly for the larger sizes of cans, with which the slowness of heat penetration becomes particularly important. Many vegetables and meats must be overcooked; some, such as cauliflower, are not canned very much in this country because the damage due to overcooking is so severe. A 50% reduction in *D* value would probably be significant in a number of processes.

However, many questions arise other than that of the degree of reduction in *D* value necessary for exploitation in specific applications. The question of safety is obvious. The physiological effects of various antibiotics, under conditions that would arise from their use in foods, have not yet been established. Practical

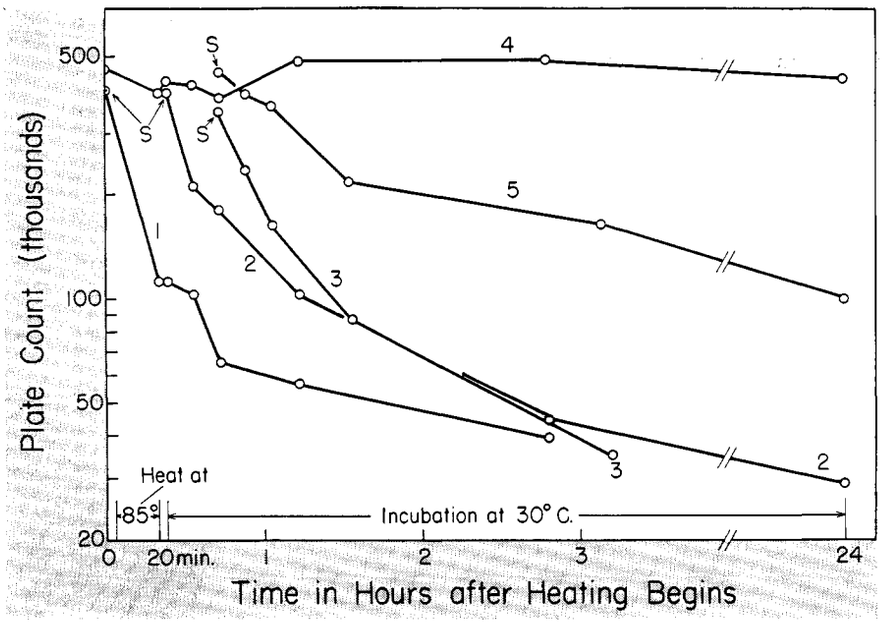

Figure 2. Effect of subtilin and heat on death of spores of *B. subtilis* ATCC 9524

1, 2, 3. Effects of adding subtilin before heating, immediately after heating, and 20 minutes after heating
4. Control heated but not exposed to subtilin
5. Control exposed to subtilin but not heated
The spore suspension was not heated prior to the experiment. During the experiment the spores were suspended in 0.025M phosphate (pH 6.7). Residual subtilin in the plates was <0.0007 p.p.m., a concentration that was without effect on either unheated or severely heated spores

use of these agents in foods is not permitted at the present time.

The cost of the additive may be considered next. On the assumption of $1.00 per gram for the additive and a requirement of 14 p.p.m., one arrives at a cost of 0.5 cent per gallon or 1.4 cents per case of No. $2^1/_2$ cans.

A very important requirement is that of reliability of action. Subtilin is the only agent that has been investigated intensively. The published data of LeBlanc, Devlin, and Stumbo (*13*) showed an average reduction of 37% in the *D* value for *C. botulinum* 62A and of 53% for P.A. 3679 in pea puree. The results were similar over the temperature range 230° to 270° F. Further investigations by Stumbo and colleagues show that equal or greater reductions are obtained with five other strains of *C. botulinum* and with a variety of vegetable media.

The frequency of occurrence and stability of resistant forms should be considered. Resistance is meant in the sense that the death of spores of a particular stock would not be accelerated at the higher temperatures by the additive. However, the situation would be different from that of chemotherapy, in that there would probably be no special tendency toward the selection of resistant variants or toward reinfection of raw materials with resistant variants. Also the question of resistance is important only with respect to the highly heat-resistant forms; a finding of resistance with a less heat-resistant form would not necessarily apply to the important spoilage organisms.

The question of the mode of action of an additive capable of accelerating death induced by moist heat is of great significance. It is necessary to consider whether the use of such an agent would invalidate or alter the basic assumptions of the calculations of processing schedules from *D* and *z* values as ordinarily determined. Although no reason why this should be so has been advanced, a more detailed understanding of the phenomenon might point to particularly important control experiments. Indeed, a detailed description of the phenomenon would probably be enlightening with respect to the nature of death at higher temperatures and of resistance to heat.

Inasmuch as germination of bacterial spores involves a loss of thermal resistance at a very early stage in the sequence of events that leads to the reproducing vegetative cell, it is of interest to see whether any of the same stages can be recognized in the sequence of events that leads to thermal death. The sequence in germination (*18*) involves first of all the activation of the spores of certain strains by heat or chemical agents. This is followed by various more or less coincidental events, including a loss of soluble nitrogenous solids and calcium to the medium and an uptake by the spore of water, a decrease in optical refractivity and an increase in permeability to stains, and a loss of heat resistance. Finally the morphological changes become evident—swelling, shedding or absorption of the spore coat, and emergence of the vegetative cell.

In the exposure of dormant spores to heat treatments of increasing severity—that is, increased duration or increased temperature—various stages can be distinguished (*7, 26*). First of all, the spores are activated. Degrees of activation are distinguishable. With the more heat-resistant strains, complete heat activation may be obtained without killing more than a small proportion of the spores. Secondly, many spores are injured to the extent that outgrowth is delayed or is rendered contingent on closer control of pH or incubation temperature, on supplying nutrilites not essential for uninjured cells, or on removing or detoxifying fatty acids or other inhibitors that are relatively ineffective against uninjured cells. Finally, all the spores are killed in the sense that outgrowth does not occur even under the best conditions that can be devised.

The question then is whether any step beyond activation is common to both the processes of germination and thermal death. Some evidence bearing on this has been reported by Michener (*16*) with a strain of *Bacillus subtilis* for which the apparent thermal death rate is increased greatly by subtilin. Inasmuch as the effect was observed under conditions such that the spores cannot complete germination—that is, lack of nutrients or too high a temperature—it was thought incorrectly that the effect was not related to germination. Subsequently it was found that subtilin increased the apparent death rate even when it was added as long as 20 minutes after the spores had been heated and cooled (Figure 2). Although spores treated with heat and subtilin were dead in the sense that they would not develop to form colonies in nutrient agar, phase microscopic observations showed that they could still undergo the decrease in refractivity characteristic of the early stage of germination soon after they were brought in contact with nutrients, even though they had previously been incubated as long as 3 days in buffer.

Another question relates to the scheduling of developmental investigations. It seems probable that agents could be found readily which would be better for accelerating death of spores under moist heat than those few that have already become available. From the diverse nature of these it would seem wise to survey other antibiotics, germination inhibitors, protein denaturants, miscellaneous biologically active compounds, synthetic organic compounds, plant extracts, and crude microbial cultures. Mechanical aids have been devised (*23*) which simplify the determination of thermal death constants to the point that such an empirical search need not be unduly laborious.

It also appears likely that accelera-

Table IV. Agents Accelerating Death of Vegetative Microorganisms at Pasteurization Temperatures

Agent and Ref.	Concn., P.P.M.	Menstruum	Temp., ° C.	Microorganism	Reduction in D Value, %
Garlic oil (*5*)	31–125	0.5% acetic acid, 5% salt	53	Yeast 7D	90–>99
Cinnamon oil (*5*)	105–420	0.5% acetic acid, 5% salt	53	Yeast 7D	90–>94
Clove oil (*5*)	105–420	0.5% acetic acid, 5% salt	53	Yeast 7D	65–>93
Allyl isothiocyanate (*6*)	20	0.5% acetic acid, 5% salt	60	Yeast 7D	64
	10–25	Buffer, pH 4.0	71	*Aspergillus niger*	84–92
	10	Apple juice, pH 3.9	71	*Aspergillus niger*	76
		10% glucose	71	*Aspergillus niger*	62
		0.75% tartaric acid	71	*Aspergillus niger*	84
		Buffer, pH 4.0	49–60	*Saccharomyces ellipsoideus*	48
		Grape juice, pH 3.8	49–60	*Saccharomyces ellipsoideus*	51
Sulfur dioxide (*17*)	20	Apple juice, pH 3.6	~55	Natural flora	~75
Sodium benzoate (*17*)	200	Apple juice, pH 3.6	~55	Natural flora	~50

tors of pasteurization play a role in certain food products and new and better agents might be useful with other foods. Table IV shows the action of some spice oils and purified chemicals on vegetative cells of various microorganisms as reported in the literature. Some of the agents show greater reductions in *D* values for pasteurization of nonsporulating microorganisms than have been observed as yet for the thermal death of bacterial spores.

A method for the processing of vegetables, reported by this laboratory some years ago (*2*), involved the use of subtilin in combination with a processing temperature of 85° to 100° C. for the preservation of vegetables. High-quality microbiologically stable products were obtained with uninoculated packs or with packs inoculated with spores of a strain of *B. stearothermophilis* that is very sensitive to subtilin. However, many other investigators have found that large inocula of spores of many other bacteria are not controlled in this process. The question that may be asked is whether control of *Clostridium* could be obtained in low-acid foods by processing for short times in this temperature range with subtilin or other additives. If the possibility be disregarded that an additive could be found which would give an extraordinary reduction in *D* value, then it would seem to be necessary to fulfill either of two conditions.

The first possibility would be to maintain a bacteriostatic concentration throughout the shelf life of the product. Because of the existence of resistant forms, this would probably require a mixture of antibiotics of extraordinary stability. The other possibility would be to obtain quantitative germination followed by bactericidal action within the period of retention of effective concentrations of the antibiotic or mixture of antibiotics used. The word "quantitative" is used in the same sense that it is used in heat sterilization; there would have to be a vanishingly low probability that any spore would germinate after effective bactericidal conditions had been lost. It appears now that the limited degree of success that was obtained with the method was due to germination of the spores with subsequent bactericidal action of subtilin such as has been reported by Sacks (*20*) and to the absence of dormant spores from the packs (*15*). The failures apparently were due either to the presence of dormant spores which germinated after the subtilin deteriorated or to the presence of a small proportion of resistant cells which not only germinated but grew in the presence of subtilin (*7*).

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