Effects of Selected Food Additives on Growth of *Pseudomonas fragi*

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Abstract

Tests were made of inhibition of *Pseudomonas fragi* in lactose–yeast extract broth by the food additives chlortetracycline, nisin, bacitracin, chloramphenicol, lysozyme, ethylenediaminetetraacetic acid (EDTA), nitrofurazone, propyl-p-hydroxybenzoate, sodium benzoate, and potassium sorbate. The additives that proved effective in broth were tested in skimmilk and half-and-half. Nisin, bacitracin, lysozyme, and nitrofurazone were ineffective in broth; chloramphenicol increased the lag period prior to development of turbidity but resulted in chloramphenicol-resistant populations; and EDTA inhibited the organism slightly in broth but not in skimmilk or half-and-half. Propyl-p-hydroxybenzoate, chlortetracycline, and a mixture of lysozyme and EDTA were effective in broth but not in skimmilk or half-and-half, a difference attributed to metal ions in dairy products that react with chlortetracycline and EDTA. Sodium benzoate retarded *P. fragi* in broth, but only at low pH. Potassium sorbate was ineffective at pH 6.5 in broth, but at pH 5.5 and 5.2 inhibited growth of *P. fragi* in broth, skimmilk, and half-and-half.

Food spoilage can occur from either chemical reactions of food constituents, often involving enzymes naturally present in food, or chemical changes brought about by enzymes produced by microorganisms growing in food. Spoilage from microorganisms can be delayed or prevented by killing undesired microorganisms before they grow extensively or by making the environment unsuitable for their growth. Milk and fresh milk products are normally refrigerated, and their spoilage usually results from microorganisms, often called psychrophiles, capable of growing more rapidly than most microorganisms in milk at refrigeration temperatures. These organisms produce such defects as ropiness, putrid or fruity odor, and bitter or rancid flavor in a variety of dairy and nondairy products. Since they are sensitive to heat, their presence in pasteurized dairy products is normally considered to indicate contamination after pasteurization.

This study was made to determine the effects of selected food additives on the growth of *Pseudomonas fragi,* a representative of the psychrophilic bacteria that are most troublesome in spoiling pasteurized milk and fresh milk products. Kristoffersen and Chakraborty (7) recently studied the effects of aureomycin, myprosine, sorbic acid, potassium sorbate, lactic acid, phosphoric acid, and citric acid on the keeping quality of Cottage cheese. They found adding aureomycin to the wash water and sorbic acid or potassium sorbate to the dressing cream most effective. Nisin has been recommended for preventing the growth of various Gram-positive bacteria in a variety of dairy products and other foods (2); tetracyclines are used to preserve fresh meat (17) and fish and poultry (4); bacitracin has been reported to delay the souring of poor-quality milk at 22 C (18); lysozyme is used as a preservative in fish sausage in Japan and has been reported to delay the souring of poor-quality milk (18); EDTA is used in a variety of foods as a preservative, color stabilizer, and chelating agent (3); nitrofurazone is used for preserving fish sausage in Japan (12); and sodium benzoate, propyl-p-hydroxybenzoate, and potassium sorbate are used in preserving a variety of foods (8).

Experimental Procedure

Organism. The culture of *Pseudomonas fragi* used was isolated from Cottage cheese spoiled by the development of slime and fruity odor. It was propagated routinely on lactose–yeast extract agar slants. Inocula for experiments were prepared by growing the organism for 12 to 16 hours at 13 C in the specific medium to be used in an experiment.

Testing procedure. Each additive was first tested to determine its influence on the growth of *P. fragi* in lactose–yeast extract broth containing 0.5% lactose, 0.5% peptone, 0.3% yeast extract, and 0.2% K_2HPO_4. The initial pH was 6.5, except when the influence of lower pH was determined in studying organic acids. Fifty-milliliter quantities of the medium were

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TABLE 1. Effect of chlortetracycline on growth of *Pseudomonas fragi* at 13 C in skimmilk and half-and-half.

Storage	Plate counts with chlortetracycline concentrations of: 0	5	10	20 μg/ml
(days)	—(Colonies/milliliter × 10^{-8})—			
Skimmilk				
0	0.07			
1	9.5	6.4	3.8	1.2
7	75.0	54.0	43.0	30.0
Half-and-half				
0	0.07			
1	1.5	1.4	0.7	0.4
7	74.0	26.0	6.0	2.0

dispensed into 300-ml Nephelo culture flasks (Bellco, Vineland, N.J.), which have a side tube for use in determining optical density, and sterilized for 12 min at 121 C. The flasks, containing medium and different quantities of the additive to be tested, were inoculated with 1% of *P. fragi* and incubated at 13 C. They were shaken during incubation with a Burrell wrist-action shaker (Burrell Corp., Pittsburgh, Pa.). Growth of *P. fragi* in the lactose–yeast extract broth was determined in most experiments by measuring turbidity in a Klett-Summerson colorimeter, Model 800-3, with Filter no. 66, and in some experiments by plate counts.

Each additive that inhibited *P. fragi* in the broth was tested for inhibition of the organism in sterile skimmilk and in half-and-half (12% milk fat), without pH adjustment except in studying organic acids. Cultures of the organism in skimmilk and half-and-half were incubated and shaken similarly to broth cultures, and growth was determined by plate counts. The plating medium was the lactose–yeast extract medium with 1.5% of Bacto-agar added. The plates were incubated for 48 ± 3 hours at 22 C.

Additives. Antibiotics, agents that hydrolyze and dissolve bacterial cell walls, and chemical preservatives were tested. The antibiotics were nisin, 10^6 units/gram (Aplin-Barrett, Ltd., Yeovil, England); tetracycline-HCl (Chas. Pfizer & Co., New York City); bacitracin, 55 units/milligram (Calbiochem, Los Angeles, Calif.); and chloramphenicol (Parke, Davis & Co., Detroit, Mich.). The hydrolytic agents were egg-white lysozyme (Calbiochem) and disodium ethylenediaminetetraacetic acid (EDTA). The preservatives were 5-nitrofurfural-semicarbazone (nitrofurazone), obtained from K & K Lab., Plainview, N.Y.; propyl-p-hydroxybenzoate (propylparaben), from Washine Chemical Co., Lodi, N.J.; sodium benzoate, from Matheson Coleman & Bell, Norwood, Ohio; and potassium sorbate, from K & K Lab.

Results

Tetracycline-HCl. A concentration of chlortetracycline of 5 μg/milliliter or greater prevented the growth of *P. fragi* in lactose–yeast extract broth (pH 6.5 and 5.5) for at least eight days. Chlortetracycline was considerably less inhibitory in skimmilk and half-and-half than it was in broth (Table 1). Apparently, metal ions in the dairy products reacted with the chlortetracycline to form an inactive complex, since milk ash or magnesium sulfate added to broth containing chlortetracycline reversed the inhibitory action of the antibiotic. Results with milk ash (Table 2) show that 0.36% or more of milk ash enabled *P. fragi* to grow in broth in the presence of 10 μg/milliliter of chlortetracycline. A concentration of magnesium sulfate of 0.01 M was also effective.

Nisin and bacitracin. Concentrations of nisin of 50, 100, and 150 μg per milliliter of the lactose–yeast extract broth, or bacitracin up to 100 μg/milliliter, had no detectable effect on length of lag period before development of turbidity by *P. fragi,* specific growth rate (k, hr^{-1}), or maximum population.

TABLE 2. Effect of milk ash on the bacteriostatic action of chlortetracycline on *Pseudomonas fragi* at 13 C in lactose–yeast extract broth.

Milk ash	Chlortetracycline	Turbidity after incubation for: 0	4	6	8	21	47	71 hr
(%)	(μg/ml)	—(Klett units)—						
0	0	0	0	0	7	210	298	294
0	10	0	0	0	0	0	4	4
0.12	10	0	0	0	0	0	0	0
0.24	10	10	47	47	47	45	50	51
0.36	10	4	116	114	116	112	138	147
0.48	10	1	119	119	157	161	160	165
0.60	10	8	150	150	166	185	188	186

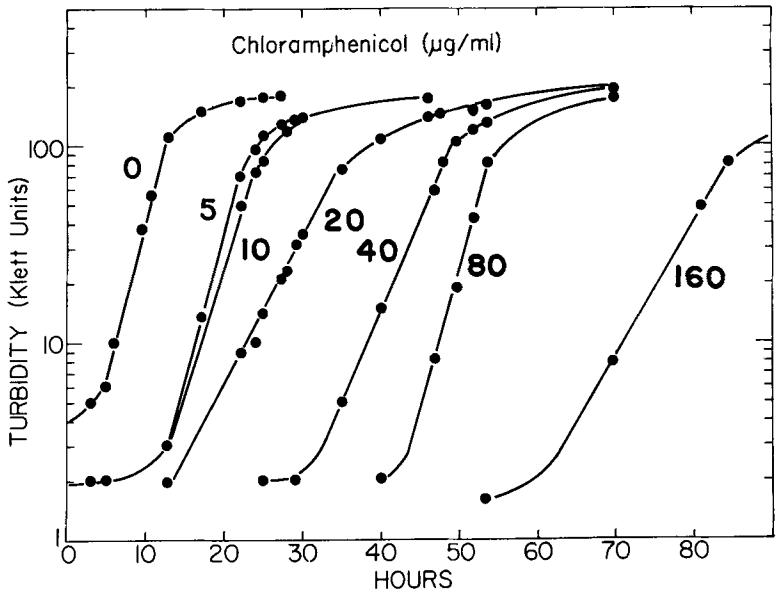

FIG. 1. Effect of chloramphenicol on growth of *Pseudomonas fragi* at 13 C in lactose–yeast extract broth.

Chloramphenicol. Concentrations of chloramphenicol of 5 to 160 μg/milliliter affected *P. fragi* primarily by extending the lag period before growth in lactose–yeast extract broth (Fig. 1). The length of the lag period varied with the concentration of chloramphenicol, and some results indicated a slight decrease in growth rate. When populations of *P. fragi* that developed in the presence of 40 and 80 μg/milliliter of chloramphenicol were subcultured (into flasks of fresh medium containing the same concentrations of chloramphenicol), they proved resistant to the antibiotic.

Lysozyme and EDTA. Concentrations of egg-white lysozyme up to 20 mg/milliliter had no detectable effect on the growth of *P. fragi* in lactose–yeast extract broth. With addition of EDTA (1 mg/milliliter), however, 2 mg/milliliter of lysozyme completely prevented growth of the organism. *P. fragi* grew in the presence of EDTA, but to a lower maximum population than in the absence of EDTA. Subsequently, the effects of EDTA of none to 0.75 mg per milliliter were determined with and without the addition of 0.2 mg of lysozyme per milliliter. This small amount of lysozyme markedly increased the influence of EDTA on the growth of *P. fragi* (Fig. 2). With 0.5 mg or more of EDTA and 0.2 mg of lysozyme per milliliter, plate counts were less at 12 days than at the start of the experiment (7.5×10^6/milliliter).

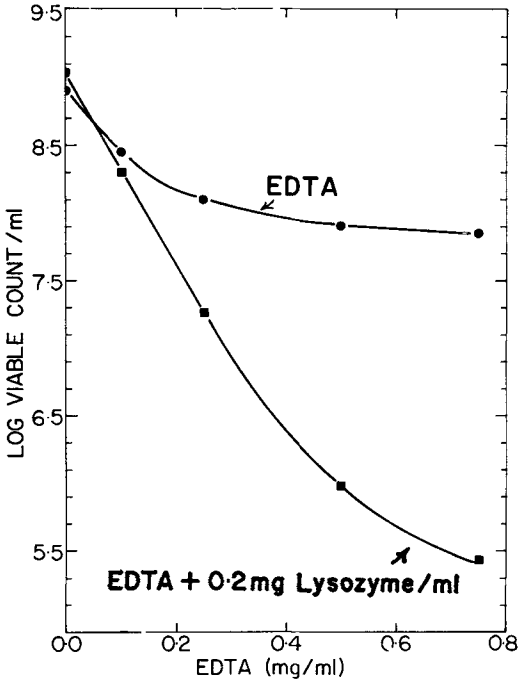

FIG. 2. Effects of EDTA and EDTA plus lysozyme on growth of *Pseudomonas fragi* at 13 C in lactose–yeast extract broth. Quantities of broth containing different concentrations of EDTA or EDTA plus 0.2 mg/milliliter of lysozyme were inoculated with the organism and incubated 12 days at 13 C on a Burrell shaker. Samples removed for plate counts were diluted in 20% sucrose to avoid osmolysis.

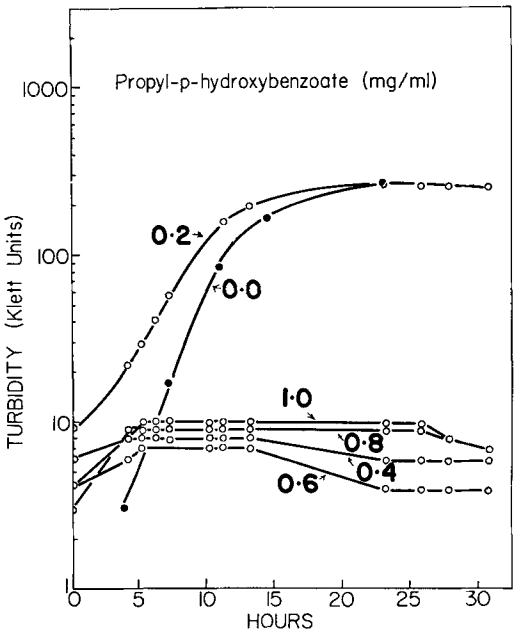

FIG. 3. Effect of propyl-p-hydroxybenzoate on growth of *Pseudomonas fragi* at 13 C in lactose–yeast extract broth.

Results were different when the growth medium was skimmilk or half-and-half in experiments similar to those reported in Fig. 2. Because of the presence of metal ions, *P. fragi* in the dairy products was not detectably inhibited by EDTA plus lysozyme. The inhibition of *P. fragi* in lactose–yeast extract broth by EDTA plus lysozyme was later prevented by adding skimmilk (2%), milk ash (0.12%), or magnesium sulfate (0.01 M).

Nitrofurazone. Nitrofurazone at concentrations of 5 and 10 μg/milliliter did not detectably inhibit *P. fragi* in lactose–yeast extract broth at pH 6.5 or 5.5.

Propyl-p-hydroxybenzoate. Concentrations of propyl-p-hydroxybenzoate of 0.4 mg/milliliter or greater were very effective in preventing the growth of *P. fragi* in lactose–yeast broth (Fig. 3). With concentrations of 0.6 and 1.0 mg/milliliter, plate counts normally decreased from about 7.5×10^6/milliliter to less than 1.0×10^3 in one day, and to none or only a few colonies per milliliter thereafter. In one experiment where growth occurred in the presence of 0.6 mg/milliliter of the additive, a resistant mutant was isolated. In skimmilk or half-and-half, however, these concentrations of propyl-p-hydroxybenzoate did not detectably inhibit *P. fragi.*

Sodium benzoate. Concentrations of sodium benzoate of 0.5, 0.75, and 1.0 mg/milliliter did not affect *P. fragi* growth in lactose–yeast extract broth at pH 6.5, but at pH 5.2 increased the lag period. Lag periods were increased two and three days, respectively, by 0.75 and 1.0 mg/milliliter of sodium benzoate at pH 5.2.

Potassium sorbate. The inhibitory effects of potassium sorbate were determined at pH 6.5, 5.5, and 5.1. Concentrations of 0.1 to 0.4% did not affect the growth of *P. fragi* in lactose–yeast extract broth at pH 6.5. At pH 5.5, 0.3% or more of the potassium sorbate retarded growth. At pH 5.1, 0.1% of the sorbate extended the lag period by 35 hr, and higher concentrations prevented visible growth for at least 100 hr (Fig. 4).

Potassium sorbate also inhibited *P. fragi* in skimmilk and half-and-half, each adjusted to pH 5.2 with hydrochloric acid. Plate counts for acidified skimmilk with 0.1, 0.2, and 0.3% potassium sorbate are shown in Fig. 5. Populations of *P. fragi* decreased for about two days and later increased to population maxima lower than in the sample without sorbate.

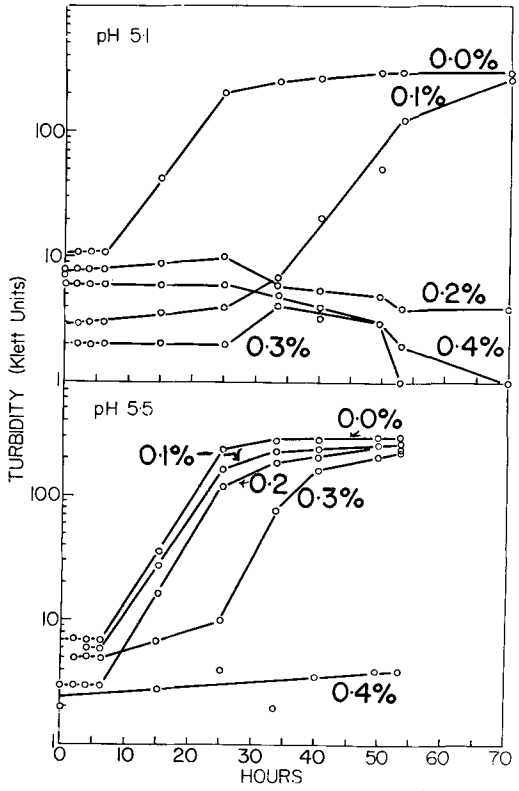

FIG. 4. Effect of potassium sorbate on growth of *Pseudomonas fragi* at 13 C in lactose–yeast extract broth at pH 5.5 and 5.1.

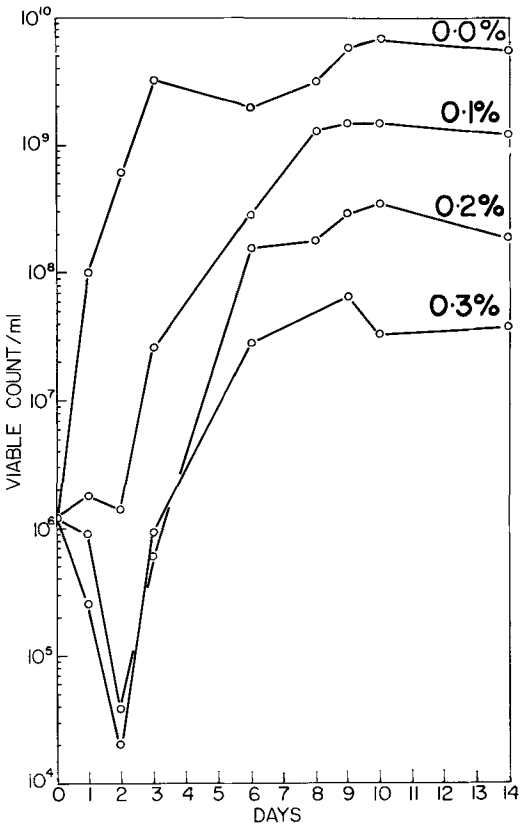

FIG. 5. Effect of potassium sorbate on growth of *Pseudomonas fragi* at 13 C in skimmilk adjusted to pH 5.2 with hydrochloric acid.

Discussion

Additives to prevent growth of microorganisms in dairy products and other foods must be approached cautiously. There seems little reason to consider additives that in comparatively large concentrations do not prevent growth of the Gram-negative microorganisms that normally spoil refrigerated pasteurized milk or fresh milk products. In this investigation, only potassium sorbate appears promising, and this additive was effective only at a low pH.

Gram-negative bacteria have more complex cell walls than Gram-positive bacteria and are less susceptible to the action of potential preservatives. The failure of lysozyme to lyse *P. fragi* is likely related to the complexity of the cell walls of this organism. Weidel and co-workers (14, 15), Kellenberger and Ryter (6), and Glauert (5) made observations indicating that another Gram-negative organism, *Escherichia coli,* has a three-layered cell wall. The inner, middle, and outer layers, respectively, consist of mucopeptide, lipopolysaccharide, and lipoprotein mosaic. The inner layer, responsible for the rigidity of the cell wall, is the substrate for lysozyme. Assuming that the cell walls of *P. fragi* are similar in structure to those of *E. coli,* it is possible that the outer layers prevented lysozyme from reaching the substrate, the mucopeptide layer.

Our results on the effectiveness of lysozyme plus EDTA agree with those of Repaske (10), Voss (13), and Salton (11), who found that the cells of several unrelated species of Gram-negative bacteria were lysed by lysozyme in the presence of EDTA. This stimulation of lysis by EDTA has been attributed to chelation of the divalent ions of the lipoprotein and lipopolysaccharide layers of the cell walls, making it easier for the lysozyme to reach the mucopeptide layer and hydrolyze the β-1-4 glucosidic linkages, with the ultimate formation of spheroplasts subject to osmotic lysis in hypertonic environment. Our results indicate that the metal ions in dairy products make lysozyme plus EDTA ineffective against *P. fragi,* presumably through formation of an EDTA-ion complex.

The metal ions in milk also drastically decreased the action of chlortetracycline on *P. fragi.* Our results are compatible with those of Oxford (9), Weinberg (16), and Albert and Rees (1). They found that under certain conditions addition of Fe^{++} or Mg^{++} reversed the bacteriostatic effect of tetracyclines. In our experiments, milk ash or magnesium sulfate added to lactose–yeast extract broth prevented the action of chlortetracycline on *P. fragi,* probably by metal ions forming an inactive complex with chlortetracycline.

The inhibitory effects of potassium sorbate and sodium benzoate against *P. fragi* were enhanced by low pH, because acids are less dissociated. Undissociated acids pass through the bacterial cell membrane by passive diffusion and active transport more readily than do dissociated ions of acids. Calculations show that 93% of the sorbic acid in a medium is undissociated at pH 3.7, and that undissociated acid decreases rapidly as the pH of the medium is increased, to only 7% at pH 5.8. At pH 5.1 to 5.2, the approximate pH of Cottage cheese, about 30%, is undissociated.

Acknowledgment

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