The Inhibiting Effect of Serine Upon the Growth of the Indigenous Flora of Cream Filling[1,2]

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It has been reported previously (Castellani, 1953) that glycine, cysteine, serine and thioglycollic acid partially inhibit the growth of food-poisoning strains of *Micrococcus pyogenes* in vanilla cream pastry filling. The present study is concerned with the effects of the above compounds, in particular serine, upon the growth of the indigenous flora of cream filling.

Experimental Procedure

Except where otherwise indicated, the composition of the filling by weight was as follows: water 300, sugar 68, starch 15, nonfat milk solids 25, fresh whole egg 50, vanilla extract 1 and salt 1.6 parts, respectively. All the ingredients were combined into a liquid mixture and 50-ml portions were dispensed into 150-ml beakers. The test compounds were added to the liquid mixture, and where required, the pH was adjusted with dilute sodium hydroxide. The fillings were then gelled by immersing in a boiling water bath. Tryptone glucose extract agar with Bacto skim milk was employed as the plating medium. The plates were incubated at the same temperature at which the fillings had been incubated as indicated in the tables. Sterile milk (1 per cent Bacto skim milk in distilled water) was employed as the plating diluent. Eleven grams of filling were initially dispersed into 99 ml of milk. The milk was used instead of distilled water because the plating of control fillings (incubated at 30 C for 24 hours) in distilled water revealed that, although an uncountable number of colonies appeared on the 10^{-4} plate, the expected number did not appear on the 10^{-5} plate. The expected decimal sequence was obtained, however, with the milk diluent. The cause and significance of these observations have not been elucidated. This discrepancy in plating, however, has not been observed in similar experiments with salmonellae, staphylococci and enterococci. All experiments presented or referred to have been duplicated, and in most instances the essentials of the various experiments have been replicated. The pH of the fillings, although not presented in every table, agreed at the time of plating with the bacteria counts, that is, a proportionately lower pH for increasing numbers of bacteria.

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Results and Discussion

Nature of indigenous flora. It was considered of interest to characterize, at least roughly, the genera of the indigenous flora. In the course of numerous experiments extending over a two-year period the flora has consisted primarily of two colonial types as indicated by appearance in pour plates. The subsurface colonies of one type are smooth punctiforms. The other type after 24 hours of incubation is rough and fuzzy. Upon further incubation, the colonies become rougher and somewhat transparent in texture. On two occasions separated by a time interval of about 6 months, eight "roughs" and six punctiforms were isolated from incubated laboratory fillings onto agar slants. All isolates were catalase positive, gram positive rods. Spores were readily observed in all of the "roughs" and in 10 of the punctiforms. As a temperature of about 80 C is readily obtained in the cooking of the laboratory fillings, it is not surprising that the indigenous flora responsible for "normal" spoilage is spore-forming bacilli.

In plating over 30 commercially distributed cream pies from five bakeries, a flora similar to that previously described has appeared to predominate on most occasions. In one instance, six "roughs" and six punctiforms were isolated from plates of each of two incubated coconut cream pies made by different bakeries. With the exception of two punctiforms from one of the fillings which were gram positive, catalase negative cocci, all the isolates were gram positive, catalase positive, spore-forming rods. The predominance of spore-formers would be expected considering the cooking process employed. In a number of observations made in a large bakery, the filling has routinely attained a temperature of 83 C and higher. The presence of nonspore-formers in commercial filling would appear to suggest either that the filling was undercooked, or more likely, that it was contaminated after the cooking. In this connection, the studies of Abrahamson *et al.* (1952) on the improvements in bacteriological quality of cream fillings following the institution of recommended practices are of interest.

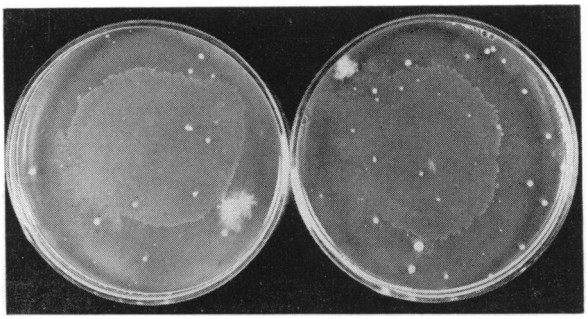

FIG. 1. Plate on left is from commercial filling. Plate on right is from laboratory filling. Note predominance of rough, fuzzy colonies. Plates incubated at 37 C for 24 hours. (Photographed by Mr. Joseph Dix, A. I. B.)

TABLE 1. *Effects of several test substances on the growth of the indigenous flora of cream filling*

Test Substance (Each Concentration 0.2 Per Cent)	Bacterial Count/g of Filling[1]	Final pH[2]
Thioglycollic acid	134,000	6.50
DL-serine	555,000	6.75
L cysteine HCl	19,500,000	6.25
Glycine	121,000,000	5.90
Glycine + DL-serine	115,000,000	5.95
None	180,000,000	5.60

[1] Fillings incubated 24 hours at 37 C.
[2] Initial pH of liquid mix 6.60.

Bacteriostatic effect of the different compounds. The compounds which had been found in earlier studies to have a partial inhibitory effect upon staphylococci were further tested for their effects upon the indigenous flora. The results of an experiment employing laboratory-prepared vanilla filling are presented in table 1. It will be observed that the bacteriostatic effect of cysteine, serine and thioglycollic acid increases in the order listed. Glycine, however, is not inhibitory, and, in fact, neutralizes the action of serine.

Bacteriostatic effects of the different serine isomers. Because cysteine and thioglycollic acid impart off-flavor, serine has been investigated more thoroughly than the other inhibitors. It was considered of interest to determine which of the isomers of serine is responsible for the bacteriostasis. The data of three experiments employing different levels of serine are presented in table 2. In order to increase the accuracy of the plate counts, duplicate 11-g aliquots of each filling were plated in experiments 1 and 2. Satisfactory duplication was obtained, and the data are the averages of the duplicate platings. In experiment 3 only a single aliquot was plated.

The bacteriostatic effect of the D-isomer is readily apparent in experiments 2 and 3 of table 2. In experiment 2, the bacteria counts with and without 0.2 per cent D-serine are, respectively, 2,000,000 and 125,000,000. The bacteriostatic effect of the L-isomer may be similarly observed in experiment 3 where the bacteria counts with and without 0.3 per cent L-serine are, respectively, 26,000,000 and 230,000,000. That the D-isomer is more bacteriostatic than the L-isomer is clearly indicated in experiments 2 and 3. It can also be observed that the racemic form is the most bacteriostatic. In experiment 1, the bacteria count for DL-serine is less than that for either D or L-serine (3,600,000–51,000,000–72,000,000, respectively). The above conclusions are actually suggested in each of the three experiments, but the above specific citations are made since some relationships are more clearly defined at one concentration of serine than at another.

The data suggesting that the racemic mixture is the most bacteriostatic entity were unexpected. Therefore, in order to establish this relationship with greater certainty, further experiments were conducted. The data of two experiments are presented in table 3. In these experiments, single 11-g aliquots were plated, but serial 10-fold dilutions were made so that each countable plate received a 1-ml plating aliquot. It can be observed in both experiments in table 3 that the bacteria counts for 0.075 per cent D-serine plus 0.075 per cent L-serine are less than those for either 0.15 per cent D or 0.15 per cent L-serine. It should be noted, furthermore, that the bacteriostatic effect of the different racemic mixtures, D + L and DL, are equivalent.

The role of the D-isomer as the bacteriostat in amino acid inhibitions is well established. That the L-isomer

TABLE 2. *Bacteriostatic effects of the different isomers of serine upon the indigenous flora of cream filling*[1, 2]

Experiment 1		Experiment 2		Experiment 3	
Per cent serine	Bacteria count/g	Per cent serine	Bacteria count/g	Per cent serine	Bacteria count/g
0	150,000,000	0	125,000,000	0	230,000,000
0.1 L	72,000,000	0.2 L	27,000,000	0.3 L	26,000,000
0.1 D	51,000,000	0.2 D	2,000,000	0.3 D	525,000
0.1 DL	3,600,000	0.2 DL	975,000	0.3 DL	230,000
0.2 DL	300,000	0.4 DL	100,000	0.6 DL	25,000
0.1 D + 0.1 L	480,000	0.2 D + 0.2 L	160,000	0.3 D + 0.3 L	7,000

[1] DL-serine was Merck's #61281, Merck & Co., Inc., Rahway, N. J. In experiments 1 and 2, D-serine was Nutritional Biochemical's #8335; in experiment 3, #6038, Nutritional Biochemicals Corp., Cleveland, Ohio. In experiments 1 and 2, L-serine was Nutritional Biochemical's #8624; in experiment 3, #4483.
[2] Fillings were incubated at 30 C for 22 to 24 hours.

TABLE 3. *The superior bacteriostatic effect of the racemic isomer of serine upon the indigenous flora of cream filling*

Per Cent Serine[1]	Final Bacteria Count/g of Filling[2]	
	Experiment 1	Experiment 2
0	177,000,000	130,000,000
0.15 L	65,000,000	102,000,000
0.15 D	39,000,000	8,700,000
0.075 L + 0.075 D	1,200,000	1,600,000
0.15 DL	1,400,000	1,100,000
0.30 DL	260,000	370,000

[1] DL-serine was Merck's #61281, Merck & Co., Inc., Rahway, N. J. L-serine was Nutritional Biochemicals #8624, Nutritional Biochemicals Corp., Cleveland, Ohio. D-serine was Nutritional Biochemicals #6038.

[2] Fillings incubated at 30 C for 22 to 24 hours.

TABLE 4. *Effects of time and temperature of incubation upon the inhibition of the indigenous flora of cream filling by* DL-*serine*

DL-Serine	Bacterial Count/g of Filling			
	30 C		37 C	
	24 hr	48 hr	24 hr	48 hr
Per cent				
0	115,000,000	185,000,000	170,000,000	220,000,000
0.2	440,000	20,800,000	6,000,000	34,200,000

can sometimes be inhibitory has also been demonstrated (Castellani 1953, Teeri 1954). Indeed, Wretland and Rose (1950) have indicated that excessive amounts of L-methionine suppress growth of rats more readily than the D-isomer. However, the present observation that racemic serine is more bacteriostatic than either isomer would not ordinarily have been predicted. An interpretation of the cause and significance of this observation must await further investigation.

Extent of preservative effect. One pertinent consideration was to assess, at least in a general way, the bacteriostatic effect of serine for different times and temperatures. Accordingly, experiments were conducted in which the fillings were incubated at 30 C and 37 C for 24 and 48 hours. Typical results are presented in table 4. It may be observed that at the end of 24 hours, the bacteria count in filling with serine at 30 C is significantly less than at 37 C. However, at the end of 48 hours the bacteria counts appear to be similar. The fillings without serine are customarily offensive after 18 hours at 37 C. The fillings with serine, on the other hand, appear and smell very fresh at the end of 24 hours. However, at 48 hours the fillings with serine at 30 C and 37 C are noticeably spoiled. In fact, even with 0.3 per cent DL-serine the fillings are spoiled at 48 hours.

Bacteriostasis in commercial fillings. The incorporation of serine into freshly cooked vanilla filling, in a commercial bakery, furnished no significant degree of bacteriostasis. A subsequent series of laboratory experiments indicated that sodium propionate, an optional ingredient in the commercial filling, neutralized the bacteriostatic effect of serine. Data demonstrating this are presented in table 5. In this experiment the initially described laboratory procedures were employed except that the filling formula was modified to contain 25 g of fresh whole egg and 25 g of fresh egg yolk in place of 50 g of fresh whole egg. This modification approximated the egg content of the commercial formula. The results of several experiments suggested also that the neutralizing effect is more decisively illustrated in this modified formula.

TABLE 5. *Neutralizing effect of sodium propionate upon the bacteriostatic effect of* DL-*serine*

Figures in millions

	With 0.25 Per Cent Sodium Propionate		Without Sodium Propionate	
	With 0.25 per cent serine	Without serine	With 0.25 per cent serine	Without serine
Bacteria count/g of filling[1]	35	50	2.8	210

[1] Laboratory formula modified to contain 25 g of fresh whole egg and 25 g of fresh egg yolk in place of 50 g of fresh whole egg; fillings incubated at 37 C for 24 hours.

Further experiments were conducted in order to evaluate the possible bacteriostatic effect under commercial conditions. In these experiments, the fillings were made with commercial ingredients and equipment on the premises of a large bakery. However, the batches were of laboratory proportions prepared by the previously described laboratory procedure. The laboratory formula was modified to contain 20 g of starch and frozen egg in place of fresh egg. DL-serine #W-2575 kindly furnished by the Monsanto Chemical Company was employed in this series of experiments. The cooked filling was dispensed in 250-g amounts into 6-inch prebaked pie shells and incubated at 37 C for 24 hours. In two experiments, the bacteria counts per g of filling with 0.3 per cent DL-serine were 4,300,000 and 4,000,000. The bacteria counts in the respective controls lacking serine were 120,000,000 and 115,000,000. In one experiment, with 0, 0.2, 0.3 and 0.4 per cent DL-serine the bacteria counts per g were, respectively, 163,000,000, 8,000,000, 1,600,000, and 600,000.

Effects of different ingredients. It has been observed in other laboratory experiments that the bacteriostatic effect of serine is lessened in coconut and chocolate fillings. Upon the addition of 2.5 g of coconut per 50 ml of liquid mix, typical bacteria counts in fillings with and without 0.2 per cent DL-serine after 24 hours at 37 C ranged between 15,000,000 to 30,000,000 and 225,000,000 to 350,000,000. Visual inspection of the colonial morphology suggested that the flora of control fillings was predominantly the indigenous type previ-

ously described, but other lens-shaped colonies were also present. Initial experiments with chocolate and cocoa indicate a similar lessening of the bacteriostatic effect of serine, but the data have been somewhat erratic.

Of even greater significance is the observation that the inhibitory effect of serine upon staphylococci is, for all practical purposes, completely neutralized by coconut and cocoa (unpublished data). The omission of milk solids from the formula does not appreciably alter these relationships. However, serine exerts a pronounced bacteriostatic effect upon staphylococci in a pineapple cream filling at pH 4.6.

The above results indicate that the bacteriostatic effect of serine must be separately evaluated for the different kinds of fillings.

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Summary

The indigenous flora responsible for "normal" spoilage of properly cooked laboratory and commercial filling appear to be several species of spore-forming bacilli.

Serine, cysteine and thioglycollic acid exert a bacteriostatic effect upon the indigenous flora of vanilla cream filling. Glycine is not inhibitory, and, in fact, counteracts the bacteriostatic effect of serine.

Racemic serine appears to be more bacteriostatic than either isomer. Both isomers, however, are individually bacteriostatic, the D-isomer more so than the L-isomer.

With regard to off-odors resulting from microbial growth in the filling, the preservative effect of 0.2 to 0.3 per cent DL-serine does not reach 48 hours at either 30 C or 37 C. However, the preservative effect is quite marked at 24 hours at 37 C.

The bacteriostatic effect of serine has been demonstrated in fillings prepared under "commercial" conditions in a large bakery.

Sodium propionate lessens the bacteriostatic effect of serine for the indigenous flora.

Coconut and cocoa completely neutralize the bacteriostatic effect of serine upon staphylococci, but serine exerts a pronounced inhibition in a pineapple cream filling.

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