Fate of Staphylococci and Enteric Microorganisms Introduced into Slurry of Frozen Pot Pies

G. M. Dack and G. Lippitz

Food Research Institute and Department of Microbiology, University of Chicago, Chicago, Illinois

Received for publication May 3, 1962

ABSTRACT

Dack, G. M. (University of Chicago, Chicago, Ill.) and G. Lippitz. Fate of staphylococci and enteric microorganisms introduced into slurry of frozen pot pies. Appl. Microbiol. **10**:472–479. 1962.—A slurry was prepared from six frozen pot pies diluted 1:5 with distilled water, two chicken, two turkey, and two beef pies of different brands. This slurry formed a reference sample and was placed in sterilized jars, frozen, and used as needed throughout the experiments. A second slurry was prepared in a similar manner from a frozen beef pot pie and a chicken pot pie, and was used as a control in only one experiment. The total count of microorganisms and the number of coliforms, *Escherichia coli*, salmonellae, and coagulase-positive staphylococci per gram were determined. Samples of slurry were inoculated in decimal dilutions with one or more of the following: *Salmonella typhimurium*, *E. coli*, and a strain of staphylococcus that causes food poisoning. The natural flora was found to exert an inhibitory effect upon the growth of the added microorganisms after incubation at 35 C for 18 hr. The inhibitory effect on growth was in part due to pH. The predominating organism isolated from the natural flora after incubation was a lactobacillus, which, when added in mixture with the test organisms in sterilized slurry, did not exert the profound inhibitory effect observed in the case of the natural flora. Some factors which may be concerned with the inhibition were investigated.

The subject of this investigation arose in a joint discussion of a committee of the Food and Drug Officials of the United States and a committee from the National Association of Frozen Food Packers held in Dallas, Texas, on 4 June 1960 concerning microbiological standards for precooked frozen foods, i.e., how many coagulase-positive staphylococci should be permitted per gram of food. After this meeting, two papers appeared by Peterson, Black, and Gunderson (1962*a*, *b*) on staphylococci in competition with the natural flora in precooked frozen foods during defrosting as well as in mixed cultures in artificial medium.

The purpose of this investigation was to determine the significance of numbers of food-poisoning staphylococci and salmonellae when in competition with the natural flora of such products. Obviously, in the frozen state no problem is involved; but, if the products were mishandled or insufficiently cooked, the question of what would happen to potential food-poisoning microorganisms is of public health significance.

Materials and Methods

To obtain samples which would represent natural flora and yet be uniform, two slurries were prepared. The first slurry, upon which most of the work was based, consisted of six frozen pot pies: two chicken, two turkey, and two beef pies of different brands. These samples were pooled, diluted to 1:5 with sterile distilled water, and blended for 2 min. All of the pie, including the crust, was used.

This stock sample was placed in four 250-ml amounts in sterile flasks and (the remainder) in 50-ml amounts in sterilized jars and frozen. Each jar contained 10 g of the pooled sample.

The second slurry was prepared from two frozen pies from different manufacturers: one a beef pot pie and the other a chicken pot pie. Slurry 2 was prepared in exactly the same manner as slurry 1, and it also represented a 1:5 dilution of the combined whole pie samples.

A detailed microbiological study was made of the two slurries to determine the total count, the number of coliform microorganisms, the number of coagulase-positive staphylococci, and the presence of *Salmonella*.

When the 250-ml frozen samples were used, they were thawed in the refrigerator at 4 C for 24 hr. Samples of the two slurries were tested after defrosting. Separate samples were defrosted and incubated for 18 hr at 35 C to compare them with the defrosted samples which were not subsequently incubated.

From the 250-ml quantities, 175 ml of slurry were removed for *Salmonella* examination; the remaining 75 ml left in the flask were diluted to make a 1:10 dilution. From this dilution, further decimal dilutions were made, up to 1:1 million. The total count was made by plating decimal dilutions in duplicate in BBL Milk Protein Hydrolysate-glucose-agar (MPH). The plates were incubated at 35 C and counted after 48 hr; the *E. coli* were determined by plating decimal dilutions in triplicate in Difco Violet Red Bile Agar (VRB); when the agar was hardened, it was coated with an additional 4 ml of VRB. The red colonies were counted after 20 hr of incubation at 35 C. Single red colonies were transferred to both

Difco Lactose Broth fermentation tubes and Difco Tryptose-Lactose-Bile Salts (EC). Fermentation tubes of Lactose Broth were incubated for 24 hr at 35 C; EC medium was incubated in a water bath at 44.5 C. If growth occurred in the EC medium and gas was produced in Lactose Broth cultures, the Lactose Broth culture was streaked on Difco Eosin Methylene Blue Agar (EMB) plates. If typical "metallic sheen" colonies developed in plates incubated at 35 C for 20 hr, the cultures were gram stained; and, if gram-negative rods, they were considered to be *Escherichia coli*.

The tests for staphylococci were made by inoculating tubes of Beef Heart medium containing 10% sodium chloride with decimal dilutions of slurry and incubating for 48 hr at 35 C. If growth occurred, these cultures were streaked on Staphylococcus Medium No. 110 (Difco) and incubated for 48 hr at 35 C. Isolated yellow or white colonies on 110 Medium were fished and placed in 1 ml of Nutrient Broth in a culture tube and incubated for 18 hr at 35 C; 0.2 ml of this culture was transferred to small sterile tubes containing 0.5 ml of diluted Coagulase Plasma (Difco). The plasma tubes were incubated for 3 hr at 35 C and observed for coagulation. Each colony used in the coagulase test was examined by use of the Gram stain.

The *Salmonella* tests were made on the 175-ml portions of slurry; three 50-ml, three 5-ml, and three 0.5-ml samples of slurry were inoculated into Lactose Broth. For the 10-g samples, a 50-ml portion was inoculated into 50 ml of double-strength Lactose Broth. For the 1-g samples, 5 ml of slurry were added to 95 ml of Lactose Broth; for the 0.1-g samples, 0.5 ml of slurry was added to 9.5 ml of Lactose Broth. After incubation for 24 hr at 35 C, 1 ml of culture was transferred to 10 ml of Selenite-F Enrichment (BBL) plus 10 μg/ml of cystine (North and Bartram, 1953). After incubation for 18 hr at 35 C, the Selenite-F Enrichment cultures were streaked on Brilliant Green Agar (BBL) plates. The plates were observed after 18 hr for the nonlactose-fermenting colonies typical of *Salmonella*.

Results

The results of the microbiological tests on the basic slurry samples are summarized in Table 1.

Most of the work, except for that reported in Tables 4 and 5, involved the use of slurry 1. Slurry 2 was used to obtain the data given in Tables 4 and 5. In the tests, the slurry was diluted and used with the natural flora which it contained. Tests were also made with slurry which had been sterilized by autoclaving at 15 psi for 45 min.

Preparation of cultures inoculated into slurry. The *E. coli* strain was obtained from the curator in the Department of Microbiology. This strain was labeled *E. coli* 1. Strain 196E of *Staphylococcus aureus* was used unless otherwise stated. This is a food-poisoning strain which is coagulase-positive. *Salmonella typhimurium* was obtained from the curator of the Department of Microbiology.

Broth cultures (0.1 ml) were transferred weekly to tubes of fresh Nutrient Broth. These broth cultures were incubated for 4 days at 35 C, after which time they were used to seed the slurry. The number of microorganisms in the inoculum was determined by periodic counts of the stock culture. The 50-ml jars of frozen stored slurry, when used, were held at room temperature for 90 min, after which they were diluted to 1:2, making the final 1:10 dilution; 10 ml of the diluted 1:10 slurries were pipetted into sterile tubes, and each tube was inoculated with test organisms. These tubes were inoculated with decimal dilutions from the test strains ranging from 10 test organisms per g to 1 million per g, and were then incubated for 18 hr at 35 C. A control slurry was autoclaved, placed in tubes, inoculated with decimal dilutions of the test organisms, and incubated for the same period. In the test involving unautoclaved slurry, an unseeded control was included. Combinations of the test organisms, as indicated in the tables, were inoculated into the slurry (Tables 2–9).

The seeded slurry cultures were tested by the following microbiological procedures in determining total count, number of coliforms, number of coagulase-positive staphylococci, and number of *S. typhimurium*.

The total count was made exactly as described for the test slurries except that it was not done in duplicate.

Since the basal slurry medium had been tested and was found to be free of *E. coli*, and since the inoculum was *E. coli*, the test for this organism involved plating decimal dilutions in VRB, using the plating method as described for the slurry. When red colonies were picked from each dilution and inoculated into EC broth and incubated for 24 to 48 hr at 44.5 C, growth on EC broth was considered indicative of *E. coli*.

Staphylococci were determined by inoculating decimal dilutions into 10 ml of Cooked Meat Medium (Difco) plus 10% NaCl, and incubating for 48 hr at 35 C. The Cooked Meat cultures were streaked onto Staphylococcus Medium No. 110 and incubated for 24 hr at 35 C. A typical staphylococcus colony from each dilution was tested for coagulase in the same manner as described for the slurry.

Salmonellae were determined by inoculating decimal dilutions into 10 ml of Lactose Broth and incubating for

TABLE 1. *Microorganisms in prepared slurries tested after thawing and after incubating for 18 hr at 35 C*

Slurry	Tested after	Total count per g	Coagulase-positive staphylococci/g	Coliforms per g	Salmonellae
1	Thawing	19,000	100	2.5	0
	Incubation	200,000,000	10,000	200	0
2	Thawing	400,000	1,000	280*	0
	Incubation	230,000,000	1,000,000	2,800*	0

* Some of these were *E. coli* but the numbers were not determined.

24 hr at 35 C; 0.1 ml of the Lactose Broth culture was transferred to 10 ml of Selenite-F Enrichment-cystine broth and incubated for 18 hr at 35 C. The resultant cultures were streaked on Brilliant Green Agar plates and incubated for 18 to 24 hr at 35 C. A typical colony from each dilution was examined serologically using group B agglutinating serum. The results of these experiments are given in Tables 2–7.

In preliminary tests, the pH of a sterilized (autoclaved) 1:10 dilution of slurry was adjusted with 0.85% phosphoric acid to the following levels: 4.0, 4.10, 4.30, 4.50, 5.00, 5.5, and 5.8. The slurry was then placed in sterile culture tubes. One series of tubes was seeded with about 100 cells of *S. typhimurium* per g of undiluted slurry, one with about 100 *E. coli*, and the third series with about 100 *S. aureus*. The tubes, after inoculation, were incubated for 18 hr at 35 C, and then plate counts were made. *Salmonella* grew slightly at pH 4.0 and 4.1, with increasing growth at pH 4.3 and 4.5. *E. coli* failed to grow at pH 4.0, 4.1, and 4.3, but did grow at 4.5 although not as well as at pH 5.0 and higher. Staphylococci failed to grow at pH 4.0, 4.1, and 4.3, and grew slightly at pH 4.5. Good growth of staphylococci occurred at pH 5.0 and above.

Experiments were repeated, inoculating tubes of slurry with known numbers of staphylococci and *E. coli*, as well as with *Salmonella* and *E. coli*. Duplicate tests were made with slurry containing the natural flora and with sterilized slurry. In these tubes, the pH was recorded after 6 and 18 hr of incubation at 35 C (Tables 8 and 9).

Experiments were carried out to elucidate further the role of the natural flora in the inhibition of the test organisms. This work was done using slurry 1 and isolating the predominating organisms after 18 hr of incubation at 35 C. The predominating colonies were studied on MPH. A lactobacillus of the homo-fermentative group predominated over all other microorganisms, and its fermentation reactions were similar to those given for the group in *Bergey's Manual*. The predominant microorganism was a gram-positive coccobacillus occurring in pairs and short chains. It was nonhemolytic on sheep blood-agar plates; it was negative for catalase, for gas production in the broth medium of Niven and Evans for lactobacilli APT (BBL), and for nitrate reduction. It grew poorly in Nutrient Broth, and the cultures used to inoculate the sterilized slurry were prepared in the same manner as other stock cultures but were isolated on MPH slants rather than Nutrient Agar slants. Three other strains were isolated from the incubated slurry cultures, even though they were not the predominant organisms.

The predominant lactobacillus fermented the following test substances: glucose, mannose, galactose, maltose, mannitol, trehalose, and raffinose. The following were not

TABLE 2. *Determination of total count, Escherichia coli and Staphylococcus aureus in pot pie slurry inoculated with E. coli and S. aureus and tested after incubation for 18 hr at 35 C (slurry 1)*

No. of staphylococci in inoculum in millions per g	Total count in millions					*S. aureus* in millions					*E. coli* in millions				
	E. coli inoculum in millions					*E. coli* inoculum in millions					*E. coli* inoculum in millions				
	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001
1.0	200	185	80	45	75	1.0	0.50	0.5	0.1	1.0	2.6	0.9	0.016	0.0007	0.0002
0.1	130	140	95	60	95	0.1	0.05	0.5	0.05	1.0	2.7	2.45	0.029	0.0009	0.0002
0.01	150	100	90	95	115	0.1	0.50	0.5	0.1	0.5	3.5	2.1	0.31	0.0062	0.0007
0.001	140	85	85	90	95	0.05	0.005	0.5	0.1	0.1	4.0	2.5	0.05	0.0291	0.0001
0.0001	150	80	95	110	155	0.005	0.005	0.05	0.05	0.05	3.0	5.0	0.34	0.0150	0.0004
0.00001	105	90	115	105	150	0.01	0.050	0.05	0.05	0.1	5.6	2.8	0.05	0.022	0.0004
None	170	105	140	120	105	0.050	0.005	0.05	0.05	0.1	6.5	2.1	0.35	0.0202	0.0045
Uninoculated slurry	140	140	115	115	135	0.01	0.01	0.005	0.005	0.01	0	0	0	0	0

TABLE 3. *Determination of total count, Escherichia coli, and Staphylococcus aureus in sterilized pot pie slurry inoculated with E. coli and S. aureus and tested after incubation for 18 hr at 35 C (slurry 1)*

No. of staphylococci in inoculum in millions per g	Total count in millions					*S. aureus* in millions					*E. coli* in millions				
	E. coli inoculum in millions					*E. coli* inoculum in millions					*E. coli* inoculum in millions				
	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001
1.0	145	650	160	135	125	5.00	5.0	5.5	50.5	10.0	110	365	55	35	22
0.1	300	600	185	150	205	5.00	2.2	50.5	5.5	5.0	190	525	75	40	55
0.01	310	610	300	190	300	5.00	50.0	5.5	5.5	5.0	130	625	205	70	110
0.001	500	500	320	265	150	0.05	0.50	50.5	5.5	5.5	290	135	190	105	70
0.0001	500	1,500	385	285	210	0.05	0.05	0.5	0.5	5.0	400	725	255	150	185
0.00001	550	1,400	500	175	265	0.005	0.05	0.05	0.05	0.05	350	820	310	260	90
None	500	500	450	120	275	—	—	—	—	—	300	535	1,200	340	145

fermented: sorbitol, lactose, salicin, arabinose, and rhamnose. Litmus milk cultures were slightly acid and the milk was not coagulated.

The predominant organism was tested in the sterilized slurry along with decimal dilutions of *E. coli*, *S. typhimurium*, and *S. aureus*.

In addition to these organisms, six food-poisoning strains of staphylococcus were tested. The results are given in Tables 10–14; it is apparent that the predominant organism, alone or in combination with the test organisms, did not exert an effect comparable with that of the natural flora. The data are not presented for the other three organisms isolated, which were outnumbered in the slurry by the predominating lactobacillus, since they did not exert any effect on the growth of *S. aureus* 196E.

TABLE 4. *Determination of total count, Escherichia coli, and Staphylococcus aureus in pot pie slurry inoculated with E. coli and S. aureus and tested after incubation for 18 hr at 35 C (slurry 2)*

No. of staphylococci in inoculum in millions per g	Total count in millions		*S. aureus* in millions		*E. coli* in millions	
	E. coli inoculum in millions		*E. coli* inoculum in millions		*E. coli* inoculum in millions	
	1.0	0.0001	1.0	0.0001	1.0	0.0001
1.0	300	500	1.0	10.0	5	0.03
0.1	400	400	1.0	1.0	80	0.8
0.01	400	600	0.1	1.0	80	0.6
0.001	400	400	0.01	1.0	60	0.4
0.0001	400	500	0.01	1.0	40	0.6
0.00001	400	400	0.01	0.1	40	2.9
None	300	700	—	—	30	30.0
Uninoculated slurry	300	260	0.1	0.1	1.5	1.5

TABLE 5. *Determination of total count, Escherichia coli, and Staphylococcus aureus in sterilized pot pie slurry inoculated with E. coli and S. aureus and tested after incubation for 18 hr at 35 C (slurry 2)*

No. of staphylococci in inoculum in millions per g	Total count in millions		*S. aureus* in millions		*E. coli* in millions	
	E. coli inoculum in millions		*E. coli* inoculum in millions		*E. coli* inoculum in millions	
	1.0	0.0001	1.0	0.0001	1.0	0.0001
1.0	80	60	10.0	~100	30	3
0.1	150	70	0.0	~100	90	5
0.01	100	70	0.1	~100	70	20
0.001	80	40	0.01	~100	60	19
0.0001	80	80	0.01	1	70	50
0.00001	90	50	<0.01	0.1	40	50
None	110	150	—	—	70	60

Discussion

The natural flora of frozen pot pies has an inhibitory effect upon the growth of a food-poisoning strain of staphylococcus, *S. typhimurium*, and *E. coli* inoculated into a slurry made from the pies and incubated for 18 hr at 35 C. The effect is greater on *S. aureus* and less marked on the growth of *E. coli*. The effect of *E. coli* on the growth of staphylococci can be demonstrated after the natural flora is eliminated by sterilizing the slurry. The inhibitory effect is not as pronounced as when the natural flora is present. Abundant growth of *E. coli* occurred when inoculated into sterilized slurry along with *S. aureus*.

In the case of *S. typhimurium*, the natural flora was inhibitory to the growth of this organism. *S. typhimurium* was not inhibited by *E. coli* unless the *E. coli* vastly outnumbered it, as indicated in Tables 6 and 7. The reason for the inhibition of *Salmonella*, *E. coli*, and *S. aureus* in a slurry containing the natural flora was at first thought to be pH but this is only one of a number of factors to be considered. The predominate organism as well as three other organisms isolated from incubated slurry, when tested with the staphylococcus, *E. coli*, and *S. typhimurium*, failed to explain the inhibitory phenomena. Levine and Tanimoto (1954) showed that certain strains of *E. coli* produce "colicines," which exert an antagonistic effect against other bacteria. Flippin and Mickelson (1960) and Mickelson and Flippin (1960), in desugaring egg-white, made use of a strain of *E. coli* which is antagonistic to the growth of *Salmonella*.

TABLE 6. *Determination of total count, Escherichia coli, and Salmonella typhimurium in pot pie slurry inoculated with E. coli and S. typhimurium and tested after incubation for 18 hr at 35 C*

No. of salmonellae in inoculum in millions per g	Total count in millions					*S. typhimurium* in millions					*E. coli* in millions				
	E. coli inoculum in millions					*E. coli* inoculum in millions					*E. coli* inoculum in millions				
	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001
1.0	130	30	90	50	60	10.0	10.0	10.0	1.0	1.0	0.29	0.04	0.001	0	0
0.1	80	30	100	50	70	1.0	0.1	10.0	1.0	10.0	0.7	0.16	0.003	0.0003	0
0.01	50	30	100	50	60	1.0	1.0	1.0	1.0	1.0	0.7	0.25	0.001	0.018	0
0.001	70	30	80	70	40	0.1	0.01	0.1	0.01	0.1	1.7	0.09	0.01	0.04	0
0.0001	110	40	90	60	50	0.1	0.01	0.01	0.001	0.001	0.3	0.27	0.018	0.025	0
0.00001	100	30	120	70	70	0.01	0.01	0.01	0.001	0.001	0.3	0.19	0.07	0.13	0.09
None	80	30	110	50	70	—	—	—	—	—	0.4	0.08	0.05	0.027	0.0011
Uninoculated slurry	80	80	80	60	60	0	0	0	0	0	0	0	0	0	0

Peterson et al. (1962*a*, *b*) found it was not possible to promote growth of appreciable numbers of staphylococci under any condition of defrost in chicken pot pies. In macaroni and cheese frozen dinners defrosted at room temperature after extended incubation, spoilage microorganisms developed and staphylococci attained large numbers but only after spoilage was advanced. These authors further studied staphylococcus multiplication in artificial medium in mixture with saprophytic and psychrophilic bacterial species, and found that staphylococci grew less as they were outnumbered in the inoculum.

The question of the number of food-poisoning staphylococci required to produce sufficient enterotoxin to cause illness has not been answered as yet, and certainly would depend on the ability of a strain to produce a potent enterotoxin. In one airline outbreak (Morbidity and Mortality Weekly Reports, 1961), 11 million staphylococci per g were reported in the incriminated food. If the data in this paper are evaluated on the assumption that staphylococci must multiply in food to at least 10 million organisms per g, to produce enough enterotoxin to cause illness when the food is eaten, it may be seen in Table 2 (slurry 1) that at no time was this number attained even when 1 million food-poisoning staphylococci per g were in the inoculum. In the uninoculated slurry 1, which was incubated at 35 C for 18 hr, the number of naturally occurring staphylococci rose from 100 per g to 10,000 per g. However, in the case of slurry 2, which had a total count of 400,000 microorganisms per g and 1,000 coagulase-positive staphylococci per g at the time of defrosting after incubation at 35 C for 18 hr, the staphylococcus count increased to 1 million per g. Slurry 2 was

TABLE 7. *Determination of total count, Escherichia coli, and Salmonella typhimurium in sterilized pot pie slurry inoculated with E. coli and S. typhimurium and tested after incubation for 18 hr at 35 C*

No. of salmonellae in inoculum in millions per g	Total count in millions					*S. typhimurium* in millions					*E. coli* in millions				
	E. coli inoculum in millions					*E. coli* inoculum in millions					*E. coli* inoculum in millions				
	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001	1.0	0.1	0.01	0.001	0.0001
1.0	300	500	400	700	1,200	100	100	1,000	1,000	100	110	16	1	2	0.02
0.1	400	400	500	500	600	100	10	1,000	100	100	70	50	5	9	0.[illegible]
0.01	400	300	400	700	1,200	100	100	100	100	100	150	120	40	3	3
0.001	300	600	300	700	1,100	10	100	100	100	1,000	200	140	100	160	25
0.0001	600	400	290	500	700	10	10	10	10	100	200	280	190	170	160
0.00001	500	300	300	500	700	0.001	1	1	100	100	190	170	170	290	160
None	500	260	400	300	270	—	—	—	—	—	250	160	250	230	170

TABLE 8. *Determination of pH in pot pie slurry inoculated with Escherichia coli and Staphylococcus aureus and tested after incubation for 6 and 18 hr at 35 C*

Incubation time	Original pH = 6.35, sterilized basal material						Original pH = 5.85, basal material with natural flora					
	No. of staphylococci in inoculum in millions per g	*E. coli* inoculum in millions					No. of staphylococci in inoculum in millions per g	*E. coli* inoculum in millions				
		0.0001	0.001	0.01	0.1	1.0		0.0001	0.001	0.01	0.1	1.0
hr												
6	1.0	5.55	5.65	5.50	5.55	5.50	1.0	5.80	5.75	5.70	5.60	5.60
	0.1	5.65	5.85	5.55	5.65	5.50	0.1	5.80	5.75	5.70	5.60	5.65
	0.01	5.65	5.85	5.55	5.65	5.50	0.01	5.85	5.75	5.70	5.60	5.65
	0.001	5.95	6.00	5.80	5.65	5.65	0.001	5.85	5.85	5.70	5.60	5.70
	0.0001	5.95	6.05	5.95	5.95	5.75	0.0001	5.85	5.85	5.85	5.75	5.70
	0.00001	6.05	6.05	6.05	5.95	5.70	0.00001	5.90	5.85	5.85	5.75	5.70
	Control	6.40	6.05	6.00	5.95	5.70	Control	5.90	5.85	5.85	5.80	5.75
							Unseeded after incubation	6.2				
18	1.0	5.35	4.90	4.85	4.75	4.90	1.0	4.45	4.50	4.45	4.40	4.35
	0.1	5.15	4.90	4.85	4.75	4.85	0.1	4.45	4.45	4.45	4.40	4.40
	0.01	4.90	4.90	4.85	4.75	4.85	0.01	4.45	4.45	4.40	4.45	4.40
	0.001	4.90	4.85	4.85	4.85	4.85	0.001	4.45	4.50	4.45	4.45	4.40
	0.0001	4.90	4.85	4.85	4.85	4.85	0.0001	4.45	4.50	4.45	4.40	4.45
	0.00001	4.85	4.85	4.85	4.75	4.80	0.00001	4.50	4.50	4.45	4.45	4.45
	Control	4.85	4.85	4.85	4.75	4.70	Control	4.50	4.50	4.50	4.45	4.45
							Unseeded after incubation	4.00				

TABLE 9. *Determination of pH in pot pie slurry inoculated with Escherichia coli and Salmonella typhimurium and tested after incubation for 6 and 18 hr at 35 C*

Incubation time	Original pH = 6.35, sterilized basal material						Original pH = 5.85, basal material with natural flora					
	No. of salmonellae in inoculum in millions per g	*E. coli* inoculum in millions					No. of salmonellae in inoculum in millions per g	*E. coli* inoculum in millions				
		0.0001	0.001	0.01	0.1	1.0		0.0001	0.001	0.01	0.1	1.0
hr												
6	1.0	5.55	5.50	5.45	5.35	5.30	1.0	5.65	5.55	5.50	5.45	5.45
	0.1	6.00	5.95	5.95	5.40	5.35	0.1	5.65	5.55	5.65	5.45	5.45
	0.01	6.10	6.00	5.95	5.40	5.35	0.01	5.70	5.65	5.70	5.45	5.45
	0.001	6.15	6.00	5.95	5.45	5.40	0.001	5.85	5.90	5.80	5.45	5.45
	0.0001	6.15	6.10	5.95	5.45	5.40	0.0001	5.85	5.90	5.85	5.45	5.50
	0.00001	6.05	6.10	6.05	5.50	5.40	0.00001	6.00	5.95	5.85	5.50	5.50
	Control	6.25	6.10	5.95	5.50	5.50	Control	6.15	6.15	5.95	5.50	5.50
							Unseeded after incubation	6.2				
18	1.0	5.25	5.15	5.00	4.95	4.80	1.0	4.25	4.25	4.10	4.25	4.10
	0.1	5.22	5.10	5.15	4.95	4.80	0.1	4.30	4.25	4.10	4.25	4.10
	0.01	5.25	5.10	5.25	4.95	4.90	0.01	4.35	4.25	4.10	4.25	4.10
	0.001	5.25	5.05	5.35	4.95	4.90	0.001	4.35	4.25	4.10	4.15	4.05
	0.0001	5.25	5.10	5.25	5.00	4.95	0.0001	4.35	4.30	4.10	4.25	4.05
	0.00001	5.25	5.25	5.10	5.05	4.95	0.00001	4.40	4.25	4.05	4.25	4.10
	Control	5.25	5.25	5.25	5.05	4.95	Control	4.40	4.25	4.05	4.25	4.10
							Unseeded after incubation	4.00				

TABLE 10. *Determination of Staphylococcus aureus 196E and total count in sterilized pot pie slurry inoculated with predominating microorganism after incubation for 18 hr at 35 C*

No. of predominating croorganisms in inoculum in millions per g	Total count in millions							*S. aureus* in millions						
	S. aureus 196E inoculum in millions							*S. aureus* inoculum in millions						
	1.0	0.1	0.01	0.001	0.0001	0.00001	None	1.0	0.1	0.01	0.001	0.0001	0.00001	None
1st determination														
0.1	100	130	100	100	100	120	80	70	70	60	10	2.6	0.3	—
None	80	120	60	60	40	9	—	40	50	40	40	11	7	—
2nd determination														
0.1	14	22	40	70	70	60	90	10	9	7	13	7	8	—
None	12	16	18	13	7	5	—	8	11	12	8	7	8	—

TABLE 11. *Determination of six strains of staphylococci tested individually and total count in sterilized pot pie slurry inoculated with predominating microorganism (0.1 million/g) after incubation for 18 hr at 35 C*

No. of predominating microorganisms in inoculum in millions per g	Total count in millions							Staphylococci in millions						
	Staphylococcal inoculum in millions							Staphylococcal inoculum in millions						
	1.0	0.1	0.01	0.001	0.0001	0.00001	None	1.0	0.1	0.01	0.001	0.0001	0.00001	None
Strain S-6														
0.1	160	80	90	90	80	70	50	40	16	6	0.7	0.3	0.16	—
None	100	80	110	90	80	11	—	80	50	100	70	70	16	—
Strain 324														
0.1	120	180	100	90	90	120	90	110	110	70	16	8	7	—
None	90	80	120	100	110	90	—	60	70	70	50	70	50	—
Strain 137														
0.1	110	110	90	120	90	100	90	40	30	9	1.3	0.3	0.06	—
None	190	180	180	180	140	80	—	120	120	160	140	100	70	—
Strain 170														
0.1	150	500	4	500	300	230	100	160	220	160	110	70	14	—
None	700	230	150	150	120	120	—	260	90	90	110	100	150	—
Strain 249														
0.1	130	120	100	110	110	100	80	130	130	100	130	40	70	—
None	90	160	100	110	80	100	—	70	130	120	110	80	100	—
Strain 340														
0.1	120	130	300	290	170	110	90	120	80	90	50	9	1.1	—
None	100	60	90	110	80	140	—	60	50	60	80	60	40	—

TABLE 12. *Determination of Escherichia coli and total count in sterilized pot pie slurry inoculated with predominating microorganism (0.1 million/g) after incubation for 18 hr at 35 C*

No. of predominating microorganisms in inoculum in millions per g	Total count in millions							*E. coli* in millions						
	E. coli inoculum in millions							*E. coli* inoculum in millions						
	1.0	0.1	0.01	0.001	0.0001	0.00001	None	1.0	0.1	0.01	0.001	0.0001	0.00001	None
0.1	130	90	110	120	90	50	50	80	60	90	100	90	220	—
None	130	110	100	100	90	120	—	110	110	140	120	170	120	—

TABLE 13. *Determination of Salmonella typhimurium and total count in sterilized pot pie slurry inoculated with predominating microorganism (0.1 million/g) after incubation for 18 hr at 35 C*

No. of predominating microorganisms in inoculum in millions per g	Total count in millions							*S. typhimurium* in millions						
	S. typhimurium inoculum in millions							*S. typhimurium* inoculum in millions						
	1.0	0.1	0.01	0.001	0.0001	0.00001	None	1.0	0.1	0.01	0.001	0.0001	0.00001	None
0.1	600	500	500	900	600	300	80	300	300	300	300	260	270	—
None	500	600	290	280	300	220	—	170	240	250	150	220	150	—

TABLE 14. *Determination of pH in pot pie slurry inoculated with the predominating microorganism, seven Staphylococcus strains, Escherichia coli, and Salmonella typhimurium after incubation for 18 hr at 35 C (initial pH of medium = 6.35)*

Test organism		Predominating microorganism	
Strain	Inoculum (in millions)	Inoculum	
		0.1 Million	None
S. aureus 196E	1.0	4.3	4.5
	None	4.9	—
S. aureus S-6	1.0	4.45	4.5
	None	5.05	—
S. aureus 324	1.0	5.1	4.4
	None	5.1	—
S. aureus 137	1.0	4.6	4.45
	None	4.9	—
S. aureus 170	1.0	4.55	4.5
	None	4.65	—
S. aureus 249	1.0	4.7	4.5
	None	4.9	—
S. aureus 340	1.0	4.5	4.5
	None	4.8	—
E. coli	1.0	5.2	4.5
	None	5.0	—
S. typhimurium	1.0	5.0	4.6
	None	5.1	—

prepared from a product which was of lower quality than slurry 1, indicating mishandling in storage, in shipping, or in its production. When this slurry was seeded with 1 million staphylococci and 100 *E. coli* per g and incubated at 35 C for 18 hr, a count of 10 million per g of coagulase-positive staphylococci was obtained (Table 4). In a similar test, except for the fact that 1 million *E. coli* were inoculated with the same number of staphylococci, the staphylococci did not increase in number (Table 4).

Although, in preliminary tests, a pH of below 4.5 was unfavorable to growth, it is evident (Tables 8 and 9) that the lower pH values are not reached until some time after 6 hr of incubation at 35 C.

The predominating lactobacillus isolated from slurry 1, when inoculated into sterilized slurry with varying numbers and strains of staphylococci (Tables 10 and 11), inhibited the growth of staphylococci, usually when it greatly outnumbered the staphylococcus inoculum, but the inhibition was much less than in those cases where the entire flora was represented. The inhibitory effect of the natural flora with a total count of 19,000 organisms per g was profound, even when vastly outnumbered by *S. typhimurium*, *E. coli*, or *S. aureus* in the inoculum.

The effectiveness of the normal flora in slurry 1 in inhibiting growth of added microorganisms may be due to synergism of organisms in the natural flora. Since microorganisms of the natural flora were isolated only after 18 hr of incubation, the inhibition and the flora pattern earlier in the incubation period may be different and may offer a clue to the cause of inhibition.

ACKNOWLEDGMENT

This work was supported, in part, by the National Association of Frozen Food Packers, Washington, D.C.

LITERATURE CITED

FLIPPIN, R. S., AND M. N. MICKELSON. 1960. Use of salmonellae antagonists in fermenting egg white. I. Microbial antagonists of salmonellae. Appl. Microbiol. 8:367-370.

LEVINE, M., AND R. H. TANIMOTO. 1954. Antagonisms among

enteric pathogens and coliform bacteria. J. Bacteriol. **67**:537–541.

MICKELSON, M. N., AND R. S. FLIPPIN. 1960. Use of salmonellae antagonists in fermenting egg white. II. Microbiological methods for the elimination of salmonellae from egg white. Appl. Microbiol. **8**:371–377.

MORBIDITY AND MORTALITY WEEKLY REPORTS, Public Health Service, U.S. Dept. of Health, Education, and Welfare, vol. 10, no. 11, March 24, 1961.

NORTH, W. R., AND M. T. BARTRAM. 1953. The efficiency of selenite broth of different compositions in the isolation of Salmonella. Appl. Microbiol. **1**:130–134.

PETERSON, A. C., J. J. BLACK, AND M. F. GUNDERSON. 1962*a*. Staphylococci in competition. I. Growth of naturally occurring mixed population in precooked frozen foods during defrost. Appl. Microbiol. **10**:16–22.

PETERSON, A. C., J. J. BLACK, AND M. F. GUNDERSON. 1962*b*. Staphylococci in competition. II. Effect of total numbers and proportion of staphylococci in mixed cultures on growth in artificial medium. Appl. Microbiol. **10**:23–30.