Staphylococci in Competition[1]

II. Effect of Total Numbers and Proportion of Staphylococci in Mixed Cultures on Growth in Artificial Culture Medium

A. C. Peterson, J. J. Black, and M. F. Gunderson

Department of Bacteriological Research, Campbell Soup Company, Camden, New Jersey

Received for publication July 11, 1961

Abstract

Peterson, A. C. (Campbell Soup Company, Camden, N. J.), J. J. Black, and M. F. Gunderson. Staphylococci in competition. II. Effect of total numbers and proportion of staphylococci in mixed cultures on growth in artificial culture medium. Appl. Microbiol. **10**:23–30. 1962.—In studies carried on in bacteriological media with selected cultures, definite repressive effects were noted on the growth of the *Staphylococcus* population by a mixture of saprophytic, psychrophilic bacterial species. This repressive effect became more pronounced as the relative proportion of the bacterial population which was staphylococcal became smaller. A varied saprophytic bacterial flora of some numbers apparently would offer definite protection to foods through repression of staphylococcal growth and by rendering the food inedible before the rise of appreciable numbers of staphylococci. It would appear that at the optimal temperature for staphylococcal growth, staphylococci could multiply rapidly in the mixed population due to the comparative shortness of the generation time of this species and because of the lengthened lag phase of the saprophytic bacterial species at this elevated temperature, especially when only cultures having psychrophilic characteristics were present. This temperature is substantially above that encountered in practical experience. With the passage of time, the staphylococcal population was completely overgrown by the saprohytes present. This effect might be eliminated in the presence of psychrophilic and mesophilic, saprophytic species. The repressive effect of competition by saprophytic, psychrophilic organisms is extremely effective up to room temperature on the staphylococcal population. Even when significant staphylococcal populations were achieved in the artificial media, such tremendous numbers of saprophytes were obtained either earlier or at the same time so that a frozen food containing this population would be organoleptically unacceptable due to the degradative action of enzymes from the saprophytic psychrophile population.

Reflection on the incidence of staphylococcal food poisoning indicated that a majority of cases occur in foods which have been treated to drastically reduce the bacterial population; staphylococci subsequently inoculated are without competition. Staphylococcal food poisoning also occurs in foods which favor selectively the growth of staphylococci while sharply inhibiting growth of other genera, or in foods which have a protective action on staphylococci through the action of substances such as eggs, starches, and lipids.

Kelly and Dack (1936) reported that meats of high salt concentration were selective for the growth of staphylococci. Staphylococci grew in concentrations of up to 10% salt. This concentration of salt prevented the growth of bacilli forms and allowed the staphylococci to overgrow other microorganisms present. Newman (1943) drew attention to the importance of high counts of mixed microbial flora in milk in making it difficult for staphylococci to gain predominance before the milk spoiled. Takahashi and Johns (1959) reported that the number of nonstaphylococci was one of the limiting factors influencing the growth of staphylococci in raw milk. Thatcher and Ross (1960), however, questioned the idea that milk with a high standard plate count was not conducive to multiplication of staphylococci.

Miller (1955), in a study of ground pork, reported that the natural saprophytic species of bacteria present outgrew added inocula of *Staphylococcus aureus* by a tremendous margin at temperatures above 18 C. Sufficient numbers of *S. aureus* to cause food poisoning were never attained. Below 18 C, *S. aureus* failed to grow significantly. In a study of creamed chicken artificially and massively inoculated with *S. aureus* by Straka and Combs (1952), the food poisoning organisms were outgrown by the saprophytic organisms present.

Borgstrom (1955), discussing food poisoning in his review on "Microbiological Problems of Frozen Food Products," noted that "the rich bacterial flora which

[1] Presented in part to the 61st Annual Meeting of the American Society for Microbiology, Chicago, Ill., April 23 to 27, 1961.

is generally found in frozen products offers the best protection against infections." The object of these experiments was to investigate the effect of the relative proportion of staphylococci in a mixed population and of total numbers of bacteria on staphylococcal growth.

Materials and Methods

A mixture of saprophytic bacteria for a growth study inoculum was obtained by breaking up the agar in ten Tryptone-glucose-meat extract (TGE) agar (BBL)[2] plates containing large numbers of organisms isolated from a chicken pie. The plates had been incubated at 5 C for 21 days. The agar was broken up in a Waring Blendor and the volume made up to 1 liter with Trypticase soy (TS) broth (BBL). Thus, the saprophytic inoculum consisted of unidentified types with psychrophilic attributes. The *Staphylococcus* inoculum consisted of a mixture of seven different coagulase-positive, pathogenic strains of *S. aureus* in about equal amounts. These cultures were obtained from Temple University Medical School. The seven cultures were plated on TS agar medium and the agar in ten plates again ground up in the blender and subsequently made up to 1 liter with TS broth. The broth suspensions were plated to determine their population levels and were kept frozen until used. Neither inoculum showed any appreciable loss in numbers on freezing or during storage.

Growth curve experiments were carried out in low form culture flasks. One thousand milliliters of TS broth per flask were used. After addition of the appropriate relative amounts of inoculum, the cultures were incubated at 0, 5, 10, 20, 30, and 37 C in thermostated incubators with the flasks partially immersed in water baths. Samples of approximately 5 ml were removed at appropriate times for plating and determination of pH on a Beckman Zeromatic[3] pH meter. Duplicate "total" counts were carried out by incubating duplicate series of TS agar plates at 37 C for 48 hr and at 5 C for 21 days. Staphylococci were enumerated on Tellurite glycine (TG) agar (BBL) as the medium of choice and the plates counted after incubation at 37 C for 24 to 36 hr. A preliminary experiment was carried out in which staphylococci were also counted on Mannitol salt (MS) agar (Difco)[4] after incubation at 37 C for 24 to 36 hrs and similarly on Trypticase soy agar (BBL).

Results and Discussion

Artificial culture medium and selected cultures of bacteria were used because of the inability to ade-

[2] Baltimore Biological Laboratory, Inc., Baltimore, Md.

[3] Beckman Instruments, Inc., Fullerton, Calif.

[4] Difco Laboratories, Inc., Detroit, Mich.

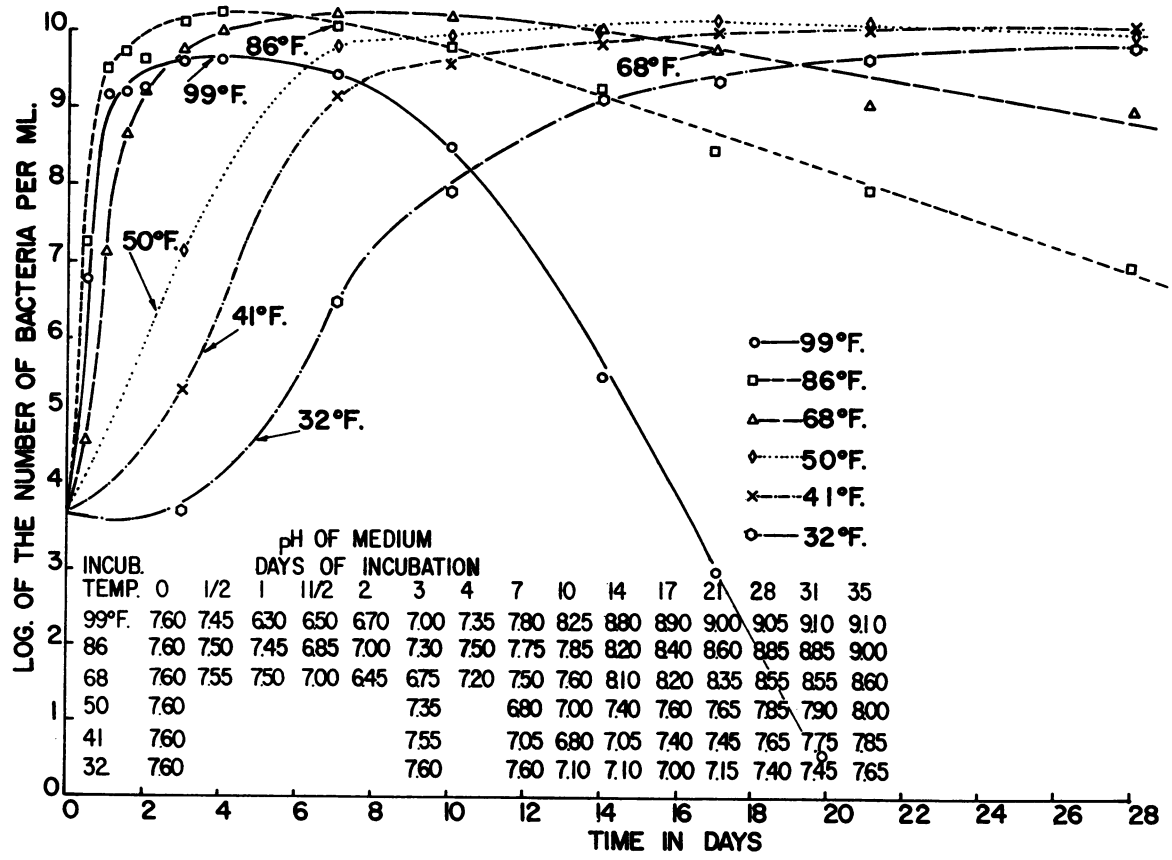


FIG. 1

quately control and vary the relative staphylococcal proportion of the bacterial population and to control and vary the total number of bacteria in a commercially prepared food product. The use of a naturally occurring saprophytic culture melange with psychrophilic tendencies duplicates some of the bacterial conditions occurring in frozen pies. The use of an inoculum from pathogenic sources duplicates the worst and most feared type of inoculation, that from an infected worker during processing.

Figure 1 is a representation of the control growth curves for the saprophytic species in the inoculum at various temperatures. For convenience, only the psychrophile population (incubation of plates at 5 C) has been plotted for the total count. Duplicate sets of plates were incubated at 37 and 5 C. Results were very similar except that somewhat longer lag phases were observed on the plates incubated at 37 C than on those incubated at 5 C. This is in accordance with the fact that the saprophytic population inoculum was primarily selected as a psychrophilic population. The experiments were generally extended to 29 or 31 days, but only the first 15 days have been reported in most of the figures for convenience.

Data on the pH of the medium have been included in all of the figures, but do not show any recognizable trend. Changes in the pH of the medium do not seem to be the cause of the termination of growth. In all cases, the medium became more acid as growth progressed. In the case of the psychrophile growth control, the lowest pH reached was 6.30 at 37 C from an initial pH of 7.60. At 21 days, the pH had risen to 9.00 and growth had ceased (Fig. 1). In the *Staphylococcus* growth control (Fig. 2), the initial pH was 7.45 and declined to a low of 5.85 at 37 C incubation temperature. At 21 days at this temperature, the pH had risen to 6.90 and growth was declining. At 7 days, a mold contaminant was detected in the *Staphylococcus* culture incubated at 30 C. No further pH readings were made on this flask. There was a general tendency in the controls for the psychrophilic saprophytes to give a higher pH value than the *Staphylococcus* inoculum at any given temperature. In the *Staphylococcus* control, a pH of about 5.90 appeared to be the limiting lower pH for growth at and above 20 C. However, termination of growth occurred at 10 C without the pH value ever going below 7.20. There was no common minimal pH observed for growth of the psychrophilic control. Similarly, no common maximal pH values were observed for either control. The pH in the mixed culture experiments never reached the lower limit at which die-off was observed in the control *Staphylococcus* culture. It would appear that pH of the medium is

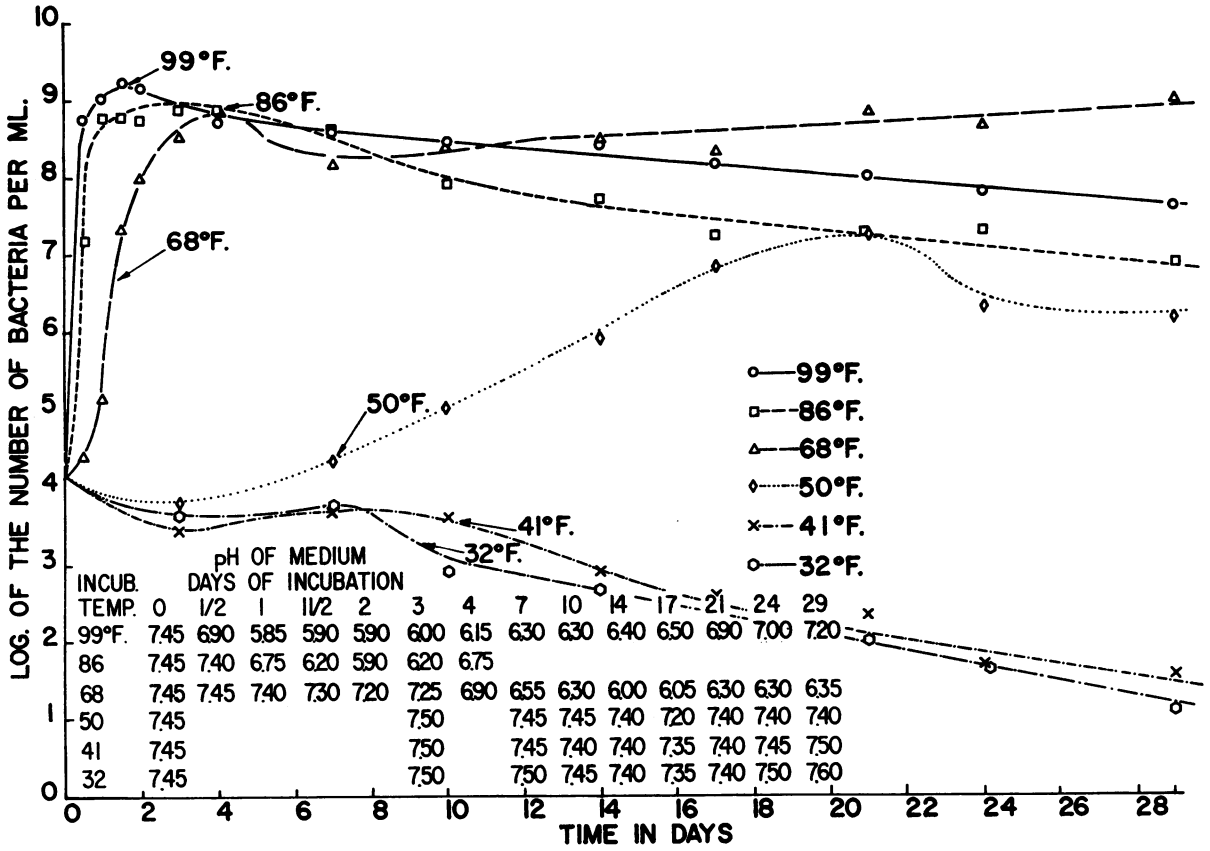


FIG. 2

closely related to the total cell crop and, except, perhaps, at extremes, is not a limiting factor, per se.

Figure 2 gives the growth curves for the staphylococcal inoculum alone, at various temperatures, and is a control for comparing staphylococcal growth in mixed populations. Table 1 gives some comparative counts for the mixed *Staphylococcus* inoculum control when three different media were used. Trypticase soy agar was used to enable the detection of the development of any appreciable nonstaphylococcal population in the *Staphylococcus* inoculum, or staphylococci which did not give typical colonies on the selective media. The effectiveness of MS agar and TG agar was compared. For the most part, the agreement between these two media was felt to be within the experimental error *in this experiment.* It will be recalled, however, that all of the strains of staphylococci employed in this study were known pathogens and coagulase positive. TG agar was selected as the medium for counting staphylococci because it gave plates which were easier to read than MS agar and this medium had been used previously (Peterson, Black, and Gunderson, 1962). Since there was no growth at 5 or 0 C, data on the counts at these incubation temperatures have not been included in this table.

Figures 1 and 2 thus provide a reference standard for comparison of growth for each of the inocula, but most especially for staphylococci in mixed cultures.

Figure 3 is the first of a related series of three experiments in which the proportion of staphylococci to the total number of bacteria was varied at roughly the same total population levels. The saprophytic population grew normally. Below 20 C, the staphylococcal population failed to grow. At 20 and 30 C, the *Staphylococcus* population was sharply repressed. At 37 C, considerable numbers of staphylococci did grow, but began to die off very early in the growth cycle. A ten fold greater number of saprophytes was obtained a

TABLE 1. *Comparison of Mannitol salt agar and Tellurite glycine agar effectiveness for enumerating growth in the Staphylococcus control*

Incubation time	Number of bacteria per ml × 10^6					
	37 C			30 C		
	TS*	MS†	TG‡	TS	MS	TG
0	0.0181	0.0130	0.0154	0.0181	0.0130	0.0154
12 hr	680	680	560	17.5	16.9	14.3
24 hr	1,830	1,860	1,070	1,020	1,000	600
36 hr	2,900	2,900	1,780	2,350	1,730	630
48 hr	2,420	2,390	1,500	1,800	1,790	580
3 days	2,080	1,830	537	1,650	1,480	780
4 days	2,030	2,120	540	1,790	1,660	760
7 days	900	840	406	750	670	466
10 days	790	650	277	555	460	79.0
14 days	990	740	280	146	130	59.0
17 days	841	716	169	100	97.0	16.7
21 days	750	720	115	76.0	61.0	18.7
24 days	640	640	72.0	59.0	52.0	25.0
29 days	350	290	49.0	21.0	19.0	8.90
	20 C			10 C		
0	0.0181	0.0130	0.0154	0.0181	0.0130	0.0154
12 hr	0.0364	0.0270	0.0245			
24 hr	0.194	0.0210	0.135			
36 hr	32.0	22.7	21.7			
48 hr	101	85.0	105			
3 days	350	350	333	0.0154	0.0120	0.0058
4 days	1,090	960	930			
7 days	206	172	158	0.0264	0.0243	0.0230
10 days	358	351	291	0.149	0.143	0.114
14 days	420	420	300	2.90	2.80	0.850
17 days	680	700	216	27.3	24.7	7.10
21 days	1,300	1,700	800	54.0	34.0	26.2
24 days	2,400	2,300	550	2.80	2.50	2.4
29 days	4,800	3,800	1,160	2.18	2.09	1.89

* Trypticase soy agar (BBL) incubated at 37 C for 24 to 36 hr.
† Mannitol salt agar (Difco) incubated at 37 C for 24 to 36 hr.
‡ Tellurite glycine agar (BBL) incubated at 37 C for 24 to 36 hr.

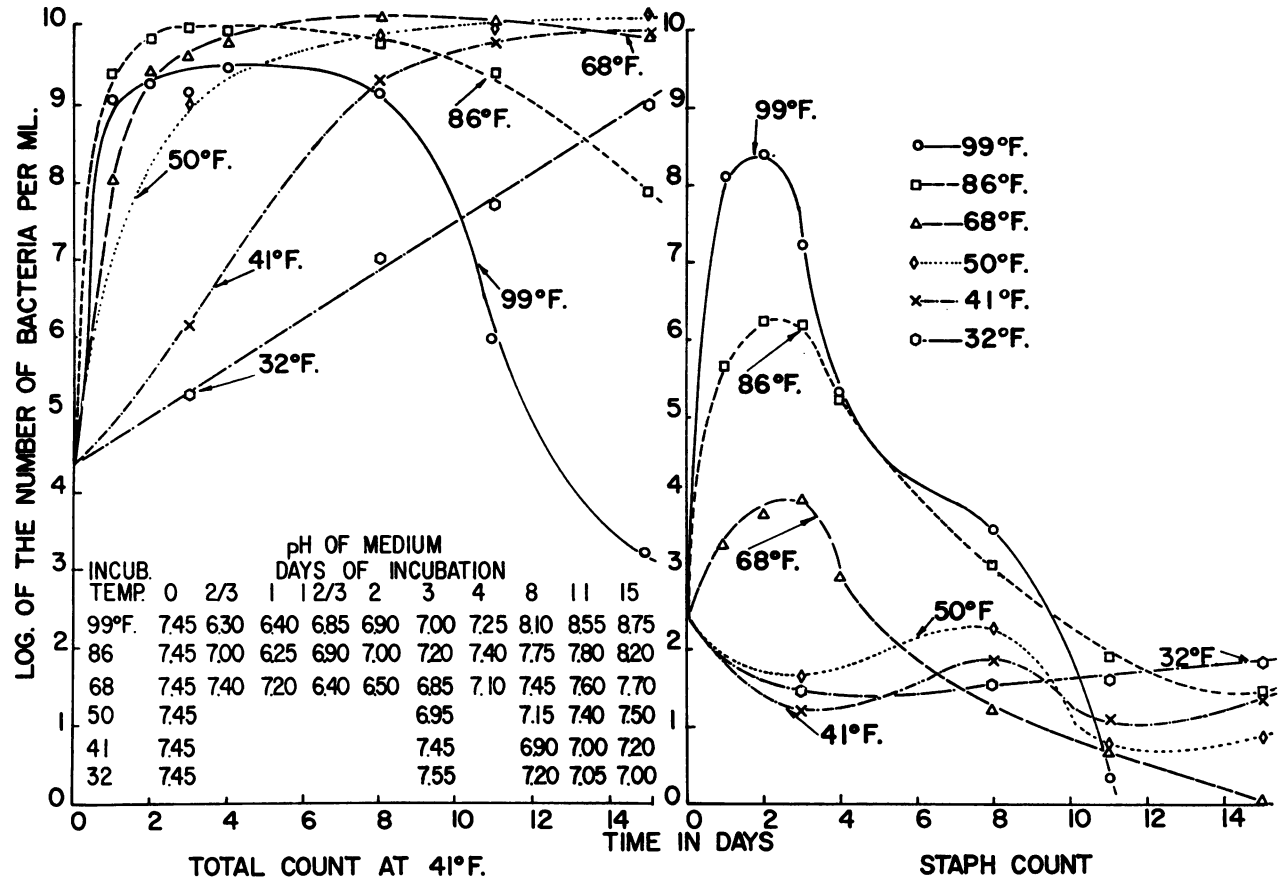

LOG. OF THE NUMBER OF BACTERIA PER ML.
68°F.
86°F.
50°F.
41°F.
99°F.
32°F.
pH OF MEDIUM
INCUB. DAYS OF INCUBATION
TEMP. 0 2/3 1 1 2/3 2 3 4 8 11 15
99°F. 7.45 6.30 6.40 6.85 6.90 7.00 7.25 8.10 8.55 8.75
86 7.45 7.00 6.25 6.90 7.00 7.20 7.40 7.75 7.80 8.20
68 7.45 7.40 7.20 6.40 6.50 6.85 7.10 7.45 7.60 7.70
50 7.45 6.95 7.15 7.40 7.50
41 7.45 7.45 6.90 7.00 7.20
32 7.45 7.55 7.20 7.05 7.00
TOTAL COUNT AT 41°F.
TIME IN DAYS
99°F.
86°F.
68°F.
50°F
32°F.
41°F.
99°F.
86°F.
68°F.
50°F.
41°F.
32°F.
STAPH COUNT

FIG. 3

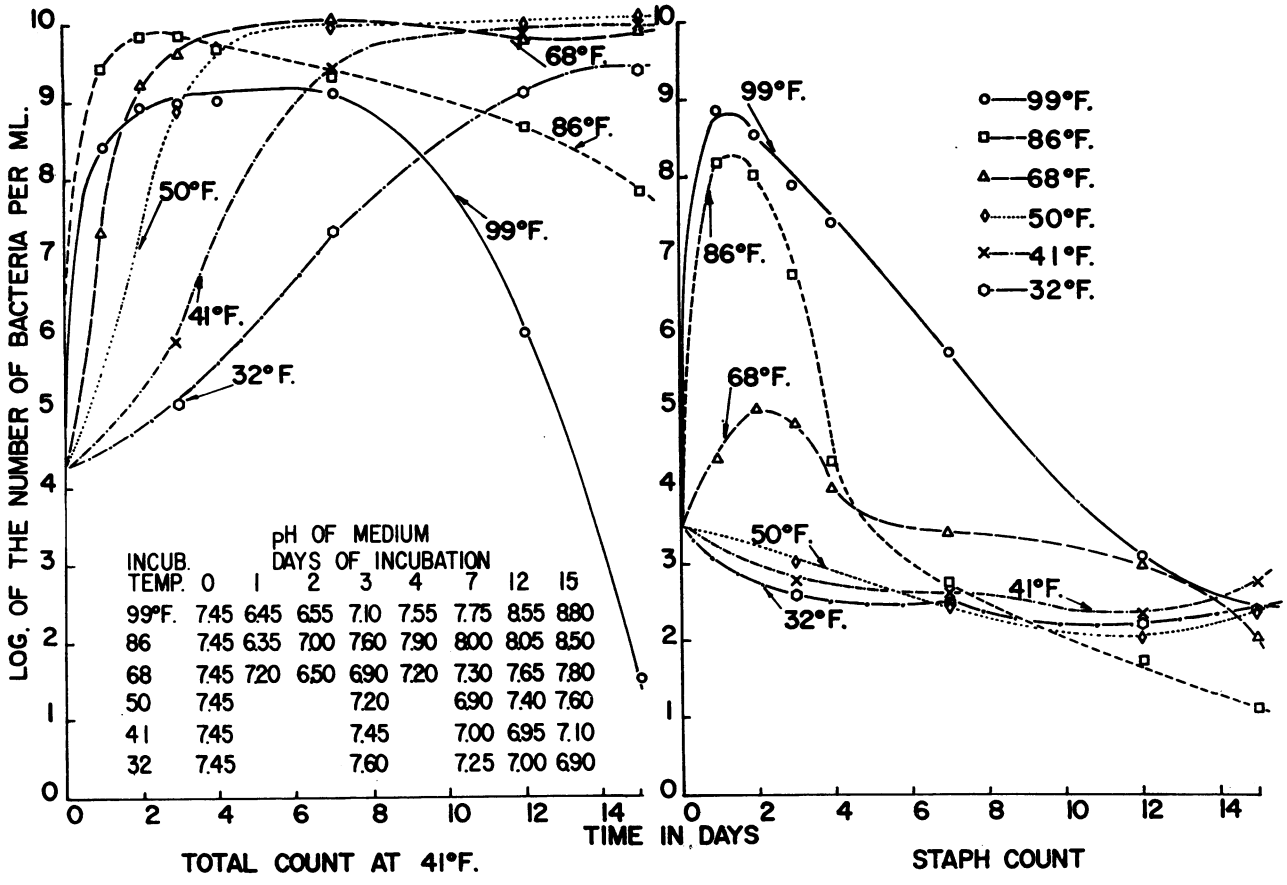
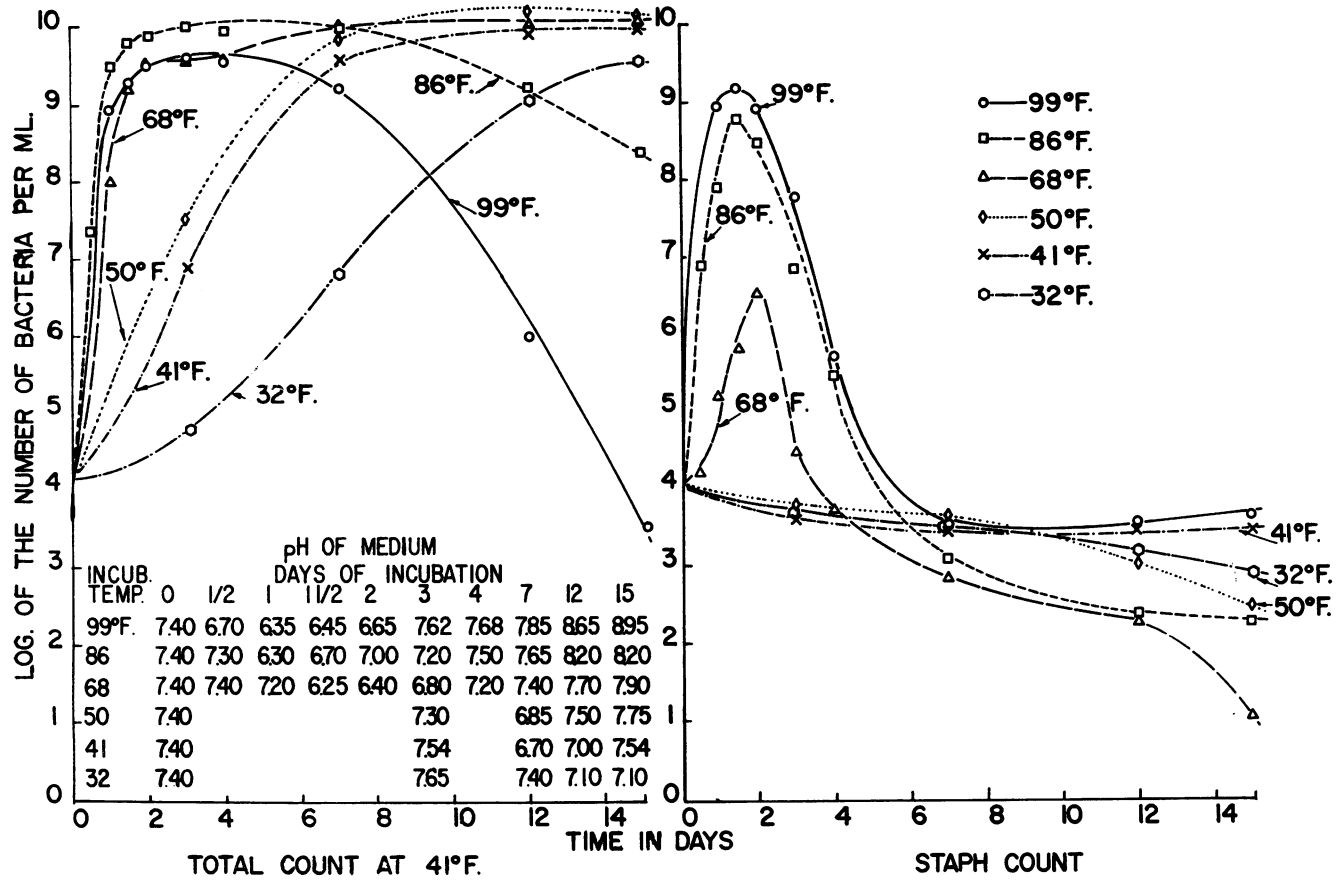

LOG. OF THE NUMBER OF BACTERIA PER ML.
68°F.
86°F.
50°F.
99°F.
41°F.
32°F.
pH OF MEDIUM
INCUB. DAYS OF INCUBATION
TEMP. 0 1 2 3 4 7 12 15
99°F. 7.45 6.45 6.55 7.10 7.55 7.75 8.55 8.80
86 7.45 6.35 7.00 7.60 7.90 8.00 8.05 8.50
68 7.45 7.20 6.50 6.90 7.20 7.30 7.65 7.80
50 7.45 7.20 6.90 7.40 7.60
41 7.45 7.45 7.00 6.95 7.10
32 7.45 7.60 7.25 7.00 6.90
TOTAL COUNT AT 41°F.
TIME IN DAYS
99°F.
86°F.
68°F.
50°F.
41°F.
32°F.
99°F.
86°F.
68°F.
50°F.
41°F.
32°F.
STAPH COUNT

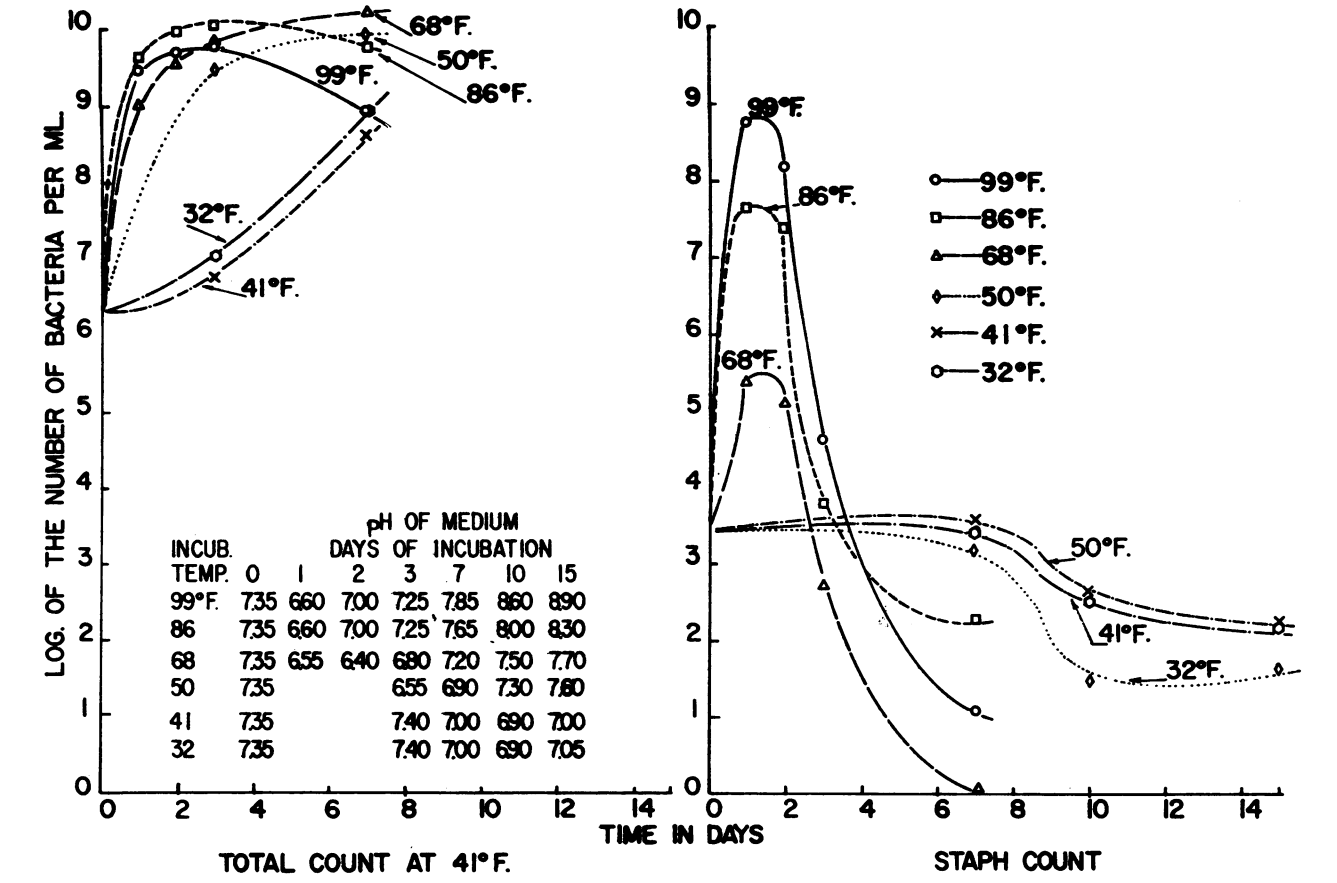
FIG. 4



pH OF MEDIUM

INCUB. TEMP.	DAYS OF INCUBATION 0	1/2	1	1 1/2	2	3	4	7	12	15
99°F.	7.40	6.70	6.35	6.45	6.65	7.62	7.68	7.85	8.65	8.95
86	7.40	7.30	6.30	6.70	7.00	7.20	7.50	7.65	8.20	8.20
68	7.40	7.40	7.20	6.25	6.40	6.80	7.20	7.40	7.70	7.90
50	7.40					7.30		6.85	7.50	7.75
41	7.40					7.54		6.70	7.00	7.54
32	7.40					7.65		7.40	7.10	7.10

FIG. 5



pH OF MEDIUM

INCUB. TEMP.	DAYS OF INCUBATION 0	1	2	3	7	10	15
99°F.	7.35	6.60	7.00	7.25	7.85	8.60	8.90
86	7.35	6.60	7.00	7.25	7.65	8.00	8.30
68	7.35	6.55	6.40	6.80	7.20	7.50	7.70
50	7.35			6.55	6.90	7.30	7.60
41	7.35			7.40	7.00	6.90	7.00
32	7.35			7.40	7.00	6.90	7.05

FIG. 6

the same temperature. Changes in the pH of the medium did not seem to be the cause of termination of growth of the staphylococci.

Figure 4 shows similar data when the total bacterial population was about the same level as before, but staphylococci constituted 14.8% of the initial population. Saprophytic bacteria grew normally as in the control. *Staphylococcus* growth was completely inhibited below 20 C and sharply repressed at this temperature. At 30 and 37 C, large numbers of staphylococci were obtained, but the death phase followed very quickly. It was difficult to determine if pH changes were a cause of termination of staphylococcal growth.

Figure 5 also shows growth curve data at approximately the same total number of bacteria. The *Staphylococcus*, however, were an appreciable (43.9%) part of the total bacterial load. Again, below 20 C staphylococcal growth was repressed. At 20 C, growth of the staphylococci was sharply repressed, but not to the same extent previously observed. At 30 and 37 C, growth of the staphylococci reached higher levels than previously observed and compared with the control curves, there seemed to be little repression. As noted in the previous experiments, very sharp and early termination of staphylococcal growth occurred at these temperatures.

Thus far, the experiments have demonstrated an antagonism against *Staphylococcus* in mixed cultures which was extremely effective in curtailing staphylococcal growth, particularly at 20 C (and below), a temperature where staphylococci alone grew well. As the number of staphylococci became a larger proportion of the initial population, the antagonism of the saprophytic bacterial species was increasingly reduced. Therefore, the staphylococcal population reached higher levels as the relative proportion of staphylococci in the total initial bacterial population increased.

The remaining two figures are related, in that, although a high total number of bacteria of roughly the same magnitude was used in each, the proportion of staphylococci was varied. The total initial bacterial load of approximately 2,000,000 bacteria per ml was substantially higher than in the preceding three experiments. Figure 6 shows some surprising results for staphylococcal growth at various temperatures in a high population level in which staphylococci were a very small proportion. Again, the saprophytic flora grew normally. Below 20 C, there was complete inhibition of staphylococcal growth. At 20 C, there was sharp repression of staphylococcal growth. At 30 C, staphylococcal growth was somewhat repressed, whereas, at 37 C, staphylococcal growth reached nearly the same

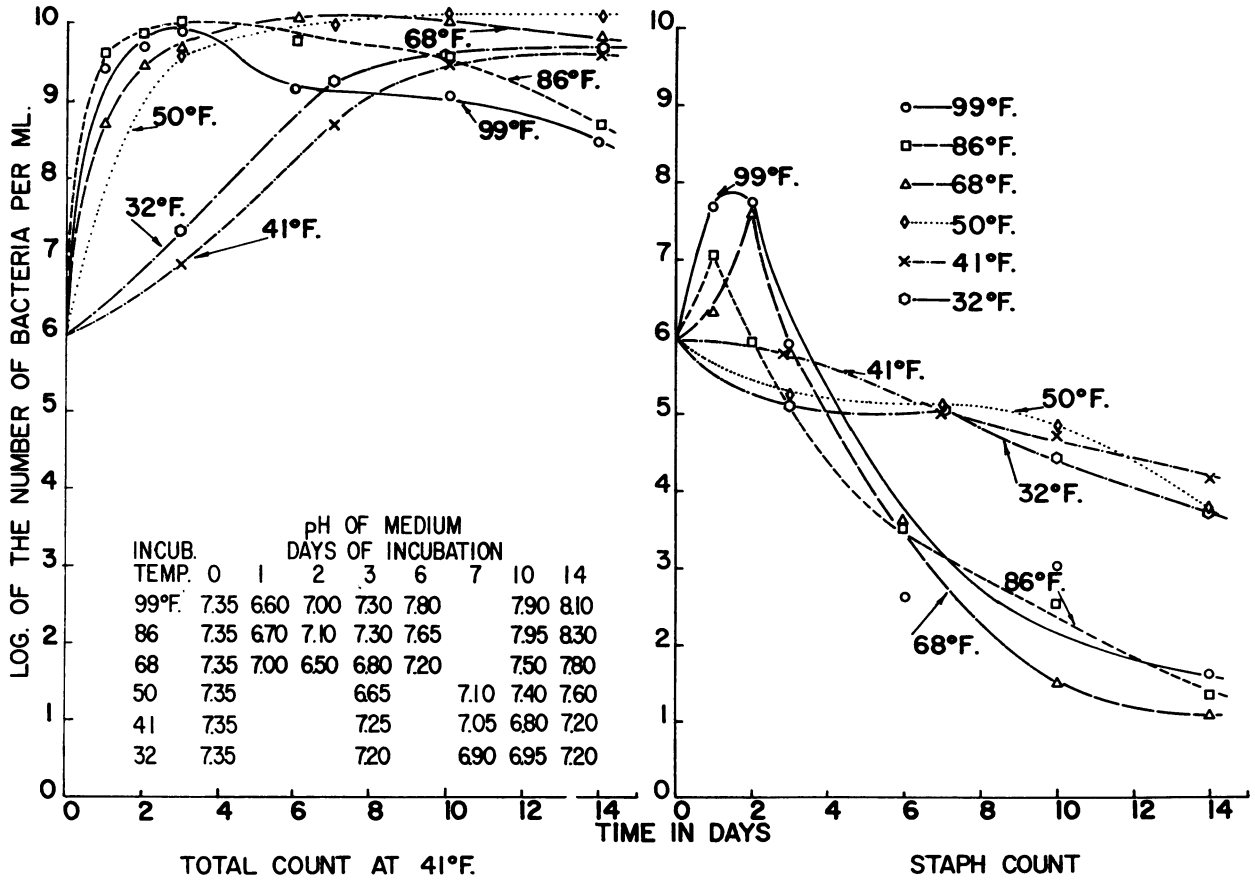


FIG. 7

proportions as the control. As previously observed, death of the *Staphylococcus* cells was accelerated and the counts dropped precipitously.

Figure 7 presents the growth curves for a total initial population of 1,920,000 bacteria per ml, of which 47.5% were staphylococci. Sharp repression of staphylococcal growth was found at all temperatures. As noted many times previously, *Staphylococcus* did not grow below 20 C. Above 20 C, there was a very sharp repression of staphylococcal growth, followed by the rapid destruction of the cells as previously observed. The saprophytic bacteria multiplied normally and tremendous numbers of these organisms were reached before any appreciable staphylococcal growth took place.

It seems clear from these experiments that the possibility of conditions for staphylococcal food poisoning occurring in the presence of a mixed bacterial population is rather limited. At temperatures from freezing to room temperature which favor the growth of cold-tolerant organisms, definite inhibition of staphylococcal growth was observed in the presence of a saprophytic bacterial population with psychrophilic characteristics. The staphylococci had little apparent effect of the growth of the saprophytes and in all cases, the growth of the saprophytic species in mixed culture was similar to the growth of these organisms without staphylococci. Therefore, although the growth of staphylococci was repressed, the concurrent growth of large numbers of saprophytes with their degradative activities would be sufficient to render a food product inedible before sufficient staphylococci grew to be a potential hazard.

Similar results might be expected at temperatures from 20 to 37 C in the presence of a selected mesophilic, saprophytic bacterial population. Definite indication of this may be had in recalling that plates incubated at 37 C for the control growth curve on the saprophytic bacterial inoculum indicated longer lag phases for bacteria growing at these temperatures than when 5 C was used as the incubation temperature. A frozen food could be expected to have a bacterial flora which normally includes both psychrophilic and mesophilic types. Previous results (Peterson et al., 1962) with frozen, precooked convenience foods indicated not only the presence of both psychrophilic and mesophilic bacterial floras, but that, depending on the temperature at which the food was maintained, each became active in preventing staphylococcal growth. This aspect will be investigated in future work. An important academic question which also remains to be studied is: Can a large population of resting, rather than actively growing, staphylococci produce sufficient enterotoxin to cause food poisoning?

LITERATURE CITED

BORGSTROM, G. 1955. Microbiological problems of frozen food products. Advances in Food Research **6**:163–230.

KELLY, F. G., AND G. M. DACK. 1936. Experimental *Staphylococcus* food poisoning. A study of the growth of a food poisoning *Staphylococcus* and the production of an enterotoxic substance in bread and meat. Am. J. Public Health **26**:1077–1082.

MILLER, W. A. 1955. Effect of freezing ground pork and subsequent storing above 32°F upon the bacterial flora. Food Technol. **9**: 332–334.

NEWMAN, R. W. 1943. The problem of staphylococci in a milk supply. Calif. Dept. Agr. Bull. **32**:42–45.

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

STRAKA, R. P., AND F. M. COMBS. 1952. Survival and multiplication of *Micrococcus pyogenes* var. *aureus* in creamed chicken under various holding, storage and defrosting conditions. Food Research **17**:448–455.

TAKAHASHI, T., AND C. K. JOHNS. 1959. *Staphylococcus aureus* in Cheddar cheese. J. Dairy Sci. **42** (No. 6): 1032–1037.

THATCHER, F. S., AND D. ROSS. 1960. Multiplication of staphylococci during cheese making. Can. J. Public Health **51**:226–234.