APPLIED MICROBIOLOGY
Vol. 12, No. 1, p. 70–76 January, 1964

Staphylococci in Competition[1]

III. Influence of pH and Salt on Staphylococcal Growth in Mixed Populations

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Received for publication 21 October 1963

ABSTRACT

PETERSON, A. C. (Campbell Soup Co., Camden, N.J.), J. J. BLACK, AND M. F. GUNDERSON. Staphylococci in competition. III. Influence of pH and salt on staphylococcal growth in mixed populations. Appl. Microbiol. **12**:70–76. 1964.—Previous results showed definite repressive effects on the growth of staphylococci in mixed cultures due to the competitive growth of psychrophilic saprophytes. This study was continued, and the influence of other environmental factors, pH and salt, on the competition between staphylococci and saprophytes was investigated. Initial pH values varied from 5 to 9. At the extremes of the pH range, staphylococci failed to grow, while the saprophytes grew under all of the conditions tested. At pH 5, the growth curves for the saprophytes were markedly altered from those obtained at neutral pH. The lag phases were greatly lengthened at and below 20 C, but normal numbers of saprophytes were reached in the stationary phase. At pH 6 and 8, staphylococcal growth showed the same inhibition observed at pH 7, at and below 20 C; normal multiplication was observed above this temperature, but with accelerated death phases. Thus, pH did not primarily effect staphylococcal growth through its influence on saprophyte growth and competition, but rather directly affected the growth of *Staphylococcus* cultures. Salt concentrations from 3.5 to 9.5% were investigated for influence on staphylococcal growth in mixed populations. Above 3.5% salt, staphylococcal inhibition at and above 20 C was not as marked as in the controls, although normal numbers were never reached. The saprophytes were increasingly inhibited, and their lag phases materially lengthened as salt concentration was increased. Salt acted directly on the *Staphylococcus* population and also, by repressing saprophyte growth, decreased competition, which allowed the staphylococci to grow.

Food poisoning from staphylococcal intoxication has been reported from many diverse food products, but there has been no authenticated case of food poisoning arising from a frozen food. Because nearly 8 billion pounds of frozen food were sold in the last year, it is extremely unlikely that there has not been sufficient opportunity for staphylococcal food poisoning to have been reported had it occurred. Therefore, it is probable that such food poisoning has not occurred due to some inherent quality characteristic of frozen food products.

[1] Presented in part to the 62nd Annual Meeting of the American Society for Microbiology, Kansas City, Mo., 6–10 May 1962.

It has been shown that staphylococci do not grow below 7 C and gradually diminish in number at 0 C. It is not known whether or not enterotoxin production occurs at these lower temperatures nor indeed at temperatures below 4.4 C (Angelotti et al., 1959). Therefore, the rapid multiplication of staphylococci necessary to produce the enterotoxin of food poisoning cannot occur in the frozen state.

Peterson, Black, and Gunderson (1962*a*) previously showed that, even under very drastic conditions of rapid defrost and incubation at elevated temperatures, frozen chicken pot pies were completely unacceptable organoleptically prior to the development of large numbers of staphylococci due to the growth of saprophytic organisms. Experiments (Peterson, Black and Gunderson, 1962*b*) were performed with artificial media to determine the effects of incubation temperatures and of composition and density of the saprophytic and staphylococcal inoculum on the resulting mixed population. It was found that, even with elevated temperatures and a heavy inoculum of staphylococci, the saprophytic bacteria present always grew better than the staphylococci, so as to render foods containing such mixed populations organoleptically unacceptable before the development of massive numbers of staphylococci. This was attributed to the retarding effect of growth competition of the saprophytic species on the staphylococci.

Post, Bliss, and O'Keefe (1961) reported an investigation of a commercially produced whipped-cream product sold as a "cream puff" from unrefrigerated home delivery trucks. The investigation was occasioned by the observation that, although the product seemed potentially hazardous, over a million of these cream puffs had been sold without even a claim for suspected food poisoning, let alone a definite report of food poisoning. The authors concluded that, in addition to elevated temperature, reduced competition from other naturally occurring organisms was necessary for enterotoxigenic *Staphylococcus aureus* growth. The authors further concluded that the product would probably be unacceptable to the consumer under conditions permitting good growth of *S. aureus*.

Prominent among the variables affecting growth in mixtures of bacteria is the effect of pH. In the previously

reported studies, the authors were not able to unravel the significance of pH changes occurring during growth of the mixed cultures, and concluded that important clues to the interactions might lie here. Hewitt (1957), in a review of the effect of hydrogen ion concentrations on bacteria, pointed out that unfavorable pH conditions produce bacteriostatic effects followed by bactericidal effects as the normal metabolism and multiplication cease. The pH of the media was shown to affect toxin production in *Corynebacterium diphtheriae* even when growth was not affected. Surprisingly, Elek (1959) had little to say about the corresponding situation, that of the effect of pH on the growth and toxin production of staphylococci. Gale and Epps (1942) found that *Escherichia coli* was able to multiply in the pH range of 4.5 to 9. They concluded that the enzyme content and constitution of the cells varied with the pH.

Porter (1947), in reviewing the effect of pH on microbial growth and enzyme activity, listed a variety of microorganisms and their minimal and maximal pH for growth. Several of these were as follows: *S. albus*, 5.6 to 8.1; *Streptococcus pyogenes*, 6.35 to 9.2; *Alcaligenes faecalis*, 6.4 to 9.7; *E. coli*, 4.4 to 9.0; *Aerobacter aerogenes*, 4.4 to 9.0; *Lactobacillus bifidus*, 3.8 to 7.2; and *Pseudomonas aeruginosa*, 5.6 to 8.0. This listing suggests that a pH range from 4.5 to 9 is, in general, limiting for bacterial growth, and that staphylococci may have an even narrower pH range for growth.

Vaughn and Stadtman (1946) found that *A. aerogenes* and *Aerobacillus macerans* were able to grow down to pH 3.9 and 3.8, respectively. These workers suggested that creation of acid tolerance in these cultures was not a function of the environment. Wodzinski and Frazier (1960) reported that *Pseudomonas fluorescens* had a minimal pH of 5.4 and a maximal pH of 8.85 for growth. These authors also found that the lag phase was increased at adverse pH values and that, as the pH and the temperature of incubation decreased, a higher available water was required for growth. This indicates the interrelation of still another variable. Carlucci and Pramer (1960) reported that pH values above pH 7.0 sharply decreased the salt tolerance of *E. coli*. Cathcart, Godkin, and Barnett (1947), in a study of staphylococcal growth in pastry fillings, found, as others had, that the type of organic acid employed was as important as pH itself in inhibiting staphylococcal growth. In cheesecake fillings, lactic acid gave complete inhibition below pH 4.67, while pH values below 5.12 gave gradual inhibition. Obviously, the effect of pH on microbial growth is a multifacet problem, and its influence on growth of bacteria in mixed cultures is a fertile field for investigation. It is known that anaerobiosis affects the minimal pH for initiation of growth by *S. aureus* (Lechowich, Evans, and Niven, 1956).

In these experiments, only the effect of initial pH on staphylococcal growth was considered. This was intended to approximate conditions in a food of the same initial pH. Other interactive phenomena, including effect of type of acid, oxidation-reduction potentials, and aeration, which also bear on the same problem, have not yet been investigated.

The effect of salt on microorganisms has received extensive study and was reviewed by Ingram (1957). Ingram listed pseudomonads and coliforms as among the weakly salt-tolerant bacteria. Staphylococci were described as moderately salt-tolerant. *Bacillus* species were listed as facultative organisms with a low tolerance, and micrococci were described as facultative organisms with a high tolerance. The nature of the cation and anion have important influences, but for the purposes of this paper and its relation to food, salt will be considered to be sodium chloride. Winslow, Walker, and Sutermeister (1932) reported that concentrations of salt up to 0.1 M (0.58%) stimulated the activities of most bacteria; beyond this concentration, activity was depressed. Staphylococci have their optimal salt concentration at about 0.6 M (3.5%), and will grow at 3 M (17.5%; Nunheimer and Fabian, 1940). Dumesh (1935) reported a temperature relationship for *E. coli* in which this species grew at 5 to 8 C in the presence of 25% salt, but not in 10% salt at 37 C or at higher salt concentrations at low temperatures. Tanner and Evans (1933) showed that tolerance to high salt concentrations is greater in rich organic media and foods, perhaps due to salt removal by the proteins.

Dienes and Sharp (1956) reported that staphylococcal colonies became small and irregular and autolyzed after 24 hr on media of high salt concentration. Hotchkiss (1923) studied extensively the effect of salt and various cations on bacterial growth. Elliott and Brant (1957) reported that *Pseudomonas* tolerated salt well up to 2.9%, and slow growth was supported up to 11.6%. Unfortunately, none of these authors investigated the effect of salt on the growth of bacterial species in competition. Again, a host of interactive variables, including osmotic pressure, pH, and water requirements, are active in determining microbial growth.

Previously reported results showed definite repressive effects on the growth of staphylococci in mixed cultures due to the competitive growth of psychrophilic saprophytes. This study was continued, and the influence of other environmental factors, pH and salt, on the competition between staphylococci and saprophytes was investigated.

MATERIALS AND METHODS

The materials and methods were fully described previously (Peterson et al., 1962*b*). Briefly, a saprophytic bacterial inoculum was obtained by blending in an Oster Blendor, with an appropriate quantity of broth medium, Trypticase Soy (TS) Agar (BBL) and attendant bacterial colonies from ten plates in which material from a defrosted chicken pie had been plated. The pie had been defrosted at 5 C for 7 davs, and the plates had been incubated at 5 C

for 21 days. Checking this inoculum on Mannitol Salt (MS) Agar (Difco) and Tellurite Glycine (TG) Agar (BBL) showed few, if any, typical coagulase-positive staphylococci. This inoculum had definite psychrophilic attributes.

The *Staphylococcus* inoculum was composed of known pathogenic and coagulase-positive cultures obtained from Temple University Medical School. In view of our previous experiences (Peterson et al., 1962*b*) with loss of some of the characteristics of pathogenicity including coagulase-positiveness and failure to give typical colonies on differential media, the stability of these features in the *Staphylococcus* inoculum was frequently and regularly determined. Cultures which did not maintain all of the indices of pathogenicity were rejected, and a highly stable *Staphylococcus* inoculum composed of five different strains was obtained. A very good correlation between total count incubated at 37 C (24 to 36 hr) on TS Agar and staphylococcal counts was obtained. The inocula were stored in the frozen state until used, and were frequently titered to determine survival and, particularly, maintenance of cultural characteristics on the part of the *Staphylococcus* inoculum. Counts on MS Agar and TG Agar at 37 C (24 to 36 hr) were in very close agreement.

The growth studies were carried out in TS broth in low-form culture flasks (No. 4422; Corning Glass Works, Corning, N.Y.) which were immersed in constant-temperature water baths. Total counts were made by plating in duplicate and in duplicate series on TS Agar. One series of duplicate plates was incubated at 37 C for 24 to 36 hr, and the other at 5 C for 21 days. Staphylococci were enumerated on TG Agar after incubation at 37 C for 24 to 36 hr. The pH values of the media were adjusted to the desired value prior to autoclaving with hydrochloric acid or sodium hydroxide solution. After sterilization, pH values were again determined with a Beckman Zeromatic pH meter (Beckman Instruments, Inc., Fullerton, Calif.). Similarly, whenever samples were taken for enumeration of bacteria present in the culture at any time interval, a pH determination was made on the sample. TS Agar normally contains 0.5% salt; thus, Fig. 2 at pH 7.4 represents the normal situation, and can be used as a control for the effect of additional salt. To this medium were added 3.0, 5.0, or 9.0% (w/v) sodium chloride to give the test medium. The maximal salt concentration used was selected because it is frequently encountered in media for the isolation of staphylococci.

RESULTS

A range of pH values from 5 to 9 in increments of approximately 1.0 pH unit was investigated. The initial pH of the broth growth medium was adjusted to the desired level, and then was allowed to respond to microbial growth, because no buffers were added to the menstruum. Reference or control growth curves for each of the inocula alone are shown in Fig. 1. The saprophytic psychrophiles had a lag phase of about 3 days at 0 C and less than 1 day at 5 C; above this temperature, maximal populations were reached very quickly. The *Staphylococcus* inoculum, when growing alone, failed to grow below 10 C, and at that temperature had a lag phase of 7 days. At 20 C, the lag phase was about 24 hr long; above this temperature, maximal populations were reached in about 16 hr.

In mixed cultures composed of the saprophytic bacterial species and the staphylococcal inoculum (Fig. 2) and at pH 7.4, definite and marked inhibition of staphylococcal growth was observed at and below 20 C. Above this temperature, inhibition of staphylococcal growth was less affected, but the staphylococci declined much more rapidly than in the controls. These inhibitions of staphylococcal growth were also observed to be directly related to the portion of the total population level which was staphy-

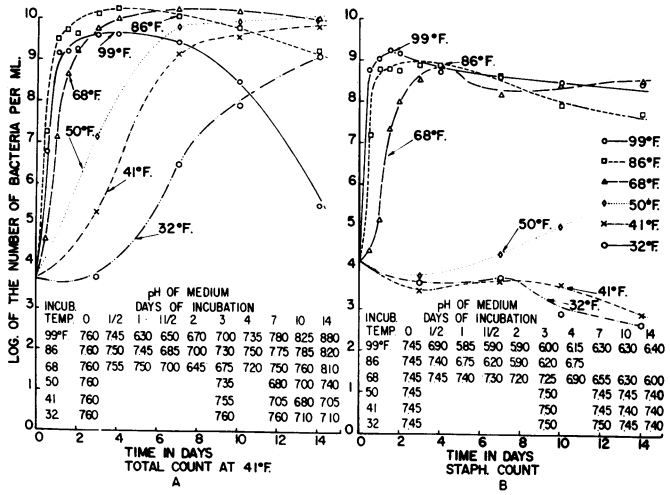

FIG. 1. (*A*) *Growth of saprophytic bacterial species inoculum (control). Initial population: 5,800 bacteria per ml.* (*B*) *Growth of Staphylococcus inoculum (control). Initial population: 15,400 staphylococci per ml.*

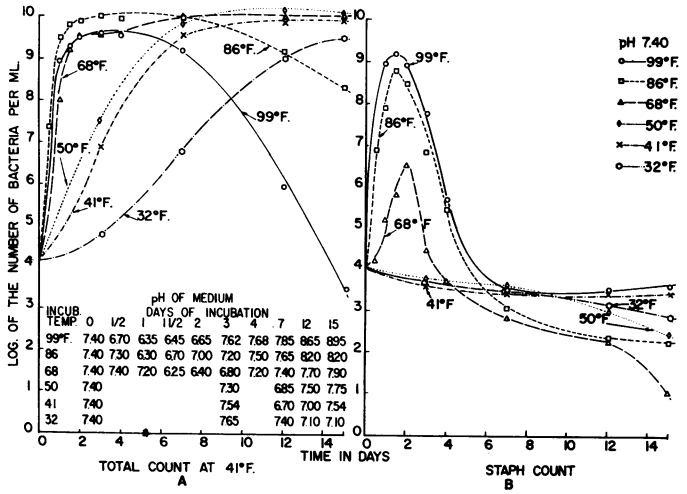

FIG. 2. *Growth of staphylococci in competition at various temperatures. Initial population: 24,800 bacteria per ml (43.9% staphylococci). Initial pH was 7.40.*

lococcal and to the total number of microorganisms seeded. Figure 2 serves both as the example for growth of these bacteria in mixed culture at pH 7.4 and as a reference, for comparing the effects of other pH values and the effect of added salt. In the series of experiments previously reported, the pH of the growth medium decreased during early stages of growth to about pH 6.0 and then increased to a maximum of pH 9.0 after prolonged growth. While there were appreciable pH changes during growth of the cultures, no significant patterns were observed.

Figure 3 shows growth of the saprophytic bacterial inoculum and of the *Staphylococcus* inoculum at pH 5.2. The initial inoculum consisted of 24,800 organisms per ml, of which 51% were staphylococci. The growth curves for the saprophytes were markedly altered from those of the control at neutral pH. At and below 20 C, the lag phases were very greatly extended. This may have been a sufficient lag to allow the selection of a bacterial population able to grow at this pH. At 30 C and above, the total numbers of bacteria reached were fewer than in the controls; staphylococci failed to grow, and the decline in numbers was quite rapid. This was not too surprising, since it was noted previously that Porter (1947) described pH 5.6 as the lower limit for staphylococcal growth. The pH of the medium during growth was between 4 and 5 and showed little variation. The lowest pH reached was 4.05.

Figure 4 shows the growth curves for saprophytes and staphylococci at pH 8.7. This pH also was outside the pH growth range cited by Porter (1947) for staphylococci. Growth of the saprophytic psychrophiles was nearly normal, with the exception of an accelerated death phase at 37 and 30 C, although the pH of the media remained in the alkaline range. Complete inhibition of staphylococcal growth was observed below 20 C. Very effective inhibition occurred above this temperature. At 37, 30, and 20 C, the pH declined to values of 7.10 to 7.40 in 2 to 3 days of incubation. At these pH values, staphylococcal growth was quite possible, but staphylococci failed to grow significantly. Obviously, this was due to the presence of the large saprophyte population. Thus, the extremes of pH at 5 and 9 directly affected staphylococcus growth to the extent that these microbes were nearly completely inhibited. Growth of saprophytic species in the mixed cultures was affected to a smaller extent, since they grew at all the incubation temperatures tested.

Figure 5a shows that at pH 6.3 the growth curves of the saprophytes were modified from those seen at pH 7. Longer times were required to reach the maximal stationary phase at 20 C and above. At 37 C, normal numbers of organisms were never obtained. At 0 C, somewhat more rapid growth was obtained, so that the growth curve coincided with the growth curve which was normal at 5 C. At pH 6.3, staphy-

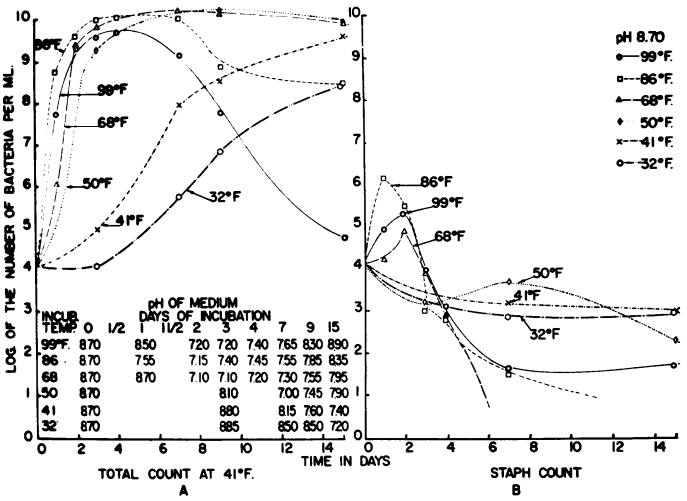

FIG. 4. *Growth of staphylococci in competition at various temperatures. Initial population: 24,200 bacteria per ml (50% staphylococci). Initial pH was 8.70.*

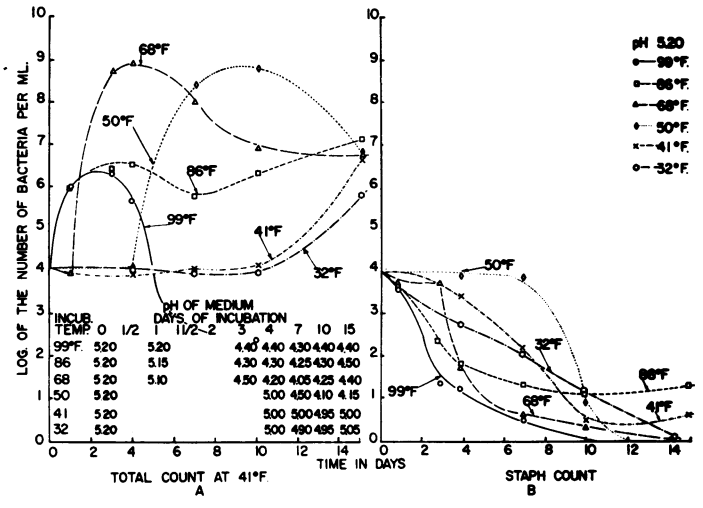

FIG. 3. *Growth of staphylococci in competition at various temperatures. Initial population: 24,800 bacteria per ml (51% staphylococci). Initial pH was 5.20.*

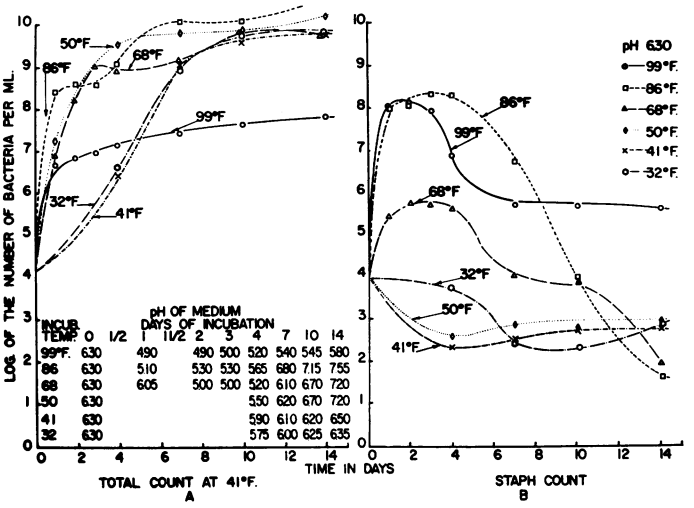

FIG. 5. *Growth of staphylococci in competition at various temperatures. Initial population: 22,200 bacteria per ml (46% staphylococci). Initial pH was 6.30.*

lococcal growth was inhibited to about the same extent seen in neutral pH, at and below 20 C. Above this temperature, staphylococci multiplied normally and had accelerated death phases, but not to the extent seen at pH 7. At pH 6.3, staphylococcal growth was affected by the concomitant growth of the saprophytic bacterial species. The pH reached a low of 4.90, while the highest pH was 7.55.

At pH 7.95, as shown in Fig. 6a, the growth curves of the saprophytic bacteria were nearly normal. The pH reached a low of 6.60 and then rose to a high of 8.35. Inhibition of staphylococcal growth was least marked (Fig. 6b). As usual, there was complete inhibition of staphylococcal growth below 20 C, and there was substantial inhibition at this temperature. There was very rapid onset of the death phases as previously observed.

Only in the intermediate pH range of 6 to 8 were staphylococci able to multiply appreciably. The growth of staphylococci in competition was also investigated at pH 6.0 in the presence of 23,600 bacteria per ml of which 2.3% were staphylococci, and at pH 6.10 in the presence of 2,040,000 bacteria per ml of which 1.3% were staphylococci. At the higher initial total inoculum, considerably more effective inhibition of staphylococcal growth was obtained. It may be that at the lower level of inoculum some particular saprophyte species was not present in large enough numbers to be as effective a growth competitor. Similar results were obtained at pH 8.0 with 23,300 bacteria per ml and 2.5% staphylococci, and at pH 7.85 and 1,900,000 bacteria per ml of which 2.3% were staphylococci.

Therefore, staphylococci did not multiply even when pH conditions interfered with the growth of the saprophytic species. On the contrary, pH appeared to affect the staphylococci more than the saprophytic species. Only in the intermediate pH range from 6 to 8, which is the normal pH growth range, were staphylococci able to grow, and even then they were exposed to the inhibiting influence of the competing saprophytic species.

Salt concentrations from 3.5 to 9.5% (w/v) were investigated for their influence on staphylococcal growth. Figure 7 shows the growth curves for staphylococci and for the saprophytic psychrophiles in 3.5% salt. Growth of the saprophytic bacterial species was nearly normal at this salt concentration. Growth at 10 C was accelerated somewhat, while at lower temperatures the growth curves resembled those of the controls. Complete inhibition of staphylococcal growth took place below 20 C. There was some inhibition at and above this temperature, and normal cell crops of staphylococci were never reached. The duration of the death phase was longer than at 0.5% salt. The pH of the medium at all temperatures declined to a low (of 5.60) and then began a gradual ascent. At the four highest temperatures, pH became alkaline, which is typical of saprophytic growth, and followed the pattern of the control. This concentration of salt allowed staphylococci to grow in the presence of saprophytes at 20 C and above. It

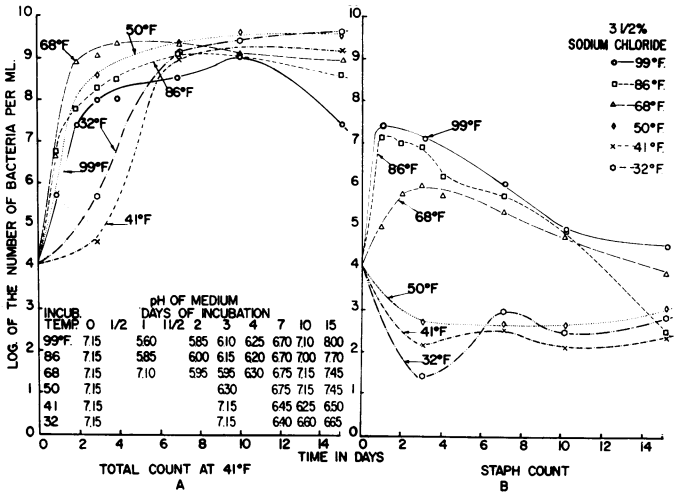

FIG. 7. *Growth of staphylococci in competition at various temperatures. Initial population: 24,400 bacteria per ml (49% staphylococci in 3.5% sodium chloride).*

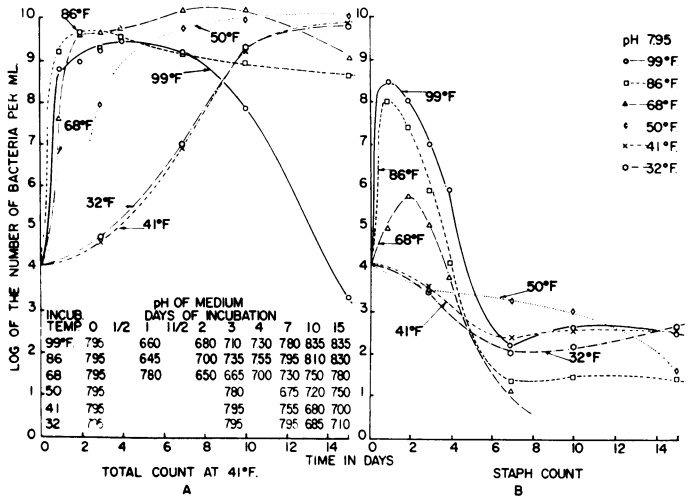

FIG. 6. *Growth of staphylococci in competition at various temperatures. Initial population: 27,400 bacteria per ml (55% staphylococci). Initial pH was 7.95.*

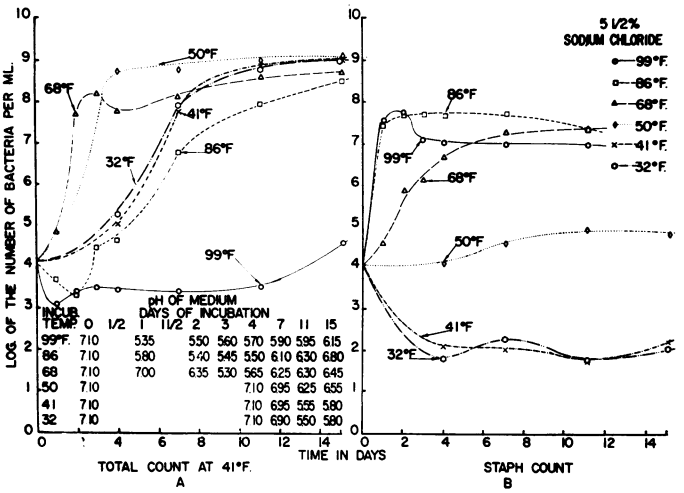

FIG. 8. *Growth of staphylococci in competition at various temperatures. Initial population: 25,400 bacteria per ml (51% staphylococci in 5.5% sodium chloride).*

did not allow staphylococci to dominate the bacterial population because the saprophytic organisms also grew well under these conditions.

At 5.5% salt (Fig. 8a), saprophyte growth was appreciably affected. Growth at 37 C was very strongly inhibited for the first 10 days of incubation. At 30 and 20 C, the lag phases were extended, but for shorter periods of time. At 10 C, growth of the saprophytes was somewhat accelerated, while below this temperature growth of these organisms resembled that of the controls. Staphylococci were inhibited at and below 10 C and, perhaps, somewhat at higher temperatures where the maximal populations were about 1 logarithm less than in the controls. The maximal stationary phase was greatly extended. The pH remained in the acid range, reached a low of 5.30, and then began to rise. The presence of 5.5% salt in the medium was sufficient to retard the growth of the saprophyte population for varying periods of time, depending on the incubation temperature. At low temperatures, it had little effect. This retardation of saprophyte growth allowed the staphylococci to dominate the bacterial population at and above 30 C and to compete effectively at 20 C.

Salt at 9.5% was very strongly inhibitory to psychrophile growth at all temperatures (Fig. 9a). An increase of 3 logs was observed only after 15 days at 10 C. Staphylococci failed to grow at 10 C and below, while the lag phase was greatly extended at 20 C. A similar increase in lag-phase duration was observed at 30 and 37 C. There was some depression of the total cell crop, and the death phases were not as sharply induced as in the controls. Throughout the experiment, pH values of the medium remained in the acid range. At higher temperatures, this was indicative of a bacterial population predominately composed of staphylococci. At lower temperature levels, this is characteristic of early psychrophile growth.

Salt at 9.5% was sufficient to retard the growth of the saprophyte population at 20 C and above, so that staphylococci dominated the bacterial population. Below 20 C, the saprophytic psychrophiles increased in number after extended incubation.

Thus, salt was seen to act directly on the staphylococcal population to reduce total cell crops and to extend the life of the cells. Also, by repressing saprophyte growth at and above 5.5% concentration, salt decreased competition and allowed the staphylococci to grow.

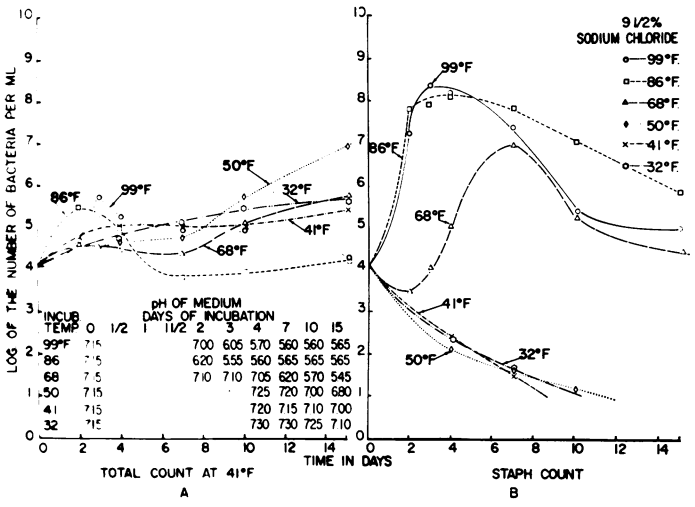

FIG. 9. *Growth of staphylococci in competition at various temperatures. Initial population: 22,900 bacteria per ml (51% staphylococci in 9.5% sodium chloride).*

DISCUSSION

Growth of saprophytic bacteria and staphylococci was directly and indirectly affected by the initial pH of the growth medium. Subsequent variations in pH of the medium were due to the amount of growth and the type of organisms growing. The variety of organisms present in the saprophytic inoculum allowed the saprophytic population to grow over a wider pH range than the staphylococci. Appreciable staphylococcal growth in mixed cultures was only possible in the pH range of 6 to 8 and then only above 20 C. The pH values of the medium were an indication of the composition of the bacterial population. Readings in the alkaline range indicate a large saprophyte population. The phenomenon of alkaline reversion was a common occurrence in the presence of large saprophyte populations. The saprophytic bacterial population was most inhibited at pH 6.0 and 37 C. This was reflected in the lower pH values of the medium during growth. At the pH values and temperatures tested, the relative composition of the inoculum was less important than the total number of bacteria seeded in affecting staphylococcal growth. Low pH values may have been responsible for the increased death rate of the staphylococci after the maximal stationary phase was reached in all of the mixed culture studies. At 0 and 5 C, 9.5% salt prevented the growth of psychrophilic organisms. Lower concentrations of salt were unable to do this. Staphylococci failed to grow at these temperatures, however. At 10 C, the psychrophilic saprophytes flourished, no matter what salt concentration was used, while staphylococci again failed to grow. At 20, 30, and 37 C, salt served to enhance the ability of staphylococci to survive and multiply and, conversely, retarded saprophyte growth. Thus, staphylococci were able to dominate the population at 30 and 37 C in 5.5% salt and at 20, 30, and 37 C in 9.5% salt.

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