EFFECTS OF INGREDIENTS USED IN CONDENSED AND FROZEN DAIRY PRODUCTS ON THERMAL RESISTANCE OF A POTENTIALLY PATHOGENIC STAPHYLOCOCCI

by

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INTRODUCTION

In recent years, several workers have reported staphylococcal food poisoning from dairy products (2, 4, 5, 13, 25, 26, 27). There is no reliable record of staphylococcal food poisoning cases in the U.S.A. (13, 14) or any other country, for any given period of time. The poisoning is not usually reported or publicized unless the outbreak is large. It is known however that many cases that are reported as "food poisoning" or food infection are usually cases of staphylococcal food poisoning.

The conditions necessary for an outbreak of staphylococcal food poisoning are contamination of food by pathogenic strains, suitability of food for their multiplication and storage of food at a temperature favorable to growth of cocci (1). The contaminated food must be held at a suitable temperature for sufficient length of time to produce enterotoxin. A common characteristic of growth of staphylococci in food is that the contaminated food is not affected in appearance, taste or smell. Hence, it is difficult for the consumer to detect any sign of contamination.

Staphylococci are noted for their ability to adapt to environmental changes (35, 36, 44, 45, 46) and are commonly found in nature (25, 36, 51). Recent surveys (23, 36, 54) have shown that more than 50% of normal human beings and cattle harbor staphylococci on some parts of the body. Latest investigations (35, 36, 46, 47, 54) show a trend to develop antibiotic resistant strains in both cattle and human beings. It is believed that streptococcal mastitis is being gradually replaced by staphylococcal mastitis and that most of the cases of mastitis are now caused by Staphylococcus aureus (36). Thus the cow's milk apart from exogenous contamination, is also a potential source of endogenous staphylococci.

The results of research work by various investigators (10, 21, 28, 59), show that dairy products are susceptible to contamination by enterotoxin producing staphylococci, which may not be destroyed by freezing or heat treatment. Milk products are most suitable media for the growth of staphylococci, if they are exposed to suitable temperatures for sufficient length of time. Under favorable conditions, toxin may be formed. Several workers (3, 19, 42, 57) have reported a protective action of sugar, serum solids and fat upon the survival of micro-organisms in general and staphylococci in particular, during heat treatment. However, they did not determine the maximum concentration of the ingredients at which the number of cells began to decrease. Neither did they determine the lowest concentration of these ingredients, at which the protective action started.

The present studies were designed to measure the protective effect of various ingredients of dairy products upon the survival of a potentially pathogenic strain of staphylococcus. It was postulated that increasing concentrations of various ingredients have increasing protective action on the thermal destruction of staphylococci. In this investigation, therefore, the concentration of ingredients studied were in the range used in the industry.

The results of this study should indicate the amount of protective action, afforded by varying concentration of each ingredient.

REVIEW OF LITERATURE

Dairy Products as a Cause of Staphylococcal Food Poisoning

Staphylococcal food intoxication is recognized as the most common type of food poisoning. Even though food poisoning in general is poorly reported

(13, 14) many dairy products like fluid milk, dry milk and cheese have often been incriminated in staphylococcal food poisoning (2, 4, 5, 13, 25, 26, 27.)

The earliest report incriminating dairy products (cheese) in food poisoning was made by Vaughan (56) in 1884. He noted that cases of severe illness followed the eating of some cheese and these cases were of frequent occurrence in North Germany and United States. He studied poisonous or "sick cheese", following a report of 30 cases of cheese poisoning. He concluded that the causative agent was a chemical poison and not a bacterial agent as staphylococcal food poisoning was not recognized at that time.

In 1914 Barber (5) isolated a white micrococcus (Staphylococcus albus) from a cow's milk, the consumption of which caused acute gastroenteritis. A significant fact was that the freshly obtained milk from the cow proved harmless when consumed fresh but it developed the toxic properties after incubation for some hr at room temperature. Barber (5) inoculated sterile milk with a culture of micrococci isolated from suspected milk. He incubated it for about 8 hr at 36°C and then drank some of the milk. Typical symptoms of the disease developed within two hr. He concluded that a poisonous substance produced by the micrococci, and not the micrococci themselves, was the cause of gastroenteritis.

There is no reliable information explaining why certain strains of staphylococci produce enterotoxin and what conditions induce them to develop this property. However many workers (6, 18, 30, 46) are of the opinion that most of the outbreaks of this type of food poisoning are caused by coagulase-positive, pigmented and haemolytic staphylococci. Coagulase-positive characteristics are generally accepted as indicative of pathogencity.

Sources of Enterotoxigenic Staphylococci

Research findings have shown a trend towards development of resistant pathogenic staphylococci in dairy cattle undergoing mastitis therapy (44, 45, 46). McCoy (36) suggested the danger of staphylococci developing resistance to some antibiotics and thereby limiting their use in treating human and cattle infections. She found that a decrease in mastitis caused by Streptococcus agalactiae has been accompanied by an increase in staphylococcal mastitis. Most of the cases of mastitis in 1959 were caused by staphylococci. She estimated that 70% of dairy cattle in U.S.A. have such infection which could not be eliminated by continued antibiotic treatment.

Schalm and Woods (46) observed a close relationship of the shedding rate of Staphylococcus aureus in milk with the incidence of clinical mastitis. Schalm and Leamonis (44) are of the opinion that the sum of coagulase-positive micrococci and coagulase-negative saprophytic micrococci remained the same during the lactation period. They further observed that the former group of organisms showed an increase in occurrence as lactation period advanced.

Spencer and Janis (51) while examining extramammary reservoirs for coagulase-positive staphylococci found that skin of the teats and milking machine teat cups were often laden with the organisms. In many cases dipping of the teat cups successively into two pails of hypochlorite solution containing 300 ppm of chlorine solution did not eliminate contamination.

It is generally recognized (23, 46) that except for pyogenic infections, the most important reservoirs of staphylococci are the nose and upper respiratory tract of human and some animals. The organisms are normally transmitted from person to person. They may be discharged to contaminate the environment and establish clinical infections where susceptible tissues

are available. Gould (23) found that large numbers of healthy persons were carriers of pathogenic Staphylococcus pyogenes, generally from autogenic infections. Continued use of penicillin and oxytetracycline, even for four weeks was not very effective in eliminating the organisms.

Mann (35) while testing 282 phage-identified, coagulase-positive strains of Staphylococcus aureus, found that 53% were resistant to tetracycline, 45% to penicillin, 11% to oleodomycin and 8% to 1:2 combination of oleodomycin and tetracycline.

Isolation of Enterotoxigenic Staphylococci

Evan and Niven (18) found that all toxigenic cultures were coagulase-positive. They concluded that most, if not all, enterotoxigenic staphylococci were members of the coagulase-positive group, though some varieties of the coagulase-negative group may also be associated with the food poisoning. Beren et al. (6) concluded that the coagulation test when used in conjunction with pigmentation and haemolysis of rabbit blood agar, permits a simple estimation of the degree of pathogencity of the strain. They also observed that the coagulase test alone, was a most reliable test for differentiation of the pathogenic strains from the non-pathogenic staphylococci.

Hussemann and Tanner (30) reported that out of 28 strains from food, incriminated in food poisoning outbreaks, 27 were coagulase-positive. Only one strain among 40 strains isolated from normal food was coagulase-positive.

George (22) noted that the addition of 7.5% sodium chloride to solid culture media effectively inhibited most bacteria other than staphylococci. Pathogenic cultures grew luxuriantly where as non-pathogenic staphylococci grew poorly. He recommended the addition of 7.5% salt to Bacto Phenol Red Mannitol (BPRM) agar, to provide an improved isolation medium for coagulase-

positive staphylococci. Busta and Jezeski (8) observed that S-110 medium (a selective medium for pathogenic staphylococci, Difco) was inhibitory to heat shocked, coagulase-positive staphylococci. They noted that thermal death time at 140°F was 12 min as measured by growth on S-110 agar, while it was about 30 min when Standard Plate (SPC) agar (52) was used. The apparently lower thermal death time was attributed to the high sodium chloride content of S-110 medium, because modification of this medium, containing decreasing amounts of sodium chloride, allowed growth of increasing numbers of heat shocked staphylococci.

Tellurite-Glycine (TG) agar was recommended by Zebovitz et al. (60). This was a selective surface plating medium, on which coagulase-positive staphylococci, after incubation at 37°C for 24 hr, produced black colonies. On further incubation, coagulase-negative staphylococci could also grow producing similar colonies.

A fluorscent antisera technique for differentiation of coagulase-positive staphylococci from coagulase-negative was described by Smith (49). He recommended the technique especially for examination of foods, in which micro-organisms have been killed by heat. Wilson et al. (58) evolved a rapid test for detecting coagulase-positive staphylococci in foods. The test was made by adding the suspected samples of the food to a selective medium (brain heart mannitol salt broth) and incubating it over a shaker at 35°C. After 6 hr of shaking, the incubated cultures were examined with Gram's stain and the coagulase test. They claimed that the sensitivity of the test was sufficient to recover staphylococci when present in numbers far below the minimum level associated with food poisoning.

Growth in Raw Milk

Smith (48) studied the multiplication of Staphylodoccus aureus in cow's milk. He concluded that the low incidence of staphylococcal poisoning originating from raw milk was due to the comparatively low temperature at which milk is normally kept and the frequent presence in the milk of bacteria, other than Staphylococcus aureus. The two factors were shown to have a limiting effect on the degree of multiplication of Staphylococcus aureus Heineman (28) concluded that raw milk was not a suitable medium for the growth of Staphylococcus aureus but that pasteurized milk was a good medium for the multiplication of enterotoxigenic strains of staphylococci. Trout et al. (55) found that the so called 'germicidal action' of fresh milk is soon dissipated unless milk is promptly and adequately cooled. According to these investigators, the promptness and adequacy of cooling milk is responsible for the difference in bacteriological and organoleptic quality rather than for any specific inhibitory factor.

Clark and Nelson (9) conducted an investigation on multiplication of coagulase-positive staphylococci in grade A raw milk. They found that these organisms did not multiply in naturally infected milk, held at 4°C for seven days. At 10°C multiplication occurred at a varying rate in different samples. The greatest multiplication was 1,000 fold. The highest count obtained was 360,000/ml.

Takashi and Johns (53) noticed a correlation between the initial standard plate count of milk and the extent of multiplication of staphylococci. They observed that staphylococci can grow actively at a low temperature, only when the bacterial count is low. Oberhofer and Frazer (38) screened 66 cultures of food micro-organisms by the spot plate test to determine their

ability to influence the growth of 4 cultures Staphylococci aureus. The most consistently inhibitory cultures for Staphylococcus aureus were Streptococcus faecium, Streptococcus faecalis var. liquefaciens, a nicin producing Streptococcus lactis and various meat lactobacilli. Other cultures were less inhibitory and some were even stimulatory. They further observed that the growth of two enterotoxigenic strains was only moderately reduced by simultaneous growth of Escherichia coli but was markedly reduced by Escherichia coli H52 especially at 15°C and 44°C.

Peterson et al. (39) while studying the effect of proportion of staphylococci in mixed cultures on growth in artificial culture media noticed a definite suppression of growth of staphylococcal population by a mixture of saprophytic and psychrophilic bacterial species. The suppression effect was more pronounced when the staphylococcal population was less than the other bacterial population and was maximum at room temperature.

Smith (48) observed a heat labile clumping factor present in raw milk which caused the clumping of staphylococci at low (about 5°C) temperature and gave an apparent lower count on plating. When dilutions were made, violent agitation of the raw milk samples, broke up the clumps and gave the original count.

Woodburn and Strong (59) studied the effect of storing in several media at -30°C and found staphylococci could survive 10 weeks of storage. Corn syrup and waxy rice flour as storage media gave better protection than phosphate buffer and egg white.

Growth During Processing of Dairy Products

Foltz et al. (20) conducted a survey on the incidence of staphylococci in all classes of dairy products at the consumer level and isolated coagulase-

positive staphylococci from 3.4% of the products examined. All of the various classes of products studied by them contained staphylococci. However, not all samples within a class contained the organisms.

In a thermal death time study of Staphylococcus aureus (196E) in raw milk, Heineman (28) noticed that this organism did not survive pasteurization at 143°F for 30 min. He found a thermal death of 24 min at 140°F and further observed that condensed milk having 42% total solids was a good medium for the multiplication of staphylococci.

Crossley and Campling (10) determined the number of staphylococci present at different stages in the processing of condensed skim milk that had been artificially infected and processed in a small pilot evaporator and spray drier. They found that in most cases the number of staphylococci decreased. One strain multiplied actively at 43°C but above 50°C the organisms were eliminated entirely. George et al. (21) observed that staphylococci grew optimally during the condensing processes at temperatures ranging from 90 - 113°F. The rate of growth under simulated vacuum condensing was less than under normal pressure. However, the growth was quite extensive excluding the possibility that subatmospheric pressure in vacuum condensing would afford any retardation.

It is generally believed that it requires 4-8 hr of steady growth (logarithmic phase) in a rich food medium at 37°C to produce enough toxin to elicit symptoms, when such food or broth is consumed. Allison (1) suggested 500,000 organisms per gram of food product as the minimum number of viable coagulase-positive staphylococci as supporting evidence of staphylococcus food poisoning. He suggested that no toxin will be formed, even by larger numbers of non-coagulase-positive staphylococci.

The most prominent physical property of the enterotoxin is its heat stability. Jordan (32) contended that boiling destroyed enterotoxin and that exposure to 60 - 65°C for half an hour either destroyed or greatly weakened it. Dack et al. (12) held that it resisted boiling for half an hour. Jordan et al. (34) confirmed the resistance of enterotoxin to boiling. Rigdon (41) found that boiling for two hours destroyed the enterotoxin and Davis and Dack (15) besides confirming the instability of the enterotoxin to prolonged heating, found that autoclaving decreased its potency. Jordan and Burrows (33) found that it was unstable to heat in acid solutions (N/100HCl).

Protective Action of Ingredients Upon Survival of Microorganisms

Anzulvic (3) reported that sugar, gelatin, serum solids and fat showed some protective action for bacteria in the order mentioned. Weiss (57) found that Bacillus botulinum was more resistant to heat in food containing heavy syrups. Robertson (42) heated Streptococcus thermophilus, Sarcina lutea, Escherichia coli and Micrococcus aureus in increasing percentages of sugar and found that as the concentration of sugar was increased, the number of surviving bacteria also increased. However, he did not determine the maximum concentration at which the number of cells surviving begin to decrease. Fay (19) observed a definite protective action of hypertonic solutions of sugar and dextrose, on the thermal destruction of microorganisms.

Read et al. (40) in a study on thermal destruction of Escherichia coli, observed a lower z value in 40% cream than in milk, chocolate milk and ice cream mix. Donald et al. (16) while studying heat resistance of Corynebacterium diphtheriae Number 2%, in ice cream mix, chocolate milk

and cream, found that these products offered protective action to the organism in the order mentioned.

Erdman et al.(17) in a study of the effect of irradiation on micro-organisms observed that staphylococcus toxin was destroyed at comparatively low levels of irradiation. Destruction of Escherichia coli by irradiation could be used as a test of adequate treatment for destruction of staphylococci in the food.

EXPERIMENTAL PROCEDURE

The Test Organism

A known culture (196E) of potentially pathogenic Staphylococcus aureus was obtained from Dr. G. M. Dack of the Department of Bacteriology, University of Chicago. It was maintained on (SPC) agar (52) slants, having the following formula.

Bacto - Tryptone	5.0 g
Bacto - Yeast Extract	2.5 g.
Bacto - Dextrose	1.0 g.
Bacto - agar	15.0 g.
Distilled water	1.0 liter

final pH 7.0 at 25°C

Cultures were transferred every fourteen days, incubated at 37°C for 24 hr and stored at 3-5°C. These served as stock culture, from which the test inocula were prepared, during the 2nd to 14th day of storage.

Preparation of Bacterial Suspension

The test inocula from stock cultures were grown for 20-24 hr on plates of (SPC) agar. The crop was harvested aseptically in about 15 ml of sterile

physiological saline. The concentrated suspension was shaken in a bacteriological tube with plastic cap to break the clumps. It was then filtered through sterile Whatman - 12 filter paper to remove any suspended portions of the media. The filtrate was standardized with physiological saline to 59% transmission as measured on the Bausch and Lomb Spectronic 20, Colorimeter at wave length of 500 mu. It was previously determined that 0.05 ml of this suspension when injected into 2 ml of sterile milk, gave about a one million staphylococcal population per ml in the milk.

Preparation of Ingredient Samples

Raw skim milk was used as a base. The different concentrations of sugar, fat, serum solids, stabilizers and emulsifiers were prepared by adding to the milk the required amount of material on a weight basis. For example, 6% sugar solution was prepared by placing 94 g of skim milk in an Erlenmeyer flask and adding to it 6 g of sugar. The sugar was disolved by gentle stirring and by warming in cases of high concentrations of sugar. The percentages of sugar tested were 6, 10, 14, 25, 30, 45, and 57.

The various fat emulsions were prepared by adding the required amount of prestandardized 40% raw cream to the skim milk. The suspension was mixed by pouring several times from one flask to another. A sample of resultant suspensions was again tested for its fat percentage by the Babcock test. Only three percentages of fat 6, 10, and 14 were tested.

Skim milk was standardized by the addition of high quality instant non fat dry milk to give 20% and 30% serum solids. The mixture was blended for about 10 min in a sterilized Waring blender. The products were stored over night in the refrigerator at 3 - 5°C to permit the escape of all incorporated

air. The percentages of serum solids tested were 9, 20, and 30. The stabilizers and emulsifiers were dissolved in skim milk to get a concentration of 0.5%.

All the ingredient samples except 20% and 30% serum solids concentration, were autoclaved at 121°C and 15 lbs pressure for 15 min. The autoclaved ingredient samples were stored over night at room temperature and used next day.

Preparation of Ingredient Samples for Heating

Two ml of sterilized ingredient sample was transferred to 13 x 100 mm bacteriological test tubes with a continuous pipetter. Forty tubes from each ingredient samples were prepared. Each tube was fitted with a sleeveless rubber diaphragm. A 22 guage needle, 1.5 inch long was inserted through the diaphragm for subsequent inoculations. The needles were kept covered with steel caps, up to the time of injection of the bacterial suspension.

Water Bath and Temperature Control

A constant temperature, electrically heated, and controlled water bath was used. A Cenco No. 99200 Dekhotinsky mercury thermometer in conjunction with a Cenco No. 99783 relay controlled the temperature within $\pm$ 0.1°C. A high speed turbine stirrer provided a constant agitation and maintained a uniform temperature in the water bath. A metal plate with several holes, 14 mm in diameter was improvised in the upper part of the water bath. The water level was maintained at the level of the plate. Rubber diaphragm stoppers, having a diameter greater than the holes, held the tube containing the

ingredient sample and at the same time agitated them, due to constant movement of water.

Heating and Cooling Rate of Ingredient Samples

The rate of heating and cooling of the ingredient samples were determined at the beginning of the study. This was determined by putting a thermometer into the tube containing 2 ml of ingredient sample and inserting the tube into the water bath. It was found that no ingredient sample took more than one min and 30 sec to reach the desired temperature. For further surety a tempering period of 5 min was followed throughout the investigation. After a desired exposure the tubes were immediately transferred to ice water, at about 5°C. This cooled the heated ingredient samples to about 25°C within 30 sec. After an additional one minute cooling, the temperature of the samples dropped to about 8°C.

Inoculation of Ingredient Samples and Timing of Exposure

The technique for inoculation and timing described by Grosche, et al. (24) was used with some modifications. The temperature of water bath was kept constant at 60°C.

To provide more comparative data, three successive ingredient samples were run in one day. Five tubes from each concentrations were transferred and tempered for 5 min. Then by means of a tuberculin syringe, a 0.05 ml suspension of the test organism was inoculated through the 22 gauge needle. Simultanously, a stop watch was actuated. Immediately after the innoculation, the needle was drawn from the stopper to avoid drainage of the residual inoculum into the milk. As the needle was withdrawn, the tubes were gently agitated to aid in the distribution of the

inoculum. The inoculum in the second tube of the series of five, was made after 30 sec. Similarly, remaining inoculations were made and ingredient samples exposed from 5 to 35 min.

To compare the effect of autoclaving of ingredient samples vs. non-autoclaving on survival, and the efficiency of (SPC) agar vs. S-110 media on recovery of staphylococci, the following experiment was designed. Three concentrations of fat emulsions (6, 10 and 14%) were prepared and further divided into two portions. One portion of each concentration was autoclaved. The unautoclaved ingredient samples were treated at 140°F and plated on (SPC) agar. The autoclaved samples were treated as usual and plated on both S-110 and (SPC) agar.

Determination of Bacterial Count

During the process of inoculation of the heating tubes, two samples of 0.5 ml of inoculum were taken in two tubes having two ml of cooled ingredient samples for each time interval. These tubes were kept in ice water up to the time of plating. Before plating the content of each tube containing 2 ml of ingredient sample and 0.05 ml of the bacterial suspension were thoroughly mixed. One ml from each tube was serially diluted and plated. (SPC) agar (52) was used as a plating medium.

To compare (SPC) agar and S-110 medium as a recovery medium three autoclaved ingredient concentrations of fat were plated on both the media. Plates were incubated for 48 hr $\pm$ 4 hr at 37°C. At the end of incubation, the colonies were counted and the number recorded. In the case of unautoclaved ingredient concentrations, the staphylococcal counts were obtained by substracting the total count of uninoculated control tubes from the

total count of inoculated tubes, for the same exposure, on (SPC) agar.

STATISTICAL PROCEDURE

The counts of surviving organisms in various ingredients at different concentration were averaged and are reported in Tables 1, 3, 6 and 7 under results. Statistical analysis was made on these average counts. In cases where some of the organisms survived 35 min of exposure, all values from 5 to 35 min of exposure were used in the calculation. Where organisms died earlier than 35 min the values up to the time when all organisms were dead were taken into account.

Examination of the data showing the relationship of bacterial count and exposure time indicated that exponential function could be used to describe this relation. Therefore the bacterial counts were converted into logs and data were plotted on ordinary graphy paper. The logs of survival were plotted on the vertical scale (Y axis) against the respective time on the horizontal scale (X axis). The scatter diagram showed that relationship was linear. D[1] values were obtained for the slope of these lines, using the suggested method of Collins and Dunkley (11).

Regression coefficients of bacterial counts on time were determined for each concentration according to the method of least squares (50). Three regression equations (for sugar, serum solids and fat concentrations) showing the relationship for the regression coefficients with the corresponding ingredient concentrations were computed by regression analysis, which are shown in figures 1-3. To test the statistical significance of these regression coefficents the "t" test (50) was performed. An analysis of

[1] D value represents the time in min required for the destruction of one log cycle or 90% of organisms at a given temperature.

variance (50) of the different regression coefficients for the same ingredients was also made and compared with the pooled regression coefficients.

The analysis of variance (50) was used to determine whether or not significant differences existed between the variables studied. Tukey's test (50) of non-additivity was performed, prior to analysis of variance, to ascertain whether or not interaction between time and concentration of ingredient was present. The test indicated that no interaction was present.

The level of significance used in all above tests, was 5%.

EXPERIMENTAL RESULTS

The results of this study are presented separately for each concentrations of sugar, serum solids, fat, stabilizers and emulsifiers.

Effect of Sugar

The effect of concentrations of sugar from 6% to 57% in skim milk, on the survival of Staphylococcus aureus (196E) at 60°C is shown in Table 1. Each number represents the average of 10 individual counts. An examination of Table 1 reveals that the number of survivors in the case of 6% sugar concentration is lower than in skim milk alone. With the increase in sugar concentration in skim milk, the number of survivors also increases regularly and are maximum at 57% sugar concentration.

A comparison of the numbers of survivors in different sugar concentrations show that up to 14%, all the organisms are killed within 30 min. The protective action at 57% sugar concentration is so marked that more than half the number of initial population survive 35 min of exposure. It may also be noticed that with the addition of even a small portion of corn sugar, the

Table 1. Effect of different concentrations of sugar in skim milk, on the survival of Staphylococcus aureus (196E) at 60°C. (Average of 10 individual counts at each concentration.)

Sugar concentration	Initial count per ml	Survivors per ml at the following time (in min)						
		5	10	15	20	25	30	35
Skim milk	1,134,000	18,590	2,476	310	56	14	0	0
6% sugar	1,351,000	13,750	2,320	69	6	0	0	0
10% sugar	1,351,000	16,060	2,820	243	15	2	0	0
14% sugar	1,351,000	28,550	2,655	452	19	6	0	0
25% sugar	1,562,000	87,550	15,310	2,550	514	109	35	5
30% sugar	1,620,000	114,100	98,000	2,330	450	112	40	28
45% sugar	1,082,000	478,000	127,000	51,400	38,000	19,800	18,280	4,600
57% sugar	1,082,000	772,000	228,400	159,400	103,000	91,400	77,000	64,000
6% sucrose - 10% corn sugar	1,002,000	4,036	50	7	0	0	0	0
10% sucrose - 6% corn sugar	1,005,000	4,174	22	7	0	0	0	0
12% sucrose - 4% corn sugar	1,214,000	3,020	84	9	0	0	0	0

number of survivors is greatly reduced. In solution containing 12% sucrose plus 4% corn sugar the organisms are eliminated within 20 min where as it took 30 min for complete destruction in 10% surcrose solution.

The regression coefficients and 'D' values obtained from these data are presented in Table 2.

Table 2. Effect of sugar concentration on regression coefficients of log of bacterial count on time and 'D' values[1].

Sugar concentration	'b' value[2]	Variance about the trend line	No. of observation	'D' value
Skim milk alone	-0.18609	0.11974	7	5.34
6% sugar	-0.24144	0.12375	6	4.11
10% sugar	-0.20340	0.17660	7	4.88
14% sugar	-0.19910	0.11940	7	5.01
25% sugar	-0.14861	0.04866	8	6.71
30% sugar	-0.13553	0.15557	8	9.11
45% sugar	-0.06548	0.01716	8	15.08
57% sugar	-0.03565	0.02783	8	42.35
6% sucrose + corn sugar	-0.32800	0.37566	5	3.04
10% sucrose + corn sugar	-0.32833	0.12925	5	3.05
12% sucrose + corn sugar	-0.32644	0.04046	5	3.08

[1] Compiled from Table 1.

[2] Regression coefficient of log of bacterial count on time indicated by 'b' value.

It may be observed in Table 2 that the regression coefficient decreases with the increase in sugar concentration, signifying that an increase in sugar concentration makes the trend line less steep. The regression coefficients are plotted with their corresponding sugar concentrations in Fig 1.

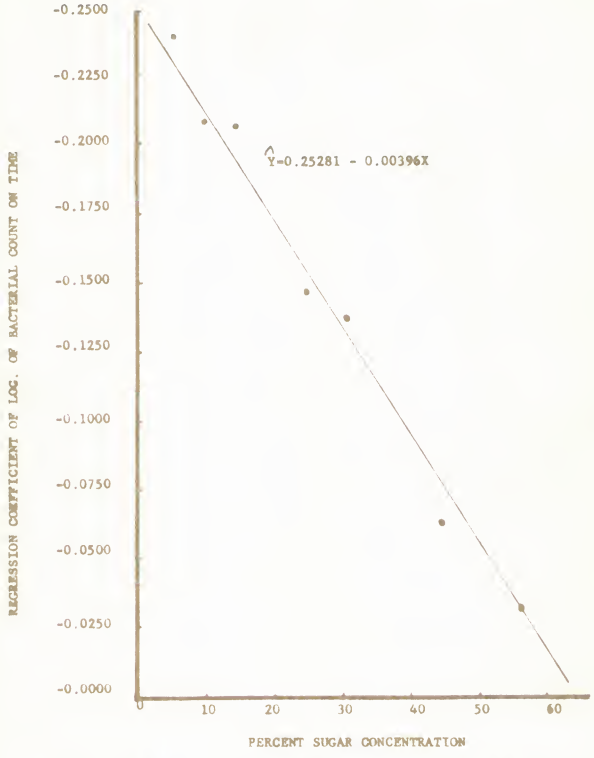

Figure 1. Effect of sugar concentration on survival rate of Staphylococcus aureus (196E), at 60° C.

This indicates that the protective action increases linearly with the increase in sugar concentration. The 'D' values also show similar results, though the relationship is not linear as they increase at an increasing rate with the corresponding sugar concentration.

The t values of the 't' test on the trend line (Fig 1) and the analysis of variance on the different regression coefficients are highly significant. The analysis of variance on the different concentrations also showed an effect of sugar concentration. If the regression coefficient for sugar concentrations is compared with that of plain skim milk (Table 2) it is observed that 'b' values increase with the addition of 6% sugar. At about 14%, the 'b' value is more or less the same as that of skim milk and is minimum at 57%.

It is therefore evident that the protective action of s gar starts at or about 14% and increases consistently with the increase in sugar concentration and is maximum at 57%. The 57% sugar concentration is a saturated solution in the water portion of the skim milk.

Effects of Serum Solids

The three serum solids concentrations studied were 9, 20 and 30%. The data on staphylococci are shown in Table 3. Each number in the table is the average of 10 individual counts. It may be observed that the number of survivals increases with the increase in serum solids concentration. The organisms were killed within 30 min in case of 9% serum solids whereas they took 35 min in 20% serum solids concentration and were not completely eliminated even after 35 min in 30% serum solids. The 'b' and 'D' values obtained are shown in Table 3.

Table 3. Effect of different concentrations of fat or serum solids in skim milk on the survival of Staphylococcus aureus (196E) at 60°C. (Average of 10 individual counts for each concentration).

Ingredient concentration	Plating media used	Initial count per ml	Survivors per ml. at the following time in min						
			5	10	15	20	25	30	35
Skim milk alone	(SPC)agar	1,134,000	18,590	2,476	310	56	14	0	0
6% fat	(SPC)agar	1,316,000	8,411	645	29	9	0	0	0
10% fat	(SPC)agar	1,316,000	5,984	456	16	5	0		0
14% fat	(SPC)agar	1,316,000	4,330	364	5	4	0	0	0
9% serum solids	S-110	960,000	1,620	166	76	39	15	0	0
20% serum solids	S-110	960,000	3,600	276	114	57	17	7	0
30% serum solids	S-110	960,000	10,600	946	454	224	32	15	5

Table 4. Effect of serum solids concentrations 'b' and 'D' values.[1]

Serum solids concentration	Plating media	'b' value	Variance about the trend line	observation	'D' value
9% serum solids	S-110	-0.16172	0.68288	7	6.20
20% serum solids	S-110	-0.14133	0.50883	8	7.12
30% serum solids	S-110	-0.13331	0.32382	8	7.51

[1]Compiled from Table 3.

A study of the above table shows that 'b' values decrease while the 'D' value increase with the increase in serum solids concentration. The 'b' values against their respective sugar concentration are plotted in Fig. 2.

The t value of the 't' test on the trend line (Fig. 2) is not insignificant, though the t value is found to approach significance. Analysis of variance of the three concentrations showed a significant effect of serum solids concentration. It should be noted that the sensitivity of the 't' test for detecting small differences in the three regression coefficients is very limited due to the smaller number of observations.

On the basis of a gradual and appreciable decrease in 'b' values, together with the significantly approaching t value and a strong serum solids concentration effect, it is apparent that increasing concentration of serum solids have a protective effect on the survival of this strain of staphylococci.

Effect of Fat

Only three concentrations of fat namely 6, 10 and 14% were studied. The number of surviving organisms for different concentrations is shown in Table 3.

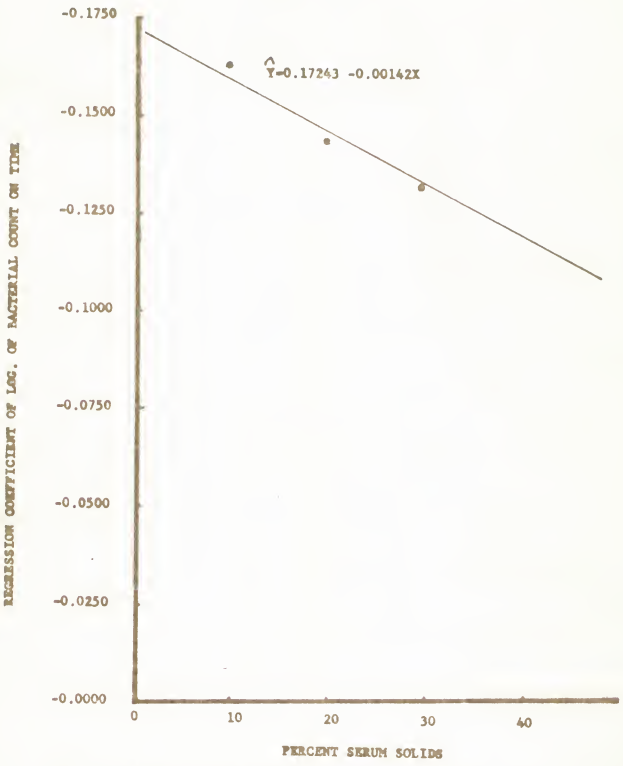

Figure 2. Effect of serum solids concentration on survival of Staphylococcus aureus (196E), At 60°C.

It may be noticed that the bacterial counts decrease with the increase in fat concentration with no survivors after 20 min. The 'b' and 'D' values calculated from Table 3 are shown in Table 5.

Table 5. Effect of fat concentration in the 'b' and 'D' values.[1]

Fat concentration	'b' value	Variance about the trend line	No. of observations	'D' value
Skim milk alone	-0.18609	0.11974	7	5.34
6% fat	-0.23380	0.27075	6	4.27
10% fat	-0.23584	0.27075	6	4.20
14% fat	-0.23742	1.55672	6	4.20

[1]Compiled from Table 3.

It may be observed (Table 5) that there is a slight gradual increase in 'b' values and decrease in 'D' values with the increase in fat concentration. The 'b' values when plotted against the fat concentration (Fig 3) show that the increasing concentration of fat is more lethal for the organism. The 't' test on the slope of this line and analysis of variance failed to show a significant effect. It is evident that concentrations of fat have no protective action.

Effect of Stabilizers and Emulsifiers

The stabilizers and emulsifiers were studied in only 0.5% concentration. Their effect on the survival of Staphylococcus aureus is shown in Table 6. Each count represents the average of 5 individual counts. A study of this table shows that in all samples except one, the staphylococci were eliminated within 25 min. A comparison of these counts with those of plain skim milk

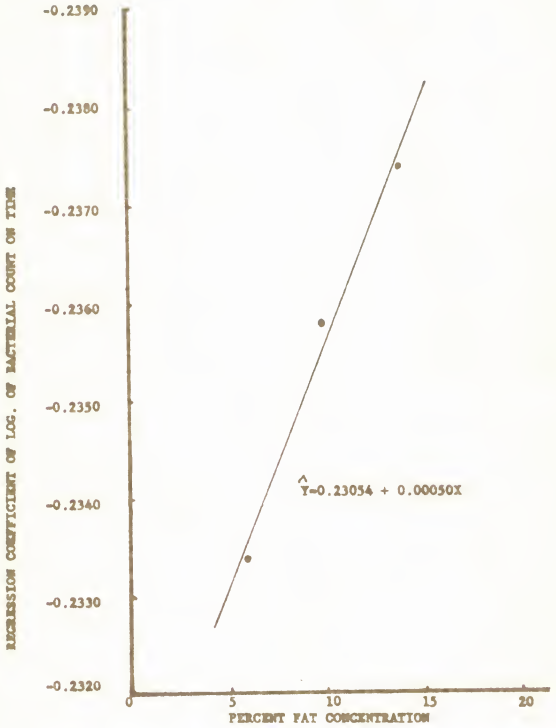

Figure 3.Effect of fat concentration on survival rate of Staphylococcus aureus (196E) , at 60°C.

Table 6. Effect of stabilizers and emulsifiers in skim milk, on the survival of Staphylococcus aureus (196E)[1] at 60°C. (Average of 5 individual counts for each ingredient)

Stabilizer or emulsifer used	Initial count per ml	Survivors per ml at the following time in min						
		5	10	15	20	25	30	35
0.5% Dariloid Kb.	1,079,000	15,911	1,172	59	3	0	0	0
0.5% egg yolk	1,079,000	13,860	804	69	6	0	0	0
0.5% gelatin	1,022,000	11,643	602	51	7	0	0	0
0.5% Gelox	1,440,000	980	38	14	0	0	0	0
0.5% ls-10s	1,440,000	3,600	44	0	0	0	0	0
0.5% Mixifier	1,024,000	6,540	190	39	23	5	0	0
0.5% Pectin	1,024,000	3,720	56	6	1	0	0	0
0.5% Tween Moss	1,024,000	1,840	40	9	6	0	0	0
0.5% Velvatex	1,440,000	7,360	204	80	0	0	0	0
0.5% Vesterine	1,440,000	7,300	128	28	0	0	0	0

[1]Plated on (SPC) agar

(Table 1) shows that the number of survivals in all the stabilizers and emulsifiers is less than in skim milk alone.

Effect of Recovery Media

In order to compare the effect of S-110 and (SPC) agar as recovery media, the same ingredient samples were plated on both media. The effect of the two plating media on the survival of heat shocked organisms is shown in Table 7. Each number represents the average of 5 individual counts. A study of the table shows that the survivors on the S-110 media are less in all the three concentrations than (SPC) agar. The 'b' and 'D' values calculated from this table are shown in Table 8.

Table 7. Effect of autoclaved vs. un-autoclaved and (SPC) agar vs. S-110 on the survival of Staphylococcus aureus (196E) in milk containing different concentration of fat at 60°C. (Average of 5 individual counts for each concentration)

Fat concentration	Plating media used	Initial count per ml	Survivors at the following time in min						
			5	10	15	20	25	30	35
6% fat autoclaved	(SPC)agar	1,486,000	11,480	272	20	5	0	0	0
6% fat autoclaved	S-110	1,486,000	1,960	16	0	0	0	0	0
6% fat unautoclaved	(SPC)agar	1,486,000	10,814	212	0	0	0	0	0
10% fat autoclaved	(SPC)agar	1,486,000	6,480	244	26	0	0	0	0
10% fat autoclaved	S-110	1,486,000	1,240	10	0	0	0	0	0
10% fat unautoclaved	(SPC)agar	1,486,000	7,724	208	0	0	0	0	0
14% fat autoclaved	(SPC)agar	1,486,000	3,300	138	6	0	0	0	0
14% fat autoclaved	S-110	1,486,000	660	8	0	0	0	0	0
14% fat unautoclaved	(SPC)agar	1,486,000	4,078	80	0	0	0	0	0

Table 8. Effect of S-110 vs. (SPC) agar and autoclaved vs. unautoclaved on the 'b' and 'D' values.

Ingredient sample	Plating media	'b' value	Variance about the trend line	No. of observation	'D' value
6% fat autoclaved	(SPC)agar	-0.24043	0.38577	5	4.16
6% fat autoclaved	S-110	-0.41208	0.35132	4	2.61
6% fat unautoclaved	(SPC)agar	-0.40449	0.03193	4	2.54
10% fat autoclaved	(SPC)agar	-0.29482	0.19499	4	3.38
10% fat autoclaved	S-110	-0.41218	0.54057	4	2.44
10% fat unautoclaved	(SPC)agar	-0.40169	0.05396	4	2.50
14% fat autoclaved	(SPC)agar	-0.29768	0.57011	4	3.33
14% fat autoclaved	S-110	-0.40864	0.74980	4	2.44
14% fat unautoclaved	(SPC)agar	-0.40444	0.08221	4	2.49

[1]Compiled from Table 7.

A study of the Table 8 shows that 'b' values on S-110 are greater than on (SPC) agar in all three ingredient concentrations. The 'D' values are also smaller indicating an inhibitory effect of S-110 media. The difference between the two media when tested by making 't' test on two 'b' values is significant except in case of 14% fat. No significant difference could be noticed by analysis of variance. It may be observed that the power of the test for detecting the difference between the two media is very limited due to the smaller number of observations.

On the basis of the 't' test and an apparently significant difference in number of survivors (Table 7) it is clear that S-110 is very inhibitory.

Autoclaved vs. Unautoclaved

It may be observed in Table 7 that the number of survivors in both autoclaved and unautoclaved samples is generally the same except that in the case of unautoclaved samples, no survivors are recorded at 15 min of exposure. However the counts when transformed into 'b' and 'D' values showed an apparent difference (Table 8). The 'b' values are greater while 'D' values are smaller in case of autoclaved ingredient samples.

No significant difference could be detected either by the 't' test or analysis of variance, between the two treatments. Evidently there is no difference between autoclaved an unautoclaved samples.

DISCUSSION

Among the various ingredients studied, it is evident that sugar in high concentrations had the greatest protective action on the survival of Staphylococcus aureus (196E). However up to about 14% of sugar concentration the thermal resistance was actually less than in skim milk alone. The earlier workers (3, 19, 42, 57) had studied the protective action of sugar in media other than milk. Their findings that sugar has protective action only partly agrees with the results of this study. The observation that equimolal solutions of different sugars do not have the same protective action is in close agreement with the work of Fay (19).

The second in order of offering protective action was found to be serum solids. The results in this case were not exactly comparable with that of sugar since a different recovery media (S-110) was employed. It was noticed that S-110 in inhibitory to heat shocked staphylococci which agrees with the results of Busta and Jezeski (8).

The fact that fat did not offer any protective action but tended to

increase the thermal destruction with the increasing concentration is contrary to the concept of Anzulvic (3) and is in agreement with the works of Read et al. (40), and Donald et al. (16). The very low thermal death rate in some of the stabilizers and emulsifiers may be due to the change in nature of these products as a result of autoclaving. For example, in the case of Dariloid Kb, Gelox and 18-105, the samples after autoclaving were separated into serum and solid portions.

Considering the combined effect of all the ingredients, upon the survival of this strain of staphylococci in ice cream mix, it is doubtful that the protective action is of any practical significance. The fact that ice cream mix is pasteurized at about 71°C (considering above the 60°C temperature used in this study) should decrease the survival rate even more. In the manufacture of sweetened condensed skim milk, the combined protective action would be much greater, as the condensing process is carried out at a lower temperature (about 50°C). Thus the proportion of surviving organisms would likely be greater than indicated in this study. Of course such heavy contamination of staphylococci is not likely to be normally encountered in the processing of dairy products. But it is generally believed (1) that staphylococci must be present or multiply to about one million/ml to secrete enough enterotoxin, so they were injected in such numbers. It is doubtful whether or not the staphylococci can multiply in sugar concentrations greater than 30%. The organisms actually present or accidently introduced after preheating as a result of contamination would probably be present in the finished product, as the high concentration of sugar and serum solids would afford protection.

The protective action of high concentrations of sugar and serum solids against the action of heat suggests that the protective action is afforded

by retardation of agglomeration and coagulation of colloidal material of the bacterial cell. As the agglomeration of the colloids becomes progressively more advanced, the cell becomes sluggish and entirely dormant. It is not clearly understood why there is less protective action at lower concentrations and why some ingredients do not offer any protection at all.

The author feels that the difference in protective action offered by varous ingredients or by different ingredient concentrations may be due to the variation in the power of agglomeration of cellular colloids. The difference in peptizing qualities of various media may also directly effect the protective action. Obviously, the susceptibility to coagulation is a relative matter and depends upon the relationship with the peptizing qualities of the medium employed. The precise mechanism by which some ingredient concentrations retards agglomeration is not very clear and requires further research.

Of necessity some conditions of this investigation differed from those that would be encountered in the normal practice. The first may be the use of autoclaved skim milk in place of raw skim milk as a base. The autoclaving might be suspected to change the nature of the milk. The autoclaved milk had to be used as there was no suitable recovery media available for heat shocked staphylococci. The S-110 media was found inhibitory for shocked organisms in the preliminary investigation. The fact was also confirmed by Busta and Jezeski (8). No significant difference as shown in part of this work could be noticed between autoclaved an unautoclaved milk as far as the protection on this strain of staphylococci was concerned. This may be due to the fact that as a result of storing of autoclaved samples overnight they return more or less to their original condition. Although it is recognized that pH tend to decrease as a result of autoclaving milk, but it

is assumed that pH relationship would be similar to commercial preheating of milk. The use of autoclaved milk has some advantages over the use of raw milk. The various advantages are that a simple recovery media may be used for plating, and the eliminations of spreaders and other counting errors.

Secondly the possibility of aerosol formation during inoculations and uneven mixing of the inocula with the ingredient samples in the test tubes could also cause variation in results. However each test on different concentrations was repeated a number of times with consistant results, these factors appear to be of minor significance.

SUMMARY AND CONCLUSIONS

The purpose of this research was to study the protective action of various ingredients used in the manufacture of condensed and frozen dairy products upon the survival of a potentially pathogenic staphylococci. The various ingredients used were in the range normally used in the industry. A total of 290 ingredient concentration samples were studied. The protective action was determined at 60°C. The number of surviving organisms, out of about one million staphylococci/ml in a 2 ml samples, was recorded at exposure intervals of 5 to 35 min. The numbers of survivors for each concentration were averaged and statistical analyses were made on these data.

It was demonstrated that increased protection occurred with increase in sugar and serum solids concentration. The thermal death time in sucrose concentration up to 14% was 30 min while it was only 20 min in case of various concentrations of fat. The staphylococci could survive up to about 30 min in plain skim milk. The equimolal solutions of different sugars did not show the same protective action. For example, in case of 14% sucrose solution the

organisms survived the 25 min of exposure while in a solution having 12% sucrose plus 4% corn sugar, all organisms were eliminated within 20 min.

The general conclusions from these data are: The addition of sugar (sucrose) to skim milk does not increase the thermal resistance of the staphylococci, until the sugar reaches about 15% concentration. At concentrations beyond 15% the protective action increases linearly with the increase in sugar concentrations. The protective action is maximum at 57% sugar concentration which is a saturated solution at room temperature. Increasing concentrations of serum solids provides a gradual increase in protective action. No protective action was shown by increased concentration of fat or usual amounts of stabilizers and emulsifiers. On the other hand, heat resistance tended to decrease with increase in concentration of fat.

On the basis of this study it may be concluded that a pasteurization temperature of 160°F for 30 min is adequate for the destruction of this strain of staphylococci in ice cream mix.

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EFFECTS OF INGREDIENTS USED IN CONDENSED AND FROZEN DAIRY PRODUCTS ON THERMAL RESISTANCE OF A POTENTIALLY PATHOGENIC STAPHYLOCOCCI

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Recent reports incriminating staphylococci in outbreaks of food poisoning in dairy products and the ability of staphylococci to adapt to environmental changes have focused attention on the need of research in this area. The increased importance of the problem and relatively little information available prompted this study of the effects of ingredients used in condensed and frozen dairy products on the thermal resistance of a potentially pathogenic staphylococci. It was postulated that the increased concentration of each ingredient has increasing protective action on the thermal destruction of staphylococci.

The different ingredient concentrations, comparable to those used commercially, were prepared by adding to the raw skim milk, the required quantities of sugar, 40% cream, high quality instant skim milk powder, stabilizers and emulsifiers on weight basis. A total of 290 ingredient samples were studied. The samples were autoclaved and held overnight for use next day except in the case of samples 20 and 30% serum solids concentration which were studied unautoclaved.

Using a continuous pipetter 2 ml quantities of the ingredient samples were transferred to a series of bacteriological tubes. The tubes were fitted with sleeveless rubber stoppers and hypodermic needles 1.5 inches long, 22-guage were inserted through them. The tubes were heated and maintained at 60°C in a constant temperature, electrically heated and controlled water bath. About two million cells of a known enterotoxigenic (196E) strain of staphylococci were inoculated through the hypodermic needles into the tubes containing ingredient samples and exposed to period ranging from 5 to 35 min. The survivors were plated on (SPC) agar and in case of unautoclaved samples on S-110 staphylococcus medium. The number of survivors for each concentration were averaged. The protective action were measured by making statistical

analyses on this data.

The thermal death times of this strain of staphylococci was 30, 25, 30 and 30 min in skim milk, 6, 10 and 14% sugar concentrations respectively. For higher concentrations of sugar the thermal death time exceeded 35 min and at about 57% slightly more than half the initial number of staphylococci survived 35 min of exposure. The organisms died in 25 min in all the three concentrations of fat. In 20% serum solids concentrations they were eliminated in 35 min. At 30% serum solids they survived 35 min of exposure. In the various stabilizers and emulsifiers the organisms were eliminated within 20 min except in the case of Mixifier where they took 30 min for complete destruction.

The addition of sugar to skim milk did not increase the protective action until it reached about 15% concentration. At concentrations beyond 15% the protective action increased regularly with the increase in sugar concentration. The protective action was maximum at 57% which is a saturated solution at room temperature. Equimolal solutions of different sugar did not have the same protective action. The replacement of even a portion of sucrose by corn sugar greatly reduced the protective action. The protective action in the case of serum solids increased regularly with the increase in concentration. No protective action was shown by increased amounts of fat or usual amounts of stabilizers and emulsifiers.

On the basis of this study it may be concluded that this strain of staphylococci does not survive the present pasteurization temperature of 160°F for 30 min in ice cream mix. It may however be noted that conclusions represents the results obtained by treating samples in a laboratory apparatus. Future work should be undertaken to verify these data with a pilot plant or actual plant equipment by study of a number of enterotoxigenic strains of staphylococci.