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Influence of certain factors on growth of bacteria in refrigerated dairy products

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Iowa State College

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INFLUENCE OF CERTAIN FACTORS ON GROWTH OF BACTERIA IN REFRIGERATED DAIRY PRODUCTS

by

Wallace Clayton Lawton

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
the Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Dairy Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1954

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INTRODUCTION

The increasing time between processing and consumption of milk has made the growth of organisms in this interval of great importance. Of particular interest are organisms which grow at the low temperatures used for milk storage. Organisms which grow at low temperatures usually have been considered slow growers and rather inert in their action on milk. Due to the large numbers of these organisms found in the samples of pasteurized milk examined after storage for 1 week at 3°C., it was decided that a more detailed investigation should be made of the growth and biological reactions of some of the organisms isolated to evaluate their role in milk quality.

The obvious criticism to the use of pure cultures would be that they would behave in a different manner in pure culture than they would in mixed flora such as would be found in pasteurized milk. While this criticism may have some merit, the information obtained under controlled conditions should be sufficiently important to overcome the undesirable effects resulting from a slightly changed environment.

The numbers of organisms and the damage done by them in a bottle of milk prior to consumption probably are influenced to some extent by fluctuating temperature of holding, such as that resulting from removal and return to the refrigerator

during use of the bottle, size of inocula provided by recontamination, optimum growth temperature of the organisms and many others. Because of this wide range of important factors it was decided to conduct this work in a manner which would check a considerable number of these variables, and if possible, provide the processor with some indication of how important these organisms are to him and what limitations he must place on his present and future operations to ensure a product with good keeping qualities.

The use of heat or chlorine, for sanitizing dairy equipment just prior to use is a common practice in the dairy industry. Some bacteria commonly escape destruction by these methods and are able to contaminate pasteurized products. The behavior of organisms subjected to sub-lethal doses of heat or chlorine may be altered, so a check of this fact may be helpful.

It is hoped that the elucidation of some of these factors and their effect on the growth of low temperature organisms in milk may be of use to the processor in his struggle to provide a good product to the consumer.

REVIEW OF LITERATURE

For the sake of convenience this section has been divided into several sub-sections, each of which covers closely related material.

Plating Temperature for Psychrophilic Bacteria

The tenth edition of Standard Methods for the Examination of Dairy Products (1) recommends that enumeration of psychrophilic bacteria be done by incubating plates for 7 days at 5°C. This procedure requires considerable time and large quantities of equipment which may not be available in some laboratories. A commercial company is interested in results as soon as possible and a long procedure would give results only after the product being tested had passed through consumer channels and further products were being processed. There are many references in the literature which show that low incubation temperatures are not necessary for enumeration of psychrophilic bacteria unless a count of them alone is required.

Nelson and Baker (44) found that the most satisfactory temperature for the enumeration of psychrophilic bacteria in stored samples was 25°C. for 3 days. They pointed out that the temperature at which a sample was held prior to plating influenced the effect that different incubation

temperatures had on the count obtained. They suggested a quick practical test for organisms that grow at refrigeration temperatures. This consisted of holding the sample at 10°C. for 3 days. Shorter incubation times gave indications of the presence of high counts. They suggested that if counts rose as high as 200,000 per milliliter after storage at 10°C. for 2 days, considerable doubt should be raised about the sanitary conditions under which that milk was produced.

Watrous et al. (78) plated pasteurized milk and incubated plates at 5, 25 and 35°C. and found that 25°C. gave consistently higher counts than 35°C.; the advantage increased as storage under refrigeration lengthened. After 6 days storage the counts on plates incubated at 5 and 25°C. approached the same magnitude. Essentially the same results were obtained when they plated chocolate milk and light cream. A somewhat similar result was observed by Atherton et al. (2), as they found that incubation of plates at 32, 26 and 20°C. resulted in nearly identical counts. At 10°C. the counts were low at first but were nearly equal to the others after 6 days of holding.

Several workers have reported (31, 54, 15) that 20-25°C. was the optimum temperature for growth of many organisms growing at refrigeration temperature.

Types of Bacteria Growing at Low Temperatures

The literature on the types of organisms growing at refrigeration temperatures is quite extensive. However, very few workers have attempted to isolate and identify organisms beyond the genera to which they belonged and many workers only classed them according to the nature of the changes they produced.

Jezeski and Macy (31) plated creamery water supplies and butter samples and isolated caseolytic and lipolytic cultures capable of vigorous growth at 8°C. The cultures were classified as to genera, 28 being Pseudomonas, five Flavobacterium, one Achromobacter and one a lactose-fermenting yeast. Some of the same genera were implicated by Thomas and Chandrasekhar (71). Garrison and Hammer (26) enriched milk and cream by holding at 5-7°C. for several days and plating. The same was done with ice cream, butter and water. They studied 496 cultures in detail and placed them all in the genus Pseudomonas.

Rogick and Burgwald (66) isolated low-temperature organisms and found that 54.47 percent were inert, 28.14 percent were acid-forming and 17.36 percent were alkali-forming. These percentages were found to change when cultures were incubated at 35°C. They concluded that only 4.19 percent were true psychrophilic bacteria and that they

were all alkali-forming, gram negative bacilli. Watrous et al. (78) reported that acid producing organisms were inhibited at 5°C. so inert forms and alkali producers predominated after prolonged storage. Thomas et al. (72) isolated 231 cultures of psychrophilic bacteria and found that 2 percent developed acid clot, 4 percent slight acid and 9 percent peptonization. Ayres and Johnson (5) found that holding raw and pasteurized milk at 10°C. resulted in an increase of peptonizing bacteria in pasteurized milk and a much smaller increase in raw milk.

Some workers identified the cultures isolated, especially when a specific defect was involved. Pennington (54) found that organisms especially resistant to cold were B. formosus, R. solitarius and B. ravenel. They were present in nearly pure culture after prolonged storage. Gainor and Wegemer (25) isolated a capsulated organism resembling Alcaligenes viscosus from ropy pasteurized milk. This organism was capable of good growth at 4.5°C. and did not grow at temperatures above 35.5°C. In England, Morgan (39) found that cans which were returned to the producers in one area were heavily contaminated with a chromogenic, curved rod which was identified as Vibrio undula and came from the can washer detergent tank which had been contaminated from the outside of cans being washed and would then contaminate clean cans. Newman (45) isolated organisms of

the Pseudomonas group from three samples of bitter milk. They grew well at 4°C. but little or not at all at 37.5°C. One culture was identified as Pseudomonas ovalis. Morrison and Hammer (41) checked the distribution of Pseudomonas fragi and found it present in 16.4 percent of 176 lots of milk delivered in Iowa. The organisms were not found in 17 lots delivered in Kentucky in June but were found in 40 percent of 40 deliveries in December at the same plant. Of the Iowa dairy plant water supplies inspected, 9.7 percent were infected. Soil and equipment likely to come in contact with milk were examined and 51.8 percent contained the organism. Pseudomonas fragi was found in 25 of 35 samples of barnyard soil examined.

Growth of Psychrophilic Bacteria

Growth of bacteria usually is considered to be greatly reduced by refrigeration. Certain organisms are known to grow at temperatures below 10°C. but their rate of growth usually is considered to be slow at first, followed by a slow, steady rise to high levels. Dahlberg (18) illustrated this point when he showed that milk stored for 4 days at 45-50°F. (7.1- 10°C.) showed a slight decrease in standard plate count after 1 day, began to rise after 3 days and had increased from 12,000 to 180,000 after 4 days. If milk was stored at 55-60°F. (12.6-15.6°C.), counts decreased the

first day, increased the second from 12,000 to 134,000 and exceeded one million thereafter. These figures do not give a completely accurate picture about the behavior of psychrophilic bacteria as the plates were incubated at 37°C., a temperature at which not all psychrophilic bacteria are able to grow. Rogick and Burgwald (65) showed that the mesophilic count in fresh milk was always higher than the psychrophilic count, while after 1 week storage at low temperature the position was reversed, although both types increased. Watrous et al. (78) showed that raw milk having a low initial contamination with psychrophilic bacteria may have a psychrophilic count which exceeds the total count at 35°C. after storage for 10-20 days in the refrigerator. Pasteurized milk from the bottler showed no psychrophilic bacteria, but the count on the same milk was extremely high after 15 days storage. Conn (16) stated that milk stored at 50°F. (10°C.) or lower would stay sweet for a long time but contain many bacteria of a more unwholesome type than if storage was at a higher temperature. Such milk was made unfit for market although perfectly sweet.

Ayres et al. (4) showed that milk with an initial count of 4,000 per milliliter showed no increase after storage for 24 hours at 4.4°C., while at 10°C. the count increased to 13,000 and at 15.5°C. it increased about 1.5 million. When milk was held at 4.4°C. the growth was slight, even

after 96 hours. When milk was held at 10°C. for 96 hours the count was just as high when the initial count of the milk was low as when it was high. Leete (36) stored 55 samples of pasteurized milk for 5 days. When storage was below 40°F. (4.4°C.), 27.2 percent of samples showed an increase in count; when storage was between 40 and 45°F. (4.4 and 7.1°C.), 37 percent of samples showed an increase; and when storage was between 45 and 50.8°F. (7.1 and 10.4°C.), 73.8 percent of the samples showed an increase in bacterial count.

Torrey and Rahe (74) showed that at ice box temperature the rate of increase of bacteria in the cream and skim milk portions was practically identical, but increase in temperature favored the latter. They reported that abrupt changes in temperature from 5 to 30°C. caused a striking bacteriolysis and suggested that it may be an expression of bacterial antagonism.

Some workers reported a rate of growth at low temperatures in excess of those usually associated with psychrophilic bacteria. Kiser (35) isolated cultures and found that 80 percent produced visible colonies in six days at 0°C. His growth curves for an Achromobacter sp. yielded generation times of 0.98 hours at 25°C., 4.8 hours at 7°C. and 30.7 hours at -4°C. Greene and Jezeski (27) found that a strain of Aerobacter aerogenes which grew at low temperatures had a generation time of 12.2 hours at 5°C., 4.1

hours at 10°C. and 2.24 hours at 15°C. Ayres et al. (3) found Pseudomonas organisms very prevalent on cut-up poultry. These bacteria were capable of fairly rapid growth at 4.4 and 10°C. Deterioration appeared in 6-8 days at the former temperature and 2-3 days at the latter.

There are indications that some coliform organisms grow at low temperatures and so influence the validity of the coliform counts used as an index of sanitation when stored samples are examined. Bardsley (8) stored 4 samples of ice cream at 8°C. for varying lengths of time. The first sample showed an increase in count after only 5 hours, while coliform bacteria and enterococci showed no increase. The second sample, stored for 18 hours, showed no increase, but after 6 days an increase in total count and in count of enterococci was noted. A third sample, with high initial count, stored for 2 days, showed an increase in coliform and total counts, while enterococci remained the same. Dahlberg (19) found that the coliform bacteria in pasteurized market milk, held at refrigeration temperatures, increased more rapidly than the total count. He also found (18) that coliforms increased in milk held at 45-50°F. (7.1-9.9°C.) and 55-60°F. (12.6-15.6°C.).

Lag Phase of Psychrophilic Bacteria

There are many conflicting reports in the literature about the lag phase encountered in bacteria at low temperatures. Palmer and McCutcheon (49) concluded that bacterial counts in milk stored at 42°F. (5.5°C.) began to increase after a total holding period of 48 hours. Chandrasekhar (14) found that 11 out of 12 samples had colony counts of less than 100,000, whereas only 6 out of 12 had less after 24 hours at 3-5°C. storage, indicating growth in that short interval. Venkataraman (77) reported the lag phase in organisms isolated from fish muscle to be 12 hours at 21°C. and 6-8 days at 0.5°C. Penfold (53) reported that if maximum growth of organisms was inhibited by placing at 2°C. for 12 minutes, growth continued without lag when returned to normal temperature, but when held cold for longer periods the lag phase tended to reappear. Greene and Jezeski (27) reported a long lag phase for organisms grown at low temperature, while increased temperatures resulted in decreased lag phases. Similar results were reported by Penfold (53) and Hess (29). Other workers reporting long lag phases for organisms at low temperatures include Chaffee (13), Trout *et al.* (76), Burgwald and Josephson (11) and Dahlberg (18).

Growth Temperatures of Psychrophilic Bacteria

The survival of organisms and their ability to grow at very low temperatures may be of importance to public health as well as being of great importance to the practical dairy man trying to produce a good product. Weinzirl and Gerdeman (79) found that storage of ice cream at -10°C. or above, did not prevent all bacterial multiplication or increase in count. Prucha and Brannan (61) reported that ice cream held in storage with an average temperature of -4°F. (-20°C.) contained viable typhoid organisms after 2 years and 4 months of storage. Jones and Lochhead (32) reported that Micrococci resisted freezing better than other bacteria, Food poisoning strains were found unable to multiply at 4.4°C. but when held for 2 months at 4°C. they did not lose their ability to produce enterotoxin. Zobel (80) concluded that marine bacteria were able to multiply in water as cold as -1.9°C. He suggested that there were indications that bacteria would withstand freezing to absolute zero. Hess (29) found that total crop of cells obtained with organisms of marine origin was greater at 0 and 3°C. than at 20 and 37°C. Prescott and Bates (38) observed that freezing reduced the numbers of organisms in water. Very small numbers persisted for some days but there was no appreciable increase over periods up

to 11 days.

Reed and Reynolds (63) added pure cultures to sterile skim milk and incubated at several temperatures. All the organisms studied grew to some extent at -1°C. Some increased for the first 3 days and then decreased. This was characteristic for bacteria having higher growth optima. The other group grew slowly at first but increased steadily. The organisms that grew best at low temperatures usually were associated with unclean practices. Pennington (54) found that bacteria in milk increased in numbers when temperatures were maintained at or a little above 0°C. Milk was kept from a few days to 2 years at 29-31°F. (-1.6 to -0.6°C.). Milk with counts as low as 300 per milliliter yielded high counts at the end of 1 week. Continuous exposure to the low temperatures resulted in a semi-frozen milk but bacterial numbers still increased into millions. Garrison and Hammer (26) studied 496 cultures isolated from milk, cream, ice cream, butter and water by enrichment at 5-7°C. In broth, all cultures grew at 7°C., most at 3°C. and all at 32°C. but a considerable number failed to grow at 37°C., although a few grew at 45°C.

Fabian and Trout (22) studied frozen, pasteurized cream for a period of 1 year when storage was at -5 to -10°F. (-20.5 to -23°C.). Bacterial counts decreased during the year. The same result was observed by Babcock et al. (6)

when they studied frozen homogenized milk. Ravenal et al. (62) found that at -9°C. there was no increase in bacteria but at 0°C. there was a marked increase. They found that storage of cream at 33-40°F. (0.5-4.4°C.) excluded the growth of lactic bacteria but did not exclude growth of putrifactive organisms. Conn and Esten (17) reported that in milk held at 10°C. the lactic bacteria were not favored, the delay in growth being 2-3 days, followed by uniform growth of all types of bacteria. The lactic acid producing bacteria did not always grow but neutral and liquefying bacteria grew rapidly. They found no difference in effect on bacteria between 10 and 1°C. except on rapidity of growth. They reported large numbers of bacteria after prolonged storage at 1°C. and pointed out that milk may not be wholesome just because it is sweet. Prescott and Bates (57) stated that certain types of spoilage organisms adapt themselves to temperatures assumed to be inhibitory to decomposition processes.

Haines (28) studied the minimum temperature of growth of several types of bacteria and found that staphylococci did not grow below 10°C., most strains of B. coli, B. proteus and micrococci stopped growing in the range 5-0°C. Some strains of B. proteus were capable of growth at 0°C. Many strains of Achromonacter, Pseudomonas and yeasts grew rapidly at 0°C. and down to -5°C.

Lochhead (37) isolated 20 distinct species of bacteria from frozen soil and found only one which grew better, three which grew equally well and 16 which grew poorer at 3°C. than at 20°C. He suggested that these bacteria were cold enduring rather than true psychrophilic bacteria. He concluded that most bacteria of frozen soil were incapable of growth at 3°C.

Morris (40) stored milk at atmospheric temperature and 4°C. overnight. He found a large increase in colony count and a shortening of methylene blue reduction time in both cases. The isolated cultures grew over a range of 4 to 37°C.

Kennedy and Weiser (34) isolated 15 pure cultures and grew them at varying temperatures with the optimum being the temperature at which there was the most rapid increase in cell numbers. They found that 7 of the 15 had their optima closer to 10 than to 20°C., 5 had optima closer to 20°C. and 3 grew best at room temperature. Erdman and Thornton (21) reported that not one of the 722 cultures isolated by them grew at 35.5°C.

Park (51) isolated 60 species of bacteria from milk and found 42 developed good growth at the end of 7 days at 39°F. (3.8°C.). He found that the bactericidal effect of clean milk acted for 12-14 hours at the low temperature but was reduced when higher temperatures of storage were used. A similar result was observed by Trout *et al.* (75) as they found the bactericidal effect was nearly lost if storage was

at 60°F. (15.6°C.). They pointed out that the germicidal effect only was of value to producers who used good production practices.

Pasteurization Resistance of Psychrophilic Bacteria

There has been much discussion on the subject of whether or not psychrophilic bacteria will withstand pasteurization temperatures. Thomas and Chandrasekhar (71) found that laboratory pasteurized raw milk produced no colonies after 21 days at 3-5°C. and that 35 pure cultures showed no survival. Further evidence for complete destruction of psychrophilic bacteria by pasteurization was presented by Rogick and Burgwald (66), Watrous et al. (78), Chandrasekhar (14) and Olson et al. (48).

Erdman and Thornton (21) found that 4 of 722 psychrophilic isolates survived pasteurization. Powell (56) flash pasteurized cream and stored for 10 days at 35°F. (1.6°C.). Counts made every 2 days yielded a flat growth curve with only a slight rise. The results can not be considered definite evidence for destruction of psychrophilic bacteria as plate incubation was at 37°C. Kennedy and Weiser (34) isolated 15 pure cultures able to grow in the cryophilic range of 5 to 25°C. Pure cultures were inoculated into sterile milk and laboratory pasteurized at 145°F. (62.8°C.)

for 30 minutes. Only one culture had no survivors, eight had reduction in numbers of 90 percent or over, five were reduced 50-90 percent and two were reduced less than 50 percent. Kaufmann and Andrews (33) found that pasteurization provided a small margin of safety with some pure cultures studied and suggested that some organisms may survive the high-temperature, short-time process when heated in whole milk.

Keeping Quality of Milk Containing Psychrophilic Bacteria

Many of the organisms which grow at low temperatures are inert in milk and produce little or no change, while others produce changes which are even more undesirable than acid production. It was suggested by Phillips and Thomas (55) that holding good quality milk on the farm at 40°F. (4.4°C.) for 24 hours improved its keeping quality, as it reduced bacterial counts. Nickolas and Anderson (46) kept pasteurized milk with counts below 94,000 per milliter at 40°F. (4.4°C.) for an average of 17.2 days before detectable spoilage occurred. Two batches of raw milk remained unspoiled for 4 and 7 days, respectively, when stored at 40°F. (4.4°C.). They pointed out that the variation in keeping time of the different samples was attributed to differences in the type of initial contamination. Trout et al. (76) found that in

some cases high plate counts were needed before off-flavors developed, but in most cases counts of 500,000 were sufficient to yield flavors such as stale, unclean, cheesy and bitter. Standard plate counts were used as a measure of numbers but no information was given about the temperature used for plate incubation and the types of organisms present in cases where a low count was found to cause the defect may not have been enumerated.

Greene and Jezeski (27) reported that keeping time of milk was increased from 1 day at 30°C. to 4 days when the temperature was lowered to 10°C. A further drop to 5°C. added 12 more days to the keeping time. The effect was even more pronounced in a further drop to 0°C., where keeping time was prolonged an additional 16 days. Kaufmann and Andrews (33) reported that 68 percent of the samples they stored at 47°F. (8.3°C.) showed evidence of spoilage on the eighth day when initial contamination was less than 10 per milliliter. Burgwald and Josephson (11) concluded from their work that milk of good keeping quality could be expected to retain excellent bacteriological and flavor qualities for at least 4 days in summer and 6-7 days in winter if stored near 40°F. (4.4°C.). They reported that psychrophilic bacteria in milk were responsible for the deterioration but that psychrophilic or mesophilic counts were not an indication of potential keeping quality during storage at 40°F. (4.4°C.).

Similarily Olson et al. (48) pointed out that psychrophilic bacteria counts on fresh milk were not a good indication of keeping quality, as low initial counts often preceded very high counts after storage. They found that plants with good bacterial records in the past usually produced a product with better keeping quality than those with poor past records.

Burgwald et al. (12) found that reconstituted milk and the concentrate from which it came, stored for 10 days at 34-44°F. (1.1-6.6°C.), resulted in psychrophilic counts higher than the mesophilic counts. They reported that the reconstituted milk had a poorer keeping quality than the concentrate and implicated the tap water used for reconstituting. The water was shown to contain both psychrophilic and mesophilic bacteria. When milk was pasteurized after processing, bottled and cooled in the bottle, keeping quality was improved over normal methods of handling. Essentially the same results were reported by Olson et al. (47), as they found that bacterial growth took place at a greater rate in recombined milk than in concentrate from which it was prepared when storage was at 4, 7 and 10°C. They used sterile distilled water for reconstituting the milk. These findings were contrary to those of Rosenberger et al. (67) as they found that dilution with sterile distilled water and in sterile equipment had no effect on changes in bacterial count on holding. Any differences found could be attributed

to dilution in preparation.

Thornton et al. (73) found that special grade cream could be obtained with twice-a-week delivery if cooling was below 50°F. (9.9°C.) and with weekly delivery if cooled below 45°F. (7.1°C.). They also reported that a 5°F. difference in storage temperature made a marked difference in the keeping quality of cream stored from 40-50°F. (4.4-9.9°C.). However, they suggested that long storage of cream at 50°F. (9.9°C.) or below probably would result in bacteriologically induced flavor defects that were not now encountered in churning cream.

Sources of Psychrophilic Bacteria

If psychrophilic bacteria are to be controlled, an understanding of their source is very important. Organisms which grow at low temperature are usually associated with water and soil. Contamination of dairy products takes place by improper procedures which allow contaminated water or soil to come into contact with dairy products or the equipment used for processing and handling.

Morris (40) found that sources of contamination included well water on farms and cans returned to the farm from the factory. Thomas et al. (72) reported that air in clean cow houses contained from 1,000 to 15,000 psychrophilic bacteria per square foot of surface (original author's terminology),

while feed and swabbings from flanks and udders of cows all showed a widely variable number of psychrophilic bacteria. Provan (60) pointed out an important fact when he stated that water for dairy use must first meet requirements for domestic use and then be further evaluated for its use in dairy plants, as types which have no significance to public health may provide a problem in dairy plants.

EXPERIMENTAL METHODS

Source and Isolation of Cultures

Pasteurized milk was obtained from commercial sources. The milk was held at 3°C. for one week of enrichment. Dilutions were made from the milk and plated on Tryptone Glucose Extract (T.G.E.) agar (20). Plates were incubated for 10 days at 3°C., after which single colonies were picked into litmus milk and grown at 5°C. for 10 days. Each culture was further purified by at least three single colony isolations prior to use. All cultures were grown in 10 percent reconstituted milk-solids-not-fat, with litmus added, which had been autoclaved for 20 minutes at 15 pounds steam pressure. Cultures were carried at 5°C. and transferred every 10 days. A culture 10 days old was used for inoculation purposes throughout this work unless specifically mentioned otherwise.

Characterization of Cultures

All cultures were characterized in an effort to obtain representatives of a variety of species. The descriptions given in Bergey's manual (10) were used as the criteria in placing the organisms in a tentative classification.

A synthetic medium containing 0.5 g. $MgSO_4$, 0.8 g. K_2HPO_4, 20.0 g. NH_4Cl and 1.0 ml. of 1.6 percent alcoholic

solution of brom cresol purple per 1,000 ml. of distilled water was used to check the organisms reaction on carbohydrates. Glucose, fructose, galactose, maltose, lactose, sucrose, mannitol, sorbitol, arabinose and raffinose were used as carbohydrate substrates. Just prior to inoculation 1 ml. of sterile carbohydrate solution, to represent 0.3 percent carbohydrate in the tube, was added to the basal medium. Tubes were inoculated, incubated for 3 days at 25°C. and read for acid production.

Routine biochemical and morpholoical tests were conducted according to the Manual of Methods for Pure Culture Study of Bacteria (70).

Flagella stains were made according to the Fisher and Conn (23) modification of the Bailey method (7).

Tyrosine and trytophan assays were made using a procedure similar to one outlined by Hull (30). The determination was made by placing 5 ml. of the sample to be tested in a test tube. To the sample was added 10 ml. of 0.72N tricholoroacetic acid, the test tube was shaken and then allowed to stand for 10 minutes. The mixture was filtered through a Whatman 42 filter paper and 1 ml. of the filtrate added to a 125 ml. Erlenmeyer flask. To the flask was added 4 ml. of distilled water and 10 ml. of sodium carbonate-quadrafos solution. (The latter solution was prepared by adding 75 g. of anhydrous sodium carbonate and 10 g. of

sodium tetraphosphate and diluting to 500 ml. with distilled water.) To the flask was added 3 ml. of Folin-Ciocalteaus' reagent, previously diluted 1:2 with water. The mixture was stirred and allowed to stand for 5 minutes to develop color. The color was measured in a Klett-Summerson colorimeter using a 645 mμ wave length filter. To convert the colorimeter readings to micrograms, a standard curve was prepared using tyrosine for the standard solution. All runs were accompanied by a blank sample of milk in which organisms had not been grown.

Growth of organisms at 37°C. was checked by inoculating cultures into nutrient broth and on T.G.E. agar slants and immersing in a water bath at 37 ± 0.1°C. The tubes were placed in the water bath so the water level was above the surface of the culture medium.

Growth Curves for Comparison of Growth of Cultures in Sterile Skim Milk and Laboratory Pasteurized Milk

Sterile skim milk was prepared by autoclaving a 10 percent suspension of milk-solids-not-fat for 20 minutes at 15 pounds pressure. The laboratory pasteurized milk was prepared by obtaining fresh, raw milk from the milk department of Iowa State College and pasteurizing at 61.7°C. for 30

minutes, followed by rapid cooling and holding at 3°C. until the following morning. When growth curves were to be run on either type of milk the milk was inoculated, shaken and divided into screw-cap test tubes so a separate tube was available for each sampling at each temperature. Uninoculated controls were run with the laboratory pasteurized milk. Holding of tubes was at 5, 10, 21, 25 and 32°C. All tubes were inverted every 24 hours to mix the cream layer. Incubation of plates was at 25°C. for 3 days, as suggested by Nelson and Baker (44).

Growth Curves for Other Purposes

All other growth curves in this work were carried out in sterile skim milk. Samples were held in a single bottle for each temperature and samples were taken at the indicated times. Agar used, temperature of holding and temperature of incubation of plates were varied to fit each experiment and will be given as each experiment is outlined in the results section.

Calculation of Generation Time

The generation time is a measure of the rate of growth of a culture and was calculated for various intervals of some of the growth curves. The formula $g = \frac{T \log 2}{\log b - \log a}$ was used, where g was generation time in minutes, T was time

interval in minutes, a was count at the beginning and b the count at the end of time T.

Heat Treatment of Cultures

Time required for cultures to reach treatment temperature.

The time required for the culture to reach water bath temperature was checked by means of a properly standardized iron-constantan thermocouple attached to a voltmeter and a galvanometer. The electrodes were placed in 10 x 75 mm. soft glass test tubes containing about 1.5 ml. of milk. One electrode was placed in cracked ice and the other in the water bath at the temperature of heating and the voltage measured. The time required for the heat to generate the voltage was considered as the time required for the tubes to reach water bath temperature.

Partial heat inactivation.

The culture to be treated was sealed in 1.5 ml. amounts into 10 x 75 mm. soft glass test tubes and completely immersed in the water bath for the required length of time. On removal from the water bath the tubes were placed in ice water. As soon as possible the tubes were opened and 1 ml. transferred to 99 ml. of sterile skim milk, plated for zero time, divided into three screw cap test tubes and held at 5, 10, and 25°C. for growth curves. When required, the heat

treated cultures were further diluted.

Chlorine Treatment of Cultures

Partial destruction of bacteria by chlorine was accomplished by adding 1 ml. of a 1:100 dilution of culture to 10 ml. of the appropriate strength of chlorine solution. The mixture was shaken and allowed to stand for 1 minute and 1 ml. was then transferred to 99 ml. of sterile skim milk and plated for zero time. To check the count of untreated culture, a control was run using sterile distilled water in place of the chlorine solution. The milk containing the treated culture was divided into three screw cap test tubes for holding at 5, 10 and 25°C. for growth curve studies.

Strength of chlorine solution was determined by the thio-sulfate method as outlined in The Milk Industry Foundation Laboratory Manual (38). The resulting amount of available chlorine was divided by two, as one atom of chlorine is equivalent to two molecules of thiosulfate. This correction was pointed out by Roadhouse and Henderson (64).

RESULTS

Before studying the behavior of the isolated organisms it was advantageous to know as much as possible about the organisms and at least tentatively identify them. The information obtained is listed under the numbers used to designate them in the balance of this work. Fifty isolates from ten sources were collected to begin the work on the project. Screening tests indicated that many of the isolates were very similar, so eight organisms were selected, on the basis of their dissimilarity, for use in the project. A ninth organism Pseudomonas fragi, was obtained from the Iowa State College Dairy Bacteriology Laboratory collection. The tests used for characterization of these organisms were carried out at 25°C. for 3 days unless other conditions are specifically mentioned.

Description of Cultures

Culture 1

Morphology. Gram negative rod, single or in pairs. Polar flagella. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Grows at 5-32°C, not at 37°C.

Fluorescence. Produced in milk.

Agar Slant. Filiform, greenish.

Agar Colonies. Circular, flat, entire, opaque, greenish white.

Broth. Turbid, slight pellicle.

Potato Slant. Dirty brown, heavy growth.

Biochemical Characteristics.

Litmus Milk. Unchanged at 3 days, alkaline after prolonged incubation.

Fermentation. Acid from glucose, fructose, mannitol, sorbitol and arabinose.

Nitrates. Not reduced.

Gelatin. Not liquefied.

Tyrosine and Tryptophan. Not released.

Tentative Classification. Pseudomonas ovalis.

Culture 3

Morphology. Gram negative rod, single or in pairs. Polar flagella. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Grows at 5-32°C., not at 37°C.

Fluorescence. Produced in milk.

Agar Slant. Thin, slightly spreading growth.

Agar Colonies. Flat, erose, rough and granular, viscid, opaque, grey.

Broth. Turbid, slight pellicle.

Potato Slant. Moist, yellowish-grey.

Biochemical Characteristics.

Litmus Milk. Proteolysis with alkaline reaction at the top of the tube.

Fermentation. Acid from glucose, fructose, galactose, mannitol, sorbitol and arabinose.

Nitrates. Not reduced.

Gelatin. Complete liquefaction.

Tyrosine and Trytophan. Released.

Tentative Classification. Pseudomonas fluorescens. This culture varied from the description in Bergey's manual in that nitrates were not reduced and gelatin liquefaction was greater than indicated.

Culture 4

Morphology. Gram negative rod, usually in pairs. Polar flagella. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Growth at 5-32°C., not at 37°C.

Fluorescence. Not produced in milk.

Agar Slant. Filiform.

Agar Colonies. Round, raised, glistening, translucent.

Broth. Turbid, sediment.

Potato Slant. Heavy, moist, brownish.

Biochemical Characteristics.

Litmus Milk. Unchanged.

Fermentation. Acid from glucose.

Nitrates. Not reduced.

Gelatin. Not liquefied.

Tyrosine and Tryptophan. Not released.

Tentative Classification. Pseudomonas arvilla. The reaction of this organism to naphthalene was not checked.

Culture 8

Morphology. Gram negative rod, pairs or single. Polar flagella. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Growth at 5-32°C, not at 37°C.

Fluorescence. Not produced in milk.

Agar Slant. Filiform, undulate.

Agar Colonies. Smooth, round, entire.

Broth. Turbid.

Potato Slant. Raised, glistening, slimy, grey-brown.

Biochemical Characteristics.

Litmus Milk. Unchanged at 3 days, turned slightly alkaline after prolonged incubation.

Fermentation. Acid from no carbohydrate tested.

Nitrates. Not reduced.

Gelatin. Not liquefied.

Tyrosine and Tryptophan. Slight release.

Tentative Classification. Pseudomonas cruciviae. The fermentation of phenol and m-cresol was not checked.

Culture 9

Morphology. Gram negative rod, single. No flagella evident. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Growth at 5-25°C., poorly at 32°C. and not at 37°C.

Fluorescence. Not produced in milk.

Agar Slant. Smooth, glistening, very slight.

Agar Colonies. Round, entire, raised, glistening, yellow when first isolated but the ability to produce pigment was lost on repeated sub-culture.

Broth. Slight, grainy growth.

Potato Slant. Slight, yellow growth.

Biochemical Characteristics.

Litmus Milk. Unchanged.

Fermentation. Acid from no carbohydrate tested.

Nitrates. Not reduced.

Gelatin. Not liquefied.

Tyrosine and Tryptophan. Not released.

Tentative Classification. Flavobacterium aquatile. The temperature optimum for this organism was found to be 5-10°C., in contrast to 25°C. reported in Bergey's manual.

Culture 10

Morphology. Gram negative rod, pairs. Polar flagella.

Non-spore forming.

Cultural Characteristics.

Growth Temperature. Growth at 5-32°C., not at 37°C.

Fluorescence. Not produced in milk.

Agar Slant. Thin, limited growth.

Agar Colonies. Round, entire, smooth, greyish-white.

Broth. Turbid, pellicle.

Potato Slant. Heavy, greyish-white, butyrous.

Biochemical Characteristics.

Litmus Milk. Complete proteolysis, alkaline.

Fermentation. Acid from glucose, fructose, galactose, mannitol, sorbitol and arabinose.

Nitrates. Not reduced.

Gelatin. Complete liquefaction.

Tyrosine and Tryptophan. Released.

Tentative Classification. Pseudomonas sp. This organism did not fit the description of any of the species recognized in Bergey's manual.

Culture 11

Morphology. Gram negative rod, short chains. Polar flagella. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Grows at 5-32°C., not at 37°C.

Fluorescence. Produced in milk.

Agar Slant. Thin, filiform.

Agar Colonies. Flat, entire, opaque, grey.

Broth. Turbid, pellicle.

Potato Slant. Slightly viscid, grey.

Biochemical Characteristics.

Litmus Milk. Complete proteolysis, alkaline.

Fermentation. Acid from glucose, fructose, galactose and mannitol.

Nitrates. Reduced beyond the nitrite stage.

Gelatin. Complete liquefaction.

Tyrosine and Tryptophan. Released.

Tentative Classification. Pseudomonas fluorescens. This organism was viscid on initial isolation so could be Pseudomonas viscosa which had lost its viscid character.

Culture 12

Morphology. Gram negative rod, single or pairs. Polar flagella. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Growth at 5-32°C., not at 37°C.

Fluorescence. Not produced in milk.

Agar Slant. Limited, filiform.

Agar Colonies. Flat, entire, circular, viscid, smooth, greyish-white.

Broth. Turbid.

Potato Slant. Thin, brownish streak, slightly viscid.

Biochemical Characteristics.

Litmus Milk. Unchanged at 3 days, alkaline after long storage.

Fermentation. Acid from glucose and arabinose.

Nitrates. Not reduced.

Gelatin. Slight liquefaction after 7 days.

Tyrosine and Trytophan. Not released.

Tentative Classification. Pseudomonas geniculata. This organism gave off the May apple odor so could be a Pseudomonas fragi which is very closely related in Bergey's manual. It did not reduce litmus milk or cause any visible coagulation.

Culture 13

Morphology. Gram negative rod, single or pairs. Polar flagella, often more than one. Non-spore forming.

Cultural Characteristics.

Growth Temperature. Grows at 5-32°C., not at 37°C.

Fluorescence. Not produced in milk.

Agar Slant. Heavy, spreading.

Agar Colonies. Convex, glistening, smooth, butyrous.

Broth. Turbid, sediment.

Potato Slant. Raised, glistening.

Biochemical Characteristics.

Litmus Milk. Unchanged in 3 days at 25°C. but slight acid coagulation at 32°C.

Fermentation. Acid from glucose, galactose and arabinose.

Nitrates. Not reduced.

Gelatin. Crateriform liquefaction.

Tyrosine and tryptophan. Not released.

Tentative Classification. Pseudomonas fragi. This organism did not produce the acid coagulation and reduction outlined in Bergey's manual, except at 32°C. It did produce the characteristic May apple odor.

All tests used for characterization in this work was carried out at 25°C. and the results were recorded after 3 days. However, the cultures also were carried at other temperatures and gross qualitative differences were noted, depending on the temperature at which the cultures were incubated. Visible proteolysis was produced in litmus milk by culture 3 when held at 5 or 25°C., but no proteolysis was evident in 3 days at 32°C. although growth did take place, as shown by growth curves at that temperature. Similarly, reduction was seen at 25°C. These variations would be of great interest in taxonomy but a comprehensive study of the differences was beyond the scope of this work.

Growth Curves in Laboratory Pasteurized Milk

Laboratory pasteurized milk was inoculated with individual pure cultures and then held in a separate screw cap test tube for each sampling period. A duplicate set of uninoculated control tubes was carried under identical conditions. All tubes were shaken every 24 hours to distribute the cream layer. Figure 1 is an example of the type of growth curve obtained with this procedure. The figure represents the results obtained when culture 3 was added to laboratory pasteurized milk. It was apparent from both sample and control that 21, 25 and 32°C. had a very similar effect on the growth of the organisms present. The control and sample were separated at these temperatures only by the amount of inoculum used, as the resulting curves rise nearly parallel to each other. This type of relationship indicated that the growth rate of the added pure culture was similar to that of the flora present in the laboratory pasteurized milk. At 5 and 10°C. the controls increased very little in count, while the inoculated samples rose to a level similar to that reached at the higher temperatures but the rate of rise was much slower. This points out a very essential difference between the surviving organisms and the inoculated ones. The surviving organisms grew very slowly at 10°C. and did not grow at 5°C., although they had a growth rate similar

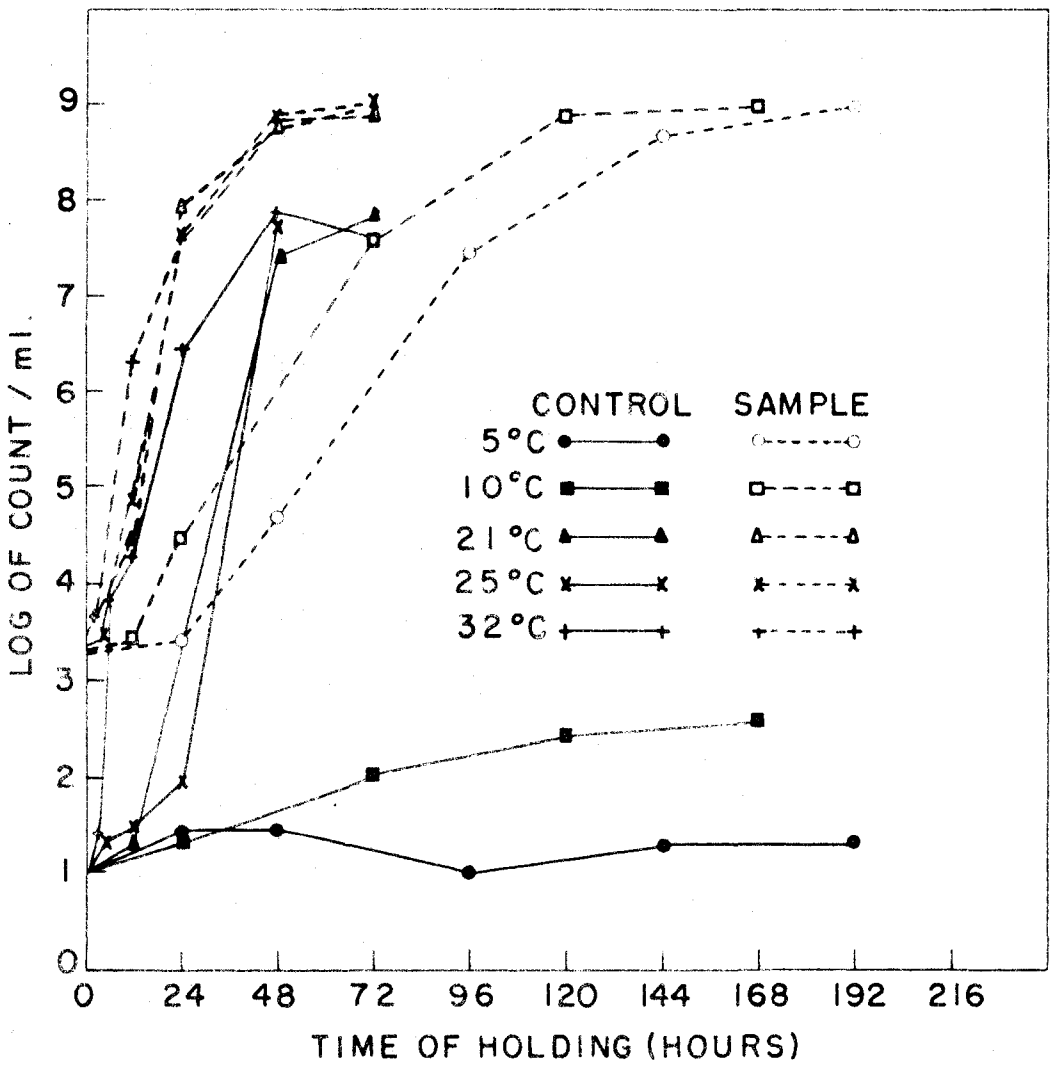

Figure 1. Counts on culture 3 grown in laboratory pasteurized milk.

to the inoculated cultures when grown at a temperature favorable to both. The lag phases in the uninoculated control at 21 and 25°C. were increased 12 and 24 hours, respectively, over the inoculated samples, while at 32°C. the lag phase was much the same in sample and control. After the initial lag period, curves at all three higher temperatures rose at the same rate in the samples as in the controls but to a somewhat lower level at the peak which appeared at about 48 hours. Figure 2 shows the results obtained with culture 1 with the same procedure. The general picture was the same as shown in Figure 1, but there were minor differences. In this case there was some increase in controls that were held at 5 and 10°C., but the increase represented less than a 50-fold increase so only represented slightly over five generations in a period of 192 hours. This difference varied when different milk sources were used.

All other cultures were checked by the same procedure and duplicate growth curves for each showed very similar results. The initial counts varied, as the experiments were spread over a considerable period of time and the milk source varied from day to day. Despite this variation in milk, the general picture of the resulting growth curves remained the same.

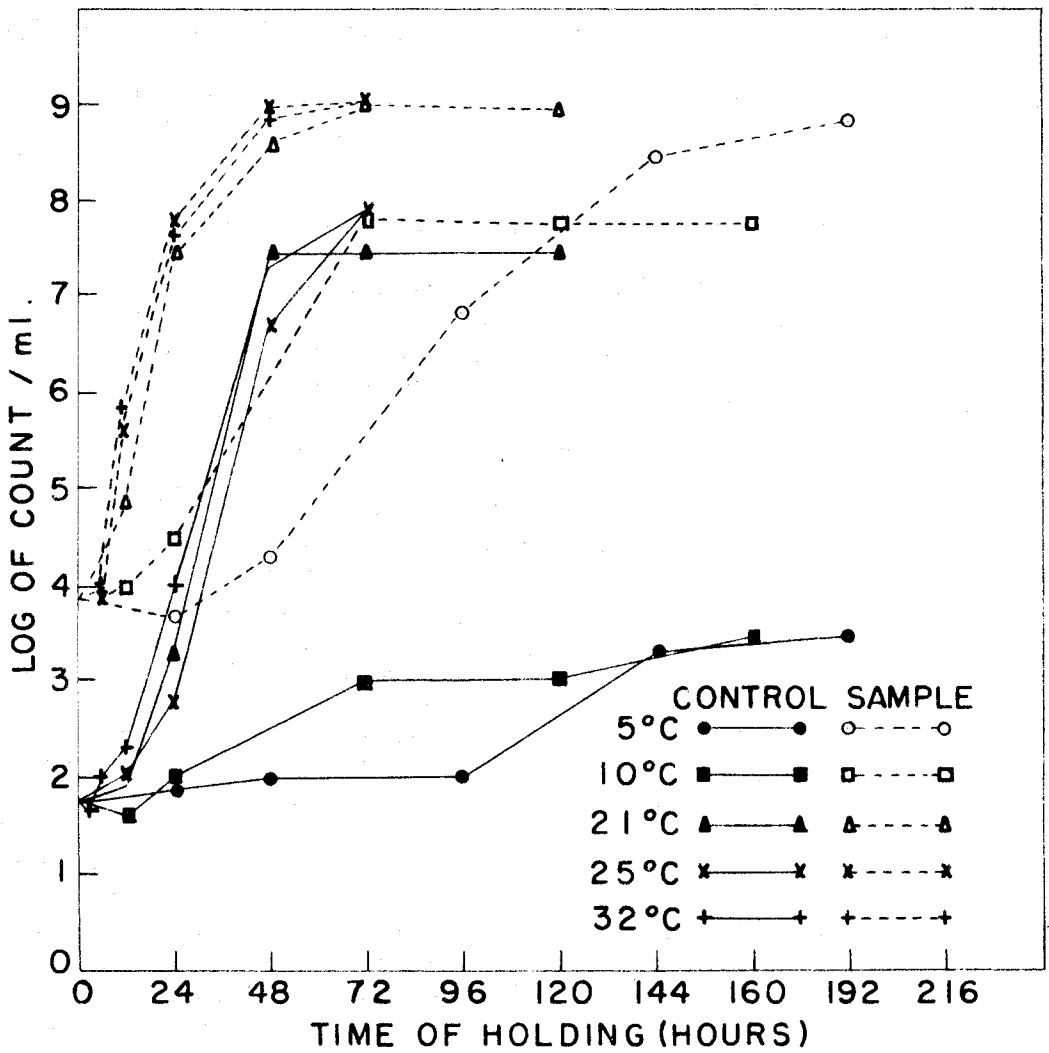

Figure 2. Counts on culture 1 grown in laboratory pasteurized milk.

Growth Curves in Sterile Skim Milk

The purified cultures were inoculated into sterile skim milk and growth curves prepared after holding at 5, 10, 21, 25 and 32°C. No controls were needed, as periodic checks for sterility of the skim milk indicated a complete lack of growth. Figure 3 shows the results for culture 3 grown in sterile skim milk. The population shows the same general trend as when the organism was grown in laboratory pasteurized milk. The curves at 21, 25 and 32°C. were nearly identical, while at 5 and 10°C. they rose to the same level but at a much slower rate. The 5 and 10°C. curves were separated by the increased lag phase at the lower temperature. When growth was initiated at these two temperatures the populations rose at nearly equal rates and to a similar height. This again is illustrated in Figure 4, which presents the growth curve obtained for culture 1 grown in sterile skim milk. The similarity of the two figures was very great and similar results also were obtained when cultures 8, 10 and 11 were grown in sterile skim milk.

Some cultures used showed variation from the general trend shown in Figure 3 and 4. Culture 9, when grown in sterile skim milk, grew very slowly, with a lag phase of about 48 hours at all temperatures tested and at a much slower rate, even in the logarithmic phase. Figure 5 shows

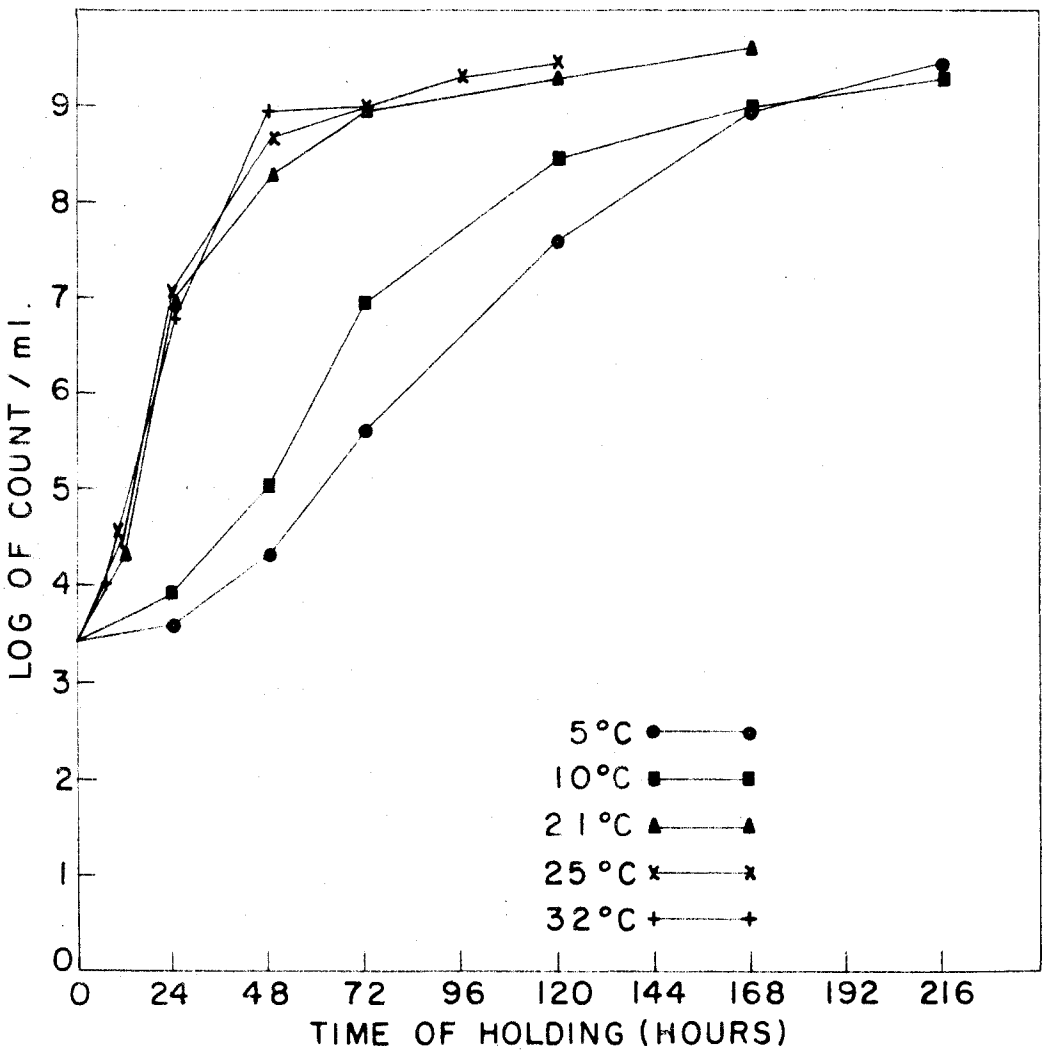

Figure 3. Counts on culture 3 grown in sterile skim milk.

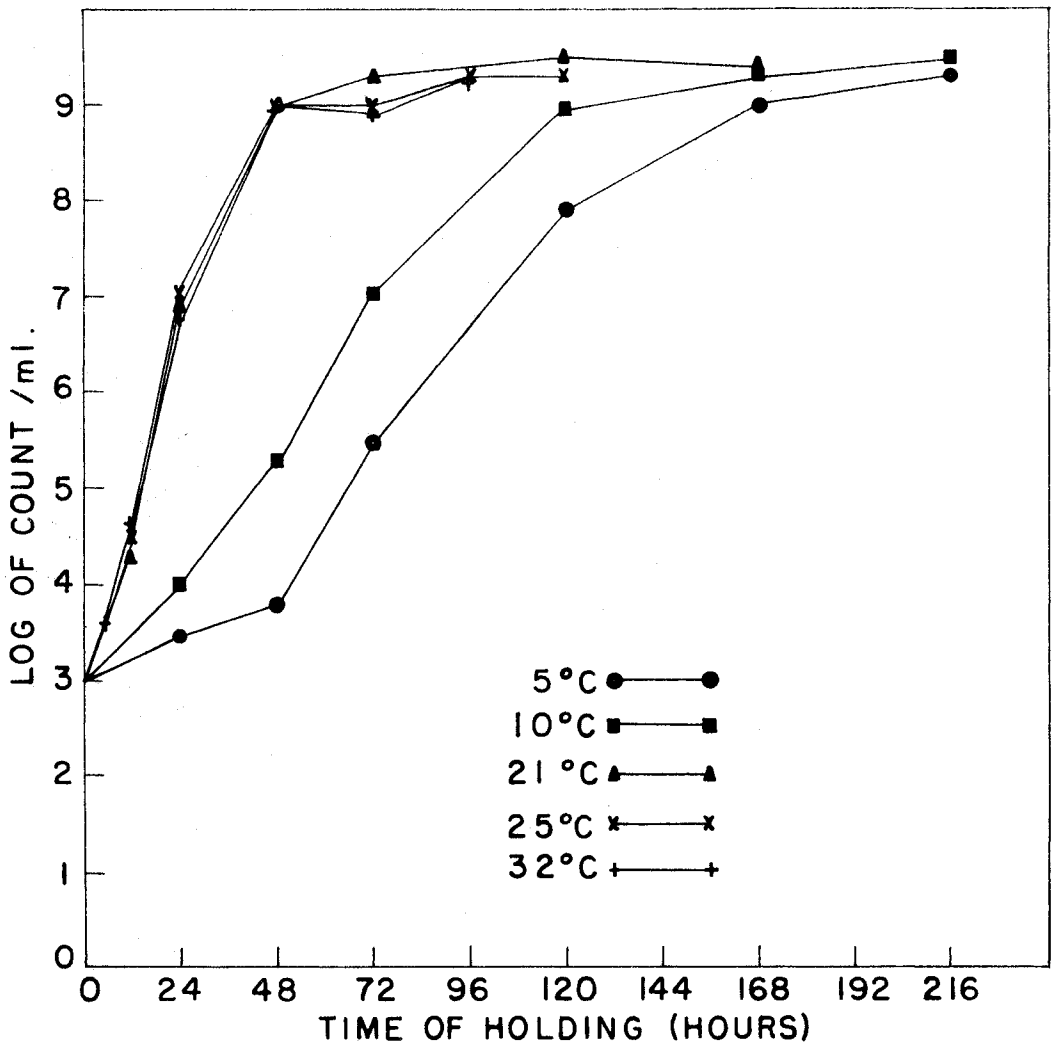

Figure 4. Counts on culture 1 grown in sterile skim milk.

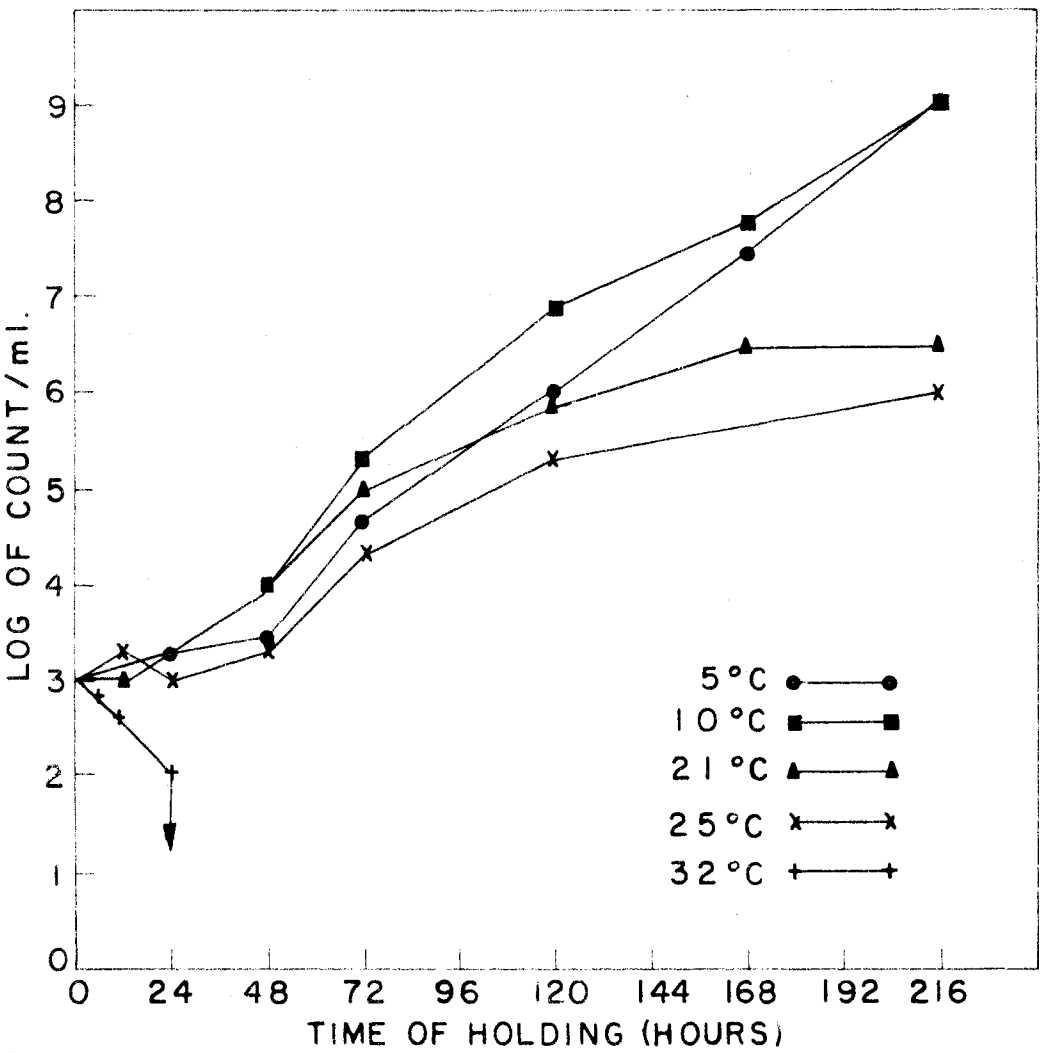

Figure 5. Counts on culture 9 grown in sterile skim milk.

that growth did not take place at 32°C., a result that was observed on three subsequent runs. At 21 and 25°C. the lag phase was about 48 hours, followed by a logarithmic phase which had a rate less than that obtained at 5 and 10°C. At the end of 9 days the count was only about 10^6 for the 21 and 25°C. cultures, as compared to 10^9 for the 5 and 10°C. cultures. Some of the poor results obtained may have been due to the poor growth of this species on most agar media, making the plate counts rather erratic.

Figure 6 shows the results obtained when culture 12 was added to sterile skim milk. This curve was fairly representative of the result obtained with cultures 4 and 13, as well as with 12. Growth was slower at 32°C. than at 21 and 25°C. This may have been because 32°C. is near the upper limit of growth for many low-temperature organisms. Growth at 5 and 10°C. rose to a higher level in these cases than that at 32°C.

In summarizing the activities of the various cultures in skim milk, on the basis of their growth curves, the cultures could be divided into three groups. Group 1 composed of cultures 1, 3, 8, 10 and 11 showed nearly identical growth rates at 21, 25 and 32°C. and in all cases one of the three temperatures was optimum for increase in numbers of the organisms. Group 2 contained only culture 9. This organism did not grow at 32°C. and had its optimum tempera-

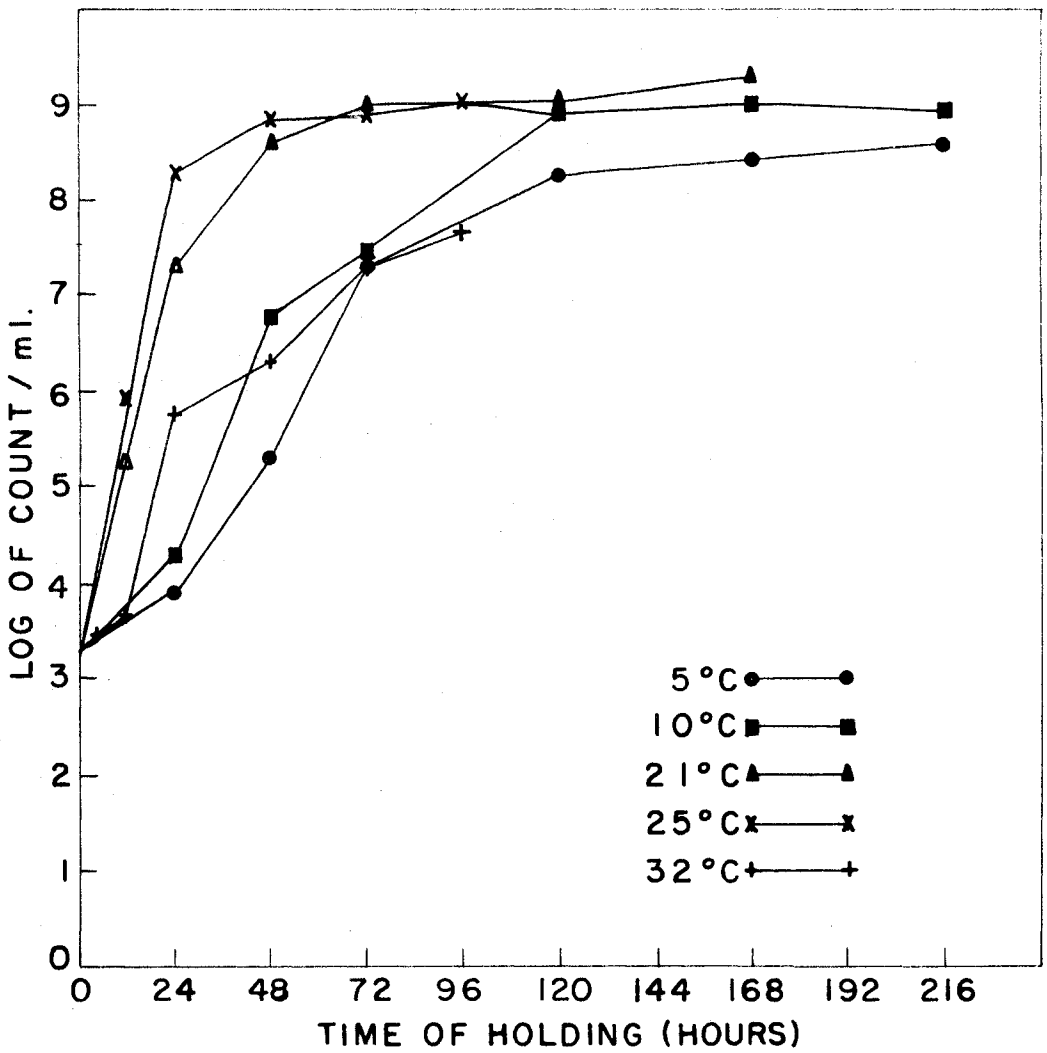

Figure 6. Counts on culture 12 grown in sterile skim milk.

ture for increase in numbers at 10°C.; it grew more rapidly and to a higher level at 5 and 10°C. than at 21 and 25°C. Group 3 included cultures 4, 12 and 13, which had growth rates greater at 21 and 25°C. than at 32°C. The final population level at 32°C. was usually surpassed by that at 5 and 10°C., although the growth rate usually was greater in the early stages when holding was at 32°C.

Generation Times of Cultures Grown in Sterile Skim Milk

The generation time for each sampling interval of each culture at each temperature was calculated from the growth curves obtained in sterile skim milk. The minimum generation time and the sampling interval from which it came are recorded in Table 1. The figures in this table indicate that 21 or 25°C. was the optimum temperature for the increase in numbers of the majority of the pure cultures studied and the logarithmic phase was in the first 24 hours after inoculation when these temperatures were used. Cultures 8 and 10 were exceptions, as the maximum rate was obtained at 32°C. with these cultures. When holding temperature was 5 or 10°C., the minimum generation time usually was not obtained until the 24-48 hour or the 48-72 hour interval.

Table 1

Generation Time of Cultures Incubated at Different Temperatures in Sterile Skim Milk

Culture Number	Incubation Temperature of Samples				
	5°C.	10°C.	21°C.	25°C.	32°C.
1	48-72[a] 255[b]	48-72 255	12-24 83	12-24 90	12-24 100
3	48-72 333	48-72 228	12-24 83	12-24 86	12-24 92
4	48-72 231	24-48 217	12-24 96	12-24 108	24-48 188
8	120-168 569	24-48 369	12-24 94	12-24 103	12-24 69
9	168-216 285	72-120 280	48-72 433	48-120 433	No Growth
10	48-72 222	24-48 221	0-24 108	0-12 82	6.12 54
11	48-72 375	48-72 285	12-24 94	12-24 108	12-24 108
12	48-72 217	24-48 175	0-24 108	0-12 83	12-24 104
13	48-72 231	24-48 199	12-24 80	0-12 86	6-12 207

[a]First row of figures for each culture is the interval in which the minimum generation time was obtained.

[b]Minimum generation time in minutes.

Effect of Varying Growth Temperature of Culture Prior to Use as Inocula for Growth Curves

All previous growth curves were made by using an inoculum which had been grown for 10 days at 5°C. In actual practice many of the contaminants which enter pasteurized milk may have grown at higher temperatures prior to entry. In order to check the effect of the growth of the inoculum at various temperatures on the growth curves obtained at 5, 10 and 25°C., cultures were grown for at least three transfers at the various temperatures before use as an inoculum. Figure 7 shows the growth curves at 5°C., obtained with culture 3, when the inocula were grown at 5, 10, and 25°C. prior to use. Figure 8 shows the results of the same inocula when holding was at 10°C. and Figure 9 shows the results when holding was at 25°C. From these figures it was not possible to detect any distinct difference in the growth curves that could be attributed to the treatment of the inocula prior to use. All nine cultures were checked in duplicate by this same procedure and no definite trend could be established. It was apparent from these data that if one of these organisms contaminated milk, the temperature at which it had been grown prior to its entry into the milk would have very little effect on its resulting growth.

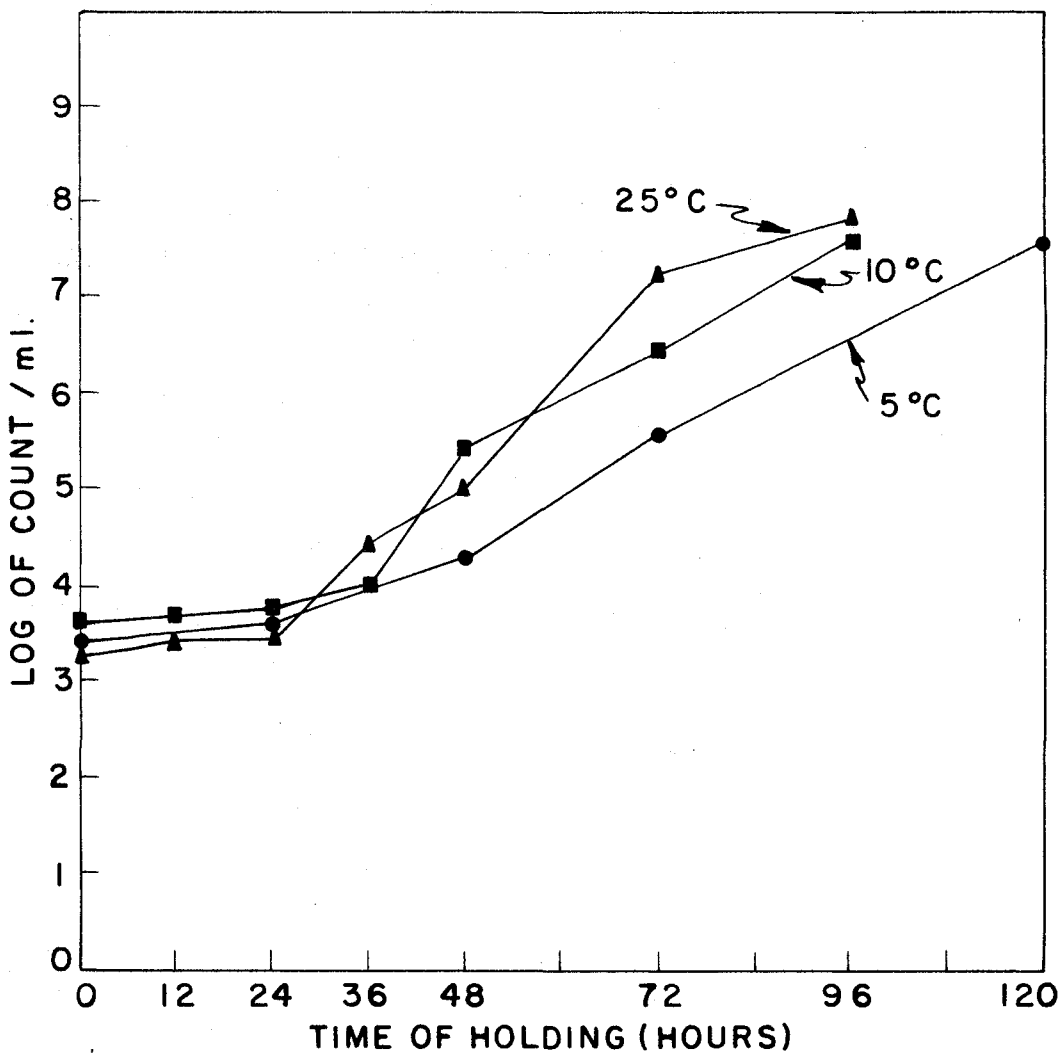

Figure 7. Counts on culture 3 held at 5°C. in sterile skim milk after inocula had been grown at 5, 10 and 25°C.

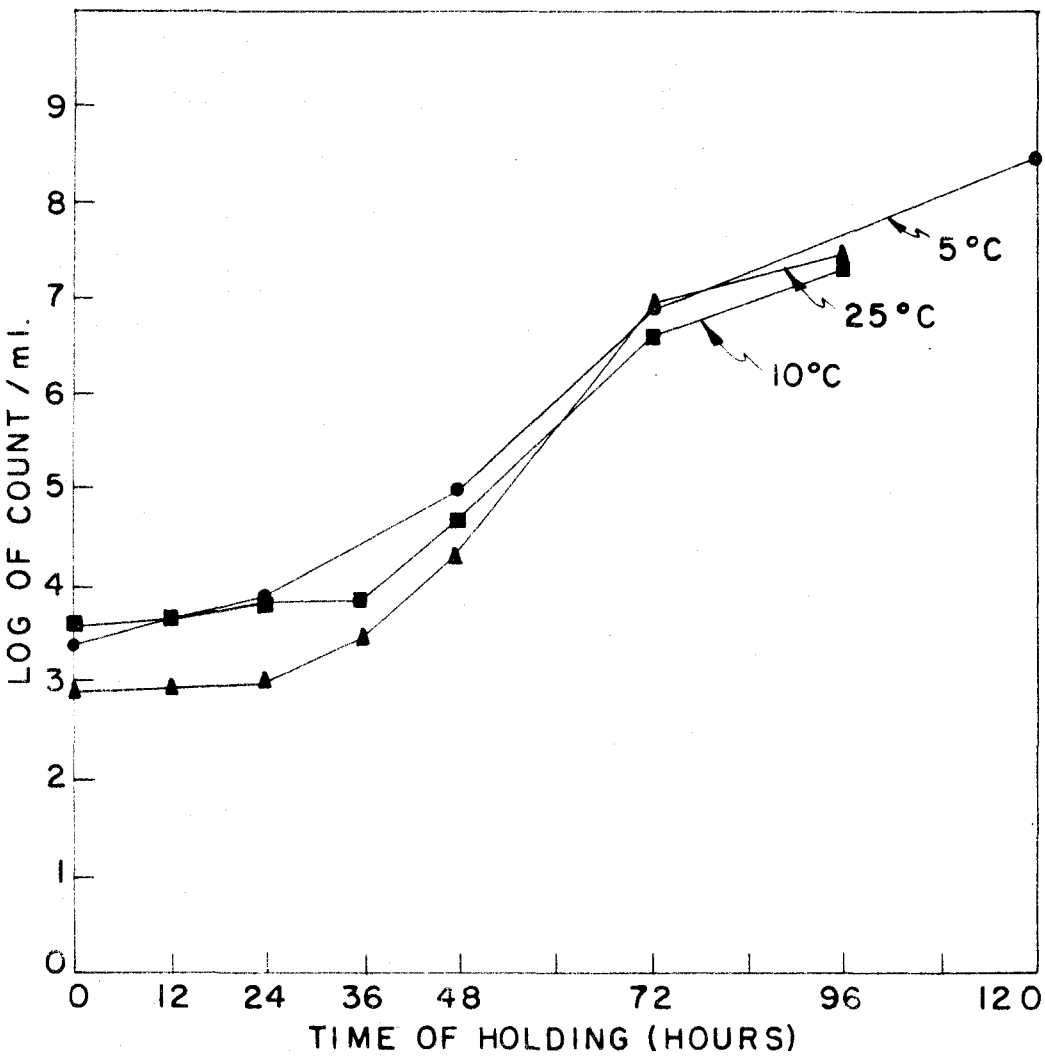

Figure 8. Counts on culture 3 held at 10°C. in sterile skim milk after inocula had been grown at 5, 10 and 25°C.

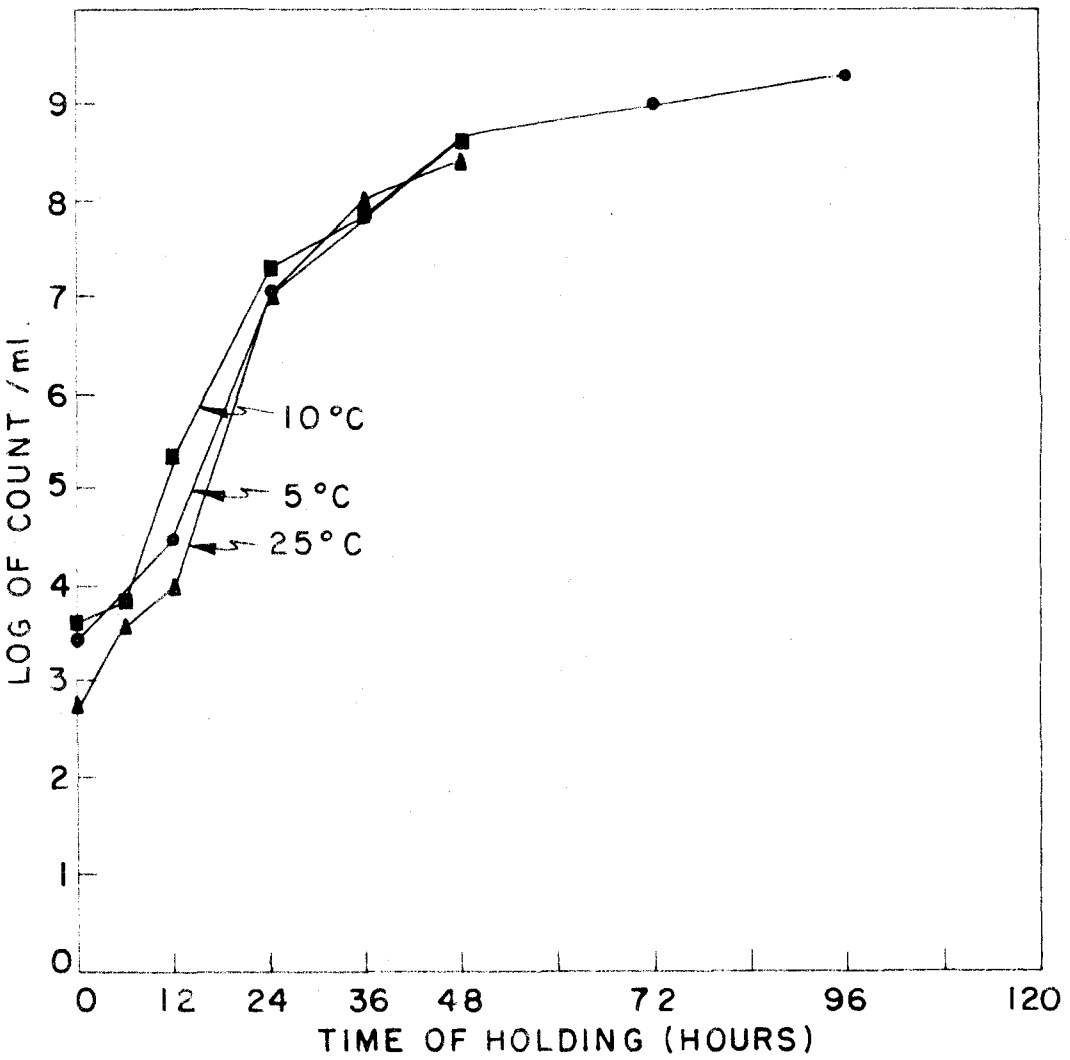

Figure 9. Counts on culture 3 held at 25°C. in sterile skim milk after inocula had been grown at 5, 10 and 25°C.

Variation in Size of Inocula

All previous growth curves were made with an initial inoculum of 100 up to a few thousand organisms per milliliter. As it is possible that the size of the inoculum may have some effect on the resulting growth durve, it was decided to test this possibility with the pure cultures used in this study.

Cultures 1, 3 and 12 were tested with three levels of inoculum, the second and third being 10-fold and 1,000-fold dilutions of the first. Results were similar with the three cultures on duplicate runs, so a single one will be used to illustrate the effect. Culture 3 was held at 5 and 25°C. and the resulting growth curves for a three inocula were plotted in Figures 10 and 11, respectively. The plot for zero time on the lowest dilution in both cases is an estimate, as the 1,000-fold dilution was great enough to indicate that the size of the inoculum had little or no effect on the rate of growth at either temperature. The three curves on each figure rise on a nearly parallel course, separated only by the initial distance brought about by the difference in size of the inoculum. The final numbers were not appreciably different for the three dilutions at either of the temperatures of holding. Placing the point for zero hour count of the lowest dilution at an arbitrary

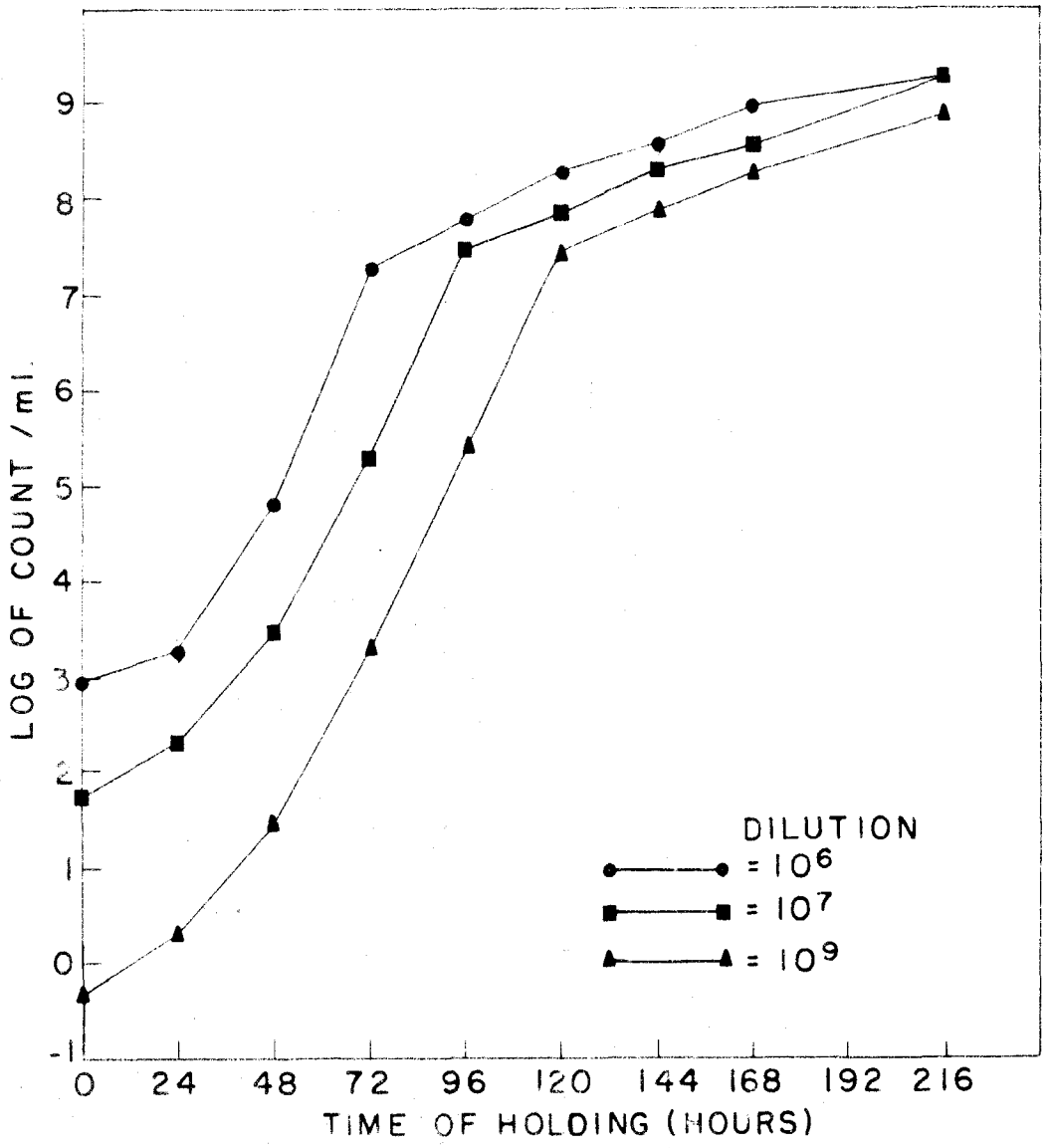

Figure 10. Growth curves of three dilutions of culture 3 when held at 5°C.

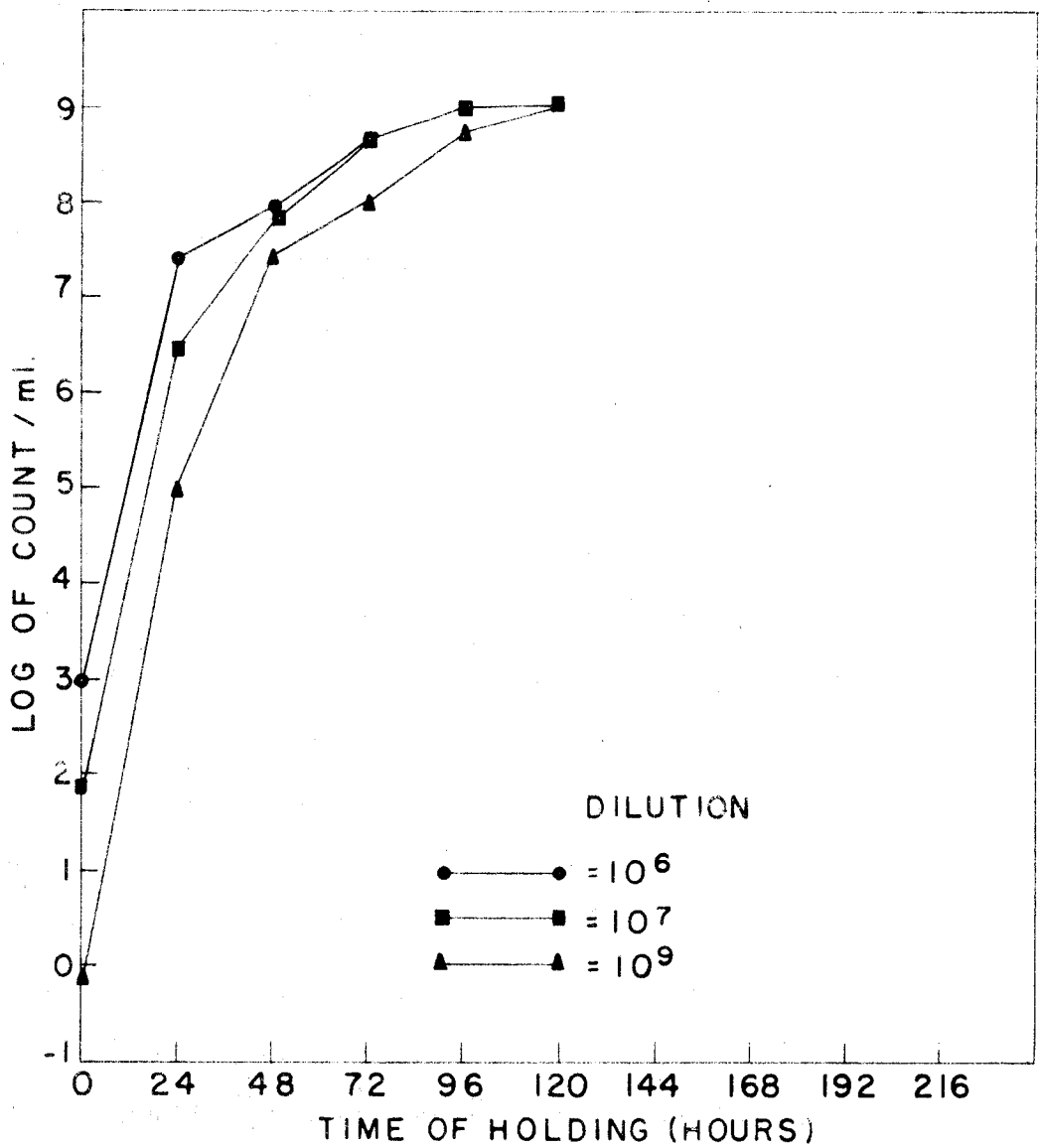

Figure 11. Growth curves of three dilutions of culture 3 when held at 25°C.

position based on dilution may not give an exact position but variation about this point likely would not be great enough to cause any appreciable change in the interpretation.

Effect of Warm-up on Growth Curves of Cultures Held at 5 and 10°C.

In the normal distribution and consumption of milk there often are periods of time when the milk is held at room temperature followed by return to low temperature storage. It was thought desirable to test the effect of a rise in temperature at different points on the growth of cultures at 5 and 10°C. Cultures 1, 3 and 12 were selected for tests in this manner. A quantity of sterile milk was inoculated with one of the pure cultures and divided into two parts. One part served as a control and one for treatment. Each member of the pair was further subdivided into screw cap test tubes so a single tube was available for each sampling period. A culture grown for 10 days at 5°C. was used as an inoculum. The samples were held in the air at room temperature for 3 hours at various times in the growth curves. All three cultures tested showed essentially the same response, so culture 12 was selected to illustrate the results. Table 2 shows the counts obtained when holding was at 5°C. with room temperature treatments at the 0-3, 12-15 and 24-27 hour intervals. When held at room tempera-

Table 2

Counts per Milliliter Obtained on Growth of Culture 12 at 5°C. when Held at Room Temperature[a] for 3 Hours at Different Times during the Holding Period

Time Sample Taken (Hours)	Intervals at Which Samples Were Held at Room Temp.					
	0 to 3 Hours		12 to 15 Hours		24 to 27 Hours	
	Sample	Control	Sample	Control	Sample	Control
0	10×10^2	10×10^2	16×10^2	16×10^2	10×10^2	10×10^2
3	13×10^2	14×10^2				
12	14×10^2	14×10^2	16×10^2	12×10^2	11×10^2	10×10^2
15			22×10^2	11×10^2		
24	43×10^2	18×10^2	46×10^2	21×10^2	19×10^2	20×10^2
27					11×10^3	23×10^2
48	17×10^4	53×10^3	18×10^4	50×10^3	12×10^4	40×10^3
72	36×10^5	13×10^5	68×10^5	28×10^5	32×10^5	24×10^5
96	35×10^6	27×10^6	42×10^6	30×10^6	45×10^6	32×10^6
120	20×10^7	83×10^6	19×10^7	98×10^6	94×10^6	78×10^6
144	38×10^7	24×10^7	32×10^7	34×10^7	16×10^7	19×10^7
168	65×10^7	27×10^7	46×10^7	43×10^7	30×10^7	46×10^7
216	75×10^7	63×10^7	72×10^7	98×10^7	82×10^7	95×10^7

[a]Room temperature was 23°C. and the samples took about 90 minutes to reach that temperature.

ture for 0-3 hours, the effect was not noticeable until the culture began to enter the logarithmic phase at 24 hours, when the sample grew at a slightly greater rate than the control for a brief interval. The spread remained fairly constant until 144 hours, when all effect of treatment was lost. When treatment was at the 12-15 hour interval, the difference again was noticeable at 24 hours and continued until 144 hours. When treatment was at the 24-27 hour interval the effect took place at once and continued to 144 hours. It was apparent from these results that the effect of the short exposure to room temperature was delayed and resulted in a slightly increased rate of growth during the transfer from the lag phase to the early logarithmic phase or a shortening of the lag phase in the treated samples. Table 3 gives the counts obtained when holding of the culture was at 10°C. and the room temperature treatment took place at the 12-15 and 24-27 hour intervals. Results at this temperature were similar to those obtained at 5°C. The effect of treatment became apparent about the same time, regardless of the time it was applied. The treatment tended to shorten the transitional interval between the lag and logarithmic phases. This apparently was due to a more rapid attainment of the logarithmic rate by the treated culture. Once the true logarithmic rate had been achieved, it was essentially the same for both control and treated culture. By the end

Table 3

Counts per Milliliter Obtained on Growth of Culture 12 at 10°C. when Held at Room Temperature[a] for 3 Hours at Different Times during the Holding Period

Time Sample Taken (Hours)	Intervals at Which Samples Were Held at Room Temp.			
	12 to 15 Hours		24 to 27 Hours	
	Sample	Control	Sample	Control
0	$28x10^2$	$28x10^2$	$29x10^2$	$29x10^2$
12	$80x10^2$	$84x10^2$	$49x10^2$	$51x10^2$
15	$30x10^3$	$12x10^3$		
24	$43x10^4$	$17x10^4$	$70x10^3$	$74x10^3$
27			$55x10^4$	$24x10^4$
36	$10x10^6$	$30x10^5$	$80x10^5$	$42x10^5$
48	$26x10^6$	$17x10^6$	$27x10^6$	$18x10^6$
72	$87x10^6$	$80x10^6$	$92x10^6$	$58x10^6$
96	25x10	18x10	L.A.[b]	L.A.

[a]Room temperature was 23°C. and the samples took about 75 minutes to reach that temperature.

[b]Laboratory accident.

of the growth period studied, the counts of the controls were nearly equal to, and in some cases exceeded, the counts of the treated samples. This effect is undoubtedly due to the early action of those factors which serve to limit final organism population.

Studies on Partial Heat Destruction

The psychrophilic bacteria are usually not considered to be pasteurization resistant, yet nearly all pasteurized products in commerce contain some of these organisms. Their presence probably must be attributed to post-pasteurization contamination from equipment with which the pasteurized product comes in contact. Much of the milk handling equipment, such as pipe lines, vats and bottle fillers, is treated by heat before use. During such treatment it is quite possible that some organisms may suffer a sub-lethal dose of heat, survive and be seeded into the pasteurized product as it passes through the equipment. The following experiments were designed to study the growth of partially heat-killed cultures and determine the difference in reaction from a control culture which had not been subjected to partial heat destruction.

Cultures 3 and 12 were selected from the initial tests in this area and were given heat treatments to kill a high percentage of the organisms. A control was run with all

trials by diluting an unheated culture so the initial concentration was in the same range as that expected for the heat treated culture. Figure 12 shows the results of heating culture 3 for 7 minutes at 52°C. The count was 46 x 10^7 per milliliter prior to heating and the survivor count was 13 x 10^2 per milliliter, or about 0.0003 percent. The surviving culture had to be diluted 1:100 to give a large enough sample to prepare growth curves at the three temperatures. The figure shows that the count of the sample held at 5°C. remained at less than 10 per milliliter for the first 72 hours and then decreased to an undetectable level with no reappearance by the end of 216 hours. Holding at 10°C. resulted in the culture remaining at or near zero count for 72 hours, followed by a fairly rapid rise to the same level as the control after 216 hours. When holding was at 25°C., the shape of the curve seemed to be unaffected by the heat destruction, except for an increase of about 12 hours in the lag phase. The surviving cells began to increase in less than 24 hours, on a course nearly parallel to that of the control and equalled it by the end of 120 hours and possibly sooner, since the datum for one point was not obtained. In many cases where destruction was not as great as that indicated in Figure 12, the culture was able to recover at 5°C. holding but the lag phase was increased to 96 hours or longer, followed by a normal rise for a culture held at 5°C. This

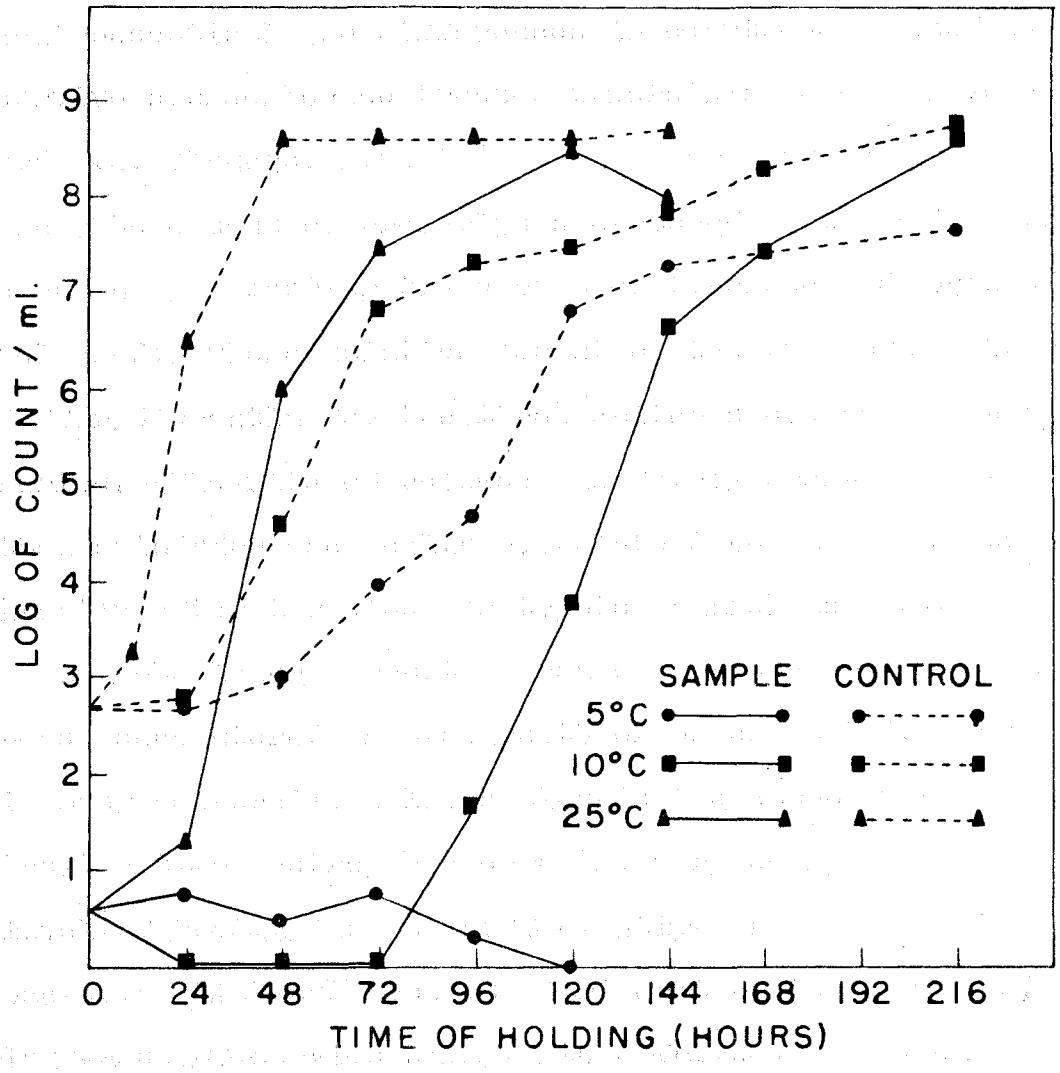

Figure 12. Growth of culture 3 at three temperatures after partial heat destruction (99.9997%).

type is illustrated in Figure 13, where at 5°C. holding there is a drop and a lag phase until 96-120 hours, after which the culture begins to rise until it reaches a level similar to the control at 216 hours. The lag at 10°C. holding extends for 48 hours or slightly longer and then rises parallel to the control until they converge at 168 hours. Holding at 25°C. apparently prolonged the lag phase slightly, followed by a rise essentially parallel to the control.

Culture 12 was more heat resistant, both as to temperature level or time required to effect partial killing and as to the modified effect on recovery at 5°C. Figure 14 illustrates the effect of heating culture 12 for 6 minutes at 60°C. The initial count was 56×10^6 per milliliter and survival was 30×10^4 per milliliter. The survival was considerably higher than that shown in Figure 12 but the effect on growth during holding at 5°C. was still quite distinct. The lag phase was prolonged for 72 hours, followed by a rise to a level slightly greater than the control at 168 hours. At 10°C. there was very little increase for the first 48 hours but the count then rose parallel to the control. Heat destruction apparently prolonged the lag phase of the culture growing at 25°C. Only a difference in slope of the growth curve furnishes a basis for this conclusion, since no count was made at 12 hours.

One case was observed where the results obtained with

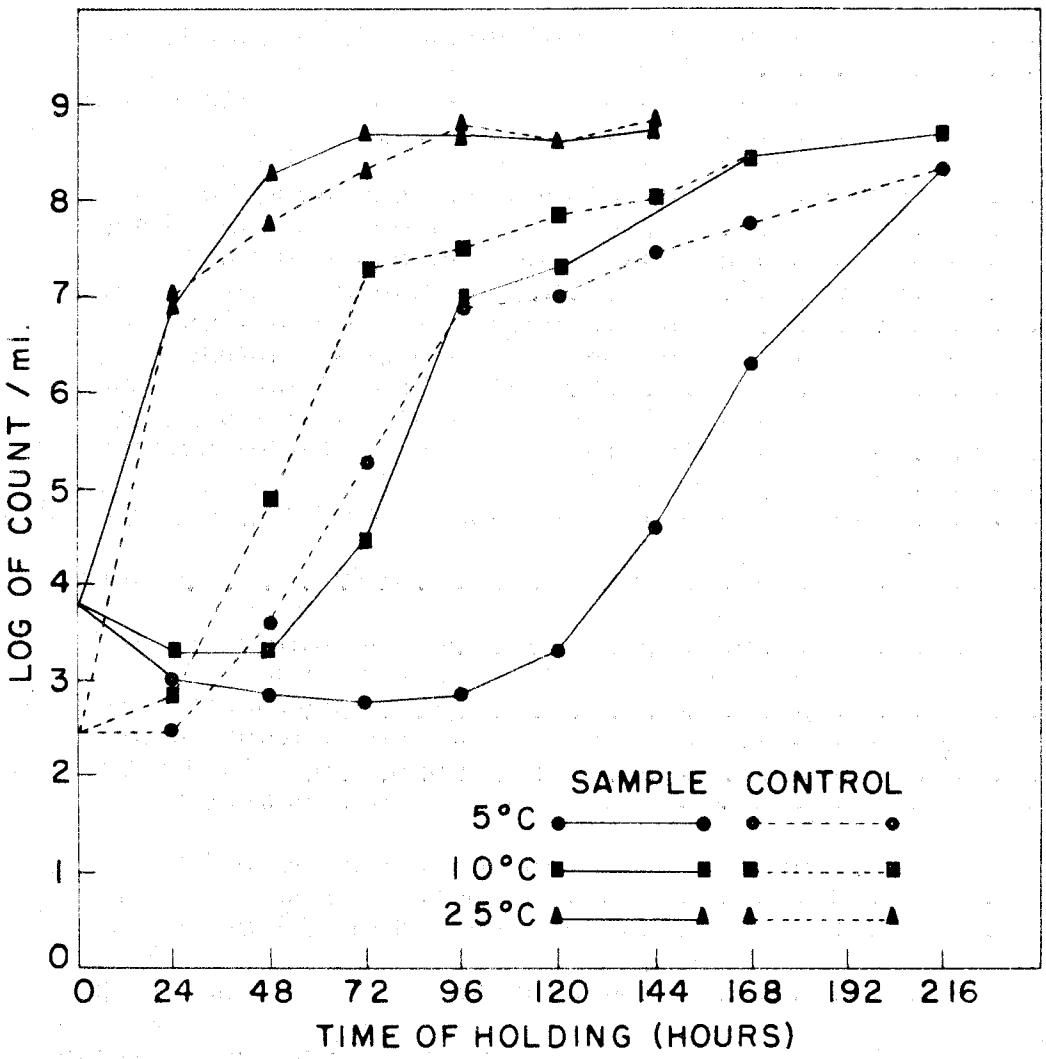

Figure 13. Growth of culture 3 at three temperatures after partial heat destruction (99.75%).

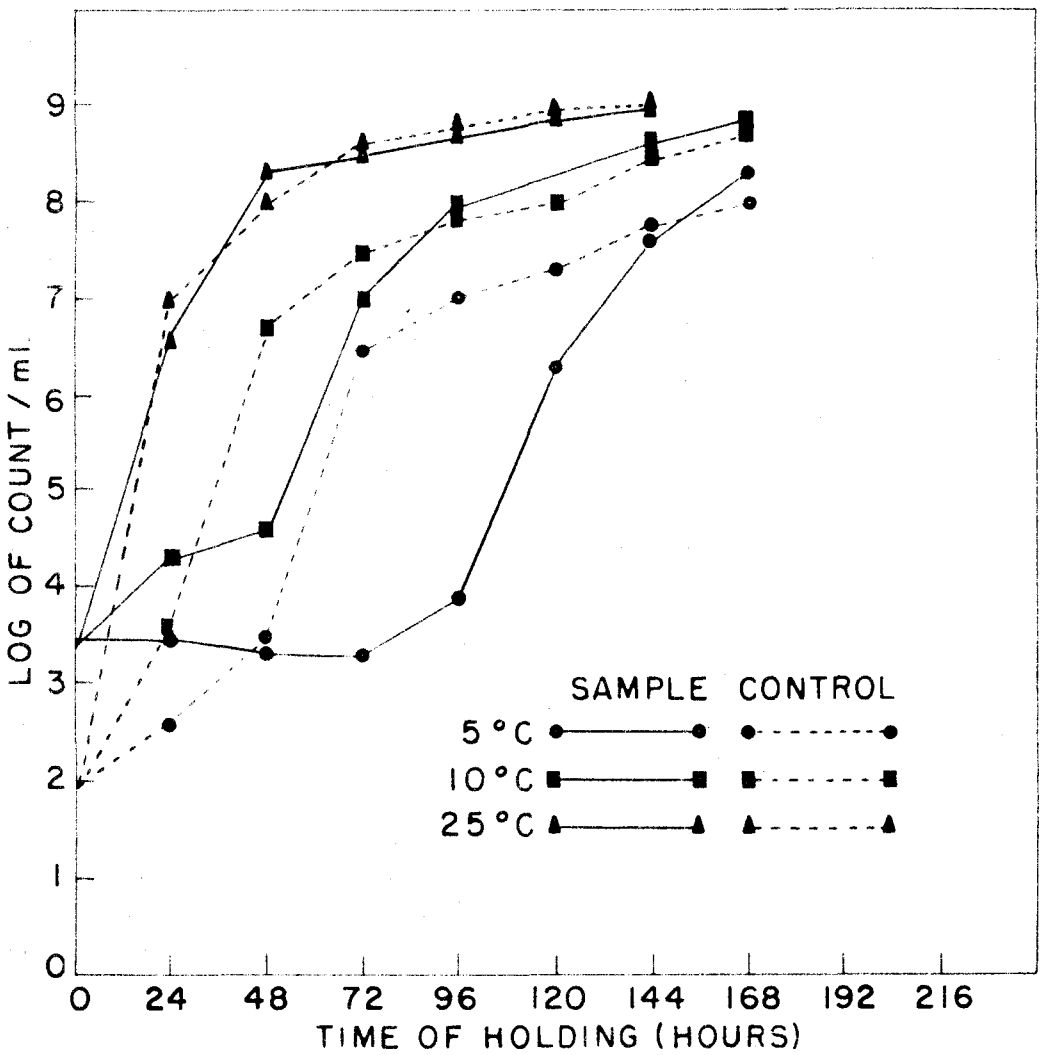

Figure 14. Growth of culture 12 at three temperatures after partial heat destruction (99.50%).

culture 12 were not so pronounced. As illustrated in Figure 15, destruction was about 99.83 percent when initial count was 95 x 10^6 and the heating was for 6 minutes at 60°C. When the culture was diluted 1:100 and then was grown at 25°C. there was an almost immediate rise of the survivors, with a lag phase apparently considerably less than 24 hours. When the heat-treated culture was inoculated and held at 10°C., some retardation beyond that found in the control was observed, indicating some prolongation of the lag phase. At 5°C. holding, the culture population rose from the beginning and increased 10-fold in the first 72 hours instead of showing the customary drop. However, the population increase was much less than that of the control, so a definite lag phase may still be said to exist. Subsequent to this, the rise was parallel to that of the control. In the many experiments, using this and other cultures, such rapid recovery from heat was not encountered again. An explanation for these different results was hard to find but the phenomenon may have been due to some inconsistancy in procedure which gave a quantitative difference.

To avoid the possibility of the phenomenon caused by heat being characteristic only of the two cultures used, cultures 1 and 10 were selected at random and subjected to partial heat destruction. Figure 16 gives the results obtained with culture 10 and these results are very similar to

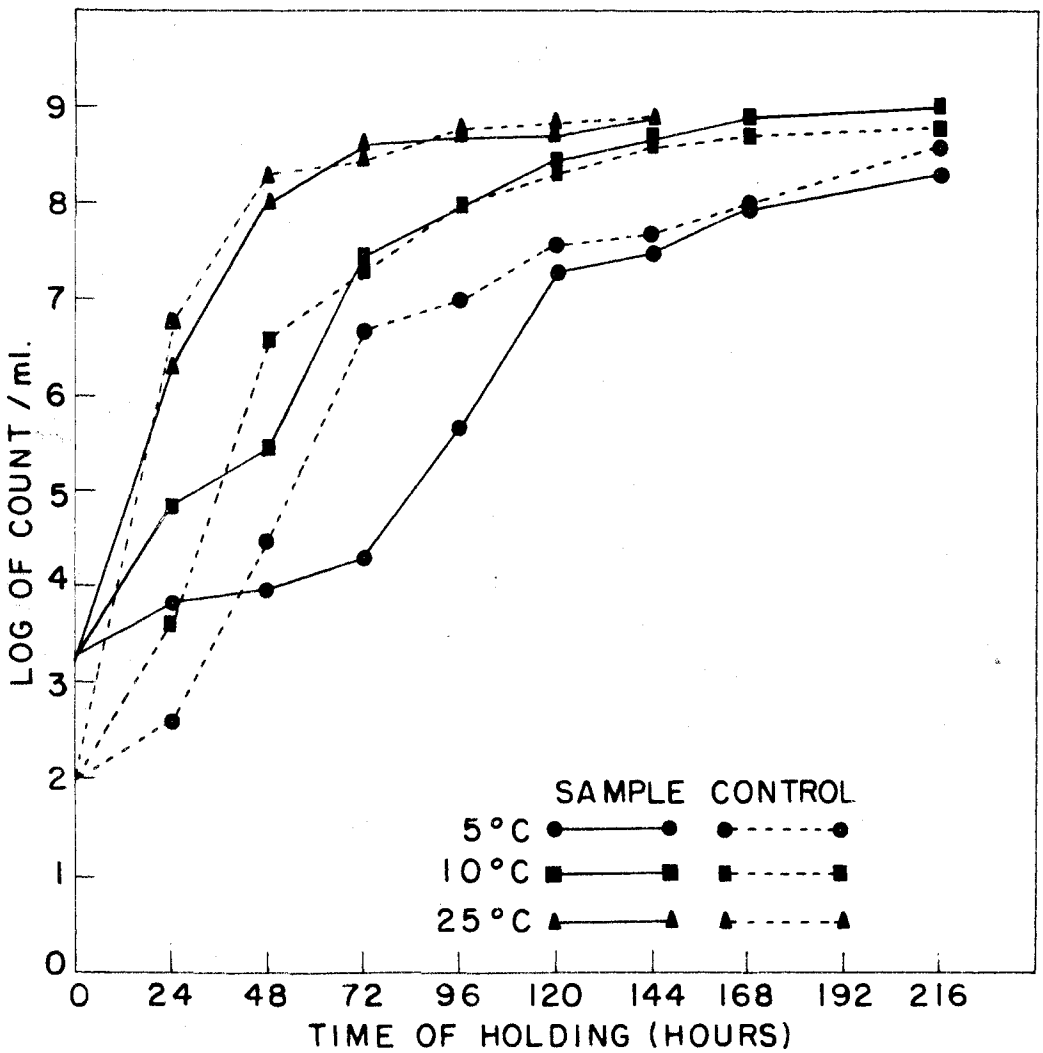

Figure 15. Growth of culture 12 at three temperatures after partial heat destruction (99.83%).

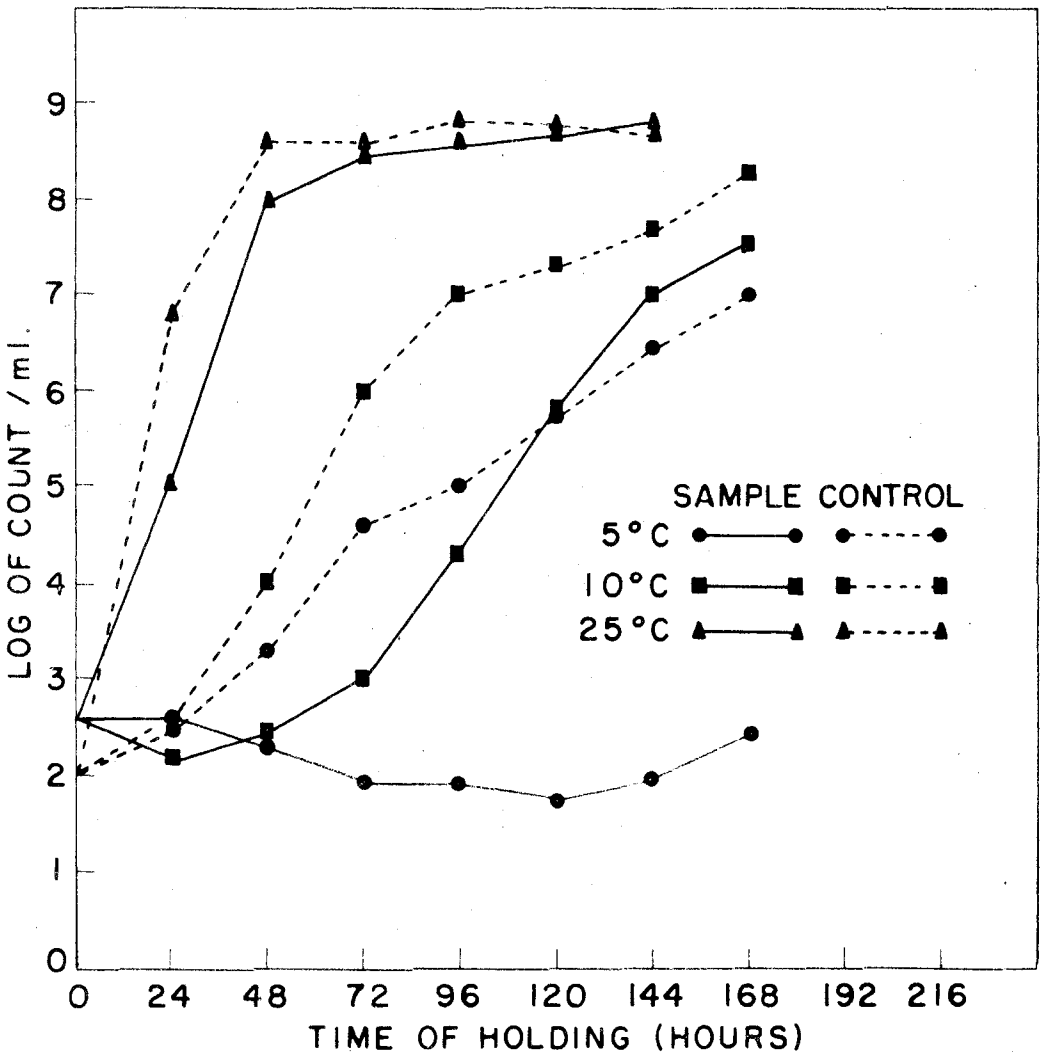

Figure 16. Growth of culture 10 at three temperatures after partial heat destruction (99.96%).

those usually obtained with cultures 3 and 12, as well as culture 1. The initial count with this culture was 14 x 10^7 per milliliter and survival was 60 x 10^3 per milliliter after heating for 9 minutes at 52°C. The results were more distinct than those shown in Figure 14, as survivors in the culture inoculated and held at 5°C. showed no increase until 144 hours. Holding at 10°C. resulted in a drop in numbers, followed by an increase after 48 hours. The level reached at both holding temperatures was lower than the control in the 168 hour test period, but may have been similar if the test had been carried longer.

In an attempt to study further the peculiar response of partially heat-killed organisms to variations in holding temperature, it was decided to further dilute the surviving organisms and see if numbers of organisms had any effect on recovery. Figure 17 shows that dilution had no more effect on the heat-treated culture than was observed in normal dilution of culture. At all three temperatures there was no noticeable difference caused by dilution of the sample.

Survival of Partially Heat-Inactivated Cultures on Two Types of Agar

During the course of this work Tryptone Glucose Yeast Extract (plate count) agar was adopted as the new standard

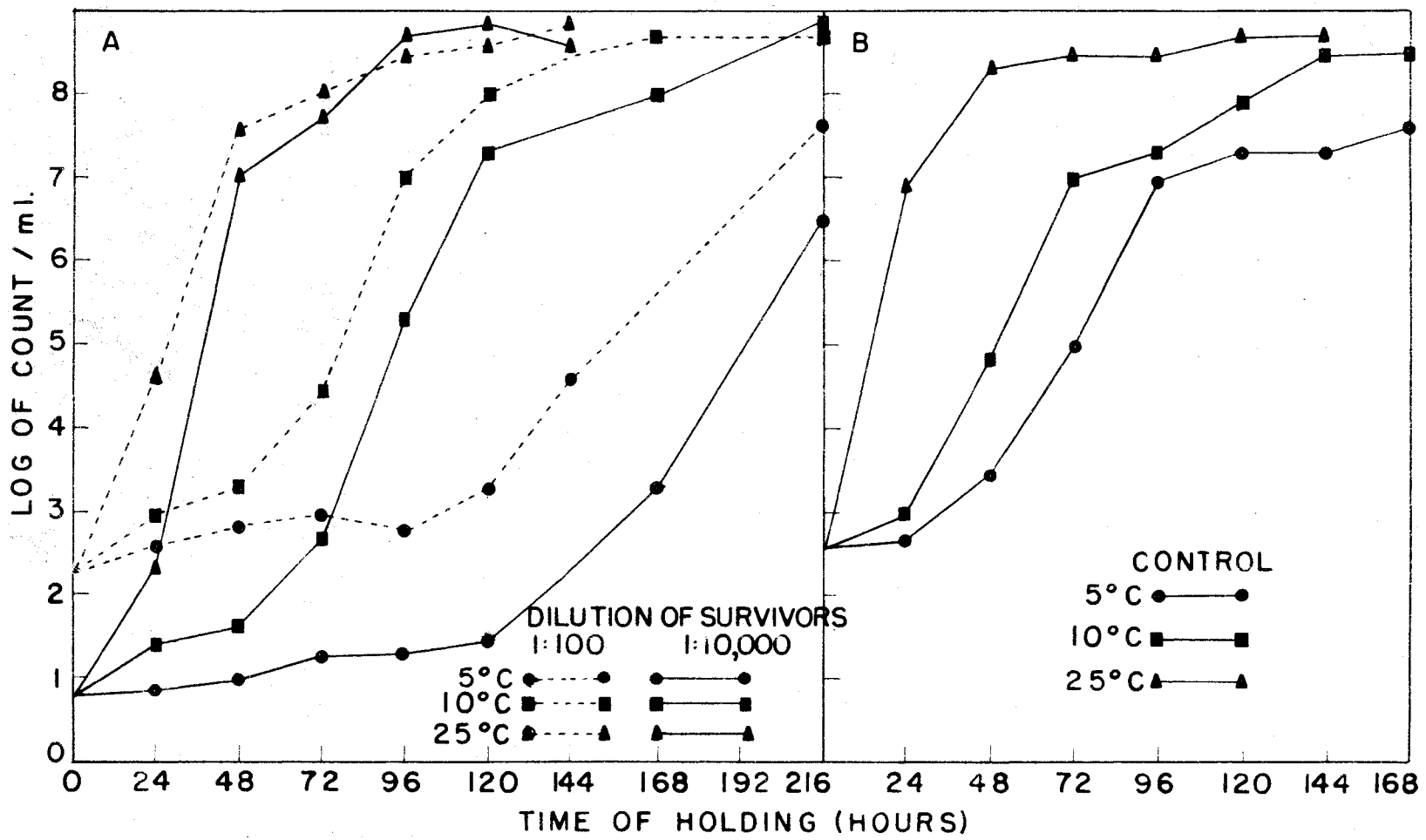

Figure 17. Effect of dilution on partially heat-killed cultures. A - curves of survivors at two dilution levels. B - curves of control samples.

plate count agar (1), so it was thought advisable to make a comparison with the T.G.E. agar used in the earlier part of this work. Duplicate plates were poured from a single set of samples and dilutions, one set for each type of agar. The results for the two agars are plotted side by side in Figure 18. An unusual condition is shown in the early stages of growth. The survival measured on the T.G.E. agar was considerably less than that obtained on the new Plate Count agar; the unheated controls showed similar curves on both types of agar. The counts on T.G.E. agar for 5°C. holding rose slightly and remained stable until after 96 hours, while the count on the Plate Count agar dropped until it was similar to that on the T.G.E. agar and remained on a fairly equal level throughout the balance of the growth period. When storage was at 10°C., the counts on the two agars were essentially the same after 24 hours and remained almost identical for the remainder of the observation period. At 25°C. the counts on the new medium indicated a 24 hour lag phase followed by rapid growth; on T.G.E. agar no such lag phase was indicated. The counts on the two agars were almost exactly the same at 24 hours and beyond. The apparent lag phase observed on Plate Count agar at 25°C could be due to enumeration, at zero time, of many organisms which were not enumerated by T.G.E. agar and were unable to grow in milk. The organisms able to grow were possibly about the same as

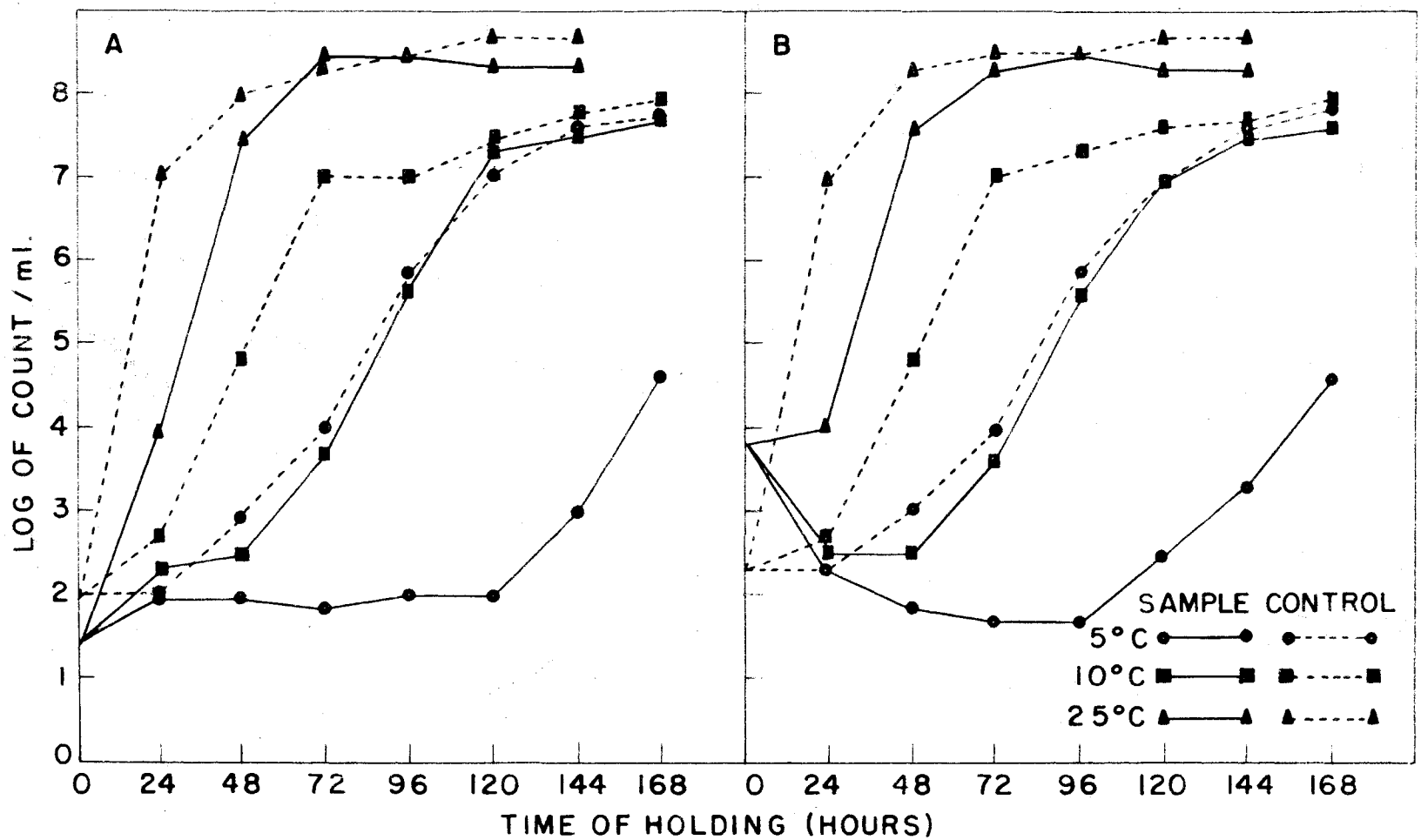

Figure 18. Growth of culture 3 after heating at 52°C. for 5 1/2 minutes when count prior to heating was 17×10^7. A - plating on T.G.E. agar. B - plating on Plate Count agar.

those enumerated on T.G.E. agar, with the remaining organisms dying off during the first 24 hours. The high initial count tended to give the false appearance of a lag phase due to the die-off nearly balancing the increase in numbers. When T.G.E. agar was used for enumeration, the high initial numbers were not counted so no die-off was observed to counteract the growth enumerated by the plate count on T.G.E. agar. Similar results were obtained when culture 12 was grown on the two agars. Five separate trials, using culture 3 and 12, gave similar results, indicating that the difference between counts on the two media was characteristic, at least for the two organisms tested.

Table 4 shows a case where initial survival was low, being about 54×10^2 when plated on T.G.E. agar and 24×10^3 when plated on Plate Count agar. After 24 hours at 5°C. the count on Plate Count agar had dropped to slightly less than that on T.G.E. agar and counts on the two media remained nearly equal and did not rise during 144 hours, although both increased slightly at 168 hours. When holding at 10°C., growth began after 72 hours, as indicated by platings on both agars. This table shows a case of relatively pronounced inhibition of a culture held at 25°C., as the increase in the first 24 hours was considerably lower, on both agars, than that of the control.

Table 5 shows the result of heat treating culture 12

Table 4

Counts per Milliliter of Partially Heat-inactivated Culture 3 and Untreated Controls Held at 5, 10, and 25°C. and Plated on Two Agars

Time Sample Taken (Hours)	Type of Agar Used	Temperature of Holding of: Heat-treated Sample 5°C.	Heat-treated Sample 10°C.	Heat-treated Sample 25°C.	Unheated Control 5°C.	Unheated Control 10°C.	Unheated Control 25°C.
0	T.G.E.	35×10^{0}	35×10^{0}	35×10^{0}	79×10^{0}	79×10^{0}	79×10^{0}
24		74×10^{0}	82×10^{0}	10×10^{2}	17×10^{1}	28×10^{1}	96×10^{5}
48		61×10^{0}	64×10^{0}	22×10^{6}	16×10^{2}	10×10^{3}	16×10^{7}
72		27×10^{0}	$<10^{2}$	77×10^{6}	10×10^{3}	27×10^{5}	16×10^{7}
96		34×10^{0}	11×10^{2}	58×10^{7}	76×10^{3}	18×10^{6}	21×10^{7}
120		20×10^{0}	47×10^{3}	32×10^{7}	88×10^{4}	26×10^{6}	22×10^{7}
144		30×10^{0}	96×10^{5}	46×10^{7}	45×10^{5}	43×10^{6}	27×10^{7}
168		97×10^{0}	37×10^{6}		13×10^{6}	58×10^{6}	
0	Plate Count (Difco)	23×10^{1}	23×10^{1}	23×10^{1}	58×10^{0}	58×10^{0}	58×10^{0}
24		32×10^{0}	38×10^{0}	78×10^{1}	13×10^{1}	22×10^{1}	12×10^{6}
48		39×10^{0}	41×10^{0}	12×10^{6}	23×10^{2}	80×10^{2}	16×10^{7}
72		22×10^{0}	$<10^{2}$	71×10^{6}	16×10^{3}	20×10^{5}	24×10^{7}
96		21×10^{0}	11×10^{2}	55×10^{7}	76×10^{3}	21×10^{6}	16×10^{7}
120		20×10^{0}	32×10^{3}	40×10^{7}	88×10^{4}	26×10^{6}	17×10^{7}
144		34×10^{0}	94×10^{5}	47×10^{7}	57×10^{5}	43×10^{6}	19×10^{7}
168		77×10^{0}	24×10^{6}		15×10^{6}	10×10^{7}	

Table 5

Counts per Milliliter of Partially Heat-inactivated Culture 12 and Untreated Controls Held at 5, 10, and 25°C. when Plated on Two Agars

Time Sample Taken (Hours)	Type of Agar Used	Temperature of Holding of: Heat-treated Sample 5°C.	Heat-treated Sample 10°C.	Heat-treated Sample 25°C.	Unheated Control 5°C.	Unheated Control 10°C.	Unheated Control 25°C.
0	T.G.E.	60×10^{0}	60×10^{0}	60×10^{0}	37×10^{0}	37×10^{0}	37×10^{0}
24		50×10^{1}	16×10^{2}	54×10^{5}	12×10^{1}	30×10^{1}	74×10^{5}
48		12×10^{2}	71×10^{2}	23×10^{7}	40×10^{2}	10^{4}	20×10^{7}
72		21×10^{2}	12×10^{4}	34×10^{7}	27×10^{4}	58×10^{5}	34×10^{7}
96		86×10^{2}	19×10^{6}	38×10^{7}	48×10^{5}	26×10^{6}	36×10^{7}
120		94×10^{3}	87×10^{6}	53×10^{7}	74×10^{5}	66×10^{6}	61×10^{7}
144		13×10^{5}	24×10^{7}	61×10^{7}	11×10^{6}	17×10^{7}	78×10^{7}
168		17×10^{6}	47×10^{7}		13×10^{7}	17×10^{7}	
0	Plate Count (Difco)	17×10^{1}	17×10^{1}	17×10^{1}	36×10^{0}	36×10^{0}	36×10^{0}
24		41×10^{1}	18×10^{2}	60×10^{5}	10×10^{1}	40×10^{2}	66×10^{5}
48		84×10^{1}	67×10^{2}	10×10^{7}	44×10^{2}	48×10^{3}	19×10^{7}
72		27×10^{2}	12×10^{4}	32×10^{7}	22×10^{4}	64×10^{5}	30×10^{7}
96		76×10^{2}	27×10^{6}	53×10^{7}	42×10^{5}	27×10^{6}	37×10^{7}
120		86×10^{3}	70×10^{6}	58×10^{7}	68×10^{5}	50×10^{6}	54×10^{7}
144		13×10^{5}	21×10^{7}	79×10^{7}	12×10^{6}	13×10^{7}	58×10^{7}
168		17×10^{6}	26×10^{7}		15×10^{7}	12×10^{7}	

for 6 minutes at 60°C., followed by plating on both T.G.E. and Plate Count agars. The initial counts were 34 x 10^6 and 33 x 10^6 for T.G.E. and Plate Count agars, respectively. The zero count on the heat-treated samples, using T.G.E. agar, was about one-third that obtained on Plate Count agar. In this case the counts on T.G.E. agar rose quickly to the level of that obtained on Plate Count agar and remained comparable throughout the trials. Again the controls were very similar for both agars.

Effect of Variation of Incubation Temperatures on Partially Heat-Inactivated Cultures

It has been shown by Nelson and Baker (44) that the counts obtained by incubation of plates at 25°C. for 3 days were equal to, or greater than, those obtained by incubation at 5°C. for 10 days, when milk had been held at low temperatures. As other abnormalities were observed with heat treated cultures, it was decided to test the effect of incubation temperature on the survival curves of heat treated cultures. Plating was done in duplicate on T.G.E. agar using the same set of samples and dilutions, one set of plates being incubated at 5°C, and the other at 25°C. Table 6 shows the results obtained at the two incubation temperatures, for both heat-treated culture 3 and a control sample not subjected to heat treatment. The treated culture was heated for 6

Table 6

Counts per Milliliter of Partially Heat-Inactivated Culture 3 and Untreated Controls when Held at 5, 10, and 25°C. with Plate Incubation at 5 and 25°C.

Time Sample Taken (Hours)	Temperature of Plate Incubation (°C.)	Temperature of Holding of: Heat-treated Sample 5°C.	Heat-treated Sample 10°C.	Heat-treated Sample 25°C.	Unheated Control 5°C.	Unheated Control 10°C.	Unheated Control 25°C.
0	5	$44x10^1$	$44x10^1$	$44x10^1$	$33x10^1$	$44x10^1$	$33x10^1$
24		$18x10^1$	$50x10^1$	$12x10^5$	$31x10^1$	$36x10^1$	$10x10^6$
48		$26x10^1$	$25x10^2$	$90x10^6$	$11x10^2$	$14x10^3$	$12x10^7$
72		$66x10^1$	$27x10^3$	$11x10^7$	$20x10^3$	$67x10^5$	$60x10^6$
96		$13x10^2$	$32x10^5$	$21x10^7$	$48x10^4$	$19x10^6$	
120		$86x10^2$	$16x10^6$	$42x10^7$	$84x10^5$	$32x10^6$	
144		$33x10^3$	$49x10^6$	$26x10^7$	$14x10^6$	$46x10^6$	
168		$46x10^4$	$18x10^7$		$26x10^6$	$10x10^7$	
0	25	$60x10^1$	$60x10^1$	$60x10^1$	$33x10^1$	$33x10^1$	$33x10^1$
24		$74x10^1$	$80x10^1$	$22x10^5$	$33x10^1$	$33x10^1$	$16x10^6$
48		$79x10^1$	$24x10^2$	$92x10^6$	$12x10^2$	$70x10^2$	$29x10^7$
72		$94x10^1$	$27x10^3$	$14x10^7$	$18x10^3$	$72x10^5$	$59x10^6$
96		$12x10^2$	$30x10^5$	$34x10^7$	$73x10^4$	$23x10^6$	
120		$56x10^2$	$22x10^6$	$37x10^7$	$76x10^5$	$31x10^6$	
144		$36x10^3$	$40x10^6$	$32x10^7$	$13x10^6$	$50x10^6$	
168		$44x10^4$	$10x10^7$		$18x10^6$	$14x10^7$	

minutes at 52°C. Initial counts obtained were 43×10^7 and 37×10^7 for 5 and 25°C., respectively, while respective survival numbers were 50×10^3 and 14×10^3. The counts obtained at the two incubation temperatures were very similar but did show somewhat lower results at the 5°C. holding temperature during the first 72 hours. The colonies, obtained at zero time and at 24 hours for 5 and 10°C. holding of samples, were very small and difficult to count when plates were incubated at 5°C., but were normal at 25°C. incubation. After 24 hours all colonies were normal at all holding temperatures and at both temperatures of incubation. The untreated controls gave very similar counts at the two temperatures of incubation. This would tend to indicate that the lower results shown in Table 6 for the early stages of growth at 5°C. holding were due to heat treatment rather than the temperature of incubation and were probably caused by the very small colonies being difficult to count when plate incubation was at 5°C. Duplication of this experiment led to the conclusion that incubation at 25°C. for 3 days gave results that were very nearly identical to those obtained by incubating plates at 5°C. for 10 days.

Studies on Partial Destruction by Chlorine

Chlorine is used extensively in the dairy industry to

sanitize equipment just prior to contact with pasteurized milk. The effect that chlorine might have on organisms which were not completely killed may be of great importance to the dairy industry, especially in view of the different type of results obtained with organisms subjected to sub-lethal doses of heat.

Cultures 3 and 12 were selected for testing in these trials. The resistance of the organisms to chlorine was found to be different for each organism. In order to get an initial count below 10 organisms per milliliter it was necessary to expose culture 3 to about 10 p.p.m. for 1 minute, while it was only necessary to expose culture 12 to 5 p.p.m. for 1 minute. Figure 19 shows the results of submitting culture 3 to 10 p.p.m. of chlorine for 1 minute, followed by inoculation into milk and holding at 5, 10 and 25°C. The initial count, due to dilution required in the procedure, was only 28×10^5 per milliliter. The figure indicates that the rates of increase of the treated samples were about the same as the controls at all the storage temperatures.

The variation of results obtained on two agars when heat-treated samples were used, led to a similar experiment for chlorine-treated cultures. Table 7 gives the results obtained when culture 12 was partially inactivated with 5 p.p.m. chlorine for 1 minute. The initial count was 34×10^4 and survival 60×10^1 when plating was done on T.G.E. agar, while it was 33×10^4 and 13×10^2, respectively, when plated

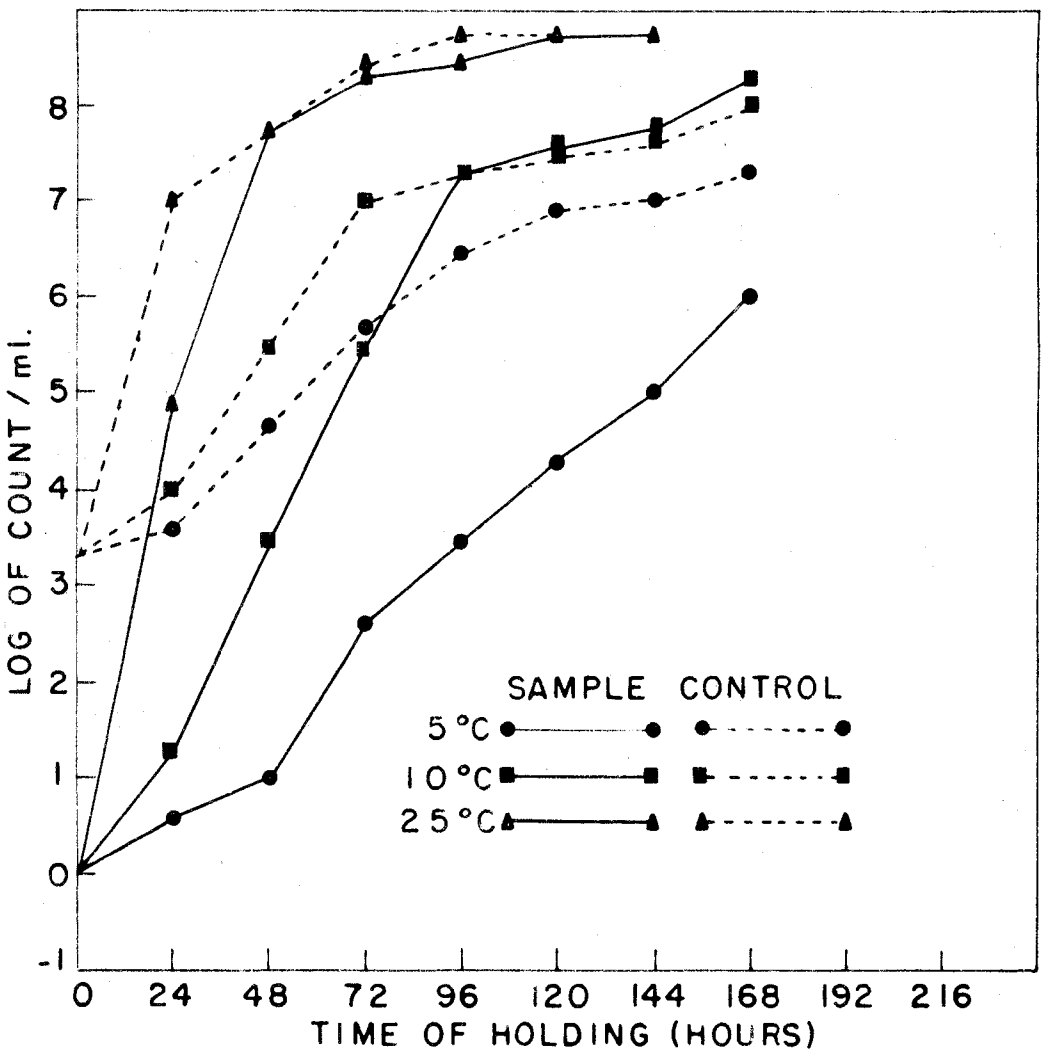

Figure 19. Growth of culture 3 at three temperatures after holding in 10 p.p.m. chlorine for 1 minute.

Table 7

Counts per Milliliter of Partially Chlorine-Inactivated Culture 12 and Untreated Controls when Held at 5, 10, and 25°C. and Plated on Two Types of Agar

Time Sample Taken (Hours)	Type of Agar Used	Temperature of Holding of: Chlorine-treated Sample 5°C.	10°C.	25°C.	Untreated Control 5°C.	10°C.	25°C.
0	T.G.E.	$6x10^0$	$6x10^0$	$6x10^0$	$48x10^1$	$48x10^1$	$48x10^1$
24		$12x10^0$	$18x10^0$	$90x10^5$	$10x10^2$	$23x10^2$	$65x10^5$
48		$13x10^1$	$30x10^2$	$88x10^6$	$53x10^3$	$91x10^4$	$33x10^7$
72		$39x10^2$	$13x10^5$	$22x10^7$	$14x10^5$	$11x10^6$	$34x10^7$
96		$87x10^3$	$24x10^6$	$62x10^7$	$37x10^5$	$37x10^6$	$48x10^7$
120		$17x10^5$	$63x10^6$	$58x10^7$	$14x10^6$	$92x10^6$	$61x10^7$
144		$13x10^6$	$19x10^7$	$87x10^7$	$23x10^6$	$21x10^7$	$70x10^7$
168		$39x10^6$	$34x10^7$		$26x10^6$	$29x10^7$	
0	Plate Count (Difco)	$13x10^0$	$13x10^0$	$13x10^0$	$38x10^1$	$38x10^1$	$38x10^1$
24		$12x10^0$	$13x10^0$	$66x10^5$	$96x10^1$	$29x10^2$	$70x10^5$
48		$12x10^1$	$26x10^2$	$96x10^6$	$62x10^3$	$11x10^5$	$29x10^7$
72		$42x10^2$	$13x10^5$	$26x10^7$	$13x10^5$	$13x10^6$	$33x10^7$
96		$87x10^3$	$21x10^6$	$69x10^7$	$36x10^5$	$38x10^6$	$53x10^7$
120		$11x10^5$	$77x10^6$	$69x10^7$	$94x10^5$	$80x10^6$	$56x10^7$
144		$13x10^6$	$24x10^7$	$86x10^7$	$14x10^6$	$17x10^7$	$79x10^7$
168		$38x10^6$	$29x10^7$		$27x10^6$	$27x10^7$	

on Plate Count agar. The two-fold difference in survival could not be duplicated. It is apparent from the table that duplication on the two agars was very close. The same close relationship was shown for culture 3. The rates of growth for treated samples were equal or nearly equal to the controls, with an indication of a slight lag period at 5 and 10°C. holding. Samples and controls reached essentially the same level by the end of the experimental period.

Relationship of Proteolysis and Plate Count

Cultures 3, 10 and 11 were proteolytic to varying degrees when measured by the amount of tyrosine and tryptophan released. The level and time of proteolysis in relation to the plate count may have some practical significance. Sterile skim milk was inoculated with the three cultures, a zero determination was made and the samples held at 5°C. Sampling for plate count and tyrosine-tryptophan assay were again made at 96 hours and each subsequent 24 hours thereafter until a final reading was made at 288 hours. A control, carried under the same conditions but with no culture added, was run at each interval to correct for substances giving false readings. A standard curve was prepared to convert Klett-Summerson colorimetric readings to micrograms of tyrosine equivalent. The curve is reproduced in Figure 20.

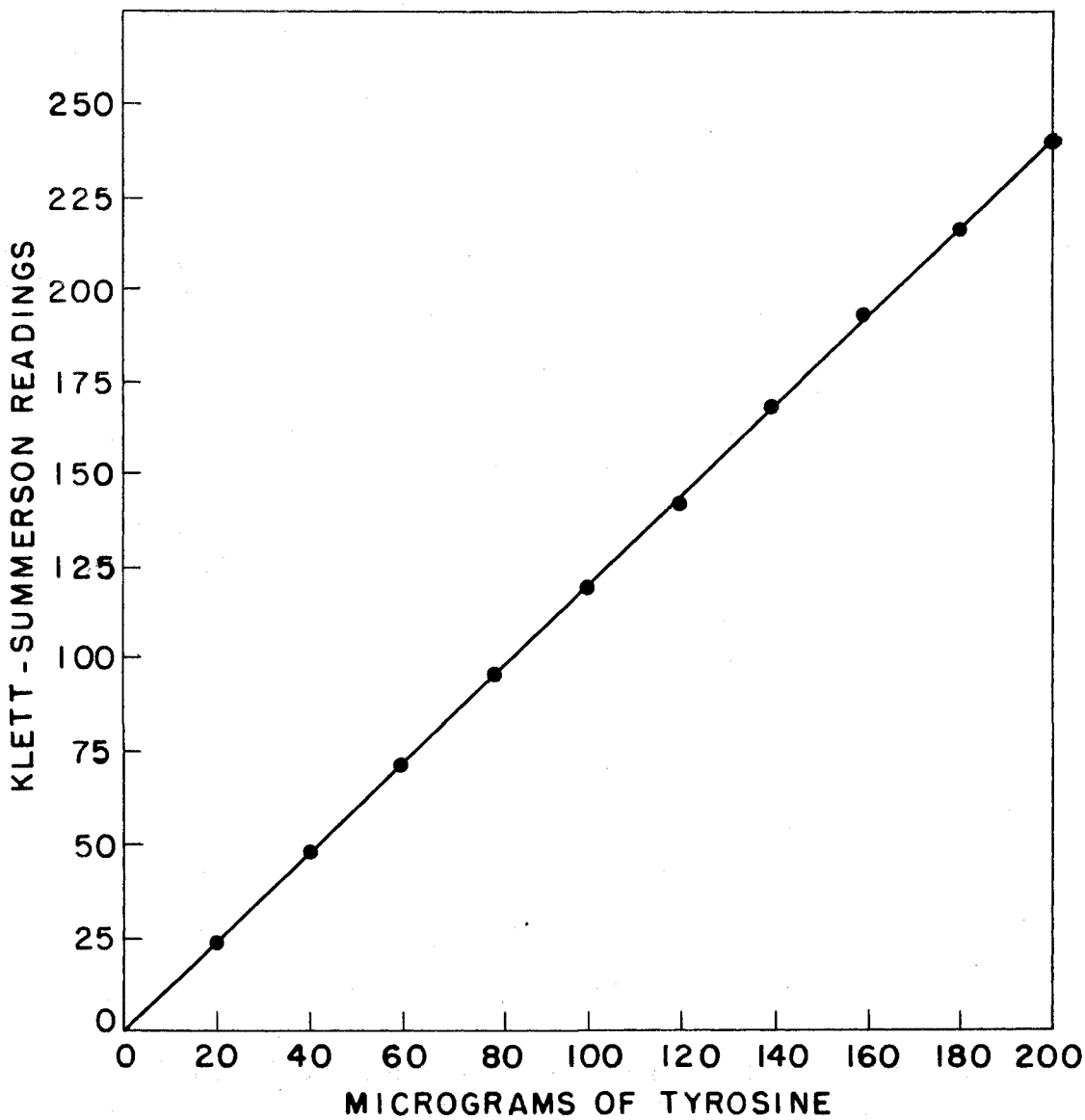

Figure 20. Standard curve to convert Klett-Summerson readings to tyrosine equivalents.

The comparison of plate counts and micrograms of tyrosine equivalent released is plotted in Figure 21 for the three proteolytic cultures. The results indicated that there was no definite minimum count above which all cultures began to release tyrosine and tryptophan, as culture 3 began to release measurable amounts 72 hours after culture 11, although the former reached the same count only about 12 hours after culture 11. The rate of proteolysis had no relationship to the rate of increase in plate count of the culture, as the three curves for growth were very close throughout the test, while the spread in amount and rate of proteolysis by the three cultures was very pronounced. Cumulative proteolysis, rather than enzyme activity during any one interval, was studied. The data do indicate proteolysis at a relatively constant rate for a considerable period of time after the populations approached maximum. This could be interpreted as indicating no additional enzyme production after maximum population was reached.

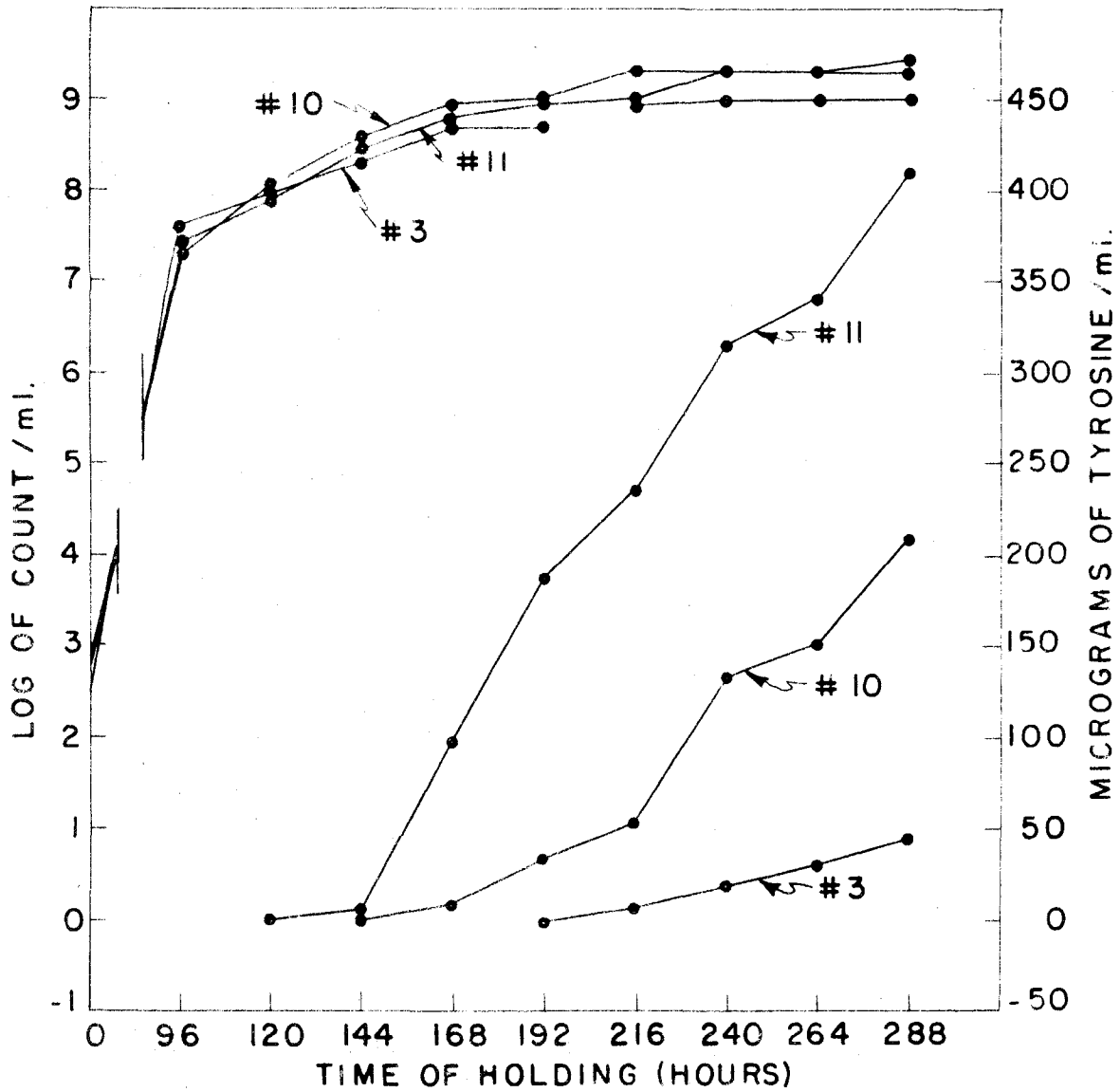

Figure 21. Plate count and cumulative proteolysis, as tyrosine equivalent, for three cultures held at 5°C.

DISCUSSION

The identification of eight different species or strains from the 50 isolates indicated the presence of a varied flora in milk. Some of the species isolated were inert and would cause little or no change in milk but some were highly proteolytic and would be undesirable contaminants. No quantitative study was made of lipolysis but some of the species were lipolytic and would be expected to cause defects in dairy products if present in large numbers. The predominance of Pseudomonas species indicated that this was one of the important genera appearing in pasteurized milk held at low temperature. A Flavobacterium species also was isolated; this organism was chromogenic and may be undesirable in some cases. The ability of all these organisms to grow in milk at a temperature of 3°C. makes them potentially very important in the storage of dairy products.

Characterization of the organisms varied in some details from those outlined in Bergey's manual (10) but the general responses of the organisms were similar enough to permit the assignment of species designations to all but one of the isolates used. One of the reasons why all characteristics did not match may have been the lack of information on the temperature at which the various reactions were studied in Bergey's classification. Sakaria and Hammer (68) pointed out

that a difference in type and rate of change produced in milk was noted with some organisms grown at room temperature, as contrasted to 37°C. They stated that specific information about temperature should be given when changes produced in milk were recorded in descriptions of organisms. All reported characteristics in the current study were for 25°C., but variations were observed when cultures were held at other temperatures. This ability to produce varied reactions depending on incubation temperature may be of practical importance. Care should be taken in making too broad a generalization about the defect-producing ability of an organism based on its action at only one temperature.

It was evident from the growth curves of all cultures isolated, with the exception of the Flavobacterium culture, that the optimum temperature of growth was at about 25°C. This tended to indicate that these organisms were only facultatively psychrophilic. On the basis of this work, the culture of Flavobacterium could be considered a true psychrophilic bacterium.

The processor of dairy products is extremely interested in the lag phase of organisms growing in milk. The lag periods of mixed flora in pasteurized milk have been reported to extend to 3-5 days by Burgwald and Josephson (11), Dahlberg (18) and Chaffee (13) when milk was held at low temperatures. A lag phase of this length provides enough

time, in most cases, for handling and comsumption before the level of organisms becomes great enough to cause spoilage.

The organisms isolated at low temperatures during this work did not have a lag phase exceeding about 24 hours, when grown in pure culture, even when held at 5°C. A similar result was reported by Kaufmann and Andrews (33), as their work indicated no lag in organisms held at 47°F. (8.3°C.). The response of organisms may be more rapid when grown in pure culture, in sterilized milk, than they would as mixed flora in commercially pasteurized milk. There is a possibility that organisms that do not normally grow at low temperatures may adapt and so cause an increase in mixed flora after a holding period for adaptation. Conn and Esten (17) reported that lactic acid bacteria sometimes developed at 10°C. but were not favored. Similarily, Bergey's manual (10) points out that lactic acid bacteria are able to grow at 10°C. In the present study, when pure cultures were subjected to heat treatment, the lag phase of the survivors was considerably extended, particularily when growth was at 5 and 10°C. This fact may account for the extended lag period observed in pasteurized milk by many workers, as the organisms found in pasteurized milk could be survivors of pasteurization or, more probably, of heat sanitizing of

equipment. Of course the lag phase could also be because a large percentage of the organisms present are not psychrophilic and only a few are able to grow at low temperature to cause a small increase in numbers in relation to a relatively high initial count. Sherman et al. (69) pointed out that bacterial growth in pasteurized milk was much slower at 0°C. than in raw milk of substantially the same bacterial content. This could mean that the majority of the organisms present in pasteurized milk were not psychrophilic, as reinoculation of pasteurized milk with minute amounts of raw milk decreased its keeping quality to that of the raw milk.

The initial numbers of organisms present in milk did not have an effect on the shape of the growth curve obtained. Low initial numbers due to dilution resulted in a growth curve, parallel to the less diluted sample but separated from it by the difference in initial count. This relationship continued to the end of the logarithmic phase. This information was considered important from a practical point of view as it pointed out that even a very low level of contamination, if growing actively, could get away to a rapid start and reach a high level, so sanitation must be thorough to be effective. In contrast to this, Penfold (53) found that, if inocula were large, variations in size were not important but, if inocula were small, the lag tended to

increase as the size of the inoculum decreased. Post-pasteurization contamination of milk with a low concentration of these organisms could result in counts, at the end of 4-5 days, which could produce undesirable effects. As this time interval is not abnormally long in relation to present processing and merchandising methods, extreme care should be taken to avoid post-pasteurization contamination.

When inocula were grown at 5, 10, and 25°C. before use in preparing growth curves at various holding temperatures, there was little or no apparent difference in the curves. Hence, the growth range of the organisms must include these temperatures. When a culture was transferred from 5 to 25°C. there was an immediate increase in the rate of growth, with little or no apparent lag phase. This pointed rather conclusively to the ability of these organisms to grow at different temperatures within this range without adaptation being necessary. Kennedy and Weiser (34) isolated 15 pure cultures able to grow in the cryophilic range of 5 to 25°C. They caution that adaptive mesophilic bacteria must be considered in this range. This condition was avoided to some extent in the present work by enrichment and isolation of cultures at 3°C. Prescott and Bates (57) reported that certain types of spoilage organisms adapted themselves to temperatures assumed to be inhibitory to decomposition processes.

The exposure of samples to room temperature for 3 hours

during the early period of growth of the culture caused an accelerated growth rate in the late lag or early logarithmic phase. The overall result was to slightly shorten the lag phase. The slight effect observed in this work was in contrast to large increases reported by Prescott et al. (59) for discontinuously refrigerated foods held at 35, 40, 45, 50 and 60°F. The effect was particularily noticeable at higher temperatures, where spoilage occurred more quickly. Baboock et al. (6) found that the decrease in bacteria observed in frozen homogenized milk was not altered materially by exposure to room temperature for 4 hours.

The use of heat, chlorine or quaternary ammonium compounds for sanitizing equipment prior to use for handling pasteurized products is a common practice in the dairy industry. The time and temperature of exposure of organisms to heat treatment may be difficult to control in sanitizing long pipe lines or assembled equipment. Therefore, the survival of organisms subjected to sub-lethal amounts of heat may be a common occurrance.

The results obtained in this work indicated that the organisms which survived heat treatment had different growth curves than the same organisms not exposed to heat. The difference occurred primarily in the lag phase, with the difference depending on the temperature of holding. In some cases when survival was low and the milk was held at 5°C.,

the population continued to decline on holding in milk until it was no longer possible to enumerate viable cells. This result could be explained by low surviving numbers with a die-off, in the early period, great enough to eliminate viable organisms. At 10°C. there was recovery after a prolonged lag phase, while at 25°C. only a slightly increased lag phase was noted and could be explained by a less severe die-off or more favorable conditions for initiation of growth so recovery took place before all viable cells were lost. This explanation was at least partially disproved by diluting the surviving organisms prior to holding. The diluted survivors recovered as readily as the undiluted ones, so absolute numbers of survivors was not the complete answer. In some cases recovery was made at 5°C. after a prolonged lag phase and in others the surviving population remained fairly stable for 8-9 days. These differences may be explained by a slight variation of heat treatment, so a slightly more severe heat treatment resulted in low survival numbers. The increased heat probably caused greater damage to the survivors and so made the adjustment necessary for resumed growth so great that they could not be made before die-off eliminated all viable cells. When heat was slightly less severe, recovery was able to take place before all viable cells were eliminated.

Frayer (24) found that holding milk cultures at 75°F.

(34°C.) for varying lengths of time before cooling to 40°F. (4.4°C.) resulted in varying rates of mortality, depending on the point in the growth cycle at which the cooling took place. This work indicated that the cooling itself likely had an effect on the mortality. If this was the case, reduction in count due to cooling after heat treatment of culture would likely be greater than when reduction was due to cooling from 75 to 40°F. Similarly, this might explain why recovery was always better at 10°C. than at 5°C., as the cooling affect was not so great.

The organisms which survived heat treatment may have been affected to different degrees, some inactivated so they could not grow in milk but could be enumerated on agar, while others were unaffected. Possibly the unaffected cultures could multiply at 5 and 10°C. but no increase was noted because it was balanced by a die-off of the injured cells. The possibility that heat-treated cells may grow on one medium and not another was shown by Nelson (42) when he found that apparent survival of heat-treated bacteria varied considerably, depending on the media used for enumeration. In a later paper (43) he showed that the presence of reducing compounds was favorable for the initiation of growth of a greater number of heat injured cells.

The acceptance of a new agar for standard plate count, made during the course of this work, necessitated a comparison of Plate Count and T.G.E. agars. Duplicate runs

using the two agars showed a consistant difference only in the very early part of the growth curve. The higher plate counts obtained on the Plate Count agar may be due to conditions in the agar being more satisfactory for the enumeration of heat-injured cells than when T.G.E. agar was used. While greater numbers were enumerated on Plate Count agar, there was possibly no chance for these organisms to survive in milk so they probably were lost during the first 24 hours of storage in milk. Subsequent counts on the two agars were very similar and subject to die-off and growth conditions as previously described. Another possible explanation could be that temperary inactivation of some enzyme system in some of the bacteria may have made it necessary to have a substrate supplied, which was present in Plate Count agar, which contains yeast extract, but was not present in T.G.E. agar or milk. The organisms requiring the substrate would quickly die out when held in milk. Thus, enumeration at 24, 48 and subsequent hours showed a similar count on both agars. This type of heat effect could be operating at the same time as the previously discussed phenomenon of die-off and recovery. Here again recovery at 10°C. was more rapid so the effect was minimized; at 25°C. it was largely masked by the rapidity of recovery at the high temperature.

Parker et al. (52) found that hypochlorite solutions were superior to quaternary ammonium compounds for destruction

of pure cultures of various Pseudomonas species. In the present work it was found that organisms surviving chlorine treatment were able to grow at all temperatures tested at a rate very similar to that of untreated cultures, with the exception of a slight lag phase noticed in the treated samples. Despite the small lag phase encountered, the results indicated that the killing effect of chlorine approached an all or nothing effect, in contrast to the effect of heat which was able to modify the growth of survivors. It could have been possible that chlorine slightly damaged portions of the survivors, resulting in a slight lag phase before multiplication began. However, the effect caused by chlorine was very small in contrast to that observed with heated cultures.

A study of proteolysis of three of the isolated cultures showed that a plate count of about 30×10^7 was sufficient for proteolysis to become measurable. Similar results were obtained by Greene and Jezeski (27), as they reported detectable proteolysis by one culture when the count had reached 1.1×10^8. The amount of tyrosine plus tryptophan released by the different cultures varied considerably and was not directly related to the population of the culture. This variation could be caused by a difference in type of enzyme produced or could be an actual difference in the

amount or activity of the enzyme produced. This indicated that proteolytic counts on milk would be of no use in predicting the time a defect would occur or the extent of such a defect.

Cases of high counts without defects occurring were obtained with some cultures in this work. Cases of high counts without noticeable defects also have been reported by Pennington (54), Conn and Esten (17) and Papadopoulos (50). Brandsaeter (9) reported that high psychrophilic counts were always associated with high proteolytic and lipolytic counts in raw milk. The varied results reported here indicated that a plate count of psychrophilic bacteria present would not be a reliable guide to the potential keeping quality of milk.

SUMMARY AND CONCLUSIONS

1. Eight organisms were isolated from commercial pasteurized milk. On identification, seven were found to belong to the genus Pseudomonas and one to the genus Flavobacterium.

2. The growth rates of seven of the eight isolated organisms and one laboratory culture of Pseudomonas fragi were similar to organisms surviving pasteurization at 61.7°C. for 30 minutes, when incubation was at 21, 25 or 32°C.

3. Eight of the nine pure cultures tested were facultative rather than truly psychrophilic organisms.

4. Size of inoculum was found to have little or no effect on the rate of growth or final numbers of organisms.

5. Plate incubation for 3 days at 25°C. was found to give counts similar to those obtained when incubation was at 5°C. for 10 days.

6. When a culture was partially killed by heat, the survivors had a greatly increased lag phase when holding was at 5 or 10°C., but only a small increase was noted at 25°C.

7. Immediately after heating, larger numbers of survivors of sub-lethal doses of heat were enumerated on Plate Count agar than on T.G.E. agar, but by 24 hours this difference had disappeared.

8. The growth curves of organisms surviving treatment with chlorine were not altered to any great extent.

9. The total cumulative proteolysis and the rate of proteolysis was found to vary for the three proteolytic cultures tested.

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