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To the Graduate Council:

I am submitting herewith a thesis written by George A. Adams entitled "Growth of Certain Psychrophilic Bacteria in Pasteurized Milk as Influenced by Previous Excessive Psychrophilic Growth in the Raw Milk." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairy Manufacturing.

W W Overcast
Major Professor

We have read this thesis and recommend its acceptance:

B. J. Demott

C. T. Miller

M. R. Johnson

Accepted for the Council:

Hilton A. Smith
Dean of the Graduate School

GROWTH OF CERTAIN PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK AS INFLUENCED BY PREVIOUS EXCESSIVE PSYCHROPHILIC GROWTH IN THE RAW MILK

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

George A. Adams

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CHAPTER I

INTRODUCTION

Undoubtedly some of the most objectionable bacteria encountered in milk are the psychrophiles. At the turn of the century it was established that these bacteria were capable of multiplying extensively at temperatures near freezing and were found in milk but little attention was paid to them because milk was normally consumed before these slow growing species were able to bring about milk spoilage (6). In recent years psychrophiles have received considerable attention because of adoption of processing and marketing procedures which tend to increase the time between processing and consumption of milk (74). The importance of psychrophiles is linked to their ability to grow at refrigeration temperatures and has been accentuated by advancements in refrigeration, increased emphasis on flavor and longer holding times (76).

Psychrophiles were invariably present in farm milk supplies, and attained exceedingly large numbers in bulk raw milk at creameries (68). Like many organisms, the psychrophiles constituted a part of the normal microflora of raw milk supplies and resulted in spoilage whenever the milk was subjected to conditions that favored their growth. Their numbers depended upon the sanitary conditions under which milk was produced, handled, and processed, the holding temperature, and the time that elapsed before final consumption.

The purpose of this investigation was to determine the significance of excessive psychrophilic bacterial growth in raw milk, held at

refrigeration temperatures, to subsequent growth of post-pasteurization contaminants in the pasteurized finished product under normal market conditions. It was felt that experimental data of this nature might be of value to processors, public health officials, and all parties concerned in locating sources of contamination, and deciding upon proper corrective measures to prevent extensive psychrophilic growth, and subsequent spoilage of refrigerated market milk prior to consumption.

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CHAPTER II

REVIEW OF LITERATURE

Definitions

Standard Methods for the Examination of Dairy Products (3) defines psychrophiles as those microorganisms which are capable of relatively rapid growth at low temperatures; commonly within the range of 35 to 50 F. To the dairy industry, this group of microorganisms includes those growing at a relatively rapid rate at refrigeration temperatures between 0 and 10 C (55, 76). Growth at a relatively rapid rate results in disagreeable flavors developing in five to ten days.

There appeared to be a common confusion concerning the growth temperatures of psychrophiles, especially of their optimum growth temperatures. Ingraham and Stokes (38) emphasized the point that many investigators have objected to the name psychrophile because it implied that the organisms preferred low temperatures, whereas the large mass of subsequent data show, almost without exception, that these bacteria grow faster at higher temperatures, generally above 20 C. Therefore, psychrophiles are cold tolerant rather than cold loving.

According to Foster et al. (25) psychrophiles are those bacteria which grow relatively rapidly in the range of 1.7 to 10 C. Without considering any of the cardinal temperatures, minimum, optimum, or maximum, Ingraham and Stokes (38) defined psychrophiles as bacteria that make significant growth at 0 C within two weeks. They concluded

that the ability to grow well at 0 C was possessed by a rather limited group of bacteria, mainly Gram-negative, nonsporeforming rods. It appears that the lowest temperature for bacterial growth was in the vicinity of minus 10 C. At this temperature multiplication was very slow and months were usually required for visible growth. Below this temperature growth was thought to be prevented by the increasing dessication and high salt concentration of the media due to the progressive removal of water by freezing.

Optimum growth temperature has been defined (38) as the temperature at which the rate of growth was most rapid, and has usually been determined roughly by visual assessment of the rate of appearance and increase of turbidity in liquid cultures or the rate of appearance and enlargement of colonies or masses of growth on solid media. Optimum growth temperatures lie in the range of 20 to 40 C and the maximum may be as high as 45 C. Psychrophiles with an optimum growth temperature below 20 C have rarely, if ever, been found. The optimum growth temperature of a number of psychrophiles, studied by Thomas and Druce (70) was about 30 C. However, Ingraham (37) has shown that psychrophiles have an 8 to 10 C lower minimum growth temperature than mesophiles and are further characterized by a markedly lower temperature coefficient for growth.

Thomas (68) defined psychrophiles as those microorganisms which are able to grow at temperatures below 5 C and do not survive heating, in milk, at 63 C for thirty minutes. Punch and Olson (58) indicated that only bacteria which are associated with spoilage and deterioration

under normal conditions of storage should be termed as psychrophilic. Gram-positive and Gram-negative bacteria normally present in milk do proliferate very slowly at temperatures of 0 to 7 C; however, various investigators (6, 53, 54, 62) have pointed out that these microorganisms are not responsible for product deterioration under normal storage conditions.

The definition of psychrophiles preferred by most industrial bacteriologists has been based upon their ability to proliferate and become the primary flora under specific conditions (58). The actual specific conditions depend upon the product under consideration.

Methods of Enumeration

There seems to be a general lack of agreement, at the present time, as to what constitutes proper methods of enumeration of psychrophiles. The methods often vary considerably depending largely upon the workers definition of these organisms (76).

According to Standard Methods for the Examination of Dairy Products (3) the psychrophilic bacterial count includes only those organisms capable of forming visible colonies on plates incubated at 5 to 7 C for seven to ten days. Many temperature and time combinations, other than those recommended by standard procedures (3) for the enumeration of psychrophilic bacteria can be found in the literature (7). Some workers maintain that the psychrophilic colony count obtained at 3 to 5 C for seven days provides information on the total number of spoilage bacteria since lactic streptococci will not grow within this

temperature range. Overcast and Skean (56) incubated plates at 5 C for seven days. Boyd et al. (10) demonstrated that incubation of agar plates at 10 C resulted in the inclusion of thermoduric organisms which were not found when plates were incubated at 5 C. Nelson and Baker (51) recommended incubation of agar plates at 21 C for four days or 25 C for three days in order to adequately include psychrophiles in viable bacteria counts. According to Baumann and Reinbold (7) one difficulty encountered with incubation at 5 C for seven days was that colonies were often so small that counting was difficult.

Boyd et al. (10) compared counts on pasteurized milk incubated at 5 and 10 C for seven, ten, fifteen, and twenty days. Seven days incubation at 5 C was discontinued because the colonies were small and difficult to count. Lower counts were obtained at 5 C than at 10 C but maximum counts were not obtained at either temperature in less than twenty days of incubation. Studying the adequacy of seven days incubation at 5 C for the determination of psychrophiles, Glen (28) noted considerably higher counts after fourteen days than after seven days incubation on many samples. The colonies were larger and more easily counted after fourteen days in all cases. Thomas et al. (71) recommended that agar plates be incubated for ten days at 7 plus or minus 0.5 C for the determination of psychrophiles.

Baumann and Reinbold (8) studied the adequacy of incubation temperatures and times (5 to 7 C for seven to ten days) for the determination of psychrophilic bacterial counts in dairy products. Their results indicated that the variation in incubation times and

temperatures could be responsible for a significant variation in psychrophilic bacterial counts. Significantly higher counts were obtained by increasing the incubation temperature from 5 to 7 C or increasing the incubation time from seven to ten days. The highest counts were obtained after incubation at 7 C for ten days. Adoption of one incubation temperature and time for the determination of psychrophilic bacterial counts was recommended.

Punch and Olson (58) utilized the common characteristic of aerobiosis in an attempt to obtain counts more rapidly at temperatures (5 to 7 C) selective for psychrophilic bacteria. Glass rods, bent to the general shape of a hockey stick, were used to spread inoculum over a poured plate containing Plate Count Agar. Surface counts were equivalent to or greater than those obtained by the standard procedure (3) after five days incubation at 6 plus or minus 0.5 C. Colonies were more easily counted, their pigmentation and fluorescence enhanced, and spreading colonies were avoided. Snyder (65) recommended more extensive use of surface plating methods for detection of psychrophilic bacteria.

Lawton and Nelson (43) observed that psychrophiles are capable of rapid growth at higher temperatures and may make significant contributions to the total plate count when made at temperatures as high as 32 C. Watrous and Doan (75) stated that most organisms growing at low temperatures are not obligate psychrophiles but grow over a rather wide range of temperatures. However, very few of these organisms will grow at 35 C.

It was shown by Ingraham (37) that most psychrophiles grow well in the "mesophilic" temperature range and accordingly their demonstration upon incubation at ordinary temperatures (25 to 30 C) may be possible when the plating substrate is selective for the organisms in question. A review by Witter (76) described several rapid methods which may be used instead of the ordinary psychrophilic count. These methods were based on the assumption that the psychrophilic flora in dairy products were predominately Gram-negative organisms and their number largely corresponded to the total number of psychrophilic organisms. The count was made on a selective substrate containing agents which inhibit the growth of Gram-positive organisms, and was completed in one or two days. Collins (14) used a culture media containing violet red bile to enumerate certain psychrophiles in forty-eight hours at 25 C. In a study by Druce et al. (17) colonies of Pseudomonas, Alcaligenes, Flavobacterium, and Achromobacter species grew on violet red bile agar in twenty to twenty-four hours at 30 C although several species of Bacillus also grew under these conditions. Also, the use of penicillin in agar medium has been suggested for the enumeration of Gram-negative bacteria (76). Olson (52) used a selective medium of Plate Count Agar with one part per million of crystal violet added for the detection of spoilage organisms in milk and cottage cheese. The counts on crystal violet agar seemed to be more effective for indicating the degree of contamination than did coliform counts.

Leesment and Dufeu (45) devised a rapid method of determining the psychrophilic count of milk by pre-incubating inoculated plates of

Tryptone Glucose Yeast Extract Agar for sixteen hours at 17 C before incubation at 7 C or 4 to 6 C. Results were obtained in four or five days using this method.

Microorganisms Involved

Organisms of the bacterial genera Pseudomonas, Achromobacter, Flavobacterium, Alcaligenes, Escherichia, and Aerobacter have been the most commonly found psychrophilic species (76). However, psychrophilic bacteria have not been limited to these six genera. Organisms of the genera Lactobacillus, Streptococcus, Micrococcus, Serratia, Proteus, Chromobacterium, Arthrobacter, and others have been isolated from milk and dairy products and observed to proliferate at low temperatures although these were exceptional cases. The general consensus of opinion has been that organisms of the Pseudomonas genus were the most commonly encountered psychrophilic species (18, 22, 23, 38, 39, 46, 62, 64, 70, 75, 76). In a study by Thomas and Druce (70) milk samples were taken from fifty-one farms. Organisms of the Pseudomonas species predominated among 235 psychrophilic bacteria isolated from these samples.

Sherman et al. (64) reported that the bacteria responsible for the spoilage of milk held just above the freezing point appeared to be mainly, if not entirely, Gram-negative, nonsporeforming rods, largely of the Pseudomonas group. Sporeforming bacteria played no part in spoilage of the milk samples studied and did not grow at low temperatures as far as could be determined. Witter (76) emphasized the

point that psycrophilic bacteria were considered to be predominantly Gram-negative, nonsporeforming rods.

The possibility of mesophilic bacteria, with an optimum temperature range of 25 to 45 C becoming adapted to growth at lower temperatures, must be accepted (41). Therefore, both psychrophilic and adaptative mesophilic bacteria may initiate undesirable biochemical changes in milk held at refrigeration temperatures. However, Scholefield (61) studying the effect of temperature on the biochemical activities of psychrophiles found that micrococci, from farm milk and dairy sources after incubation at 5 C for seven days, in contrast to certain pseudomonads were unable to metabolize actively at 5 C. Thus they were considered to be of minor significance as true psychrophiles or as contaminants of refrigerated milk.

Sources of Contamination

Psychrophilic bacteria were a part of the normal flora of raw milk, constituting a problem whenever milk was subjected to conditions that favored their growth (25). The natural sources of the prominent psychrophiles are water and soil (76). These organisms were known in older literature as water bacteria and they have been found in every conceivable water source; dairy plant, farm and municipal water supplies, ditches, lakes, streams, salt water, and fresh water ice. When such water comes in contact with a dairy product it may cause serious psychrophilic contamination and result in rapid spoilage of the product. Water supplies, otherwise satisfactory, may be a major source of psychrophilic bacteria (39).

An experiment by Thomas (72) designed to determine the effect of rinsing a sterilized milk can with contaminated water, containing about one thousand bacteria per milliliter, showed an active build up within forty-eight hours of a milk spoilage microflora dominated by psychrophiles and coliform organisms. Aside from contaminated water supplies, poorly washed farm utensils and equipment and coats of cows have been incriminated as sources of psychrophilic contamination (33). According to Ingraham and Stokes (38) psychrophilic bacteria were isolated, often in large numbers, from soil, air, fresh and salt water, common foods, and other habitats. One of the most frequently encountered sources of psychrophilic contamination was improperly cleaned utensils and equipment (76). Pipelines, milk cans, holding tanks, cream pumps, valves, and milking machines have all been cited as some of the specific sources of contamination. Thomas (68) and Thomas et al. (73) cited unclean dairy equipment as the main direct source of contamination of milk with psychrophiles. Erdman and Thornton (21) concluded that unclean plant equipment was responsible for the majority of psychrophiles in freshly pasteurized milk, and their presence indicated inefficient plant sanitation. Elliker et al. (20) concluded that contaminated equipment between the pasteurizer and the final container was the most important source of bacteria in pasteurized milk. This may result in excessively high bacterial counts in the finished product as used by the consumer and may qualify as the number one sanitation problem in the dairy industry of the day.

In pasteurized milk psychrophilic bacteria have been considered to be post-pasteurization contaminants (10, 21, 25, 44, 68, 69, 71, 75, 76). Psychrophiles were shown to be present in most bottles of pasteurized milk, after low temperature storage, due to their introduction at one or more points during post-pasteurization handling of the milk (54, 60, 75). In a study by Rogick and Burgwald (60) psychrophiles were never found in 4.1 milliliters of pasteurized milk from the vat or high-temperature, short-time pasteurizer, but were present in large numbers after one week of storage at 4 to 7 C. Watrous et al. (75), examining milk sold in Pennsylvania, observed that pasteurized milk which was well protected against contamination showed little, if any, multiplication of psychrophiles after nine days of storage at 5 C.

Elliker et al. (20) recommended milk plant use of in-line sampling techniques in order to determine sources of contamination. The procedures involved removal of samples by insertion of sterile, disposable hypodermic syringes through rubber or neoprene gaskets between in-line joints, or through rubber stoppered nipples welded into lines at different locations in the system. Certain plants have been successfully using these techniques.

Dahlberg et al. (16) found that psychrophiles grew more rapidly at 6.7 C in winter milk than in summer milk. They postulated that this was due to winter time development of a bacterial microflora on unclean equipment composed of naturally selected or mutant strains of psychrophiles.

Factors Affecting Keeping Quality

Within limits, reduction in temperature reduced the growth rate and biochemical activity of bacteria. The keeping quality of milk was therefore logically increased by a reduction in storage temperature (5, 76). However, during storage at low temperatures psychrophiles increased markedly, replaced the normal flora, and ultimately caused spoilage (76). These observations have led to the conclusion that keeping quality and psychrophilic contamination were somewhat closely related. The first evidence of poor keeping quality in milk and cream was the development of off-odors and flavors (20). Such defects were often preceeded by bacterial growth numbering in the millions in a product stored at an average temperature of 45 F within five to seven days. By the time bacterial populations became excessive, resulting in flavor defects, the product was in the hands of the consumer.

Olson et al. (54) found that pasteurized milk kept sweet for seven to ten days. The rapidity at which deterioration took place depended not only upon the initial number of organisms present and their rate of multiplication but also on their ability to cause an organoleptically detectable change in the milk.

Kaufmann and Andrews (40) concluded that an initial psychrophilic count of less than ten per milliliter was obtainable under conditions of sanitary plant operation. Milk, even at this low level of contamination, did not retain its good quality for periods longer than six or seven days at 8.3 C. With adequate attention to fundamentals and a good quality raw milk, all pasteurized bottled

milk could be processed to maintain good flavor for twelve days at 4.4 C (6, 75).

During the past decade an increasing trend toward bulk cooling and storage of raw milk has occurred. However, bacterial counts in raw milk, at refrigeration temperatures, did increase and this increase was due to the growth of psychrophiles (76). Higginbottom (35) studied the effect of the change from cans to bulk farm cooling. The average bacterial count was observed to be lower in bulk cooled milk for one or two days, the difference disappearing on further storage.

Some of the criticisms, odors, and flavors that develop as a result of psychrophilic growth have been described as fruity, putrid, ropy, discoloration, cheesy, soapy, bitter, "sweaty feet odor", "skunk-like", potato odor, fishy taint, and aroma loss (76). Erdman and Thornton (22) described some of the predominating flavor defects as stale, oxidized, sour, and bitter. Hussong et al. (36) indicated the ability of Pseudomonas fragi to cause ropiness, may-apple odor, and later rancidity in milk. Atherton et al. (6) encountered fermented or stale flavors of a proteolytic nature in aged pasteurized milk held at 4.4 C. Sherman et al. (64) noted that spoilage of milk and cream, held at low temperatures, was characterized mainly by proteolysis and development of bitter flavors.

Little specific relationship appeared to be evident between total psychrophilic population and off-flavor development in milk stored at refrigeration temperatures (6, 27, 54, 69, 75). A tremendous increase in numbers of psychrophiles in pasteurized milk may not change the

physical qualities of the product until the numbers have become astronomical.. According to Olson et al. (54) psychrophiles have attained a count exceeding ten million per milliliter without any detectable flavor deterioration. Glazier (27) found that samples of commercially pasteurized milk did not reflect a lower flavor score until the psychrophilic count exceeded ten million per milliliter. Several workers (7) have attempted to correlate numbers of organisms with flavor changes in milk or some metabolic activity of the psychrophiles. At a given temperature the rate of deterioration may depend on the types of organisms as well as initial numbers.

In a study by Punch et al. (59) commercially pasteurized and homogenized whole milks from the cooling section of a high-temperature, short-time pasteurizer were inoculated with pure cultures of psychrophiles to give an initial population of approximately five hundred per milliliter. Off-flavors were detected at the following population levels: Pseudomonas, 5.2 to 200 million; Alcaligenes, 2.5 to 14 million; Flavobacterium, 8.3 to 120 million; coliforms, 2.7 to 150 million; and yeasts, 2.5 to 14 million. Certain cultures of Alcaligenes caused ropiness before the detection of a flavor change.

Post-pasteurization contamination not only represented an important source of contamination of pasteurized milk with psychrophilic bacteria but also had a pronounced effect on the keeping quality of the finished product. Ford and Babel (24) indicated that the extent of post-pasteurization contamination had a greater influence on the keeping quality of milk than conditions encountered during distribution.

Seitz et al. (63) told of an outbreak of violet discoloration in refrigerated milk and cream in two milk plants. The cause was traced to the presence of an organism identified as Chromobacterium lividium which was thought to have occurred as a result of post-pasteurization contamination. Gyllenberg et al. (31) developed a plating method, based on the use of Ammonium Lactate Crystal Violet Agar, for the demonstration of recontaminant pseudomonads in milk and also as a test for keeping quality of the sample.

In a study by Evans et al. (23) raw milk was processed in a plate type heat exchanger at 160 to 260 F in increments of 10 F with a 0.6 second holding time. Samples processed at 160 to 170 F showed a significant initial decrease in total bacterial population in the processed milk as compared to the raw milk. However, a significant increase was noted in the psychrophilic and mesophilic count after one week of storage at 40 F. The psychrophilic population increased at a greater rate than did the mesophilic population. There was thought to have been a prolonged lag phase in the growth curve or supression of the thermophiles, or both, by the more rapid development of the psychrophiles and mesophiles during low temperature storage. Samples processed within the range of 180 to 210 F gave similar results initially, but the psychrophilic and mesophilic organisms surviving the process were able to render the milk bacteriologically unacceptable (twenty-five thousand organisms per milliliter) within two to three weeks of storage at 40 F. Samples processed within the range of 220 through 260 F showed a drastic reduction in both psychrophilic and

mesophilic counts which remained at a low level throughout four weeks of storage at 40 F. Samples processed at 240, 250, and 260 F remained acceptable bacteriologically throughout eight weeks of storage at 40 F.

Glazier (27) comparing the quality of pasteurized milks found that samples of milk pasteurized at 172 F for sixteen seconds exhibited higher psychrophilic counts, in most cases, than those pasteurized at 193 F for one second after twenty-one and twenty-eight days of storage at 36 F. Sherman et al. (64) found that bacterial growth in pasteurized milk was much slower at 0 C than in raw milk at the same temperature, and the keeping quality was two to three times as long.

Burgwald and Josephson (12) demonstrated that psychrophiles were primarily responsible for deterioration of pasteurized milk held at low temperatures. Boyd et al. (9, 11) showed that deterioration of flavor was closely associated with psychrophilic growth in pasteurized milk and that keeping quality at low temperatures was poorer in autumn and winter than in summer.

In a study by Gyllenberg et al. (32) the spoilage of refrigerated milk samples of poor keeping quality was attributed to the simultaneous activity of several types of psychrophilic bacteria rather than one predominant organism, although enterobacteria and *Pseudomonas aeruginosa* were more frequently found in poor than in good samples. Dahlberg (15) found that coliform bacteria in pasteurized milk stored at refrigeration temperatures increased more rapidly than total count. This may have been due to psychrophilic coliforms or to the fact that they were recontaminants, not subjected to pasteurization temperatures, and so were more actively growing than those organisms which survived heat treatment.

Gyllenberg et al. (30) concluded that the keeping quality of refrigerated milk was correlated with the growth of bacteria on ammonium lactate agar (AL bacteria) and that a distinct increase in the growth rate of the "AL bacteria" was always associated with the appearance and predominance of pseudomonads. The conclusion was drawn that the initial occurrence and rapid growth of pseudomonads accelerated the multiplication of acidogenic bacteria.

The growth of psychrophilic bacteria was generally quite slow when fresh milk was first placed at a low temperature but later the growth was very rapid (33). The temperature at which refrigerated milk was stored had a marked effect on the growth of psychrophilic bacteria and the keeping quality of the milk. Dahlberg et al. (16) observed that psychrophilic bacteria grew quite readily in pasteurized milk at 44 F while growth was very slow or the numbers actually decreased at 33 F during a seven-day period. Ingraham (37) found that only two strains out of fifty-four pure cultures of psychrophilic bacteria showed distinct growth in three days or less at 1 C. Andrey and Frazier (4) isolated two-hundred and twenty strains of psychrophilic bacteria from dairy farm sources. Not all of the species were able to grow within three days in pasteurized milk at 38 F, but most were able to grow at 45 F.

Studying the growth characteristics of the most frequent saprophytic bacteria in milk, Gyllenberg et al. (29) observed that Pseudomonas, Aerobacter, and Micrococcus species increased rapidly to high numbers in milk at both 5 and 10 C. Alcaligenes viscolactis,

and Alcaligenes tolerans increased rapidly to high numbers at 10 C but required longer times for growth in milk at 5 C.

Marth and Frazier (48) studied the effect of numbers of bacteria in the original milk samples containing less than fifty thousand organisms per milliliter compared to those giving colony counts of over fifty thousand at 35 C. The milk was subsequently stored at 2.2, 3.3, and 7.2 C for one to four days. More samples of the high count milks showed appreciable bacterial growth within one, two, three, or four days than of the low count milks, but psychrophiles often grew faster in the low count milk, especially in two days.

Marth and Frazier (47) held raw milk samples from farm sources at 2.2, 3.3, and 7.2 C for four days and daily determined standard, thermoduric, and psychrophilic colony counts. Little increase in numbers was noted in three days at 2.2 C but at higher temperatures significant increases occurred.

In a study by Harmon et al. (34) homogenized milk was stored at various temperatures and examined frequently for organoleptic quality, psychrophilic, lipolytic, proteolytic, and coliform bacterial counts; titrable acidity, acid degree values, and proteolysis. All samples were found to be satisfactory after twenty-one days at 33 F, seven days at 43 F, three days at 53 F, and one day at 68 F. The shelf life was related to bacterial counts, but the maximum numbers of microorganisms attained at spoilage were approximately the same regardless of storage temperatures. Sour flavors were encountered at spoilage when the milk

was stored at 53 F and 68 F, whereas at 33 and 43 F, bitter, cheesy, and fruity flavors were common.

Lawton and Nelson (43) studying the growth of psychrophiles of the genera Pseudomonas and Flavobacterium observed that there was a lag phase of twenty-four hours when the cultures were held at 5 and 10 C, but little or no lag phase at 21, 25, and 32 C. The optimum growth temperature appeared to be about 21 to 32 C for most isolates.

El-Farekh (18) studied the reproductive cycles of some common psychrophilic bacteria of the genera Pseudomonas and Brevibacterium in pure culture. Generation times at 4 C were calculated to be 5.55 hours for Pseudomonas fragi, 7.22 hours for each of two cultures of Pseudomonas fluorescens, regardless of size of inoculum, and 11.24 hours for Brevibacterium lipolyticum. Of all cultures studied, Pseudomonas fragi was observed to be the most actively growing with the shortest generation time, the highest counts after seven days of storage, and the shortest lag phase.

Vanderzant and Moore (74), using pure cultures of several pseudomonads observed an increased lag phase in skimmilk when stored at 5 C in contrast to storage at higher temperatures. However, after eleven days of storage the population at 5 C surpassed that at 25 C and 21 C and continued to increase to a much higher level. The same was true after seven days for a sample held at 10 C.

Dahlberg et al. (16) found bacterial growth in milk at refrigeration temperatures to be quite seasonal. The average psychrophilic

count was shown to be 1,940,000 per milliliter in winter, and 156,000 per milliliter in summer in pasteurized milk, after seven days of storage at 44 F.

According to Jezeski and Macy (39) the presence of large numbers of psychrophiles, in certain circumstances, caused milk to show unsatisfactory methylene blue test results. However, generally very slow dye reduction was exhibited by psychrophiles and methylene blue and resazurin tests were not suitable for detecting their growth in milk (5, 6, 42, 69, 76). These tests were designed as indexes of keeping quality, prior to the advent of bulk cooling systems, and are more closely correlated to the Standard Plate Count than to the psychrophilic count (76). Atherton et al. (6) found resazurin reduction in pasteurized milk at 20 or 37 C was very slow, even when psychrophilic counts had reached levels of one million per milliliter or more. Atherton and Bradfield (5) confirmed that the resazurin test was of little value for the detection of milk spoilage at refrigeration temperatures.

The Standard Plate Count and the psychrophilic count have been shown to be of little value in predicting the keeping quality of milk (20, 76). Presumably this was because a low level of contamination with a biochemically active, rapidly-growing psychrophile resulted in a poorer keeping quality than that resulting from a relatively large number of biochemically inert psychrophiles (76). Also, according to Olson et al. (53) the type of contaminating organism was more important than the number.

The titrable acidity and pH of pasteurized milk, under refrigeration, did not change significantly as a result of psychrophilic growth and has been observed to be of little value in following bacterial increases and flavor deterioration (6, 69).

Elliker et al. (20) suggested that refrigerated samples of commercially pasteurized milk be subjected to plate counts after storage for five days at 45 F. Even though by the time results are obtained the product may have already been consumed, corrective measures can be taken to prevent a recurrence of high bacterial counts, thus, improving and maintaining keeping quality.

Effect of Pasteurization

Agreement was found, in general, that psychrophilic bacteria commonly encountered in milk were destroyed by proper pasteurization (6, 18, 21, 22, 25, 37, 54, 60, 69, 71, 75, 76) although some doubt seemed to exist that this was always true (1, 29, 39, 41, 43, 46, 66, 75).

Watrous et al. (75) observed that samples exhibited a complete absence of psychrophiles after laboratory pasteurization at 62.8 C for thirty minutes. There was no evidence of them after twenty days of storage at 5 C. These workers proposed that these organisms could be used as an index for post-pasteurization contamination. Thomas et al. (71) found no organisms grew at 7 C in laboratory pasteurized samples. He concluded that incubation at this temperature was adequate for detecting post-pasteurization contamination with psychrophilic organisms. Rogick and Burgwald (60) reported that no psychrophilic

bacteria were found in 4.1 milliliters of pasteurized milk taken from the vat or high-temperature, short-time pasteurizer. However, psychrophilic bacteria were found in some of the bottled milk, particularly in the first milk bottled, indicating post-pasteurization contamination. In a study by Thomas and Druce (70) no psychrophiles survived laboratory pasteurization in milk at 145 to 146 F for thirty minutes.

In an investigation by Chaudhary et al. (13) the thermal resistance characteristics of Pseudomonas viscosa, Pseudomonas fluorescens, and Pseudomonas fragi were determined in sterile skimmilk. Pseudomonas fragi was shown to be the most heat resistant of the three organisms, however, results obtained provided evidence that these microorganisms would not survive normal pasteurization of milk or skimmilk.

In a study by Maculay et al. (46) the effect of pasteurization (73 C for sixteen seconds) on survival in milk of four strains of psychrophilic pseudomonads and one strain of Alcaligenes, (Alcaligenes tolerans) was determined. All strains were killed in Trypticase Soy Broth when one hundred, but not one-hundred thousand cells per milliliter, were present. The pseudomonads were not as heat resistant as Alcaligenes tolerans. Results indicated that the initial number of organisms present was the most important variable in determining survival of psychrophilic bacteria on pasteurization.

Isolates of Alcaligenes tolerans were found by Gyllenberg et al. (29) to be highly resistant to heat. Abd-el-Malek and Gibson (1) reported that Alcaligenes tolerans survives heating in milk for thirty minutes at 63 C. Measuring the survival rate was difficult due to

erratic growth on agar, especially when heated suspensions were plated. In a study by Stark and Scheib (66) 188 cultures of Gram-negative rods were isolated, four of which were said to be able to survive laboratory pasteurization at 145 F for 30 minutes.

Kennedy and Weiser (41) inoculated fifteen flasks of sterile milk with pure cultures of psychrophiles. Each inoculated flask of milk was laboratory pasteurized at 145 F for thirty minutes. Eight of the fifteen cultures had a reduction in numbers of 90 percent or over. Five had reductions of 50 to 90 percent. The remaining two cultures, showing greater thermoduric characteristics, had 24 and 4.8 percent reductions in numbers.

Biochemical Activity

Some of the psychrophilic microorganisms have been shown to produce lipase at refrigeration temperatures, and these lipases were active at temperatures below freezing and especially active at 40 F (55). Some of these lipases were extremely temperature stable and required heating to boiling temperatures for twenty minutes for complete destruction. Proteolytic enzymes causing hydrolysis of proteins into proteoses, peptones, and other products were also produced. These two types of enzymes were common causes of milk spoilage at low temperatures.

Certain organisms hydrolyze fat through the production of lipase with the liberation of free fatty acids and glycerol (33). This change is referred to as lipolysis. Butterfat contains short chain fatty acids,

such as butyric and caproic acids (25). These are usually objectionable when present in free form in dairy products because they impart a rancid flavor. Since fatty acids arise from the hydrolytic action of lipase on fats, rancid flavors may result especially when butyric acid is a component of the fat as is the case with butterfat. Bacterial lipolysis of milk and cream was frequently noted when held at temperatures as low as 45 F (33). Under these conditions lactic acid organisms were inhibited and certain lipolytic species developed rapidly due to their psychrophilic nature. The chemical reaction involved in fat decomposition generally takes place in two stages (19). The fat is first split by the lipase enzyme resulting in the liberation of fatty acids and glycerol. The fatty acids have an undesirable odor and flavor and the combined effect is one of rancidity. Next decomposition of fatty acids and glycerol takes place. Microorganisms often accomplish only the first step of hydrolyzing the fat.

Psychrophiles are more active in the production of lipase at low temperatures but the activity of the lipase enzyme is generally more pronounced at higher temperatures. Alford and Elliot (2) reported lipase production per cell in Pseudomonas fluorescens to be the same at 5 and 20 C, but very slight at 30 C, in spite of good growth. On the other hand, lipase actively was optimal at 40 C regardless of growth temperature. Nashif and Nelson (49) found that the lipase production of Pseudomonas fragi was high between 8 and 15 C, but practically absent at 30 C and above. Psychrophiles were generally responsible for the same biochemical reactions at both low and high

temperatures, but the reactions proceeded more slowly at low temperatures (38).

In a study by Gelpi (26) psychrophilic bacteria were isolated from pasteurized milk, cottage cheese, and light cream obtained from retail stores. Lipase activity which was present in most of the cultures was correlated with increases in acid degree value and off flavors in milk and product samples. In a study by Overcast and Skean (56) freshly pasteurized whole milk was inoculated with twenty-five pure cultures of lipolytic microorganisms and stored at 4 plus or minus 1 C for twelve days. Growth of eighteen cultures was good and was accompanied by an increase in free fat acidity. Rancidity and sometimes bitterness were detected but the flavor of milks inoculated with slow growing cultures remained unchanged. Data accumulated by Hussong *et al*. (36) indicate a distinct difference in the lipolytic activity of the variants of *Pseudomonas fragi*. A difference in the lipolytic activity of the same colony types obtained from different cultures was noted.

A majority of psychrophilic isolates have been observed to be proteolytic and a common spoilage of milk, held at low temperatures, was due to proteolysis (76). The activity of both extracellular and endocellular proteolytic enzymes increased with increasing temperatures, while the liberation of these enzymes was greatest at temperatures close to 0 C. Peterson and Gunderson (57) observed that production of proteolytic enzymes by a strain of *Pseudomonas fluorescens* was higher at lower growth temperatures.

Proteolysis has been described as a process by which casein or some insoluble casein derivative is broken down into water soluble compounds (33). The decomposition products were usually a mixture of various groups of compounds including peptones and related compounds, amino acids, ammonia, and even free nitrogen. Proteolysis of milk and cream was often accompanied by the development of a bitter flavor due to the presence of peptones. At times bitterness was evident before proteolysis was conspicuous. This occurred most frequently when the products were held at relatively low temperatures.

CHAPTER III

EXPERIMENTAL METHODS

Preparation of Materials and Equipment

The psychrophilic bacteria used in this study were *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Brevibacterium lipolyticium*, *Alcaligenes viscolactis*, *Pseudomonas putrefaciens*, *Flavobacterium sauveolens*, and a psychrophilic organism from bulk raw milk collected at the University of Tennessee Creamery. Stock cultures of these organisms were maintained in screw cap tubes on Milk-Protein Hydrolyzate (M-PH) agar slants at 5 C. The psychrophilic organism was an isolated colony from a sample of raw milk transferred into Trypticase Soy Broth and incubated at 21 C. After twenty-four hours this culture was transferred to an M-PH agar slant and hereafter referred to as the psychrophilic isolate. Working cultures were prepared by transferring a loopful of each stock culture to a tube of Trypticase Soy Broth incubating at 21 C, and transferring to a fresh tube of Trypticase Soy Broth at the end of each twenty-four hour period. Two exceptions were *Flavobacterium sauveolens* which differed only in that it was transferred every forty-eight hours and *Alcaligenes viscolactis* which differed only in that it was incubated in sterile skim milk. The number of organisms in each working culture was determined by diluting one part culture to ten thousand parts of sterile skim milk and counting microscopically by the standard procedure (3). Dilution blanks were a gelatin-phosphate

diluent composed of 0.2 percent Difco gelatin, 0.725 percent $NaH_2PO_4 \cdot H_2O$, and 0.37 percent Na_2HPO_4 (50). The inoculum for each sample was prepared by suspending one milliliter of each working culture in an appropriate volume of the diluent, so that 0.5 milliliter would provide approximately one thousand cells per milliliter to each milk sample.

Experimental Procedure

For this study fresh raw milk at a temperature of 40 F was collected from a bulk farm cooling tank at the University of Tennessee Dairy Farm. Milk was placed in 400 milliliter quantities in sterile 500 milliliter Erlenmeyer flasks.

A Standard Plate Count (3), was made on the original milk samples on M-PH agar incubated at 35 C. Original milk samples were then divided into 50 milliliter quantities in sterile glass containers which were closed with aluminum foil covered rubber stoppers in such a manner as to aid in the prevention of external contamination. Each 50 milliliter sample was numbered and designated for comparison with one other 50 milliliter sample, providing a two sample growth comparison for each trial. The two samples were designated as sample A and sample B as shown in Figure 1. Sample A was laboratory pasteurized in a continuously circulating water bath maintained at 63 C for thirty minutes, a total plate count* was performed, and the sample was then stored at 0 C.

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*The total plate count differed from the Standard Plate Count in that plates containing less than thirty colonies per plate were counted in the former.

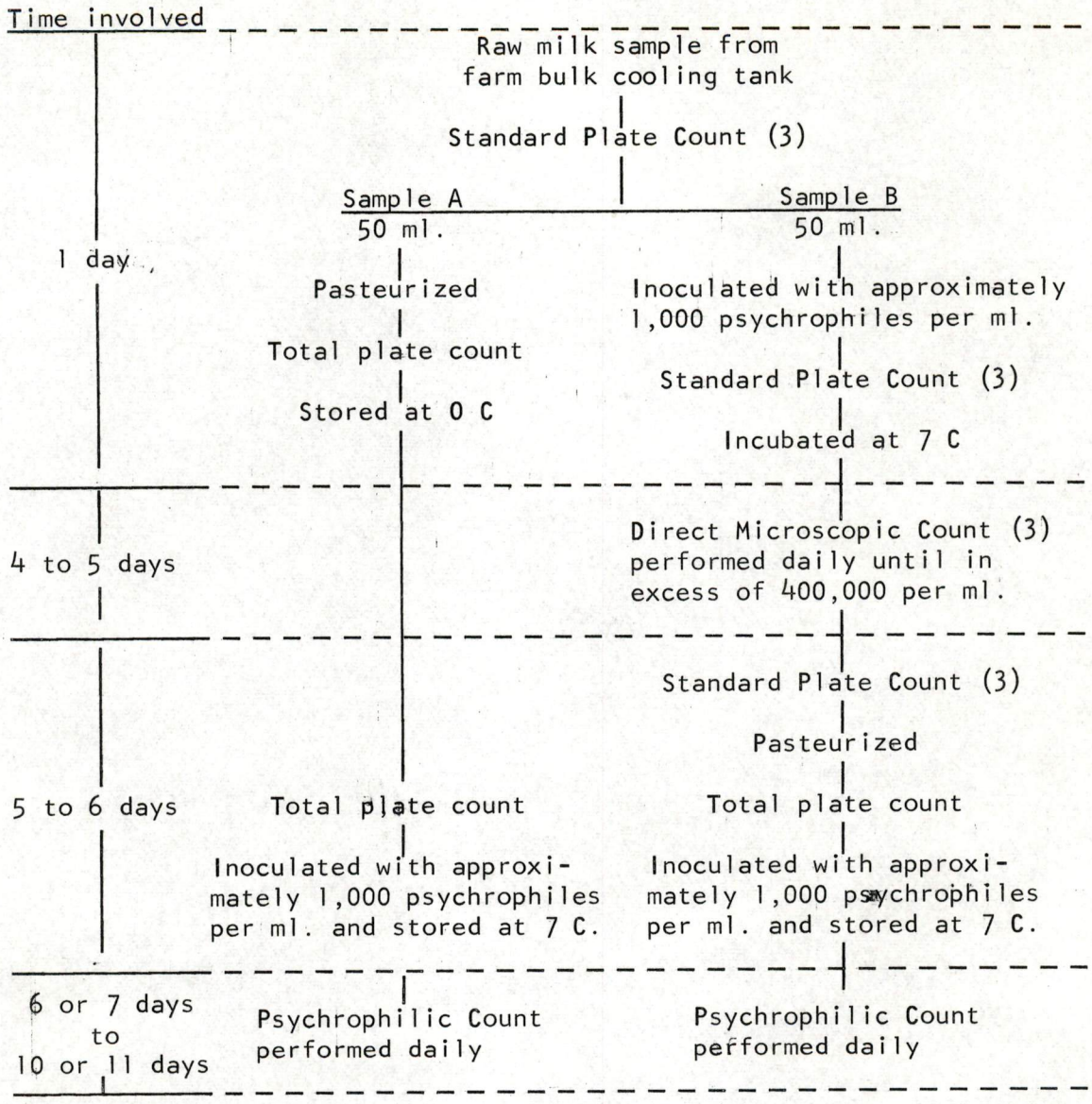

Figure 1. Experimental procedure used for comparing psychrophilic growth in pasteurized milk samples prior to and after excessive psychrophilic growth in the raw milk.

Sample B was inoculated with approximately one thousand psychrophiles per milliliter, plated for the Standard Plate Count (3), and incubated at 7 C. Growth of organisms in sample B was determined daily, beginning one day after inoculation by microscopic examination (3) until the count exceeded 400,000 per milliliter. Sample B was then plated for the Standard Plate Count (3) and laboratory pasteurized at 63 C for thirty minutes. Total plate counts were then performed on both samples A and B. Sample A was inoculated and sample B re-inoculated with approximately one thousand psychrophiles per milliliter and both samples were incubated at 7 C. For four successive days, beginning one day after the final inoculation, psychrophilic plate counts were made on samples A and B using the surface plate method described by Punch and Olson (58), with incubation at 7 C for five days. The logarithms of the psychrophilic counts obtained from samples A and B four days after the final inoculation were determined and statistical analysis applied using the F test as described by Steel and Torrie (67).

CHAPTER IV

RESULTS AND DISCUSSION

A series of trials were performed with each of six pure cultures of psychrophilic bacteria and one psychrophilic isolate from bulk raw milk from the University of Tennessee Creamery. An attempt was made to determine what effect, if any, excessive psychrophilic growth in raw milk had upon the growth of psychrophilic bacteria re-introduced into the milk after pasteurization. A series of steps had to be performed in order to prepare milk samples for trials with each organism. These steps included a control (sample A) representing high quality raw milk which was pasteurized prior to excessive psychrophilic growth and sample B, pasteurized after psychrophilic growth in excess of 400,000 organisms per milliliter. Some difficulty was encountered in determining the exact time to pasteurize sample B because of very slow growth for one to three days followed by extremely rapid growth by the next day. In some instances Direct Microscopic Counts (3) increased from 200,000 per milliliter to about 200,000,000 per milliliter during a twenty-four hour period.

The microscopic count was much higher on inoculated samples just prior to pasteurization than was the Standard Plate Count (3) in most, but not all cases. Generally, there appeared to be little correlation between the Direct Microscopic Count and the Standard Plate Count (3) when working with psychrophilic organisms. This was

probably because the microflora in the samples were predominated by psychrophiles which did not grow well at 35 C.

The data in Table I present a series of eleven trials performed with Pseudomonas fluorescens. These organisms did not increase to great numbers on the first day after inoculation as the highest count on any sample was 10,000 per milliliter. No growth occurred in either sample in trial one, and only one-hundred organisms per milliliter in trial nine. On the second day, growth became more pronounced but there was still no growth in trial one, sample B, and only 3,000 per milliliter in sample A. However, organisms numbered in the millions in at least one sample in trials four and nine. On the third day a drastic increase in numbers of organisms occurred in all trials, except sample B, trial five, and the organisms continued to increase to a much greater number by the fourth day. Organisms numbered in the millions in all samples on the fourth day. The data of trial six show that the count on sample B was greater than that on sample A in three days and the reverse was true on the fourth day. But in trials seven, eight, nine, and ten the counts on sample A were greater than those on sample B in three days, but the reverse was true on the fourth day. Both samples in trials six and nine revealed their maximum counts on the third day. The logarithms of all counts obtained on the fourth day were determined and statistical analysis applied using the F test (67). These data are presented in Table V. The mean logarithmic values were 7.21 for the samples inoculated prior to excessive growth (samples A), and 7.24 for the samples inoculated after excessive

TABLE I

GROWTH OF PSEUDOMONAS FLUORESCENS IN PASTEURIZED MILK PRIOR TO AND AFTER EXCESSIVE PSYCHROPHILIC GROWTH IN THE RAW MILK

	Counts[a] after inoculation[b] and storage at 7 C							
	1 day		2 days		3 days		4 days	
Trial	A[c]	B[d]	A	B	A	B	A	B
	(in thousands)							
1	NG[e]	NG	3.0	NG	45.5	21.5	2,000	1,425
2	2.3	0.7	169.9	3.2	29,450	153.5	49,550	9,450
3	1.1	1.7	13.7	17.6	4,265	370	29,050	20,025
4	4.2	3.6	42.5	1,950	8,115	3,400	27,450	10,000
5	5.9	5.3	638	569.5	3,045	655	10,600	4,450
6	0.3	0.8	500	491	80,500	125,000	11,500	10,550
7	0.7	0.8	3.4	20.2	11,540	520	35,200	48,500
8	9.3	10	111	334	10,350	9,150	14,600	42,750
9	0.1	0.1	5,110	476	151,750	150,250	17,650	75,500
10	2.2	2.3	14.7	2.4	170	140	4,100	31,750
11	2.0	2.2	6.0	9.8	725	4,150	50,000	77,500

[a]Average of duplicate surface plates with incubation at 7 C for 5 days.
[b]Samples were inoculated with approximately 1,000 organisms per milliliter.
[c]Pasteurized prior to excessive growth and held at 0 C until inoculated.
[d]Pasteurized after excessive growth and then re-inoculated.
[e]No visible growth on agar plates.

psychrophilic growth (samples B). Even though growth was generally greater in the samples inoculated after excessive psychrophilic growth (samples B) this growth was not great enough to be statistically significant. The F value of 0.04 indicated no significant difference at the 0.10 level of probability.

The data of Table II present a series of ten trials performed with Pseudomonas fragi. With this organism, growth was generally more pronounced than with Pseudomonas fluorescens on the first day after inoculation although a wide variation existed. In both samples of trial four, very rapid growth occurred in one day. However, other samples; A in trial one, five, seven, and eight, and B in trial one and seven grew very little, if any. The organisms had materially increased in numbers on the second day and by the third day all samples except trial one, sample B, had counts in the millions. The counts on sample A were greater than those on sample B in the first eight trials on the third and fourth days after inoculation but the reverse was true in trial nine. Sample A of trial ten had a count higher than sample B on the third day, but the reverse was true on the fourth day. It was generally true in most, but not all, cases throughout this series of trials that the sample inoculated prior to excessive psychrophilic growth (sample A) had counts higher than the sample inoculated after excessive psychrophilic growth in the raw milk (sample B). Maximum numbers of organisms were never attained at three days with Pseudomonas fragi. Statistical analysis (67) of the logarithms of the psychrophilic counts on the fourth day showed a mean

TABLE II

GROWTH OF PSEUDOMONAS FRAGI IN PASTEURIZED MILK PRIOR TO AND AFTER EXCESSIVE PSYCHROPHILIC GROWTH IN THE RAW MILK

	Counts[a] after inoculation[b] and storage at 7 C							
	1 day		2 days		3 days		4 days	
Trial	A[c]	B[d]	A	B	A	B	A	B
	(in thousands)							
1	0.4	0.3	14.5	11.5	24,950	570	110,650	34,450
2	57.1	4.1	4,680	416	61,110	37,700	179,500	94,500
3	5.0	5.7	16,380	12,380	91,000	78,975	238,038	183,040
4	244.3	142.3	8,651	6,695	74,945	46,150	205,400	146,350
5	0.5	5.5	37,804	17,602	172,000	97,000	250,000	135,500
6	5.5	6.0	5,725	1,495	100,250	25,250	175,000	99,500
7	NG[e]	0.6	78.5	238	28,250	20,900	124,500	92,000
8	0.2	2.2	136	134	26,300	7,500	126,500	56,000
9	17.9	36.0	13,000	6,450	132,000	144,250	245,500	265,500
10	87.8	34.8	7,600	15,750	170,950	169,000	266,000	354,000

[a]Average of duplicate surface plates with incubation at 7 C for 5 days.
[b]Samples were inoculated with approximately 1,000 organisms per milliliter.
[c]Pasteurized prior to excessive growth and held at 0 C until inoculated.
[d]Pasteurized after excessive growth and then re-inoculated.
[e]No visible growth on agar plates.

logarithmic value of 8.26 for the samples inoculated prior to excessive psychrophilic growth (samples A) and 8.06 for the samples inoculated after excessive psychrophilic growth in the raw milk (samples B). These data are presented in Table V. An F value of 3.33 indicated a significant difference at the 0.10 level of probability. In general, this organism initiated growth, in the milk pasteurized prior to excessive psychrophilic growth, at a very rapid rate. This would indicate that post-pasteurization contamination with Pseudomonas fragi could lead to lowered quality and possibly off-flavors of milk in two or three days even though the milk was stored at refrigeration temperatures.

The data of Table III present a series of nine trials performed with Brevibacterium lipolyticum. The counts on some samples in this series of trials were low on the first day after inoculation with no growth in either sample of trial one while samples B of trials two, six, seven, eight and nine had counts ranging from 17,200 to 26,300 per milliliter and sample A of trial seven had a count of 28,500. The data of Table III also show that the counts were higher in the samples inoculated after psychrophilic growth (samples B) than those inoculated prior to excessive psychrophilic growth in the raw milk (samples A) in all trials except one and seven on the first day after inoculation. This was true in all trials except one, five, and seven on the second day but by the third day counts were higher in the samples inoculated prior to excessive growth (samples A) in all trials but three, six, and nine. On the fourth day counts were higher in the samples inoculated prior to

TABLE III

GROWTH OF BREVIBACTERIUM LIPOLYTICUM IN PASTEURIZED MILK PRIOR TO AND AFTER EXCESSIVE PSYCHROPHILIC GROWTH IN THE RAW MILK

Trial	Counts[a] after inoculation[b] and storage at 7 C							
	1 day		2 days		3 days		4 days	
	A[c]	B[d]	A	B	A	B	A	B
	(in thousands)							
1	NG[e]	NG	6.5	1.5	180	93.5	33,930	3,055
2	5.0	17.2	1,400	3,680	97,500	94,250	107,900	164,200
3	1.7	6.1	50.0	229	1,000	2,220	30,450	49,500
4	0.1	1.2	380	1,600	47,750	25,500	184,000	112,000
5	0.6	0.8	214	18.6	7,425	265	146,750	36,000
6	8.7	18.3	850	1,788	5,050	19,929	85,250	156,000
7	28.5	21.2	2,034	1,287	19,400	7,900	97,500	170,625
8	5.4	20.3	865	1,825	28,600	23,400	243,750	203,750
9	8.9	26.3	825	1,505	29,737	35,035	227,500	270,400

[a]Average of duplicate surface plates with incubation at 7 C for 5 days.
[b]Samples were inoculated with approximately 1,000 organisms per milliliter.
[c]Pasteurized prior to excessive growth and held at 0 C until inoculated.
[d]Pasteurized after excessive growth and then re-inoculated.
[e]No visible growth on agar plates.

excessive psychrophilic growth (samples A) in four out of nine trials. These data indicated that excessive growth of psychrophilic organisms in raw milk stimulated the growth of Brevibacterium lipolyticum when re-introduced into the milk after pasteurization for one or two days but the stimulatory effect disappeared in three or four days. Maximum numbers of organisms were never attained at three days of this series of trials. The logarithms of all counts obtained on the fourth day were determined and statistical analysis applied (67). These data are presented in Table V. The mean logarithmic values were 8.02 for the samples inoculated prior to excessive growth (samples A) and 7.90 for the samples inoculated after excessive psychrophilic growth in the raw milk (samples B). Even though the logarithmic mean value was higher for the samples inoculated prior to excessive psychrophilic growth (samples A) this growth was not great enough to be statistically significant. The F value of 0.25 showed no significant difference at the 0.10 level of probability.

The data of Table IV present a series of four trials performed with the psychrophilic isolate. Counts were quite high throughout this series of trials even on the first day after inoculation. Also, counts on the first day after inoculation were all higher on samples B than on samples A indicating a stimulatory effect of excessive psychrophilic growth in raw milk for this organism. This could possibly be some essential metabolite that enables these organisms to initiate growth in the milk after pasteurization. By the second day, only half of the counts on samples B were higher than those on samples A and three

TABLE IV

GROWTH OF THE PSYCHROPHILIC ISOLATE IN PASTEURIZED MILK PRIOR TO AND AFTER EXCESSIVE PSYCHROPHILIC GROWTH IN THE RAW MILK

	Counts[a] after inoculation[b] and storage at 7 C							
	1 day		2 days		3 days		4 days	
Trial	A[c]	B[d]	A	B	A	B	A	B
	(in thousands)							
1	250	2,535	28,795	27,182	118,000	116,000	222,000	194,000
2	39	734	45,240	40,950	127,100	103,500	239,500	200,000
3	6,175	6,240	21,645	26,325	111,150	70,850	157,820	191,000
4	438.8	1,008	21,586	23,400	79,300	104,650	165,000	171,000

[a]Average of duplicate surface plates with incubation at 7 C for 5 days.
[b]Samples were inoculated with approximately 1,000 organisms per milliliter.
[c]Pasteurized prior to excessive growth and held at 0 C until inoculated.
[d]Pasteurized after excessive growth and then re-inoculated.

trials out of four had higher counts on samples A than on samples B on the third day, indicating a disappearance of the stimulatory effect. Maximum counts were never attained at three days in this series of trials. Statistical analysis (67) of the logarithms of the psychrophilic counts on the fourth day (Table V) indicated a mean logarithmic value of 8.28 for the samples inoculated prior to excessive psychrophilic growth (samples A) and 8.27 for the samples inoculated after excessive psychrophilic growth in the raw milk (samples B). Even though the logarithmic mean value was slightly higher for the samples inoculated prior to excessive psychrophilic growth (samples A) this growth was not great enough to be statistically significant. The F value of 0.14 showed no significant difference at the 0.10 level. This tended to further substantiate the disappearance of any stimulatory factor resulting from pre-pasteurization psychrophilic growth.

The mean values of the logarithms of all counts obtained on the fourth day of each tabulated trial (Table V) were statistically analyzed (67). The logarithmic mean values for this analysis were 7.94 for the samples inoculated prior to excessive psychrophilic growth (samples A) and 7.87 for those inoculated after excessive psychrophilic growth in the raw milk (samples B). The F value of 0.05 indicated no significant difference at the 0.10 level of probability.

Four trials each were conducted with *Pseudomonas putrefaciens*, *Alcaligenes viscolactis* and *Flavobacterium sauveolens*. These organisms grew very poorly at 7 C in five days and would not constitute a

TABLE V

LOGARITHMIC MEAN VALUES AND STATISTICAL SIGNIFICANCE OF PSYCHROPHILIC COUNTS FOUR DAYS AFTER INOCULATION OF PASTEURIZED MILK SAMPLES PRIOR TO AND AFTER EXCESSIVE PSYCHROPHILIC GROWTH IN THE RAW MILK

Organism	A[a]	B[b]	F value[c]	Statistical Significance
Ps. fluorescens	7.21	7.24	0.04	NS[d]
Ps. fragi	8.26	8.06	3.33	*[e]
Brevi. lipolyticum	8.02	7.90	0.25	NS
Psychrophilic isolate	8.28	8.27	0.14	NS
Average mean value	7.94	7.87	0.05	NS

[a]Pasteurized prior to excessive growth and held at 0 C until inoculated.
[b]Pasteurized after excessive growth and then re-inoculated.
[c]The ratio of the variances for among treatments and within treatments.
[d]No significant difference at the 0.10 level of probability.
[e]Significant difference at the 0.10 level of probability.

psychrophilic problem in the dairy industry. Therefore, these results were not included in the analysis of data.

CRANESBILL CREST

CHAPTER V

SUMMARY AND CONCLUSIONS

The effect of excessive psychrophilic bacterial growth in raw milk upon the growth of psychrophilic organisms re-introduced into the milk after pasteurization was studied.

A series of growth trials were performed with each of six pure cultures of psychrophilic bacteria and one psychrophilic isolate from bulk raw milk. Samples of pasteurized milk inoculated prior to and after excessive psychrophilic growth in the raw milk were incubated at 7 C and counted daily for four successive days using the surface plate method described by Punch and Olson (58).

Results indicated that excessive psychrophilic growth in raw milk had a stimulatory effect on the ability of Brevibacterium lipolyticum and the psychrophilic isolate to initiate growth in the milk after pasteurization and grow more rapidly than in the normal pasteurized milk for one or two days. Statistical analysis (67) of the logarithms of the psychrophilic counts four days after inoculation indicated no significant difference at the 0.10 level of probability. Evidently the stimulatory effect that resulted in rapid initiation of growth had disappeared by the fourth day.

Results of trials with Pseudomonas fragi indicated that the effect of excessive pre-pasteurization psychrophilic growth was inhibitory on the growth of this organism in the milk after pasteurization.

Statistical analysis (67) of growth in four days indicated a significant difference between numbers of organisms in the pasteurized samples inoculated prior to excessive growth (samples A) and numbers in those inoculated after excessive growth in the raw milk (samples B) at the 0.10 level of probability.

Results of trials with Pseudomonas fluorescens indicated a slight but statistically insignificant stimulatory effect from excessive pre-pasteurization growth on the growth of this organism in four days.

Statistical analysis (67) of the means of the logarithms of the psychrophilic counts, in each series of trials on the fourth day after inoculation, indicated that the inhibitory effect on growth from excessive pre-pasteurization psychrophilic growth was generally not significant. However, specifically this depended upon the organism involved as Pseudomonas fragi received no detectable stimulatory effect from pre-pasteurization psychrophilic growth, but was significantly inhibited after four days of growth in the milk that had excessive growth of psychrophiles prior to pasteurization.

Pseudomonas putrefaciens, Alcaligenes viscolactis, and Flavobacterium sauveolens grew very poorly at 7 C in five days indicating that they would not constitute a serious psychrophilic problem in the dairy industry.

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