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To the Graduate Council:

I am submitting herewith a thesis written by Man Mohan Singh Sandhu entitled "Contributions of the Heat-Resistant Lipase of Psychrophilic Bacteria to Off-Flavors in Pasteurized 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 Dairying.

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We have read this thesis and recommend its acceptance:

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CONTRIBUTIONS OF THE HEAT-RESISTANT LIPASE OF PSYCHROPHILIC BACTERIA TO OFF-FLAVORS IN PASTEURIZED 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

---

by

Man Mohan Singh Sandhu

June 1967

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CHAPTER I

INTRODUCTION

The importance of psychrophilic bacteria is linked to their ability to grow at refrigeration temperature. Their importance to the dairy industry has been accentuated by the advancements in refrigeration facilities, increased regard for keeping quality, and longer holding times resulting from every-other-day pickup, less frequent home deliveries, and transporting over greater distances. The psychrophiles have long received the attention of butter and cottage cheese manufacturers and much of the present knowledge of the psychrophiles was received from these products; recently much concern has been shown to market milk processing with regard to the contamination by the psychrophiles.

The psychrophiles are primarily responsible for limiting the keeping quality of milk and fluid milk products in which they may produce a wide variety of spoilage defects. Despite the obvious and long importance of lipolytic activity of bacteria in dairy products, other than demonstration of the presence of lipolytic bacteria (81) or the observation that a particular bacterium produced rancidity, very little work has been directed toward the study of lipolysis by psychrophilic bacteria. Some of the species of the genus Pseudomonas produced lipase which was found to be heat stable (52).

The present study was undertaken to determine the effects of the heat-resistant lipase of the psychrophilic bacteria on the flavor of pasteurized milk stored at refrigeration temperature (4°C.).

CHAPTER II

REVIEW OF LITERATURE

Definitions

The term psychrophile was first used by Schmidt-Nielsen (81) in 1902 to describe bacteria which grew at 0°C. The first recorded knowledge of bacteria which grow at low temperatures was credited to Forester, 1887 (39). Different names have been given to bacteria that can grow at low temperatures. These bacteria are called commonly psychrophiles, a word derived from the Greek psychros meaning cold and philos meaning loving (cold loving). These organisms are cold tolerant rather than cold loving with an optimum growth temperature of 20°C. or above (9).

Bacteria are divided into three classes (36) according to their optimum temperature requirement for growth--namely thermophiles, mesophiles, and psychrophiles. Other terms used in the literature are as follows: cryophiles, psychrotolerant, and thermophobic (39). No absolute agreement for the definitions of psychrophiles and their growth temperatures occurs in the literature. Standard Methods for the Examination of Dairy Products (3) defines psychrophilic bacteria as "those which are capable of relatively rapid growth at low temperatures, commonly within the range of 35° to 50°F." Olson et al. (57) pinpointed this common range as 35° to 45°F. Thomas (77) 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 30 minutes. Ingraham

(38) concluded that "probably the best definition of a psychrophile is an organism that grows reasonably well at 0°C. with generation time of less than 48 hours."

Ingraham and Stokes (39) defined the psychrophilic bacteria as those that form easily visible colonies on solid media within two weeks at 0°C. Considering all the viewpoints of several authors, psychrophilic bacteria may be defined as those bacteria which grow relatively rapidly at refrigeration temperatures.

Temperature Relationships

Ingraham and Stokes (39) pointed out that most strains have a maximum growth temperature of 30°C., also Witter (81) reported that a maximum growth temperature for psychrophilic bacteria was 30°C. Zobell (83) observed that the optimum temperature was within the range of 18° to 22° C., whereas Thomas (77) pointed out the optimum temperature range as 10° to 20°C. for psychrophiles. Ingraham and Stokes (39) stated the optimum temperature was the temperature where the rate of growth was most rapid and further that for psychrophilic bacteria this temperature was near 32°C.

Thomas (77) noted that the minimum range of growth temperature is -5° to 0°C. Witter (81) stated that the growth of bacteria below -10°C. was unlikely, and Berry and Magoon (9) concurred.

Methods of Enumeration

According to Standard Methods for the Examination of Dairy Products (3), the psychrophilic bacterial count include only those

organisms capable of forming visible colonies on plates incubated at 5° to 7°C. for seven to ten days. Overcast and Skean (58) incubated plates at 5°C. for seven days. Nelson and Baker (54) 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. Leesment and Dufeu (48) 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.

Type and Ecology of Psychrophilic Bacteria

The term psychrophile does not describe a particular taxonomic group of microorganisms, although most of them are Gram-negative, non-spore-forming rods (72). The general consensus has been that organisms of the *Pseudomonas* genus were the most commonly encountered psychrophilic species (21,22,24,26,39,40,49,69,72,78,80,81).

Thomas and Druce (78) summarized the results obtained by various workers and listed the organisms as belonging to genera *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Flavobacterium*, *Arthrobacter*, *Micrococcus*, *Chromobacterium* besides coli-aerogenes. Davis and Mattick (20) stated that the commonly encountered psychrophilic organisms belong to genera *Pseudomonas*, *Achromobacter*, *Chromobacterium*, and *Micrococcus*. Sherman *et al*. (72) 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, nonspore-forming rods, largely of the Pseudomonas group. In addition, spore-forming bacteria played no part in spoilage of the milk samples studied and did not grow at low temperatures.

Witter (81) emphasized that psychrophilic bacteria were considered to be predominantly Gram-negative, nonspore-forming rods.

Schultze and Olson (69), in a study isolated 586 cultures from pasteurized samples of milk, cream, chocolate drink, and cottage cheese after storage at 4°C. for seven days (excepting the cottage cheese which was stored at 4°C. for fourteen days). The characterization of the organisms showed that 70.6 percent were Pseudomonas species, 7.9 percent were Alcaligenes species, 9.2 percent were Achromobacterium species, while 10.8 percent were coliforms and the remaining 0.8 percent were yeasts. Foter and Rahn (31) observed the growth of Streptococcus faecalis and Streptococcus glyceranicus at 0°C., but no explanation for the growth at this low temperature was given. Witter (81) stated that some organisms belonging to genera Pseudomonas, Achromobacter, Alcaligenes, Flavobacterium, Escherichia, Aerobacter, Serratia, Proteus, Chromobacterium, Arthrobacter, Lactobacillus, Streptococcus, Micrococcus, Bacillus, and Sarcina were psychrophilic bacteria.

Sources of Contamination

The natural sources of psychrophilic bacteria are soil and water (15,38,81). These organisms, in the older literature, were known as water bacteria and have been repeatedly and consistently found in every conceivable water source, from water supplies, dairy plant water supplies,

municipal water supplies, ditches, lakes, streams, salt water, and fresh water ice. When such water comes in direct contact with a dairy product, as the washing of butter and cottage cheese or residual rinse water on equipment, serious psychrophilic contamination of the dairy product may result in rapid spoilage (40). While the initial source of psychrophiles in the dairy industry is the water supply, a secondary, and perhaps more frequently encountered source of contamination, is improperly cleaned equipment and utensils. Usually equipment and utensils have been implicated in a general way but occasionally specific equipment has been cited as the source of psychrophilic contamination: pipelines, milk cans, butter churns, holding tanks, cream pumps, valves and milking machines (81). Johns (42) observed that the usual treatment of water supplies killed coliforms but not psychrophiles, and the equipment coming in contact with such water was contaminated by the latter bacteria. Olson et al. (57) in their study observed that a treated water supply, otherwise satisfactory, may be a major source of psychrophilic bacteria.

Erdman and Thornton (24) found 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. (23) in their study 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. Rogick and Burgwald (66) reported that the bottles were a serious factor in contamination and found that fifteen out of thirty-four freshly washed half-pint bottles contained psychrophilic bacteria.

Labots et al. (46) showed that air was the major important factor in contaminating pasteurized milk while processing. The bacterial content of the surrounding air during bottling varied in several experiments from 20,000 to 60,000 per liter. The number of bacteria in the empty bottles was two to three times higher than the bacterial content of the corresponding air. This large difference was explained, tentatively, as a result of sedimentation of bacteria in the bottles. Such findings might stimulate dairy industries to processing and filling milk under proper vacuum and under more sanitized conditions.

Balaprakasam (8) determined the microbiological content of the air in two dairy plants by using the Andersen Air Sampler (4) and found that the bacterial content of the air from the bottle washing room and cottage cheese filling room of the first plant was significantly higher than counts from other rooms. The bacterial content of air of the second plant was much less compared to the first plant, and the explanation was the absence of undue air movements, spacious accommodation and only two workers in the plant.

Effect of Pasteurization

The preponderance of evidence shows that psychrophiles do not survive either laboratory or commercial pasteurization (6,41,66,77,78). Watrous et al. (80) observed that psychrophilic bacteria were destroyed

by pasteurization at 62.8°C. for thirty minutes. Thomas and Druce (78) studied 235 psychrophilic cultures and none of them survived laboratory pasteurization in milk at 63.5°C. for thirty minutes. None of the Pseudomonas organisms of the 199 green-fluorescent organisms isolated by Seleen and Stark (71) survived heating for thirty minutes at 60°C. Rogick and Burgwald (67) could not detect any psychrophilic bacteria in 4.1 milliliters of milk samples taken in sterile bottles directly from the vat or holding tubes.

Only 4 out of 722 psychrophilic isolates of Erdman and Thornton (25) survived laboratory pasteurization at 142°F. for thirty minutes. These four were Gram-negative, nonfluorescent cocci which did not liquify gelatin. Jezeski and Macy (40) noted the survival of six out of forty-one cultures when heated at 150°F. for thirty minutes and these surviving organisms showed considerable caseolytic activity.

Abd-el-Malek and Gibson (2) reported that Alcaligenes tolerans survived heating in milk at 63°C. for thirty minutes. In a study by Stark and Scheib (74) 188 cultures of Gram-negative rods were isolated, four of which were able to survive laboratory pasteurization at 145°F. for thirty minutes.

In an investigation by Chaudhary et al. (14) the thermal resistance characteristics of Pseudomonas viscosa, Pseudomonas fluorescens, and Pseudomonas fragi were determined in sterile skim milk. Pseudomonas fragi was shown to be most heat-resistant of the three organisms; however, results obtained provided evidence that these microorganisms would not survive normal pasteurization of milk or skim milk.

Kennedy and Weiser (44) 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.

Keeping Quality

Both the growth rate and biochemical activity of bacteria are reduced by a reduction in temperature. The keeping quality of milk and dairy products was, therefore, logically increased by reduction in storage temperature and this has been uniformly supported by the literature (15,35,45); however, at low temperature storage psychrophiles may increase markedly, replacing the normal flora, and ultimately the cause of spoilage. From this the conclusion was drawn that keeping quality and psychrophilic contamination are closely related (81,11). Nicholas and Anderson (55) studying the keeping quality of pasteurized milk with a bacterial content ranging from 3,000 to 92,000 per milliliter, found that such milk kept in the home refrigerator at 40°F. would retain high quality from ten days to two weeks. This milk was removed from the refrigerator daily, mixed and permitted to stand at room temperature for one hour before returning to storage. Boyd et al. (10) reported that the keeping quality of commercially pasteurized and homogenized milk stored at 40°F. was retained 13 to 18 days based on a 50,000 per

milliliter psychrophilic bacteria count. When stored at 33°F., the average keeping quality of the milk was extended an additional eleven to fourteen days. Flavor deterioration was correlated with the growth of psychrophilic bacteria at both storage temperatures. Burgwald and Josephson (13) found that initial bacterial counts of the milk did not always indicate its potential keeping quality. One lot of pasteurized milk samples which contained bacterial count of 61,000 per milliliter remained sweet for twenty-five days at temperatures below 40°F. and for twenty-one days at a temperature about 40°F. in the half-pints and for thirteen to fifteen days in the quarts. Another lot of samples held below 40°F. and having an initial bacterial count of 7,150 per milliliter remained sweet for only sixteen days. The half-pints and quarts held at 40°F. remained sweet for only twelve days.

Watrous et al. (80) and Rogick and Burgwald (66) found psychrophiles in all commercially pasteurized milk of their study by the end of one week of storage at refrigeration temperature.

Conn (17) investigated the development of defects in dairy products due to the growth of psychrophiles and observed that milk preserved at 50°F. or lower will keep sweet for a long time, but it becomes "filled" with bacteria of a more unwholesome type than those that grow at higher temperature. Numerous defects may be attributed to their activity.

Dahlberg (18) in a study held freshly pasteurized milk at room temperature for six hours before refrigerating at 35° to 40°F. to simulate the failure of prompt refrigeration of delivered milk and found

very slight increase in total bacterial count and coliform count in some samples. The flavor scores of the milk held at room temperature for six hours before refrigeration were good for seven days, and he concluded from this study that the keeping quality of pasteurized milk was good enough at all seasons of the year to permit every other day delivery of milk. Olson et al. (56) in a study on the keeping quality of pasteurized milk, found commercially pasteurized milk frequently kept seven to ten days, and the rapidity with which deterioration took place depended upon the initial number of organisms present. The rate of growth and the capability to cause organoleptic defects were influenced largely by the initial number of these organisms.

Lawton and Nelson (47) stated that the milk containing as low as one thousand psychrophilic organisms per milliliter increased to ten million per milliliter in three to four days even when stored at 5°C.

Marth and Frazier (50) stored raw milk at 36°, 38°, and 45°F. for four days and made Standard Plate Counts, thermoduric and psychrophilic counts every day. On the average, at 36°F., there was a decrease in the numbers of bacteria during the first two days, the psychrophiles increased at the end of three days and both Standard Plate Counts and psychrophilic counts increased after four days. A storage temperature of 36°F. was found to be satisfactory for holding raw milk for three days. These research workers observed that the psychrophilic bacteria grew more in a raw milk sample held at 38° and 45°F. than those held at other temperatures and they grew at a greater rate in summer than in winter. During the past decades an increasing trend toward bulk cooling

and storage of raw milk has occurred. However, bacterial counts of such milk have increased and this increase was due to the growth of psychrophiles.

Broitman et al. (12) indicated the impossibility of predicting the keeping quality of milk by considering the initial psychrophilic counts, and further noted that milk samples with less than ten organisms per milliliter in initial psychrophilic counts stored well for a longer time in refrigerators than did other samples. The other samples had ten to ten thousand psychrophilic organisms per milliliter and these samples, in turn, stored longer than the samples with ten thousand to one hundred million psychrophilic organisms per milliliter. However, the authors stated that it would be very difficult to predict the shelf life based on psychrophilic counts.

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 (80). Ford and Babel (29) indicated that the extent of post-pasteurization contamination had a greater influence on the keeping quality of milk than conditions encountered during distribution.

Studies on the reproductive cycles of some common psychrophilic bacteria of the genera Pseudomonas and Brevibacterium in pure cultures by El-Farekh (22) showed that the 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 the size of initial inoculum, and 11.24 hours for Brevibacterium lipolyticum. 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.

The Standard Plate Count and the psychrophilic count have been shown to be of little value in predicting the keeping quality of milk (81,23). Presumably this was because a low level of contamination with a biochemically active, rapidly growing psychrophile resulted in a poorer keeping quality than resulting from a relatively large number of biochemically inert psychrophiles (81). Also, according to Olson et al. (57) the type of contaminating organism was more important than the number. The ultimate failure of keeping quality due to spoilage by psychrophilic bacteria was manifested in a large and varied number of defects. A few of the odors and flavors that developed were fruity, rancid, putrid, cheesy, soapy, bitter, sweaty feet odor, potato odor, fishy taint and aroma loss (56,57,78). These were coupled with physical defects such as soapy or discoloration, whereas Erdman and Thornton (25) described some of the predominating flavor defects as stale, oxidized, sour and bitter.

Pseudomonas fragi was found to cause ropiness, May apple odor and later rancidity in milk (37), and Sherman et al. (72) observed the spoilage of milk and cream held at low temperatures mainly as proteolysis and development of bitter flavor.

No specific relationship between total psychrophilic population and off-flavor development occurred in milk stored at refrigeration

temperatures (56,77,80). 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. Olson et al. (56) stated that psychrophiles have attained a count exceeding ten millions per milliliter without any detectable flavor deterioration.

In a study by Punch et al. (65) with psychrophilic cultures to determine population levels present at a time a flavor or physical change in pasteurized milk was detected, populations associated with a detectable change varied among genera and among species within a genus. Generally, these levels were in a range of 5 to 20 million per milliliter at the time a change was observed; more active cultures were below and less active ones were above this range, and further the most commonly observed flavor defect was unclean; one culture of Alcaligenes viscolactis produced ropiness with no detectable flavor change which occurred at population levels below 5 million per milliliter.

Ingraham and Stokes (39) observed that the psychrophilic bacteria produce diffusable and nondiffusable pigments even at low temperatures, although the pigment production was not a common characteristic of psychrophilic bacteria but pigment production was an important factor in spoilage of fish, dairy and other products; whereas psychrophilic cultures produced slime on cottage cheese held at 4.4°C. in seven days (21).

In a study by Harmon et al. (35) homogenized milk was stored at various temperatures for organoleptic quality, psychrophilic, lipolytic, proteolytic and coliform bacterial counts, titratable acidity, acid degree value, 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 in 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.

Biochemical Activity

Psychrophiles are considered to be reasonably inactive in the reduction of dyes (32), but rapidly cause the destruction of small amounts biacetyl presumably by reduction to acetyl-methyl carbionol and possibly even to 2, 3-butylene glycol (60). Some psychrophiles are active phosphatase producers, notably Pseudomonas putrefaciens, but do not appear to increase the phosphatase in milk samples (7).

A number of psychrophiles are oxidase-producing but are not responsible for oxidized flavors in milk. In fact, when large inoculums of these organisms were added to milk, the development of oxidized flavor was markedly or completely inhibited (16). Psychrophiles are generally characterized as being inert toward carbohydrates, yet they are in a large part responsible for the elucidation of the pathways of carbohydrate degradation (82). The fact that psychrophiles usually produce little or no change in litmus milk is probably responsible for their being considered biochemically inert. When psychrophiles do produce a reaction in litmus milk, they usually cause an increase in alkalinity (66). Jones and Thomas (43) observed a psychrophilic Flavobacterium

species that produced no visible change in sterile milk in seven days at 22°C. Further, psychrophiles sometimes are slow to produce spoilage defects.

Psychrophilic bacteria are usually strongly lipolytic, proteolytic, or both. Roughly 90 percent of the psychrophiles isolated by Schultze and Olson (68) were either lipolytic or proteolytic and 66 percent were both.

Proteolytic Activity

The majority of isolates of psychrophilic bacteria have been observed to be proteolytic (74,75). It was observed very early that a common failure of milk held at low temperature was due to proteolysis (61). Pure culture studies of proteolytic bacteria, however, have shown that the activity of proteolytic enzyme increases with increasing temperature.

Peterson and Gunderson (62) studied the proteolytic enzymes from Pseudomonas fluorescens and reported that the liberation of the extracellular proteolytic enzymes was greatest at 0°C. and decreased with increasing temperature. The activity of the endocellular proteolytic enzymes from the bacteria grown at low temperatures was greater per milligram of protein than when the cells were grown at higher temperatures. After elaboration, both the extracellular and endocellular systems increased in activity with an increase in temperature. Casein and some insoluble casein derivative was broken down into water soluble compounds in the process of proteolysis and 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 found with the development of a bitter flavor due to the presence of peptones, and sometimes bitterness was evident before proteolysis was conspicuous. This occurred most frequently when the products were held at relatively low temperatures (34, 81).

Lipolytic Activity

Some organisms produce lipase which hydrolyse fat with the liberation of free fatty acids and glycerol (34), and this is called lipolysis. Butterfat contains short chain fatty acids, such as butyric and caproic acids (30), and these are usually objectionable when present in free form in dairy products because they impart a rancid flavor. Some of the psychrophilic microorganisms have been found to produce lipase at low temperatures and these lipases were active at temperatures below freezing and especially active at 40°F. (59).

Nashif and Nelson (52) studied in detail the lipase of Pseudomonas fragi and demonstrated that this lipase was preferentially produced at low temperatures and none was produced by these organisms when growing at 30°C. or higher. The lipase once produced, had a temperature optimum of 40°C., had a pH optimum of 7.0 to 7.2, exhibited a specificity for tricaprylin, was relatively inactive towards tributyrin and triolein and was heat stable requiring heating to 99°C. for twenty minutes to completely inactivate.

Of immense practical significance, butter containing residual lipase from Pseudomonas fragi undergoes considerable fat degradation during storage at -10°C., but especially at 5°C. and above (52). Recently Pinheiro et al. (63) determined the heat stability of lipases of selected psychrophilic bacteria in milk and Purdue Swiss-type cheese and found that the enzyme produced by Pseudomonas fluorescens was inactivated using the commercial heat treatment applied to milk, whereas the enzymes produced by Pseudomonas mucidolens and Pseudomonas fragi were more heat stable and caused problems in Purdue Swiss-type cheese, making it unmarketable due to the off-flavors developed. The ability to produce lipase varies considerably between different genera, between different species of the same genus, and even between strains of the same species (81). The optimum temperature of lipase production also varies markedly with the producing organism (53).

The lipases produced exhibit different specificities toward triglycerides and have different pH optima for activity. In a study by Overcast and Skean (58) freshly pasteurized whole milk was inoculated with twenty-five pure cultures of lipolytic microorganisms and stored at 4°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. A general disagreement exists when lipases from different sources are compared with respect to heat stability, substrate specificity, optimum temperature for production and activity, adaptive characteristics and inactivating agents. The existing

knowledge of microbial lipases, however, was only fragmentary and further study may resolve many of these disagreements. One point of agreement was that lipolytic psychrophiles are frequently encountered (75,76,79) and that many, if not most, of the fat-splitting bacteria are psychrophilic (81).

CHAPTER III

EXPERIMENTAL METHODS

The lipolytic Pseudomonas cultures used in this study were obtained from the stock culture collection of the Dairy Microbiology Laboratory at the University of Tennessee. Stock cultures were carried on Trypticase Soy Agar Slants which were stored at 2° to 3°C. Transfers from these slants were made into Trypticase Soy Broth and incubated at 21°C. for forty-eight hours, and this culture was used as an inoculum in the milk samples. Inocula were standardized by a turbidity measurement using a "Spectronic-20" colorimeter manufactured by Bausch and Lomb. One milliliter of the inoculum of the culture was diluted in 69 milliliters of sterile water. This dilution gave a transmission of 87 percent at 600 mu wave length, and repeatedly had bacterial count of 24 million organisms per milliliter. Each inoculum was prepared for the different cultures of Pseudomonas species on this basis. The cultures used in this experiment were transferred every other day in order to keep them ready for the day-to-day research. Dilution blanks were the gelatin-phosphate diluent of Naylor and Smith (51).

Fresh raw milk of individual cows used in this study was collected from the University of Tennessee Dairy Farm. Percentage of butter fat was determined by the Babcock method. Milk brought in the laboratory was used within an hour from the milking time, according to procedure outlined in Figure 1. All the cultures used in the study were pure.

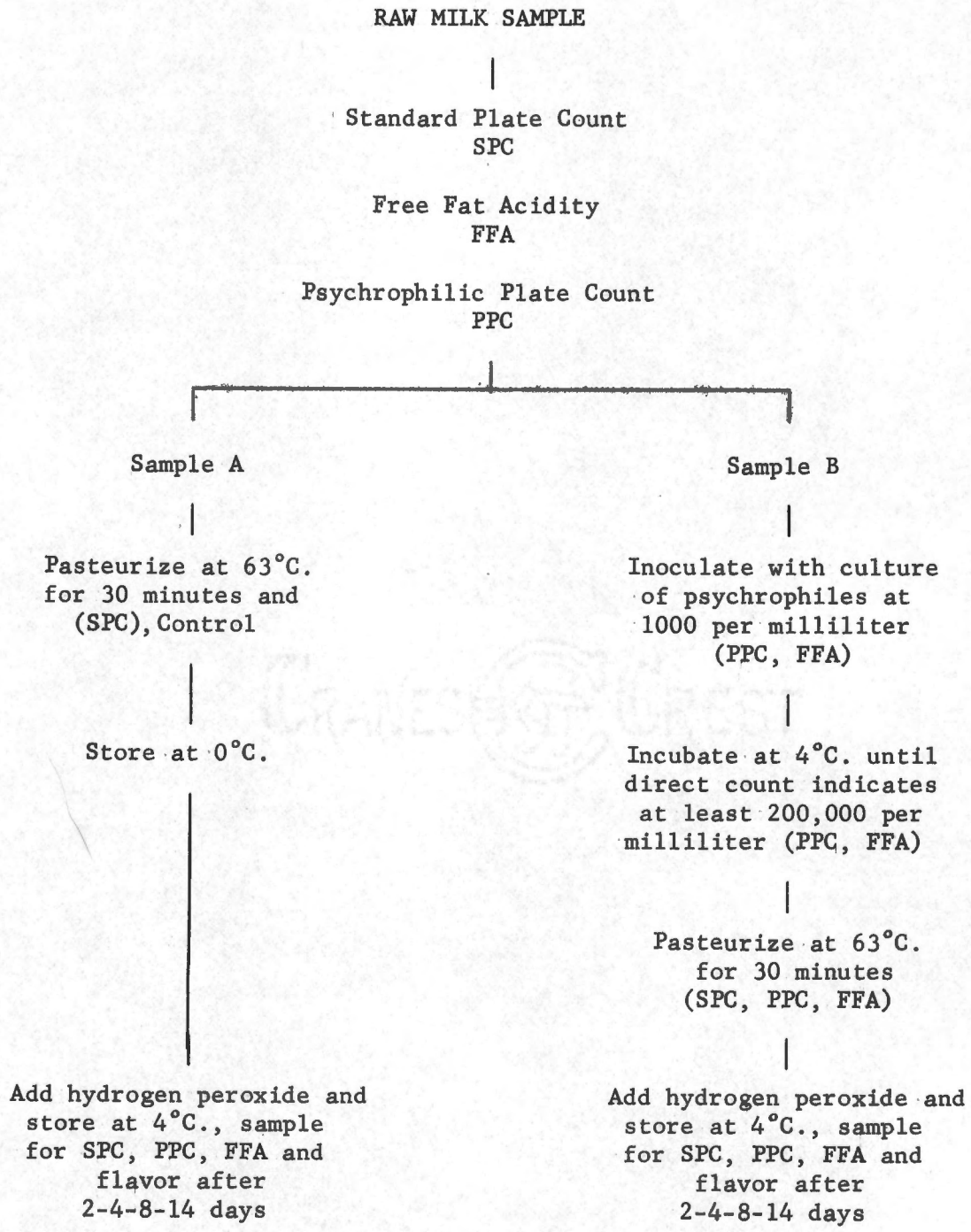


FIGURE 1

EXPERIMENTAL PROCEDURE USED IN EVALUATING THE CONTRIBUTIONS OF THE HEAT RESISTANT LIPASE OF PSYCHROPHILIC BACTERIA TO OFF-FLAVORS IN PASTEURIZED MILK

A Standard Plate Count (3) was made on the raw milk using Plate Count Agar incubated at 32°C. for forty-eight hours. A psychrophilic count was also made on the raw milk using Plate Count Agar but incubated at 21°C. for seventy-two hours. The free fat acidity was measured on fresh raw milk by the extraction-titration method (28). Original raw milk samples of 700 milliliters each were placed in the 1000 milliliter sterile culture bottles and labeled A and B and closed with rubber stoppers.

Sample A was pasteurized in the laboratory at 63°C. for thirty minutes, cooled immediately and plated for Standard Plate Count and then stored at 0°C. for two days. Sample B containing 700 milliliters of the same raw milk was inoculated with the pure culture of the Pseudomonas species (approximately 1,000 cells per milliliter) and incubated at 4°C. for forty-eight hours.

The psychrophilic count and free fatty acidity was determined on this inoculated milk. Growth of organisms in Sample B was determined daily, beginning one day after inoculation by microscopic examination and continued until the count exceeded 200,000 per milliliter. Sample B was then plated for psychrophilic count and the free fat acidity determined and pasteurized at 63°C. for thirty minutes.

This Sample B was then cooled immediately and plated for the Standard Plate and psychrophilic counts and free fat acidity was again determined. To both Samples A and B, H_2O_2 was added at 0.03 percent level to kill any surviving bacteria. Both Samples A and B were stored at 4°C. for fourteen days and examined for Standard Plate Count,

psychrophilic count, free fat acidity, and flavor changes after two, four, eight, and fourteen days interval.

The heat-resistant lipase activity of the psychrophilic bacteria inoculated in Sample B was determined by the net increase in free fat acidity produced during the fourteen days of storage. Standard Plate Count and psychrophilic count were made repeatedly during storage and found to be nil even in one milliliter quantities.

Raw milk used in the study was tested organoleptically and if any pronounced off-flavor was found, this milk was rejected. Sample B was again tasted before and after pasteurization in order to be sure that off-flavor detected within two days after inoculation of psychrophiles would not jeopardize the evaluation of the organoleptic quality due to the activity of the heat-resistant lipase during fourteen days of storage. The organoleptic evaluations were made by experienced judges of dairy products. The flavor of Samples A and B was recorded with no attempt being made to assign a numerical score.

CHAPTER IV

RESULTS AND DISCUSSION

Twenty-three cultures of lipolytic Pseudomonas species were used in the study. The data pertaining to the Standard Plate Counts, psychrophilic plate counts, free fat acidity, net free fat acidity and organoleptic evaluations are presented in appropriate tables. In order to check the precision and the reproducibility in the titration-extraction method (28) used to calculate the free fat acidity, Standard deviation (73) of the free fat acidity was determined with eight replicates of a fresh pasteurized milk sample with 4 percent butterfat (Table XVII, in the Appendix). The Standard deviation of $\pm$ 0.034 confirmed that the method used in the determination of free fat acidity did give precise results.

Since the Standard Plate Count and the psychrophilic plate count approached zero, the possibility of the psychrophiles producing proteolysis or lipolysis was eliminated. Therefore, the assumption was that the rancidity in Sample B was due to the heat-resistant lipase produced by the psychrophiles.

In spite of the fact that bacterial and psychrophilic counts were nil, still an increase was found in free fat acidity in all controls (Sample A). Hydrogen peroxide was added in both Samples A and B before storage in order to inactivate completely the organisms that may have survived pasteurization. Hydrogen peroxide used did not inhibit the

lipase activity (27). The increase in free fat acidity of all controls (Sample A) could be due to the reactivation of the lipase enzyme of milk or heat resistant lipase. This was in conformity with the findings of Shahani (70) regarding certain milk samples in market channels or during storage, particularly those pasteurized by the high temperature methods had shown an increase in the free fat acidity content. Shahani (70) further reported that such a phenomenon was observed in sterilized concentrated milk products, including ice milk mix and eggnog drink, whether such an increase was due to spontaneous hydrolysis of milk fat or reactivation of the lipase system or to the heat stable acid lipase was not known.

Oxidized flavor occurred in almost all of the controls (Sample A). This may have been due to the presence of oxygen in the milk which was not utilized by bacteria during the fourteen days of storage and also milk from cows on dry feed is more susceptible to oxidized flavors (33, 64). Guthrie (33) found that copper and oxygen are very important factors in influencing the rate of oxidation of ascorbic acid and the development of the oxidized flavors in milk. The possibility of the stored milk being oxidized by the sunlight or certain metal containers did not arise because adequate precautions were taken.

Culture 3

The free fat acidity in Sample A increased from 2.90 to 3.32 during fourteen days of storage at 4°C. (Tables I and II). This was the control milk to be compared in free fat acidity with the inoculated

TABLE I

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 3, 4, AND 6 OF PSYCHROPHILIC BACTERIA STORED AT 4°C.

Culture Number	Raw Milk			A	B After Inoculation		B After Incubation		B		
				Pasteurized					Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
3	10	6	2.90	---	10	2.93	310	4.03	.03	---	4.0
4	19	7.5	2.90	.03	1500	2.95	2600	4.69	.03	---	4.6
6	120	90	2.12	.30	500	2.13	2500	3.0	.30	.35	3.10

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE II

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 3, 4, AND 6 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 3 Sample A	Culture 3 Sample B	Culture 4 Sample A	Culture 4 Sample B	Culture 6 Sample A	Culture 6 Sample B
2 days	SPC	---[b]	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.09	4.12	3.09	4.87	2.25	3.31
	Flav.	Co.	Co.	Sl. Ox. Sl. Co.	Sl. Ran.	Sl. Co.	Sl. Co.
4 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.19	4.40	3.18	5.06	2.31	3.45
	Flav.	Co. Sl. Ox.	Sl. Ox. Sl. Un.	Sl. Ox. Co.	Sl. Ran. Sl. Bit.	Sl. Co.	Sl. Ox.
8 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.28	4.59	3.28	5.39	2.37	3.56
	Flav.	Sl. Ox. Co.	Sl. Bit. Sl. Ran.	Ox. Co.	Sl. Ran. Sl. Bit.	Sl. Ox.	Sl. Un. Sl. Ox.
14 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.32	4.96	3.37	5.62	2.43	4.30
	Flav.	Ox.	Sl. Ran. Sl. Bit.	Ox.	Ran. Sl. Bit.	Ox.	Sl. Ran. Sl. Bit.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit. = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

Sample B. Sample B increased in free fat acidity from 2.90 to 4.96 with a net increase of 1.64 from the control or 1.64 free fat acidity produced by the heat resistant lipase. The change in flavor of Sample B was found on the fourth day of storage at 4°C., compared to a slight change in flavor of Sample A. The off-flavor rancidity and bitterness was developed on the eighth and fourteenth day of storage.

Culture 4

Sample B was slightly rancid on the second day compared to Sample B of Culture 3 which did not become rancid until the eighth day of storage (Tables I and II). The rapid increase in the free fat acidity of Sample B with Culture 4 may have been due to many times more psychrophilic organisms before pasteurization as compared to Culture 3, Sample B. Sample B from Culture 4 was slightly rancid on the fourth day, slightly rancid and bitter on the eighth day, slightly rancid and bitter on the fourteenth day of storage. The psychrophilic count and the bacterial count was nil on the fourteenth day of storage of Sample B. The control (Sample A) developed an oxidized flavor during the fourteen days of storage.

Culture 6

The flavor detected in Sample B was slightly rancid and bitter and in Sample A oxidized only, and 43 percent more free fat acidity was found in Sample B compared with Sample A during the fourteen days of storage at 4°C. (Tables I and II).

Culture 7

An increase in free fat acidity in Sample B compared to Sample A with a net free fat acidity of 1.51 was produced by the heat resistant lipase (Tables III and IV). Sample B developed slight rancidity on the second day, rancidity and bitterness on the eighth day, and slight bitterness, rancidity and unclean flavor on the fourteenth day of storage at 4°C.

Culture 26

In Sample B 33 percent more free fat acidity was produced than in Sample A on the fourteenth day of storage (Tables III and IV). Sample B was bitter and rancid compared to Sample A which was slightly oxidized. With this culture bitterness occurred on the fourth day whereas with Culture 7 bitterness occurred on the eighth day of storage which might have been due to higher psychrophilic count before pasteurization of Sample B.

Culture 31

Sample B possessed a slight oxidized flavor on the second day, slight rancidity on the fourth day and bitterness and rancidity on the fourteenth day of storage at 4°C. (Tables III and IV). The possibility of the bitterness produced solely as a result of fat hydrolysis was doubtful. Usually if microbial degradation of the milk constituents continued the milk developed an extreme bitterness. But nil bacterial count was found on the fourteenth day of storage. The question of microbial degradation of the milk constituents did not arise and

TABLE III

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 7, 26, AND 31 OF PSYCHROPHILIC BACTERIA STORED AT 4°C.

Culture Number	Raw Milk			A	B After Innoculation		B After Incubation		B		
				Pasteurized					Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
7	15	9	3.0	.4	250	3.11	1520	3.75	.03	---	3.77
26	19	30	3.01	.1	200	3.10	2200	4.50	.15	.03	4.52
31	30	25	2.81	.2	30	2.85	500	3.47	---	---	3.46

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE IV

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 7, 26, AND 31 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 7 Sample A	Culture 7 Sample B	Culture 26 Sample A	Culture 26 Sample B	Culture 31 Sample A	Culture 31 Sample B
2 days	SPC	40	40	40	40	200	200
	PPC	---[b]	---	---	30	120	100
	FFA	3.18	4.03	3.37	4.87	3.0	3.67
	Flav.	Co.	Sl. Ran.	Co.	Sl. Ran.	Co.	Sl. Ox.
4 days	SPC	---	40	---	30	150	110
	PPC	---	30	---	30	100	60
	FFA	3.20	4.22	3.42	5.06	3.10	4.04
	Flav.	Co. Ox.	Sl. Ran. Sl. Ox.	Ox.	Bit. Ran.	Sl. Ox.	Sl. Ran.
8 days	SPC	30	---	---	30	100	100
	PPC	---	---	---	30	60	50
	FFA	3.31	4.70	3.55	5.16	3.15	4.22
	Flav.	Co. Ox.	Bit. Sl. Ran.	Sl. Ox.	Bit., Ran., Un.	Sl. Ox. Co.	Sl. Ran.
14 days	SPC	300	---	---	---	50	40
	PPC	250	---	---	---	---	---
	FFA	3.65	5.16	3.59	5.43	3.20	5.03
	Flav.	St. Ox.	Sl. Bit. Ran., Un.	St. Ox.	Bit., Ran.	Ox.	Bit., Ran.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit. = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

therefore the bitterness in Sample B might have been due to the presence of heat resistant lipase or proteolytic enzyme produced by the psychrophiles.

Davis (19) believed that bitterness in milk held at low temperature might well be the result of butterfat degradation rather than formation of "peptones," which also agreed with Anderson's and Hardenberg's (5) views. Most of the cultures used in the study produced rancidity which was accompanied or followed by bitterness on the eighth and fourteenth day of storage at 4°C.

Cultures 60, 63, and 75

Sample B of Cultures 60, 63, and 75 had 3 million, 201,000 and 250,000 psychrophilic count per milliliter and 4.03, 3.37, and 3.93 free fat acidity, respectively, before pasteurization (Table V). Sample B of Culture 60 became rancid and bitter, whereas with Cultures 63 and 75 only slightly oxidized flavor occurred on the fourth day of storage (Table VI). On the fourteenth day of storage, Sample B with Cultures 60, 63, and 75 had an increase in net free fat acidity (see Table XVII in the Appendix), 1.92, 1.05, and 1.07, respectively. The flavor changes were strongly bitter, rancid and unclean; slightly rancid, and rancid, slightly unclean in Samples B, strongly oxidized, slightly oxidized and strongly oxidized in Samples A on the fourteenth day of storage at 4°C.

Tables V and VI show that Sample B with Culture 60 had more distinct changes in the flavor when compared to Sample B with Cultures 63 and 75, which might be due to fourteen times higher psychrophilic count before the pasteurization of Sample B.

TABLE V

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 60, 63, and 75 OF PSYCHROPHILIC BACTERIA STORED AT 4°C.

Culture Number	Raw Milk			A Pasteurized	B After Innoculation		B After Incubation		B Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
60	65	32	3.07	.30	500	3.12	3000	4.03	.30	.03	4.01
63	80	35	2.06	---	37	2.09	201	3.37	.12	.03	3.33
75	85	50	2.90	.03	55	2.94	250	3.93	.15	---	3.90

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE VI

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 60, 63, AND 75 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 60 Sample A	Culture 60 Sample B	Culture 63 Sample A	Culture 63 Sample B	Culture 75 Sample A	Culture 75 Sample B
2 days	SPC	100	50	---[b]	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.20	4.40	2.40	3.42	3.10	4.12
	Flav.	Co. Sl. Ox.	Sl. Ran. Sl. Bit.	Co.	Sl. Co.	Co.	Sl. Co. Sl. Ox.
4 days	SPC	30	40	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.23	4.60	2.50	3.55	3.35	4.21
	Flav.	Co. Ox.	Sl. Ran. Bit. Sl. Un.	Sl. Ox.	Sl. Ox.	Ox.	Sl. Ox.
8 days	SPC	30	40	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.30	4.95	2.60	3.70	3.45	4.40
	Flav.	Co. Ox.	Ran., Bit., Sl. Un.	Co. Sl. Ox.	Sl. Ox.	Ox.	Sl. Ran.
14 days	SPC	---	30	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.42	5.34	2.77	3.82	3.52	4.59
	Flav.	St. Ox.	Ran., Un., St. Bit.	Sl. Ox.	Sl. Ran.	St. Ox.	Ran. Sl. Un.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit., = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

Cultures 94, 98, and 99

Results with Cultures 94, 98, and 99 used in the study are shown in Tables VII and VIII.

Cultures 94, 98, and 99 had 240,000, 200,000, and 300,000 psychrophilic count per milliliter; 3.82, 3.30, and 3.93 free fat acidity, respectively, before pasteurization of Sample B. Sample B with Culture 99 became slightly rancid on the fourth day, and with Culture 98 rancidity occurred on the eighth and the fourteenth days and with Culture 94 on the fourteenth day of storage. Sample A developed only oxidized flavor on the fourteenth day of storage. Nil bacterial and psychrophilic counts were found on the fourteenth day of storage.

Although Sample B with Culture 94 possessed a greater net increase in free fat acidity compared with Culture 98 on the eighth day of storage, yet Sample B with Culture 98 showed rancidity in the flavor, whereas Sample B with Culture 94 was only slightly oxidized (Table XVII, in the Appendix). This may have been due to some differences in ratio of volatile short chain free fatty acids present in the milk. Al-Shabibi et al. (1) reported that of the sixteen free fatty acids added to milk only caproic, caprylic, capric, and lauric acids were associated with rancidity, whereas Foster et al. (30) stated that rancid flavor was due to free butyric acid.

Cultures 100, 101, and 125

Cultures 100, 101, and 125 had 210,000, 270,000, and 290,000 psychrophilic counts per milliliter, respectively, before pasteurization

TABLE VII

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 94, 98, AND 99 OF PSYCHROPHILIC BACTERIA STORED AT 4°C.

Culture Number	Raw Milk			A Pasteurized	B After Innoculation		B After Incubation		B Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
94	18	8.2	2.92	.04	13	2.95	240	3.82	.06	.04	3.81
98	30	20	2.55	.06	30	2.59	200	3.30	.30	.10	3.26
99	40	30	3.18	.07	40	3.20	300	3.93	---	---	3.90

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE VIII

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 94, 98, AND 99 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 94 Sample A	Culture 94 Sample B	Culture 98 Sample A	Culture 98 Sample B	Culture 99 Sample A	Culture 99 Sample B
2 days	SPC	80	30	---[b]	---	---	---
	PPC	30	---	---	---	---	---
	FFA	3.07	3.90	2.70	3.37	3.28	4.21
	Flav.	Co. Sl. Ox.	Co.	Sl. Co.	Sl. Co.	Ox.	Co.
4 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.15	4.05	2.77	3.52	3.37	4.40
	Flav.	Co. Sl. Ox.	Co. Sl. Ox.	Sl. Ox.	Sl. Co. Sl. Ox.	Ox. Co.	Sl. Ran. Sl. Un.
8 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.20	4.20	2.85	3.67	3.45	4.59
	Flav.	Co. Ox.	Sl. Ox.	Ox.	Sl. Ran.	Co. Ox.	Sl. Ran. Sl. Bit.
14 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.35	4.50	2.92	4.35	3.56	5.06
	Flav.	Ox.	Sl. Ran. Sl. Un.	Ox.	Ran. Sl. Un.	St. Ox.	Ran. Bit.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit., = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

of Sample B. No serious detectable change in flavor was found in Sample B during the second, fourth, and eighth day of storage with the exception of cooked and slightly oxidized flavor (Tables IX and X). On the fourteenth day of storage all three Samples B with Cultures 100, 101, and 125 became slightly rancid; in addition Culture 101 had slight bitterness. Net free fat acidity produced by the heat resistant lipase of Cultures 100, 101, and 125 was 0.97, 1.42, and 0.96, respectively (Table XVII, in the Appendix), on the fourteenth day of storage. The bitterness produced in Sample B with Culture 101 may have been due to excessive free fat acidity compared to Sample B of Cultures 100 and 125. As far as Sample A was concerned there was no change in flavor except oxidized on the fourteenth day of storage. Tables IX and X with data of three cultures show that Sample B within a range of 210,000 to 290,000 of psychrophiles per milliliter could produce enough heat-resistant lipase to cause slight rancidity and bitterness on the fourteenth day of storage after the milk was pasteurized where no growth of bacteria occurred after pasteurization.

Cultures 131, 135, and 145

Psychrophilic counts in Sample B before pasteurization for Cultures 131, 135, and 145 were 300,000, 230,000 and 250,000, respectively. The free fat acidity before pasteurization was 4.78, 4.03, and 4.46, respectively (Tables XI and XII). Sample B was slightly rancid on the second day of storage with all three of these cultures. On the eighth day of storage Sample B with Cultures 131 and 145 showed rancidity

TABLE IX

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 100, 101, AND 125 OF PSYCHROPHILIC BACTERIA STORED AT 4°C.

Culture Number	Raw Milk			A Pasteurized	B After Innoculation		B After Incubation		B Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
100	75	45	2.77	.15	48	2.79	210	3.15	.20	.10	3.13
101	5	2.5	2.43	.03	8	2.45	270	3.52	---	.03	3.50
125	12	6.5	3.15	.03	10	3.16	290	3.82	---	---	3.80

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE X

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 100, 101, AND 125 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 100 Sample A	Culture 100 Sample B	Culture 101 Sample A	Culture 101 Sample B	Culture 125 Sample A	Culture 125 Sample B
2 days	SPC	---[b]	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	2.85	3.30	2.50	3.60	3.22	3.82
	Flav.	Sl. Co.	Sl. Co.	Sl. Co.	Sl. Ox. Sl. Un.	Co.	Sl. Co.
4 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	2.90	3.52	2.60	3.90	3.27	3.97
	Flav.	Sl. Ox.	Sl. Co.	Co.	Sl. Co.	Sl. Ox.	Co.
8 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	2.95	3.67	2.70	4.05	3.30	4.12
	Flav.	Ox.	Sl. Ox.	Co. Ox.	Sl. Un.	Ox.	Co.
14 days	SPC	---	---	---	---	---	---
	PPC	---	---	---	---	---	---
	FFA	3.0	3.97	3.0	4.42	3.39	4.35
	Flav.	Ox.	Sl. Ran.	Co. Ox.	Sl. Ran. Sl. Bit.	St. Ox.	Sl. Ran. Sl. Un.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit., = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

TABLE XI

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 131, 135, AND 145 OF PSYCHROPHILIC BACTERIA STORED AT 4°C.

Culture Number	Raw Milk			A Pasteurized	B After Innoculation		B After Incubation		B Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
131	13	30	3.56	---	40	3.60	300	4.78	---	---	4.72
135	8.5	6	3.09	---	10	3.10	230	4.03	---	---	4.02
145	7	4	3.14	---	35	3.19	250	4.46	---	---	4.46

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE XII

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 131, 135, AND 145 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 131 Sample A	Culture 131 Sample B	Culture 135 Sample A	Culture 135 Sample B	Culture 145 Sample A	Culture 145 Sample B
2 days	SPC	---[b]	---	60	---	30	---
	PPC	---	---	50	---	---	---
	FFA	3.84	4.96	3.28	4.31	3.40	4.77
	Flav.	Co. Sl. Ox.	Sl. Ran. Sl. Un.	Co.	Sl. Ox. Sl. Ran.	Co. Sl. Ox.	Sl. Ox. Sl. Ran.
4 days	SPC	---	---	30	---	---	---
	PPC	30	---	30	---	---	---
	FFA	3.93	5.15	3.37	4.59	3.50	4.96
	Flav.	Co. Sl. Ran.	Ran. Sl. Sour	Co. Ox.	Sl. Ran. Sl. Ox.	Sl. Ox. Co.	Sl. Ran. Sl. Ox.
8 days	SPC	30	---	30	---	---	---
	PPC	---	---	---	---	---	---
	FFA	4.03	5.34	3.46	4.78	3.59	5.56
	Flav.	Sl. Ran.	Ran. Bit.	Co. Ox.	Sl. Ran. Ox.	Ox.	Ran. Sl. Bit.
14 days	SPC	---	---	50	---	---	---
	PPC	---	---	---	---	---	---
	FFA	4.12	5.84	3.56	4.96	3.65	5.80
	Flav.	Sl. Ran. Sl. Un.	St. Ran. Bit.	St. Ox.	Sl. Bit. Ran.	Ox.	Bit. Sl. Ran.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit., = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

and bitterness whereas on the fourteenth day of storage with all three cultures had enough rancidity and bitterness to make this milk unsuitable to drink, compared to other Samples B with different cultures used in the study. The net free fat acidity of Sample B on the fourteenth day of storage was 1.72, 1.40, and 2.15 for Cultures 131, 135, and 145, respectively (Table XVII, in the Appendix). The control Sample A used for Culture 131 became slightly rancid on the fourth day of storage, whereas the controls for Cultures 135 and 145 had oxidized flavor only. The psychrophilic count and bacterial count of Sample B on the fourteenth day of storage approached zero, and therefore the change in the flavor could be only due to the action of heat resistant lipase or proteolytic enzyme produced by the inoculated psychrophiles. Unclean flavor was detected occasionally in Sample B which may have been due to the by-products liberated in the development of bitterness and rancidity. Possibly the effect of different fatty acids liberated by the lipase was associated with the unclean flavor.

Cultures 147, 149, and 158

Tables XIII and XIV show the result of Cultures 147, 149, and 158 used in the study. Also, see Table XVII in the Appendix.

Psychrophilic counts for Cultures 147, 149, and 158 were 1 million, 360,000, and 850,000 per milliliter and the free fat acidity was 3.44, 3.46, and 3.49, respectively, before pasteurization of Sample B. Flavor deterioration resulting from Sample B with Culture 147 ranged from slight bitterness on the second day to strong bitterness and slight rancidity

TABLE XIII

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 147, 149, AND 158 OF PSYCHROPHILIC BACTERIA STORED AT 4°C.

Culture Number	Raw Milk			A	B After Innoculation		B After Incubation		B		
				Pasteurized					Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
147	33	23.5	3.09	.3	240	3.10	1000	3.44	.3	.4	3.45
149	17	8	2.82	.3	10	2.90	360	3.46	.3	---	3.44
158	18.8	17	3.10	.3	180	3.13	850	3.49	.3	.4	3.48

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE XIV

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 147, 149, AND 158 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 147 Sample A	Culture 147 Sample B	Culture 149 Sample A	Culture 149 Sample B	Culture 158 Sample A	Culture 158 Sample B
2 days	SPC	300	100	50	40[b]	300	100
	PPC	100	50	30	---	40	30
	FFA	3.11	3.89	2.84	3.50	3.15	3.52
	Flav.	Co. Sl. Ox.	Sl. Bit. Sl. Un.	Sl. Co.	Sl. Co.	Co.	Sl. Co. Sl. Ox.
4 days	SPC	300	300	30	---	300	300
	PPC	---	---	---	---	---	---
	FFA	3.26	4.03	2.88	3.80	3.18	4.03
	Flav.	Sl. Ox. Co.	Bit. Sl. Ran.	Sl. Co.	Sl. Ox. Sl. Co.	Co. Sl. Ox.	Sl. Ran.
8 days	SPC	300	100	---	---	300	300
	PPC	40	30	---	---	---	---
	FFA	3.37	4.40	2.89	3.93	3.21	4.30
	Flav.	Sl. Ox. Sl. Un.	Sl. Ran. Bit.	Sl. Ox.	Sl. Bit.	Ox.	Sl. Bit. Sl. Ran.
14 days	SPC	60	30	---	---	250	200
	PPC	---	40	---	---	---	---
	FFA	3.40	4.79	3.16	4.85	3.53	4.78
	Flav.	St. Ox.	St. Bit. Sl. Ran.	Co. St. Ox.	Bit. Sl. Ran.	St. Ox.	St. Bit. Sl. Ran.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit., = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

on the fourteenth day of storage, with a net free fat acidity of 1.39.

Sample B with Culture 149 developed slight bitterness on the eighth day, bitterness and slight rancidity on the fourteenth day of storage with a net free fat acidity of 1.69. Sample B with Culture 158 developed slight rancidity onthe fourth day, strong bitterness and slight rancidity on the fourteenth day of storage with a net free fat acidity of 1.25. Although Culture 149 had the lowest number of psychrophiles before pasteurization and produced more net free fat acidity when compared to Cultures 147 and 158, yet off-flavors were produced more slowly than with the other two cultures. This would indicate that only specific fatty acids would cause the milk to be rancid or bitter, the production of which depended on the ability of lipase to catalyze the action (53, 81).

Cultures 169 and 178

Cultures 169 and 178 (Tables XV and XVI) had a psychrophilic count of 260,000 and 200,000 and a free fat acidity of 4.52, 3.41, respectively, before the pasteurization. Sample B with Culture 169 developed a slight rancidity on the second day; slight bitterness and slight rancidity on the fourth day; bitterness on the eighth day; and bitterness and rancidity on the fourteenth day of storage with a net free fat acidity of 1.84 (Table XVII, in the Appendix).

Sample B with Culture 178 developed a slight bitterness on the eighth and slight bitterness and slight rancidity on the fourteenth day of storage, and a net free fat acidity of 2.21 (Table XVII) which was

TABLE XV

STANDARD PLATE COUNTS,[a] PSYCHROPHILIC PLATE COUNTS,[a] AND FREE FAT ACIDITIES OF MILK WITH CULTURES 169 AND 178 OF PSYCHROPHILIC BACTERIA STORED AT 40°C.

Culture Number	Raw Milk			A	B After Innoculation		B After Incubation		B		
				Pasteurized					Pasteurized		
	SPC	PPC	FFA[b]	SPC	PPC	FFA	PPC	FFA	SPC	PPC	FFA
169	19	11	3.06	---	20	3.07	260	4.52	---	---	4.51
178	15	5	2.32	.10	6	2.33	200	3.41	---	---	3.42

[a]Bacteria count in thousands with a dash indicating no colonies developed from 1 ml. samples.

[b]Free fat acidity expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

TABLE XVI

OFF-FLAVORS AND FREE FAT ACIDITIES PRODUCED BY CULTURES 169 AND 178 OF PSYCHROPHILIC BACTERIA IN PASTEURIZED MILK STORED AT 4°C.

After Stor.	Test[a]	Culture 169 Sample A	Culture 169 Sample B	Culture 178 Sample A	Culture 178 Sample B
2 days	SPC	---[b]	---	---	---
	PPC	---	---	---	---
	FFA	3.52	4.69	2.36	3.46
	Flav.	Co.	Ox. Sl. Ran.	Sl. Co.	Sl. Co.
4 days	SPC	---	---	---	---
	PPC	---	---	---	---
	FFA	3.60	4.75	2.38	3.49
	Flav.	Co. Sl. Ox.	Sl. Bit. Sl. Ran.	Sl. Ox.	Sl. Co. Sl. Un.
8 days	SPC	30	---	---	---
	PPC	---	---	---	---
	FFA	3.65	5.15	2.40	3.75
	Flav.	St. Ox.	Bit.	Sl. Ox.	Sl. Bit.
14 days	SPC	40	---	---	---
	PPC	---	---	---	---
	FFA	3.75	5.59	2.49	4.70
	Flav.	St. Ox.	Bit. Ran.	Sl. Ox.	Sl. Bit. Sl. Ran.

[a]SPC--Standard Plate Counts; PPC--Psychrophilic Plate Counts; FFA--Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat; Flav.--flavors (Co. = cooked, Sl. = slightly, St. = strongly, Ran. = rancidity, Bit. = bitter, Ox. = oxidized, Un. = unclean).

[b]Dash indicates no colonies developed from 1 ml. of sample.

much higher than Culture 169, yet was slow in producing rancidity which showed the variability in the activity of the lipase toward triglycerides (53). Results from this study showed that the heat resistant lipase produced by the psychrophilic microorganisms (Pseudomonas species) with counts of 250,000 to 300,000 per milliliter in the milk before pasteurization could bring about a severe change in the form of rancid and bitter flavor on the eighth and fourteenth day of storage at 4°C. and a slightly rancid flavor as early as two days after storage. This points out the seriousness of the presence of psychrophiles in the raw milk which could contribute to the off-flavors and shorten the shelf life thereby affecting quality and ultimately consumption.

Further research on other species of different genera of psychrophiles might be useful in order to come up with a definite idea of either setting up minimum standards for psychrophiles in the raw milk or some method to inactivate the lipase completely without affecting the flavor during the storage period of milk.

In summary, of the twenty-three lipolytic psychrophilic cultures (which produced heat resistant lipase) used, seven cultures produced slight rancidity and one culture slight bitterness on the second day of storage, whereas twelve cultures produced rancidity and five cultures bitterness on the fourth day of storage. Fourteen cultures produced rancidity and thirteen bitterness on the eighth day of storage. All cultures produced slight rancidity to rancid, seventeen cultures bitterness on the fourteenth day of storage (Table XVII, in the Appendix).

The net free fat acidity varied from 0.96 to 2.25 and flavor changes that occurred ranged from slight rancidity to strong rancidity, bitterness and a few times unclean on the fourteenth day of storage of Sample B compared to control Sample A which developed oxidized flavors only. Also on two occasions a net increase in free fat acidity as low as 0.96 and 0.97 (Cultures 125 and 100) produced a rancid flavor on the fourteenth day of storage (Table XVII, in the Appendix) which agreed to the findings made by Overcast and Skean (58) in which they found that one culture produced a net free fat acidity as low as 0.96, yet rancidity and bitterness in the flavor of pasteurized milk occurred on the twelfth day of storage.

CHAPTER V

SUMMARY AND CONCLUSION

The heat resistant lipase produced by twenty-three lipolytic, psychrophilic cultures belonging to the Pseudomonas species and its contribution to flavor in the pasteurized milk upon storage was studied. Fresh raw milk was divided into Sample A (control) and Sample B, inoculated with the cultures in the study. Sample A was pasteurized immediately and stored at 0°C. and Sample B inoculated with psychrophiles and incubated at 4°C. for the growth of psychrophiles to various levels and then pasteurized. Hydrogen peroxide was added in both Samples A and B as an added precaution against bacterial growth after pasteurization and both samples were examined for off-flavor, free fat acidity, Standard Plate Count, and psychrophilic count after two, four, eight, and fourteen days of storage at 4°C.

The net free fat acidity (FFA Sample B minus FFA Sample A) measured was the free fat acidity produced by the heat-resistant lipase of the psychrophiles inoculated in the raw milk. Of the twenty-three cultures used in the study, seven cultures produced rancidity on the second day, twelve on the fourth, fourteen on the eighth, and all cultures produced rancidity on the fourteenth day of storage of pasteurized milk at 4°C., whereas the control Sample A produced only oxidized flavor on the fourteenth day of storage. The bacterial count and psychrophilic count approached zero at the end of fourteen days of storage of both

Samples A and B. The net increase in free fat acidity produced by the twenty-three cultures studied varied from 0.96 to 2.25 and flavor varied from slight rancidity to strong rancidity and bitterness on the fourteenth day of storage (Table XVII, Appendix). Most of the psychrophilic cultures in the range of 200,000 to 300,000 per milliliter in the raw milk before pasteurization produced enough heat-resistant lipase to cause rancid and bitter flavor in the pasteurized Sample B on the eighth and fourteenth day of storage at 4°C. This brought up a serious question as to whether the present Standard Plate Count for raw milk should be lowered or a new psychrophilic plate count standard should be set up with less than 200,000 per milliliter so that there may not be left enough heat-resistant lipase to cause the milk to be rancid on the eighth and fourteenth day of storage. This raises further question with regard to the usefulness of the present study as to whether more research should be pursued using less than 200,000 psychrophiles per milliliter in the raw milk with different species within the same genus or species of different genera and determined the heat resistance lipase activity with pasteurization at 145°F. for thirty minutes or 163°F. for fifteen seconds.

REFERENCES

REFERENCES

1. Al-Shabibi, M. M. A., Langner, E. H., Tobias, J., and Tuckey, S. L. 1964. Effects of Added Fatty Acids on the Flavor of Milk. J. Dairy Sci., 47:295.

2. Abd-el-Malek, Y., and Gibson, T. 1952. Studies in the Bacteriology of Milk. IV. The Gram-Negative Rods of Milk. J. Dairy Research, 19:294.

3. American Public Health Association. 1960. Standard Methods for the Examination of Dairy Products. 11th Ed. American Public Health Association, Inc., New York, New York.

4. Andersen, A. A. 1958. New Sampler for the Collection, Sizing and Enumeration of Viable Airborne Particles. J. Bacteriol., 76:471.

5. Anderson, J. A., and Hardenberg, J. G. 1932. The Deterioration of Cream by a Lipolytic Bacterium. J. Bacteriol., 23:59.

6. Andrews, R. H. 1953. The Destruction of Psychrophilic Bacteria in Milk by H.T.S.T. Pasteurization. J. Dairy Sci., 36:570.

7. Atherton, H. V., Doan, F. J., and Watrous, G. W. 1953. Observations on Bacterial Population and Characteristics of Bottled Milk Under Refrigerated Holding. Pa. Agr. Expt. Sta. Bull. 575.

8. Balaprakasam, R. A. 1961. Microbiological Determination of the Air of a Dairy Plant with Andersen Sampler. M.S. Thesis. University of Tennessee, Knoxville.

9. Berry, J. A., and Magoon, C. A. 1934. Growth of Microorganisms at and Below 0°C. Phytopathology, 24:780.

10. Boyd, J. C., Smith, C. K., and Trout, G. M. 1953. The Role of Psychrophilic Bacteria in the Keeping Quality of Commercially Pasteurized Milk. J. Dairy Sci., 36:571.

11. Boyd, J. C., and Smith, C. K. 1955. The Role of Psychrophilic Bacteria in the Keeping Quality of Commercially Pasteurized and Homogenized Milk. J. Milk and Food Technol., 18:32.

12. Broitman, S., Mallman, W. L., and Trout, G. M. 1958. A Simple Test for Detecting Keeping Quality for Milk. J. Milk and Food Technol. 21:280.

13. Burgwald, L. H., and Josephson, D. V. 1946. Considerations on the Keeping Quality of Pasteurized Milk. Am. Milk Review, 8:46.

14. Chaudhary, R. A., Tuckey, S. L., and Witter, L. D. 1960. Heat Resistance of Three Strains of Psychrophilic Organisms Added to Skim Milk for Cottage Cheese Manufacture. J. Dairy Sci., 43:1774.

15. Collins, E. B. 1955. Factors Involved in the Control of Gelatinous Curd Defects of Cottage Cheese. J. Milk and Food Technol., 18:169.

16. Cone, J. F., and Babcock, C. J. 1943. The Role of Oxidase-Producing Bacteria in the Development of Oxidized Flavor in Milk. J. Dairy Sci., 26:1.

17. Conn, E. W. 1903. The Relation of Temperature to the Keeping Property of Milk. Connecticut (Storrs) Agr. Expt. Sta. Res. Bull., 319.

18. Dahlberg, A. C. 1946. The Keeping Quality of Pasteurized Milk. Cornell Uni. Agr. Expt. Sta. Bull., 838.

19. Davis, P. A. 1951. The Effect of Cold on Microorganisms in Relation to Dairying. Proc. Soc. Appl. Bacteriol., 14:216.

20. Davis, J. G., and Mattick, A. T. R. 1929. Rusty Spot in Cheddar and Other Cheese. I. Description of the Causative Organisms. J. Dairy Research, 1:50.

21. Davis, P. A., and Babel, F. J. 1954. Slime Formation on Cottage Cheese. J. Dairy Sci., 37:176.

22. El-Farekh, T. A. 1962. Quantitative Study of Growth of Some Dairy Psychrophiles. M.S. Thesis. University of Tennessee, Knoxville.

23. Elliker, P. R., Sing, E. L., Christensen, L. J., and Sandine, W. E. 1964. Psychrophilic Bacteria and Keeping Quality of Pasteurized Dairy Products. J. Milk and Food Technol., 27:69.

24. Erdman, I. E., and Thornton, H. R. 1951. Psychrophilic Bacteria in Edmonton Milk and Cream. I. Numbers Can. J. Technol., 29:232.

25. Erdman, I. E., and Thornton, H. R. 1951. Psychrophilic Bacteria in Edmonton Milk and Cream. II. Kinds Can. J. Technol., 29:238.

26. Evans, D. A., and Lachman, E. L. 1963. Some observations on the Bacteriological Keeping Quality of Milk Processed by High Temperature with a 0.6 Second Holding Time. J. Milk and Food Technol., 26:232.

27. Frankel, E. N., and Tarassuk, N. P. 1959. Inhibition of lipase and lipolysis in Milk. J. Dairy Sci., 42:409.

28. Frankel, E. N., and Tarassuk, N. P. 1955. An Extraction-Titration Method for the Determination of Free Fatty Acids in Rancid Milk and Cream. J. Dairy Sci., 38:751.

29. Ford, H. F., and Babel, F. J. 1959. Milk Quality Problems Associated with Present-Day Marketing. J. Milk and Food Technol., 22:141.

30. Foster, E. M., Nelson, F. E., Speck, M. L., and Olson, J. C. 1957. Dairy Microbiology. Prentice-Hall, New Jersey.

31. Foter, M. J., and Rahn, O. 1936. Growth and Fermentation of Bacteria. J. Bacteriol., 32:485.

32. Greene, V. W., and Jamison, R. M. 1959. Influence of Bacterial Interaction on Resazurin Reduction Times. J. Dairy Sci., 42:1099.

33. Guthrie, E. L. 1955. Role of Ascorbic Acid in Development of Oxidized Flavors in Dairy Products. Cornell Univ. Agr. Expt. Sta. Res. Bull., 340.

34. Hammer, B. W., and Babel, F. J. 1957. Dairy Bacteriology. 4th Ed. John Wiley & Sons, New York.

35. Harmon, L. G., Trout, G. M., and Hedrick, T. I. 1959. Relationship Between Storage Temperatures and the Rate of Development of Various Organisms on the Shelf-Life of Homogenized Milk. Milk Dealer, 48:40.

36. Hampil, B. 1932. The Influence of Temperature on the Life Processes and Death of Bacteria. Quart. Rev. Biol., 7:172.

37. Hussong, R. V., Long, H. F., and Hammer, B. W. 1937. Classification of the Organisms Important in Dairy Products. II. Pseudomonas fragi. Iowa Agr. Expt. Sta. Res. Bull., 225.

38. Ingraham, J. L. 1958. Growth of Psychrophilic Bacteria. J. Bacteriol., 76:75.

39. Ingraham, J. L., and Stokes, J. L. 1959. Psychrophilic Bacteria. Bacteriol. Revs., 23:97.

40. Jezeski, J. J., and Macy, H. 1946. Cryophilic Organisms in Water and Butter. J. Dairy Sci., 29:439.

41. Jezeski, J. J. 1954. The Microbiology of Dairy Products. Ann. Rev. Microbiol., 8:429.

42. Johns, C. K. 1959. Detecting Recontamination of Pasteurized Products. Can. Dairy and Ice Cream J., 38(4):34.

43. Jones, G. E., and Thomas, S. G. 1950. The Occurrence of a Flavobacterium Species in Chilled Water. Proc. Soc. Appl. Bacteriol., 13:13.

44. Kennedy, L., and Weiser, H. 1950. Some Observations on Bacteria Isolated from Milk That Grow Within the Psychrophilic Temperature Range. J. Milk and Food Technol., 13:353.

45. Leete, C. S. 1931. Some Bacteriological and Temperature Studies in Milk Plants. Milk Plant Monthly, 20(1):72.

46. Labots, H., Galesloot, Th. E., and Ubbels, J. 1961. Airborne Contamination of Milk During Bottling, Using Filling Valves of Different Design. Neth. Milk and Dairy J., 15:123.

47. Lawton, W. G., and Nelson, F. E. 1954. The Effect of Storage Temperature on the Growth of Psychrophilic Organisms in Sterile and Laboratory Pasteurized Milk. J. Dairy Sci., 37:1164.

48. Leesment, H., and Dufeu, J. 1962. A Rapid Method for Determining the Psychrophilic Count in Milk. Proceedings of XVI International Dairy Congress, Vol. C:392.

49. Maculay, D. M., and Hawirko, R. Z. 1963. Effect of Pasteurization on Survival of Certain Psychrophilic Bacteria. Appl. Microbiol., 11:90.

50. Marth, E. H., and Frazier, W. C. 1957. Bacteriology of Milk Held at Farm Bulk Cooling Tank Temperature. I. Effect of Time and Temperature During Storage. J. Milk and Food Technol., 20:72.

51. Naylor, H. B., and Smith, P. A. 1946. Factors Affecting the Viability of Serratia marcescens During Dehydration and Storage. J. Bacteriol., 52:565.

52. Nashif, S. A., and Nelson, F. E. 1953. The Lipase of Pseudomonas fragi. I. Characterization of the Enzymes. J. Dairy Sci., 36:459.

53. Nashif, S. A., and Nelson, F. E. 1953. The Extracellular Lipases of Some Gram-Negative Non-Spore Forming Rod-Shaped Bacteria. J. Dairy Sci., 36:698.

54. Nelson, F. E., and Baker, M. P. 1954. The Influence of Time and Temperature of Plate Incubation Upon Bacterial Counts of Market Milk Related Products. J. Milk and Food Technol., 17:95.

55. Nicholas, J. E., and Anderson, T. G. 1962. Keeping Qualities of Milk. Refrig. Engineering, 44:370.

56. Olson, J. C., Willoughby, D. S., and Thomas, E. L. 1953. The Keeping Quality of Pasteurized Milk as Influenced by the Growth of Psychrophilic Bacteria and the Addition of Aureomycin. J. Milk and Food Technol., 16:213.

57. Olson, J. C., Parker, R. B., and Mueller, W. S. 1955. The Nature, Significance and Control of Psychrophilic Bacteria in Dairy Products. J. Milk and Food Technol., 18:200.

58. Overcast, W. W., and Skean, J. D. 1959. Growth of Certain Lipolytic Microorganisms at 4°C. and Their Influence on Free Fat Acidity and Flavor of Pasteurized Milk. J. Dairy Sci., 42:1479.

59. Overcast, W. W. 1964. Problems with Psychrophiles. Paper presented at 12th Annual Dairy Institute, University of Tennessee, Knoxville. (Mimeographed.)

60. Parker, R. B., and Elliker, P. R. 1953. Effect of Spoilage on Biacetyl Content and Flavor of Cottage Cheese. J. Dairy Sci., 36:843.

61. Pennington, M. E., and Hepburn, J. S. 1913-14. Bacterial and Enzymic Changes in Milk and Cream at 0°C. J. Biol. Chem. 16:331.

62. Peterson, A. C., and Gunderson, M. F. 1960. Some Characteristics of Proteolytic Enzymes from Pseudomonas fragi. Appl. Microbiol., 8:98.

63. Pinheiro, A. J. R., Liska, B. J., and Parmelee, C. E. 1965. Heat Stability of Lipases of Selected Psychrophilic Bacteria in Milk and Purdue Swiss-type Cheese. J. Dairy Sci., 48:983.

64. Pont, E. G. 1952. Studies on the Origin of Oxidized Flavor in Whole Milk. J. Dairy Res., 19:316.

65. Punch, J. D., Olson, J. D., and Thomas, E. L. 1965. Psychrophilic Bacteria. III. Population Levels Associated with Flavor or Physical Change in Milk. J. Dairy Sci., 48:1179.

66. Rogick, F. A., and Burgwald, L. H. 1952. Some Factors Which Contribute to the Psychrophilic Bacterial Count in Market Milk. J. Milk and Food Technol., 15:181.

67. Rogick, F. A., and Burgwald, L. H. 1950. A Study on Psychrophilic Bacteria in Market Milk. J. Dairy Sci., 33:403.

68. Schultz, W. D., and Olson, J. C. 1957. The Distribution of Psychrophilic Bacteria in Commercial Dairy Products. J. Dairy Sci., 40: 602.

69. Schultz, W. D., and Olson, J. C. 1960. Studies on Psychrophilic Bacteria. J. Dairy Sci., 43:346.

70. Shahani, K. M. 1966. Milk Enzymes. J. Dairy Sci., 49:907.

71. Seleen, W. A., and Stark, C. N. 1943. Some Characteristics of Green-Fluorescent Pigment-Producing Bacteria. J. Bacteriol., 46: 491.

72. Sherman, J. M., White, J. C., and Cameron, G. M. 1941. The Bacteriological Spoilage of Milk Held Near the Freezing Point, J. Dairy Sci., 24:526.

73. Snedecor, G. W. 1956. Statistical Methods. 5th ed. The Iowa State College Press, Ames, Iowa.

74. Stark, C. N., and Scheib, B. J. 1936. A Study of Fat Splitting and Casein Digesting Bacteria Isolated from Butter. J. Dairy Sci., 19:191.

75. Thomas, S. B., and Thomas, B. F. 1947. Some Observations on the Bacterial Flora of Farm Water Supplies. Proc. Soc. Appl. Bacteriol., 2:65.

76. Thomas, S. B. 1949. Psychrophilic Microorganisms in Milk and Dairy Products. Dairy Sci. Abstract, 20:355.

77. Thomas, S. B. 1958. Psychrophilic Microorganisms in Milk and Dairy Products. Dairy Sci. Abstract, 20:448.

78. Thomas, S. B., and Druce, R. G. 1963. Types of Psychrophlic Bacteria in Milk. Dairy Eng., 80:378.

79. Van-Derzant, W. C., and Moore, A. V. 1955. Some Factors Affecting Bacterial Counts of Soft Ice Milk. Food Tech., 9:285.

80. Watrous, G. H., Doan, F. J., and Josephson, D. V. 1952. Some Bacteriological Studies on Refrigerated Milk and Cream. Pa. Agr. Expt. Sta. Bull. 551.

81. Witter, L. D. 1961. Psychrophilic Bacteria. A Review. J. Dairy Sci., 44:983.

82. Wood, W. A. 1955. Pathways of Carbohydrate Degradation in Pseudomonas fluorescens. Bacteriol. Revs., 19:222.

83. Zobell, C. E. 1940. Studies on the Thermal Sensitivity of Marine Bacteria. J. Bacteriol., 40:223.

APPENDIX

TABLE XVII

LABORATORY RESULTS OF TWENTY-THREE CULTURES OF PSYCHROPHILIC BACTERIA

Culture Number	Raw Milk Butterfat %	After 14 Days Storage at 4°C. Sample B FFA[a]	Sample A FFA	Net FFA[b]	Flavor After Storage[c] Sample B 8 Days	Sample B 14 Days	Sample A 8 Days	Sample A 14 Days
3	4.0	4.96	3.32	1.64	Sl. Bit. Sl. Ran.	Sl. Ran. Bit.	Sl. Ox. Co.	Ox.
4	4.0	5.62	3.37	2.25	Sl. Ran. Sl. Bit.	Ran. St. Bit.	Ox. Co.	Ox.
6	6.0	4.30	2.43	1.87	Sl. Un. Sl. Ox.	Sl. Ran. Sl. Bit.	Sl. Ox.	Ox.
7	4.0	5.16	3.65	1.51	Bit. Sl. Ran.	Sl. Bit. Ran. Un.	Co. Ox.	St. Ox.
26	4.0	5.43	3.59	1.84	Bit. Ran. Un.	Bit. Ran.	Sl. Ox.	Sl. Ox.
31	4.0	5.03	3.20	1.83	Sl. Ran.	Bit. Ran.	Sl. Ox. Co.	Ox.
60	4.0	5.34	3.42	1.92	Ran. Bit. Sl. Un.	St. Bit. Ran. Un.	Co. Ox.	St. Ox.

TABLE XVII (continued)

Culture Number	Raw Milk Butterfat %	After 14 Days Storage at 4°C. Sample B FFA[a]	Sample A FFA	Net FFA[b]	Flavor After Storage[c] Sample B 8 Days	Sample B 14 Days	Sample A 8 Days	Sample A 14 Days
63	6.0	3.82	2.77	1.05	Sl. Ox.	Sl. Ran.	Co. Sl. Ox.	Sl. Ox.
75	4.0	4.59	3.52	1.07	Sl. Ran.	Ran. Sl. Un.	Ox.	St. Ox.
94	5.0	4.50	3.35	1.15	Sl. Ox.	Sl. Ran. Sl. Un.	Co. Ox.	Ox.
98	5.0	4.35	2.92	1.43	Sl. Ran.	Ran. Sl. Un.	Ox.	Ox.
99	4.0	5.06	3.56	1.50	Sl. Ran. Sl. Bit.	Ran. Bit.	Co. Ox.	St. Ox.
100	5.0	3.97	3.00	0.97	Sl. Ox.	Sl. Ran.	Ox.	Ox.
101	5.0	4.42	3.00	1.42	Sl. Un.	Sl. Ran. Sl. Bit.	Co. Ox.	Co. Ox.
125	5.0	4.35	3.39	0.96	Co.	Sl. Ran. Sl. Un.	Ox.	St. Ox.
131	4.0	5.84	4.12	1.72	Ran. Bit.	St. Ran. Bit.	Sl. Ran.	Sl. Ran. Sl. Un.

TABLE XVII (continued)

Culture Number	Raw Milk Butterfat %	After 14 Days Storage at 4°C. Sample B FFA	Sample A FFA	Net FFA[b]	Flavor After Storage[c] Sample B 8 Days	Sample B 14 Days	Sample A 8 Days	Sample A 14 Days
135	4.0	4.96	3.56	1.40	Sl. Ran. Ox.	Sl. Bit. Ran.	Co. Ox.	St. Ox.
145	3.7	5.80	3.65	2.15	Ran. Sl. Bit.	Bit. Sl. Ran.	Ox.	Ox.
147	4.0	4.79	3.40	1.39	Sl. Ran. Bit.	St. Bit. Sl. Ran.	Sl. Ox. Sl. Un.	St. Ox.
149	4.0	4.85	3.16	1.69	Sl. Bit.	Bit. Sl. Ran.	Sl. Ox.	St. Ox.
158	4.0	4.78	3.53	1.25	Sl. Bit. Sl. Ran.	St. Bit. Sl. Ran.	Ox.	St. Ox.
169	4.0	5.59	3.75	1.84	Bit.	Bit. Ran.	St. Ox.	St. Ox.
178	4.0	4.70	2.49	2.21	Sl. Bit.	Sl. Bit. Sl. Ran.	Sl. Ox.	Sl. Ox.

[a]Free Fat Acidity, expressed as milliliters of 1 N alkali required to neutralize an ether extract of milk containing 100 grams of fat.

[b]Net free fat acidity or free fat acidity produced by the psychrophilic lipase in Sample B.

[c]Sl. Bit. = slightly bitter, Sl. Ran. = slightly rancid. Sl. Ox. = slightly oxidized, Ox. = oxidized, Bit. = bitter, Ran. = rancid, Co. = cooked, Un. = unclean, St. = strongly.

TABLE XVIII

STANDARD DEVIATION OF FREE FAT ACIDITY FROM EIGHT REPLICATES OF A PASTEURIZED MILK SAMPLE WITH 4 PERCENT BUTTERFAT

Milk Sample Replicates (N)	Milliliter 0.025N Alcoholic KOH per 10 Milliliters Milk	Free Fat Acidity (X)	(X^2)
1	0.196	3.67	13.47
2	0.198	3.71	13.76
3	0.197	3.69	13.62
4	0.196	3.67	13.47
5	0.200	3.75	13.06
6	0.195	3.65	13.32
7	0.198	3.71	13.37
8	0.197	3.69	13.62
		ΣX 29.54	ΣX^2 109.08

$$\text{Standard Deviation (73)} = \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N}} = \sqrt{\frac{109.08 - 109.07}{8}} = \pm 0.0034$$