IDENTIFICATION AND PHYSIOLOGICAL ACTIVITIES OF PSYCHROPHILIC MICROORGANISMS IN MILK

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This is to certify that the

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IDENTIFICATION AND PHYSIOLOGICAL

ACTIVITIES OF PSYCHROPHILIC MICROORGANISMS IN MILK

by

Frederick Weber, Jr.

AN ABSTRACT

Submitted to the School of Special Graduate Studies of Michigan
State University of Agriculture and Applied Science
in partial fulfillment of the requirements
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Department of Microbiology and Public Health

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Frederick Weber, Jr.

A series of experiments were conducted which indicated the following:

1. Bacterial counts from plates incubated at 35 and 20 C do not adequately evaluate milk quality when the milk has been held in cold storage.

2. The counts obtained from plates incubated at 20 C are a better criterion for determining the bacteria in milk than the 35 C plate counts.

Growth curves of milk stored at 4.5 C showed that psychrophilic microorganisms increased 10 to 100 fold during one day of cold storage. When the psychrophilic population approached 10,000,000, an off-flavor could be detected in the milk. The psychrophilic growth occurring in raw milk adversely affects the keeping quality of the pasteurized product. Based on these findings, raw and pasteurized milk standards were proposed, designed to insure the sale of good quality milk.

Evidence was presented to show that psychrophilic counts are a better criterion for determining post-pasteurization contamination than the coliform index. Suggestions were given to interpret psychrophilic counts obtained from commercially pasteurized milk.

Certain species of gram negative rods and micrococci are predominantly found in milk held at 4.5 C. These organisms are generally feebly saccharolytic and readily killed by pasteurization. A mechanical key was prepared for identifying most of these psychrophilic organisms.

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I. INTRODUCTION

At the present time, the dairy industry, as well as most other agricultural industries, is faced with a problem unique in the United States; namely, surpluses. Probably the best solution to this unusual dilemma is in finding new products to make use of our excess commodities. But while this long range research finds answers to our problems, an attempt should be made to increase consumption of these products.

The sale of milk is still the most direct and best method of handling dairy surpluses. Almost everyone appreciates the value of this beverage, has made use of it, and can be sold on the idea that some of his ills could be cured if he made more use of it. The cost of milk is moderate, and it is readily available. Our society has accepted its taste and we have designed many foods which use milk as a required additive, such as breakfast cereals, bakery products, desserts, etc.

Much emphasis has been and is being placed on selling more milk. For the sake of economy, every other day delivery of pasteurized milk to the consumer is now almost universal, and bulk handling of raw milk at the farm is advancing so that within a few years the milk can will be largely replaced by the refrigerated holding tank. In the area of distribution advances have also been made to sell milk. Automatic dispensers and vending machines now distribute milk 24 hours a day without the need of constant supervision.

All these methods for selling milk are dependent upon refrigeration. Although this method has long been used by the dairies to control bacterial growth, its use has been greatly extended so that it becomes necessary to determine if we have not gone beyond its limits to prevent the growth of microorganisms.

In studying the effect of cold storage on milk, the once accepted bacterial standards cannot adequately be relied upon to insure good quality milk. For this reason, an incubation temperature below 35 C is recommended for determining standard plate counts on milk.

Thus far we have concerned ourselves with the desire of the dairy industries to sell good quality milk to the consumer. We should not overlook the interest of the customer who purchases the milk. When he buys milk, he has the right to expect a wholesome product free from potential hazards. Spreading disease by milk is a primary concern to all those who are involved in dairy work, but herd inspections, pasteurization, routine barn and dairy inspections and systematic laboratory analysis, combined with adequate local laws and trained personnel, drastically reduces the possibility of disease transmission by market milk. Whereas the consumer is reasonably protected from the possibility of ingesting pathogens with his milk, he is not insured against a poor quality milk or one that will lose its quality in a short time. Local and state laws are designed to make sure that market milk has the minimum butter fat content and contains no additives, but they fail to protect the purchaser from buying milk that either has or shortly will have a sour, bitter, putrid or other off-flavor.

The purpose of the present investigation is to obtain more information about organisms which grow at refrigeration temperature. The knowledge gained from this work will, it is hoped, clarify some of the conflicting statements appearing in publications dealing with psychrophiles and lead to greater interest in establishing psychrophilic standards comparable to the standards now followed with the standard plate count.

In short, the ultimate aim of this work is to make the dairy industry and the governmental agencies become more concerned with the deleterious effect of bacterial growth at refrigeration temperatures. Once the attitude is accepted that bacteria growing at cold temperatures are as harmful to milk quality as is the bacterial multiplication in unrefrigerated milk, techniques can be developed and standards adopted which will aid the dairy in selling milk and insure the consumer against receiving poor quality milk.

II. LITERATURE REVIEW

A. General characteristics of psychrophilic microorganisms

1. Temperature relationships

Since the term psychrophile indicates a temperature relationship, a psychrophile is defined as an organism which grows best at the lower limits of the temperature scale. To make a useful distinction among the psychrophile, mesophile and thermophile, arbitrary temperature ranges are to be established for each of the groups. At present there are no universally accepted standards at which an organism must show optimum growth in order to be classified as a psychrophile; hence, the definition of the term rests with the individual concept of the particular investigator. Some of the suggested temperature ranges are shown in table 1.

These authors have attempted to establish a range as well as an optimum temperature for each of the groups. In so doing, an organism is readily characterized, but such characterizations are not very meaningful in the dairy industry. For this reason, the dairy bacteriologist refers to psychrophiles as microorganisms which grow in milk during cold storage. Since the storage temperature of most dairy products is above the minimal temperature for mesophiles, the organisms which are classified as psychrophilic by the dairy bacteriologist are considered by most bacteriologists as mesophiles with a low minimal temperature requirement.

TABLE 1.

Some cardinal temperatures given in the literature for characterizing microorganisms

Groups	Minimum Temperature (C)	Optimum Temperature (C)	Maximum Temperature (C)	Reference
Psychrophilic	-7			Haines (1934)
	0	5-10	35	Tanner (1938)
				Erdman & Thornton (1951a)
		20-22		Morrison & Hammer (1941)
	0	20-21	32	Roadhouse & Henderson (1941)
	0	15-20	30	Salle (1943)
	0-5	10-20	25-30	Porter (1946)
		18-22		ZoBell & Conn (1950)
		5-25		Kennedy & Weiser (1950)
		18-20		Michell (1951)
	>3	>20		Davis (1951)
Mesophilic	5-25	37	43	Salle (1943)
	10-25	20-40	40-50	Porter (1946)
Thermophilic	25-45	50-55	60-90	Salle (1943)
	25-45	50-60	70-80	Porter (1946)

The different cardinal temperatures reported in the literature can often be attributed to the habitat studied. Papers dealing with marine and fresh water microorganisms generally study bacteria whose optimum temperature is below the microorganisms found in dairy products. Since the marine microbiologist uses the term psychrophile to differentiate those organisms whose optimum temperature is below 37 C, the optimum temperature he sets for psychrophiles is lower (ZoBell, 1946) than that adopted by the dairy microbiologist who is concerned with differentiation of organisms whose optimum temperature ranges up to 50 or 60 C. Attempting to clarify the terminology of both groups of investigators, some propose the addition of more descriptive terms. Frobisher (1944) suggests the term obligate psychrophile to describe the marine forms whose optimum is near 5 C and whose maximum temperature is near 30 C. Sekhar and Walker (1947) use the term facultative psychrophile to designate those microorganisms which grow at 3 C, but whose optimum is above 20 C. Both Sekhar and Walker (1947) and Davis (1951) reserve the term psychrophile to correspond to Frobisher's obligate psychrophile, but Davis (1951) would prefer the term psychroduric to facultative psychrophile.

Although the terminology used is not worthy of heated debate, standardizing incubation time and temperature is necessary before general properties can be ascribed to psychrophilic microorganisms. Much of the disagreement regarding psychrophiles is caused by the variety of incubation time and temperature used by the several authors. For our purpose, the term psychrophile will be used to include those organisms predominantly found in milk stored at 4.5 C and grown in pour plates incubated at 4.5 C in 14 days. Essentially, Greene and Jezeski (1954), as well as others, have used the term psychrophile to imply a similar meaning.

The methods of estimating the optimum temperature for the growth of an organism is generally by plate counts (Dorn and Rahn, 1939), although Hess (1934) feels that total cell mass is a more accurate method. The optimum temperature of an organism determined by cell volume is generally 10 C below the value obtained by plate counts (Foter and Rahn, 1936; Dorn and Rahn, 1939; Michell, 1950).

2. Thermal resistance

The lack of an established standard for defining psychrophiles according to temperature requirements results in difficulties to characterize these organisms by other criteria. In the dairy industry where psychrophilic and thermoduric organisms are of importance, there is an understandable reason for studying the heat tolerance of psychrophiles. Most investigators feel that they cannot withstand pasteurization (Sherman, Stark and Gunsalus, 1938; Sherman, Cameron and White, 1941; Claydon, 1943; Thomas and Sekhar, 1946; Thomas, Thomas and Ellison, 1949; Moore, Tracy and Ordal, 1951; Rogick and Burgwald, 1952; Watrous, Doan and Josephson, 1952; Olson, Willoughby, Thomas and Morris, 1953; Olson, Parker and Mueller, 1955), while others believe that psychrophiles are not always destroyed by pasteurization. The early observations of Ravenal,

Hastings and Hammer (1910) and Ayers and Johnson (1910), that psychrophiles could be isolated from commercially pasteurized milk, has been shown repeatedly, but whether all these organisms pass through the pasteurization process or are a result of post-pasteurization contamination, has not been established. Further- more, the difficulties of completely pasteurizing a batch of milk, either in the laboratory or in the dairy, are numerous so that only when extreme care is used will a milk sample be effectively pasteurized.

Assuming that the pasteurization methods have been effective, and no post-pasteurization contamination occurs, two explanations are available for the presence of psychrophilic bacteria in the pasteurized product: some psychrophiles are thermoduric (Roadhouse and Henderson, 1941; Jezeski and Macy, 1946; Kennedy and Weiser, 1950; Ashton, 1950; Erdman and Thornton, 1951a) or mesophiles that have adapted to grow at lower temperatures (Prescott, Bates and Needle, 1931; Burgwald and Josephson, 1947; Rogick and Burgwald, 1950; Egdell and Bird, 1950).

The more recent work seems to substantiate the concept that psychrophiles are killed during pasteurization and the detection of these organisms in pasteurized milk represents post-pasteurization contamination (Watrous, Doan and Josephson, 1952; Rogick and Burgwald, 1952) from water, poorly cleaned dairy equipment (Thomas, Thomas and Ellison, 1949) and milk bottles (Rogick and Burgwald, 1952).

B. Types of psychrophilic microorganisms found in milk

Thus far the literature reviewed deals with the thermal characteristics of psychrophiles. A more precise definition of psychrophiles could be obtained by examining the types of microorganisms which are characterized as psychrophiles, but in this task taxonomic problems are confronted.

The limited knowledge and interest in classification and the numerous incomplete descriptions of species have not contributed to stabilizing bacterial taxonomy. Furthermore, the psychrophiles belong to a group of microorganisms lacking the attention of the medical or industrial investigators; hence, their morphological and physiological characteristics are less well defined than those of their cousins who have readily ascribed harmful or beneficial effects upon our lives. Although the genera and species which have been designated as psychrophiles are subject to errors, enough agreement exists so that some of the morphological and physiological characteristics of psychrophiles can be used to define this group of microorganisms.

Rogick and Burgwald (1950) found that cocci and gram negative rods predominate in cold storage milk. They (Rogick and Burgwald, 1952) found that of 167 cultures, about 64 per cent were cocci and 36 per cent were non-spore-forming rods. Erdman and Thornton (1951b) reported that of 722 cultures, 45 per cent were rods and 55 per cent were cocci. About 65 per cent of the total isolates were gram negative, including 70 per cent of the rods and 62 per cent of the cocci.

Thomas and Thomas (1947) found that 93 per cent of the psychrophilic bacteria from farm water were gram negative rods, three per cent were gram positive non-spore-forming rods and that over 50 per cent of all the cultures were chromogenic belonging to the genus Pseudomonas or Flavobacterium.

From a similar source Jones and Thomas (1950) also showed that species of Flavobacterium predominated.

The gram negative psychrophilic rods reported in the literature belong to the genera Pseudomonas, Alcaligenes, Achromobacter and Flavobacterium. Pseudomonas species, grown at 0 to 3 C, were isolated by Forster (1887; 1892), Fischer (1888) and Schmidt-Nielsen (1902) from water, milk, soil and food products. Their optimum growth was between 5 and 10 C at which temperature visible growth appeared in six to eight days. Some of the strains reported were halophilic. Similar halophilic psychrophiles were recovered by Castell and McDermott (1942), Anderson (1942), Castell and Mapplebeck (1952) and Tobin, Alford and McCleskey (1941) from salt water fish using 2 to 3 C incubation. Sherman, Cameron and White (1941), Garrison and Hammer (1942), Jezeski and Macy (1946), Hammer (1948), and Erdman and Thornton (1951b) and others have isolated Pseudomonas cultures from milk and dairy products having off-flavors and odors. The temperature of incubation used by Morris (1942) was 17.8 C, but the other investigators cited above used 0 to 8 C incubation. Erdman and Thornton (1951b) found that their isolates did not grow at temperatures above 35.5 C. Psychrophilic Pseudomonas cultures were also isolated from fresh, frozen and stored meats by Sulzbacker (1950), Sulzbacker and McLean (1951) and Kirsch, Berry, Baldwin and Foster (1952), using plates incubated at 0 to 2 C.

Pseudomonas aeruginosa, *Pseudomonas fluorescens*, *Pseudomonas schuylkilliensis*, *Pseudomonas chlororaphis*, *Pseudomonas fragi* and *Pseudomonas geniculata* were isolated by Ely (1954). *Pseudomonas fragi* has also been cited by Hussong, Long and Hammer (1937), Morrison and Hammer (1941) and others as causing off-flavors in dairy products. *Pseudomonas putrifaciens* has been reported (Claydon and Hammer, 1939; Derby and Hammer, 1931; Long and Hammer, 1941; Wagenaar, 1952) as well as *Pseudomonas graveolens*, *Pseudomonas mucidolens* (Olson and Hammer, 1934) and *Pseudomonas nigrificans* (White, 1940). All of these species were grown at 4 to 10 C and were involved with off-flavors and odors in dairy products. Lawton and Nelson (1954) used *Pseudomonas ovalis*, *Pseudomonas fluorescens*, *Pseudomonas cruceviae*, *Pseudomonas aquatile*, *Pseudomonas geniculata* and *Pseudomonas fragi* as representative psychrophiles.

Organisms belonging to the genus *Alcaligenes* have been observed by Castell and McDermott (1942) in water, Sulzbacker and McLean (1951) and Tanaka, Nozaki and Yoshida (1951) in fresh and stored pork sausage and Anderson and Hardenbergh (1932), Thomas and Sekhar (1946), Jezeski and Macy (1946) and Alexander and Higginbottom (1953) in dairy products at incubation temperatures of 0 to 10 C. Anderson and Hardenbergh (1932) found that no growth occurred at 34 to 35 C.

The species which have been reported in the genus Alcaligenes are Alcaligenes metalcaligenes and Alcaligenes faecalis found by Bly (1954), Alcaligenes viscosus (Erdman and Thornton, 1951b) and Alcaligenes tolerans (Abd-el Malek and Gibson, 1952), which was found in 60 per cent of the samples analyzed.

Achromobacter have been isolated in dairy products by Anderson and Hardenbergh (1932), Thomas and Sekhar (1946), Jezeski and Macy, (1946) and Alexander and Higginbottom (1953). These cultures grew below 18.5 C but not at 34 to 35 C (Anderson and Hardenbergh, 1932). They were associated with lipolytic spoilage of cream and off-flavor of milk and butter.

Achromobacter have also been found in frozen meat (Sulzbacker, 1950), fresh pork sausage (Sulzbacker and McLean, 1951), stored pork sausage (Tanaka, Nozaki and Yoshida, 1951) and market hamburg (Kirsch, Berry, Baldwin and Foster, 1952). All of these organisms grew within the psychrophilic temperature range. Tobin, Alford and McCleskey (1941), Anderson (1942), Castell and McDermott (1942) and Castell and Mapplebeck (1952) isolated Achromobacter from spoiled salt water fish. The species reported by Bly (1954) from dairy products isolated at 5 C are: Achromobacter butyri, Achromobacter delmarvae, Achromobacter stationis and Achromobacter superficiale.

Psychrophilic members of the genus Flavobacterium have been reported by Thomas and Sekhar (1946), Jezeski and Macy (1946) and Alexander and Higginbottom (1953) from dairy products. They have also been observed in spoiled salt water fish (Castell and

Mapplebeck, 1952). Ely (1954) lists one species, Flavobacterium devorans.

Aerobacter has also been reported as a psychrophile isolated from dairy products (Erdman and Thornton, 1951b; Ely, 1954).

Hammer and Yale (1932) found Aerobacter aerogenes, Aerobacter cloaca and Aerobacter oxytocum. Claydon (1943) also reported finding Aerobacter aerogenes. Aerobacter lipolyticum was found by Hammer (1948).

Other gram negative psychrophiles which have been found are: Escherichia and Lactobacillus (Erdman and Thornton, 1951b).

Both streptococci and micrococci have been found as psychrophiles in dairy products (Alexander and Higginbottom, 1953). Foter and Rahn (1936), incubating at 0 to 5 C, found Streptococcus fecalis, Streptococcus lactis and Streptococcus glycerinaceus. Sherman and Stark (1931), using 10 C incubation, found Streptococcus fecalis, Streptococcus glycerinaceus, Streptococcus liquefaciens and Streptococcus zymogenes.

Ely (1954) found Micrococcus candidus, Micrococcus varians, Micrococcus conglomeratus and Micrococcus caseolyticus when incubating at 5 C.

Several other bacteria have been reported as psychrophilic including members of the genus Corynebacterium, Lactobacillus and Serratia (Ely, 1954) as well as Bacillus and Microbacterium (Alexander and Higginbottom, 1953), but these are apparently seldom encountered.

Yeasts have been found to grow at low temperatures in dairy products (Jezeski and Macy, 1946; Alexander and Higginbottom, 1953); as well as several molds and <u>Actinomycetes</u> (Schmidt-Nielsen, 1902; Müller, 1903; Berry and Magoon, 1934).

Some authors have characterized psychrophilic cultures on their physiological action in milk. Pennington (1908) found that inert and proteolytic organisms predominated in milk stored near 0 C.

Ayers and Johnson (1910) , however, found more acid formers than proteolytic types. They also reported that inert types were most common and alkaline producers least common. Black, Prouty and Graham (1932) found 73 per cent of their isolates were inert and alkaline producers, 26 per cent produced acid and one per cent was proteolytic.

Thomas and Sekhar (1946), using 231 cultures, found that 85 per cent were inert in litmus milk, two per cent produced an acid curd, six per cent produced acid feebly or not at all and nine per cent peptonized milk.

Watrous, Doan and Josephson (1952) also found inert forms predominated while proteolytic types were seldom found. Upon incubation, alkaline producers were more common than those forming acid.

Rogick and Burgwald (1950) characterize most of their psychrophilic cultures mainly as inert forms or acid producers. They (Rogick and Burgwald, 1952) found over half of the psychrophilic cultures were inert in litmus milk, 28 per cent produced acid and 17 per cent caused litmus milk to become alkaline when incubation was at 4 to 7 C, but at 35 C incubation only 13 per cent were inc

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inert and 69 per cent produced acid. The alkaline-forming group remained constant in regard to numbers and action in litmus milk. Of the 167 cultures they used in their study, only about four per cent were considered to be "true" psychrophiles; i.e., they grew at 4 to 7 C but not at 35 C. The effect of lowering the incubation temperature below the optimum on the bacterial physiology has not received significant attention to warrant generalizations, but it is often assumed that proteolytic activities are increased at the expense of carbohydrate digestion, that pigmentation is enhanced, that larger populations and greater amounts of end products are obtained and that cold storage increases the heat tolerance of bacteria (Anderson and Meanwell, 1936; Foter and Rahn, 1936; Dorn and Rahn, 1939; Thiel, 1940a, 1940b; Jordan and Jacobs, 1947).

C. Influence of psychrophilic microorganisms on the keeping quality of milk

In the discussion dealing with the types of psychrophilic microorganisms found in milk, some mention was made as to the off-flavors and off-odors they produced. Boyd (1953) and Boyd, Smith and Trout (1953) have shown a direct relationship between milk spoilage and the psychrophilic plate count, although they were unable to evaluate the keeping quality of a milk when samples of freshly pasteurized milk were analyzed due to its low bacterial population. Atherton (1953) has emphasized the fact that a total psychrophilic count is not as closely related to milk deterioration as is the specific type of psychrophile present.

The deleterious effects produced by the psychrophiles in milk has generally been attributed to proteolytic and lipolytic action (Black, Prouty and Graham, 1932; Olson and Hammer, 1934; Anderson, 1938; Powell, 1938; Garrison and Hammer, 1942; Jezeski and Macy, 1946; Jezeski, 1952; Babel, 1953). An extensive interest has been shown in the lipase activity of psychrophiles (Lubert, Smith and Thornton, 1949; Kesta, Nelson and Peters, 1953; Nashif and Nelson, 1953a, 1953b, 1953c). In connection with their proteolytic activities, it is possible that some psychrophiles are capable of producing toxin-like compounds in dairy products (Ravenal, Hastings and Hammer, 1910; Hammer, 1948; Thomas, Thomas and Ellison, 1949). In addition to these general methods of deterioration, certain organisms are capable of causing specific undesirable effects, such as ropiness (Anderson, 1942), medicinal or phenolic tastes (Claydon, 1943), slime formation (Parker, Smith and Elliker, 1951; Davis and Babel, 1954), or off-colors, as fluorescense, (Garrison and Hammer, 1942) and pigmentation (Hiscock, 1936; Elliker, Smith and Parker, 1951; Jezeski, 1952).

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III. EXPERIMENTAL METHODS

A. Characterization of psychrophilic microorganisms

The procedure for plating milk samples used in this research is that recommended by the American Public Health Association (1953).

The number of psychrophilic bacteria was obtained by plating suitable dilutions of each milk sample. All platings were made in triplicate. Where counts were made from the one ml plating, the number was recorded as less than 10 unless the 1:10 dilution showed one or more colonies. This was done to avoid inaccuracies in counting dense plates containing milk particles from one ml of undiluted milk.

Replicate plates were incubated at 55, 35, 20 and 4.5 C for 1, 2, 5 and 14 days.

1. Fresh raw milk

The raw milk samples came from several college barns. Barn A contains modern dairy equipment allowing for a maximum degree of efficiency in maintaining high standard milk. Barn B is more typical of Michigan barns. Its equipment is modest, but in excellent condition. Barn C is primarily an experimental barn used to study nutritional problems. From the past records of these three barns, the milk from barn A is unusually clean in the bacteriological sense. Barn B produces good quality milk although below that of barn A. The milk from barn C is generally similar in bacterial quality to that of barn B. Dusty conditions in barn C occasionally cause the bacterial count in the milk to be greater from barn C than from barn B.

Daily raw milk samples were collected from these three barns for two weeks. Determinations for total and psychrophilic microorganisms were made within three hours after milking. Ninety-six raw milk samples were collected from barn C with great care to prevent contamination during milking. The samples, collected from individual cows during milking, were plated in the laboratory. and incubated at 35 and 4.5 C within one hour of collection. The 96 raw milk samples were obtained over a 16 day period; daily collection being made. The 96 samples represent milk from six cows, 16 samples per cow.

2. Stored raw milk

Fresh raw milk samples were obtained from barns A and B. These samples were stored at 4.5 C for 10 days and plated every two days to determine the thermophilic (55 C), total (35 C), 20 C and psychrophilic (4.5 C) counts.

The 96 raw milk samples from individual cows previously described were initially plated to obtain a total and a psychrophilic count. These milk samples were then stored at 4.5 C and psychrophilic counts were determined for 2, 4, 6 and 8 days.

3. Pasteurized milk

a. Laboratory

Two fresh raw milk samples were obtained from barns A and B. To compare the bacterial counts from pour plates incubated at various temperatures, the bacterial populations of two raw milk samples were determined from plates incubated at 55, 35, 20 and 4.5 C. The milk was then heated to 62.8 C (± 0.1) for 5, 10, 20 and 30 minutes and the populations were again determined. The pasteurized samples (62.8 C for 30 minutes) were then stored at 4.5 C for 2, 4, 6, 8 and 10 days. At each storage interval, populations were again determined.

b. Commercial

Eighty-one commercial milk and cream samples were obtained. These samples were collected from 28 Michigan dairies. Total counts at 35 C, coliform, 20 C and psychrophile counts were made. These samples represented: cream-line milk, homogenized milk, skim milk, chocolate milk, homogenized frozen milk, skim frozen milk, coffee cream and whipping cream.

B. Isolation of pure cultures

1. Sampling

Twenty fresh raw milk samples from barns A, B and C were selected at random to determine the predominant types of microorganisms obtained from plates incubated at 55, 35, 20 and 4.5 C. The samples were stored at 4.5 C for 10 days. At intervals of 0, 2, 4, 6 and 10 days, platings were made and incubated at 4.5 C. Representative colonies were picked from plates prepared from the highest dilution, inoculated into broth and incubated at the appropriate temperature until visible signs of growth were evident. The purity of the cultures was determined by checking the appearance of the colonies in pour plates and the morphology of the gram stain.

Another set of 20 samples was collected from barn C. These samples were obtained from individual cows. Cultures were picked from the plates incubated at 4.5 C.

2. Taxonomy

The characteristics which have been used to classify the cultures were those prescribed by Bergey's Manual (Breed, et.al., 1948), but it was soon found that the natural key given in Bergey's Manual was not suited for satisfactorily identifying a large number of organisms. For this reason, the mechanical key prepared by Skerman (1949) for determining the genera of organisms was tried with moderate success. After careful study and noting the occasional deviation of Skerman's mechanical key from the natural key of Bergey's Manual, a modified key was prepared (see Appendix A). The key was greatly simplified by including only the gram negative rods of the four genera which had been found in the largest numbers. The coliform group was not included in the key since only a few were detected; they were identified by their ability to ferment lactose, their characteristic colonies on EMB agar and the IMViC test. The gram positive rods and cocci were classified to their genera wherever possible, with the aid of Skerman's key.

The predominant gram negative rods and gram positive cocci were further classified in groups resembling the species described in Bergey's Manual. Using the descriptions in the manual, a mechanical key was prepared (Appendix A) for this task. Since the descriptions were often not adequate for positive identification, the key occasionally does not allow for the separation of closely related species. A further simplification was effected in the key by omitting such species which were exotic in their habitat. When an identification was completed, other characterizations were made so as to confirm the identification with the description given in Bergey's Manual. Where no identification could be found according to the descriptions given in Bergey's Manual, the organisms were placed in groups and their characteristics noted in Appendix B.

The methods used to identify the isolated cultures are those given in the Manual of Methods for Pure Culture Study of Bacteria (committee on bacteriological technique, Society of American Bacteriologists).

3. Temperature studies

A study was undertaken to determine the influence of incubation temperature on the plate count of milk. Representative cultures were grown in sterile skim milk at 4.5 C for six days. Triplicate sets of plates were prepared and incubated at 35, 20 and 4.5 C.

To determine the heat tolerance of the isolates, 10 ml of 48 hour cultures grown in skim milk at 20 C was placed in sterile test tubes and heated to 62.8 C for 30 minutes. After rapidly cooling, the cultures were placed in a 20 C incubator for three days after which pour plates were made and incubated at 20 C for five days to determine the presence of organisms. The same procedure was followed using a five day old culture grown at 4.5 C in skim milk.

C. Influence of psychrophilic microorganisms on the keeping quality of milk

From five to 10 gallon lots of fresh raw milk were obtained from barn B.

One group of raw milk samples was pasteurized at 62.8 C for 30 minutes. A portion of the fresh pasteurized milk was inoculated with a 0.2 per cent amount of a rapidly growing psychrophilic culture known to produce off-flavors in milk. Both the inoculated and uninoculated pasteurized milk samples were stored at 4.5 C and examined every other day for any off-flavors and for their bacterial content.

A second group of raw milk samples was stored at 4.5 C. A portion was removed every other day and pasteurized. The pasteurized portions were divided into two sets; one set was inoculated. Both the inoculated and the uninoculated samples were stored at 4.5 C and tested every other day.

All of the pasteurized milk samples were tasted for off-flavors and plated for total and psychrophilic counts at two day intervals until deterioration was evident. The flavor testing was done under the direction of Dr. G. Malcolm Trout from the Dairy Department of Michigan State University. The milk was scored satisfactory (indicated by a 0 in the tables) or unsatisfactory (+ to ++++ depending upon the extent of flavor deterioration). This type of scoring was used in place of the conventional method to overcome confusion in evaluating such flavors in milk which are not attributable to bacterial growth.

IV. RESULTS

A. General characteristics of psychrophilic microorganisms

1. Fresh raw milk

The average numbers of bacteria found in 14 fresh raw milk samples from three barns are recorded in table 2. The results obtained from these samples indicate that the total counts (35 C incubation) were highest in the milk from barn B. With two exceptions, the individual counts made over the two week period, were consistently higher from barn B than from either barns A or C. The individual and average bacterial counts from barns A and C were not significantly different. The psychrophilic counts of the fresh raw milk from these three barns indicated that the raw milk from barn B had relatively more psychrophilic microorganisms; barn C had a moderate number and barn A an insignificant number.

When milk samples were collected directly from individual cows (table 3) and immediately plated in the laboratory, an average of 54 psychrophilic and 3,600 total colonies were counted. These counts are similar to those obtained from barn C in the previously mentioned study (table 2), although the total count is somewhat higher. No individual cow was observed to produce milk consistently low in bacteria.

2. Stored raw milk

The counts obtained from raw milk stored at 4.5 C for 10 days, recorded in table 4, indicate that the storage period does

TABLE 2

Average total (35 C) and psychrophilic (4.5 C) bacterial counts of 14 samples of fresh raw milk obtained from three dairy barns

Source	Total (35 C) count	Psychrophile (4.5 C) count	
	Average	Average	Range
Barn A	3,400	10	0-8
Barn B	21,000	2,000	50-4,800
Barn C	1,300	57	10-110

TABLE 3

Average total (35 C) and psychrophilic (4.5 C) counts of 96 milk samples from six cows in barn C

Designation of cow	Number of samples	Total count	Psychrophilic count Average	Psychrophilic count Range
K 109	16	5,800	24	10-80
K 17	16	3,400	62	10-150
K 19	16	1,900	56	10-130
K 134	16	3,200	74	10-140
K 144	16	4,200	78	20-210
K 126	16	3,000	31	10-120
Average		3,600	54	

TABLE 4

Bacterial populations from plates incubated at various temperatures of two raw milk samples stored at 4.5 C for 10 days

1. Barn A

Days storage at 4.5 C	Plates incubated at (C) 55	35	20	4.5
0	<10	3,300	4,300	10
2	<10	3,100	5,000	100
4	<10	2,300	7,100	10,000
6	<10	1,100	1,800,000	380,000
8	<10	790	16,000,000	5,000,000
10	<10	1,000	56,000,000	30,000,000

2. Barn B

0	180	24,000	25,000	170
2	160	16,000	36,000	5,000
4	260	6,300	310,000	40,000
6	100	3,100	17,000,000	960,000
8	130	4,500	100,000,000	100,000,000
10	90	6,400	350,000,000	350,000,000

not allow growth of those organisms which are normally found on 55 and 35 C plates. The psychrophilic population, however, increases logarithmically as indicated in the counts from the 20 and 4.5 C plates. The 20 C counts indicate an initial lag period.

Later, in a study of keeping quality, data are presented that show a slight variation from the 35 C counts recorded in table 4, but a similar marked increase in psychrophilic counts occurs.

A log average of the 96 stored raw milk samples is presented in figure 1 which is typical of the psychrophilic growth rate in all the milk samples examined; i.e., a 10 to 100 fold increase generally occurs after two days of storage at 4.5 C.

3. Pasteurized milk

a. Laboratory

The bacterial counts of two fresh raw milk samples that were heated at 62.8 C for 30 minutes are presented in table 5. The thermophilic bacteria were not affected by the pasteurization temperatures while the remaining types of bacteria were materially decreased within the first five minutes of heating. The psychrophilic population was rapidly destroyed as indicated by both the plate counts shown in table 5 as well as in subsequent plates from the heat treated milk after six days storage at 4.5 C. In all cases where the initial psychrophile count was below 10 per ml, no growth could be detected in the six day stored milk. In

FIGURE I.

A PSYCHROPHILIC GROWTH CURVE.

AN AVERAGE OF 96 RAW MILK SAMPLES STORED AT 4.5 C.

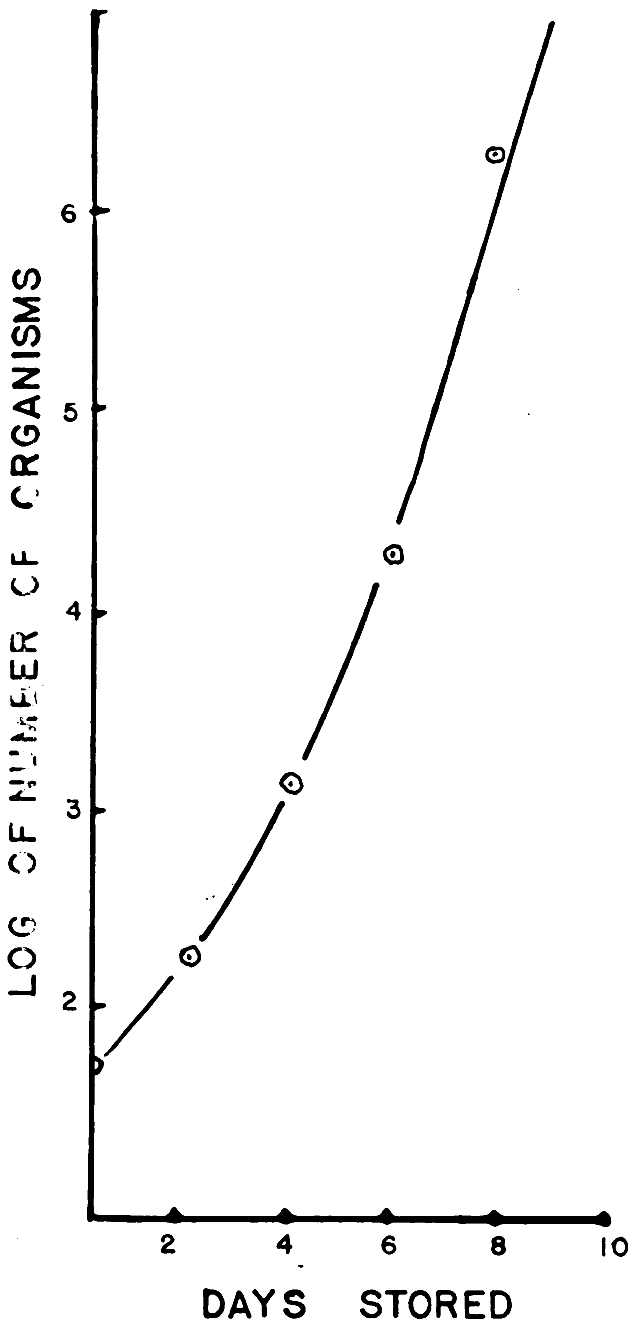

TABLE 5

The effect of holding fresh raw milk at 62.8 C for various time intervals on the bacterial population

1. Barn A

Time held at 62.8 C (min.)	Plate counts Incubation temperature (C) 55	35	20	4.5
0	14	2,900	3,500	10
5	12	27	42	<10
10	20	13	20	<10
20	21	6	34	<10
30	17	12	26	<10

2. Barn B

0	180	18,000	20,000	500
5	130	1,000	660	350
10	200	450	330	200
20	210	400	320	<10
30	200	330	130	<10

the milk sample from barn B, which was treated for 10 minutes and showed a psychrophile count of 200 bacteria per ml, the psychrophile count after six days storage was 95.

When the pasteurized (62.8 C for 30 minutes) milk was stored at 4.5 C, none of the plate counts, regardless of the incubation temperature, indicated that bacterial growth had occurred in the sample. The plate counts of these samples stored for 10 days are shown in table 6.

When pasteurized milk was prepared from fresh raw milk, the bacterial counts (table 6) are not significantly different from the counts of pasteurized milk obtained from 2, 4, 6 or 8 day old raw milk (tables 7-11). However, the pasteurized milk obtained from 10 day old raw milk of barn B had an initially higher bacterial population (table 11) which increased during storage. This difference is probably a result of incomplete pasteurization which resulted from the presence of curds in the raw milk.

b. Commercial

Plate counts of the commercially pasteurized milk samples are given in table 12. Of the 81 samples tested,22 have 35 C counts above the maximum allowable by the State regulation, i.e., 50,000 bacteria per ml or above (Michigan Allied Dairy Association, 1949). Forty-eight samples contained coliforms. Based on the 35 C count and the coliform plates, 58 samples could be rejected, and since this represents over 70 per cent of the samples, we are reasonably safe in assuming that these samples do not represent a particularly good milk supply.

TABLE 6

Bacterial populations of two laboratory pasteurized milk samples obtained from fresh raw milk and stored for 10 days at 4.5 C

1. Barn A

Days stored	Plate counts Incubation temperature (C) 55	35	20	4.5
0	17	12	26	< 10
2	< 10	20	30	< 10
4	< 10	30	28	< 10
6	12	21	21	< 10
8	21	26	20	< 10
10	< 10	31	38	< 10

2. Barn B

Days stored	55	35	20	4.5
0	200	330	130	< 10
2	140	400	240	< 10
4	90	580	210	< 10
6	30	600	230	< 10
8	20	550	220	< 10
10	40	540	250	< 10

TABLE 7.

Bacterial populations of two laboratory pasteurized milk samples obtained from two day old raw milk and stored for 10 days at 4.5 C

1. Barn A

Days stored	Plate counts Incubation temperature (C) 55	35	20	4.5
2	16	40	<10	<10
4	10	<10	10	<10
6	10	20	14	<10
8	<10	20	20	<10
10	11	60	50	<10

2. Barn B

Days stored	55	35	20	4.5
2	10	100	180	<10
4	12	300	150	<10
6	11	300	220	<10
8	13	540	280	<10
10	10	800	340	<10

TABLE 8

Bacterial populations of two laboratory pasteurized milk samples obtained from four day old raw milk and stored for 10 days at 4.5 C

1. Barn A

Days stored	Plate counts Incubation temperature (C) 55	35	20	4.5
2	<10	20	10	<10
4	<10	20	20	<10
6	<10	30	20	<10
8	<10	<10	10	<10
10	<10	20	20	<10

2. Barn B

Days stored	55	35	20	4.5
2	10	300	90	<10
4	30	300	160	<10
6	50	310	170	<10
8	40	400	270	<10
10	50	450	370	<10

TABLE 9

Bacterial populations of two laboratory pasteurized milk samples obtained from six day old raw milk and stored for 10 days at 4.5 C

1. Barn A

Days stored	Plate counts Incubation temperature (C) 55	35	20	4.5
2	< 10	20	40	< 10
4	< 10	40	30	< 10
6	< 10	20	40	< 10
8	< 10	20	20	< 10
10	< 10	10	20	< 10

2. Barn B

Days stored	55	35	20	4.5
2	50	300	370	< 10
4	90	490	290	< 10
6	70	600	300	< 10
8	50	550	380	< 10
10	40	500	400	< 10

TABLE 10

Bacterial populations of two laboratory pasteurized milk samples obtained from eight day old raw milk and stored for 10 days at 4.5 C

1. Barn A

Days stored	Plate counts Incubation temperature (C) 55	35	20	4.5
2	10	50	30	<10
4	20	40	40	<10
6	10	20	40	<10
8	20	60	50	<10
10	10	30	30	<10

2. Barn B

Days stored	55	35	20	4.5
2	14	200	200	<10
4	10	240	170	<10
6	10	190	150	<10
8	20	180	120	<10
10	50	210	120	<10

TABLE 11

Bacterial populations of two laboratory pasteurized milk samples obtained from 10 day old raw milk and stored for 10 days at 4.5 C

1. Barn A

Days stored	Plate counts Incubation temperature (C) 55	35	20	4.5
2	<10	10	40	<10
4	<10	20	20	<10
6	<10	50	20	<10
8	<10	60	20	<10
10	<10	50	20	<10

2. Barn B

Days stored	55	35	20	4.5
2	70	560	820	500
4	80	700	900	430
6	80	930	1,400	1,000
8	50	1,200	1,600	1,900
10	70	1,700	3,000	2,900

TABLE 12

Bacterial populations of milk and cream from several Michigan milk plants

Type of sample	Dairy	Plate counts 35 C	20 C	coliform	4.5 C
Cream-line milk	A	24,000	54,000	0	61,000
	B	49,000	110,000	4	40
	C	27,000	3,200	0	14
	D	9,400	880	0	30
	E	74,000	30,000	0	82
	F	31,000	37,000	45	60
	G	16,000	23,000	0	33
	H	16,000	24,000	1	20
	I	14,000	23,000	0	24
	J	51,000	43,000	0	20
	K	63,000	94,000	0	200
	L	<3,000	200	0	300
	M	10,000	1,800	2	<10
	N	15,000	34,000	39	20
	O	<3,000	14,000	1	21
	P	<3,000	3,200	1	30
	Q	44,000	41,000	0	1,800
	R	34,000	52,000	0	<10
	S	600,000	850,000	0	180,000
	T	12,000	14,000	1	700
	U	14,000	23,000	0	20
Homogenized milk	A	48,000	>3,000,000	0	>3,000,000
	B	22,000	26,000	10	50
	C	7,500	7,600	2	41
	D	33,000	19,000	6	40
	E	150,000	190,000	170	60
	F	>3,000,000	>3,000,000	25	>3,000,000
	G	35,000	52,000	0	200
	H	8,400	>3,000,000	0	10
	I	30,000	79,000	125	12
	J	35,000	61,000	20	18,000
	K	100,000	140,000	8	<10
	L	<3,000	1,000	0	800
	M	<3,000	2,700	0	<10
	N	18,000	6,000	>300	13
	O	8,000	6,900	26	20
	P	<3,000	3,900	6	90
	Q	<3,000	4,300	5	1,300
	R	<3,000	15,000	0	20

TABLE 12 (continued)

Bacterial populations of milk and cream from several Michigan milk plants

Type of sample	Dairy	Plate counts 35 C	20 C	coliform	4.5 C
Homogenized milk	S(bottle)	<3,000	11,000	72	5,300
	S(carton)	<3,000	1,700	0	<10
	T	4,000	8,200	1	1,000
	V	28,000	35,000	5	13,000
	W	>3,000,000	>3,000,000	0	>3,000,000
	X	10,000	230,000	43	63,000
	Y	44,000	90,000	0	43
	Z	380,000	1,300	2	60
	AA	38,000	100,000	2	<10
	AA(frozen)	23,000	39,000	14	<10
Skim milk	R	3,300	14,000	0	3,300
	S	<3,000	10,000	3	9,700
	T	<3,000	14,000	1	<10
	U	<3,000	4,900	6	34
	V	10,000	5,200	12	20
	X	>3,000,000	>3,000,000	>300	>3,000,000
	AA	18,000	61,000	0	10
	AA(frozen)	18,000	26,000	0	41
	AB	<3,000	6,000	0	20
Chocolate milk	R	200,000	710,000	0	530,000
	T	3,000	5,400	8	2,500
	U	70,000	270,000	260	160,000
	AA	<3,000	6,400	0	14
Coffee cream	G	17,000	11,000	0	11
	J	410,000	>3,000,000	>300	>3,000,000
	K	170,000	46,000	64	3,700
	L	5,000	9,000	41	2,000
	M	<3,000	2,500	40	12
	N	220,000	>3,000,000	>300	>3,000,000
	O	5,000	700	35	20
	P	<3,000	34,000	18	3,500
	R	60,000	320,000	15	190,000
	U	<3,000	>3,000,000	4	700
	V	>3,000,000	>3,000,000	0	>3,000,000

TABLE 12 (concluded)

Bacterial populations of milk and cream from several Michigan milk plants

Type of sample	Dairy	Plate counts 35 C	20 C	coliform	45 C
Whipping cream	A	>3,000,000	>3,000,000	>300	> 3,000,000
	B	29,000	85,000	140	25,000
	D	21,000	53,000	54	19,000
	E	>3,000,000	>3,000,000	0	>3,000,000
	F	13,000	>3,000,000	24	>3,000,000
	Q	>3,000,000	>3,000,000	>300	>3,000,000
	V	500,000	>3,000,000	0	>3,000,000
	W	>3,000,000	>3,000,000	0	>3,000,000

The results of counts obtained from plates incubated at 20 C are generally higher than comparable results from 35 C incubated plates. Eighteen samples had 50,000 or more bacteria per ml as indicated in counts from plates incubated at both temperatures. Only four samples gave counts of 50,000 or more at 35 C incubation but not at 20 C, while the remaining 16 samples were shown to have 50,000 or more bacteria per ml at 20 C incubation but not at 35 C. Since the dilutions made to determine plate counts from 35 C incubation were 1:100, 1:1,000 and 1:10,000, the results from 35 C incubation include counts between 3,000 and 3,000,000. When less than 30 colonies were counted on plates made from the 1:100 dilution, the counts were recorded as less than 3,000 bacteria per ml of sample. Similarly, when more than 300 colonies were counted on plates prepared from the 1:10,000 dilution, the counts were estimated as greater than 3,000,000. However, 68 samples counted from plates incubated at 35 C had counts between 3,000 and 3,000,000, and these samples, when compared to similar counts from 20 C incubation, were generally lower than the latter. Only in 14 cases, about 20 per cent, were the counts from 35 C incubation higher than the comparable 20 C counts.

The psychrophilic microorganisms were present in 73 of the 81 milk samples while coliform bacteria were found in 48 samples.

4. Isolation of pure cultures

a. Fresh raw milk

The types of microorganisms most commonly found in the pour plates from the 20 fresh raw milk samples are shown in tables 13, 14, 15 and 16.

In table 13 are recorded the results obtained from the 55 C incubated plates. The predominant organisms were gram positive spore-forming rods and Actinomycetes. Three gram positive rods could not be identified; they may have been members of the genus Bacillus, but no spores were detected. The five cocci which could also not be positively identified were gram positive tetrads.

The isolates from the 35 C incubated plates are classified in table 14. Bacillus and Actinomycetes were still predominant, but no Streptococcus was found. Contrary to the 55 C isolates, 13 gram negative rods were isolated as well as 11 cultures belonging to the genus Micrococcus.

From the 20 C plates (table 15) predominantly gram negative rods and gram positive cocci were isolated. These types were also isolated from the 35 C plates but less frequently. Actinomycetes and Bacillus cultures were evident, but all of the types isolated from the 35 C plates were also found in the 20 C plates.

Of the 106 cultures isolated from the psychrophilic plates (table 16), 65 or about 61 per cent belonged to the genera Alcaligenes, Achromobacter, Flavobacterium and Pseudomonas. Alcaligenes was most frequently found in the milk from barn C. The cultures obtained from the milk of barns A and B were too few to indicate the predominant genus, but members of the genus Flavobacterium appeared to be found less frequently than the other genera. The largest number of gram positive organisms belonging to

TABLE 13

Microorganisms isolated from 55 C pour plates made from fresh raw milk

Barn	Number of samples	Types of microorganisms Bacillus	Streptococcus	Actinomycetes	Gram ≁ rods	Misc. cocci
A	5	9	3	4	2	2
B	7	8	2	9	1	0
C	8	9	3	12	0	3
Total	20	26	8	25	3	5

TABLE 14

Microorganisms isolated from 35 C pour plates made from fresh raw milk

Barn	Number of samples	Types of microorganisms: *Bacillus*	*Actinomycetes*	*Micrococcus*	*Alcaligenes*	*Achromobacter*	*Flavobacterium*	*Pseudomonas*	Molds
A	5	4	5	4	2	1	1	0	2
B	7	11	6	4	0	1	1	0	0
C	8	5	10	3	3	1	2	1	3
Total	20	20	21	11	5	3	4	1	5

TABLE 15

Microorganisms isolated from 20 C pour plates made from fresh raw milk

Barn	Number of samples	Types of microorganisms: *Bacillus*	*Actinomycetes*	*Micrococcus*	*Alcaligenes*	*Achromobacter*	*Flavobacterium*	*Pseudomonas*	Molds	Gram + rods
A	5	4	6	8	2	3	1	1	2	0
B	7	8	3	6	2	2	5	1	1	1
C	8	3	0	8	4	1	2	2	2	2
Total	20	15	9	22	8	6	8	4	5	3

TABLE 16

Microorganisms isolated from 4.5 C pour plates made from fresh raw milk

Barn	Number of samples	Numbers belonging to the genera: *Micrococcus*	*Alcaligenes*	*Achromobacter*	*Flavobacterium*	*Pseudomonas*	Others
A	5	2	2	4	1	2	3
B	7	2	6	3	1	8	0
C	8	5	17	5	10	6	29
Total	20	9	25	12	12	16	32

a single genus were found to be members of genus *Micrococcus*. Organisms listed as "others" in table 16 were as follows: barn A- two *Aerobacter*, one mold; barn C- 20 yeasts and nine molds.

The 20 fresh raw milk samples from individual cows in barn C gave similar results (table 17). Members of the "Big Four" genera were again most frequently found while the yeasts and molds were not uncommon. Of all the genera listed in table 17, *Achromobacter* and *Pseudomonas* were found in the fewest samples.

b. Stored raw milk

The 20 samples of stored raw milk from the three barns gave the results shown in table 18.

As would be expected, the same organisms appeared after 10 days as were originally present. However, it is interesting to note that, the gram negative rods, particularly *Pseudomonas*, remained prominent. The yeasts, molds and *Aerobacter* strains were not as prevalent after 10 days storage as they were in the fresh milk, while members of the genus *Micrococcus* were commonly found throughout the storage period. Within the groups listed as *Micrococcus* and *Bacterium* were several strains of gram negative or gram variable organisms. On subsequent transfers at 20 C all of the cultures listed as *Micrococcus* became more gram positive than gram negative. Some of the strains listed as *Bacterium* were decidedly gram negative, but could not accurately be placed in any of the gram negative genera defined by Breed et.al. (1948). The gram positive or gram variable strains failed to produce endospores.

TABLE 17

Psychrophilic microorganisms isolated from fresh raw milk taken from individual cows in barn C

Designation of cow	Number of samples	Numbers belonging to the genera: Alcaligenes	Achromobacter	Flavobacterium	Pseudomonas	Micrococcus	Yeasts	Molds
K 19	2	1	0	1	0	0	1	0
K 134	2	2	0	2	5	0	2	2
K 17	2	1	2	0	0	0	1	1
K 109	2	0	0	2	0	1	1	2
K 144	2	2	0	1	0	2	4	0
K 126	2	0	1	0	0	0	0	2
K 132	1	0	0	1	0	1	0	2
K 105	2	0	0	2	0	0	1	1
K 205	1	1	0	0	0	0	0	1
K 101	2	0	0	0	0	1	2	0
K 211	1	0	0	0	0	0	2	1
K 129	1	1	0	0	0	0	0	0

TABLE 18

Psychrophilic microorganisms isolated from 20 samples of aged raw milk

Age of milk (days)	Numbers belonging to the genera: Alcaligenes	Achromobacter	Flavobacterium	Pseudomonas	Aerobacter	Micrococcus	Bacterium	Yeasts	Molds
0*	25	12	12	16	2	9	0	20	10
2	6	7	7	7	1	4	0	6	4
4	11	11	6	12	1	4	2	4	2
6	10	2	5	16	0	2	2	1	0
10	9	6	3	17	0	4	3	1	1
Totals	61	38	33	68	4	23	7	32	17

* Data from table 17

B. Taxonomy

1. *Micrococcus*

Sixty-one micrococci were isolated from 35, 20 and 4.5 C plates. Forty-seven of these cultures came from fresh raw milk while the remaining 14 were from stored raw milk. All of the 14 cultures from stored raw milk were picked from 4.5 C incubated plates. Fourteen other colonies were picked from 4.5 C incubated plates prepared from fresh raw milk. Eleven and 22 cultures came from 35 and 20 C incubated plates respectively.

Thirty-six of these isolates were readily classified according to the description given by Breed et.al. (1948) while 25 were not. The 36 cultures which had the characteristics to match the descriptions given by Breed et.al. (1948) belonged to 10 species, while the remaining 25 cultures were placed into 11 groups. The distribution of the 61 cultures is shown in table 19.

The predominant types of micrococci were *Micrococcus conglomeratus* and *Micrococcus epidermidis*; the former found in plates incubated at 20 and 4.5 C, the latter found in the 35 and 20 C incubated plates. Micrococci belonging to groups D and K were also frequently encountered, but never in 35 C incubated plates. The cultures identified as *Micrococcus freudenreichii*, *Micrococcus caseolyticus* and those in groups B, D, E, F, H and I did not initially grow at 35 C but after several transfers at 20 C, all cultures except one strain of *Micrococcus freudenreichii* and three strains of *Micrococcus caseolyticus* grew at 35 C.

TABLE 19

Type of micrococci isolated from pour plates incubated at various temperatures

Name of organism	Number of cultures	Source Plates incubated at (C) 35	20	4.5
Micrococcus luteus	2	0	1	1
Micrococcus ureae	2	0	2	0
Micrococcus freudenreichii	2	0	1	1
Micrococcus flavus	1	1	0	0
Micrococcus conglomeratus	9	0	4	5
Micrococcus varians	1	1	0	0
Micrococcus caseolyticus	6	0	1	5
Micrococcus aurantiacus	2	1	0	1
Micrococcus epidermidis	10	4	6	0
Micrococcus roseus	1	1	0	0
Group A *	3	1	0	2
Group B	2	0	0	2
Group C	1	0	1	0
Group D	6	0	0	6
Group E	2	0	0	2
Group F	1	0	0	1
Group G	1	1	0	0
Group H	1	0	0	1
Group I	1	0	0	1
Group J	3	1	2	0
Group K	4	0	4	0

* For a description of each group, see appendix B.

Strains of *Micrococcus epidermidis*, groups G, J and K could not be detected after 14 days incubation at 4.5 C.

2. *Alcaligenes*

Eighty-two cultures of *Alcaligenes* were obtained, five from 35 C, eight from 20 C and 69 from 4.5 C incubated plates. All of the cultures were readily characterized as shown in table 20.

Alcaligenes faecalis was only picked from 35 C plates; the remaining five species were found on the 4.5 C plates. *Alcaligenes bookeri* was isolated most frequently.

3. *Achromobacter*

The 50 *Achromobacter* cultures were classified as shown in table 21. All of the cultures except one group were comparable to the descriptions given by Breed et.al. (1948). The description of this group is given in appendix B. Both this unidentified group and *Achromobacter superficiale* were the predominant *Achromobacter*.

4. *Flavobacterium*

The descriptions which are given by Breed et.al. (1948) to distinguish members of the genus *Flavobacterium* are not complete enough to allow for adequate separation as is indicated in table 22. The 54 cultures did fit into 10 groups; six of which are comparable to the descriptions given by Breed et.al. (1948). The remaining four groups are probably also given in *Bergey's Manual*, but too incompletely described to warrant conclusive identification.

TABLE 20

Types of Alcaligenes isolated from pour plates incubated at various temperatures

Name of organism	Number of cultures	Source Plates incubated at (C) 35	20	4.5
Alcaligenes faecalis	3	3	0	0
Alcaligenes viscosus	13	0	2	11
Alcaligenes metalcaligenes	19	1	5	13
Alcaligenes bookeri	34	1	1	32
Alcaligenes recti	9	0	0	9
Alcaligenes marshallii	4	0	0	4

TABLE 21

Types of Achromobacter isolated from pour plates incubated at various temperatures

Name of organism	Number of cultures	Source Plates incubated at (C) 35	20	4.5
Achromobacter liquefaciens	3	0	0	3
Achromobacter iophagum	1	1	0	0
Achromobacter delicatulum	2	0	2	0
Achromobacter cycloclastes	4	1	0	3
Achromobacter superficiale	13	0	0	13
Achromobacter butyri	6	0	2	4
Achromobacter eurydice	5	0	0	5
Achromobacter delmarvae	3	1	0	2
Group A *	13	0	2	11

* For a description of this group, see appendix B

TABLE 22

Types of Flavobacterium isolated from pour plates incubated at various temperatures

Name of organism	Number of cultures	Source Plates incubated at(C) 35	20	4.5
Flavobacterium devorans	2	1	0	1
Flavobacterium suaveolens	3	0	1	2
Flavobacterium invisibile	6	1	0	5
Flavobacterium lactis	7	0	1	6
Flavobacterium esteroaromaticum	2	0	0	2
Flavobacterium ferrugineum	1	1	0	0
Group A *	9	0	0	9
Group B	2	0	0	2
Group C	5	0	0	5
Group D	2	0	2	0
Group E-1	7	0	1	6
Group E-2	5	0	3	2
Group E-3	3	1	0	2

* For a description of each group, see appendix B

5. Pseudomonas

Since 73 out of 78 *Pseudomonas* cultures were obtained from plates incubated at 4.5 C, the results of classifying this group of organisms applies particularly to psychrophilic *Pseudomonas*. The findings recorded in table 23 indicated that two groups, *Pseudomonas pavonacea* and *Pseudomonas ovalis* accounted for nearly 75 per cent of all *Pseudomonas* cultures.

C. Temperature studies

The representative psychrophile cultures subjected to 62.8 C in skim milk failed to show any growth after the heat treatment.

Most of the 23 cultures (table 24) grew better at 20 C than at either 35 or 4.5 C. Whereas the 4.5 C counts were generally comparable to the 20 C plate counts, the 35 C plate counts in seven cases fell far below the counts from the 20 and 4.5 C incubated plates. Although the cultures may have grown at 35 C, the colonies on the pour plates were often too small to count with any degree of accuracy.

D. Influence of psychrophilic microorganisms on the keeping quality of milk

The plate counts of four raw milk samples stored at 4.5 C are recorded in table 25. In a similar run previously described, the 35 and 20 C plate counts were initially higher than the 4.5 counts, but within a short storage period, the psychrophilic count equaled the other counts. The population determined in 35 C

TABLE 23

Types of Pseudomonas isolated from pour plates incubated at various temperatures

Name of organisms	Number of cultures	Source Plates incubated at (C) 35	20	4.5
Pseudomonas chlororaphis	2	0	2	0
Pseudomonas synxantha	3	0	0	3
Pseudomonas fluorescens	5	1	1	3
Pseudomonas pavonacea	37	0	0	37
Pseudomonas geniculata	4	0	0	4
Pseudomonas syncyanea	1	0	0	1
Pseudomonas iodina, *Pseudomonas smaragdina*	2	0	0	2
Pseudomonas ovalis	22	0	1	21
Pseudomonas convexa	1	0	0	1
Pseudomonas immobilis	1	0	0	1

TABLE 24

Bacterial count of psychrophilic organisms in skim milk after six days incubation at 4.5 C

Designation of organisms	Bacterial counts from plates incubated at : 35 C	20 C	4.5 C
Micrococcus A	1,500,000	28,000,000	3,400,000
Micrococcus B	1,000	9,200,000	8,200,000
Micrococcus D	2,100	6,700,000	6,500,000
Micrococcus E	10	160,000	270,000
Micrococcus H	10	130,000	270,000
Micrococcus conglomeratus	4,500,000	4,400,000	4,300,000
Micrococcus caseolyticus	2,700,000	8,700,000	4,000,000
Alcaligenes viscosus	1,800,000,000	2,800,000,000	3,000,000,000
Alcaligenes metalcaligenes	110,000,000	68,000,000	77,000,000
Alcaligenes bookeri	270,000,000	230,000,000	220,000,000
Alcaligenes recti	110,000,000	200,000,000	970,000
Achromobacter A	10	2,500,000	3,100,000
Achromobacter liquefaciens	25	3,700,000	4,700,000
Achromobacter superficiale	130,000,000	590,000,000	140,000,000
Flavobacterium A	610,000,000	1,600,000,000	470,000,000
Flavobacterium E-1	18,000,000	59,000,000	240,000,000
Flavobacterium E-2	400	4,200,000	10,000,000
Flavobacterium lactis	4,200,000	5,600,000	3,800,000
Pseudomonas pavonacea	700,000	190,000,000	240,000,000
Pseudomonas ovalis	1,600,000	970,000,000	1,300,000,000
Pseudomonas convexa	150,000,000	98,000,000	270,000,000
Pseudomonas mobilis	200,000,000	350,000,000	470,000,000
Yeast	500,000	400,000	300,000

TABLE 25

Bacterial count of stored raw milk using several incubation temperatures

Days stored at 4.5 C	Bacterial count Incubation temperature (C) 35	20	4.5
1. Milk sample A			
1	14,000	15,000	2,400
2	33,000	30,000	29,000
4	1,500,000	2,200,000	1,100,000
6	4,700,000	28,000,000	15,000,000
8	43,000,000	65,000,000	66,000,000
9	100,000,000	200,000,000	240,000,000
13	970,000,000	2,900,000,000	4,500,000,000
2. Milk sample B			
0	8,400	5,600	170
2	35,000	30,000	8,700
4	440,000	400,000	360,000
6	2,400,000	9,500,000	13,000,000
8	49,000,000	210,000,000	57,000,000
12	660,000,000	560,000,000	720,000,000
3. Milk sample C			
0	280,000	310,000	18,000
2	190,000	940,000	870,000
4	360,000	8,200,000	7,600,000
6	1,700,000	39,000,000	42,000,000
8	2,400,000	190,000,000	110,000,000
10	12,000,000	1,400,000,000	980,000,000
4. Milk sample D			
0	83,000	87,000	57
2	22,100	49,000	110
4	19,400	150,000	28,000
6	16,000	470,000	400,000
8	180,000	5,400,000	6,000,000
10	2,900,000	31,000,000	27,000,000
12	26,000,000	490,000,000	430,000,000

plates, as opposed to the 20 and 4.5 C plates, failed to increase. From the data in table 25, a slightly different trend can be noted. The initial 35 and 20 C plate counts were well above the psychrophile count, but within two to four days, the psychrophile count was as high as the counts obtained from the other plates. With milk samples A and B, except for an occasional variation, all plates gave similar counts. The 35 C plate counts from milk samples C and D indicate a definite lag, especially in the latter case, but the increase in the psychrophile count is eventually reflected in the counts from the 35 C plates.

A portion of milk sample C, which was pasteurized immediately, was divided into five parts. One part was stored without inoculation while the other four parts were inoculated with an actively growing pure culture. The plate counts and taste characteristics of these milk samples are recorded in table 26. The uninoculated milk did not deteriorate after 16 days, although the psychrophile count increased slightly. The inoculated milk samples had off-flavors when the psychrophile count was 10 to 100 million, which occurred at varying times of storage depending on the amount of inoculum and the rate of growth of the particular organism.

The milk samples A and B were pasteurized in two day intervals as shown in table 25 and inoculated with 0.2 ml of a raw milk sample containing 830,000 psychrophiles. The subsequent growth of psychrophiles and the deterioration of milk

TABLE 26

Plate counts and taste of a pasteurized raw milk sample from fresh raw milk (Sample C; table 25)

Days stored at 4.5 C	Plates incubated at (C) 35	4.5	Off-taste
1. Uninoculated			
0	1,300	10	0
2	1,100	10	0
4	1,200	10	0
6	2,400	10	0
8	3,000	10	0
10	4,800	11	0
12	5,900	65	0
14	6,100	95	0
16	6,700	700	0
2. Inoculated with *Flavobacterium* (E-2)			
0	1,300	970	0
4	1,700	1,000	0
8	3,800	9,000	0
12	6,400	270,000	0
16	16,000	2,500,000	0
3. Inoculated with *Pseudomonas ovalis*			
0	1,700	330,000	0
4	1,400	2,800,000	0
8	1,400	4,500,000	0
12	950	40,000,000	+
16	1,300	470,000,000	++++
4. Inoculated with *Alcaligenes viscosus*			
0	1,400	3,000,000	0
4	2,000	42,000,000	+
8	4,000	49,000,000	++
12	40,000	28,000,000,000	++++
16	370,000		

TABLE 26 (continued)

Plate counts and taste of a pasteurized raw milk sample from fresh raw milk (Sample C; table 25)

Days stored at 4.5 C	Plates incubated at (C) 35	4.5	Off-taste
5. Inoculated with Flavobacterium (A)			
0	2,400	1,700,000	0
4	2,000	2,900,000	/
8	30,000	150,000,000	///
12	300,000	200,000,000	////
16	46,000,000	370,000,000	curd
6. Inoculated with Micrococcus conglomeratus			
0	1,900	126,000	0
4	3,600	370,000	0
8	10,000	16,000,000	0
12	59,000	39,000,000	/
16	1,100,000	49,000,000	///

proceeded as indicated in table 27. Both raw milk samples, having a psychrophile count above 50 million, produced pasteurized milk which was unfit for consumption. Likewise, the pasteurized milk produced from raw milk with a low psychrophile count, was able to withstand more psychrophilic growth before the milk developed an off-flavor.

TABLE 27

The rate of deterioration of pasteurized milk prepared from raw milk stored at 4.5 C for various times and inoculated with a mixed population of psychrophiles

Pasteurized milk stored (days)	Psychrophile count *	Age of raw milk at time of pasteurization (days) 0	1	2	4	6	8	9	12	13
1. Milk sample A										
0	700,000	--[1]	0[2]	0	0	0	++[3]	+++	--	++++
1	1,300,000	--	0	0	0	+	++	++++	--	--
2	7,000,000	--	0	0	0	+++	++++	--	--	--
3	19,000,000	--	0	0	+	++++	--	--	--	--
4	79,000,000	--	0	++	++	--	--	--	--	--
5	130,000,000	--	++	+++	+++	--	--	--	--	--
2. Milk sample B										
0	960,000	0	--	0	0	0	+	--	++++	--
1	1,200,000	0	--	0	0	+	++	--	--	--
2	12,000,000	0	--	0	0	++	+++	--	--	--
3	24,000,000	0	--	0	0	++	++++	--	--	--
4	90,000,000	0	--	0	+	+++	--	--	--	--
5	220,000,000	+	--	+	++	++++	--	--	--	--
6	1,100,000,000	++++	--	++++	++++	--	--	--	--	--

* The psychrophile count is an average of plates prepared from all raw milk stored for various times The individual counts from milk stored from 0 to 13 days varied to the same extent as did the triplicate plates from a single milk sample

1 dash (--) indicates that no examinations were made

2 a zero (0) indicates that the sample had no objectionable off-flavor resulting from bacterial growth

3 plus signs (+ to ++++) indicate that the sample had an objectionable off-flavor resulting from bacterial growth

V. DISCUSSION

A. Relationship between total (35 C) and psychrophile (4.5 C) plate counts

In evaluating the bacterial quality of fresh raw milk from three barns (table 2), the total count would indicate that the milk from barns A and C was superior to barn B, with the milk from barn C being slightly better than the milk from barn A. Using the psychrophilic count, the milk samples from barn A had a lower psychrophilic population than similar samples from barn C; the latter containing a psychrophile population equal to about four per cent of its total count. The psychrophile population of the milk from barn B is equivalent to about 10 per cent of its total count. The data in table 3 also support the contention that the total count cannot be used to evaluate the psychrophilic population. In fact, the average of 16 milk samples which had the highest total count was found to have the lowest psychrophilic population.

One of the serious defects in basing conclusions on the data in both tables 2 and 3 is the relatively low bacterial populations of the milk samples. This is especially true of the psychrophilic microorganisms. However, studies showing the differences in the total and psychrophilic populations over a period of cold storage (tables 4, 12, 24, 25 and 26) clearly indicated the fallacy of using bacterial counts obtained from plates incubated at 35 C to evaluate the bacterial quality of milk. The total counts in both samples used to obtain the results given in table 4 actually decreased upon storage, while the psychrophilic microorganisms multiplied logarithmically. Although such a drastic difference cannot be demonstrated in the values shown in table 25, those data also indicate that (1) the total count of fresh raw milk is greater than the initial psychrophilic count, (2) the total and psychrophilic counts approximate each other in four days of cold storage and (3) after four days the psychrophilic population is higher than counts obtained from plates incubated at 35 C. These findings are in general agreement with those reported by Ayers, Cook and Clemmer (1918) and Babel (1953) who found that raw milk held at 4.4 C for four to five days showed only a 10 to 100 fold increase by counting plates incubated at 32 C.

The data in table 12 are useful in showing the errors which can be expected when the 35 C plate count alone is relied upon to judge the bacterial quality of milk. In discussing these data, the milk samples have been arranged into four general groups as suggested in <u>Standard Methods for the Examination of Dairy Products</u> (American Public Health Association, 1953). These groups are characterized as follows:

Group 1- milk containing a bacterial content, by the standard plate count, with a good safety margin. Arbitrarily the bacterial count for this group was set at 10,000 per ml or below;

Group 2- milk containing a bacterial population above 10,000 but below 50,000 per ml. This group represents marginally acceptable milk, only slightly below the maximum limits of the bacterial content;

Group 3- milk which is marginally in violation of the accepted standard of 50,000 per ml, but not grossly contaminated, the upper limits being 100,000 bacteria per ml;

Group 4- milk indicating gross contamination by its bacterial population of over 100,000 per ml.

In separating the 81 commercial milk samples into these four groups, 30 met the requirements of group 1, 28 of group 2, 7 of group 3 and 16 of group 4. The 30 samples in group 1 included 18 which had a total plate count below 3,000 per ml. The psychrophilic populations in these 18 samples were generally below 100 per ml (11 samples), but three milk samples had a psychrophilic population above 3,000 per ml. Of the remaining 12 milk samples in group 1, seven had a psychrophile content below 100, one was below 1,000 and the remaining four samples had a psychrophile content between 1,000 and 63,000.

The bacterial counts recorded in table 28 indicate that milk with a low 35 C count generally had a lower psychrophilic population although a significant number of samples had psychrophile counts above 100. A few samples had a higher psychrophilic population than is indicated by the total count. The milk samples placed into group 2 generally contained few psychrophiles, but in this group, as with subsequent groups, the milk samples contained either very few or a large number of psychrophiles. The milk

TABLE 28

Classification of several commercially pasteurized milk and cream samples into groups based on their bacterial population obtained from plates incubated at 35 C

Group	Number of samples having psychrophile populations Less than 100	Less than 1,000	Less than 10,000	Greater than 10,000
Group 1- Not over 10,000 bacteria per ml as determined by counts from plates incubated at 35 C	18	3	8	1
Group 2- between 10,000 and 49,000 bacteria per ml	18	2	1	7
Group 3- between 50,000 and 100,000 bactoria per ml	4	1	0	2
Group 4- over 100,000 bacteria per ml	2	0	1	13

in group 4 generally contained a psychrophilic population as high or higher than the count from plates incubated at 35 C.

A reason for the dissimilarity between counts made from plates incubated at 35 C and 4.5 C is obtainable from tables 25 and 26. All of the 23 psychrophilic cultures grown in skim milk for six days at 4.5 C, reached a population above 100,000 as determined by the psychrophilic plate count. However, the counts from the 35 C incubated plates revealed that one-third of the cultures attained counts below 100,000 and only in two cases were the counts obtained from plates incubated at 35 C significantly higher than from plates incubated at 4.5 C. Similarly, the growth rate of psychrophilic cultures as indicated by plates incubated at 35 and 4.5 C (table 26) showed slight resemblance to each other.

The unpredictable counts from cold storage milk, using 35 C incubated plates, can also be shown from the data reported by Chaffee (1952) who stored 16 commercially pasteurized milk samples for four days and found that the counts , upon storage, decreased in two samples, remained about the same in 11 samples and increased in three of the samples. When Olson, Willoughby, Thomas and Morris (1953) stored pasteurized milk at 45 F for seven days, they also obtained results from the 35 C incubated plates which in one case increased, as did the psychrophilic population as determined by incubating plates at 7 C, while in another case, the 35 C plates gave results which indicated a lag in growth of the bacteria in the stored milk. The data of Leet (1930) and Mikolajcik and Burgwald (1953) also indicate the unreliability of determining the bacterial count of milk stored under refrigeration by 35 C incubation.

That some psychrophilic microorganisms do not grow at 37 C has been shown by Haines (1934) who found little or no growth at 37 C by *Escherichia coli*, one strain of *Flavobacterium*, two strains of *Pseudomonas*, five strains of *Achromobacter* and two cultures of yeast.

B. Relationship between counts from plates incubated at 20 C and the other (35 and 4.5 C) incubation temperatures

The bacterial counts given in tables 4 and 25 would indicate that the psychrophile population approximates the counts of the plates incubated at 20 C. This condition existed particularly in milk samples which had been stored under refrigeration for a period of time. Counts of fresh milk obtained from plates incubated at 20 C, however, are influenced by the bacterial population counted on plates incubated at 35 C. The similarity of the counts from the plates incubated at 20 C and 35 C can best be shown when the psychrophilic population is negligible, as is the case in fresh raw milk (tables 4 and 25) and in the laboratory pasteurized milk samples (tables 5 to 11). The relationship between the counts of plates incubated at 20 and 4.5 C, from the data in table 12, can be illustrated when the milk samples are placed into groups based on the counts obtained at 20 C (table 29). In general, the agreement between these counts was were better than those from 35 and 4.5 C incubation (table 28). This fact can be shown by noting that the number of acceptable milk samples based on the 35 C plate count (groups 1 and 2 of table 28) is 58, while the number of acceptable milk samples based on counts from 20 C incubated plates (groups 1 and 2 of table 29) is 47. If the classification of milk samples based on the psychrophilic population, as indicated in tables 28 and 29, is accepted, 48 samples fit into the first two groups which is similar to the results obtained from the plates incubated at 20 C. Counts from plates incubated at 35 and 20 C showed moderate agreement when the milk samples were placed into general classes based on their bacterial counts as was done in table 30. Counts from 20 C incubation indicated that 47 samples were acceptable, while 58 samples were acceptable according to the counts from plates incubated at 35 C. Based on these data, the 20 C incubation is more favorable for bacterial growth than 35 C. Nelson and Baker (1954), basing their conclusion on extensive data collected to determine the optimum incubation conditions for enumerating bacteria in milk, recommend 21 C for four days or 25 C for three days which is in accord with our practice of incubating at 20 C for five days. From pure culture studies, Lawton and Nelson (1954) also conclude that the optimum temperature for psychrophiles found in milk is in the range of 21 to 32 C.

TABLE 29

Classification of several commercially pasteurized milk and cream samples into groups based on the bacterial population obtained from plates incubated at 20 C

Group	Number of samples having psychrophile populations			
	Less than 100	Less than 1,000	Less than 10,000	Greater than 10,000
Group 1- not over 10,000 bacteria per ml	17	2	4	0
Group 2- between 10,000 and 49,000 bacteria per ml	16	1	6	1
Group 3- between 50,000 and 100,000 bacteria per ml	5	2	0	4
Group 4- over 100,000 bacteria per ml	4	1	0	18

TABLE 30

Relationship between the classification of several commercially pasteurized milk and cream samples by counts from 20 and 35 C incubated plates

Groups based on 35 C incubation	Number of samples in groups based on 20 C incubation 1	2	3	4
Group 1	21	6	0	3
Group 2	2	14	9	3
Group 3	0	2	2	3
Group 4	1	1	0	14
Total samples 1. From plates incubated at 20 C	24	23	11	23
2. From plates incubated at 35 C	30	28	7	16

C. Significance of the psychrophilic count

Most investigators agree that the initial psychrophilic population of milk is too low to warrant placing any significance upon it. Furthermore, it is often assumed that determining particular types of psychrophiles in milk is a better criterion for evaluating milk quality than plate counts. That the initial psychrophile count is low in fresh raw milk is generally substantiated by the results presented in tables 2 and 3 and the initial counts shown in tables 4 and 25.

Two exceptions, however, do exist as indicated in the average count of 2,000 psychrophiles per ml of raw milk from barn B (table 2) and the 18,000 count from milk sample C shown in table 25. That the initial psychrophile count is probably not significant in determining milk quality is indicated in the data from the stored milk samples given in tables 4 and 25. The initial count has no particular affect on the growth of psychrophiles in milk during cold storage. In this respect, the data here presented agree with the conclusions drawn by other investigators.

Whether only particular species of psychrophiles are involved in producing off-flavors or lowering the keeping quality of milk, as implied in the literature, cannot be established in our findings. When the psychrophilic population increased above ten million, the milk sample showed signs of deterioration (tables 26 and 27), regardless of the types of psychrophiles multiplying in the milk. A study of the published data further supports this contention. The data given by Day and Doan (1956), for example, show that off-flavors developed in milk when the 25 C plate counts were from 7 to 320 million or had an average population of about 90,000,000. The psychrophilic bacteria reached a population of ten million in raw milk within four to eight days of storage at 4.5 C (tables 4 and 25). The effect of using stored raw milk in which varying amounts of psychrophilic growth had occurred was indicated in table 27. When the raw milk sample A was stored six days at 4.5 C, it developed a psychrophilic count of 57,000,000 under the same storage conditions. Both of these raw milk samples with the above mentioned psychrophilic counts, and raw milk samples with higher counts, produced pasteurized milk which had an undesirable taste. Similarly, the data in table 27 indicated that raw milk samples with a high psychrophilic population will affect the keeping quality of the pasteurized milk sample if the latter is contaminated with psychrophiles.

That commercially pasteurized milk and cream are generally contaminated with psychrophiles was evident from the data in table 12. Only eight of the 81 samples had less than 10 psychrophiles; 42 samples had less than 100 psychrophiles per ml of milk or cream tested.

The data in tables 5, 6, 7, 8, 9, 10 and 11 together with the pure culture studies indicated rather clearly that pasteurization of milk was very effective in killing psychrophilic bacteria even in high populations, and that the thermoduric population, counted on plates incubated at 20 and 35 C, did not grow at 4.5 C.

When colonies were not found on 4.5 C incubated pour plates containing one or 0.1 ml of milk sample, it was not safe to assume that psychrophilic microorganisms were absent in the milk sample, but after the milk was stored for six to 10 days and no psychrophilic growth could be detected, there was no reason to suspect that psychrophiles were initially in the milk sample. Babel (1953) also failed to show any growth of organisms in six samples of laboratory pasteurized milk when stored at 4.4 C for five days using counts from plates incubated at 32 C.

With the above factors in mind, a series of standards is proposed based on the psychrophilic count of raw and pasteurized milk:

1. Raw milk should contain less than 100,000 psychrophiles per ml of sample at the time of pasteurization. In terms of time, it is suggested that the dairy collect the raw milk from the farms every other day, and pasteurize the milk within three days after milking. Furthermore, the raw milk must be rapidly cooled and held at 4.5 C or lower.

2. The pasteurized milk should contain less than 10 bacteria per ml if samples are collected at the dairy, and less than 100 at the time it reaches the consumer.

3. A series of grades based on the psychrophilic count is recommended, comparable to the grading recommended by the American

Public Health Association (1953) for the standard plate count. The grading suggested is as follows:

Grade 1. Good quality pasteurized milk with satisfactory keeping quality; pasteurized milk from fresh raw milk and which has a psychrophile count of less than 10 bacteria per ml.

Grade 2. Marginally acceptable pasteurized milk; similar to grade 1, but with a psychrophilic count between 10 and 100 bacteria per ml.

Grade 3. Marginally unacceptable pasteurized milk; commercial milk with a psychrophilic population between 100 and 1,000 bacteria per ml.

Grade 4. Unacceptable pasteurized commercial milk with a psychrophilic count above 1,000 bacteria per ml.

These standards are proposed more to stimulate collection of additional data to test the validity of the results obtained in this research than as a tool to be applied without further work. The proposed standards are designed to supplement the presently accepted bacteriological methods rather than replace them. They are designed to fill the gap which now exists. The direct count of raw milk, standard plate count and coliform count of pasteurized milk are well established, mainly on the basis of preventing the spread of disease. But no standards have as yet been developed relative to the keeping quality of milk; the psychrophilic standards given above are proposed for this purpose. In connection with these standards, it is interesting to note the statement made by Davis, Twigg and Wright (1941):

> "Bulk raw milk would keep about three days, commercially pasteurized milk about seven days and laboratory pasteurized milk about 30 days when held in the region of 2 C."

The findings when the psychrophilic population are used as an indicator for post-pasteurization contamination do not agree with the results obtained when coliform organisms are used for the same purpose. To compare the two tests with the data shown in table 12, table 31 was prepared. In discussing these results the following criteria were used:

1. Although it is possible, and undoubtedly desirable, to produce milk with less than 10 psychrophile colonies per ml, only 10 per cent of the samples met this condition. Even though a psychrophilic population less than 10 may indicate post-pasteurization, for the purpose of this discussion it will be interpreted in the negative.

2. A psychrophilic population greater than 10 colonies per ml will be accepted as representing post-pasteurization contamination.

3. Psychrophilic populations between 10 and 100 colonies per ml, group 2, will be interpreted as showing no signs of psychrophilic growth.

4. A psychrophilic population between 100 and 1,000 colonies per ml, group 3, will be assumed to represent moderate psychrophilic growth during a short (about one day at 4.5 C) storage period.

TABLE 31

Differences in using the psychrophilic and coliform populations as indicators of post-pasteurization contamination

Psychrophile population (bacteria per ml)	Number of samples	Coliform population (bacteria per ml)	Number of samples
Group 1 less than 10	8	Less than 1	3
		Less than 10	4
		Greater than 10	1
Group 2 between 10 and 100	42	Less than 1	18
		Less than 10	13
		Greater than 10	11
Group 3 between 100 and 1,000	6	Less than 1	4
		Less than 10	2
Group 4 more than 1,000	33	Less than 1	11
		Less than 10	5
		Greater than 10	17

5. When the psychrophilic population is between 1,000 and 10,000 bacteria per ml, group 4, it will be interpreted as representing growth equivalent to three days of storage at 4.5 C.

6. A psychrophilic population above 10,000 colonies per ml of sample will be considered to represent extensive psychrophilic growth over an extended storage period.

7. Less than one coliform per ml of sample will be interpreted as indicating no contamination.

8. A coliform count between one and 10 will be interpreted as representing moderate post-pasteurization contamination.

9. When the coliform population is greater than 10, gross contamination will be assumed.

Using the above criteria in examining the data in table 31, out of eight milk samples which show no signs of post-pasteurization contamination by their psychrophilic population, five were indicted according to the coliform test. One of these five samples showed signs, based on its coliform content, of gross contamination. No explanation can be given for this discrepancy. Neither were the counts from plates incubated at 35 or 20 C substantially different in milk containing coliforms than in the samples with less than one coliform per ml. Likewise, it is hardly conceivable that milk can be contaminated after pasteurization by bacteria which do not exhibit psychrophilic characteristics.

Only about 60 per cent of the milk and cream samples, indicating post-pasteurization contamination by their psychrophilic population, contained coliform organisms. Based on the coliform test 40 per cent of the samples, incriminated by their psychrophilic count as being contaminated after pasteurization, gave no indication of post-pasteurization contamination. In view of these findings, the psychrophilic populations should be determined routinely on pasteurized milk to determine post-pasteurization contamination and the extent of storage of the milk.

Some argument may arise against using the psychrophilic population because it fails to evaluate the extent of contamination. This argument is justified assuming that the coliform test is able to make such an evaluation. The extensive work of Sherman and Wing (1933), Dahlberg (1945, 1946a, 1946b) and Nelson and Baker (1954) supports the view that coliform organisms multiply in milk and, therefore, their numbers in milk cannot be used to determine the degree of post-pasteurization contamination.

Nelson and Baker (1954) conclude that the psychrophile count is probably a better indicator of post-pasteurization contamination than the coliform test, but they suggest retaining the latter because it gives quicker results. The validity of their argument cannot be disputed. Even in the short incubation time recommended by the American Public Health Association (1953), the psychrophile population could not be obtained until five days after the sample was received in the laboratory while a coliform index could be determined in one day. In addition to the delay, determinations of psychrophilic counts would involve equipment for cold storage incubation, more laboratory equipment, as Petri dishes and dilution bottles, and more space. These are problems that will retard the use of psychrophilic counts as indicators of post-pasteurization contamination, but no one has convincing evidence that the psychrophilic count is not a good criterion. On the contrary, there is every indication to believe that measuring the psychrophilic population is superior to measuring the coliform content of a dairy sample for evaluating post-pasteurization contamination.

The only argument in favor of retaining the coliform index to measure post-pasteurization contamination is the speed in which results can be obtained. The question to be answered in making a choice between these two methods is, how significant is post-pasteurization contamination? If the people concerned with dairy products feel that this type of contamination is not an important consideration, then the coliform test should probably be maintained. For three basic reasons, the adoption of a method based on the psychrophilic content of commercial milk for determining post-pasteurization contamination would be more appropriate:

1. the test is more accurate than the one presently employed,

2. a psychrophilic index would give some idea of the potential keeping quality of the milk and

3. a psychrophilic count would indicate the relative age of the milk.

D. Characteristics of psychrophilic microorganisms

The term psychrophilic microorganism is used throughout this paper as it applies to the dairy industry. That is, microorganisms which predominate in dairy products after cold storage. Thus far, evidence has been presented to show that the psychrophilic microorganisms have an optimum temperature nearer 20 C than either 35 or 4.5 C, that some species have a wide temperature range in which growth occurs and that they are killed by pasteurization. Their growth rate in milk at 4.5 C incubation approximates an increase of a log in one or two days (figure 1). When these psychrophiles have grown in milk to the extent that their population is from 1,000,000 to 100,000,000, they impart an off-flavor to the milk.

The types of bacteria normally encountered (table 16) were feebly saccharolytic gram negative rods and gram positive cocci. The gram negative rods belong to the genera Alcaligenes, Achromobacter, Flavobacterium and Pseudomonas. Some dairy barns may, however, exhibit their own particular psychrophilic population, as is indicated in the results obtained from barn C (tables 16 and 17). Based on their prevalence in stored milk (table 18), Pseudomonas species tended to be the most prolific of the gram negative group. Aerobacter, as well as yeasts and molds, appear to be more transient than agents to be considered significant in causing off-flavors.

The investigations dealing with taxonomy primarily indicate the need for a better method to identify organisms. Even where an adequate description of an organism is available, the descriptive manuals do not suit the investigator determining the types of organisms in a particular habitat.

Three strains of micrococci appeared to predominate (table 19) as psychrophiles: Micrococcus conglomeratus, Micrococcus caseolyticus and a strain designated as Micrococcus D. Of the gram negative psychrophiles (tables 20, 21, 22 and 23), Pseudomonas pavonacea is most commonly found followed by Alcaligenes bookeri and Pseudomonas ovalis. Alcaligenes metalcaligenes and Achromobacter superficiale as well as Alcaligenes viscosus and an Achromobacter strain designated as group A are also not uncommon. Flavobacteria were less common than the other gram negative groups, and no one particular species was predominant.

The increased predominance of Pseudomonas during storage, as indicated in the results given in table 18, was particularly noteworthy in light of the work reported by Tobin, Alford and McCleskey (1941) and Castell and Mapplebeck (1952). Studying fish deterioration, Tobin and his co-workers initially isolated cocci and a few organisms belonging to the genera Bacillus, Achromobacter, Flavobacterium, Aerobacter, Escherichia and Pseudomonas, but as the fish deteriorated in cold storage, practically pure cultures of Pseudomonas and Achromobacter remained. Studying this shifting population further, Castell and Mapplebeck (1952) show that in pure culture at 2 to 3 C incubation, _Pseudomonas_ cultures grow 56 times faster than cultures of _Flavobacterium_, but in mixed cultures, the ratio of _Flavobacterium_ to _Pseudomonas_ is 1: >10,000. They conclude, quite logically, that _Pseudomonas_ probably inhibits the development of _Flavobacterium_ strains.

The differences in the types of psychrophilic organisms given in this paper and those reported in the literature can be partly explained by the individual techniques employed. Some investigators have used special media and methods (e.g., Wagenaar and Jezeski, 1952) to find a particular organism suspected as being the causative agent of spoilage. This is particularly true in the papers dealing with _Pseudomonas fragi_ and _Pseudomonas putrifaciens_. Furthermore, the incubating temperature is extremely important in determining the types of organisms which will predominate in stored milk. The temperature of storage at 4.5 C was selected because it approximates the temperature recommended by the American Public Health Association (1953) and, it corresponds to the temperature at which milk should be stored. Deviation, however, has been made from the incubation time recommended by the American Public Health Association (1953) which suggests five days. In using a 14 day incubation period, only slightly higher counts were obtained, but the colonies were larger making the plates easier to count. Other incubation periods and temperatures which have been used are: 10.5 C for 13 days, 4.5 C for seven days (Erdman and Thornton, 1951a); 4-7 C for 12 days (Rogick and Burgwald, 1952);

5 C for 10 days (Watrous, Doan and Josephson, 1952); 6.7 C for 10 days (Dahlberg, Adams and Held, 1953); 7 C for 10 days (Olson, Willoughby, Thomas and Morris, 1953); 17 C for five days (Prouty, 1955); 4.4 C for 20 days (Boyd, 1953).

VI. CONCLUSIONS

A series of experiments has been conducted which indicate the following:

1. Only bacterial counts from plates incubated at 4.5 C gave a true picture of the psychrophilic population in milk.

2. When the psychrophilic population of raw milk multiplied, it imparted a characteristic to the raw milk which decreases the keeping quality of the pasteurized milk.

3. Psychrophiles were killed by pasteurization.

4. Pasteurized milk developed an off-flavor when the psychrophile population reached a particular level of growth.

5. Certain species tended to predominate in milk stored at 4.5 C for several days.

VII. SUMMARY

From the discusssion on the relationship between plate counts incubated at 35 and 4.5 C, counts from plates incubated at 35 C did not reflect the psychrophilic population of the sample.

The initial count from the 35 C incubated plates is higher than the psychrophilic population. With cold storage, the counts from plates incubated at 35 C may decrease or increase slightly. The psychrophilic population increases rapidly with storage , and after four days of cold storage, the psychrophilic population is greater than indicated by counts from plates incubated at 35 C. Some commercially pasteurized milk samples, which would be judged good or satisfactory by the count obtained from plates incubated at 35 C, would not be considered satisfactory on the basis of the psychrophilic population. An insignificant number of the organisms which are predominantly found on plates incubated at 4.5 C grew better at 35 C; most grew practically as well at both temperatures. One-third of the cultures studied did poorly at 35 C as compared to the counts obtained from the 4.5 C incubated plates. When milk is stored at 4.5 C and no psychrophilic growth is evident from plates incubated at 4.5 C, the counts from 35 C incubated plates also will not show signs of bacterial growth during storage.

In short, the counts from plates incubated at 35 C can be used to evaluate careless handling of milk or improper refrigeration, but should not be the criterion for judging the general bacterial quality of milk.

An examination of counts obtained from the plates incubated at 20 C and those at 35 C indicate that the latter temperature is more nearly optimum for growth of bacteria found in milk. The plate counts obtained upon incubation at 20 C are influenced both by the organisms found on plates incubated at 35 C and 4.5 C, which explains the higher counts obtained at 20 C incubation. This fact invalidates the use of 20 C incubation for determining the psychrophilic population in milk even though 20 C more closely approximates the optimum temperature for the group of organisms. However, the fact that at 20 C incubation higher counts are obtainable than at 35 C validates the use of incubation temperatures below 35 C.

Raw milk generally contains a small population of psychrophilic microorganisms. This population increases to about 10,000,000 in four to eight days. If this milk is pasteurized, the pasteurized product will probably have an off-flavor. Pasteurized milk does not contain psychrophilic microorganisms unless post-pasteurization contamination occurs. Most commercial milk contains a low psychrophilic population. Pasteurized milk obtained from fresh raw milk will generally not have any off-flavors due to psychrophilic multiplication until the psychrophilic population approaches 10,000,000 bacteria per ml. If the raw milk, from which the pasteurized product is obtained, is stored for a period of time, the pasteurized product will deteriorate before the psychrophilic population reaches 10,000,000 per ml. A series of standards is proposed, based on the psychrophilic population of raw and pasteurized milk which will safeguard the dairy and the consumer from selling and receiving poor quality milk respectively. The maximum psychrophilic count recommended for raw milk is 100,000 bacteria per ml and 100 for pasteurized milk; the latter being probably too conservative a figure.

From the discussion on the relationship between psychrophilic and coliform plate counts as indices of post-pasteurization, data have been cited indicating that the psychrophilic population is probably a more sensitive measure. Rather than measure the extent of contamination, the psychrophilic population is suggested to be used as an evaluation of whether or not post-pasteurization contamination has occurred and the relative holding time of the milk.

The following characteristics are proposed to designate psychrophilic microorganisms in milk:

1. They generally have an optimum temperature above 4.5 C and below 35 C; some may grow at 35 C.

2. Their growth rate approximates a log increase every two days of storage at 4.5 C in milk.

3. They impart an off-flavor to milk when their population has developed to 1,000,000- 100,000,000 bacteria per ml.

4. They are feebly saccharolytic and predominantly gram negative, consisting of the genera <u>Alcaligenes</u>, <u>Achromobacter</u>, <u>Flavobacterium</u> and <u>Pseudomonas</u>.

5. Members of the genus *Micrococcus* are the most common gram positive group.

6. Yeasts and molds may be present in large numbers depending on the particular barn.

7. Psychrophiles are killed by pasteurization.

8. Certain species of the genera *Micrococcus*, *Pseudomonas*, *Alcaligenes* and *Achromobacter* tend to predominate.

A mechanical key has been prepared to aid in classifying psychrophilic bacteria associated with milk.

APPENDIX A

Mechanical key for the identification of microorganisms isolated from milk

1. Generic key to the gram nebative rods

2. Key to the genus *Micrococcus*

3. Key to the genus *Alcaligenes*

4. Key to the genus *Achromobacter*

5. Key to the genus *Flavobacterium*

6. Key to the genus *Pseudomonas*

Appendix A

1. Generic key to the gram negative rods*

1.	Motile	2
	Non-motile	7
2.	Polar flagella	Pseudomonas
	Peritrichate flagella	3
3.	Litmus milk alkaline	4
	Litmus milk not alkaline	6
4.	Yellow to orange pigment	5
	No yellow to orange pigment	Alcaligenes
5.	Acid from glucose	Flavobacterium
	No acid from glucose	Alcaligenes
6.	Yellow to orange pigment	Flavobacterium
	No yellow to orange pigment	Achromobacter
7.	Green fluorescent water soluble pigment	Pseudomonas
	No green fluorescent water soluble pigment	8
8.	Litmus milk alkaline	9
	Litmus milk not alkaline	11
9.	Yellow to orange pigment	10
	No yellow to orange pigment	Alcaligenes
10.	Nitrate reduced to nitrite	Flavobacterium
	Nitrate not reduced to nitrite	Alcaligenes
11.	Yellow to orange pigment	Flavobacterium
	No yellow to orange pigment	Achromobacter

* Coliform group not included

2. Key to the genus *Micrococcus*

1.	Pigment formed	2
	No pigment formed	20
2.	Gelatin liquefied	3
	Gelatin not liquefied	10
3.	Nitrate reduced to nitrite	4
	Nitrate not reduced to nitrite	9
4.	Litmus milk acid	5
	Litmus milk alkaline	8
5.	Litmus milk coagulated	6
	Litmus milk not coagulated	*Micrococcus conglomeratus*
6.	Pigment yellow	7
	Pigment red to orange	*Micrococcus pyogenes var. aureus*
7.	Litmus milk peptonized	*Micrococcus caseolyticus*
	Litmus milk not peptonized	*Micrococcus citreus*
8.	Pigment yellow	Group A
	Pigment red to orange	*Micrococcus roseus*
9.	Litmus reduced	*Micrococcus flavus*
	Litmus not reduced	*Micrococcus freudenreichii*
10.	Nitrate reduced to nitrite	11
	Nitrate not reduced to nitrite	18
11.	Pigment yellow	12
	Pigment red to orange	14
12.	Litmus milk coagulated	*Micrococcus varians*
	Litmus milk not coagulated	13
13.	Litmus milk acid	*Micrococcus aurantiacus*
	Litmus milk alkaline	Group B
14.	Litmus milk acid	15
	Litmus milk alkaline	*Micrococcus cinnabareus-rhodochrous*
	No reaction in litmus milk	*Micrococcus cinnabareus*
15.	Litmus milk coagulated	16
	Litmus milk not coagulated	17

16.	Acid from lactose	*Micrococcus cinnabareus*
	No acid from lactose	*Micrococcus rubens*
17.	Motile	*Micrococcus agilis*
	Non-motile	*Micrococcus cinnabareus*
18.	Pigment yellow	*Micrococcus luteus*
	Pigment red to orange	19
19.	Litmus milk acid	Group C
	Litmus milk alkaline	*Micrococcus rhodochrous*
	No reaction in litmus milk	Group D
20.	Gelatin liquefied	21
	Gelatin not liquefied	25
21.	Nitrate reduced to nitrite	22
	Nitrate not reduced to nitrite	24
22.	Litmus milk peptonized	*Micrococcus caseolyticus*
	Litmus milk not peptonized	23
23.	Litmus milk acid	*Micrococcus epidermidis*
	Litmus milk alkaline	Group E
	No reaction in litmus milk	Group F
24.	Litmus milk acid	*Micrococcus freudenreichii*
	Litmus milk alkaline	*Micrococcus liquefaciens*
	No reaction in litmus milk	Group G
25.	Nitrate reduced to nitrite	26
	Nitrate not reduced to nitrite	27
26.	Litmus milk acid	*Micrococcus aurantiacus*
	Litmus milk alkaline	Group H
	No reaction to litmus milk	Group I
27.	Litmus milk acid	28
	Litmus milk alkaline	*Micrococcus ureae*
	No reaction to litmus milk	Group J
28.	Litmus milk coagulated	Group K
	Litmus milk not coagulated	*Micrococcus candidus*

3. Key to the genus *Alcaligenes*

1.	Gelatin liquefied		2
	Gelatin not liquefied		4
2.	Motile		3
	Non-motile	*Alcaligenes marshallii*	
3.	Nitrate reduced to nitrite	*Alcaligenes recti*	
	Nitrate not reduced to nitrite	*Alcaligenes bookeri*	
4.	Motile		5
	Non-motile	*Alcaligenes metalcaligenes*	
5.	Ropiness produced in milk	*Alcaligenes viscosus*	
	Ropiness not produced in milk		6
6.	Growth better at 4.5 C than 35 C	*Alcaligenes viscosus var. dissimilis*	
	Growth better at 35 C than 4.5 C	*Alcaligenes faecalis*	

4. Key to the genus *Achromobacter*

1.	Motile	2
	Non-motile	7
2.	Gelatin liquefied	3
	Gelatin not liquefied	6
3.	Nitrate reduced to nitrite	4
	Nitrate not reduced to nitrite	5
4.	Some reaction (generally acid, reduction and peptonization) in litmus milk	*Achromobacter delicatulum*
	No reaction in litmus milk	*Achromobacter iophagum*
5.	Litmus milk acid	*Achromobacter superficiale*
	No reaction in litmus milk	*Achromobacter liquefaciens*
6.	Nitrate reduced to nitrite	*Achromobacter cycloclastes*
	Nitrate not reduced to nitrite	*Achromobacter superficiale*
7.	Nitrate reduced to nitrite	8
	Nitrate not reduced to nitrite	9
8.	Gelatin liquefied	Group A
	Gelatin not liquefied	*Achromobacter delmarvae*
9.	Gelatin liquefied	*Achromobacter butyri*
	Gelatin not liquefied	*Achromobacter eurydice*

5. Key to the genus *Flavobacterium*

1.	Motile	2
	Non-motile	9
2.	Gelatin liquefied	3
	Gelatin not liquefied	8
3.	Nitrate reduced to nitrite	4
	Nitrate not reduced to nitrite	6
4.	Indole formed	*Flavobacterium suaveolens*
	Indole not formed	5
5.	Litmus milk alkaline	*Flavobacterium rhenanus*
	No reaction in litmus milk	*Flavobacterium diffusum* or *Flavobacterium rigense*
6.	Litmus milk alkaline	7
	No reaction in litmus milk	*Flavobacterium devorans*
7.	Litmus milk peptonized	*Flavobacterium harrisonii*
	Litmus milk not peptonized	*Flavobacterium marinum*
8.	Litmus milk acid	*Flavobacterium lactis*
	Litmus milk alkaline	Group A
	No reaction in litmus milk	*Flavobacterium invisible*
9.	Gelatin liquefied	10
	Gelatin not liquefied	23
10.	Nitrate reduced to nitrite	11
	Nitrate not reduced to nitrite	20
11.	Litmus milk acid	12
	Litmus milk alkaline	15
	No reaction in litmus milk	18
12.	Litmus reduced	*Flavobacterium dormitator*
	Litmus not reduced	13
13.	Litmus milk peptonized	*Flavobacterium esteroaromaticum*
	Litmus milk not peptonized	14
14.	Acid from lactose	*Flavobacterium ferrugineum*
	No acid from lactose	*Flavobacterium balustinum*

15.	Litmus milk peptonized	16
	Litmus milk not peptonized	Group B (*Flavobacterium lutescens* or *Flavobacterium ferrugineum*)
16.	Acid from glucose	*Flavobacterium ferrugineum*
	No acid from glucose	17
17.	Nitrate reduced to nitrite	*Flavobacterium fucatum*
	Nitrate not reduced to nitrite	*Flavobacterium esteroaromaticum*
18.	Acid from glucose	19
	No acid from glucose	*Flavobacterium esteroaromaticum*
19.	Yellow pigment	*Flavobacterium flavotenue*
	Orange pigment	*Flavobacterium ferrugineum*
20.	Litmus milk peptonized	21
	Litmus milk not peptonized	22
21.	Acid from glucose	*Flavobacterium ferrugineum*
	No acid from glucose	*Flavobacterium esteroaromaticum*
22.	Litmus reduced	Group C (*Flavobacterium arborescens* or *Flavobacterium ferrugineum*)
	Litmus not reduced	*Flavobacterium aquatile* or *Flavobacterium ferrugineum*
23.	Nitrate reduced to nitrite	24
	Nitrate not reduced to nitrite	25
24.	Acid and gas from glucose	*Flavobacterium proteus* or *Flavobacterium brevi*
	Only acid from glucose	Group D (*Flavobacterium brevi* or *Flavobacterium flavotenue*)
25.	Litmus milk acid	Group E-1 (*Flavobacterium brevi* or *Flavobacterium solare*)
	Litmus milk alkaline	Group E-2 (*Flavobacterium brevi* or *Flavobacterium solare*)
	No reaction in litmus milk	Group E-3 (*Flavobacterium brevi* or *Flavobacterium solare*)

6. Key to the genus <u>Pseudomonas</u> *

1.	Gelatin liquefied		2
	Gelatin not liquefied		16
	No growth on gelatin	*Pseudomonas erythra*	
2.	Motile		3
	Non-motile		15
3.	Grows better at 35 C than at 20C		4
	Grows better at 20 C than at 35 C		5
4.	Litmus milk acid	*Pseudomonas caviae*	
	Litmus milk alkaline	*Pseudomonas effusa*	
5.	Litmus milk acid		6
	Litmus milk alkaline		8
	No reaction in litmus milk	*Pseudomonas syncyanea*	
6.	Nitrate reduced to nitrite	*Pseudomonas perolens*	
	Nitrate not reduced to nitrite		7
7.	Indole formed	*Pseudomonas fairmountensis*	
	Indole not formed	*Pseudomonas fragi*	
8.	Indole formed		9
	Indole not formed		10
9.	Nitrate reduced to nitrite	*Pseudomonas myxogenes*	
	Nitrate not reduced to nitrite	*Pseudomonas schuylkilliensis*	
10.	Distinct yellow to orange soluble pigment produced in cream	*Pseudomonas synxantha*	
	No distinct yellow to orange pigment in cream		11
11.	Nitrate reduced to nitrite		12
	Nitrate not reduced to nitrite		14

* The terminology and descriptions of the genus Pseudomonas are based largely on the recommendation of Haynes (1951a, 1951b). According to Haynes, this is " essentially as it will appear in the next edition of <u>Bergey's Manual</u>", i.e., the seventh edition.

12.	Litmus milk coagulated	*Pseudomonas chlororaphis*
	Litmus milk not coagulated	13
13.	Litmus reduced	*Pseudomonas mephitica*, *Pseudomonas putrifaciens*, *Pseudomonas cohaerens*
	Litmus not reduced	*Pseudomonas fluorescens*
14.	Litmus milk peptonized	*Pseudomonas pavonacea*
	Litmus milk not peptonized	*Pseudomonas geniculata*
15.	Nitrate reduced to nitrite	*Pseudomonas fluorescens* (non-motile var.)
	Nitrate not reduced to nitrite	*Pseudomonas iodina*, *Pseudomonas smaragdina*
16.	Motile	17
	Non-motile	*Pseudomonas immobilis*
17.	Grows better at 35 C than 20 C	18
	Grows better at 20 C than 35 C	31
18.	Nitrate reduced to nitrite	19
	Nitrate reduced to nitrogen	*Pseudomonas stutzeri*
	Nitrate not reduced	29
19.	Acid from glucose	20
	No acid from glucose	27
20.	Starch hydrolyzed	21
	Starch not hydrolyzed	24
21.	Indole formed	22
	Indole not formed	23
22.	Litmus milk alkaline	*Pseudomonas striata*
	Litmus milk not alkaline	*Pseudomonas rathonis*
23.	Litmus milk coagulated	*Pseudomonas rathonis*, *Pseudomonas tralucida*
	Litmus milk not coagulated	*Pseudomonas putida*
24.	Indole formed	25
	Indole not formed	26
25.	Litmus milk alkaline	*Pseudomonas striata*
	Litmus milk not alkaline	*Pseudomonas desmolytica*

26.	Litmus milk acid	*Pseudomonas desmolytica*	
	Litmus milk not acid	*Pseudomonas putida*, *Pseudomonas desmolytica*	
27.	Starch hydrolyzed		28
	Starch not hydrolyzed	*Pseudomonas striata*, *Pseudomonas dacunhae*	
28.	Litmus milk alkaline	*Pseudomonas striata*	
	Litmus milk not alkaline	*Pseudomonas oleovorans*, *Pseudomonas lasia*	
29.	Litmus milk acid	*Pseudomonas ambigua*	
	Litmus milk alkaline	*Pseudomonas ovalis*	
	No reaction in litmus milk		30
30.	Acid in glucose	*Pseudomonas arvilla*, *Pseudomonas salopia*	
	No acid in glucose	*Pseudomonas cruciviae*	
31.	Nitrate reduced to nitrite		32
	Nitrate reduced to nitrogen	*Pseudomonas mephitica*	
	Nitrate not reduced		33
32.	Litmus milk acid	*Pseudomonas incognita*	
	Litmus milk alkaline	*Pseudomonas mira*	
33.	Litmus milk acid	*Pseudomonas rugosa*	
	Litmus milk alkaline	*Pseudomonas mildenbergii*, *Pseudomonas convexa*	

APPENDIX B

Description of groups of microorganisms not found in *Bergey's Manual*

1. *Micrococcus*
2. *Achromobacter*
3. *Flavobacterium*

Appendix B

1. Micrococcus

Group A. Gram positive cocci occurring in pairs and clusters. Non-motile. Appears to be identical with Micrococcus roseus except it produces a yellow pigment. Slow liquefaction of gelatin. Colonies on agar are circular, entire, smooth, convex and yellow. In broth, slight turbidity and yellow sediment develops. Litmus milk turns slightly alkaline and coagulates. No peptonization or reduction was detected. Acid produced from glucose but not in lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is used as the sole nitrogen source. Nitrate is reduced to nitrite. Grows at 4.5 C, 20 C and 35 C; best at 20 C.

Group B. Gram positive cocci occurring singly and in pairs. Non-motile. Morphologically similar to Micrococcus varians. Yellow pigment produced. Gelatin not liquefied. Colonies on agar are circular, entire, smooth, convex and yellow. In broth moderate turbidity and yellow sediment develops. Litmus milk turns alkaline; the litmus is reduced. No coagulation or peptonization was detected. Acid produced from glucose and lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is used as the sole nitrogen source. Nitrate is reduced to nitrite. Grows at 4.5 C and 20 C; poorly at 35 C.

Group C. Gram positive cocci occurring singly and in clusters. Non-motile. Similar to Micrococcus candidus except it produces an orange pigment. Gelatin not liquefied. Colonies on agar are circular, entire, smooth, convex and orange. In broth slight pellicle, heavy turbidity and slight yellow sediment develops. Litmus milk turns acid; no reduction of litmus or coagulation and peptonization of the milk detected. Acid produced from glucose and lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is not used as the sole nitrogen source. Nitrate is not reduced to nitrite. Grows at 4.5 C, 20 C and 35 C; best at 20 C.

Group D. Gram positive cocci occurring in clusters. Non-motile. Appears to be similar to group 3 reported by Ely (1954) except it produces a pink pigment. Gelatin not liquefied. Colonies on agar are circular, entire, smooth, convex and pink. In broth moderate turbidity and pink sediment develops. Litmus milk is unchanged. No acid produced in glucose or lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is used as the sole source of nitrogen. Nitrate is not reduced to nitrite. Grows at 4.5 C and 20 C; poorly at 35 C.

Group E. Gram positive cocci occurring in pairs and clusters. Non-motile. Appears to be similar to group 1 reported by Ely (1954) except no pigment was observed. Gelatin liquefied. Colonies on agar are punctiform, entire, smooth, raised and translucent. In broth, moderate turbidity and granular sediment develops. Litmus milk turns alkaline. No coagulation, peptonization or reduction was detected. Acid is produced from glucose but not from lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is used as the sole nitrogen source. Nitrate is reduced to nitrite. Grows at 4.5 C and 20 C; not at 35 C.

Group F. Gram positive cocci occurring singly and in pairs. Non-motile. Gelatin liquefied. Colonies on agar are punctiform, entire, smooth, convex and opaque. In broth, moderate turbidity and granular sediment develops. Litmus milk turns slightly acid without coagulation, peptonization or reduction. Acid produced from glucose but not from lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is not used as the sole nitrogen source. Nitrate is reduced to nitrite. Grows at 4.5 C and 20 C; not at 35 C.

Group G. Gram variable cocci occurring in clusters. Non-motile. Morphologically similar to Micrococcus ureae. Gelatin liquefied. Colonies on agar are circular, entire, smooth, raised and translucent. In broth, moderate turbidity and viscid sediment develops. Litmus milk remains unchanged; no coagulation, peptonization or reduction was detected. No acid produced from glucose or lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is used as the sole nitrogen source. Nitrate is not reduced to nitrite. Grows at 20 C and 35 C; not at 4.5 C.

Group H. Similar to group G except that gelatin is not liquefied, nitrate is reduced to nitrite and growth occurs at 4.5 C and 20 C; not at 35 C.

Group I. Similar to group H except that litmus milk turns alkaline and acid is produced from glucose.

Group J. Gram variable cocci occurring singly, in pairs and in clusters. Non-motile. Appears to be identical to group 3 by Ely (1954). Gelatin not liquefied. Colonies on agar are circular, entire, smooth, convex, glistening and white. In broth, moderate turbidity and viscid sediment develops. Litmus milk remains unchanged; no coagulation, peptonization or reduction was detected. No acid produced from glucose or lactose. Starch is not hydrolyzed. Indole is not formed. Ammonium acid phosphate is used as the sole nitrogen source. Nitrate is not reduced to nitrite. Grows at 20 C and 35 C; not at 4.5 C.

Group K. Gram positive cocci occurring in pairs and in clusters. Non-motile. Most closely resembles Micrococcus candidus. Gelatin not liquefied. Colonies on agar are punctiform, entire, smooth, convex and white. In broth moderate turbidity and pellicle develops.

Litmus milk turns acid; is coagulated and peptonized. Reduction of the litmus occasionally occurs. Acid is produced from glucose and lactose. Starch is not hydrolyzed. Indole is not produced. Ammonium acid phosphate is not used as the sole nitrogen source. Nitrate is not reduced to nitrite. Grows at 20 C and 35 C; not at 4.5 C.

2. Achromobacter

Group A. Gram negative, non-motile rods occurring singly. Gelatin not liquefied. Colonies on agar are punctiform, entire, smooth, glistening, translucent and gray. In broth, moderate turbidity, gray pellicle and viscid sediment develops. Litmus milk turns acid, coagulates and peptonizes. The litmus is reduced. Acid is produced from glucose but not from lactose. Starch is not hydrolyzed. Indole is not formed. Nitrate is reduced to nitrite. Grows at 4.5 C and 20 C; not at 35 C.

3. Flavobacterium

Group A. The cultures isolated appear to have characteristics identical to *Flavobacterium lactis* except that it causes litmus milk to turn alkaline.

Group B. This group has characteristics which fit both *Flavobacterium lutescens* and *Flavobacterium ferrugineum*.

Group C. This group has characteristics which fit both *Flavobacterium arborescens* and *Flavobacterium ferrugineum*.

Group D. This group has characteristics which fit both *Flavobacterium brevi* and *Flavobacterium flavotenue*.

Group E. This group has characteristics which fit both *Flavobacterium proteus* and *Flavobacterium flavotenue*.
Group E has been further subdivided on its action in litmus milk. Group E-1 turns litmus milk acid; Group E-2 turns litmus milk alkaline; Group E-3 does not change litmus milk.

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