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Enumeration of thermoduric bacteria in milk

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IN MILK.

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ENUMERATION OF THERMODURIC BACTERIA IN MILK

by

William Robb Thomas

A Dissertation Submitted to the
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INTRODUCTION

Recent changes in milk production and handling practices have necessitated reappraisal of certain bacteriological tests. With the growth of bacteria in raw milk greatly retarded by more efficient cooling procedures, tests applied directly to the raw milk are not always effective in detecting faulty production practices. As a consequence, tests for specific groups of bacteria as indices of insanitary production procedures are being viewed with heightened interest.

Among these tests for particular groups of bacteria, the laboratory pasteurized count of raw milk has been advocated as a means of evaluating certain production procedures on the farm. The time honored method for the enumeration of bacteria in milk has been the Standard Plate Count. The thermoduric count of milk is determined in the same manner as the Standard Plate Count except that the milk is subjected to laboratory pasteurization previous to plating.

Although generally conceded to be the most precise procedure for estimating the bacterial population of milk, the agar plate method is not without limitations. No one medium incubated at a given temperature for a given period of time can be expected to initiate or promote growth of all bacterial types present in milk. Furthermore, preliminary investigations have indicated that conditions satisfactory for growth of bacteria in their normal state are not equally satisfactory for growth

of organisms which have been subjected to heat treatment of sublethal intensity.

From the foregoing, it is evident that standard plating procedures may not reveal the true thermoduric flora of laboratory pasteurized milk. Therefore, further knowledge of some factors which might influence the enumeration of heat-treated bacteria seemed desirable. This study was undertaken to determine the effect of the plate incubation temperature, length of the incubation period, pH of the plating medium and type of bacteriological peptone used in the plating medium upon the enumeration of pasteurization-resistant bacteria in milk. The effect of these various factors upon the enumeration of specific types of thermoduric bacteria was considered as well as their effect upon the quantitative determination of these organisms.

REVIEW OF LITERATURE

The general subject of thermoduric bacteria in milk has been reviewed extensively by Hileman (42), Thomas et al. (90) and Foster et al. (35). Some of the applications and limitations of tests for thermoduric bacteria in milk have been reviewed by Johns (50). The following review will be restricted primarily to the enumeration of thermoduric bacteria in milk.

Sources and Significance of Thermoduric Bacteria in Milk

Hileman et al. (44) believed that micrococci, found in the cow's udder, contributed to the thermoduric flora of milk. However, Gibson and Abd-El-Malek (36) were unable to support this view. They failed to find any pasteurization resistant bacteria in milk aseptically drawn from the udder. In later confirmatory work (3,37) these workers concluded that the udder was not a primary source of thermoduric micrococci. Mallmann and Bryan (55) reported that thermoduric bacteria were present only in negligible numbers in aseptically drawn milk.

The presence of thermoduric bacteria in milk has been reported by many investigators (10,57,59,92,93) to be primarily a matter of improperly cleaned and sanitized dairy farm utensils. After reviewing the subject, Thomas et al. (90) stated that a high incidence of thermoduric bacteria in raw milk was an index of persistently poor production methods at the farm as opposed to an occasional omission of efficient

sterilization. Barnum (16) reported a marked decrease in the number of farm violations on the items of cleanliness and sanitizing of equipment in the Denver, Colorado area. He attributed this improvement to a combination of laboratory pasteurization tests and follow-up by the Health Department. He further reported that dairy producers in the Denver market were monetarily penalized for milk with thermoduric counts in excess of 10,000 per ml. Raw milk supplies showing two successive thermoduric counts in excess of 30,000 per ml. were excluded from the market for 5 days.

Egdell et al. (29) stated that, where effective cleansing and sterilization of dairy utensils have been carried out, the thermoduric plate count should not exceed 1,000 per ml. of milk. They concluded that a thermoduric count exceeding 10,000 per ml. was indicative of a build-up of thermoduric organisms on parts of the utensils or equipment. McKenzie et al. (57) suggested that thermoduric counts of raw milk exceeding 10,000 per ml. provided evidence of unsatisfactory production methods but Johns (50) decided that such a standard was unduly lenient.

The incidence of thermoduric bacteria in the raw milk supply has been shown to be influenced by the season of the year. Thomas et al. (90) observed that the thermoduric colony count of milk produced under poor to fair hygienic conditions was generally much higher in summer than in winter. Where milk was produced under extremely good hygienic conditions the thermoduric count was occasionally slightly higher in winter than in

summer, but the magnitude of the count was much lower in this case.

The influence of cooling on the thermoduric count of milk was studied extensively by Thomas et al. (94). Numbers of thermoduric bacteria increased slowly in milk held at a temperature of 3° to 5° C. The combined effects of milk cooling and utensil cleanliness upon the thermoduric count of raw milk supplies was investigated by Thomas et al. (92). They observed that milk inadequately cooled did not have higher thermoduric counts than properly cooled milk when utensil sterilization was good. When utensil sterilization was poor, milk cooled efficiently did not show lower thermoduric counts than milk cooled inadequately.

Clegg et al. (23) observed that samples of milk could be held up to 24 hours after production without significant change in the thermoduric count if the storage temperature did not exceed 20° C. They failed to detect any changes in the thermoduric flora of raw milk during periods of storage up to 24 hours at 18° C.

It is generally agreed that thermoduric bacteria do not adversely affect the keeping quality of pasteurized milk in normal trade channels (77,90,101). However, Smythe (83) reported the keeping quality of the pasteurized product to be considerably reduced when milk was held at 5° to 35° C. for 10 hours prior to pasteurization. He attributed the reduction in keeping quality to the fact that the thermoduric count of the

milk increased during storage.

Types and Distribution of Thermoduric Bacteria in Milk

According to Thomas et al. (90), the thermoduric organisms commonly found in milk are limited to a few species of five bacterial groups: microbacteria; micrococci; streptococci, aerobic spore-bearing rods and Gram-negative rods. This author pointed out, after reviewing the subject, that the types of thermoduric bacteria reported in milk depended a great deal upon the plating procedures used in their recovery from the pasteurized product. He stated that microbacteria generally dominated the types isolated from plates incubated at 30° C. Streptococci were predominant on plates incubated at 37° C. Micrococci were sub-dominant at both temperatures.

Abd-El-Malek (1) laboratory pasteurized bulked and certified milk samples and incubated the plates at 37° and 30° C. Microbacteria, although appearing in large numbers at 30° C., were uniformly absent on plates incubated at 37° C. Micrococci appeared in significant numbers in all samples when plates were incubated at 30° C. but were encountered only in two of twelve samples at 37° C. Gibson and Abd-El-Malek (36) observed that the predominant flora of bulked milk appearing on plates prepared after laboratory pasteurization and incubated at 30° C. for 5 to 6 days were streptococci, micrococci and corynebacteria (microbacteria). These authors suggested that the occurrence of large numbers of corynebacteria in

pasteurized milk probably had been overlooked as a result of incubating plates at 37° C. This view has been supported by other investigators (22,27,35,86,96).

Although failure of microbacteria to form colonies on solid media at 37° C. has been generally reported, some workers (58,61) have noted that certain strains will grow at this temperature. McKenzie and Morrison (58) laboratory pasteurized 698 samples of raw milk and plated them on Yeastrel Milk Agar at 37° C. A few of the pasteurization-resistant organisms were isolated and identified. They were found to consist of 16 cultures of corynebacteria (microbacteria), 25 cultures of micrococci and sarcina and 16 cultures of streptococci. In a later study, McKenzie et al. (59) determined the types of thermophilic bacteria present in farm milk cans. Laboratory pasteurized can rinses were plated and incubated at 30° C. The thermophilic types present in a total of 654 colonies, statistically picked, were: microbacteria, 73.1 percent; micrococci, 10.2 percent; streptococci, 2.7 percent and miscellaneous types (Gram-negative and filamentous rods), 13.9 percent.

Hucker (47) noted that laboratory pasteurized milk held at 20° to 30° C. for 4 hours previous to pasteurization contained relatively large numbers of Streptococcus thermophilus. The same milk held at 10° C. for any length of time previous to pasteurization tended to yield a mixed flora and rarely yielded S. thermophilus after pasteurization.

The thermoduric streptococci of milk were studied extensively by Abd-El-Malek and Gibson (2). Milk samples were examined for thermoduric flora after laboratory pasteurization at 63° C. for 30 minutes. Of 54 samples of pasteurized milk examined, 33 yielded streptococci. S. thermophilus was detected in 28 of the samples, Streptococcus bovis in 25, Streptococcus faecalis in 6 and Streptococcus kefir in 5. Pasteurized milk held at temperatures of 10° to 22° C. until becoming tainted yielded S. kefir and S. faecalis at the lower temperatures and S. thermophilus and S. bovis at the higher temperatures.

Of the non-sporulating bacteria present in milk, those belonging to the species Microbacterium lacticum have been reported to be the most heat resistant (76,98). Doetsch and Pelczar (27) determined the heat resistance of 18 cultures of microbacteria by heating broth suspensions of the organisms to 85° C. for 2.5 minutes. Of the 18 cultures, three, classified as M. lacticum, survived the heat treatment. The heat resistance of 18 strains of M. lacticum and three strains of Microbacterium liquefaciens was determined by Turbett et al. (97) by heating in both broth and milk media. A temperature of 68° C. for 30 minutes was suggested for differentiating between species of the genus Microbacterium. This time-temperature combination was found to be above the critical zone for M. liquefaciens and below that for M. lacticum.

Speck (86) isolated 49 strains of microbacteria from milk

handling equipment, commercially pasteurized milk, laboratory pasteurized milk, raw milk and Cheddar cheese and examined them for various characteristics including heat resistance. Five ml. of litmus milk were inoculated with 1 drop of a 4-day culture grown at 30° C. and immediately laboratory pasteurized. All cultures were found to survive 62.5° C. for 30 minutes. Microbacteria, isolated from a variety of dairy products by Nashif and Nelson (61), never constituted a large percentage of the countable bacteria in unpasteurized products. However, microbacteria commonly accounted for a majority of the countable colonies from pasteurized products. These authors were able to isolate only microbacteria identifiable as M. lacticum and called attention to the possible close relationship of the microbacteria and members of the Micrococcaceae family.

Abd-El-Malek and Gibson (3) made a detailed study of staphylococci and micrococci isolated from milk and milk utensils. They devised a classification system by which the various strains of this group could be arranged in a continuous series. At one extreme were placed the pathogenic staphylococci and at the other the thermoduric, saprophytic micrococci. The series was divided into three main groups, one of which was designated as the dairy micrococci. Members of this group were described as thermoduric sugar fermenters which occurred frequently on dairy equipment and in pasteurized milk. Two species, conforming to Micrococcus luteus Cohn emend Lehmann and Neumann and Micrococcus varians (Dyar) Migula, were in-

cluded in the dairy micrococci group.

In a study of the thermoduric flora of 100 samples of bulk cooled milk, Buchanan (22) found that micrococci ordinarily were the predominant thermoduric bacteria in milk having a thermoduric count of more than 10,000 per ml. Streptococci accounted for 20 to 30 percent of the cultures isolated from samples having a thermoduric count of more than 1,000 per ml. Microbacteria accounted for less than 12 percent of the thermoduric population in all samples. Other thermoduric bacteria reported by this author were spore-bearing rods, actinomycetes and "Arthrobacter types."

Foster et al. (35) stated that spore-bearing organisms were practically always present in raw milk but usually in low numbers. Other workers (1,22,36) have reported that spore-bearing rods constituted a small portion of the thermoduric flora of milk with a high thermoduric count. The low heat-resistance of the vegetative cell as compared with that of the spore for bacteria of the genus Bacillus has been noted (102).

Certain rather inert and ill-defined Gram-negative bacteria that eventually produce an alkaline reaction in litmus milk, a variety of anaerobic spore-formers and lactobacilli have been reported in negligible numbers among the thermoduric flora of milk (22,35,90). After examining the heat resistance of lactobacilli found in Cheddar cheese, Slatter and Halvorson (82) reported that the majority of these bacteria were killed by pasteurization at 143° F. for 30 minutes or 160° F. for 15

seconds. However, these workers concluded that, occasionally, lactobacilli might contribute significantly to the thermoduric flora of milk.

Some confusion has existed concerning the occurrence of thermoduric coliform organisms in milk. In a recent study, Glenn and Olson (38) isolated 67 coliform cultures from milk before or during processing and found that only three of the cultures survived laboratory pasteurization of 143° F. for 30 minutes. Only the most heat-resistant strains survived this time-temperature combination in commercial holder pasteurization. These authors stated that a count of less than 1 per ml. could be expected in the cooled milk.

Effect of Pasteurization Methods on the Bacterial Count of Milk

The particular method employed in pasteurization of milk has been shown to affect the bacterial count of the finished product. Parfitt (69) and Quin and Burgwald (75) observed that laboratory pasteurization tended to give lower counts than commercial pasteurization at either 162° F. for 15 seconds or 143° F. for 30 minutes. These reports have been confirmed by Varma et al. (99). Comparison of milk pasteurized in the laboratory at 145° C. for 30 minutes, commercially by the high-temperature short-time (HTST) method at 162° F. for 16 seconds and commercially by the holder method at 145° F. to 150° F. for 30 minutes showed that laboratory pasteurized milk had the lowest

count. Laboratory pasteurized milk had better keeping quality than milk pasteurized by the HTST method but not as good keeping quality as that pasteurized by the holder method.

Some reports (42,90) have revealed that milk pasteurized by the holder method usually had a lower bacterial count than milk pasteurized by the HTST method. After examining the bacterial flora surviving both methods of pasteurization, Hileman et al. (44) concluded that the higher survival of bacteria in milk pasteurized by the HTST method was due primarily to the ability of certain micrococci to survive this method in greater numbers.

Methods for Enumerating Thermoduric Bacteria in Milk

Although the thermoduric count of milk is commonly determined by an agar plate count of laboratory pasteurized milk (9), several simpler and less expensive methods have been suggested. These include the oval tube method of Myers and Pence (60), the bottle culture method of Heinemann and Rohr (40), the Astell roll tube method (11), the Bacto-strip technique (51) and microscopic examination of pasteurized milk with incorporation of special stains for differentiation of living and dead bacteria (43,55,56).

Although generally conceded to be less accurate than the agar plate method, the modified methods have been proposed as routine screening tests for detecting thermoduric bacteria in

milk. Egdell et al. (30), using an incubation temperature of 30° C. for 72 hours, reported that thermoduric colony counts of laboratory pasteurized milk determined by the roll tube or agar strip methods were lower than those obtained by the standard agar plate method. Fischer and Johns (33), after comparing various methods for detecting thermoduric bacteria in milk, found that the oval tube method yielded counts in much closer agreement with the plate count method than did microscopic methods.

Factors Influencing the Thermoduric Plate Count

Temperature of plate incubation

Incubation at 37° C. for 2 days was adopted in the early 19th century in most countries for the official grading of milk. This incubation temperature was used because it was believed that animal pathogens that might be present in milk would have an opportunity to develop at their optimum temperature (105). Although the incubation temperature of 37° C. for the Standard Plate Count was used universally for a period of over 20 years, it was realized that it did not give the maximum colony count and that many of the more common bacteria from milk developed slowly at this temperature. As early as 1908, Heinemann and Glenn (41) suggested that since pathogenic bacteria were always difficult, and in most cases impossible, to find in milk, an incubation temperature of 37° C. had no real advantage over

room temperature incubation. They reported that incubation at 20° C. was superior to 37° C. because both a higher count and a better differential count were obtained.

With the introduction of improved plating media and the demonstration that slight variations in incubator temperatures around 32° C. affected the counts of replicate plates much less than around 37° C. (70,71,87), the whole question of incubation temperatures was reopened for discussion and investigation.

After making comparative counts on replicate plates made from pasteurized milk and incubated at various temperatures ranging from 20° to 47° C. for 48 hours, Pederson et al. (71) concluded that the maximum number of colonies developed at an incubation temperature of 31.3° C. They found that the average count at 37.2° C. was only 45 percent of the average count at 31.3° C. and the average count at 35.7° C. was only 69.2 percent of the count at 31.3° C. In a more detailed investigation, Pederson and Yale (70) studied the effect of incubation temperatures of 21°, 25°, 30°, 32°, 35°, 37°, 45° and 55° C. for 48 hours on replicate plate counts of 50 pasteurized milk samples. They found that the average maximum plate count occurred at 32° C. and recommended the adoption of this temperature of incubation as the standard.

Wilson et al. (103) took exception to the recommendation of Pederson and Yale (70) and stated that an incubation temper-

ature of 32° C. was optimum only if the incubation period was limited to 2 days. They concluded that, in this sense, a fictitious picture of the real incubation requirements of milk organisms was given.

Realizing that an incubation temperature of 37° C. was not optimum for growth of many of the more common bacteria in milk, numerous investigators compared this temperature with lower ones. Most of the comparisons were made between plate incubation temperatures of 37° and 32° C. Bowers and Hucker (19) examined 77 samples of pasteurized milk and found, in a majority of cases, a larger number of colonies developed on standard Nutrient Agar plates incubated at 32° C. for 2 days than at the standard temperature of 37° C. These results were confirmed by Bradfield (20), Abele (4), Fay and Howard (32) and Hileman et al. (45). Kelly (53) pointed out that lowering the temperature of plate incubation from 37° to 32° C. resulted in a greater percentage increase in count with pasteurized milk than with raw milk.

Yale (104), after summarizing 42 reports by 56 laboratories, concluded that the use of the lower incubation temperature of 32° C. did not result merely in a constant percentage increase in count. He stated that incubation at 32° C. produced a greater spread between counts of good and poor quality milk than did incubation at 37° C. As a result of these studies, the eighth edition of Standard Methods for the Examination of Dairy Products (6), published in 1941, recognized

the optional use of either 32° or 37° C. for 48 hours as temperatures of incubation for the agar plate method.

Workers in England, focussing their attention upon the enumeration of pasteurization-resistant bacteria in milk, compared incubation temperatures below 32° C. with the standard 37° C. temperature. Gibson and Abd-El-Malek (36) have been given much of the credit for introducing a lower incubation temperature for the agar plate method in that country. They used an incubation temperature of 30° C. for 5 to 6 days and suggested that the occurrence of large numbers of thermoduric bacteria in pasteurized milk probably had been overlooked as a result of incubating plates at 37° C. After comparing colony counts of raw and pasteurized milk plated on Yeastrel Milk Agar, Thomas and Jenkins (95) concluded that incubation for 3 days at 30° C., as compared with 3 days at 37° C., gave an increase in count of approximately two times for raw and six times for pasteurized milk.

In a study of the thermoduric flora of pasteurized milk, Abd-El-Malek (1) reported that the ratio of colony counts on plates incubated at 37° C. for 2 to 3 days and 30° C. for 5 to 6 days varied between 1.2 and 45.7. Thomas (89) found that thermoduric colony counts on Yeastrel Milk Agar incubated for 4 days at 30° C. were, on the average, 12 times those on the same medium incubated for 2 days at 37° C. Using the same plating and incubation procedures, Thomas et al. (91) reported

that 80 percent of the milk samples tested gave a higher thermoduric plate count with 30° C. incubation. Many European workers (25,29,54) now recognize an incubation temperature of 30° C. for 3 to 4 days as being optimum for the agar plate method for enumeration of thermoduric bacteria in milk.

Some investigations have shown that incubation temperatures lower than 30° C. resulted in agar plate counts comparable to those obtained at higher temperatures. Rowlands and Provan (77), after studying the bacteriological aspects of heat-treated milk, noted that there was little difference between counts on plates incubated at 22° C. for 5 days and those incubated at 30° C. for 3 days. These findings were confirmed by Egdell and Bird (28).

Incubation temperatures found to be optimum for growth of bacteria on plates prepared from commercially pasteurized milk may not prove to be optimum for growth of bacteria from laboratory pasteurized milk. Commercially pasteurized milk may contain bacterial contaminants of a non-thermoduric nature in addition to truly pasteurization-resistant bacteria. However, investigations with commercially pasteurized milk cannot be excluded entirely when reviewing the subject of thermoduric bacteria. Watrous et al. (100) and Atherton et al. (12) obtained appreciably higher counts on commercially pasteurized milk when plates were incubated at 25° C. for 3 days than at 35° C. for 2 days. Nelson and Baker (67) used incubation temperatures ranging from 5° to 35° C. in studying the influence

of time and temperature of plate incubation upon bacterial counts of market milk. They found that incubation at 25° C. for 3 days commonly gave the maximum count while incubation of plates at 35° C. for 2 days frequently failed to detect samples which gave high counts upon incubation at 32° C. or below.

Comparative studies (18,88) of plate counts on raw and commercially pasteurized milk following incubation of plates at 37°, 35° and 32° C. for 48 hours showed that counts were somewhat higher at 35° C. and still slightly higher at 32° C. As a result of these studies, incubation of agar plates at 32° or 35° C. for 48 hours was recognized in 1948 as standard procedure in this country (7).

Length of the incubation period

It is recognized that the effect which time of incubation might have upon the agar plate count is dependent upon the incubation temperature employed. However, since there are indications that the incubation period is more critical for the enumeration of thermoduric bacteria than for other bacteria in milk, the significance of the length of the incubation period upon the plate count will be briefly reviewed.

Although Standard Methods (9) call for incubation of plates at either 32° or 35° C. for 48 hours, several investigators have suggested that longer incubation at these and other temperatures would be advantageous especially for the enumeration of thermoduric bacteria. Heinemann and Glenn (41) stated

as early as 1908 that, since milk is normally consumed before the results of bacterial examinations are available, bacteriological tests should have as their principal objects the improvement and control of the general milk supply. They concluded that accuracy is of greater importance than quick results and the loss of a day, in the interest of accuracy, is irrelevant.

Wilson et al. (103) found that, with raw milk, plates incubated at 37° C. for 3 days had only a slightly higher count than those incubated for 2 days. However, with pasteurized milk, an average increase of 22 percent was observed. Rowlands and Provan (77) reported that, with pasteurized milk, colony counts on plates incubated at 30° and 37° C. were increased by prolonging the period of incubation from 48 to 72 hours. Colony counts in excess of 30,000 per ml. were not influenced to the same extent as lower counts. At an incubation temperature of 22° C. a minimum incubation period of 5 days was found to be necessary to obtain a maximum colony count.

Nelson and Baker (67) observed that incubation periods shorter than 3 days at 25° C. and 4 days at 21° C. resulted in lower plate counts on certain samples of pasteurized milk. Also, the smaller colonies formed in the shorter incubation times were more difficult to count. In a study of the effect of time and temperature of incubation on the Standard Plate Count of milk, Babel et al. (14) found that, with raw milk, incubation for 3 days at 32° or 35° C. gave no increase in count over 2 days of

incubation. However, with pasteurized milk, the additional day of incubation resulted in an increased count. These workers concluded that the development of colonies on plates prepared from pasteurized milk was slower than on plates prepared from raw milk. The same incubation period of 48 hours is specified in Standard Methods (9) for both raw and pasteurized milk. Johns (50) stated that the International Dairy Federation has recommended an extra 24 hours of incubation for plates prepared from pasteurized milk. This is in recognition of the fact that many bacteria which survive pasteurization exhibit a prolonged lag phase and fail to form discernible colonies on agar plates in 48 hours.

The fact that bacteria undergo a prolonged lag phase following heat treatment has been demonstrated by several investigators. Included among these were Tobias et al. (96) who observed that heat treatment affected the recovery of a strain of Micrococcus sp. Slow recovery was found with this strain even when the initial level of survivors was greater than 100 per ml. This led them to believe that heat treatment rather than low survivor level induced the prolonged lag. Using the same strain of Micrococcus sp., Kaufmann et al. (52) heated cultures in sterile whole milk at 76° C. for 17 seconds and 82° C. for 5 seconds. They compared growth of unheated and heated cultures having the same cell population and concluded that heated cells exhibited a longer lag phase of development than did unheated cells. The lag period was not markedly reduced with unheated

or heated cells when the cell level was increased from 2,000 to 20,000 per ml.

The literature appears to be lacking information pertaining to specific types of thermoduric bacteria contributing to higher counts after extended plate incubation. Cuthbert et al. (25), using an incubation temperature of 30° C. reported that the number of colonies on plates from laboratory pasteurized milk showed a marked increase between the second and third day of incubation. They found no appreciable difference between the flora developing on agar plates after 2 days incubation at 30° C. and that developing subsequently.

Composition of plating media with special reference to bacteriological peptones

Sherman (80) in 1916 pointed to the advantages of a carbohydrate containing medium for the enumeration of bacteria in pasteurized dairy products. Supplee et al. (87) plated 100 samples of market milk on plain Nutrient Agar, Nutrient Agar containing 1 percent dextrose and Nutrient Agar containing 1 percent lactose. They concluded that, for developing maximum colony counts, the medium containing dextrose was superior to the others. Fay (31) plated 55 cultures of pasteurization-resistant bacteria on various types of media. He concluded that sugar was necessary for their growth on agar and growth would occur on Nutrient Agar only if the dilution was not greater than 1:100.

Ayres and Mudge (13) in 1920 reported that appreciably higher plate counts were obtained from pasteurized dairy products by the use of a plating medium containing skim milk powder. These results were confirmed later by Safford and Stark (78,79).

It is appreciated that bacteriological peptones are only a portion of the nutrient complex of a medium. However, this review will be restricted, essentially, to the role of bacteriological peptones in media used for the enumeration of bacteria in milk.

Prior to the publication of the seventh edition of Standard Methods (5) in 1939, Nutrient Agar containing beef extract and peptone as nutrient sources was recommended for use as a plating medium. Previously, little regard had been given to the particular type of peptone to be used. Bowers and Hucker (19) suggested the use of an improved plating medium containing 0.5 percent tryptone (a casein digest peptone), 0.1 percent glucose, 0.5 percent skim milk and agar. Comparing this medium with Nutrient Agar, plate counts were found to be 36 percent higher on 134 samples of raw milk and 350 percent higher with 77 samples of pasteurized milk. Similar results were obtained by Bradfield (20) and Curtis and Hileman (24) when plate counts on the two media were compared.

Yale (104) summarized 42 reports by 56 laboratories on 23,715 samples of dairy products plated on standard Nutrient

Agar and Tryptone-Glucose-Milk Agar. He noted that the modified medium gave a higher count in the majority of cases than Nutrient Agar. The average increase in count was greater with pasteurized milk than with raw milk. However, in some instances counts on Nutrient Agar were higher than counts on the Tryptone-Glucose-Milk Agar. Abele (4) suggested that such instances could be reduced by the inclusion of beef extract. Consequently, a plating medium containing tryptone, glucose, beef extract, skim milk and agar replaced Nutrient Agar as the official plating medium in 1939 (5).

Several investigators (34,45,62,63) demonstrated that the Tryptone-Glucose-Beef Extract-Milk agar (TGEM agar) was superior to Nutrient Agar for the enumeration of bacteria in milk, especially in the case of pasteurized milk. Nelson (64) compared the colony productivities of Nutrient Agar and TGEM agar. Unheated and heated pure cultures of Escherichia coli, Bacillus subtilis, Streptococcus liquefaciens, Streptococcus zymogenes, Streptococcus durans and Staphylococcus aureus were plated on both media. He observed that only in rare instances were differences in plate count of the unheated controls possibly attributable to differences in the media. However, the TGEM agar was definitely superior to Nutrient Agar, except for one strain of S. liquefaciens, for development of the heat-treated bacteria. Bacteria which had been subjected to heat at sublethal levels were said to be more demanding in their requirements for growth than were the unheated control organisms.

In a later study, Nelson (65) reported that variations in the tryptone content of the plating medium and the time of addition of the bacteriological peptone in the preparation of the medium influenced colony development by heat-treated bacteria.

Because of difficulties encountered with precipitation of the skim milk in the TGEM medium, studies were undertaken to find a suitable milk-free medium to replace TGEM agar. Pessin and Black (73) compared several substitute media for enumeration of bacteria in milk. They suggested that media without skim milk could be prepared which produced colony counts at least as great as the standard TGEM agar.

Pessin and Robertson (74) compared colony productivity of five media with that of TGEM medium on 337 samples of raw milk and 349 pasteurized milk samples. They reported that the greatest colony productivity, particularly for pasteurized milk, was afforded by a milk-free medium containing 0.35 percent yeast extract, 0.5 percent tryptone, 0.1 percent dextrose and 1.5 percent agar. As a result of these studies, Plate Count Agar containing tryptone (a pancreatic digest of casein), yeast extract and dextrose was officially recommended in 1953 (8) and currently is recognized (9) for the Standard Plate Count of milk.

The role of bacteriological peptones in bacterial growth has been investigated by many workers. Bowers and Hucker (19) stated that hydrolyzed casein served as an excellent source of nitrogen for bacterial growth, particularly for those organisms

associated with milk. They assumed that the efficiency of hydrolyzed casein depended upon the large amount of tryptophan in casein which became available when the casein was hydrolyzed either with trypsin or pepsin. Shrader (81), compared ten milk plating media prepared with various peptones. Peptones were found to vary in their cultural effects and media of different nitrogen composition were said to affect the cultural characteristics of various bacteria. He further stated that bacterial culture media must possess the lower nitrogen compounds for proper growth and that amino acids in proper concentration were generally the most important nitrogen compounds for culture media.

Black (17) reported that 50 different modifications of milk plating media had been used in comparing the better peptones generally available for bacteriological use. He concluded that the better peptones gave comparable results. Barkworth and Davis (15) compared various amounts of peptones in media for the plate count of milk. They reported that, due to the minute colony size, ease of counting could not be obtained in a medium containing less than 0.2 percent peptone.

According to Hook and Fabian (46), peptones are prepared by hydrolysis of proteins by enzymes, acids or alkalies. The composition of the peptone is influenced by many variables including the kind of protein hydrolyzed, type of hydrolysis and extent of hydrolysis. They stated that it was doubtful

that a standardized peptone would ever be produced by present methods. Pelczar and Brown (72), noted that different lots of the same brand of milk plating agar showed considerable irregularity in colony counts of the same samples of milk. They devised a reproducible synthetic medium in dry form which might serve as a reference standard against which different lots of peptone media could be evaluated. These authors concluded that such a reference medium was needed if the irregular results inherent in peptone media were to be avoided.

Reaction (pH) of the plating medium

- The literature failed to reveal any comprehensive studies relating to the influence of the pH of the plating medium upon the enumeration of thermoduric bacteria in milk. Cooledge, as cited by Fay (31, p. 352), believed the appearance of pin-point colonies formed by thermodurics was associated with the reaction of the plating medium. The same sample of milk plated on two media with reactions of pH 6.6 and 7.3, respectively, resulted in counts of 15,400 per ml. on the former and 317,000 per ml. on the latter medium. Wilson et al. (103) plated 22 raw milk samples and 23 pasteurized milk samples on Yeastrel Milk Agar adjusted to pH levels of 6.0, 6.8 and 7.6. They reported that for both raw and pasteurized milk, a medium reaction of pH 6.0 was too acid. For raw milk a medium of pH 6.8 was more favorable than one of pH 7.6, but for pasteurized milk a medium of pH 7.6 was more favorable than one adjusted to pH 6.8.

Nelson (66) studied the effect of media pH upon growth of pure cultures of Escherichia coli, Pseudomonas aeruginosa, Streptococcus durans, Micrococcus pyogenes var. aureus and Bacillus subtilis before and after heat treatment. In general, the unheated cultures grew on solid media over a much wider pH range than did the heated cultures. Heat treated S. durans gave a maximum count when the plating medium was adjusted to pH 6.5 to 8.0, while the unheated control gave a maximum count from pH 5.6 to 9.0.

The effect of pH of the medium in which thermoduric cultures of streptococci were heated was investigated by Iyengar et al. (49). The cells were inoculated into tryptone broth, adjusted to different pH levels, and then heated to 63° C. for various periods of time. A heating medium at pH 8.0 enhanced the survival of cultures after heating at 63° C. for 30 minutes over that obtained in a medium at pH 7.0.

The current edition of Standard Methods (9) recommends a plating medium of pH 6.9 to 7.1 for the enumeration of thermoduric bacteria in milk by the agar plate method.

EXPERIMENTAL METHODS

Collection of Samples

Samples of raw milk, representing can and bulk cooled manufacturing grade and bulk cooled grade A supplies, were collected over a period of one year. Samples were gathered and handled in an aseptic manner in accordance with Standard Methods (9). After collection, samples were stored at temperatures of 32° to 40° F. for a period not exceeding 24 hours prior to examination. The individual samples represented either composites of one or more milkings from individual farms or blends of milk from several farms. A total of 76 samples collected at five processing plants in Iowa were examined.

General Plating and Laboratory Pasteurization Procedures

Except for certain modifications necessitated by the nature of this study, the plating methods employed were those outlined in Standard Methods (9). In order to reduce the time required for preparing replicate plates, 1.0 ml. and 10.0 ml. pipettes graduated in tenths of a milliliter were used. The 1.0 ml. pipettes were used for delivery of 0.1 ml. quantities. The 10.0 ml. pipettes were used for delivery of 1.0 ml. quantities. A standard plate count at 32° C. was made on each milk sample prior to laboratory pasteurization.

The laboratory pasteurization technique employed was the

complete immersion method of Anderson and Meanwell (10) as modified by Clegg et al. (23). Ten ml. of milk were pipetted aseptically into a sterile 125 x 15 mm. test tube. The tube was closed with a sterile rubber stopper and was completely immersed in an electrically heated water bath. Pasteurization was accomplished by heating the sample to $62.5 \pm 0.1^\circ \text{C}$. and holding at this temperature for 30 minutes. The time required for the sample to reach pasteurization temperature was determined by the use of a pilot tube of milk with an inserted thermometer. This time never exceeded 5 minutes. Immediately following laboratory pasteurization, the tube of milk was cooled by complete immersion in ice water. Before pipetting milk from the tube for preparation of serial dilutions, the tube was inverted 12 times. The upper part of the tube was thoroughly flamed to guard against possible contamination from the cooling bath.

Determination of the Effect of Time and Temperature of Incubation upon the Thermoduric Plate Count

Twelve samples of bulk cooled and 20 samples of can cooled manufacturing grade milk were examined. Following laboratory pasteurization of the milk samples, a single series of dilutions was prepared for each sample. The required number of plates were prepared from each sample dilution. Plates were poured with Plate Count Agar (8) adjusted to $\text{pH } 7.0 \pm 0.1$.

Duplicate plates for each dilution were incubated as follows: 35° C. for 2, 3 and 4 days; 32° C. for 2, 3 and 4 days; 28° C. for 3, 4 and 5 days; 21° C. for 4, 5 and 7 days and 10° C. for 7, 14, 21 and 28 days. Plates prepared from seven of the bulk cooled samples were incubated at the additional temperature of 5° C. and colonies were counted at intervals up to 131 days. Temperature variations did not exceed $\pm 1.0^{\circ}$ C. during incubation of the plates.

At the end of each incubation period, colonies were counted with the aid of a Quebec colony counter and the location of each colony was marked with ink on the bottom of the plate. Various colored inks were used to designate colonies appearing after each respective incubation period. This system of color-coding facilitated subsequent isolation and identification of bacteria relevant to their ability to form colonies during incubation.

Extreme care was exercised in counting colonies. In some instances this possibly led to counting colonies which, due to their minute size, might have been overlooked in routine laboratory practice.

The thermoduric plate counts were computed by arithmetically averaging colony counts on duplicate plates and multiplying by the reciprocal of the dilution used (9). Except where noted, only plates having 30 to 300 colonies were selected for counting. Inclusion of plates showing less than 30 colonies

was necessary particularly for plates incubated at the lower temperatures.

Determination of the Effect of Various Bacteriological Peptones in the Plating Medium upon the Thermoduric Plate Count

Ten samples of bulk cooled grade A milk, ten samples of can cooled manufacturing grade milk and six samples of blended bulk and can cooled manufacturing grade milk were examined. Following laboratory pasteurization of the milk samples, a single series of dilutions was prepared for each sample. The required number of plates were prepared from each sample dilution. Duplicate plates for each dilution were poured with each of seven media prepared with various bacteriological peptones.

With the exception of the type of bacteriological peptone used in formulation, the composition of all media was identical to that of Plate Count Agar as outlined in Standard Methods (8). The bacteriological peptones used were as follows:

- I. Bacto-Tryptone, B-123 (pancreatic digest of casein)
- II. N-Z-Amine Type AS (pancreatic digest of casein)
- III. N-Z-Amine Type YT (enzymatic digest of casein)
- IV. Edamin (lactalbumin hydrolysate)
- V. Soy Peptone Powder (enzymatic digest of soybean meal)
- VI. N-Z-Case (tryptic digest of casein)

VII. Hy-Case SF (acid hydrolysate of casein)

Bacto-Tryptone, B-123, the recommended peptone for Plate Count Agar, was obtained from Difco Laboratories (26). The other bacteriological peptones were obtained from Sheffield Chemical.¹ Analyses of these, as supplied by Sheffield Chemical, are given in the Appendix. Plates were incubated at 32° C. The colonies were counted after 2, 3 and 4 days of incubation. Colonies were counted and marked in the same manner as outlined previously (p. 30).

Determination of the Effect of pH of the Plating
Medium upon the Thermoduric Plate Count

Six samples of bulk cooled grade A milk, six samples of can cooled manufacturing grade milk and six samples of blended bulk and can cooled manufacturing grade milk were examined. Following laboratory pasteurization of the milk samples, a single series of dilutions was prepared for each sample. The required number of plates were prepared for each sample dilution. Duplicate plates of each dilution were poured with each of five Plate Count Agars (8) adjusted to final pH levels of 6.5, 7.0, 7.5, 8.6 and 9.1. The six laboratory pasteurized samples of bulk cooled grade A milk were plated using Plate

¹Sheffield Chemical Company, Inc., Division of National Dairy Products Corporation, Norwich, N. Y.

Count Agars adjusted to additional pH levels of 5.6 and 8.0.

All media were prepared from the same lot of dehydrated base medium. Except for the variation in pH, the media were identical in composition and method of preparation. A Beckman potentiometer¹ was used for making all pH determinations. Ten percent solutions of NaOH or HCl were used in adjusting the pH of the media prior to autoclaving. The final pH of each medium was determined just prior to plating at a temperature of 45° C. in accordance with Standard Methods (9). Storage, melting and tempering did not cause the final pH of any medium to vary by more than 0.1 pH unit from the level to which it had been adjusted.

Plates were incubated at 32° C. and colonies counted after 2, 3 and 4 days of incubation. Colonies were counted and marked in the same manner as previously outlined (p. 30).

Characterization of the Thermoduric Flora

Milk samples showing a wide variation as well as some of those showing no variation in thermoduric count as a result of the various plating procedures were selected for bacterial flora study. Immediately after the colonies were counted, representative colonies from suitable plates were picked into tubes of sterile litmus milk. Colonies were picked according

¹Model 96 "Zeromatic".

to the random sampling method suggested by Harrison (39). This technique was used in an effort to obtain a truly random sampling of colonies from each plate. The inoculated tubes of litmus milk were incubated at 32° C. for 3 to 5 days. Following incubation, a loopful of the contents of each tube was streaked onto plates of TGEM agar (Plate Count Agar plus 0.25% non-fat milk solids). Surface colony characteristics were noted on the streaked plates after incubation for 72 hours at 32° C.

To assure the isolation of pure cultures, a single colony was picked from each streak plate into a tube containing 5 ml. of sterile litmus milk. These tubes were incubated at 32° C. and the reaction noted at intervals over a 14-day period. TGEM agar slants were inoculated from the litmus milk tubes and incubated at 32° C. for 24 hours. Immediately following incubation smears were prepared from the slants, stained, using Hucker's modification of the Gram stain (85), and examined microscopically.

A total of 1272 pure cultures of thermoduric bacteria were isolated. The isolates were preliminarily classified into genera on the basis of cell morphology, Gram stain, reaction in litmus milk and colony characteristics. An attempt was made to verify the genus classification and to classify some of the isolates into species by additional testing of representative isolates. Further cultural and biochemical testing procedures

used were essentially those outlined in the Manual of Microbiological Methods (85). Unless otherwise stated, tests were carried out at 32° C.

Representative streptococci were tested for production of ammonia from arginine according to the method of Niven et al. (68), production of acid from mannitol, growth at 10° C. in tryptone glucose yeast broth (9) and production of acid in litmus milk at 10° and 45° C.

Representative micrococci were tested for production of acid from glycerol and glucose, ability to utilize $\text{NH}_4\text{H}_2\text{PO}_4$ as the sole source of nitrogen (21) and catalase production on Plate Count Agar slants.

Representative microbacteria were tested for liquefaction of gelatin within 7 days at 28° C., catalase production on Plate Count Agar slants, growth in tryptone glucose yeast broth (9) at 37° C. and hydrolysis of starch within 7 days.

Representative cultures of bacteria preliminarily classified as belonging to the Arthrobacter genus were tested for growth on Plate Count Agar slants at 37° and 10° C., production of catalase on Plate Count Agar slants, fermentation of glucose, growth in Plate Count Agar deep shake cultures and gelatin liquefaction at 21° C. Motility was determined by the hanging drop method on 12 and 24 hour cultures grown in tryptone glucose yeast broth (9). Spore production by these bacteria was tested at intervals over a 30-day period on Plate Count

Agar and Tomato Juice Agar (26). Cells were stained using Conklin's modification of the Wirtz method (85).

Representative lactobacilli were tested for fermentation of lactose, maltose, fructose, sucrose and glycerol, catalase production on Plate Count Agar, liquefaction of gelatin and growth in Plate Count Agar deep shake cultures. -

No cultural or biochemical tests, other than reaction in litmus milk, Gram-stain, cell and colony characteristics, were conducted on the spore-bearing rods. Also, no further testing was carried out on a few isolates which could not be categorized to genus after preliminary testing.

Effect of the Various Plating Procedures upon the
Recovery of Pure Cultures of Thermoduric Bacteria
Before and After Laboratory Pasteurization

Four isolates, representative of the predominant genera of thermoduric bacteria found, were selected for this portion of the study. These cultures were classified as Microbacterium lacticum, Micrococcus varians, Streptococcus sp. and Arthrobacter sp. Stock cultures, from TGEM agar slants, were inoculated into sterile litmus milk and incubated for 24 hours at 32° C. One ml. of the litmus milk culture was added to 100 ml. of sterile reconstituted skim milk containing 10 percent non-fat milk solids. After the mixture was thoroughly shaken, 10 ml. were transferred to a sterile test tube for laboratory pasteurization.

A single series of dilutions was prepared for both the pasteurized and non-pasteurized cultures. The required number of plates were prepared from each culture dilution. Duplicate plates of each dilution were poured with each of the seven "peptone" media (p. 31) and each of the seven "pH" media (p. 32). These plates were incubated at 32° C. and colonies counted after 2, 3 and 4 days incubation. Replicate plates, prepared at the same time for both pasteurized and non-pasteurized cultures, were poured with standard Plate Count Agar. Duplicates of each dilution were incubated at 35° C. for 2, 3 and 4 days, 32° C. for 2, 3 and 4 days, 28° C. for 2, 3, 4 and 5 days, 21° C. for 3, 4, 5 and 7 days and 10° C. for 7, 14, 21 and 28 days.

A portion of the non-pasteurized diluted culture was refrigerated at 38° to 40° F. for 24 hours, shaken thoroughly and 10 ml. transferred to a sterile test tube for laboratory pasteurization. Again, both the pasteurized and non-pasteurized diluted cultures were plated and incubated as outlined above for non-refrigerated cultures.

RESULTS

The presentation, in concise mathematical form, of a summary of data developed from a study of this type offers many problems. The effects of different plating procedures on the mixed flora of milk are so diverse and the results obtained so extreme that it is difficult to reduce them to terms that will portray their true significance. To state, for example, that a certain time and temperature of incubation will result in a higher count 90 percent of the time is meaningless if nothing is said about the magnitude of the increases. On the other hand, to say that one method shows a higher average count than another means little unless data are presented to indicate whether the higher average count is a result of many small increases or a few increases of large magnitude. Finally, a discussion of the effects of various plating procedures upon counts is incomplete unless something can be said about the particular types of bacteria responsible for the variance in counts.

Because of the inadequacy of average figures alone to express the comparative effects of different plating methods, most of the data are presented on an individual sample basis. Plate count results and thermoduric flora distribution data are presented either in the same table or in adjacent tables

for easy reference.

Classification of Thermotolerant Flora

It should be emphasized that exhaustive tests were not conducted on each isolate. As a rule, isolates were grouped into probable genera on the basis of certain preliminary tests. Then, more exhaustive tests were performed on representatives of a given group to verify genus or species classification.

Isolates classified as belonging to the genus Arthro-
bacter showed the following characteristics:

Morphology: Rods, generally 0.6 to 0.8 by 0.8 to 1.2 microns, occasionally up to 5.0 microns in length. Cells occur characteristically in pairs. Ends of cells are well rounded. Gram-variable. Gram-negative cells generally predominate a 24-hour culture grown on TGEM agar at 32° C. Gram-negative cells tend to be coccoidal in shape while Gram-positive cells tend to be rod shaped; both forms appear frequently in the same culture. Gram-negative cells may appear as diplococci or short rods with lightly stained centers.

Agar Colonies: Circular, slightly raised, entire to slightly undulating margin, 0.8 to 1.5 mm. in diameter. Grey-white to pale cream, butyrous texture, glistening.

Agar slant (TGEM agar, 24 hours at 32° C.): Usually filiform, grey-white to pale cream, glistening.

Litmus milk: Alkaline after 4 to 7 days, slight reduction at base of tube, occasionally slight clearing after 4 days, slight flocculent surface growth.

Non-motile.

Non-sporulating.

Catalase-positive.

Growth on Plate Count Agar at 10° and 37° C.

Alkaline reaction with no gas in glucose broth.

Gelatin not liquefied within 2 weeks at 21° C.

Aerobic growth in Plate Count Agar deep shake tubes.

Isolates classified as belonging to the genus Microbacterium displayed the following characteristics:

Morphology: Small rods occurring characteristically in pairs or singly, 0.3 to 0.6 by 0.8 to 1.5 microns. In pairs, the cells tend to form a "V" arrangement. Gram-positive. Granulation or uneven staining is common.

Agar colonies: Circular, convex and entire. Generally less than 1.0 mm. in diameter. Grey-white to greenish-yellow on Plate Count Agar. With milk added to the Plate Count Agar, colonies tend to be grey-white. Colonies characteristically appear as "dew-drops", glistening and slightly transparent.

Litmus milk: Generally acid with occasional coagulation after 7 days.

Catalase-positive.

Gelatin not liquefied after 7 days at 28° C.

Growth at 37° C. variable.

Starch hydrolyzed.

The characteristics of the isolates placed in the genus Microbacterium agree closely with those listed in Bergey's Manual (21) for Microbacterium lacticum Orla-Jensen, 1919.

Isolates included in the Micrococcus genus displayed the following characteristics:

Morphology: Cocci, 0.6 to 1.2 microns in diameter, occurring in pairs, irregular clusters and occasionally in tetrads. Cells occasionally slightly elongated. Gram-positive to Gram-variable.

Agar colonies: Circular, convex and entire, occasional rough forms, 1.0 to 2.0 microns in diameter. Pale yellow to bright yellow, occasionally white.

Litmus milk: Generally acid, sometimes with weak coagulation or proteolysis after 7 days.

Acid produced from glucose. Fermentation of glycerol variable.

Utilization of $\text{NH}_4\text{H}_2\text{PO}_4$ as sole source of nitrogen variable.

Catalase positive.

No attempt was made to classify the micrococci into

species. However, on the basis of the few tests that were conducted, a majority of these isolates appeared to closely resemble Micrococcus varians according to the classification of Abd-El-Malek and Gibson (3).

Isolates placed in the genus Lactobacillus displayed the following characteristics:

Morphology: Rods, 0.4 to 0.8 by 0.6 to 3.0 microns, occurring in pairs and short chains. Gram-positive with granulation or uneven staining.

Agar colonies: Circular, convex and entire. Less than 1.0 mm. in diameter. White to golden-yellow.

Litmus milk: Acid followed by reduction in the lower portion of the tube. Coagulation after 4 to 7 days.

Acid from lactose, maltose and fructose. No acid from sucrose and glycerol.

Catalase-negative.

Microaerophilic growth in Plate Count Agar deep shake culture.

Gelatin not liquefied.

Isolates classified as belonging to the genus Streptococcus showed the following characteristics:

Morphology: Spherical to ovoid cells, 0.6 to 1.0 micron in diameter, occurring in pairs to long chains.

Gram-positive.

Agar colonies: Circular, convex and entire, occasionally with slight undulate margins and depressed centers.

Glistening grey to dull white. Generally less than 1.0 mm. in diameter.

Litmus milk: Usually acid followed by reduction and then acid coagulation within 3 days. Occasionally coagulated prior to reduction.

Acid produced in litmus milk at 45° C. Growth at 10° C. variable.

Ammonia occasionally produced from arginine.

Mannitol occasionally fermented.

Unidentified isolates showed the following characteristics:

Morphology: Rods, curved and filamentous, 0.6 to 1.0 by 1.0 to 4.0 microns, occurring singly, in pairs and short chains. Gram-positive. Granulation or uneven staining common.

Agar colonies: Circular, convex and entire, pale yellow to golden yellow, 0.5 to 2.0 mm. in diameter.

Litmus milk: Unchanged after 4 days. Slight reduction followed by soft coagulation and proteolysis after 7 days. Yellow sediment common.

Isolates included in the group of spore-bearing rods showed the following characteristics:

Morphology: Rods of variable size, usually 0.6 to 1.0 by 1.0 to 4.0 microns, occurring singly, in pairs and chains, occasionally showing swollen spores.

Gram-positive to Gram-variable.

Agar colonies: Large irregular or spreading, white to pale cream, occasionally transparent.

Litmus milk: Rapid reduction followed by proteolysis. Proteolysis sometimes preceded by coagulation.

Effect of Time and Temperature of Incubation
upon the Thermoduric Plate Count

The thermoduric plate counts of seven bulk cooled manufacturing grade milk samples obtained by incubation of plates at various temperatures are given in Table 1. The distribution of thermoduric bacteria in these samples is shown in Table 2.

For this series of samples, colonies were picked only from plates incubated at 32° C. for 2 days. Consequently, bacteria responsible for counts obtained at other temperatures of incubation could differ somewhat in proportionate numbers and types. However, certain trends or relationships between counts obtained at the various incubation temperatures and types of thermoduric bacteria contributing to the counts at 32° C. can be noted.

Referring to Table 1, sample K showed a definite increase in colony count as the plate incubation temperature was decreased from 35° to 28° C. This sample, as shown in Table 2, contained a thermoduric flora of micrococci and microbacteria. Sample P, also containing a thermoduric flora of micrococci

Table 1. Thermoduric plate counts of bulk cooled manufacturing grade milk samples obtained by incubation of plates at various times and temperatures

Sample no. (SPC) ^a	Incubation time, days	Incubation temperature, °C.			
		35	32	28	21
				Count/ml.	
K (2,400)	2	29,000	58,000		
	3			120,000	
	4			140,000	130,000
	5			140,000	160,000
	7				170,000
L (1,700)	2	29,000	43,000		
	3			65,000	
	4			68,000	37,000
	5			68,000	42,000
	7				50,000
M (1,400)	2	930	13,000		
	3			20,000	
	4			21,000	15,000
	5			21,000	18,000
	7				18,000
N (5,700)	2	84,000	98,000		
	3			84,000	
	4			91,000	84,000
	5			94,000	94,000
	7				100,000
O (1,700)	2	14,000	21,000		
	3			25,000	
	4			26,000	17,000
	5			28,000	20,000
	7				22,000

^aStandard Plate Count ($\times 10^3$) at 32° C. on raw milk samples.

Table 1. Continued

Sample no. (SPC) ^a	Incubation time, days	Incubation temperature, °C.			
		35	32	28	21
Count/ml.					
P (4,700)	2	71,000	88,000		
	3			160,000	
	4			200,000	130,000
	5			200,000	200,000
	7				250,000
T (1,100)	2	950	640		
	3			550	
	4			580	360
	5			620	390
	7				490

Table 2. Distribution of thermoduric bacteria in bulk cooled manufacturing grade milk samples (Table 1) determined by isolation from plates incubated at 32° C. for 2 days

Sample no.	No. of isolates	Distribution of isolates			
		Micro-bacteria	Micro-cocci	Strepto-cocci	Spore-bearing rods
K	11	4	7		
L	11		2	9	
M	11	11			
N	9			9	
O	12		1	11	
P	12	2	10		
T	10				10

and microbacteria, showed the same general trend in count as did sample K. However, the difference between counts obtained at incubation temperatures of 35° and 32° C. was not as great with sample P as with sample K. At the same time, sample P contained proportionately fewer microbacteria than did sample K.

The thermoduric colony count obtained for sample M at an incubation temperature of 32° C. was about 14 times the count obtained at 35° C. The flora of this sample was found to consist entirely of microbacteria as indicated in Table 2.

An incubation temperature of 28° C. produced the maximum thermoduric plate counts for samples L and O. As indicated in Table 2, each of these samples contained a thermoduric flora of streptococci and micrococci with streptococci being present in proportionately greater numbers in both samples. Colony counts for both samples decreased as the incubation temperature was increased from 28° to 35° C.

Sample N, containing a thermoduric flora of streptococci only, gave colony counts of relatively the same magnitude at all four incubation temperatures.

Of the seven samples examined in this series, only sample T gave a higher thermoduric plate count at 35° C. than at the lower incubation temperatures. The thermoduric flora of this sample was found to consist entirely of spore-bearing rods.

Thermoduric plate counts of five bulk cooled manufacturing grade milk samples obtained by incubation of plates at

various times and temperatures are presented in Table 3. The distribution of thermoduric bacteria in these samples is shown in Table 4. For this group of samples, colonies were picked only from plates incubated at 32° C. However, colonies appearing on the plates at 2 days were distinguished from those appearing at 3 to 4 days of incubation.

As shown in Table 3, an appreciable increase in colony count was obtained for samples AG, AI and AJ when the plate incubation period was extended beyond 2 days. Reference to Table 4 shows that each of these samples contained a mixed thermoduric flora of arthrobacters, micrococci and streptococci. In each case, arthrobacters predominated the 2-day colony count at 32° C. However, the increase in colony count after 3 and 4 days of incubation at 32° C. was attributable primarily to micrococci.

Sample AH, having relatively low thermoduric counts at all incubation temperatures, interestingly showed a higher colony count at 35° and 28° C. incubation than at 32° C. This sample displayed a thermoduric flora of microbacteria, micrococci and spore-bearing rods.

It will be noted in Table 3 that sample AK showed no appreciable difference in thermoduric colony counts at the various incubation temperatures. The thermoduric flora of this sample was found to consist entirely of micrococci.

The thermoduric plate counts of ten samples of can cooled manufacturing grade milk obtained by incubation of plates at

Table 3. Thermoduric plate counts of bulk cooled manufacturing grade milk samples obtained by incubation of plates at various times and temperatures

Sample no. (SPC) ^a	Incubation time, days	Incubation temperature, °C.			
		35	32	28	21
		Count/ml.			
AG (670)	2	17,000	13,000		
	3	26,000	23,000	23,000	
	4	27,000	24,000	25,000	23,000
	5			25,000	23,000
	7				24,000
AH (9)	2	360	140		
	3	440	150	320	
	4	480	250	370	260
	5			400	270
	7				330
AI (400)	2	11,000	9,900		
	3	15,000	16,000	17,000	
	4	17,000	16,000	18,000	11,000
	5			18,000	12,000
	7				12,000
AJ (320)	2	8,700	10,000		
	3	15,000	19,000	20,000	
	4	17,000	20,000	21,000	18,000
	5			21,000	19,000
	7				20,000
AK (330)	2	26,000	27,000		
	3	29,000	30,000	37,000	
	4	29,000	30,000	37,000	26,000
	5			38,000	30,000
	7				33,000

^aStandard Plate Count ($\times 10^3$) at 32° C. on raw milk samples.

Table 4. Distribution of thermoduric bacteria in bulk cooled-manufacturing grade milk samples (Table 3) determined by isolation from plates incubated at 32° C.

Sample no.	Incubation time, ^a days	No. of isolates	Distribution of isolates				
			Arthro-bacters	Micro-bacteria	Micro-cocci	Strepto-cocci	Spore-bearing rods
AG	2	13	9		3	1	
	3-4	5	1		4		
AH	2	12		5	2		5
	3-4	5		3	2		
AI	2	10	7		1	1	1
	3-4	5			5		
AJ	2	11	9		1	1	
	3-4	5			5		
AK	2	12			12		
	3-4	5			5		

^aTime at which colonies had developed on plates.

various times and temperatures are summarized in Table 5. The distribution of thermoduric bacteria in these samples is shown in Table 6. By comparing the two tables it can be seen that differences among colony counts at the various incubation temperatures for any one sample was generally attributable to the heterogeneous thermoduric flora of the particular sample. At the same time, increases in colony counts obtained by extending the plate incubation period were sometimes the result of failure of certain thermoduric types to produce colonies at

Table 5. Thermoduric plate counts of can cooled manufacturing grade milk samples obtained by incubation of plates at various times and temperatures

Sample no. (SPC) ^a	Incubation time, days	Incubation temperature, °C.			
		35	32	28	21
Count/ml.					
DA (5,800)	2	1,200	1,700		
	3	1,500	1,900	1,900	
	4	1,500	2,000	2,000	1,700
	5			2,000	1,900
	7				2,000
DB (1,600)	2	49,000	56,000		
	3	63,000	62,000	84,000	
	4	64,000	63,000	84,000	72,000
	5			84,000	82,000
	7				84,000
DC (3,800)	2	23,000	29,000		
	3	26,000	36,000	37,000	
	4	28,000	36,000	43,000	26,000
	5			43,000	31,000
	7				35,000
DD (380)	2	6,300	9,000		
	3	8,500	9,700	11,000	
	4	9,100	9,800	11,000	8,600
	5			11,000	9,200
	7				9,500
DE (1,600)	2	11,000	12,000		
	3	12,000	12,000	12,000	
	4	12,000	12,000	12,000	13,000
	5			12,000	13,000
	7				13,000

^aStandard Plate Count ($\times 10^3$) at 32° C. on raw milk sample.

Table 5. Continued

Sample no. (SPC) ^a	Incubation time, days	Incubation temperature, °C.			
		35	32	28	21
Count/ml.					
DF (48,000)	2	5,200	7,400		
	3	6,900	11,000	15,000	
	4	7,100	11,000	16,000	19,000
	5			16,000	24,000
	7				25,000
DG (1,600)	2	200	570		
	3	400	610	550	
	4	430	630	570	470
	5			570	510
	7				540
DH (180)	2	15,000	19,000		
	3	17,000	22,000	23,000	
	4	18,000	22,000	24,000	19,000
	5			24,000	23,000
	7				24,000
DI (390)	2	13,000	18,000		
	3	17,000	19,000	21,000	
	4	17,000	19,000	21,000	17,000
	5			21,000	19,000
	7				20,000
DJ (16,000)	2	550,000	1,000,000		
	3	780,000	1,200,000	1,200,000	
	4	870,000	1,200,000	1,200,000	810,000
	5			1,200,000	1,100,000
	7				1,200,000

Table 6. Distribution of thermoduric bacteria in can cooled manufacturing grade milk samples (Table 5) determined by isolation from plates incubated at various times and temperatures

Sample no.	Incubation temp., °C.	Incubation time, ^a days	No. of isolates	Distribution of isolates				
				Arthro-bacters	Lacto-ba-cilli	Micro-bac-teria	Micro-cocci	Others
DA	32	2	10			3	3	4 ^b
		3	3			3		
DB	35	2	7			1	6	5 ^c
		3-4	4			2	2	
	32	2	8			3	5	
	28	3-4	7			1	1	
DC	35	2	6	2		1	3	1 ^d
		3-4	7		7			
	32	2	8	2			6	
		3	7	1		5	1	
	28	3	5			1	3	
		4	4			4		
DD	35	2	4			1	3	
		3-4	6			2	4	
	32	2	10			1	9	
	28	3-4	3			3		
DE	35	2	9	9				
		32	2	9	9			
		28	3	9	9			

^aTime at which colonies had developed on plates.

^bSpore-bearing rods.

^cUnidentified.

^dStreptococci.

Table 6. Continued

Sample no.	Incubation temp., °C.	Incubation time, ^a days	No. of isolates	Distribution of Isolates				
				Arthro-bacters	Lacto-bacilli	Micro-bacteria	Micro-cocci	Others
DF	35	2	6	3		2	1	
		3-4	6			6		
	32	2	8			5	3	
		3-4	4			4		
	28	3	6			6		
		4	4			3		1 ^d
21	4	7		6	1			
	5	4		1	3			
DG	32	2	6		2	4		
		3-4	4		4			
DH	32	2	8			8		
		3	5		5			
DI	35	2	6			6		
		3	5		5			
	32	2	9		2	7		
		3	1			1		
28	3	6		3	3			
DJ	35	2	6			6		
		3	5			5		
	32	2	13			13		
3		2						

the shorter incubation times.

Sample DB gave a thermoduric colony count of 49,000 per ml. at 35° C. for 2 days compared with a count of 84,000 per ml. at 28° C. for 3 days. Table 6 reveals that only one-seventh of the colonies developing within 2 days at 35° C. were microbacteria while half of those developing within 3 days at

28° C. were microbacteria. The thermoduric micrococci in this sample apparently produced colonies equally well at both incubation temperatures. The increase in colony count upon extended incubation at 35° C. was attributed to both microbacteria and micrococci. However, the increase in count after extended plate incubation at 32° C. was the result of delayed growth of unidentified thermoduric bacteria in addition to microbacteria and micrococci.

Sample DC was the only sample in this study from which thermoduric lactobacilli were isolated. Table 5 shows that the thermoduric colony count for this sample increased upon extended incubation at each incubation temperature. Although this sample contained quite a heterogeneous thermoduric flora (Table 6), the increase in colony count upon extended plate incubation at 35°, 32° and 28° C. was due almost entirely to lactobacilli. It should be noted also that lactobacilli accounted for none of the colonies appearing on plates incubated for 2 days at 35° or 32° C. Only one-fifth of the colonies appearing on plates incubated at 28° C. for 3 days could be attributed to lactobacilli.

Neither temperature nor time of incubation appeared to influence the thermoduric colony count for sample DE (Table 5). As shown in Table 6, this sample contained a thermoduric flora comprised entirely of bacteria of the Arthrobacter genus.

The thermoduric colony count for sample DF increased as the plate incubation temperature was decreased from 35° to 21°

C. The thermoduric flora of this sample consisted essentially of microbacteria, micrococci and arthrobaeters. Table 6 shows that, as the plate incubation temperature was decreased, microbacteria accounted for a greater portion of the first colonies to appear on the plates. Appreciable increases in colony count also were obtained for sample DF when the incubation time at the various temperatures was prolonged. At incubation temperatures of 35°, 32° and 28° C. the increase in count with extended incubation was attributable essentially to microbacteria. However, at the 21° C. incubation temperature the increase in count upon extended plate incubation was due primarily to micrococci.

Colonies requiring longer than 2 days to appear on plates for samples DG and DH at 32° C. incubation and for sample DI at 35° C. incubation were found to be produced by microbacteria. Micrococci in these samples were capable of forming colonies within 2 days at the incubation temperatures of 35° and 32° C.

Sample DJ gave a substantially higher thermoduric colony count at a plate incubation temperature of 32° C. than at 35° C. (Table 5). The colony count obtained at each temperature was attributable to micrococci. However, it should be pointed out that, on the basis of the limited tests performed on these isolates, the predominant flora on plates at 35° C. differed in several respects from the flora predominating at 32° C. Insufficient tests were performed on these isolates to permit

classification to species.

The average distribution of thermoduric bacteria in samples DB, DC, DD, DF and DI is presented in Table 7. Colony development by the thermoduric microbacteria was enhanced at the lower incubation temperatures. Only 17.2 percent of the colonies that formed on plates incubated at 35° C. for 2 days were microbacteria. Of the colony counts obtained at 32° C. for 2 days and 28° C. for 3 days, 25.6 and 45.2 percent, respectively, were attributable to microbacteria. Microbacteria also accounted for an appreciable share of the colonies developing on plates after extended incubation at 35°, 32° and 28° C. The increase in the portion of the colony count attributable to microbacteria as the incubation temperature was decreased was accompanied by an increase in total thermoduric colony count.

Table 7 shows that thermoduric lactobacilli were slow in forming colonies at both 35° and 32° C. incubation. None of the colonies formed at 2 days of incubation at 35° and 32° C. were attributable to lactobacilli. However, an appreciable number of the colonies developing upon extended plate incubation at 35° and 32° C. were the result of growth of lactobacilli. There was some indication that these bacteria were capable of producing colonies after 3 days of incubation at 28° C., but colony development was favored by extended incubation even at this temperature.

Micrococci and microbacteria accounted for a major portion

Table 7. Average distribution of thermoduric bacteria in five samples^a of can cooled manufacturing grade milk as affected by time and temperature of plate incubation

Incubation temp., °C.	Incubation time, ^b days	Average thermoduric count per ml.	No. of isolates	Distribution of isolates, %				
				Arthro-bacters	Lacto-bacilli	Micro-bacteria	Micro-cocci	Others
35	2	19,000	29	17.2	0.0	17.2	65.6	0.0
	3-4	25,000 ^c	28	0.0	25.0	53.6	21.4	0.0
32	2	24,000	43	4.7	0.0	25.6	69.7	0.0
	3-4	28,000 ^c	22	4.6	22.7	36.4	13.6	22.7 ^d
28	3	34,000	31	0.0	3.2	45.2	48.4	3.2 ^e
	4	35,000	8	0.0	50.0	37.5	0.0	12.5 ^e
10	7-28	24,000 ^c	30	13.3	0.0	43.3	43.3	0.0

^aSamples, DB, DC, DD, DF and DI.

^bTime at which colonies had developed on plates.

^cAverage count at maximum incubation time.

^dUnidentified.

^eStreptococci.

of the colonies which appeared on plates within 28 days at 10° C. It is noteworthy that the average thermoduric colony count after 28 days at 10° C., for samples included in Table 7, approximated the average count obtained at 35° C. for 3 days and 32° C. for 2 days.

The thermoduric counts of ten samples of can cooled manufacturing grade milk obtained by incubation of plates at various times and temperatures are summarized in Table 8. The distribution of thermoduric bacteria in these samples is presented in Table 9. Sample DL showed no appreciable difference in thermoduric colony count at plate incubation temperatures of 35°, 32°, 28° and 21° C. Extended incubation of plates failed to materially increase the colony count at each of the four incubation temperatures. This sample contained a thermoduric flora consisting entirely of micrococci as indicated in Table 9.

The thermoduric colony count obtained for sample DN at an incubation temperature of 35° C. was approximately ten percent of each count obtained for this sample at 32°, 28° and 21° C. Examination of Table 9 shows that, while micrococci were the only thermoduric bacteria recovered from plates incubated at 35° C., microbacteria were prominent among the thermoduric flora on plates incubated at the lower temperatures. Even the micrococci in this sample preferred the lower incubation temperatures as evidenced by their delay in forming colonies at 35°

Table 8. Thermoduric plate counts of can cooled manufacturing grade milk obtained by incubation of plates at various times and temperatures

Sample no. (SPC) ^a	Incubation time, days	Incubation temperature, °C.			
		35	32	28	21
Count/ml.					
DK (1,100)	2	260	300		
	3	410	540	630	
	4	480	560	710	280
	5			710	630
	7				930
DL (17,000)	2	20,000	19,000		
	3	20,000	20,000	21,000	
	4	20,000	20,000	22,000	19,000
	5			22,000	20,000
	7				21,000
DM (420)	2	10,000	13,000		
	3	12,000	15,000	17,000	
	4	12,000	15,000	17,000	14,000
	5			17,000	16,000
	7				17,000
DN (10,000)	2	130,000	1,500,000		
	3	160,000	1,600,000	1,800,000	
	4	180,000	1,600,000	1,900,000	1,800,000
	5			1,900,000	1,900,000
	7				1,900,000
DO (3,400)	2	180,000	310,000		
	3	180,000	310,000	370,000	
	4	180,000	310,000	370,000	360,000
	5			370,000	360,000
	7				360,000

^aStandard Plate Count ($\times 10^3$) at 32° C. on raw milk samples.

Table 8. Continued

Sample no. (SPC) ^a	Incubation time, days	Incubation temperature, °C.			
		35	32	28	21
		Count/ml.			
DP (3,100)	2	290,000	360,000		
	3	300,000	390,000	370,000	
	4	300,000	390,000	380,000	350,000
	5			380,000	390,000
	7				410,000
DQ (900)	2	37,000	69,000		
	3	52,000	81,000	84,000	
	4	55,000	84,000	89,000	63,000
	5			91,000	87,000
	7				95,000
DR (1,500)	2	37,000	68,000		
	3	62,000	79,000	81,000	
	4	67,000	84,000	87,000	16,000
	5			88,000	72,000
	7				84,000
DS (6,600)	2	22,000	65,000		
	3	55,000	130,000	150,000	
	4	90,000	140,000	320,000	78,000
	5			350,000	150,000
	7				240,000
DT (1,400)	2	2,400	2,400		
	3	2,600	2,400	2,500	
	4	2,700	2,500	2,600	2,500
	5			2,600	2,600
	7				2,600

Table 9. Distribution of thermoduric bacteria in can cooled manufacturing grade milk samples (Table 8) determined by isolation from plates incubated at various times and temperatures

Sample no.	Incubation temp., °C.	Incubation time, ^a days	No. of isolates	Distribution of isolates			
				Arthro-bacters	Micro-bacteria	Micro-cocci	Unidenti-fied
DL	35	2	10			10	
	32	2	10			10	
	28	3	10			10	
	21	4	10			10	
DN	35	2	10			10	
		3-4	5			5	
	32	2	10		5	5	
	28	3	10		6	4	
	21	4	10		6	4	
DG	35	2	10	10			
	32	2	10	10			
	28	3	10	10			
	21	4	10	10			
DR	35	2	5			2	3
		3	5				5
	32	2	8			2	6
		3	2				2
	28	3	9			3	6
	21	4	5			5	
5		5				5	
DS	35	2	5		2	3	
		3	5		4	1	
	32	2	5		2	3	
		3	5		4	1	
	28	3	5		3	2	
		4	7		7		
	21	4	5		1	4	
		5-7	10		8	2	

^aTime at which colonies had developed on plates.

C. plus the apparent increase in colony count attributable to them at 32°, 28° and 21° C.

Sample DO did not show an increase in thermoduric colony count at any of the four incubation temperatures upon extension of the plate incubation time. However, the count obtained at an incubation temperature of 35° C. was approximately half that obtained at the lower temperatures. Table 9 indicates that this sample contained a thermoduric flora consisting wholly of bacteria of the Arthrobacter genus.

Differences in thermoduric colony counts obtained at the various incubation temperatures for sample DR (Table 8) were attributed to unidentified organisms. These bacteria were slow in forming colonies, especially on plates incubated at 35° and 21° C. as indicated in Table 9.

Sample DS gave a substantial increase in colony count as the plate incubation temperature was lowered from 35° to 28° C. and as the incubation period was extended at each of these temperatures. This sample contained a thermoduric flora composed of microbacteria and micrococci. Colony productivity by both the micrococci and microbacteria was increased at the lower incubation temperatures and colony production by the microbacteria was particularly enhanced by prolonged incubation at all temperatures.

The average distribution of thermoduric bacteria in samples DL, DN, DO, DR and DS is summarized in Table 10. Arthrobacters accounted for a rather constant percentage of

Table 10. Average distribution of thermoduric bacteria in five samples^a of can cooled manufacturing grade milk as affected by time and temperature of plate incubation

Incubation temp., °C.	Incubation time, ^b days	Average thermoduric count per ml.	No. of isolates	Distribution of isolates, %			
				Arthro-bacters	Micro-bacteria	Micro-cocci	Unidenti-fied
35	2	78,000	40	25.0	5.0	62.5	7.5
	3-4	107,000 ^c	15	0.0	26.7	40.0	33.3
32	2	390,000	43	23.2	16.3	46.5	14.0
	3	430,000	7	0.0	57.1	14.3	28.6
28	3	480,000	44	22.7	20.5	43.2	13.6
	4	540,000	7	0.0	100.0	0.0	0.0
21	4	450,000	40	25.0	17.5	57.5	0.0
	5-7	520,000 ^c	15	0.0	55.3	13.3	33.3

^aSamples DL, DN, DO, DR and DS.

^bTime at which colonies had developed on plates.

^cAverage count at maximum incubation time.

the thermoduric colony count obtained at incubation temperatures of 35°, 32°, 28° and 21° C. However, these bacteria did not contribute to increases in colony count upon extended plate incubation at the four temperatures.

Only 5 percent of the thermoduric colonies produced on plates incubated at 35° C. for 2 days were attributable to microbacteria. However, microbacteria accounted for 20.5 percent of the count obtained at 28° C. for 3 days. The magnitude of this difference becomes even greater when it is noted that the average thermoduric colony count at 35° C. for 2 days was only 78,000 per ml. as compared with an average count of 480,000 per ml. at 28° C. for 3 days. The microbacteria also contributed substantially to increases in thermoduric colony counts upon extended plate incubation at the various temperatures.

As indicated in Table 10, micrococci accounted for approximately equal portions of the colony count obtained at 32°, 28° and 21° C. incubation. Micrococci not only constituted a major portion of the colony count at 35° C. for 2 days but also contributed substantially to the increase in count upon prolonged plate incubation at this temperature. Unidentified thermoduric bacteria also contributed to increases in colony counts obtained after extended plate incubation at 35°, 32° and 21° C.

The mean thermoduric colony counts of 25 milk samples obtained by incubation of plates at various temperatures and

times are presented in Table 11. Samples K through T were not included in this analysis due to incomplete count data. The mean thermoduric colony counts obtained after 2 days of incubation at 35° and 32° C. were 31.0 and 73.7 percent, respectively, of the mean count obtained at 28° C. for 4 days. Although the count increased upon prolonged incubation at 35° and 32° C., the maximum count obtained at each of these temperatures was appreciably lower than that obtained at 28° C. for 4 days.

The maximum mean count was obtained after 7 days of incubation at 21° C. as well as at 28° C. for 4 days. The mean thermoduric count obtained at 10° C. for 28 days exceeded the mean count at 35° C. for 2 days and was almost half of the maximum mean count obtained at 28° C. for 4 days. However, as indicated in Table 11, thermoduric bacteria, on the average, were slow in forming colonies at a plate incubation temperature of 10° C.

Thermoduric bacteria rarely formed colonies on plates incubated at 5° C. prior to 21 days of incubation. Even after 130 days of incubation at 5° C. the thermoduric count rarely exceeded 10 percent of the count obtained at 28° C. for 4 days. No attempt was made to identify thermoduric bacteria which formed colonies at 5° C. but colony characteristics indicated that a majority of these were probably micrococci.

Analysis of variance (84) of thermoduric counts showed that counts obtained after 3 days of incubation at 35°, 32°

Table 11. Mean thermoduric plate counts of 25 samples of manufacturing grade milk obtained by incubation of plates at various times and temperatures

Incubation time, days	Incubation temperature, °C.				
	35	32	28	21	10
2	59,000 (31.0) ^a	140,000 (73.7)			
3	74,000 (38.9)	160,000 (84.2)	180,000 (94.7)		
4	81,000 (42.6)	160,000 (84.2)	190,000 (100.0)	150,000 (78.9)	
5			190,000 (100.0)	180,000 (94.7)	
7				190,000 (100.0)	240 (0.1)
14					6,400 (3.4)
21					52,000 (27.4)
28					81,000 (42.6)

^aPercent of the mean count obtained at 28° C. for 4 days.

and 28° C. were significantly different ($P < 0.01$). The same significant difference was obtained when counts after 4 days at 35°, 32° and 28° C. were compared. Thermoduric counts obtained by incubating plates at 35° C. for 2 days were significantly lower than counts obtained at 32° C. for 2 days ($P < 0.01$).

Effect of Various Bacteriological Peptones in the Plating Medium upon the Thermoduric Plate Count

The thermoduric colony counts of ten samples of grade A milk obtained with media containing various bacteriological peptones are presented in Table 12. Most samples showed a slight variation in colony count with the various "peptone" media. However, the variation in count was slight and was not considered to be significantly different for any one medium when compared against the others.

With the possible exception of sample EB, an increase in colony count was obtained with each medium when the plate incubation period was extended beyond 2 days. Sample EB contained a thermoduric flora consisting entirely of micrococci. Although no study of the thermoduric flora of the other grade A samples was made, colony characteristics indicated that the flora was predominantly spore-bearing rods.

The thermoduric colony counts of ten manufacturing grade samples obtained with media containing various bacteriological peptones are summarized in Table 13. The distribution of

Table 12. Thermoduric plate counts^a (32° C.) of grade A milk samples obtained with media containing various bacteriological peptones

Sample no. (SPC) ^b	Incubation time, days	Bacteriological peptone						
		Bacto-Tryptone	N-Z-Amine Type AS	N-Z-Amine Type YF	Edamin	Soy Peptone Powder	N-Z-Case	HY-Case SF
Count/ml.								
DU (11)	2	500	260	370	390	520	330	230
	3	570	410	440	510	560	510	350
	4	620	460	450	510	590	610	400
DV (17)	2	170	110	120	140	120	150	130
	3	250	160	160	190	150	160	190
	4	260	180	160	210	160	170	200
DW (4)	2	40	10	10	30	30	20	10
	3	50	20	20	30	30	20	60
	4	50	30	20	30	30	20	60
DX (13)	2	540	520	350	400	410	510	450
	3	590	560	390	460	440	530	500
	4	620	580	420	460	460	530	520
DY (58)	2	50	60	50	30	40	30	20
	3	90	70	60	110	60	60	30
	4	110	70	60	112	70	70	30
DZ (3)	2	20	40	40	30	30	20	40
	3	50	70	70	30	50	30	70
	4	60	30	30	80	50	30	70

^a1:10 dilution plates having less than 30 colonies per plate were included for those samples showing counts of less than 300 per ml.

^bStandard Plate Count ($\times 10^3$) at 32° C. on raw milk sample.

Table 12. Continued

Sample no. (SPC) ^b	Incubation time, days	Bacteriological peptones						
		Bacto-Tryptone	N-Z-Amine Type AS	N-Z-Amine Type YT	Edamin	Soy Peptone Powder	N-Z-Case	HY-Case SF
Count/ml.								
EA (14)	2	270	260	340	300	280	260	270
	3	300	300	360	340	300	300	320
	4	300	300	360	350	310	320	320
EB (86)	2	46,000	46,000	43,000	46,000	45,000	45,000	46,000
	3	46,000	46,000	43,000	46,000	45,000	46,000	46,000
	4	46,000	46,000	43,000	46,000	45,000	46,000	46,000
EC (52)	2	7,400	7,200	7,100	7,100	7,300	7,800	8,100
	3	7,600	7,500	7,600	7,300	7,800	8,300	8,500
	4	7,600	7,500	7,700	7,300	7,800	8,400	8,500
ED (35)	2	350	520	430	320	330	490	250
	3	430	570	470	350	410	560	360
	4	450	610	490	390	410	590	360

thermoduric bacteria in some of the samples is presented in Table 14. Samples EH and EJ showed considerable fluctuation in colony count with the various "peptone" media. Each of these samples contained a thermoduric flora essentially comprised of arthrobacters and micrococci. The lowest colony counts for both samples were obtained with media prepared with N-Z-Amine Type AS and Edamin. Examination of Table 14 reveals that bacteria of the genus Arthrobacter were absent or contributed insignificantly to the 2-day colony count with both media.

Table 13. Thermoturc plate counts (32° C.) of manufacturing grade milk samples obtained with media containing various bacteriological peptones

Sample no. (SPC) ^a	Incubation time, days	Bacteriological peptone						
		Bacto-Tryptone	N-Z-Amine Type AB	N-Z-Amine Type YF	Edamin	Soy Peptone Powder	N-Z-Case	HY-Case SF
Count/ml.								
EE (6,000)	2	4,400	5,700	6,600	7,100	7,500	6,100	7,400
	3	5,700	7,100	8,200	8,200	9,000	7,000	8,900
	4	6,300	7,800	8,500	8,400	9,100	7,400	10,000
EF (1,400)	2	260,000	260,000	270,000	270,000	280,000	210,000	280,000
	3	290,000	290,000	300,000	290,000	300,000	270,000	310,000
	4	290,000	300,000	310,000	290,000	300,000	280,000	320,000
EG (3,000)	2	33,000	31,000	31,000	30,000	40,000	36,000	40,000
	3	34,000	32,000	32,000	30,000	42,000	37,000	43,000
	4	34,000	32,000	33,000	35,000	42,000	40,000	44,000
EH (96)	2	4,800	2,900	6,900	3,300	4,000	3,900	4,000
	3	6,300	3,700	8,500	3,700	5,900	5,200	6,400
	4	6,600	3,900	8,800	3,800	6,200	5,800	7,000
EI (680)	2	25,000	54,000	59,000	58,000	58,000	59,000	73,000
	3	32,000	55,000	60,000	58,000	59,000	62,000	74,000
	4	39,000	56,000	61,000	58,000	59,000	62,000	74,000

^aStandard Plate Count (X10³) at 32° C. on raw milk samples

Table 13. Continued

Sample no. (SPC) ^a	Incubation time, days	Bacteriological peptone						
		bacto-Tryptone	N-Z-Amine Type AS	N-Z-Amine Type YT	Edamin	Soy Peptone Powder	N-Z-Case	HY-Case SF
Count/ml.								
EJ (20,000)	2	10,000	6,500	12,000	6,100	7,600	7,900	7,500
	3	11,000	7,500	13,000	6,900	8,800	9,000	11,000
	4	12,000	7,600	14,000	7,100	8,900	9,000	12,000
EK (1,400)	2	150,000	160,000	150,000	140,000	150,000	160,000	150,000
	3	160,000	170,000	160,000	140,000	160,000	180,000	160,000
		160,000	170,000	170,000	140,000	160,000	180,000	160,000
EL (540)	2	9,200	8,500	7,500	8,400	8,800	8,900	7,900
	3	9,800	8,700	8,100	8,600	9,100	9,000	8,100
	4	9,800	8,700	8,100	8,600	9,200	9,000	8,100
EM (7,300)	2	17,000	300,000	210,000	100,000	340,000	280,000	260,000
	3	25,000	300,000	210,000	100,000	340,000	280,000	260,000
	4	26,000	300,000	210,000	100,000	340,000	280,000	260,000
EN (170)	2	9,800	16,000	16,000	14,000	14,000	14,000	13,000
	3	13,000	16,000	17,000	14,000	14,000	14,000	14,000
	4	13,000	16,000	17,000	14,000	14,000	14,000	14,000

Table 14. Distribution of thermoduric bacteria in manufacturing grade milk samples (Table 13) determined by isolation from plates prepared with media containing various bacteriological peptones

Sample no.	Bacteriological peptone ^a	Incubation time, ^b days	No. of isolates	Distribution of isolates			
				Arthro-bacters	Micro-bac-teria	Micro-cocci	Strepto-cocci
EH	I	2	6	3		3	
		3-4	4	2		2	
	II	2	5	1		4	
		3-4	4	3		1	
	III	2	7	5		2	
		3-4	4	2		2	
	IV	2	7	3		4	
3		2			2		
VII	2	6	3		3		
	3-4	4	4				
EI	I	2	6		1	5	
		3-4	4			4	
	II	2	6		2	4	
	III	2	6		2	4	
	IV	2	6		2	4	
	VII	2	7		3	4	
		3	1		1		

^aBacteriological peptone used in medium: I, Bacto-Tryptone; II, N-Z-Amine Type AS; III, N-Z-Amine Type YT; IV, Edamin; V, Soy-Peptide Powder; VI, N-Z-Case; VII, HY-Case SF.

^bTime at which colonies had developed on plates at 32° C.

Table 14. Continued

Sample no.	Bacteriological peptone ^a	Incubation time, ^b days	No. of iso-lates	Distribution of isolates			
				Arthro-bacters	Micro-bac-teria	Micro-cocci	Strepto-cocci
EJ	I	2	9	5		4	
		3	1			1	
	II	2	8			8	
	III	2	11	3		8	
		3	2			2	
	IV	2	5	1		4	
		3	2			2	
VII	2	6	4	1	1		
	3-4	5	1	1	3		
EM	I	2	7			6	1
		3	3			3	
	II	2	8			1	7
	III	2	6				6
	IV	2	5			5	
	V	2	10			1	9
	VI	2	8				8
VII	2	8				8	

However, these organisms accounted for a major portion of the colony count obtained with the other media.

The 2-day colony count for sample EI was approximately three times greater with medium prepared with HY-Case SF than with medium prepared with Bacto-Tryptone. Table 14 reveals that the difference in count between the two media was due to the proportionally greater number of microbacteria forming colonies on the medium prepared with HY-Case SF.

There was practically no difference among thermoduric colony counts obtained with the various media for sample EK. The thermoduric flora of this sample was composed entirely of micrococci.

As shown in Table 13, the colony count obtained with the Bacto-Tryptone medium for sample EM was only about one-tenth that obtained with most of the other media. The thermoduric flora of this sample consisted of streptococci and micrococci (Table 14). The medium prepared with Bacto-Tryptone failed almost entirely to support growth of the thermoduric streptococci in this sample. However, with the exception of the count obtained with the medium containing Edamin, the colony counts obtained with the other media were attributable almost entirely to streptococci. Although it did not support growth of streptococci, the Edamin medium apparently enhanced colony productivity by the thermoduric micrococci.

Table 15 summarizes the thermoduric colony counts of six samples of manufacturing grade milk obtained with media

Table 15. Thermoduric plate counts (32° C.) of blended^a manufacturing grade milk samples obtained with media containing various bacteriological peptones

Sample no. (SPC) ^b	Incubation time, days	Bacteriological peptone						
		Bacto-Tryptone	N-Z-Amine Type AS	N-Z-Amine Type YT	Edamin	Soy Peptone Powder	N-Z-Case	HY-Case SF
Count/ml.								
EU (3,500)	2	80,000	88,000	85,000	79,000	96,000	100,000	76,000
	3	94,000	100,000	92,000	87,000	100,000	110,000	91,000
	4	95,000	100,000	94,000	88,000	100,000	110,000	93,000
EV (6,000)	2	210,000	270,000	240,000	210,000	270,000	250,000	260,000
	3	220,000	270,000	250,000	230,000	280,000	250,000	270,000
	4	220,000	270,000	250,000	230,000	280,000	250,000	270,000
EW (4,700)	2	190,000	240,000	240,000	200,000	240,000	240,000	220,000
	3	200,000	240,000	250,000	210,000	250,000	250,000	230,000
	4	200,000	250,000	260,000	220,000	250,000	250,000	230,000
EX (2,700)	2	86,000	130,000	130,000	90,000	84,000	140,000	100,000
	3	100,000	130,000	130,000	92,000	93,000	150,000	120,000
	4	100,000	130,000	140,000	92,000	93,000	150,000	130,000

^aEach sample is a blend of can and bulk cooled milk from several farms.

^bStandard Plate Count ($\times 10^3$) at 32° C. on raw milk sample.

Table 15. Continued

Sample no. (SPC) ^b	Incubation time, days	Bacteriological peptone						
		Bacto-Tryptone	N-Z-Amine Type AS	N-Z-Amine Type YT	Edamin	Soy Peptone Powder	N-Z Case	HY-Case SF
Count/ml.								
EY (6,800)	2	190,000	160,000	170,000	160,000	190,000	190,000	190,000
	3	190,000	170,000	130,000	170,000	190,000	200,000	200,000
	4	200,000	170,000	130,000	170,000	190,000	200,000	210,000
EZ (10,000)	2	300,000	380,000	300,000	270,000	360,000	340,000	310,000
	3	310,000	380,000	320,000	270,000	360,000	360,000	320,000
	4	310,000	390,000	320,000	280,000	360,000	370,000	330,000

containing various bacteriological peptones. The distribution of thermophilic bacteria for two of the samples is presented in Table 16. Samples EV and EZ contained a thermophilic flora composed almost entirely of micrococci and streptococci. The lowest thermophilic colony counts for both samples were obtained with media prepared with Bacto-Tryptone and Edamin. Examination of Table 16 reveals that streptococci accounted for an appreciable portion of the colonies on media other than those prepared with Bacto-Tryptone and Edamin. However, with the medium containing Edamin as the bacteriological peptone, streptococci apparently failed to produce colonies. Streptococci accounted for only a minor portion of the colony count obtained with the Bacto-Tryptone medium. Micrococci accounted for a major portion of the thermophilic colony counts obtained with media prepared with Bacto-Tryptone and Edamin.

The mean thermophilic colony counts of 26 milk samples obtained by plating with media containing various bacteriological peptones are summarized in Table 17. Although variation among mean counts obtained with the various media was not great, the data indicate that Bacto-Tryptone and Edamin media produced the lowest mean colony counts. Mean colony counts obtained with the other media were similar, especially after 4 days of incubation.

Table 18 summarizes the distribution of thermophilic bacteria in eight samples of milk obtained by plating with media

Table 16. Distribution of thermoduric bacteria in manufacturing grade milk samples (Table 15) determined by isolation^a from plates prepared with media containing various bacteriological peptones

Sample no.	Bacteriological peptone	No. of isolates	Distribution of isolates		
			Micro-bacteria	Micro-cocci	Streptococci
EV	Bacto-Tryptone	7		6	1
	N-Z-Amine Type AS	6		3	3
	N-Z-Amine Type YT	6			6
	Edamin	6		6	
	Soy Peptone Powder	6		3	3
	HY-Case SF	6			6
EZ	Bacto-Tryptone	6		4	2
	N-Z-Amine Type AS	6	1	2	3
	N-Z-Amine Type YT	7	1	2	4
	Edamin	6	1	5	
	Soy Peptone Powder	6		3	3
	HY-Case SF	6		2	4

^aAll isolations were made after 2 days of incubation at 32° C.

Table 17. Mean thermoduric plate counts (32° C.) of 26 milk samples obtained with media containing various bacteriological peptones

Bacteriological peptone	Incubation time, days		
	2	3	4
Bacto-Tryptone	63,000 (100.0) ^a	68,000 (107.9)	68,000 (107.9)
N-Z-Amine Type AS	83,000 (131.7)	86,000 (136.5)	87,000 (138.1)
N-Z-Amine Type YT	76,000 (120.6)	80,000 (127.0)	82,000 (130.2)
Edamin	65,000 (103.2)	68,000 (107.9)	69,000 (109.5)
Soy Peptone Powder	85,000 (134.9)	88,000 (139.7)	88,000 (139.7)
N-Z-Case	81,000 (128.6)	87,000 (138.1)	87,000 (138.1)
HY-Case SF	79,000 (125.4)	84,000 (133.3)	86,000 (136.5)

^aPercent of the mean count obtained with the "Bacto-Tryptone" medium at 2 days.

Table 18. Average distribution of thermoduric bacteria in eight samples^a of milk obtained with media containing various bacteriological peptones

Bacteri- ological peptone	Incu- bation time, ^b days	Average thermo- duric count per ml.	No. of iso- lates	Distribution of isolates, %			
				Arthro- bacters	Micro- bac- teria	Micro- cocci	Strepto- cocci
Bacto- Tryptone	2	95,000	59	13.6	1.7	77.9	6.8
	3-4	100,000 ^c	14	14.3		85.7	
N-Z-Amine Type AS	2	150,000	57	1.8	5.3	70.1	22.8
	3-4	160,000 ^c	6	50.0		50.0	
N-Z-Amine Type YT	2	130,000	61	13.1	4.9	55.7	26.3
	3-4	140,000 ^c	8	25.0		75.0	
Edamin	2	100,000	55	7.3	5.4	87.3	
	3-4	110,000 ^c	4			100.0	
HY-Case SF	2	140,000	57	12.3	7.0	49.1	31.6
	3-4	150,000 ^c	8	25.0	25.0	50.0	

^aSamples EB, EH, EI, EJ, EK, EM, EV and EZ.

^bTime at which colonies had developed on plates incubated at 32° C.

^cAverage count at maximum incubation time.

containing various bacteriological peptones. The data indicate that bacteria of the Arthrobacter genus preferred media prepared with Bacto-Tryptone, N-Z-Amine Type YT and HY-Case SF. The medium prepared with N-Z-Amine Type AS did not substantially support colony productivity by these bacteria after 2 days of plate incubation.

Table 18 illustrates the inadequacies of the Bacto-Tryptone and Edamin media for supporting growth of the thermoduric streptococci. However, these organisms accounted for a substantial portion of the colony counts obtained with media prepared with N-Z-Amine Type AS, N-Z-Amine Type YT and HY-Case SF.

Effect of the pH of the Plating Medium upon the Thermoduric Plate Count

Table 19 illustrates the effect of the pH of Plate Count Agar upon the thermoduric colony counts of blended manufacturing grade milk samples. Generally, the thermoduric colony count obtained at pH 8.6 was equal to or exceeded that obtained at pH 7.0. The thermoduric count for five of the six samples was greater after 3 days of incubation at pH 9.1 than at pH 6.5. The counts obtained at pH 7.0 were higher than counts obtained at pH 6.5 for all samples in this group. The distribution of thermoduric bacteria in two of the samples is shown in Table 20. Micrococci accounted for a major portion of the colony counts obtained with media of pH 6.5 and 7.0. However,

Table 19. Thermoduric plate counts (32° C.) of blended^a manufacturing grade milk samples obtained with Plate Count Agar at various pH levels

Sample no. (SPC) ^b	Incubation time, days	Plating medium pH				
		6.5	7.0	7.5	8.6	9.1
Count/ml.						
EO (10,000)	2	210,000	300,000	370,000	370,000	220,000
	3	220,000	310,000	390,000	380,000	290,000
	4	230,000	310,000	400,000	380,000	300,000
EP (6,800)	2	110,000	190,000	210,000	190,000	88,000
	3	120,000	190,000	220,000	200,000	130,000
	4	120,000	220,000	220,000	210,000	140,000
EQ (2,700)	2	60,000	86,000	86,000	78,000	37,000
	3	66,000	100,000	90,000	90,000	53,000
	4	69,000	100,000	91,000	91,000	54,000
ER (4,700)	2	160,000	190,000	240,000	240,000	200,000
	3	170,000	200,000	240,000	240,000	220,000
	4	170,000	200,000	240,000	250,000	220,000
ES (6,000)	2	120,000	210,000	250,000	250,000	180,000
	3	120,000	220,000	250,000	260,000	200,000
	4	130,000	220,000	250,000	260,000	200,000
ET (3,500)	2	59,000	80,000	98,000	85,000	65,000
	3	62,000	94,000	100,000	93,000	82,000
	4	68,000	95,000	110,000	95,000	82,000

^aEach sample is a blend of can and bulk cooled milk from several farms.

^bStandard Plate Count ($\times 10^3$) at 32° C. on raw milk sample.

Table 20. Distribution of thermoduric bacteria in manufacturing grade milk samples (Table 19) obtained by isolation from plates prepared with Plate Count Agar at various pH levels

Sample no.	Plating medium pH	Incubation time, ^a days	No. of isolates	Distribution of isolates		
				Micrococci	Streptococci	Others
EO	6.5	2	6	5	1	
	7.0	2	6	4	2	
	7.5	2	7	2	4	1 ^b
	8.6	2	7	3	4	
	9.1	2 3	5 2	1 2	4	
ES	6.5	2	5	3	2	
	7.0	2	7	6	1	
	7.5	2	7	1	6	
	8.6	2	7	1	5	1 ^c
	9.1	2 3	6 1	1	5 1	

^aTime at which colonies had developed on plates incubated at 32° C.

^bArthrobacter sp.

^cMicrobacterium sp.

streptococci were the prevailing thermoduric bacteria recovered with the alkaline media. This was especially true for sample ES.

The thermoduric colony counts of six samples of manufacturing grade milk obtained with Plate Count Agar at various pH levels are summarized in Table 21. The distribution of thermoduric bacteria in these samples is shown in Table 22. The maximum colony count for sample FA was obtained at pH 7.5. This sample contained a thermoduric flora of arthrobacters, micrococci and streptococci. Bacteria of all three groups contributed to the colony count at pH 7.5. However, streptococci were not isolated from media below pH 7.5 and arthrobacters apparently did not contribute to counts obtained with media above pH 7.5. Micrococci were detected at all levels except pH 9.1.

The thermoduric colony count for sample FB fell sharply with media above pH 7.5 (Table 21). Examination of Table 22 reveals that bacteria of the Arthrobacter genus dominated the colony count obtained with media adjusted to pH levels of 6.5, 7.0 and 7.5. However, these organisms were not detected at pH 9.1, whereas, streptococci comprised a major portion of the colonies produced at pH 9.1.

Approximately equal thermoduric colony counts were obtained with media of pH 7.0, 7.5 and 8.6 for sample FC (Table 21). With media of pH 6.5 and 9.1, however, this sample showed an appreciably lower count at 2 days of incubation than that

Table 21. Thermoduric plate counts (32° C.) of can cooled manufacturing grade milk samples obtained with Plate Count Agar at various pH levels

Sample no. (SPC) ^a	Incubation time, days	Plating medium pH				
		6.5	7.0	7.5	8.6	9.1
		Count/ml.				
FA (1,800)	2	120,000	190,000	240,000	190,000	190,000
	3	120,000	190,000	240,000	190,000	200,000
	4	120,000	200,000	240,000	200,000	200,000
FB (3,200)	2	390,000	510,000	530,000	100,000	32,000
	3	390,000	510,000	540,000	110,000	44,000
	4	400,000	510,000	540,000	110,000	44,000
FC (1,300)	2	32,000	210,000	220,000	200,000	35,000
	3	140,000	210,000	230,000	210,000	160,000
	4	160,000	220,000	230,000	210,000	170,000
FD (620)	2	23,000	27,000	29,000	16,000	9,100
	3	23,000	28,000	29,000	16,000	10,000
	4	23,000	28,000	29,000	16,000	10,000
FE (4,900)	2	3,100	3,000	3,800	3,500	3,400
	3	3,300	3,200	4,000	3,800	3,900
	4	3,400	3,200	4,100	3,900	4,000
FF (630)	2	9,000	12,000	12,000	6,000	1,500
	3	10,000	12,000	12,000	6,200	2,700
	4	11,000	12,000	12,000	6,200	2,900

^aStandard Plate Count ($\times 10^3$) at 32° C. on raw milk sample.

Table 22. Distribution of thermophilic bacteria in manufacturing grade milk samples (Table 21) obtained by isolation from plates prepared with Plate Count Agar at various pH levels

Sample no.	Plating medium pH	Incubation time, a	No. of isolates	Distribution of isolates			
				Arthro-bacters	Micro-bac-teria	Micro-cocci	Strepto-cocci
FA	6.5	2	6	1		5	
	7.0	2	6	3		3	
	7.5	2	7	2		2	3
	8.6	2	6			2	4
	9.1	2	6				6
FB	6.5	2	6	6			
	7.0	2	6	6			
	7.5	2	6	6			
	8.6	2	6	4	1		1
	9.1	2	6		1	1	4
FC	6.5	2	3			2	1
		3-4	3			3	
	7.0	2	7		1	6	
	9.1	2	3			3	
3		4			4		
FD	6.5	2	7	4		3	
	7.0	2	6	3		1	2
	9.1	2	6			3	3
FE	7.0	2	6	1		5	
FF	7.0	2	6	3		1	2
		3	6	2	1	1	2
	9.1	2	3		1	1	1
		3	3		1	2	

^aTime at which colonies had developed on plates at 32° C.

obtained at 2 days at the other pH levels. At the same time, extended incubation of plates prepared with media of pH 6.5 and 9.1 resulted in a substantial increase in colony count. The thermoduric flora of this sample was predominantly micrococci (Table 22).

Samples FD and FF showed the same trend in colony counts at the various pH levels (Table 21). For each of these samples the colony counts obtained with media of pH 6.5, 7.0 and 7.5 were approximately equal but counts at higher pH levels were substantially reduced. Examination of Table 22 reveals that arthrobacters contributed appreciably to the colony counts at the lower pH levels but were not detected at pH 9.1. Micrococci and streptococci contributed to the colony count at pH 9.1 for sample FD while micrococci, streptococci and microbacteria appeared to be responsible for the count obtained at this pH for sample FF.

The thermoduric colony counts of six grade A milk samples obtained with Plate Count Agar at various pH levels are summarized in Table 23. With the exception of sample FK, the thermoduric flora of these samples was predominantly spore-bearing rods. The thermoduric colony count for sample FK remained rather constant over a range of pH 6.5 to 9.1. The thermoduric flora of this sample was composed entirely of micrococci. No attempt was made to classify these micrococci to species but a limited number of tests indicated that they constituted a homogeneous group. No discernible colonies were

Table 23. Thermoduric plate counts^a (32° C.) of bulk cooled grade A milk samples obtained with Plate Count Agar at various pH levels

Sample no. (SPC) ^b	Incubation time, days	Plating medium pH					
		6.5	7.0	7.5	8.0	8.6	9.1
Count/ml.							
FG (37)	2	530	430	540	620	540	710
	3	560	450	550	690	630	730
	4	600	490	580	730	640	740
FH (44)	2	400	470	460	260	290	260
	3	520	580	530	290	310	290
	4	590	610	570	350	380	310
FI (10)	2	180	120	210	230	170	140
	3	180	140	220	250	180	160
	4	210	170	280	310	210	170
FJ (21)	2	330	400	340	420	390	350
	3	410	540	430	470	350	370
	4	460	560	440	490	400	400
FK (50)	2	37,000	38,000	38,000	38,000	37,000	34,000
	3	37,000	38,000	38,000	38,000	37,000	35,000
	4	37,000	38,000	38,000	38,000	38,000	35,000
FL (6)	2	190	260	290	290	250	150
	3	220	300	300	300	260	160
	4	240	310	310	310	270	160

^a1:10 dilution plates having less than 30 colonies per plate were included for those samples showing counts of less than 300 per ml.

^bStandard Plate Count ($\times 10^3$) at 32° C. on raw milk samples.

produced with any of the samples with Plate Count Agar at pH 5.6.

Table 24 indicates that the maximum mean thermoduric colony count of 18 samples was obtained with Plate Count Agar at pH 7.5. Insufficient data were obtained to include counts on medium of pH 8.0 in the summary table. However, the mean 4-day thermoduric count obtained at pH 8.6 equaled the mean count obtained after 2 days of incubation at pH 7.0. Mean colony counts obtained at the various incubation times with Plate Count Agar at pH 9.1 were only slightly lower than those obtained at pH 6.5.

Table 25 summarizes the average distribution of thermoduric bacteria in nine milk samples obtained with Plate Count Agar at various pH levels. Arthrobacters accounted for approximately equal portions of the isolates obtained from media of pH 6.5, 7.0 and 7.5. However, colony development by these bacteria was restricted at pH 8.6 and entirely absent at pH 9.1.

The number of microbacteria isolated from samples included in Table 25 was inadequate to demonstrate the effect of medium pH upon their ability to produce colonies. Micrococci accounted for an appreciable portion of the mean thermoduric colony count obtained at each pH level.

Table 25 reveals that as the pH of the plating medium progressively increased from 6.5 to 9.1, the proportionate share of the mean colony count attributable to streptococci at

Table 24. Mean thermoduric plate counts (32° C.) of 18 milk samples obtained with Plate Count Agar at various pH levels

Plating medium pH	Incubation time, days		
	2	3	4
6.5	74,000 (67.3) ^a	82,000 (74.5)	86,000 (78.2)
7.0	110,000 (100.0)	120,000 (109.1)	120,000 (109.1)
7.5	130,000 (118.2)	130,000 (118.2)	130,000 (118.2)
8.6	98,000 (89.1)	100,000 (90.9)	110,000 (100.0)
9.1	61,000 (55.5)	80,000 (72.7)	81,000 (73.6)

^aPercent of the mean count obtained at pH 7.0 for 2 days.

each pH level also increased. The most drastic increase in the contribution of streptococci to the colony count occurred with an increase in pH from 7.0 to 7.5.

The pH of the plating medium influenced the size of individual colonies as well as the thermoduric plate count. Although there were some exceptions, colony size generally was largest with those media yielding the highest count for a

Table 25. Average distribution of thermoduric bacteria in nine samples^a of milk obtained with Plate Count Agar at various pH levels

Plating medium pH	Average thermoduric count ^b per ml.	No. of isolates	Distribution of isolates, %			
			Arthro-bacters	Micro-bacteria	Micro-cocci	Strepto-cocci
6.5	100,000	41	26.8		63.4	9.8
7.0	170,000	58	27.6	1.7	58.7	12.0
7.5	190,000	35	25.8		37.1	37.1
8.6	130,000	39	15.4	7.7	35.9	41.0
9.1	78,000	43		4.7	41.9	53.5

^aSamples EO, -ES, FA, FB, FC, FD, FE, FF and FK.

^bPlates incubated for 2 days at 32° C.

particular sample. This was especially true for samples containing appreciable numbers of streptococci and microbacteria. Although the thermoduric colony count for certain samples was not influenced substantially by variance in pH, levels above pH 7.0 usually produced the largest and most easily discernible colonies.

Effect of the Various Plating Procedures upon Colony
Counts of Pure Cultures of Thermophilic Bacteria
Before and After Laboratory Pasteurization

Table 26 shows the effect of time and temperature of plate incubation upon the colony counts of some pure cultures of thermophilic bacteria isolated during this study. Approximately equal colony counts were obtained for an unheated culture of Arthrobacter sp. DO-31-1 when plates were incubated for 2 days at 35°, 32°, 28° and 21° C. However, colony counts for the laboratory pasteurized culture showed considerable variation after two days of incubation at the various temperatures. The pasteurized culture showed a definite preference for incubation temperatures of 32° and 28° C. when plates were incubated for two days. The colony count was noticeably suppressed at a plate incubation temperature of 35° C. Although the colony count at 21° C. for 2 days was appreciably less than that obtained at 32° and 28° C. for 2 days, the count at 21° C. increased substantially upon extended plate incubation.

Both unheated and pasteurized cultures of Microbacterium lacticum DN-32-1 failed to produce countable colonies at an incubation temperature of 35° C. Differences in colony counts obtained at 32°, 28° and 21° C. for both unheated and pasteurized cultures were not attributable to differences in incubation temperature. However, a substantially higher count was obtained for the refrigerated M. lacticum culture after pasteurization.

Table 26. Effect of incubation time and temperature upon plate counts of pure cultures of thermophilic bacteria

Organism	Series ^a	Incubation temp., °C.	Raw count ^b per ml.	Laboratory pasteurized count per ml. after plate incubation for				
				2 days	3 days	4 days	5 days	7 days
				In thousands				
<u>Arthrobacter</u> sp. DO-32-1	1	35	690	34	35	35		
		32	690	400	420	420		
		28	760	610	610	610	610	
		21	770	200	500	500	500	500
	2	35	730	36	36	36		
		32	830	610	610	610		
		28	750	570	610	610	610	
		21	840	360	590	600	600	600
<u>Microbacterium</u> lacticum DN-32-1	1	35	<1	<1	<1	<1		
		32	1,500	1,500	1,600	1,600		
		28	1,400	1,500	1,800	1,800	1,800	
		21	1,500 ^c		1,700	1,700	1,700	1,700

^aSeries 1 cultures were incubated at 32° C. for 24 hours prior to testing while series 2 cultures were incubated at 32° C. for 24 hours and then refrigerated at 38-40° F. for 24 hours prior to testing.

^bRaw count at 2 days of plate incubation except where noted. Count did not change with extended plate incubation.

^cRaw count at 3 days of incubation.

Table 26. Continued

Organism	Series ^a	Incubation temp., °C.	Raw count ^b per ml.	Laboratory pasteurized count per ml. after plate incubation for				
				2 days	3 days	4 days	5 days	7 days
				In thousands				
<u>Microbacterium lacticum</u>	2	35	<1	<1	<1	<1		
		32	1,700	2,500	2,600	2,600		
		28	1,300	2,300	2,500	2,500	2,500	
		21	1,600 ^c		2,800	2,800	2,800	2,800
<u>Micrococcus varians</u> EM-1V-2	1	35	<1	<1	<1	<1		
		32	110	<1	32	37		
		28	110	<1	40	60	64	
		21	110 ^c		<1	24	48	61
	2	35	<1	<1	<1	<1		
		32	180	<1	30	34		
		28	160	<1	9	15	17	
		21	170 ^c		<1	5	13	20
<u>Streptococcus</u> sp. FA-F-1	1	35	15,000	6,000	6,100	6,100		
		32	15,000	7,100	7,300	7,300		
		28	16,000	6,600	7,300	7,500	7,500	
		21	13,000 ^d			510	1,400	2,100
	2	35	14,000	4,400	4,400	4,400		
		32	13,000	5,000	5,100	5,200		
		28	16,000	3,300	4,200	4,200	4,200	
		21	14,000 ^d			290	760	1,200

^dRaw count at 4 days of incubation.

zation than before pasteurization. This phenomenon did not occur with cultures which were not refrigerated prior to pasteurization. Repeated trials showed similar increases in colony count after pasteurization for refrigerated cultures of this organism. Microscopic examination of stained smears of the refrigerated M. lacticum culture before and after pasteurization revealed that pairs and small clumps of cells were common in the unheated culture, but single cells predominated in the pasteurized culture.

Both unheated and pasteurized cultures of Micrococcus varians EM-IV-2 failed to produce countable colonies at an incubation temperature of 35° C. Differences in colony counts obtained at 32°, 28° and 21° C. for the unheated cultures were not attributable to differences in incubation temperatures. Although no increase in colony count was obtained for the unheated culture beyond 2 days of incubation at 32° and 28° C., plates prepared with pasteurized culture required extended incubation for colony development.

Unheated cultures of Streptococcus sp. FA-F-1 showed no appreciable difference in colony counts at plate incubation temperatures of 35°, 32°, 28° and 21° C. (Table 26). However, colony development for pasteurized cultures was definitely inhibited more at an incubation temperature of 21° C. than at 35°, 32° and 28° C. Colony development at 28° C. appeared to be slower for the refrigerated culture than for the non-refrigerated culture.

Table 27 shows the effect of various bacteriological peptones used in the plating medium upon colony counts of pure cultures of thermoduric bacteria before and after pasteurization. Only in rare instances were differences in colony count of the unheated cultures possibly attributable to differences in the composition of the media employed. However, differences in colony counts of the pasteurized cultures were commonly attributable to differences in bacteriological peptones used in the plating media. This was especially true for cultures of Arthrobacter sp. DO-32-1 and M. varians EM-IV-2.

Pasteurized cultures of M. lacticum DN-32-1 demonstrated no differences in colony counts attributable to variation in media composition. However, as noted earlier, for refrigerated cultures of this organism, the laboratory pasteurized count was appreciably higher than the count for the unheated culture.

Pasteurized cultures of Streptococcus sp. FA-F-1 showed some variation in colony count with the various media. Colony counts of pasteurized cultures of this organism were substantially lower with medium containing Edamin than with the other media. There was some indication that the colony count also was inhibited with the Bacto-Tryptone medium.

The effect of pH of Plate Count Agar upon the colony counts of pure cultures of thermoduric bacteria before and after laboratory pasteurization is shown in Table 28. Unheated cultures of Arthrobacter sp. DO-32-1 produced colonies equally

Table 27. Effect of plating media containing various bacteriological peptones upon the plate count (32° C.) of pure cultures of thermoduric bacteria

Organism	Series ^a	Bacteriological peptone ^b	Raw count ^c per ml.	Laboratory pasteurized count per ml. after plate incubation for		
				2 days	3 days	4 days
----- In thousands -----						
<u>Arthrobacter</u> sp. DO-32-1	1	I	690	400	420	420
		II	740	68	69	69
		III	650	230	230	230
		IV	710	2	2	2
		V	710	110	110	110
		VI	740	26	26	26
		VII	710	86	86	86
	2	I	830	610	610	610
		II	740	28	31	31
		III	740	300	300	300
		IV	740	15	15	15
		V	760	270	300	300
		VI	770	25	25	25
		VII	780	240	240	240
<u>Microbacterium</u> <u>lacticur</u> DN-32-1	1	I	1,500	1,500	1,600	1,600
		II	1,500	1,300	1,500	1,500
		III	1,300	1,500	1,700	1,700
		IV	1,400	1,500	1,600	1,600
		V	1,400	1,600	1,700	1,700
		VI	1,500	1,400	1,600	1,600
		VII	1,400	1,400	1,500	1,500

^aSeries 1 cultures were incubated at 32° C. for 24 hours prior to testing while series 2 cultures were incubated at 32° C. for 24 hours and then refrigerated at 38°-40° F. for 24 hours prior to testing.

^bI, Bacto-Tryptone; II, N-Z-Amine Type AS; III, N-Z-Amine Type YT; IV, Edamin; V, Soy Peptone Powder; VI, N-Z-Case; VII, HY-Case SF.

^cRaw count (at 2 days of plate incubation) did not increase upon extended plate incubation.

Table 27. Continued

Organism	Series ^a	Bacteri- ological peptone ^b	Raw count ^c per ml.	Laboratory pasteurized count per ml. after plate incubation for		
				2 days	3 days	4 days
				----- In thousands -----		
<u>Microbacterium</u> <u>lacticum</u> DN-32-1	2	I	1,700	2,500	2,600	2,600
		II	1,700	2,500	2,600	2,600
		III	1,800	2,500	2,600	2,600
		IV	1,500	2,300	2,400	2,400
		V	1,800	2,200	2,300	2,300
		VI	1,700	2,400	2,400	2,400
		VII	1,600	2,200	2,300	2,400
<u>Micrococcus</u> <u>variens</u> EM-IV-2	1	I	110	<1	32	37
		II	110	31	62	67
		III	120	30	70	73
		IV	100	5	28	29
		V	110	88	100	100
		VI	120	38	79	81
		VII	110	26	53	54
	2	I	180	<1	30	34
		II	170	42	85	88
		III	180	24	58	60
		IV	180	7	49	51
		V	not determined			
		VI	170	65	110	120
		VII	180	51	91	94
<u>Streptococcus</u> sp. FA-F-1	1	I	15,000	7,100	7,300	7,300
		II	15,000	9,000	9,100	9,100
		III	13,000	9,400	9,500	9,500
		IV	15,000	5,900	5,900	5,900
		V	14,000	8,500	8,500	8,500
		VI	14,000	7,900	7,900	7,900
		VII	13,000	6,300	6,300	6,300
	2	I	13,000	5,000	5,100	5,200
		II	13,000	9,000	9,200	9,200
		III	15,000	6,900	7,100	7,200
		IV	13,000	3,300	3,300	3,300
		V	14,000	7,400	7,400	7,400
		VI	13,000	5,300	5,300	5,300
		VII	13,000	5,800	6,200	6,200

Table 28. Effect of pH of Plate Count Agar upon the plate count (32° C.) of pure cultures of thermophilic bacteria

Organism	Series ^a	Plating medium pH	Raw count ^b per ml.	Laboratory pasteurized count per ml. after plate incubation for		
				2 days	3 days	4 days
				In thousands		
<u>Arthrobacter</u> sp. DO-32-1	1	5.6	<10	<1	<1	<1
		6.5	690	100	100	100
		7.0	690	400	420	420
		7.5	700	240	240	240
		8.0	670	96	96	96
		8.6	760	<1	<1	<1
		9.1	<10	<1	<1	<1
	2	5.6	<10	<1	<1	<1
		6.5	710	330	340	340
		7.0	830	610	610	610
		7.5	790	550	550	550
		8.0	720	45	47	47
		8.6	780	<1	<1	<1
		9.1	<10	<1	<1	<1
<u>Microbacterium</u> <u>lacticum</u> DN-32-1	1	5.6	<1	<1	<1	<1
		6.5	1,500	890	1,500	1,600
		7.0	1,500	1,500	1,600	1,600
		7.5	1,400	1,700	1,800	1,800
		8.0	1,400	1,600	1,700	1,700
		8.6	1,400	1,500	1,600	1,600
		9.1	<10	960	1,600	1,700
	2	5.6	<10	<1	<1	<1
		6.5	1,600	700	1,600	1,700
		7.0	1,700	2,500	2,600	2,600
		7.5	1,600	2,300	2,300	2,300
		8.0	1,700	2,300	2,400	2,400
		8.6	1,600	2,300	2,400	2,400
		9.1	100 ^c	1,700	2,400	2,400

^aCultures used in series 1 were incubated at 32° C. for 24 hours prior to testing while cultures used in series 2 were incubated at 32° C. for 24 hours and then refrigerated at 38°-40° F. for 24 hours prior to testing.

^bRaw count (at 2 days of plate incubation) did not increase upon extended plate incubation except where noted.

^cCount increased to 200,000 after 4 days of incubation.

Table 28. Continued

Organism	Series ^a	Plating medium pH	Raw count ^b per ml.	Laboratory pasteurized count per ml. after plate incubation for		
				2 days	3 days	4 days
				----- In thousands -----		
<u>Micrococcus varians</u> EM-IV-2	1	5.6	<1	<1	<1	<1
		6.5	48 ^d	<1	<1	<1
		7.0	110	<1	32	37
		7.5	110	2	37	45
		8.0	110	59	96	98
		8.6	110	15	33	34
		9.1	110	<1	<1	<1
	2	5.6	<1	<1	<1	<1
		6.5	150	<1	<1	<1
		7.0	180	<1	30	34
		7.5	180	3	56	61
		8.0	180	63	100	110
		8.6	190	38	58	60
		9.1	190	<1	<1	<1
<u>Streptococcus</u> sp. FA-F-2	1	5.6	<100	<10	<10	<10
		6.5	14,000	<10	<10	<10
		7.0	15,000	7,100	7,100	7,100
		7.5	14,000	9,400	9,400	9,400
		8.0	14,000	10,000	10,000	10,000
		8.6	14,000	8,800	8,800	8,800
		9.1	14,000	9,500	9,500	9,500
	2	5.6	<100	<10	<10	<10
		6.5	13,000	<10	<10	<10
		7.0	13,000	5,000	5,100	5,200
		7.5	13,000	6,800	6,800	6,800
		8.0	14,000	8,900	9,000	9,000
		8.6	13,000	8,200	8,200	8,200
		9.1	14,000	9,100	9,100	9,100

^dCount increased to 56,000 after 4 days of incubation.

well over a range of pH 6.5 to 8.6. However, colony counts for the pasteurized cultures were decreased substantially above or below pH 7.0.

Unheated cultures of M. lacticum DN-32-1 gave essentially the same colony count with Plate Count Agar at levels of pH 6.5 to 8.6. The 2-day colony counts for pasteurized cultures were considerably less at pH 6.5 than at pH levels of 7.0, 7.5, 8.0 and 8.6. Colony productivity by this organism at pH 9.1 appeared to be enhanced by pasteurization (Table 28).

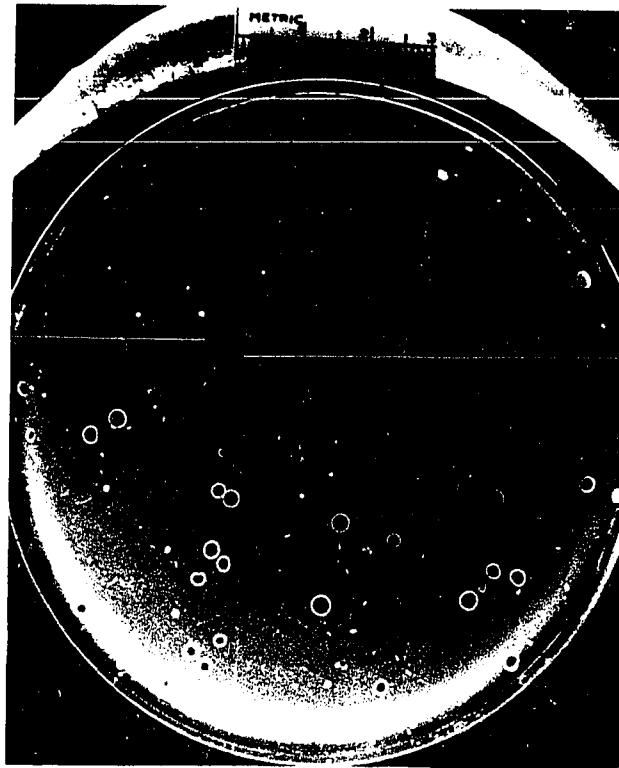
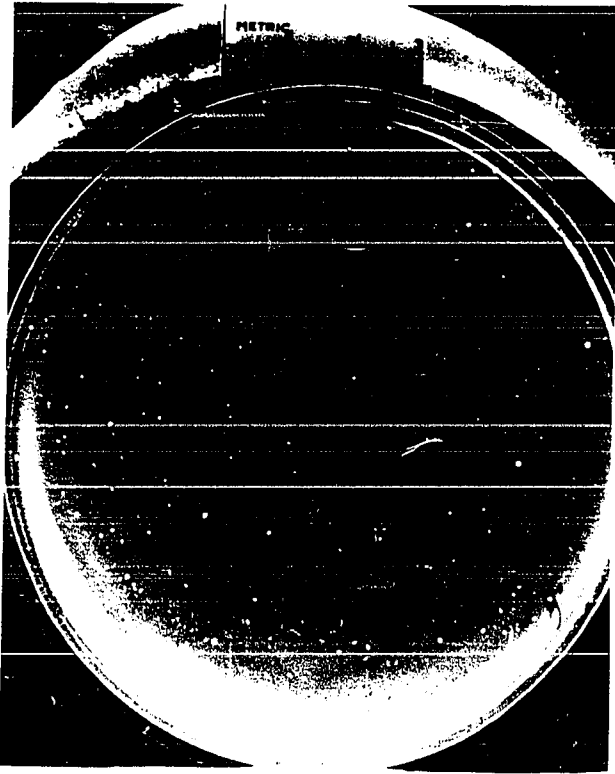
Unheated cultures of M. varians EM-IV-2 showed essentially the same degree of colony development over a range of pH 7.0 to 9.1. With pasteurized cultures of this organism, however, the maximum colony count was obtained at pH 8.0. As the pH of the medium was raised or lowered from pH 8.0, the laboratory pasteurized count decreased noticeably (Table 28).

Unheated cultures of Streptococcus sp. FA-F-2 gave essentially the same degree of colony development over a range of pH 6.5 to 9.1. Pasteurized cultures of this organism, however, failed to produce countable colonies at pH 6.5 and colony development was somewhat suppressed at pH 7.0.

Colony size, as well as colony count, was influenced by pH of the Plate Count Agar. As a general rule, the pH level producing the greatest laboratory pasteurized count for a par-

ticular culture also produced the largest and most easily discernible colonies. Although colony counts for pasteurized cultures of M. lacticum DN-32-1 did not differ appreciably at pH levels of 7.0 to 8.6, colony size varied considerably. Colonies produced at pH 7.0 were generally less than 1.0 mm. in diameter after 3 days of incubation at 32° C. However, with pH levels of 7.5 to 8.6, colonies averaged about 2 mm. in diameter after incubation at 32° C. for 3 days. Figure 1 illustrates the comparative size of colonies produced by M. lacticum DN-32-1 with Plate Count Agar at pH levels of 7.0 and 8.0.

Figure 1. Appearance of colonies of Microbacterium
lacticum on Plate Count Agar of pH 7.0 (top)
and pH 8.0 (bottom) after 3 days of incu-
bation at 32° C.



DISCUSSION

No single medium incubated at a given temperature for a given period of time can be expected to initiate and sustain growth of all bacterial types present in milk. This does not preclude, however, the fact that a plating method for the enumeration of organisms in milk should have the objective of determination of maximum numbers. There is always the question of time versus accuracy and a balance of the two, commensurate with the objectives of the test, should be achieved.

A second and perhaps more important objective of a plating procedure is that it reveal a true picture of the types of viable bacteria in the milk. This does not mean that one should be able to determine the types of bacteria in milk by visual inspection of colonies formed on the solid medium. It simply implies that a plating procedure should afford equal opportunity for all types of viable bacteria in milk to manifest themselves. A plating procedure that would discriminate against a certain group of bacteria commonly found in milk would fall short of this second objective.

A third objective of a plating procedure should be the production of easily discernible and countable colonies. The fact that minute or "pin-point" colonies might be overlooked in counting is a constant source of error. This is especially true in the case of the thermoduric colony count. Many thermoduric bacteria are noted for their production of colonies of

minute size.

This study was not undertaken in an attempt to develop an optimum plating procedure for the enumeration of thermoduric bacteria in milk. On the contrary, its primary purpose was the determination of the effects of certain basic modifications of the plating procedure commonly in use upon the thermoduric plate count. However, the findings of this study should contribute to a better understanding of the physiological nature of thermoduric bacteria and, consequently, aid in the development of a more adequate method for their detection and enumeration in pasteurized milk.

Effect of Time and Temperature of Incubation upon the Thermoduric Plate Count

For the agar plate method of enumerating bacteria in milk, Standard Methods (9) recommends incubation of plates for 2 days at 35° or 32° C. This standard applies for raw, commercially pasteurized and laboratory pasteurized milk. However, results of this study have indicated that incubation temperatures lower than 35° C. and even 32° C. for slightly longer incubation periods may have some distinct advantages for the enumeration of pasteurization resistant bacteria in milk.

A comparison of various plate incubation times and temperatures showed that the average thermoduric colony count obtained at 35° C. for 2 days was only 31 percent of the average count obtained at 28° C. for 4 days. Even at an incubation

temperature of 32° C. for 2 days, the average count was more than double that obtained at 35° C. for 2 days. These results become even more significant when it is noted that of 32 samples examined, 31 gave a higher count at 28° C. for 4 days than at 35° C. for 2 days. Twenty-seven of the 32 samples gave a higher count at 32° C. for 2 days than at 35° C. for 2 days.

Examination of the thermoduric flora of the milk samples revealed that the higher counts obtained at the lower incubation temperatures were the result of certain of the thermoduric bacteria being able to produce colonies much better at the lower temperatures. This was especially true of the highly heat-resistant microbacteria. These organisms commonly accounted for an appreciably larger share of the colony count obtained at 32° and 28° C. than at 35° C. These findings are in accord with those of other workers (27,35,36,86,96) who suggested that large numbers of microbacteria in pasteurized milk probably had been overlooked as a result of incubating plates at 37° C. The results of this study indicate that 35° C. is also too high an incubation temperature for accurate enumeration of microbacteria in pasteurized milk.

Buchanan (22) suggested that where large number of microbacteria are present in milk, plate incubation temperatures lower than 35° C. and possibly even lower than 32° C. may be necessary for maximum estimation of the thermoduric population. Results of this study tend to confirm that an incu-

bation temperature of 28° C. favors colony productivity by microbacteria more than a temperature of 32° C. These organisms accounted for a substantially greater portion of the colony counts obtained at 28° C. than at 32° C.

There was some indication that micrococci and arthro-bacters also contributed to the higher counts obtained with incubation temperatures below 35° C. Although these organisms usually accounted for a major portion of the count obtained at 35° C. for 2 days, they contributed even more substantially to the higher counts obtained at 32° C. for 2 days.

Extending the plate incubation period to 3 or 4 days at 35° and 32° C. resulted in an increased thermoduric count in a majority of cases. This was especially true for milk samples containing lactobacilli or microbacteria. Apparently microbacteria not only prefer incubation temperatures lower than 35° C. but also are slow in developing colonies even at the lower temperatures. An incubation temperature of 30° C. for 3 to 6 days has been advocated by European workers (1,25,29,36,-54) for thermoduric counts. In most cases, microbacteria have been found to constitute 60 to 80 percent of the thermoduric flora growing on plates incubated under these conditions.

Lactobacilli have not been reported to contribute significantly to the thermoduric count of milk. As shown in this study, this could possibly be a result of failure to incubate plates for a sufficient period of time to permit their growth on solid media. The fact that lactobacilli grow poorly under

aerobic conditions and require a rather complex medium for growth has been reported (21,34). In this study, lactobacilli were detected on plates incubated for 3 to 4 days at 35° and 32° C. but not on plates incubated at these temperatures for only 2 days. This would lend credence to the fact that the types of thermoduric bacteria found in milk will depend a great deal upon the plate incubation procedure used in their recovery from the pasteurized product. Unidentified bacteria, showing characteristics dissimilar to those of the more common thermoduric bacteria also contributed substantially to increased counts for some samples upon extended plate incubation at 35° and 32° C. Again, these organisms may have been overlooked by other workers due to failure to incubate plates for a sufficient period of time.

A thermoduric count in excess of 10,000 per ml. has been suggested (16,29,57) as providing evidence of unsatisfactory milk production methods. On the basis of this standard, it is noteworthy that in this study, 45 percent of the samples which met this standard when plates were incubated at 35° C. for 2 days failed to meet the standard when plates were incubated at 28° C. for 3 days. Of the samples showing a thermoduric count of 10,000 per ml. or less at 32° C. for 2 days, 40 percent gave counts in excess of 10,000 per ml. at 28° C. for 3 days. All samples exceeding the standard when plates were incubated at 35° or 32° C. for 2 days also exceeded the standard when plates were incubated at 28° C. for 3 days. Assuming that the

standard of 10,000 per ml. is valid, this would indicate that the lower incubation temperatures would do a better job of detecting milk of poor quality.

The average thermoduric count obtained at 21° C. for 5 days exceeded the average counts obtained at 35° and 32° C. for 4 days. However, 7 days of incubation at 21° C. were necessary for the average count to equal the maximum average count which was obtained at 28° C. for 4 days. Of 32 samples examined, 21 gave counts at 28° C. for 4 days equal to or higher than those obtained at 21° C. for 7 days. However, in no case was the difference between counts obtained at 28° C. for 4 days and 21° C. for 7 days of extreme magnitude. Although incubation at 21° C. might have some advantage over incubation at 35° or 32° C., the results of this study indicate that it would offer no real advantage over 28° C. incubation.

It is noteworthy that an appreciable portion of the thermoduric bacteria present in the milk samples included in this study were capable of forming colonies on Plate Count Agar at an incubation temperature of 10° C. if the incubation period was of sufficient length. Notable among these bacteria were those belonging to the genera Arthrobacter, Microbacterium and Micrococcus. However, an incubation period of 21 to 28 days at 10° C. was necessary for any appreciable colony development. Even after 28 days of incubation at 10° C. the average colony count of 25 samples was only 42.6 percent of the maximum aver-

age count obtained at 28° C. for 4 days.

Incubation of plates at 5° C. revealed that thermoduric bacteria rarely formed colonies in less than 21 days at this temperature. These results indicate that thermoduric bacteria do not ordinarily possess psychrophilic properties. This is in agreement with the observation of Thomas et al. (94). These authors found that thermoduric bacteria multiplied slowly in milk held at 3° to 5° C.

Of the thermoduric bacteria present in milk examined in this study, spore-bearing rods were the only ones indicating a preference for an incubation temperature of 35° C. over lower temperatures. However, these organisms failed to appear in large numbers in any of the samples tested. They would sometimes account for a major portion of the thermoduric flora of samples showing a thermoduric count of less than 3,000 per ml. However, they failed to contribute appreciably to the thermoduric flora of milk showing a thermoduric count in excess of 3,000 per ml. These results support reports by other workers (1,35,36) that only a small proportion of spore-bearing rods are found in milk having a high thermoduric count.

Buchanan (22) observed that spore-bearing rods were seldom the cause of high thermoduric counts. He suggested that their improved growth after laboratory pasteurization on plates incubated at 35° C. rather than 32° C. was possibly due to their average optimum growth temperature being closer to 35° C. than 32° C. Another possible explanation for this would be that the

higher temperature is more conducive to spore germination and consequent colony formation.

Effect of Various Bacteriological Peptones in the Plating Medium upon the Thermoduric Plate Count

Standard Methods (9) recommends a pancreatic digest of casein conforming to the specifications outlined in the Manual of Microbiological Methods (85) for the preparation of milk plating medium. Bacto-Tryptone, supplied by Difco Laboratories, Inc. (26), is perhaps one of the most commonly employed bacteriological peptones. This bacteriological peptone along with six others of varying types (Appendix) were used in the preparation of plating media to determine their effect upon the thermoduric plate count.

Due to their extremely complex nature, it would be difficult to evaluate the suitability of bacteriological peptones for bacterial growth on the basis of chemical analysis alone. However, it has been suggested (81) that the nutritive value of various peptones for microorganisms in milk is directly related to their amino nitrogen content. The amino nitrogen content of the bacteriological peptones included in this study ranged from 1.8 percent for Soy Peptone Powder to 6.9 percent for Edamin. Results for 25 milk samples showed that the highest average thermoduric count was obtained with medium prepared with Soy Peptone Powder which contained the least amino nitrogen. In contrast, the average thermoduric count

obtained with medium prepared with Edamin was appreciably less than that obtained with all other media except for the one containing Bacto-Tryptone. Considering that the only variation among the media was the type of peptone used in preparation, these results indicate that the value of bacteriological peptones for growth of thermoduric bacteria cannot be established on the basis of amino nitrogen content alone.

Of the seven media, the one prepared with the standard Bacto-Tryptone gave the lowest average thermoduric colony count. However, it should be noted that the average counts obtained with the various media showed a rather narrow range of variation of from 63,000 to 88,000 per ml. Nevertheless, for certain samples of milk, the thermoduric counts obtained with media prepared with Bacto-Tryptone and Edamin were substantially lower than counts obtained with other media. These discrepancies apparently were due primarily to the inability of some of the thermoduric streptococci to produce colonies on the Bacto-Tryptone and Edamin media.

The thermoduric microbacteria, micrococci and spore-bearing rods apparently grew equally well on all media prepared with the various peptones. However, bacteria of the genus Arthrobacter failed to produce colonies as well on the medium containing N-Z-Amine Type AS as with the other media.

It is appreciated that the bacteriological peptones included in this study represent but a small portion of the possible number of peptones that could be utilized in the pre-

paration of plating media. However, the results obtained with this limited number of peptones illustrate their important role in milk plating media. Perhaps of greatest significance is the observation that some peptones commonly employed in plating medium may fail entirely to support growth of certain types of thermoduric bacteria in milk. If certain groups of thermoduric bacteria, such as streptococci, go undetected in pasteurized milk, then the thermoduric plate count has lost much of its meaning.

As indicated earlier, one of the important objectives of a plating medium is the production of discernible and easily countable colonies. Colonies of minute size might be inadvertently overlooked in the counting process. Generally, the media producing the highest counts in this study also produced the largest sized colonies. Colonies produced on media containing Bacto-Tryptone and Edamin were as a rule noticeably smaller than those produced on the other media.

Effect of the pH of the Plating Medium upon the Thermoduric Plate Count

The current edition of Standard Methods (9) recommends a pH of 7.0 ± 0.1 for the plating medium used in determining the Standard Plate Count of milk. This pH level is recommended for the examination of raw, commercially pasteurized and laboratory pasteurized milk. Results of this study, however, have indi-

cated that a pH of 7.0 for Plate Count Agar is suboptimal for colony production by most thermoduric bacteria.

As a rule, the maximum thermoduric counts were obtained with Plate Count Agar adjusted to pH 7.5 and 8.0. The reason for the higher counts at these pH levels can be explained partially by the increased growth of the thermoduric streptococci. As the pH of the plating medium was progressively raised from pH 6.5 to 9.1, the portion of the mean colony count attributable to streptococci also increased. However, at levels above pH 8.0 and, in some cases, pH 7.5 the growth of certain other thermoduric bacteria was inhibited as evidenced by a decrease in total colony count.

Thermoduric bacteria of the genus Arthrobacter apparently were primarily responsible for decreases in count obtained at levels above pH 7.5. Of all the thermoduric bacteria isolated in this study, only those of the Arthrobacter genus showed a definite preference for a medium of pH 7.0 to 7.5. There were some indications that certain of the thermoduric micrococci preferred a neutral medium rather than an alkaline one. However, this group of bacteria exhibited no definite preference and often times colony productivity was as prolific at pH 7.5 to 8.6 as at pH 7.0.

An increase in the thermoduric plate count was usually obtained when the reaction of the plating medium was elevated from pH 7.0 to pH 7.5. On the other hand, lowering the pH to 6.5 almost always resulted in a substantial decrease in thermo-

duric count from that obtained at pH 7.0. Using a level of pH 7.0 as the basis, the average increase in count obtained at pH 7.5 was considerably less than the average decrease in count obtained at pH 6.5. This observation offers further support in favor of the use of a slightly alkaline medium for obtaining a more nearly maximal thermoduric count of milk.

The results of this study tend to confirm observations made by a limited number of other investigators with respect to the influence of the pH of the medium upon the plate count of pasteurized milk. Cooledge, as cited by Fay (31, p. 352), suggested that the formation of pin-point colonies by thermoduric bacteria was associated with the reaction of the plating medium. He plated the same sample of milk on two media with reactions of pH 6.6 and 7.3 and obtained counts, respectively, of 15,400 and 317,000 per ml. Wilson et al. (103) plated raw and pasteurized milk samples on Yeastrel Milk Agar adjusted to pH levels of 6.0, 6.8 and 7.6. They observed that a medium adjusted to pH 6.0 was too acid for both raw and pasteurized milk. A medium having a pH of 6.8 was found to be more favorable for plating raw milk than one adjusted to pH 7.6, but for pasteurized milk a medium of pH 7.6 was more favorable than one adjusted to pH 6.8.

The fact that the pH of the plating medium influenced the size of individual colonies as well as the number of colonies appearing on the medium should not be overlooked. As noted

earlier, the accuracy of a plating procedure is determined not only by its ability to recover the maximum number of viable bacteria in a product but also by its ability to produce distinct and easily countable colonies. As a general rule, the pH level displaying the highest thermoduric colony count for a particular sample also produced colonies of the largest size. This relationship held true especially for those samples containing thermoduric streptococci. The thermoduric count for some samples was not influenced appreciably by variance in pH of the plating medium. For these samples, however, the most easily discernible colonies were usually produced at levels above pH 7.0. This was most notable with samples containing microbacteria.

Effect of the Various Plating Procedures upon Colony
Counts of Pure Cultures of Thermoduric Bacteria
Before and After Laboratory Pasteurization

In the previous portion of this study no attempt was made to compare the effects of the various plating procedures upon the plate count of milk before and after laboratory pasteurization. Since the bulk of the bacterial flora of most milk is usually of a non-thermoduric nature, such a comparison would have revealed little with respect to the growth characteristics of thermoduric bacteria prior to heating. In this portion of the study the effects of the various plating procedures upon the plate count of non-pasteurized and pasteurized

cultures of thermoduric bacteria were compared. The thermoduric cultures selected were representative of the predominant genera of thermoduric bacteria found in the milk examined in this study. It is recognized that the cultures selected might not show identically the same reactions as other members of their respective genus. However, they should typify, in a general manner, the characteristics of other thermoduric members of their genus.

Time and temperature of plate incubation

The results indicate that thermoduric bacteria generally are more exacting in their growth temperature requirements after they have been subjected to laboratory pasteurization than prior to heat treatment. This was found to be true for cultures of Arthrobacter sp., Micrococcus varians and Streptococcus sp. These bacteria grew well over a rather wide temperature range before pasteurization. After subjection to laboratory pasteurization they exhibited a definite preference for a much narrower temperature range.

These results help to explain why earlier investigations (45,53,95) showed that lowering the temperature of plate incubation from 37° to 32° or 30° C. resulted in a greater percentage increase in count with pasteurized milk than with raw milk. An apparent explanation is that certain of the thermoduric bacteria in the raw milk grew equally well at all temperatures. However, after being subjected to pasteurization,

colony productivity was favored more at the lower incubation temperatures.

Comparative studies (18,88) of plate counts of raw milk as well as pasteurized milk following plate incubation at 37°, 35° and 32° C. for 48 hours have shown that counts were somewhat higher at 35° C. and still slightly higher at 32° C. As revealed by this study, the increase in colony count at the lower temperatures resulted from the failure of certain bacteria to produce colonies at the higher temperatures. Notable among these is Microbacterium lacticum. A culture of this organism failed to grow at 35° C. before as well as after laboratory pasteurization. However, excellent colony productivity was exhibited by both unheated and pasteurized cultures of this organism at a plate incubation temperature of 32°C.

Although micrococci as a rule were found to grow fairly well at 35° C. throughout this study, there are apparently some exceptions to this general rule. Colony productivity by unheated and pasteurized cultures of Micrococcus varians was favored by an incubation temperature of 32° C. in contrast to 35° C. These results are in agreement with observations made by Buchanan (22). He found that some of the micrococci, particularly M. varians, grew better at 32° C. than at 35° C. after laboratory pasteurization.

The same plate incubation period of 48 hours is specified in Standard Methods (9) for both raw and pasteurized milk. Results of this study indicate that incubation in excess of 2

days is necessary for colony formation by certain of the pasteurization-resistant bacteria. The maximum colony count for an unheated culture of M. varians was obtained at 2 days of incubation at 32° and 28° C. However, colonies did not appear on plates prepared with a pasteurized culture of this organism at 2 days of incubation at 32° or 28° C. Appreciable colony formation was evident only after 3 days of incubation.

Hussong and Hammer (48) observed that the count obtained for some milk samples after laboratory pasteurization was higher than that obtained initially. They did not attempt to identify the bacterial flora of these samples but indicated that the increases in count could not be interpreted as indicating growth during pasteurization. As shown in the present study, the highly heat-resistant flora encountered by these workers could very well have been composed largely of M. lacticum. Not only were cultures of this organism found to be highly heat resistant but occasionally demonstrated higher colony counts after laboratory pasteurization than initially. Growth of M. lacticum cultures during pasteurization was ruled out by virtue of their failure to produce colonies on Plate Count Agar at an incubation temperature of 35° C.

Robertson (76) isolated nine cultures of M. lacticum from milk. All of these cultures survived pasteurization at 145° F. for 30 minutes and several determinations of percentage survival showed increases of 10 to 120 percent. Growth was not observed when laboratory strains of these organisms were inocu-

lated into sterile skimmilk and held at pasteurizing temperatures. An attempt to explain the results by assuming that clumps of cells were broken up sufficiently to cause the entire percentage increase following pasteurization was unsatisfactory. As shown in this study, if laboratory cultures had been refrigerated prior to pasteurization, disintegration of clumps during pasteurization might have been more evident.

Type of bacteriological peptone contained in the plating medium

The results indicate that thermoduric bacteria are generally more exacting in the type of bacteriological peptone required for growth after being subjected to laboratory pasteurization than prior to pasteurization. This was found to be true especially for cultures of Arthrobacter sp., Micrococcus varians and Streptococcus sp. Prior to pasteurization, cultures of these bacteria grew equally well on all media prepared with the seven peptones (Appendix) examined in this study. Following pasteurization, however, the organisms grew much better on certain of the media than on others.

The relative suitability of the different media for determining viable numbers after laboratory pasteurization varied according to the particular organism employed. For example, laboratory pasteurized cultures of Arthrobacter sp. exhibited maximum colony productivity on the medium which contained Bacto-Tryptone. In contrast, laboratory pasteurized cultures of M. varians exhibited minimum colony production on this medi-

um. These results would indicate that if maximum viable population of pasteurized milk is to be determined, plating medium must be more adequate than that currently recommended.

These results also indicate that the relative heat resistance of a given organism cannot always be accurately determined by plating on a single medium. The common definition of thermoduric bacteria, "bacteria that survive pasteurization in considerable numbers," becomes meaningless if the plating medium employed in determining degree of survival is not adequate for supporting growth of heat-treated bacteria. To illustrate this point, a pasteurized culture of M. varians showed survival of less than 1 percent when plated with medium prepared with the recommended Bacto-Tryptone. When plated with medium prepared with Soy Peptone Powder, however, this same culture showed a pasteurization survival of 80 percent. Additional examples of similar results obtained with pure cultures of other thermoduric bacteria could be cited.

The observation that heat-treated bacteria are more exacting in their requirements for initiation of growth than are bacteria in their normal state cannot be overemphasized. It is noteworthy that apparently few investigators have taken cognizance of this when studying the pasteurization-resistant flora of milk or in formulating media for making plate counts on pasteurized dairy products. As a rule, the same medium has been employed for determining the viable numbers of bacteria

in both raw and pasteurized milk.

The observations made in this study are in accord with those of Nelson (64). Although not restricting his studies to thermoduric bacteria, this author noted that bacteria which had been subjected to heat at partially lethal levels were more demanding in their requirements of media for growth than were unheated control organisms. He concluded that this should be considered in the formulation of media for the enumeration of bacteria in heated food products and in experiments concerned with the effect of heat upon microorganisms.

Reaction (pH) of the plating medium

The results indicate that thermoduric bacteria after being subjected to laboratory pasteurization are more exacting in their medium pH requirements than prior to pasteurization. Unheated cultures of a strain of M. varians, for example, gave essentially the same count with Plate Count Agar at pH levels from 7.0 to 9.1 after 2 days of incubation. After being subjected to laboratory pasteurization, however, this strain failed to produce colonies at 2 days of incubation with Plate Count Agar of pH 7.0 and 9.1 and exhibited a pronounced maximum count at pH 8.0.

These observations, in general, agree with those of Nelson (66). After studying the effect of sub-lethal heat treatment on several bacteria of a non-thermoduric nature, he concluded that unheated cultures grew over a much wider pH

range than did the heated cultures. The only exception to this general rule found in the present study was the result obtained with a strain of Microbacterium lacticum. Colony productivity by unheated cultures of this organism was definitely inhibited on Plate Count Agar at pH 9.1. However, after the cultures were subjected to laboratory pasteurization, colonies were produced quite well at pH 9.1. No explanation can be offered for this occurrence. However, it appears logical that the process of heating altered the character of the organism in such a manner as to render it more tolerant to the higher pH. This points out the apparent need for additional work with respect to the effects of heat upon the physiological characteristics of thermoduric bacteria.

As mentioned previously, the current edition of Standard Methods (9) recommends that the plating medium used for the enumeration of bacteria in milk have a pH of 7.0 ± 0.1 . This standard applies for the examination of both raw and pasteurized milk. Results of this study have indicated that a pH level of 7.0, while being adequate for the enumeration of bacteria in milk prior to pasteurization, is sub-optimal for maximum colony production by some of the thermoduric bacteria after being subjected to pasteurization. This should be considered in the preparation of media for the enumeration of bacteria in pasteurized products and in experiments concerned with the effect of heat upon microorganisms in dairy products.

SUMMARY AND CONCLUSIONS

The effect of the plate incubation temperature, length of the incubation period, pH of the plating medium and type of bacteriological peptone included in the plating medium upon the enumeration of pasteurization-resistant bacteria in milk was studied. The effect of these various factors upon the detection of specific types of thermoduric bacteria as well as their effect upon quantitative enumeration of this group of organisms was determined. The effect of the various plating procedures upon the recovery of pure cultures of some thermoduric bacteria before and after laboratory pasteurization also was examined. A total of 76 samples of raw milk, representing can and bulk cooled manufacturing grade and bulk cooled grade A supplies, were included in the study. A total of 1272 pure cultures of pasteurization-resistant bacteria were isolated and classified to genus and in some cases to species.

Of the plate incubation temperatures of 35°, 32°, 28° and 21° C., incubation at 28° C. for 4 days was found, on the average, to be the optimum for determining the maximum bacterial population of laboratory pasteurized milk. The mean thermoduric colony counts obtained after 2 days of incubation at 35° and 32° C. were 31.0 and 73.7 percent, respectively, of the mean count obtained after 4 days of incubation at 28° C. Colony counts tended to increase upon extended plate incubation at 35° and 32° C., but even after 4 days of incubation they

were significantly lower ($P < 0.01$) than counts obtained at 28° C. for 4 days. The mean thermoduric colony count obtained at 21° C. for 7 days equalled that obtained at 28° C. for 4 days. Pasteurization-resistant bacteria formed colonies slowly on plates incubated at 10° C. with the mean count after 28 days of incubation being only 42.6 percent of the mean count obtained at 28° C. for 4 days. Extremely slow colony formation on plates incubated at 5° C. indicated that thermoduric bacteria do not, as a rule, possess psychrophilic properties.

Colony production on Plate Count Agar by the thermoduric microbacteria was notably inhibited at an incubation temperature of 35° C. Colony productivity by these bacteria was increased at 32° C. and was further increased at an incubation temperature of 28° C. There was some indication that colony production by bacteria of the genera Arthrobacter and Micrococcus was favored more at incubation temperatures of 32° and 28° C. than at 35° C. Pasteurization-resistant lactobacilli were able to produce colonies on Plate Count Agar only after 3 to 4 days of incubation at 35° and 32° C. In addition to the lactobacilli, microbacteria and micrococci also contributed appreciably to increases in thermoduric colony counts upon extended plate incubation at 35° and 32° C.

The type of bacteriological peptone employed in the plating medium materially influenced the thermoduric colony count in several instances. The effect was most pronounced with those milk samples containing appreciable numbers of

thermoduric streptococci. Bacto-Tryptone, a recommended peptone for use in the preparation of Plate Count Agar, was found to be deficient for the initiation of colony development by thermoduric streptococci. Plating medium containing Edamin, a lactalbumin hydrolysate, failed entirely to support growth of these organisms. Thermoduric streptococci produced colonies quite well on media prepared with five other bacteriological peptones. No relationship could be established between the colony productivity and amino nitrogen content or other characteristics of the various bacteriological peptones employed in the plating media.

As a rule, thermoduric bacteria were more tolerant of pH levels above than below 7.0. The maximum mean thermoduric colony count was obtained with Plate Count Agar adjusted to pH 7.5. The mean count obtained at pH 8.6 was only slightly less than that obtained at pH 7.0 and considerably greater than that obtained at pH 6.5. Thermoduric bacteria of the genus Arthrobacter accounted for approximately equal portions of the isolates from Plate Count Agar at pH levels of 6.5, 7.0 and 7.5. However, colony development by these organisms was restricted at pH 8.6 and entirely absent at pH 9.1. Micrococci comprised an appreciable portion of the mean thermoduric colony count obtained with Plate Count Agar at all pH levels. As the pH progressively increased from 6.5 to 9.1, the proportionate

share of the mean thermoduric colony count attributable to streptococci at each pH level also increased. The greatest increase in the contribution of streptococci to the thermoduric colony count occurred with an increase in pH from 7.0 to 7.5.

The pH and type of bacteriological peptone used in formulation of the plating medium influenced the colony size as well as the thermoduric colony count. Although there were some exceptions, those media yielding the highest count generally produced the largest and most easily discernible colonies.

Thermoduric cultures of Arthrobacter sp., Micrococcus varians and Streptococcus sp. grew over a much wider temperature and pH range prior to than after being subjected to laboratory pasteurization. No appreciable differences in colony counts were observed when the unheated cultures were plated with media prepared with various bacteriological peptones. After laboratory pasteurization, however, colony counts obtained with the various media differed substantially.

The same plating procedure is currently recommended for the enumeration of bacteria in both raw and pasteurized milk. Results of this study have indicated that certain of these standards, while being adequate for the enumeration of bacteria in milk prior to pasteurization, may be sub-optimal for

detection of the maximum viable population of pasteurized milk. Thermoduric bacteria which have been subjected to pasteurization are more exacting in their growth requirements than are unheated bacteria. This should be considered in the preparation of media for the enumeration of bacteria in pasteurized products and in experiments concerned with the effect of heat upon bacteria in dairy products.

BIBLIOGRAPHY

1. Abd-El-Malek, Y. Further studies on the bacterial flora of pasteurized milk. Proc. Soc. Agr. Bacteriologists 1943:23-27. 1943.
2. Abd-El-Malek, Y. and Gibson, T. Studies in the bacteriology of milk. I. The streptococci of milk. J. Dairy Research 15:233-248. 1948.
3. Abd-El-Malek, Y. and Gibson, T. Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. J. Dairy Research 15:249-260. 1948.
4. Abele, C. A. Results of bacterial plate counts of milk on three media and two temperatures of incubation. Am. J. Public Health 29:821-846. 1939.
5. American Public Health Association, Inc. Standard methods for the examination of dairy products. 7th ed. New York, N. Y. Am. Public Health Assn. 1939.
6. American Public Health Association, Inc. Standard methods for the examination of dairy products. 8th ed. New York, N. Y. Am. Public Health Assn. 1941.
7. American Public Health Association, Inc. Standard methods for the examination of dairy products. 9th ed. New York, N. Y. Am. Public Health Assn. 1948.
8. American Public Health Association, Inc. Standard methods for the examination of dairy products. 10th ed. New York, N. Y. Am. Public Health Assn. 1953.
9. American Public Health Association, Inc. Standard methods for the examination of dairy products. 11th ed. New York, N. Y. Am. Public Health Assn. 1960.
10. Anderson, E. B. and Meanwell, L. J. Studies in the bacteriology of low-temperature pasteurization. Part I. The significance of heat-resisting organisms in raw milk supplies. J. Dairy Research 4:213-225. 1933.
11. Astell Roll Tube Equipment. Lab. Practice 6:635-640. 1957.
12. Atherton, H. V., Doan, F. J. and Watrous, G. H., Jr. Changes in the bacterial population and characteristics of bottled market milk during refrigeration holding. Pa. Agr. Expt. Sta. Bull. 575. 1954.

13. Ayers, S. H. and Mudge, C. S. Milk powder agar for the determination of bacteria in milk. *J. Bacteriol.* 5:565-588. 1920.
14. Babel, F. J., Collins, E. B., Olson, J. C., Peters, I. I., Watrous, G. H. and Speck, M. L. The standard plate count of milk as affected by the temperature of incubation. *J. Dairy Sci.*, 38:499-503. 1955.
15. Barkworth, H. and Davis, J. G. A cheap and efficient medium for the plate count of milk. *J. Hyg.* 42:218-223. 1942.
16. Barnum, H. J. Laboratory pasteurization test to detect thermodurics. *Am. Milk Review and Milk Plant Monthly* 21 (2):110-111, 122-123. 1959.
17. Black, L. A. Current investigations of laboratory methods used in milk and food utensil sanitation. *J. Milk and Food Technol.* 11:5-12, 34. 1948.
18. Black, L. A. Effects of contemplated changes in standard methods. *J. Milk and Food Technol.* 11:200-205. 1948.
19. Bowers, C. S. and Hucker, G. J. The composition of media for the bacteriological analysis of milk. N. Y. (Geneva) *Agr. Expt. Sta. Tech. Bull.* 228. 1935.
20. Bradfield, A. Bacterial content of milk as affected by the use of different plating methods. *Vt. Agr. Expt. Sta. Bull.* 417. 1937.
21. Breed, R. S., Murray, E. G. D. and Smith, N. R. *Bergey's manual of determinative bacteriology.* 7th ed. Baltimore, Md. The Williams and Wilkins Co. 1957.
22. Buchanan, R. A. Thermoduric microorganisms in bulk-tank farm milk in Iowa. Unpublished M.S. Thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1960.
23. Clegg, L. F. L., Egdell, J. W., Thomas, S. B., McKenzie, D. A. and Cuthbert, W. A. Thermoduric organisms in milk. Part II. Preliminary studies on testing techniques. *Proc. Soc. Appl. Bacteriol.* 13:124-131. 1950.
24. Curtis, L. R. and Hileman, J. L. A comparison of media and incubation temperatures for making bacteria counts of spray-process dry skim milk. *Food Research* 2:73-88. 1937.

25. Cuthbert, W. A., Thomas, S. B., Egdell, J. W., Nichols, A. A. and Cox, C. P. Thermoduric organisms in milk. Part IV. Colony counts of thermoduric organisms obtained with different incubation times at 30°. *J. Appl. Bacteriol.* 17:182-189. 1954.
26. Difco Laboratories, Inc. *Difco manual of dehydrated culture media and reagents.* 9th ed. Detroit, Mich. Difco Laboratories, Inc. 1953.
27. Doetsch, R. N. and Pelczar, M. J., Jr. The microbacteria. I. Morphological and physiological characteristics. *J. Bacteriol.* 56:37-49. 1948.
28. Egdell, J. W. and Bird, E. R. Some aspects of the bacteriological quality of pasteurized and heat treated milk in the West of England. *J. Soc. Dairy Technol.* 2: 144-148. 1949.
29. Egdell, J. W., Thomas, S. B., Clegg, L. F. L. and Cuthbert, W. A. Thermoduric organisms in milk. Part III. Provisional standard technique for the laboratory pasteurization test for milk, and rinses and swabs of dairy equipment, with suggestions for interpretation of results. *Proc. Soc. Appl. Bacteriol.* 13:132-134. 1950.
30. Egdell, J. W., Thomas, S. B., Cuthbert, W. A., Jones, G. E. and Hosking, Z. D. Thermoduric organisms in milk. Part V. A simplified testing technique. *J. Appl. Bacteriol.* 19:256-263. 1956.
31. Fay, A. C. Thermo-tolerant organisms as a cause of so-called pin-point colonies. *J. Bacteriol.* 13:347-377. 1927.
32. Fay, A. C. and Howard, A. W. A comparison of the results obtained from incubating bacteriological plates at 32° C. and 37° C. on the bacterial counts of milk. *J. Dairy Sci.* 1069-1072. 1940.
33. Fischer, J. B. and Johns, C. K. A comparison of various methods for detecting thermoduric bacteria in milk. *J. Milk Technol.* 5:269-275. 1942.
34. Foltz, V. D. and Bushnell, L. D. Growth of certain organisms of importance to the dairy industry on new standard milk agar. *J. Dairy Sci.* 23:973-975. 1940.

35. Foster, E. M., Nelson, F. E., Speck, M. L., Doetsch, R. N., and Olson, J. C., Jr. Dairy microbiology. Englewood Cliffs, New Jersey. Prentice-Hall, Inc. 1957.
36. Gibson, T. and Abd-El-Malek, Y. The effect of low temperature pasteurization on the bacterial flora of milk. Proc. Soc. Agr. Bacteriologists 1940:19-22. 1940.
37. Gibson, T. and Abd-El-Malek, Y. The micrococci of milk. Proc. Soc. Agr. Bacteriologists 1941:31. 1941.
38. Glenn, W. E. and Olson, H. C. Sources, heat-resistance and growth at refrigeration temperatures of coliforms in pasteurized milk. Milk Dealer 48(6):54, 86, 88, 90, 92. 1959.
39. Harrison, J. Numbers and types of bacteria in cheese. Proc. Soc. Agr. Bacteriologists 1938:12-14. 1938.
40. Heinemann, B. and Rohr, M. R. A bottle agar method for bacterial estimates. J. Milk and Food Technol. 16:133-135. 1953.
41. Heinemann, P. G. and Glenn, T. H. A comparison of practical methods for determining the bacterial content of milk. J. Infect. Diseases 5:412-420. 1908.
42. Hileman, J. L. Thermoduric bacteria in milk. A review of literature. J. Dairy Sci. 23:1142-1160. 1940.
43. Hileman, J. L. and Leber, H. High-temperature, short-time pasteurization and its practical application to the dairy industry. J. Milk Technol. 4:128-137. 1941.
44. Hileman, J. L., Leber, H. and Speck, M. L. Thermoduric bacteria in pasteurized milk. II. Studies of the bacteria surviving pasteurization, with special reference to high-temperature, short-time pasteurization. J. Dairy Sci. 24:305-315. 1941.
45. Hileman, J. L., Moss, C. and Stead, B. Thermoduric bacteria in milk. III. The effect of changing agar and temperature of incubation for plate counts on the problem of thermoduric bacteria in milk. J. Dairy Sci. 24:799-806. 1941.
46. Hook, A. E. and Fabian, F. W. Chemical and bacteriological studies on peptones. Mich. Agr. Expt. Sta. Tech. Bull. 185. 1943.

47. Hucker, G. J. Studies on the Coccaceae. VIII. A study of the cocci resisting pasteurization temperatures. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull. 134. 1928.
48. Hussong, R. and Hammer, B. W. The pasteurization efficiencies secured with milk from individual farms. Iowa Agr. Expt. Sta. Bull. 286. 1931.
49. Iyengar, M. K. K., Laxminarayana, H. and Iya, K. K. Studies on the heat-resistance of some streptococci. Indian J. Dairy Sci. 10*90-99. 1957.
50. Johns, C. K. Applications and limitations of quality tests for milk and milk products. A review. J. Dairy Sci. 42:1625-1650. 1959.
51. Johns, C. K. and Berzina, I. The bacto-strip technique for making counts of bacteria, molds and yeast. J. Milk and Food Technol. 20:345-347. 1957.
52. Kaufmann, O. W., Harmon, L. G., Pailthorp, O. C. and Pflug, I. J. Effect of heat treatment on the growth of surviving cells. J. Bacteriol. 78:834-838. 1959.
53. Kelly, E. Report of collaborator on cooperative work with proposed changes in medium and temperature of incubation. Proc. Intern. Assn. Milk Dealers. Lab. Sect. 29:50-75. 1936.
54. Madelung, P. Research on tests for content of thermoduric organisms in bulk milk. Nord. Mejeritidsskr. 24(8): 137-138, 140. 1958. (Original not available for examination; abstracted in Dairy Sci. Abstracts 21:420. 1959.)
55. Mallmann, W. L. and Bryan, C. S. Use of thermoduric bacteria test with special reference to a new rapid method. Proc. Intern. Assn. Milk Dealers 35:175-188. 1943.
56. Mallmann, W. L., Bryan, C. S. and Fox W. K. A new microscopic procedure for detecting and locating the source of thermoduric organisms in milk. J. Milk Technol. 4:195-199. 1941.
57. McKenzie, D. A., Druce, R. G. and Lambert, J. Routine resazurin test results and thermoduric content of milk samples as a measure of milk production conditions. Proc. Soc. Appl. Bacteriol. 13:19-25. 1950.
58. McKenzie, D. A. and Morrison, M. The occurrence of thermoduric organisms in raw milk. Proc. Soc. Appl. Bacteriol. 1945:26-27. 1945.

59. McKenzie, D. A., Morrison, M. and Lambert, J. Further studies of thermoduric bacteria in milk. *Proc. Soc. Appl. Bacteriol.* 1946:37-39. 1946.
60. Myers, R. P. and Pence, J. A. A simplified procedure for the laboratory examination of raw milk supplies. *J. Milk Technol.* 4:18-22. 1941.
61. Nashif, S. A. and Nelson, F. E. Some studies on micro-bacteria from Iowa dairy products. *Appl. Microbiol.* 1: 47-52. 1953.
62. Nelson, F. E. The effect of the new standard agar on the plate counts of dairy products. *J. Dairy Sci.* 22:421-422. 1939.
63. Nelson, F. E. The effect of the new standard milk agar on the plate count of dairy products. *J. Bacteriol.* 39: 263-272. 1940.
64. Nelson, F. E. Factors which influence the growth of heat-treated bacteria. I. A comparison of four agar media. *J. Bacteriol.* 43:395-403. 1943.
65. Nelson, F. E. Factors which influence the growth of heat-treated bacteria. II. Further studies on media. *J. Bacteriol.* 48:473-477. 1944.
66. Nelson, F. E. The influence of the pH of the plating medium upon enumeration of heat-treated bacteria. *Soc. Am. Bacteriologists. Bacteriological Proc.* 1956:40. 1956.
67. Nelson, F. E. and Baker, M. P. The influence of time and temperature of plate incubation upon bacterial counts of market milk and related products, particularly after holding under refrigeration. *J. Milk and Food Technol.* 17: 95-100. 1954.
68. Niven, C. F., Jr., Smiley, K. L. and Sherman, J. M. The hydrolysis of arginine by streptococci. *J. Bacteriol.* 43:651-660. 1942.
69. Parfitt, E. H. Some bacteriological problems involved in high-temperature short-time pasteurization. *Annual Report N. Y. State Assn. Dairy and Milk Inspectors* 13:195-219. 1939.
70. Pederson, C. S. and Yale, M. W. Effect of temperature of incubation upon agar plate count of milk. *Am. J. Public Health* 24:477-484. 1934.

71. Pederson, C. S., Yale, M. W. and Eglinton, R. Temperature variations in bacteriological incubators. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull. 213. 1933.
72. Pelczar, M. J., Jr. and Brown, J. H. Synthetic culture media for reference in dairy bacteriology. J. Milk and Food Technol. 14:90-91, 97. 1951.
73. Pessin, V. and Black, L. A. A comparative study of six agars proposed for bacterial plate counts of milk. J. Milk and Food Technol. 14:98-102. 1951.
74. Pessin, V. and Robertson, A. H. Observations on the colony productivity of six milk plating media. J. Milk and Food Technol. 15:104-109. 1952.
75. Quin, J. D. and Burgwald, L. H. High short holding and low long holding. Milk Plant Monthly 22(2):26-31. 1933.
76. Robertson, A. H. Thermophilic and thermoduric microorganisms with special reference to species isolated from milk. III. Description of the non-spore-forming thermoduric organisms isolated. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull. 131. 1927.
77. Rowlands, A. and Provan, A. L. Pasteurized milk. III. The relationship between the colony count at various temperatures and keeping quality. Proc. Soc. Agr. Bacteriologists 1939:23-26. 1939.
78. Safford, C. E. and Stark, C. N. Advantages of a skim-milk agar for the determination of the sanitary quality of market milk. J. Dairy Sci. 18:539-546. 1935.
79. Safford, C. E. and Stark, C. N. Further studies on skim-milk agar for routine milk counts. J. Dairy Sci. 20:577-581. 1937.
80. Sherman, J. M. The advantages of carbohydrate medium in the bacterial examination of milk. J. Bacteriol. 1:481-488. 1916.
81. Shrader, J. H. Media composition and bacteria counts of milk. Annual Report Intern. Assn. Dairy and Milk Inspectors 15:208-221. 1926.
82. Slatter, W. L. and Halvorson, H. O. The heat resistance of lactobacilli found in American Cheddar cheese. J. Dairy Sci. 30:231-243. 1947.

83. Smythe, V. R. Significance of thermoduric bacteria in milk supplies. 15th Intern. Dairy Congr., London, 1959. Proc. 3:1898-1903. 1959.
84. Snedecor, G. W. Statistical methods. 5th ed. Ames, Iowa, Iowa State College Press. 1956.
85. Society of American Bacteriologists. Manual of microbiological methods. New York, N. Y. McGraw-Hill Book Co., Inc. 1957.
86. Speck, M. L. A study of the genus Microbacterium. J. Dairy Sci. 26:533-543. 1943.
87. Supplee, G. C., Whiting, W. A. and Downs, P. A. Variations in bacteria counts from milk as affected by media and incubation temperature. N. Y. (Ithaca) Agr. Expt. Sta. Mem. 43. 1921.
88. Thomas, R. C., Levine, B. S. and Black, L. A. Studies showing the effect of changes in the new (9th) edition of standard methods in relation to the bacteriological analysis of milk. Am. J. Public Health 38:233-238. 1948.
89. Thomas, S. B. Some factors which influence the thermoduric colony counts of raw milk. Dairy Inds. 14:586-589. 1949.
90. Thomas, S. B., Egdell, J. W., Clegg, L. F. L. and Guthbert, W. A. Thermoduric organisms in milk. Part I. A review of the literature. Proc. Soc. Appl. Bacteriol. 13:27-64. 1950.
91. Thomas, S. B., Ellison, D., Griffiths, D. G., Jenkins, E. and Morgan, K. J. Heat resistant bacteria in raw milk. I. Comparison of thermoduric colony counts on yeastrel milk agar incubated for 2 days at 37° C. and 4 days at 30° C. J. Soc. Dairy Technol. 3:187-190. 1950.
92. Thomas, S. B., Griffiths, D., Davies, E., Charlton, J. A. and Jones, R. G. Heat resistant bacteria in raw milk. Part V. Influence of milk cooling. J. Soc. Dairy Technol. 4:245-250. 1951.
93. Thomas, S. B. and Hobson, P. M. A survey of methods of milk production in relation to the incidence of thermoduric organisms in farm milk supplies. Proc. Soc. Appl. Bacteriol. 1948:7-12. 1948.

94. Thomas, S. B., Hobson, P. M. and Bird, E. R. Psychrophilic bacteria in milk. 15th Intern. Dairy Congr., London, 1959. Proc. 3:1334-1340. 1959.
95. Thomas, S. B. and Jenkins, E. Colony counts on milk agar incubated at 25°, 30° and 37° C. Proc. Soc. Agr. Bacteriologists 1940:38-40. 1940.
96. Tobias, J., Kammann, O. W. and Tracy, P. H. Pasteurization equivalents of high-temperature short-time heating with ice cream mix. J. Dairy Sci. 38:959-968. 1955.
97. Turbett, P. A., Seaman, A. and Woodbine, M. Microbiological aspects of the genus Microbacterium. Proc. Soc. Appl. Bacteriol., J. Appl. Bacteriol. 22:v. 1959.
98. Turbett, P. A., Seaman, A. and Woodbine, M. Microbiological aspects of the thermoduric Microbacterium genus. Dairy Sci. Abstracts 22:543-548. 1960.
99. Varma, K. Nambudripad, V. K. N. and Laxminarayana, H. Studies on pasteurization of milk in India. 15th Intern. Dairy Congr., London, 1959 Proc. 1:385-390. 1959.
100. Watrous, G. H., Doan, F. J. and Josephson, D. V. Some bacteriological studies on refrigerated milk and cream. Pa. Agr. Expt. Sta. Bull. 551. 1952.
101. Williams, D. J. The rates of growth of some thermoduric bacteria in pure culture and their effects on tests for the keeping quality of milk. J. Appl. Bacteriol. 19: 80-94. 1956.
102. Williams, O. B. and Zimmerman, C. H. Studies on heat resistance. III. The resistance of vegetative cells and spores of the same organism. J. Bacteriol. 61:63-65. 1950.
103. Wilson, G. S., Twigg, R. S., Wright, R. C., Hendry, C. B., Cowell, M. P. and Maier, I. The bacteriological grading of milk. Medical Research Council Special Report 206. London. His Majesty's Stationery Office. 1935.
104. Yale, M. W. Standard agar counts as compared with counts on improved agars at 32° C. Am. J. Public Health 28:148-154. 1938.
105. Yale, M. W., Pederson, C. S. and Breed, R. S. Questions and answers on incubation of agar plates in milk analysis. N. Y. (Geneva) Agr. Expt. Sta. Circ. 143. 1934.

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APPENDIX

Analyses of bacteriological peptones as supplied by
Sheffield Chemical.

N-Z-AMINE TYPE AS

Description: A pancreatic digest of casein specially
processed so it can be used for highly
concentrated clear solutions and whenever
a high solubility is desirable.

Typical Analysis:	Moisture	3.52%
(as is basis)	Ash	5.05%
	Total Nitrogen	12.58%
	Amino Nitrogen	6.83%
	Amino N/Total N	54.3 %
	Sodium	2.25%
	Chloride	0.28%
	Total Phosphate	2.25%
	Calcium	0.28%
	pH (2% solution)	6.55
	Solubility (clear, 30° C.)	210 g./l.
Amino Acid Assay:	Valine	6.0 %
(total)	Leucine	7.2%
	Isoleucine	6.2 %
	Serine	1.4 %
	Lysine	9.04%
	Tryptophane	1.2 %
	Phenylalanine	3.5 %

Amino Acid Assay:	Tyrosine	1.4 %
(total)	Methionine	2.3 %
(The additional amino acids have not been assayed.)		

N-Z-AMINE TYPE YT

Description: A broad spectrum bulk peptone resulting from the enzymatic digestion of casein. Its superior growth-promoting qualities compared to many widely used peptones, mark it as the peptone choice for wide microbiological use.

Typical Analysis:	Moisture	3.7 %
(as is basis)	Ash	6.0 %
	Total Nitrogen	13.4 %
	Amino Nitrogen	5.3 %
	Amino N/Total N	39.4 %
	Iron	33 ppm
	Copper	5 ppm
	pH (2% solution)	6.9
Vitamin Content:	(micrograms per gram)	
(as is basis)	Biotin	0.11
	B-12	0.005
	Choline	400.0
	Folic Acid	0.60
	Inositol	244.0

Vitamin Content:	(micrograms per gram)	
(as is basis)	Niacin	20.5
	Pantothenic Acid	6.7
	Pyridoxine	3.1
	Riboflavin	8.4
	Thiamin	1.7

EDAMIN

Description: A lactalbumin hydrolysate in which the protein has been reduced to its constituent amino acids and peptides. Conforms to the degree of hydrolysis acceptable to the American Medical Association.

Typical Analysis:	Moisture	6.51%
(as is basis)	Ash	5.31%
	Total Nitrogen	11.9 %
	Amino Nitrogen	6.9 %
	Amino N/Total N	58.0 %
	Sodium	1.82%
	Chloride	1.34%
	Iron	80 ppm
	Total Phosphates	1.13%
	pH (2% solution)	6.8
	Solubility (clear, 30° C.)	15 g./l.
Amino Acid Assay:	Glycine	1.7 %
(total)	Alanine	3.8 %

Amino Acid Assay:	Valine	4.1 %
(total)	Leucine	9.95%
	Isoleucine	5.4 %
	Serine	5.6 %
	Threonine	3.8 %
	Lysine	10.0 %
	Arginine	3.1 %
	Aspartic Acid	8.2 %
	Glutamic Acid	17.5 %
	Proline	6.3 %
	Histidine	1.9 %
	Tryptophane	2.1 %
	Phenylalanine	3.4 %
	Tyrosine	3.3 %
	Cystine	2.16%
	Methionine	1.84%

SOY PEPTONE POWDER

Description: An enzymatic digest of soybean meal of
U.S.P. quality.

Typical Analysis:	Moisture	4.86%
(as is basis)	Ash	14.0 %
	Nitrogen	9.2 %
	Amino N	1.8 %
	Amino N/Total N	19.8 %
	Carbohydrate (by difference)	37.2 %

Typical Analysis: (as is basis)	Sodium	3.25%
	Chloride	2.68%
	Total Phosphates	1.27%
	Iron	52 ppm
	pH (2% solution)	7.1
	Solubility (clear, 30° C.)	120 g./1.
	Amino Acid Assay: (total)	Glycine
	Alanine	2.3 %
	Valine	2.0 %
	Leucine	3.8 %
	Isoleucine	2.5 %
	Serine	4.1 %
	Threonine	1.9 %
	Lysine	3.7 %
	Arginine	4.6 %
	Aspartic Acid	5.3 %
	Glutamic Acid	12.1 %
	Proline	3.1 %
	Histidine	1.8 %
	Tryptophane	0.72%
	Phenylalanine	3.35%
	Tyrosine	1.9 %
	Cystine	0.58%
	Methionine	0.82%

N-Z-CASE

Description: A new and improved tryptic digest of casein of U.S.P. quality. This uniform peptone is designed for general application as a bacteriological nutrient with excellent solubility and clarity in solution.

Typical Analysis:	Moisture	3.5 %
(as is basis)	Ash	5.9 %
	Total Nitrogen	12.7 %
	Amino Nitrogen	5.1 %
	Amino N/Total N	40.2 %
	Sodium	2.45%
	Chloride	0.30%
	Iron	35.4 ppm
	Total Phosphates	2.37%
	pH (2% solution)	7.2
	Solubility (clear, 30° C.)	25 g./l.
Amino Acid Assay:	Glycine	1.8 %
(total)	Alanine	3.3 %
	Valine	5.5 %
	Leucine	10.3 %
	Isoleucine	4.5 %
	Serine	6.7 %
	Threonine	3.7 %
	Lysine	6.5 %

Amino Acid Assay:	Arginine	3.6 %
(total)	Aspartic Acid	5.9 %
	Glutamic Acid	22.8 %
	Proline	10.6 %
	Histidine	3.05%
	Tryptophane	1.4 %
	Phenylalanine	4.6 %
	Tyrosine	3.5 %
	Cystine	0.51%
	Methionine	2.35%

HY-CASE SF

Description: An acid hydrolysate of casein in which the protein molecule has been substantially reduced to its constituent amino acids. It has been specially processed to remove essentially all the salt.

Typical Analysis:	Moisture	4.4 %
(as is basis)	Ash	2.2 %
	Total Nitrogen	13.1 %
	Amino N/Total N	76.7 %
	Sodium Chloride	0.30%
	Iron	26 ppm
	pH (2% solution)	6.0-6.5
	Solubility (clear, 30° C.)	25 g./l.

Amino Acid Assay:	Valine	4.5 %
(total)	Leucine	5.3 %
	Isoleucine	5.3 %
	Threonine	2.9 %
	Lysine	8.5 %
	Arginine	2.9 %
	Histidine	3.1 %
	Tryptophane	none
	Phenylalanine	2.2 %
	Methionine	1.4 %

(The additional amino acids have not been assayed.)

Vitamin Content:	(micrograms per gram)	
(as is basis)	B-12	0.00014
	Biotin	0.006
	Folic Acid	0.01
	Niacin	0.20
	Pantothenic Acid	0.066
	Pyridoxine	0.034
	Riboflavin	0.11
	Thiamin	0.12