EFFECT OF TEMPERATURE AND TIME OF PLATE INCUBATION ON THE ENUMERATION OF PASTEURIZATION-RESISTANT BACTERIA IN MILK

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ABSTRACT

This study was undertaken to determine the effect of temperature and time of plate incubation upon the count of thermoduric bacteria in milk. Specific types of thermoduric bacteria in pure culture, as well as those present in the mixed flora of commercial milk samples, were enumerated. Plate incubation at 28 C for 4 days was the temperature-time combination that produced the highest thermoduric bacterial count with laboratory-pasteurized milk. Incubation at 21, 32 or 35 C gave lower counts. Thermoduric bacteria subjected to pasteurization were more exacting in their growth temperature requirements than were unheated bacteria. Cultures of Arthrobacter sp., Micrococcus varians and Streptococcus sp. grew over a much wider temperature range before laboratory pasteurization than after the heat treatment. The incubation temperature and time currently recommended for the standard plate count of bacteria in raw milk, may not be equally satisfactory for the determination of the maximum viable bacterial population of pasteurized milk.

Recent changes in milk production and handling practices have necessitated reappraisal of certain bacteriological tests. Tests applied directly to raw milk are not always effective in detecting faulty production practices because the growth of bacteria in raw milk is greatly retarded by efficient cooling. Therefore, tests for specific groups of organisms that might serve as indices of contamination are now receiving considerable attention.

The count of laboratory-pasteurized samples has been suggested as an index of unsanitary milk handling. The most common procedure for determining the viable bacterial population of milk is the agar plate method as outlined in Standard Methods for the Examination of Dairy Products (6). In this procedure, plates are incubated at 35 or 32 C for 48 hr for both the standard plate count of raw or commercially-pasteurized milk and the thermodic bacterial count of laboratory-pasteurized milk.

Incubation at 37 C for 2 days was originally employed to give pathogens that might be present in milk an opportunity to develop at their optimum growth temperature. This procedure had been followed for over 20 years before studies (2, 13, 16, 19) revealed that 32 C was nearer the optimum for many bacteria found in milk. The eighth edition of Standard Methods for the Examination of Dairy Products (3) recognized incubation at either 37 or 32 C for 48 hr for the agar plate method. Later reports (8, 24) concerning plate counts on raw and commercially pasteurized milk showed that counts after 48 hr of incubation of plates were somewhat higher at 35 than at 37 C and still slightly higher at 32 C than at 35 C. Plate incubation at 32 or 35 C for 48 hr was recognized in 1948 as standard procedure in this country (4).

Although "Standard Methods" (6) continues to stipulate that plates be incubated at 35 or 32 C for 48 hr, some investigations (7, 18, 21) have suggested that longer incubation at these and other temperatures might be advantageous for the enumeration of bacteria in pasteurized milk. These investigations point out that the development of colonies on plates inoculated with pasteurized milk tended to be slower than on plates prepared from raw milk. Incubation for 3 days is required for plate counts on dried milk (6).

The present study was undertaken to determine the effect of temperature of plate incubation and length of the incubation period upon the enumeration of pasteurization-resistant (thermoduric) bacteria in milk. The identification of specific types of thermodic bacteria was carried out, as well as their enumeration both in pure cultures and as the mixed flora of commercial milk samples.

EXPERIMENTAL METHODS

Except for certain indicated modifications, the methods employed were those outlined in "Standard Methods" (6). 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 for delivery of 0.1 ml and 1.0 ml quantities.

Twelve samples of manufacturing grade milk, cooled in bulk tanks, and 20 can-cooled samples were examined. A standard plate count at 32 C was determined for each sample prior to laboratory pasteurization. A "complete immersion" laboratory pasteurization technique was employed. Ten ml of raw
milk were pipetted aseptically into a sterile 125 x 15 mm test tube, using care to avoid contamination of the upper portion of the tube. The tube was closed with a sterile rubber stopper and was completely immersed in an electrically heated water bath. The pasteurization temperature was 62.5 ± 0.1 C, and the pasteurization time was 30 min. Less than 5 min were required for the sample to reach pasteurization temperature. Immediately following pasteurization, the tube of milk was cooled in ice water. The tube was completely inverted 12 times; the upper part was thoroughly flamed before aliquots were removed for plating.

Duplicate plates for each dilution were incubated at the following temperatures and times:

<table>
<thead>
<tr>
<th>Incubation temperature (C)</th>
<th>Incubation time (days)</th>
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<tbody>
<tr>
<td>35</td>
<td>2 3 4</td>
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<tr>
<td>32</td>
<td>2 3 4</td>
</tr>
<tr>
<td>28</td>
<td>3 4 5</td>
</tr>
<tr>
<td>21</td>
<td>4 5 7</td>
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<td>10</td>
<td>7 14 21 28</td>
</tr>
</tbody>
</table>

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. Plates were protected from air-borne contamination by counting, unopened, in an inverted position. 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 according to their ability to form colonies during incubation at the various temperatures.

Milk samples showing either a wide variation or no variation in thermodynamic count, among the various incubation temperatures and times, were selected for study of the bacterial types encountered. Immediately after the colonies were counted, representative colonies from suitable plates were picked by a random method (15) and inoculated into tubes of sterile litmus milk. Following incubation at 32 C for 3 to 5 days, a loopful of milk from each tube was streaked onto a plate containing Plate Count Agar plus 0.25% non-fat milk solids. Surface colony characteristics were noted on the streaked plates after 72 hr at 32 C.

To assure the isolation of pure cultures, a single colony picked from each streak plate was inoculated into a tube containing 5 ml of sterile litmus milk. The reaction was noted at intervals during a 14-day period at 32 C. Slants inoculated from the litmus milk tubes were incubated at 32 C for 24 hr; smears were prepared from the slants, Gram stained and examined microscopically.

A preliminary classification of the isolates into genera was based upon cell morphology, Gram staining characteristics, reaction in litmus milk and colony characteristics. Additional cultural and biochemical testing of representative isolates verified classification into genera and, in some cases, into species.

Four isolates, representative of the predominant genera of thermodynamic bacteria found, were selected for study of the effect of temperature and time of plate incubation upon the growth of pure cultures before and after laboratory pasteurization. The selected isolates were classified as Microbacterium lacti-cum, Micrococcus varians, Streptococcus sp. and Arthrobacter sp. Stock cultures were prepared by inoculating sterile litmus milk with representative colonies from agar slants and then incubating the milk for 24 hr at 32 C. One ml of each culture was added to 100 ml of sterile reconstituted skim milk containing 10% non-fat milk solids. After thorough mixing, 10 ml of the inoculated skim milk were transferred to a sterile test tube for laboratory pasteurization. A second portion of the nonpasteurized, inoculated skim milk was refrigerated at 3.3 to 4.4 C for 24 hr before laboratory pasteurization. The pasteurized and nonpasteurized cultures were plated and incubated in the manner previously outlined for pasteurized samples of raw milk.

RESULTS

The effect of various incubation temperatures and times upon the average arithmetic mean thermodynamic plate count of 25 manufacturing grade milk samples is shown in Figure 1. The greatest mean count was obtained at incubation temperatures of 28 C for 4 days and 21 C for 7 days. The mean thermodynamic colony counts obtained after 2 days at 35 and at 32 C were 31.0 and 73.7%, 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 mean thermodynamic count obtained at 10 C for 28 days exceeded the mean count at 35 C for 2 days and was almost half of the highest mean count obtained at 28 C for 4 days. However, as indicated in Figure 1, these bacteria, as a rule, were slow in forming colonies at 10 C.

Analysis of variance showed that counts obtained after 3 days of incubation at 35, 32 and 28 C were significantly different (P < 0.01). Differences of the same significance were obtained when counts after 4 days at 35, 32 and 28 C were compared. The counts at 32 C for 2 days were significantly lower (P < 0.01) than counts at 32 C for 3 days.
The distribution of bacteria that survived pasteurization of milk, as influenced by the temperature and time of plate incubation, is presented in Table 1. As the incubation temperature was decreased from 35 to 28°C, microbacteria accounted for a greater share and micrococci for a lesser share of the thermoduric count. Microbacteria also accounted for a greater share than did micrococci of the colonies developing on plates after extended incubation at 35, 32, 28 and 21°C. Incubation temperature and time did not appreciably affect the proportion of thermoduric bacteria of types other than microbacteria and micrococci.

Thermoduric lactobacilli were slow in forming colonies at both 35 and 32°C. None of the colonies counted after 2 days at 35 and 32°C were composed of lactobacilli. An appreciable number of the colonies developing upon extended plate incubation at 35 and 32°C were, however, the result of growth of lactobacilli. There was some indication that these bacteria are capable of producing colonies after 3 days of incubation at 28°C, but colony development was favored by longer incubation even at this temperature.

Figure 2 shows the effect of temperature of plate incubation upon the colony counts of a pure culture of Arthrobacter sp. before and after laboratory pasteurization. Approximately equal counts were obtained for the unheated culture when plates were incubated at 35, 32, 28 and 21°C for 2 days. The counts on the pasteurized culture were definitely higher at 32 and 28°C than at 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 incubation. After 3 days, the count at 21°C approximated the count obtained at 32 and 28°C after 2 days.

Both unheated and pasteurized cultures of Microbacterium lacticum failed to produce countable colonies at an incubation temperature of 35°C. If non-

Table 1. Average Distribution of Thermoduric Bacteria in Milk* as Determined by Incubation of Plates at Various Temperatures and Times

<table>
<thead>
<tr>
<th>Incubation temp, °C</th>
<th>Incubation time, days</th>
<th>Distribution of bacteria (% of total)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Microbacteria</td>
</tr>
<tr>
<td>35</td>
<td>2</td>
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</tr>
<tr>
<td>32</td>
<td>4</td>
<td>30.4</td>
</tr>
<tr>
<td>32</td>
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<td>21</td>
<td>7</td>
<td>44.5</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>59.5</td>
</tr>
</tbody>
</table>

*Pasteurized at 62.5 ± 0.1°C for 30 min.
*bArthrobacters, lactobacilli, streptococci and unidentified bacteria.

Figure 1. Mean thermoduric plate counts of 25 milk samples obtained by incubation of plates at various times and temperatures. (Pasteurization was at 62.5 ± 0.1°C for 30 min.)
Effects of Temperature and Time

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 types and physiological states of bacteria present in milk. A plating method for the enumeration of organisms in milk should nevertheless have the objective of determining the greatest possible proportion of the bacteria present. Relative to the question of time versus maximum recovery, a balance should be achieved commensurate with the objectives of the test.

A second objective of a plating procedure should be the production of easily discernible and countable colonies. Minute or "pinpoint" colonies overlooked in counting afford a source of error. This is especially true of the thermotolerant colony count. Many thermotolerant bacteria are known to produce colonies of minute size.

For the agar plate method of enumerating bacteria in milk, "Standard Methods" recommends incubating plates for 2 days at 35 or 32°C. This standard applies for raw, commercially-pasteurized and laboratory-pasteurized milk. However, results of the present study have indicated that incubation temperatures lower than 35°C and even lower than 32°C for incubation periods longer than 2 days may have some distinct advantages for the enumeration of thermotolerant organisms in milk.

A comparison of various incubation temperatures and times showed that the average thermoduric colony count obtained at 35°C for 2 days was only 31% of the average count obtained at 28°C for 4 days. With incubation at 32°C for 2 days, the average arithmetic count was more than double that
obtained at 35°C for 2 days. Significantly, of 32 samples examined, 31 had 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.

Highly heat-resistant microbacteria commonly accounted for an appreciably larger share of the colony count at 32 and 28°C than at 35°C. These findings are in accord with those of other workers (11, 14, 23) 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 also is too high an incubation temperature for accurate enumeration of microbacteria in pasteurized milk.

Buchanan (9) suggested that, when large numbers of microbacteria are present in milk, plate incubation temperatures lower than 35°C, and possibly even lower than 32°C, may be necessary for estimation of the total thermoduric population. Results of the present study confirm that incubation at 28°C frequently results in larger numbers of colonies of microbacteria than does 32°C. These organisms accounted for a substantially greater portion of the colonies obtained at 28°C than at 32°C.

Micrococci and arthrobacters also frequently contributed to the higher counts obtained with incubation 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. Seemingly, 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 (10, 12, 14) for thermoduric counts. In most cases, microbacteria have been found to constitute 60 to 80% of the thermoduric flora that grow 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 be a result of failure to incubate plates long enough to permit lactobacilli to grow on solid media. Many lactobacilli grow poorly under aerobic conditions and require a rather complex medium for growth. 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 indicates that the types of thermoduric bacteria found in milk depend 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. These organisms also may have been overlooked by other workers who did not incubate the plates long enough.

A thermoduric count in excess of 10,000 per ml has been suggested (12, 20) as providing evidence of unsatisfactory milk handling. In this study, 45% 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% gave counts in excess of 10,000 per ml at 28°C for 3 days. All samples with counts above this standard when plates were incubated at 35 or 32°C for 2 days also exceeded this standard when plates were incubated at 28°C for 3 days. Assuming that the standard of 10,000 per ml is valid, these results indicate that the lower incubation temperature would do a better job of detecting milk of poor quality.

The average thermoduric count obtained using incubation at 21°C for 5 days exceeded the average counts obtained at 35 and 32°C for 4 days. However, 7 days incubation at 21°C was necessary for the average count to equal the maximum average count obtained after 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 there no difference between the counts obtained at 28°C for 4 days and 21°C for 7 days of great magnitude. Although incubation at 21°C might have some advantage over incubation at 35 or 32°C, the results of this study indicate that 21°C incubation would offer no real advantage over 28°C incubation.

Many of the thermoduric bacteria observed in this study were capable of forming colonies on Plate Count Agar during incubation at 10°C if the incubation period was long enough. Notable among these were Arthrobacter, Microbacterium, and Micrococcus. However, at 10°C, an incubation period of 21 to 28 days was necessary for any appreciable colony development. Even after 25 days at 10°C, the average arithmetic colony count of 25 samples was only 42.6% of the maximum average count obtained at 28°C for 4 days.

Of the thermoduric bacteria examined in this study, spore-bearing rods were the only ones that grew better at 35°C than at lower temperatures. However, they did not appear in large numbers in any of the samples tested. Spore-bearing rods sometimes
accounted for a major portion of the thermoduric flora of samples with a thermoduric count of less than 3,000 per ml but did not contribute appreciably to the thermoduric flora of milk with a thermoduric count of more than 3,000 per ml. These results support reports by other workers (1, 14) that few spore-bearing rods are found in milk having a high thermoduric count.

Studies with pure cultures indicated that thermoduric bacteria generally are more exacting in their growth temperature requirements after they have been subjected to laboratory pasteurization than before. 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 laboratory pasteurization, they exhibited a definite preference for a much narrower growth temperature range.

These results help to explain why earlier investigations (16, 19, 25) 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 explanation is that certain of the thermoduric bacteria in the raw milk grew equally well at all temperatures. However, after pasteurization, colonies developed more readily at the lower incubation temperatures.

Comparative studies (8, 24) of plate counts of raw milk and pasteurized milk, following plate incubation at 37, 35 and 32°C for 48 hr, have shown that counts were somewhat higher at 35°C and were higher yet at 32°C. As revealed by this study, the increase in colony count at the lower temperatures results from 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 development was exhibited by both unheated and pasteurized cultures of this organism at a plate incubation temperature of 32°C.

Although micrococci generally were found to grow fairly well at 35°C throughout this study, there were some exceptions to this general rule. Colony formation 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 (9), who found some micrococci, particularly *M. varians*, grew better at 32°C than at 35°C after laboratory pasteurization.

A plate incubation period of 48 hr is specified in "Standard Methods" (6) 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, appreciable colony formation by a pasteurized culture was evident only after 3 days of incubation.

Hussong and Hammer (17) 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 due to growth during pasteurization. As shown in the present study, the highly heat-resistant flora encountered by these workers could have been composed largely of *M. lacticum*. Not only were cultures of this organism highly heat resistant, but occasionally they produced higher colony counts after laboratory pasteurization than before. Growth of *M. lacticum* cultures during pasteurization was ruled out by their failure to produce colonies on Plate Count Agar at an incubation temperature of 35°C.

All nine cultures of *M. lacticum* which Robertson (22) isolated from milk, survived pasteurization at 145°F for 30 min; determinations of percentage survival showed increases of 10 to 120%. Growth was not observed when laboratory strains of these organisms were inoculated into sterile skim milk 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. The present study showed that if laboratory cultures had been refrigerated prior to pasteurization, disintegration of clumps during pasteurization might have occurred to a greater degree.

**Summary**

Incubation at 28°C for 4 days was the temperature-time combination that most frequently produced the highest colony counts with laboratory pasteurized milk. The mean arithmetic thermoduric colony counts obtained after 2 days of incubation at 35 and 32°C were 31.0 and 73.7%, 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°C 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 equaled that obtained at 28°C for 4 days. Pasteurization-resistant bacteria formed colonies slowly on plates incubated at 10°C, and the mean count after 28 days of incubation was only 42.6% of the mean count after 4 days at 28°C.

Colony production on Plate Count Agar by those microbacteria surviving pasteurization was notably
inhibited at an incubation temperature of 35°C. Colonies produced by these bacteria were increased at 32°C and were further increased at an incubation temperature of 28°C. Colony production by *Arthrobacter* and *Micrococcus* was favored more at 32 and 28°C than at 35°C. Pasteurization-resistant lactobacilli produced colonies on Plate Count Agar only after 3 to 4 days of incubation at 35 and 32°C. Microbacteria and micrococci also contributed appreciably to increases in thermodic colony counts upon extended plate incubation at 35 and 32°C.

Thermodic bacteria that have been subjected to pasteurization are more exacting in their growth temperature requirements than are unheated bacteria. Thermodic cultures of *Arthrobacter* sp., *Micrococcus varians* and *Streptococcus* sp. grew over a much wider temperature range prior to than after laboratory pasteurization.

Results of this study have indicated that the temperature and time currently recommended for the standard plate count, while adequate for the determination of bacteria in raw milk, may not be satisfactory for the determination of the maximum viable population of pasteurized milk.

**REFERENCES**


