THE ENUMERATION OF PSYCHROPILIC MICROORGANISMS IN DAIRY PRODUCTS

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ABSTRACT

The eleventh edition of Standard Methods for the Examination of Dairy Products sanctions optional use of incubation temperatures and times (5-7°C for 7-10 days) for determination of the psychrophilic bacterial count of dairy products. This study shows that this 2 degree difference in incubation temperature, the 3 day difference in incubation time and a combination of these factors could be responsible for a significant variation in psychrophilic bacterial counts. A total of 67 raw milk, 58 pasteurized milk, 19 ice cream and eight cottage cheese samples were plated at 5 and 7°C for 7 and 10 days. Significantly higher counts were obtained after 10 days than after 7 days incubation at both temperatures; however, greater increases in counts resulted from raising the temperature from 5 to 7°C. Highest counts were obtained at 7°C for 10 days. A total of 559 isolates were picked from plates of 12 milk samples that had been incubated at 5 and 7°C for 7 days and 7°C for 10 days. Classification of the isolates indicated that variations in counts were due to differing abilities of organisms within genera to grow at low temperatures and not to preferential growth of different genera. Adoption of one incubation temperature and time for the determination of psychrophilic bacterial counts is recommended.

Organisms capable of growing at refrigeration temperatures long have been recognized as a primary cause of dairy product spoilage. The problem of enumeration of these organisms has been considered by many workers. Numerous incubation temperature and time combinations for psychrophilic bacterial counts can be found in the literature. Thomas (9) listed 25 such combinations used by various workers, with temperatures ranging from 0 to 25°C and times ranging from 3 to 28 days. Problems arising in the enumeration of psychrophilic bacteria have been reviewed by Davis (5), Ingraham and Stokes (6), Witter (12) and Baumann and Reinbold (2).

Holding plates at 5-7°C for 7-10 days is suggested by the eleventh edition of Standard Methods for the Examination of Dairy Products (1). This work was undertaken to determine if the difference in incubation temperature from 5 to 7°C, the difference in incubation time from 7 to 10 days or a combination of both factors would be sufficient to cause a significant variation in the psychrophilic bacterial count of dairy products.

EXPERIMENTAL PROCEDURE

A total of 67 raw milk samples, representing both can and bulk tank manufacturing grade and grade A milk, were obtained from six different Iowa dairy plants. Nineteen samples of ice cream, eight samples of cottage cheese and 58 samples of commercially pasteurized milk were obtained from quality control samples received by the Food Products Analysis Laboratory.

Procedures for preparing dilutions and plating were those outlined in “Standard Methods” (1). Sextuplet platings of each dilution were poured with Standard Methods agar (1); three plates of each dilution were placed at 5 and 7°C. Indicating thermometers, previously calibrated against a standard thermometer, were placed in each incubator. Temperatures were checked by a recording thermometer and were found to vary less than ± 0.5°C.

Colonies were counted with the aid of a Quebec colony counter after 7 and 10 days incubation. Plates were protected from airborne contamination by being counted, unopened, in an inverted position. The location of each colony was marked with ink on the bottom of each plate. Different colored inks were used for each incubation time to designate those colonies that were countable at 7 days and those that...
Values became countable by 10 days. Plates were counted at the end of 7 days incubation, marked with ink and retained in the incubator to be recounted at the end of 10 days. Counting was done in the incubator to prevent moisture condensation from obscuring colonies and to avoid warming of the plates which could have influenced the appearance of colonies between 7 and 10 days. Psychrophilic bacterial counts were computed by arithmetically averaging colony counts from triplicate plates and multiplying by the reciprocal of the dilution used. Usually, only plates with between 30 and 300 colonies were selected for counting. Plates with fewer than 30 colonies at 5 C were selected for counting if the corresponding plates at 7 C had more than 30 colonies. Plates with fewer than 30 colonies after 7 days incubation were counted if it was felt that the counts after 10 days incubation would surpass 30. Except where differences in counts were more than tenfold, plates of the same dilution at 5 and 7 C were selected for counting and picking to reduce errors resulting from plating procedure.

Six samples of raw milk and six samples of pasteurized milk were selected at random from among those used for psychrophilic bacterial counts. Colonies which had appeared at 5 and 7 C in 7 days and at 7 C between 7 and 10 days were picked into tubes of sterile litmus milk. Single plates representing each temperature treatment were selected. Where possible, plates with 15-30 colonies were used to reduce error resulting from unequal distribution of colonies of different genera within plates. Otherwise, approximately 20 colonies from each plate were selected by picking all colonies within a predesignated zone. After purity had been determined, inoculations were made into media suggested primarily by the Manual of Microbiological Methods (8) for classification of the isolates. Berger's Manual of Determinative Bacteriology (4) was consulted for identification of the organisms.

### Results

**Effect of time and temperature of incubation upon the psychrophilic bacterial count.**

Logarithmic means of psychrophilic bacterial counts of various dairy products obtained at 5 C and 7 C for 7 and 10 days are shown in Table 1. This table also presents the logarithmic mean ratios between counts at 7 C for 10 days compared with counts at 5 C for 7 days. The values presented reflect the range in ratios for individual samples: cottage cheese, 4.5 to 600; ice cream, 1.5 to 720; pasteurized milk, 0.9 to 156; raw milk, 1.02 to 1,570.

The analysis of variance of the counts of raw and pasteurized milk samples is shown in Table 2. Values of F for variations in counts resulting from temperature of incubation and days of incubation were significant at the 1% level. Increasing the temperature from 5 to 7 C was found to increase counts more than increasing the incubation time from 7 to 10 days. In addition, results of "t" tests showed greater differences in counts obtained at 5 C at 7 and 10 days.
than in counts obtained at 7°C at 7 and 10 days. Interaction between temperature and type of product and between days and type of product was significant at the 5% level of F.

Types of microorganisms contributing to counts.

In an attempt to determine whether differences in counts obtained from plates incubated at 5 or 7°C for 7 or 10 days were due to the types of microorganisms present, 559 isolates representing six samples of raw milk and six samples of pasteurized milk were identified as to genus. The number of organisms representing each genus was considered in relation to the total psychrophilic bacterial counts of the samples. These results are shown in Tables 3 and 4.

Members of ten different genera were picked from the plates. *Pseudomonas* appeared in nine samples, *Micrococcus* in seven and *Achromobacter* were found in five different samples.

Tables 3 and 4 reveal a general tendency for members of the various genera to increase in number as the plate incubation temperature and time are increased. Considering the calculated numerical response by individual genera per sample, 26 increases in count occurred as the plate incubation temperature and time were changed from 5°C for 7 days to 7°C for 7 days. In eight instances, the count per genus decreased; in one instance, there was no change in count. As plate incubation conditions were changed from 5°C for 7 days to 7°C for 10 days, 28 increases and seven decreases in count of an individual genus per sample occurred. Using a 7°C incubation temperature, an increase of plate incubation time from 7 to 10 days produced 17 increases, 12 decreases and six instances of no change in count of individual genera per sample.

**Discussion**

**Effect of time and temperature of incubation upon the psychrophilic bacterial count.**

As the temperature of incubation approaches the optimum temperature for a group of microorganisms, plate counts of a culture of these organisms are expected to increase to a maximum. Similarly, as the time of incubation of a culture increases, counts are expected to eventually reach a maximum. The temperature yielding highest counts of most psychrophilic microorganisms has been established in the vicinity of 20°C (5, 7). Therefore, counts obtained at 5°C

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Genus</th>
<th>Incubation temperature and time</th>
<th>5°C - 7 days</th>
<th>7°C - 7 days</th>
<th>7°C - 10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 1</td>
<td><em>Pseudomonas</em></td>
<td>(Organisms/ml)</td>
<td>3,140 (28)</td>
<td>2,600 (8)</td>
<td>2,820 (3)</td>
</tr>
<tr>
<td></td>
<td><em>Micrococcus</em></td>
<td></td>
<td>240 (2)</td>
<td>2,280 (7)</td>
<td>2,820 (4)</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus</em></td>
<td></td>
<td>150 (1)</td>
<td>320 (1)</td>
<td>250 (1)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>3,500 (29)</td>
<td>5,200 (16)</td>
<td>5,900 (7)*</td>
</tr>
<tr>
<td>R 2</td>
<td><em>Pseudomonas</em></td>
<td></td>
<td>320 (10)</td>
<td>360 (1)</td>
<td>350 (1)</td>
</tr>
<tr>
<td></td>
<td><em>Micrococcus</em></td>
<td></td>
<td>220 (7)</td>
<td>3,220 (9)</td>
<td>7,740 (13)</td>
</tr>
<tr>
<td></td>
<td><em>Flavobacterium</em></td>
<td></td>
<td>720 (2)</td>
<td>1,060 (1)</td>
<td>9,500 (15)</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus</em></td>
<td></td>
<td>60 (2)</td>
<td>4,300 (12)</td>
<td>350 (1)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>600 (19)</td>
<td>4,300 (12)</td>
<td>9,500 (15)</td>
</tr>
<tr>
<td>R 3</td>
<td><em>Pseudomonas</em></td>
<td></td>
<td>353,000 (21)</td>
<td>471,000 (19)</td>
<td>493,000 (3)</td>
</tr>
<tr>
<td></td>
<td><em>Micrococcus</em></td>
<td></td>
<td>17,000 (1)</td>
<td>49,000 (2)</td>
<td>67,000 (1)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>370,000 (22)</td>
<td>520,000 (21)</td>
<td>560,000 (4)</td>
</tr>
<tr>
<td>R 4</td>
<td><em>Pseudomonas</em></td>
<td></td>
<td>420,000 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Micrococcus</em></td>
<td></td>
<td>60,000 (1)</td>
<td>700,000 (1)</td>
<td>500,000 (1)</td>
</tr>
<tr>
<td></td>
<td><em>Aerobacter</em></td>
<td></td>
<td>600,000 (10)</td>
<td>2,800,000 (4)</td>
<td>5,500,000 (7)</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia</em></td>
<td></td>
<td>120,000 (2)</td>
<td>10,500,000 (15)</td>
<td>12,000,000 (9)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>1,200,000 (20)</td>
<td>14,000,000 (20)</td>
<td>18,000,000 (16)</td>
</tr>
<tr>
<td>R 5</td>
<td><em>Pseudomonas</em></td>
<td></td>
<td>77,000 (3)</td>
<td>528,000 (3)</td>
<td>440,000 (1)</td>
</tr>
<tr>
<td></td>
<td><em>Achromobacter</em></td>
<td></td>
<td>104,000 (4)</td>
<td>880,000 (5)</td>
<td>734,000 (7)</td>
</tr>
<tr>
<td></td>
<td><em>Aerobacter</em></td>
<td></td>
<td>207,000 (8)</td>
<td>1,060,000 (6)</td>
<td>1,330,000 (3)</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia</em></td>
<td></td>
<td>52,000 (2)</td>
<td>355,000 (2)</td>
<td>2,350,000 (14)</td>
</tr>
<tr>
<td></td>
<td><em>Flavobacterium</em></td>
<td></td>
<td>177,000 (1)</td>
<td></td>
<td>148,000 (1)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>440,000 (17)</td>
<td>3,000,000 (17)</td>
<td>5,000,000 (17)</td>
</tr>
<tr>
<td>R 6</td>
<td><em>Micrococcus</em></td>
<td></td>
<td>1,800,000 (16)</td>
<td>3,700,000 (17)</td>
<td>35,000,000 (13)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>1,800,000 (16)</td>
<td>3,700,000 (17)</td>
<td>35,000,000 (13)</td>
</tr>
</tbody>
</table>

* a, b *Theoretical no. of organisms in each genus calculated from actual psychrophilic bacterial count/ml
*的实际菌数，每ml计算从实际psychrophilic细菌计数/ml
* actual no. of isolates picked from plate incubated at 5°C-7 days
*实际从培养皿中挑取的细菌数，在5°C-7天培养
* actual no. of isolates picked from comparable plate incubated at 7°C-7 days
*实际从类似的培养皿中挑取的细菌数，在7°C-7天培养
* actual no. of isolates picked that had developed on same plate in 3 days after the 7°C-7 day count
*实际从相同的培养皿中挑取的细菌数，在7°C-7天后计数
natural would be expected to be lower than those obtained at 7 C, since the lower temperature is further from that generally considered optimum for most psychrophilic organisms. It also would seem reasonable that, at either of these temperatures, higher counts will be obtained after 10 days than after 7 days incubation. Even greater differences would be expected between counts obtained at the lower temperature and shorter time than those obtained at the higher temperature and longer time.

The results presented show statistically significant differences among counts obtained within the range of temperature and time of incubation specified in "Standard Methods" (I). In many instances, colony size was responsible for these count differences, particularly between 5 and 7 C. Frequently, colonies were too small to be counted accurately at the lower temperature or shorter incubation time. Although it was possible to detect the presence of colonies, accurate counting was impossible at the magnification provided by a Quebec colony counter. Incubation at 7 C for 7 days, or at either 5 or 7 C for 10 days, usually resulted in colonies large enough to be counted. Van der Zant and Moore (11) found colonies on plates incubated at 5 C for 7 days difficult to count because of the small size. Boyd et al. (3) discontinued using incubation at 5 C for 7 days because the resulting colonies were small and difficult to count.

Table 1 shows that differences in counts were greater in some types of products than in others. Cottage cheese samples showed the greatest differences in counts, followed by ice cream and raw milk. The smallest differences among counts were observed in pasteurized milk samples. This suggests that different types of organisms may contribute to the psychrophilic count. The data, however, indicate that the types of organisms usually are alike. The high counts on the pasteurized milk were due to their age, since they were commercial samples obtained through the Food Products Analysis Laboratory from local grocery stores after considerable storage. Because of the small numbers of samples, comparisons of counts of ice cream and cottage cheese should be made with caution.

The products which showed the greatest increases in count with increasing temperature and time of incubation were found to have the lowest count at any given temperature and time. Disregarding types of products, samples with lower psychrophilic populations demonstrated greater increases in counts as temperature or time were increased. This agrees with the findings of Thomas et al. (10) who compared counts obtained at 3-5 C for 10 days with counts obtained at 7 C for 10 days. Higher ratios (7 C, 10 days/3-5 C, 10 days) were obtained with samples giving low counts at 3-5 C. Although there usually
are wide variations between counts, samples with higher counts at 5°C for 7 days show smaller increases. This may account for the differences among products in Table 1.

Of the 58 pasteurized milk samples, 21 had the same count at 7°C for both 7 and 10 days. The results, which show a significant difference in counts obtained at 7 and 10 days, must be interpreted carefully. The average difference in count was found to be statistically significant. Therefore, one would expect differences in counts among a large number of samples. For any one sample, however, it cannot be said that incubation at 7°C for 10 days will result in a higher count than incubation at 7°C for only 7 days. Thirty-seven of the 58 samples did show increases large enough to significantly affect the average increase. This indicates that, for any one sample, one may obtain a higher count at 10 days than at 7 days. In some instances, the count at 10 days may be considerably higher than at 7 days. To obtain the highest counts, 7°C for 10 days should be used in the method suggested by "Standard Methods" (1). The range of logarithmic mean ratios for individual products has been given to emphasize the extreme variability that could exist between counts on the same sample obtained by different laboratories, both operating within recommendations made by "Standard Methods" (1).

Types of microorganisms contributing to the psychrophilic bacterial count.

As mentioned previously, differences in counts obtained at different incubation temperatures may result from preferential growth of different types of organisms. Some organisms may grow better at lower temperatures than others. Thermoduric organisms have been known to appear on psychrophilic plates incubated at 10°C. Thomas et al. (10), however, found no thermoduric bacteria contributing to counts obtained at 7°C for 10 days. If this is true, thermoduric microorganisms should not influence the count when plates are incubated at 7°C for 10 days to obtain maximum counts.

The results in Tables 3 and 4 indicate no tendency for one type of organism to occur more frequently at the lower temperature or the shorter incubation time than at the higher temperature or longer time. Where decreases in the count of an individual genus occurred with an increase in plate incubation temperature or time, the decreases were usually insignificant, resulting only from mathematical calculations based on relatively few colonies.

Tables 3 and 4 show that no single genus caused most of the differences between counts. Each of the ten different genera, in one sample or another, increased progressively in number as the incubation temperature and time were increased. In some samples, the increase in numbers of a particular genus was striking, in others, negligible. Variations in counts, therefore, appeared to be due to differing abilities of organisms within each genus to grow at low temperatures.

Summary

Psychrophilic bacterial counts were determined for samples of cottage cheese, ice cream and raw and pasteurized milk. Plates were incubated at 5 and 7°C for 7 and 10 days.

Results obtained showed significantly higher counts after 10 days than after 7 days incubation at both temperatures. Counts obtained at 7°C for 7 days were higher than counts obtained at 5°C for 10 days. Highest counts were obtained at 7°C for 10 days. Greater increases in counts resulted from raising the temperature from 5 to 7°C rather than from lengthening the time from 7 to 10 days.

Identification of organisms isolated from plates of raw and pasteurized milk samples indicated that the significant differences in counts had not resulted from preferential growth of different genera of organisms because of different temperature and time treatments. Instead, variations in counts were due to differing abilities of organisms within each genus to grow at low temperatures.

In the interest of obtaining greater reproducibility among testing laboratories, it is recommended that one time and temperature combination for plate incubation be adopted for determination of the "Psychrophilic Bacterial Count."

Conclusions

The conditions of incubation selected for psychrophilic bacterial counts depend largely upon the worker's definition of psychrophilic microorganisms. Predilection for some definitions would require incubation below 5°C; other definitions allow incubations above 7°C. In any case, maximum counts of microorganisms included in the definition usually are desired. If the plating procedure in "Standard Methods" (1) is followed, organisms producing colonies on plates within 7-10 days at 5-7°C would be considered as psychrophilic. Among these temperatures and times, 7°C for 10 days yielded highest counts. Whether this is the "best" temperature and time for enumeration of psychrophilic organisms may be debatable. Within the suggested range of incubation conditions, however, significant variation in counts occurs. If one particular temperature and time combination were adopted, reproducibility of counts among laboratories would be more likely than at present.
ENUMERATION OF PSYCHROPHILIC MICROORGANISMS

REFERENCES