EFFECT OF pH OF PLATING MEDIUM ON ENUMERATION OF PASTEURIZATION-RESISTANT BACTERIA IN MILK[1]

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SUMMARY

Plating media of different pH were studied as to effects on enumeration of pasteurization-resistant bacteria in milk. After heat treatment, thermoduric bacteria were more tolerant of pH levels above rather than below 7, and maximum mean thermoduric counts were obtained at pH 7.5. There were some exceptions, but usually the pH levels that yielded the highest counts also produced the largest and most easily discernible colonies. Pure cultures of thermoduric bacteria grew over a much wider range of pH before than they did after laboratory pasteurization. Results indicate that, although seemingly adequate for enumeration in raw milk, the medium pH currently recommended for the standard plate count may not be satisfactory for the determination of the maximum viable bacteria population of pasteurized milk.

Reappraisal of certain bacteriological methods has directed attention to tests for specific groups of bacteria which might serve as indices of milk contamination. Pasteurization-resistant bacteria have been considered as such an index of unsanitary production practices. The most common procedure for determining the viable population of milk, both raw and commercially-pasteurized, is the agar plate method as described in *Standard Methods for the Examination of Dairy Products (2)*. This procedure, also recommended for determining the thermoduric bacterial count of laboratory-pasteurized milk, calls for the use of a plating medium of pH 6.9 to 7.1.

The literature lacks comprehensive studies related to the influence of pH of the plating medium upon the enumeration of thermoduric bacteria in milk. Cooledge, as cited by Fay *(3)*, believed that 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 at pH 6.6 and 7.3 resulted in counts of 15,400 and 317,000 per ml, respectively. Wilson et al. *(7)* 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 desirable.

Nelson *(5)* 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 present study was undertaken to determine the effect of plating medium pH upon the enumeration of pasteurization-resistant (thermoduric) bacteria in milk.

EXPERIMENTAL METHODS

Except for certain indicated modifications, the methods employed were those outlined in Standard Methods *(2)*. 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.

Six samples each of bulk-cooled grade A milk, can-cooled manufacturing grade milk and blended bulk and can-cooled manufacturing grade milk were examined. A standard plate count at 32 C was determined for each sample before laboratory pasteurization. A "complete immersion" laboratory pasteurization technique as described by Thomas et al. *(6)* was employed. Samples were pasteurized at 62.5 C for 30 min. Less than 5 min was required for the sample to reach pasteurization temperature. Immediately following pasteurization, samples were cooled in ice water.

Following pasteurization of each milk sample, a single series of dilutions was prepared. The required number of replicate plates was prepared for each sample dilution. Duplicate plates of each dilution were poured with each of five Plate Count agars *(1)* adjusted to final pH levels of 6.5, 7.0, 7.5, 8.6 and 9.1. Grade A milk samples were plated by using Plate Count agars adjusted to additional pH levels of 5.6 and 8.0.

All media were prepared from the same lot of dehydrated basal medium. Except for the variation in pH, the media were identical in composition and method of preparation. A Beckman Model 96 potentiometer was used for all pH determinations. Ten-percent solutions of NaOH or HCl

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TABLE 1. AVERAGE DISTRIBUTION OF THERMODURIC BACTERIA IN EIGHT SAMPLES OF MILK[a] OBTAINED WITH MEDIA CONTAINING VARIOUS BACTERIOLOGICAL PEPTONES

Bacteriological peptone	Average thermoduric count[b]/ml	Distribution of bacteria (% of total)			
		Arthrobacters	Microbacteria	Micrococci	Streptococci
Bacto-Tryptone	95,000	13.6	1.7	77.9	6.8
N-Z-Amine Type AS	150,000	1.8	5.3	70.1	22.8
N-Z-Amine Type YT	130,000	13.1	4.9	55.7	26.3
Edamin	100,000	7.3	5.4	87.3	00.0
HY-Case SF	140,000	12.3	7.0	49.1	31.6

[a]Pasteurized at 62.5 ± 0.1 C for 30 min.

[b]Plates incubated at 32 C for 48 hr.

were used in adjusting the pH of all media prior to autoclaving. The final pH of each medium was determined at a temperature of 45 C just before plating in accordance with Standard Methods (2). 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.

Milk samples showing either a wide variation or no variation in thermoduric count, when plated at the various pH levels at 32 C for 4 days, 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 (4) and inoculated into tubes of sterile litmus milk. After 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 thermoduric bacteria found, were selected for study of the effect of pH of the plating medium upon the growth of pure cultures before and after laboratory pasteurization. The selected isolates were classified as *Microbacterium lacticum*, *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 at 32 C for 24 hr. One ml of each culture was added to 100 ml of sterile reconstituted skimmilk containing 10% non-fat milk solids. After thorough mixing, 10 ml of the inoculated skimmilk were transferred to a sterile test tube for laboratory pasteurization. A second portion of the nonpasteurized, inoculated skimmilk 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. Plate Count agars adjusted to pH 5.6, 6.5, 7.0, 7.5, 8.0, 8.6 and 9.1 were used; duplicate plates were incubated at 32 C and were counted after 2, 3 and 4 days.

RESULTS

The effect of plating medium reaction upon the average arithmetic thermoduric plate count of 18 milk samples is shown in Figure 1. The maximum mean count was obtained with Plate Count agar adjusted to pH 7.5. Insufficient data were obtained to include counts on a medium of pH 8 in Figure 1, but the limited data available indicate that average counts with a medium of pH 8 would at least equal those obtained with a medium of pH 7.5. No discernible colonies were produced with any of the samples with Plate Count agar at pH 5.6. Of the various media mentioned in Figure 1, only that of pH 7.5 produced average thermoduric counts that did not increase when plates were incubated beyond 2 days. Limited data would suggest similar results with media of pH 8.0. The mean 4-day thermoduric count obtained at pH 8.6 was equal to the 2-day count obtained at pH 7. Mean colony counts obtained with Plate Count agars at pH 6.5 and pH 9.1 were comparable to but considerably lower than counts obtained with media of intermediate pH.

The distribution of thermoduric bacteria in nine samples of milk obtained with Plate Count agar at various pH levels is presented in Table 1. The proportionate share of the mean colony count attributable to micrococci tended to decrease as pH of the medium was increased. The greatest number of micrococci, however, was obtained with a medium of pH 7.0. In contrast to the trend shown by micrococci, the percentage of the mean colony count attributed to streptococci increased at each pH level as the plating medium pH progressively increased from 6.5 to 9.1. The highest average streptococcus colony count was obtained at pH 7.5. The counts

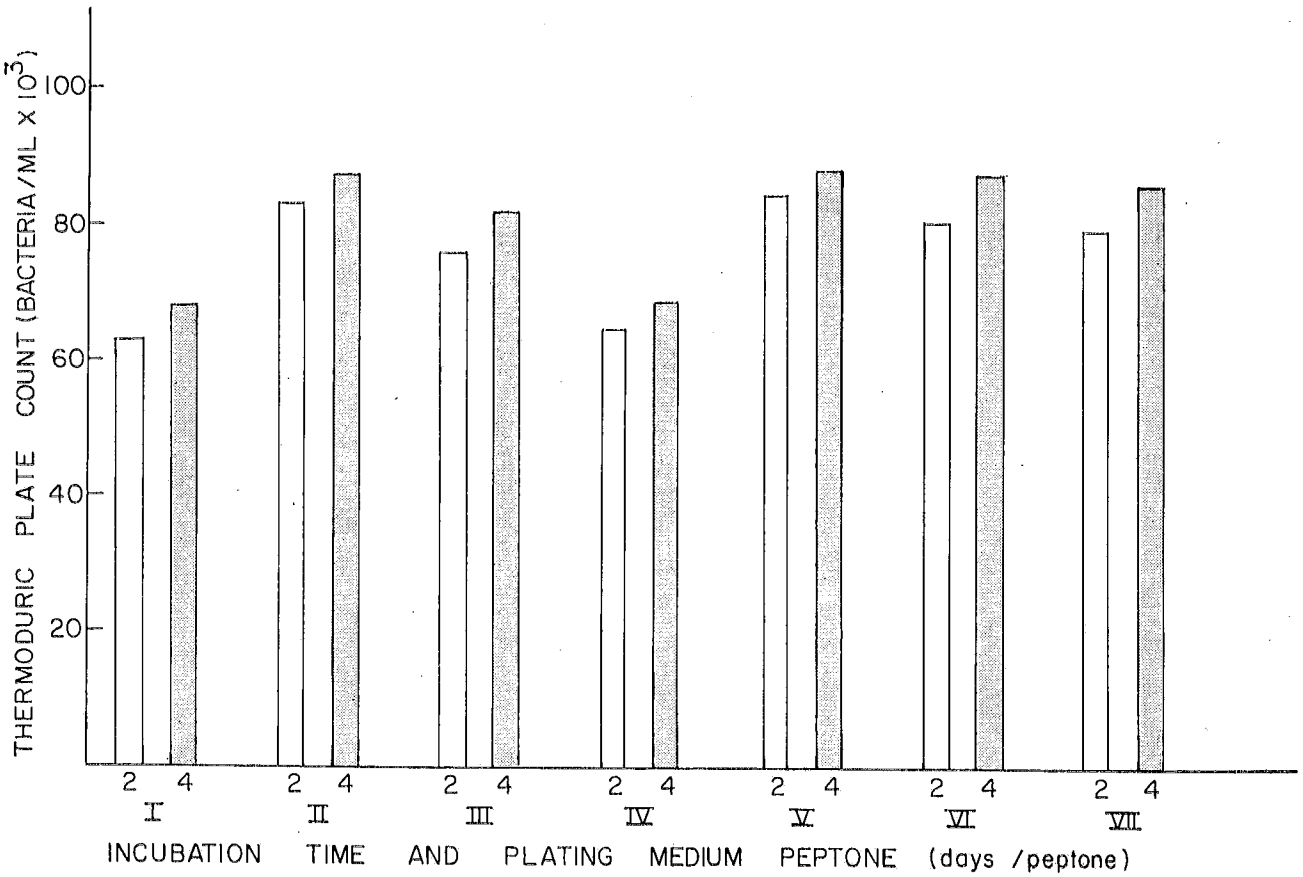

Figure 1. Mean thermoduric plate counts (32 C) of 26 milk samples obtained with media containing various bacteriological peptones: I, Bacto-Tryptone; II, N-Z-Amine Type AS; III, N-Z-Amine Type YT; IV, Edamin; V, Soy Peptone Powder; VI, N-Z-Case; VII, HY-Case SF.

obtained at pH 8.6 and 9.1 were more than double the count of streptococci obtained with a medium of neutral reaction.

Arthrobacters accounted for approximately equal portions of the counts obtained with media of pH 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 1 was inadequate to demonstrate the effect of the medium pH upon their ability to produce colonies. The limited data suggested, however, that growth of microbacteria that survived pasteurization was favored by the higher pH media.

In addition to influencing the thermoduric plate count, the pH of the plating medium influenced the size of individual colonies. Although there were some exceptions, colony size generally was largest with those media yielding the highest count for a 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 variations in pH, levels above pH 7 usually produced the largest and most easily discernible colonies.

The effect of plating medium pH upon the plate count of a pure culture of *Arthrobacter* sp. before and after laboratory pasteurization is shown in Figure 2. The unheated culture produced colonies equally well over a range of pH 6.5 to 8.6. However, the laboratory pasteurized culture showed a definite preference for a medium of pH 7.0 and colony counts were depressed substantially above and below this level. Incubation of plates beyond 48 hr failed to change the results shown in Figure 2.

An unheated culture of *M. varians* showed essentially the same degree of colony development over a range of pH 6.5 to 9.1 as shown in Figure 3. With a pasteurized culture of this organism, however, the maximum 48-hr colony count was obtained at pH 8 with noticeably decreased colony production on media below or above pH 8. Extending the plate incubation period to 96 hr permitted colony development at pH 7.5 and 7, but the counts were considerably less than those obtained at pH 8.

Figure 4 shows that an unheated culture of *Streptococcus* sp. gave essentially the same degree of colony development over a range of pH 6.5 to 9.1.

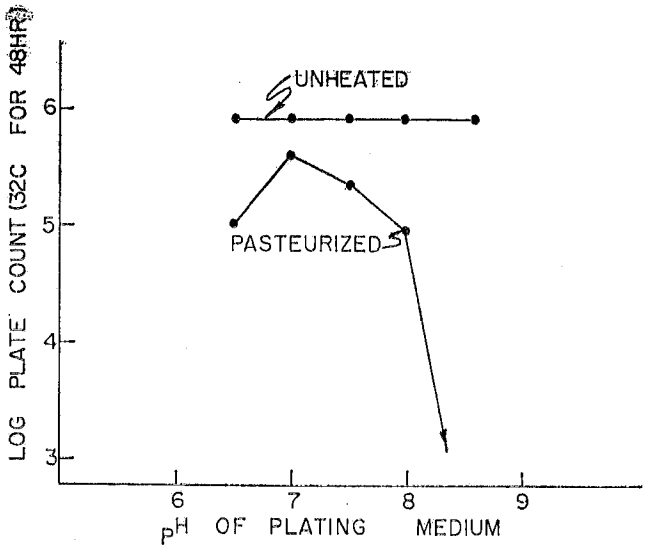

Figure 2. Effect of pH of Plate Count agar upon the plate count of a culture of *Arthrobacter* sp. before and after laboratory pasteurization.

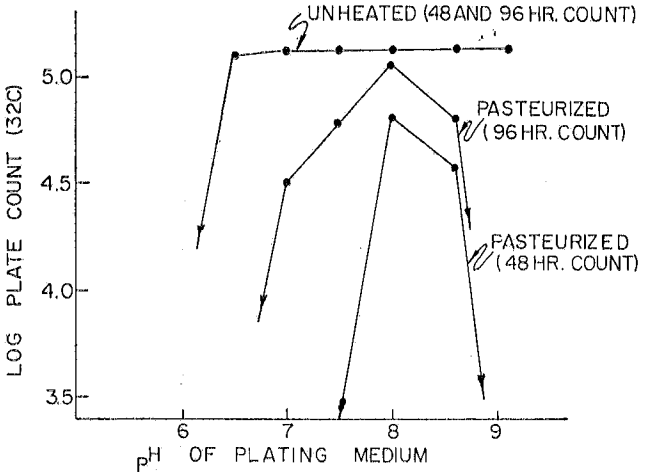

Figure 3. Effect of pH of Plate Count agar upon the plate count of a culture of *Micrococcus varians* before and after laboratory pasteurization.

A laboratory pasteurized culture of this organism failed to produce countable colonies at pH 6.5, and colony development increased from pH 7 to 8.6.

Unheated cultures of *M. lacticum* gave essentially the same colony count with Plate Count agar at levels of pH 6.5 to 8.6. The colony counts for pasteurized cultures were considerably less at pH 6.5 than at pH levels of 7, 7.5, 8 and 8.6. Colony productivity by this organism was substantially enhanced by laboratory pasteurization.

Colony size, as well as colony count, was influenced by pH of the Plate Count agar. Although colony counts for pasteurized cultures of *M. lacticum* did not differ appreciably at pH levels of 7 to 8.6, colonies produced at pH 7 were generally less than 1 mm in diameter after incubation at 32 C for 3 days. With pH levels of 7.5 to 8.6, colonies averaged about 2 mm in diameter after incubation at 32 C for 3 days.

DISCUSSION

The current edition of Standard Methods *(2)* recommends a pH of 7.0 ± 0.1 for the plating medium used in determining the Standard Plate Count of raw, commercially pasteurized and laboratory pasteurized milk. Results of this study, however, have indicated that a pH of 7 for Plate Count agar is suboptimal for colony production by most thermoduric bacteria.

As a rule, maximum thermoduric counts were obtained with Plate Count agar adjusted to pH 7.5 and 8. 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 and, in some cases, above 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* evidently 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 genus *Arthrobacter* showed a definite preference for a medium of pH 7 to 7.5. There were some indications that certain of the thermoduric micrococci preferred a neutral medium rather than

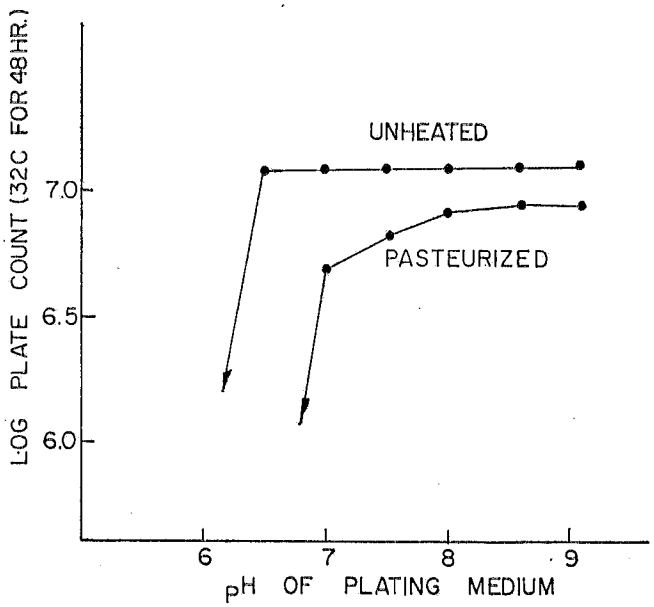

Figure 4. Effect of pH of Plate Count agar upon the plate count of a culture of *Streptococcus* sp. before and after laboratory pasteurization.

an alkaline one. However, this group of bacteria exhibited no definite preference, and colony productivity often was as great at pH 7.5 to 8.6 as at pH 7.

An increase in the thermoduric plate count was usually obtained when the reaction of the plating medium was elevated from pH 7 to pH 7.5. On the other hand, lowering the pH to 6.5 almost always resulted in a substantial decrease in thermoduric count from that obtained at pH 7. With a level of pH 7 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.

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 *(6)*, 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 variations in pH of the plating medium. For these samples, however, the most easily discernible colonies were usually produced at levels above pH 7. This was most noticeable with samples containing microbacteria.

The results indicate that thermoduric bacteria subjected to laboratory pasteurization are more exacting in their pH requirements for growth than they were before pasteurization. Unheated cultures of a strain of *M. varians*, for example, gave essentially the same count with Plate Count agar at pH levels from 6.5 to 9.1 after 2 days of incubation. After being subjected to laboratory pasteurization, however, this strain did not produce colonies at 2 days of incubation with Plate Count agar of pH 7 and 9.1 and yielded a pronounced maximum count at pH 8.

These observations generally agree with those of Nelson *(5)*. 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 in the present study were the results 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. After the cultures were subjected to laboratory pasteurization, however, colonies were produced quite well at pH 9.1. No explanation can be offered for this occurrence, but it seems logical that the process of heating altered the character of the organism so 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 *(2)* recommends that the plating medium used for the enumeration of bacteria in milk have a pH of 7.0 $\pm$ 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, although adequate for the enumeration of bacteria in milk before pasteurization, is sub-optimal for maximum colony production by some of the thermoduric bacteria after these have been 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.

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