EFFECT OF THE TYPE OF BACTERIOLOGICAL PEPTONE IN THE PLATING MEDIUM UPON THE ENUMERATION OF PASTEURIZATION-RESISTANT BACTERIA IN MILK[1]

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SUMMARY

Various bacteriological peptones were studied as to effects on enumeration of pasteurization-resistant bacteria in milk. No appreciable differences were observed in colony counts of unheated cultures of thermoduric *Micrococcus varians* and *Arthrobacter* and *Streptococcus* sp. when plated with media containing different peptones. After laboratory pasteurization, colony counts differed substantially. Usually, media producing the highest counts yielded the largest and most easily discernible colonies. Bacto-Tryptone was deficient for colony development of thermoduric streptococci. Results indicate that, although seemingly adequate for enumeration in raw milk, the bacteriological peptone currently recommended for the standard plate count may not be satisfactory for the determination of the maximum viable bacteria population of pasteurized milk.

Before publication of the seventh edition of *Standard Methods for the Examination of Dairy Products (2)* in 1939, Nutrient agar, containing beef extract and peptone as nutrient sources, was recommended as a plating medium. Previously, little regard had been given to the particular type of peptone used. Bowers and Hucker *(5)* suggested the use of an improved plating medium containing 0.5% tryptone (a casein-digest peptone), 0.1% glucose, 0.5% skimmilk and agar. Comparing this medium with Nutrient agar, plate counts were 36% higher on 134 samples of raw milk and 350% higher on 77 samples of pasteurized milk. Similar results were reported by other investigators *(6, 7, 20)* when plate counts on the two media were compared. Abele *(1)* suggested further refinement of the proposed medium by the inclusion of beef extract. Consequently, a plating medium containing tryptone, glucose, beef extract, skimmilk and agar replaced Nutrient agar as the official plating medium in 1939 *(2)*. Several investigations *(9, 10, 11, 12, 13)* demonstrated that the newly adopted medium, TGEM agar, was superior to Nutrient agar for the enumeration of bacteria in milk, especially for pasteurized milk and heat-treated bacteria. Nelson *(14)* also 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 with precipitation of the skimmilk in the TGEM medium, studies *(15, 16)* were undertaken to find a suitable milk-free medium to replace TGEM agar. As a result, Plate Count agar, containing tryptone (a pancreatic digest of casein), yeast extract, dextrose and agar, was officially recommended in 1953 *(3)* and currently is recognized *(4)* for the standard plate count of milk.

The role of bacteriological peptones in bacterial growth has been investigated by many workers. Bowers and Hucker *(5)* stated that hydrolyzed casein was an excellent source of nitrogen for bacterial growth, particularly for those microorganisms 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 *(17)* compared 10 milk plating media prepared with various peptones. Peptones varied 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 lower nitrogen compounds for proper growth and that amino acids in proper concentration were generally the most important nitrogen compounds for culture media.

This study was undertaken to explore the effect of various bacteriological peptones in the plating medium upon the enumeration of pasteurization-resistant bacteria in milk. It is appreciated, however, that bacteriological peptones constitute only a portion of the nutrient complex of a medium.

EXPERIMENTAL METHODS

Except for certain indicated modifications, the methods employed were those outlined in Standard Methods *(4)*. 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.

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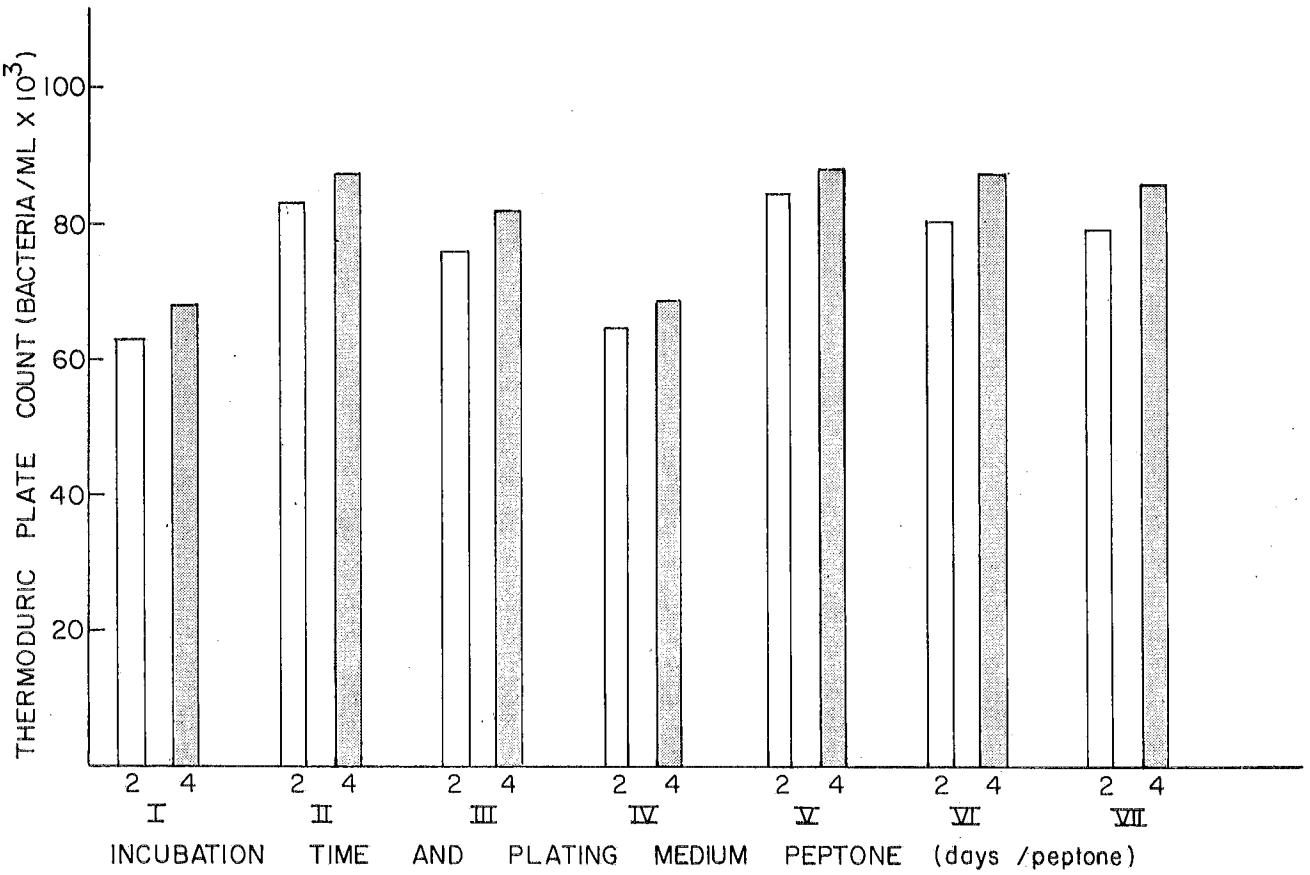

Figure 1. Mean thermoduric plate counts (32 C) of 26 milk samples obtained with media containing various bacteriological peptones: I, Bacto-Tryptone; II, N-Z-Amine Type AS; III, N-Z-Amine Type YT; IV, Edamin; V, Soy Peptone Powder; VI, N-Z-Case; VII, HY-Case SF.

Ten samples of bulk-cooled grade A milk, 10 samples of can-cooled manufacturing grade milk and six samples of blended bulk and can-cooled manufacturing grade milk were examined. A "complete immersion" laboratory pasteurization technique as described by Thomas et al. *(19)* was employed. Samples were pasteurized at 62.5 ± 0.1 C for 30 min. Less than 5 min. were required for samples to reach pasteurization temperature, and they were cooled in ice water immediately following pasteurization.

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 peptones used in formulation, the composition of all media was identical to that of Plate Count agar as outlined in Standard Methods *(3)*. The bacteriological peptones were:

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, a recommended and commonly used peptone for Plate Count agar, was obtained from Difco Laboratories *(8)*. The other bacteriological peptones were obtained from Sheffield Chemical[4]. Analyses of these materials were supplied by Sheffield Chemical. Plates were incubated at 32 C, and colonies counted after 2, 3 and 4 days of incubation. Counting and marking of colonies were conducted in the manner outlined by Thomas et al. *(19)*.

Milk samples showing wide variations as well as no variation in thermoduric count, among the various media, were selected for study of the bacterial types encountered. Isolation, classification and study of the growth of pure cultures on the various media before and after laboratory pasteurization were conducted as described in an earlier study *(19)*.

RESULTS

Arithmetic mean thermoduric colony counts obtained by plating 26 milk samples with media containing various bacteriological peptones are summarized in Figure 1. Although variation among mean counts obtained with the various media was not great, the data indicate that media prepared from Bacto-Tryptone and Edamin produced the lowest mean colony counts. Mean colony counts obtained with the other media were similar, especially after 4 days of incubation.

Table 1 summarizes the distribution of thermoduric bacteria, obtained with media prepared with the various peptones, in eight samples of milk. The data indicate that bacteria of the *Arthrobacter* genus preferred media prepared with Bacto-Tryptone, N-Z-

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.

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 according to the 2-day colony count. Table 1 illustrates the apparent inadequacies of the Bacto-Tryptone and Edamin media for supporting growth of 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.

A study of the effect of various peptones used in the plating medium upon colony counts of pure cultures of thermoduric bacteria revealed that only in rare instances were differences in colony count of the unheated cultures possibly attributable to differences in the composition of the media. In contrast, 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. and *Micrococcus varians* (Figures 2 and 3).

A pasteurized culture of *Microbacterium lacticum* demonstrated no differences in colony counts attributable to variation in medium composition. Pasteurization conditions employed did not result in any killing effect upon this organism. As noted in an earlier study *(19)* for refrigerated cultures of this organism, the laboratory pasteurized count was appreciably higher than the count for the unheated cultures.

Although unheated cultures of *Streptococcus* sp. showed no appreciable variation in count among the different media, pasteurized cultures showed noticeable variation in colony count. Colony counts of pasteurized cultures were substantially lower with a medium containing Edamin than with other media. There was some indication that the colony count also was inhibited with Bacto-Tryptone medium.

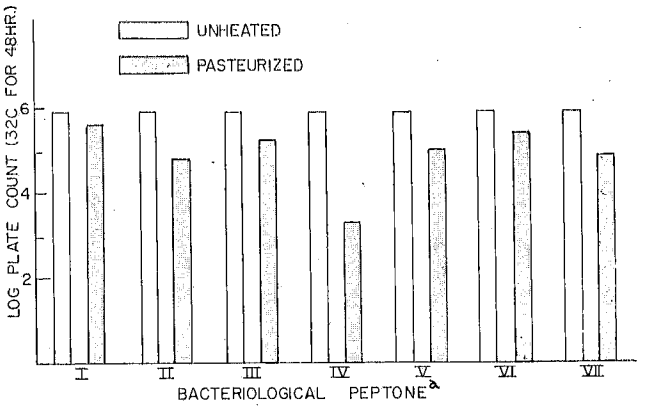

Figure 2. Effect of Plate Count agar bacteriological peptone upon the plate count of a culture of *Arthrobacter* sp. before and after laboratory pasteurization. I, Bacto-Tryptone; II, N-Z-Amine Type AS; III, N-Z-Amine Type YT; IV, Edamin; V, Soy Peptone Powder; VI, N-Z-Case; VII, HY-Case SF.

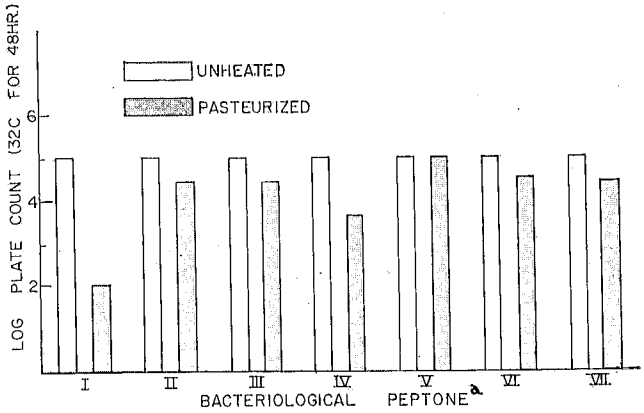

Figure 3. Effect of Plate Count agar bacteriological peptone upon the plate count of a culture of *Micrococcus varians* before and after laboratory pasteurization. I, Bacto-Tryptone; II, N-Z-Amine Type AS; III, N-Z-Amine Type YT; IV, Edamin; V, Soy Peptone Powder; VI, N-Z-Case; VII, HY-Case SF.

DISCUSSION

Standard Methods *(4)* recommends a pancreatic digest of casein conforming to the specifications outlined in the *Manual of Microbiological Methods (18)* for the preparation of milk plating medium. Bacto-Tryptone, supplied by Difco Laboratories, Inc. *(8)*, is perhaps one of the most commonly employed bacteriological peptones. This bacteriological peptone and six others of varying types were used in the preparation of plating media to determine their effect upon the thermoduric plate count.

Because of 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 *(17)* 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% for Soy Peptone Powder to 6.9% for Edamin. Results for 26 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. Because 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, 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 differences seemingly resulted from the inability of some of the thermoduric streptococci to produce colonies after heat treatment on the Bacto-Tryptone and Edamin media.

The bacteriological peptones included in this study represent but a small portion of the possible number of peptones that could be utilized in the preparation 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 media may fail entirely to support growth of certain types of thermoduric bacteria after heat treatment. If certain thermoduric bacteria, such as streptococci, go undetected in pasteurized milk, then the thermoduric plate count has lost much of its meaning.

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, usually, noticeably smaller than those produced on the other media.

The results with pure cultures indicate that thermoduric bacteria are generally more exacting in the type of bacteriological peptone required for growth after being subjected to laboratory pasteurization than before pasteurization. This was 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 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 medium. These results indicate that, if the maximum viable bacterial population of pasteurized milk is to be determined, the plating medium currently recommended may not be adequate.

The 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% 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%. 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 overlooked. Few investigators seem to have taken cognizance of this in studying the pasteurization-resistant flora of milk or in formulating media for making plate counts on pasteurized dairy products. Usually, 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 *(13)*. Although not restricting his studies to thermoduric bacteria, he 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.

ADDENDUM

Attention of the reader is directed to the "Erratum," contained in this (June) issue of the Journal, to the paper "Effect of pH of plating medium on enumeration of pasteurization resistant bacteria in milk," by Thomas, Reinbold, and Nelson, which appeared in the May 1966 issued of this Journal.

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SPEEDING LITTERBUG A RARITY, SAYS KAB

Speed has a direct bearing on the accumulation of litter on the country's roadways, according to Keep America Beautiful, Inc.

Professional and voluntary litter fighters from all 50 states overwhelmingly agreed in a KAB survey that the slower-traveled secondary highways, country roads and city streets are suffering most at the hands of the litterbug.

Heavy accumulation of litter on the faster-traveled limited access thruways and turnpikes was reported by only three per cent of those responding to the survey, said KAB.

Allen H. Seed, Jr., executive vice president of KAB, noted that refuse thrown from a car is a safety hazard in addition to being unsightly and the fact that motorists appear to be more conscious of the safety element when traveling at higher speeds accounts for the lower incidence of "toss-out" on the high-speed routes.

"But even the slow-driving litterbug can be eliminated by installation and use of an auto litterbag," said Mr. Seed.