PRELIMINARY INCUBATION OF RAW MILK SAMPLES AS AN AID IN EVALUATING BACTERIOLOGICAL QUALITY[1]

M. N. Desai[2] and T. J. Claydon

Department of Dairy Science
Kansas State University, Manhattan

(Received for publication April 24, 1964)

A total of 194 milk samples were obtained from 35 bulk tank grade-A milk producers in the Manhattan, Kansas, milk-shed during different seasons, and examined for standard, coliform, and psychrophile plate counts both before and after preliminary incubation (P.I.). Samples also were tested for inhibitory substances. Farms were ranked I, II, or III in general sanitation on the basis of visible conditions, with rank I most desirable. Except for one doubtful sample, all tests for inhibitory substances were negative. With standard plate counts, when no consideration was given to initial count level, there was no statistically significant relation between P.I. counts and season or sanitation rank, although summer growth ratios were always lowest. When data were grouped on the basis of initial count range, P.I. counts and growth ratios increased with increased sanitation rank in the lower count ranges. In initial count ranges $> 50{,}000$/ml the relationship between growth ratio and sanitation rank declined. With coliform counts, considerable inconsistency occurred in P.I. counts among seasons and sanitation ranks. Initial and P.I. coliforms counts showed little relationship with standard plate counts (except in summer) or with psychrophile counts. With psychrophile counts, growth ratios during P.I. were influenced by season, being highest in winter and lowest in summer. P.I. counts generally increased with increase in sanitation rank. The relation between growth ratios and sanitation rank was statistically significant ($P < 0.05$).

Bulk tank handling of milk on producing farms has introduced certain problems relating to quality. Among them is the altered status of the standard plate count as a quality index. Efficient cooling may mask insanitary production practices by contributing to low bacterial counts *(2, 3, 4, 5, 6, 7)*. The psychrophilic flora is favored by longer holding at low temperatures. Standard bacteriological tests may fail to indicate insanitary conditions or the true bacteriological quality of the milk. As a partial solution, it has been proposed that raw milk samples be incubated prior to making initial plate counts. Johns *(4, 5)* proposed incubation of samples at 55 F for 18 hr. He contended that such preliminary incubation (P.I.) would encourage growth of contaminants and differentiate between milk that had been produced under sanitary conditions and that with bacterial numbers suppressed by efficient cooling. Hence the procedure would be a more reliable measure of care in production and handling and would contribute to better control of raw milk quality. Other investigators *(2, 6)* endorse a P.I. procedure as an index of sanitation.

Investigations of the merits of the P.I. procedure have been limited to a few geographical areas, and have included only limited information on seasonal effects. Further evaluation of the proposal is needed. Work reported here was undertaken to study effects of the procedure on standard plate, psychrophile, and coliform counts during different seasons in north-central Kansas.

Experimental Procedure

Collection of Samples

Milk samples were collected from 35 bulk tank grade-A producers in the Manhattan, Kansas, milk-shed. Most samples were obtained by the county milk sanitarian and some were taken by a milk hauler trained and licensed to do such sampling. Although most of the samples were known to represent four milkings, in some cases this information was not obtained. More samples were obtained during some seasons than others. Samples were obtained aseptically after operating the bulk tank agitator for several minutes. They were iced immediately after collection and brought to the laboratory. In almost all cases, samples were analyzed for inhibitory substances and for bacteriological quality promptly after arrival. When it was necessary to hold samples, they were kept at 0-2 C for not more than 24 hr before analysis. General sanitary conditions of each producer's milk handling facilities were also judged and rated visually by the sanitarian as rank I, II and III with rank I most satisfactory.

Analyses of Samples

Milk samples were analyzed for inhibitory substances by the rapid disc assay screening method *(1)*. Standard plate counts (S.P.C.), coliform counts, and psychrophile counts were made initially as well as after incubating the milk samples at 13 C (approximately 55 F) for 18 hr. The counts made after incubation were termed P.I. counts. Plating methods used were the standard procedures recommended by the American Public Health Association *(1)*, using 35 C incubation temperature for standard plate counts.

[1]Contribution No. 326, Department of Dairy Science, Kansas Agricultural Experiment Station. Part of dissertation submitted by the senior author as partial fulfillment of the requirements for the M. S. degree.

[2]Present address: Jalalpore, Guparat, India.

TABLE 1. INCREASE IN STANDARD PLATE COUNT DUE TO PRELIMINARY INCUBATION OF MILK SAMPLES EXAMINED DURING DIFFERENT SEASONS

Season	No. of samples	Geometric means of standard plate counts/ml Initial (A)	P.I. (B)	Growth ratio B/A
Fall	28	11,000	50,000	4.5
Winter	36	14,000	68,000	4.9
Spring	38	9,000	44,000	4.9
Summer	92	24,000	59,000	2.5
Summary	194	14,000[a]	55,000[a]	3.7

[a]Geom. avg. of seasonal means

Violet red bile agar medium was used for coliform counts. For psychrophile counts, plates were incubated at 7 C for 7 days.

In summarizing the data, standard plate counts and psychrophile counts were averaged logarithmically as recommended in the Milk Ordinance and Code *(8)* to even discrepancies. These counts are presented in results as geometric means. With coliform counts, arithmetic averages were used since the count range was much lower and some zero counts were obtained. Values reported for counts are on a per ml basis.

In studying the effect of season on preliminary incubation of raw milk samples, the different seasons were identified as follows: Fall—September, October, and November; Winter—December, January and February; Spring—March, April and May; Summer—June, July and August.

Statistical evaluations were made by analysis of variance and linear regression.

RESULTS AND DISCUSSION

Tests for inhibitory substances

All milk samples except one gave negative test results for inhibitory substances. The one exception was classed as doubtful. It was concluded that inhibitory agents, as measured by the test employed, were not a factor in influencing bacterial development during preliminary incubation of milk samples.

Influence of P.I on standard plate counts during different seasons

Data are summarized and grouped in Table 1 by season. Since the plate incubation temperature was 35 C, counts probably averaged lower, especially after P.I., than if a 32 C temperature had been used. The overall geometric average of initial counts of 14,000/ml supports the contention of Johns *(4)* that present bacterial limits (S.P.C. 200,000/ml) are not consistent with changed conditions of producing grade-A milk. Initial counts averaged highest in summer and lowest in spring. Statistical analyses of the logarithms of individual counts (analysis of variance) showed that season had a significant effect ($P<0.01$) on initial counts, with summer counts considerably higher than counts in other seasons.

After P.I., it is significant that the highest average count (68,000/ml) was obtained in winter and that relative differences in counts during different seasons tended to even out. Average growth ratios were generally similar for fall, winter, and spring but were markedly lower for summer. Apparently the P.I. treatment was less favorable for the prevailing flora in summer than in other seasons. Growth ratios on individual samples varied from slightly more than 1 to >100, and analysis of variance of the individual ratios indicated that effect of season on growth ratio was not statistically significant. Nevertheless it appears that the P.I. count is more effective in fall, winter, and spring than in summer, in revealing organisms that do not show up in initial counts.

Influence of P.I. on coliform counts during different seasons

Data on coliform counts, summarized by arithmetic means, are presented on a seasonal basis in Table 2. Relatively more seasonal variation occurred in initial coliform counts than in initial standard plate counts. This may be due partly to the use of arithmetic averages rather than geometric averages. Counts were considerably higher in summer than in other seasons and correlated with the high initial standard plate counts obtained in summer. However, the relatively low coliform counts obtained in winter raise some question as to whether initial coliform counts would be a suitable measure of bacteriological quality during all seasons.

P.I. treatment reduced the seasonal variation observed in initial coliform counts. In winter, when initial coliform counts were low, average growth ratio on P.I. was highest. This suggests that the

TABLE 2. INCREASE IN COLIFORM COUNTS DUE TO PRELIMINARY INCUBATION OF MILK SAMPLES EXAMINED DURING DIFFERENT SEASONS

Season	No. of samples	Arithmetic means of coliform counts/ml. Initial (A)	P.I. (B)	Growth ratio B/A
Fall	27	313	2,100	6.9
Winter	32	55	830	15.1
Spring	37	220	1,400	6.4
Summer	92	1,700	4,000	2.3
Summary	188	570[a]	2,100[a]	3.7

[a]Arith. avg. of seasonal means

TABLE 3. INCREASE IN PSYCHROPHILIC COUNTS DUE TO PRELIMINARY INCUBATION OF MILK SAMPLES EXAMINED DURING DIFFERENT SEASONS

Season	No. of samples	Geometric means of psychrophilic counts/ml Initial (A)	P.I. (B)	Growth ratio $\frac{B}{A}$
Fall	19	1,900	45,000	23.7
Winter	36	4,000	190,000	47.5
Spring	37	1,700	46,000	27.0
Summer	92	1,600	22,000	13.8
Summary	184	2,100[a]	54,000[a]	25.7

[a]Geom. avg. of seasonal means

P.I. coliform count would be a better measure of quality than the initial coliform count, since the P.I. count correlated better with the standard plate count in winter. As with standard plate counts, the growth ratios were lowest when initial counts were highest.

Influence of P.I. on psychrophile counts during different seasons

The geometric mean of initial psychrophile counts was greater in winter than in the other seasons, which were essentially equal (Table 3). The higher winter counts are not unexpected since prevailing lower temperatures tend to favor psychrophilic types in the environment. However, analysis of variance of individual count data indicated that observed differences were not statistically significant.

After P.I., the overall average count of 54,000/ml was about equal to the standard plate P.I. count. The P.I. procedure had a much greater effect on psychrophiles than on standard plate counts. This indicates that psychrophiles multiplied more rapidly during P.I. than did other types. The high standard plate counts obtained after P.I. in winter (Table 1) probably were due to greater increases in psychrophiles. Although these obviously increased during P.I. (13 C for 18 hr), many would fail to grow at the 35 C standard plate count incubation temperature.

It is significant that the average P.I. psychrophile count was much higher in winter than in other seasons and also much higher than the P.I. standard plate count. Obviously even the P.I. standard plate count fails to measure a large part of the flora in winter. In contrast to standard plate counts and coliform counts, highest initial psychrophile counts also produced highest growth ratios.

Since the initial psychrophile counts averaged much lower in all seasons than the initial standard plate counts, initial psychrophile counts might be misleading as an index of the bacteriological quality of raw milk. However, after P.I., the psychrophile counts were about the same as standard plate counts in fall and spring and considerably higher in winter. This suggests that during winter, a P.I. psychrophile count would be a better quality index than a P.I. standard plate count. On the other hand, in summer a P.I. psychrophile count did not reflect the bacteriological condition as well as a P.I. standard plate count.

Growth ratios were much higher in winter than in other seasons and statistical analysis of individual count data showed that season had a significant effect ($P < 0.01$) on growth ratios of psychrophilic bacteria.

Relationship of sanitary rank of producing farms to P.I. standard plate counts

In Table 4, data have been grouped in an attempt to show the effect of sanitary rank of farms on P.I. counts during different seasons. Such tabulation greatly reduced the number of samples in each group.

The overall geometric means of initial counts for sanitation ranks I, II and III were 12,000, 12,000 and 24,000 per ml of milk, respectively. This suggests that the rank III farms were less sanitary. The similar overall averages for ranks I and II may reflect the fact that farms were ranked by visual inspection and that there were no real differences in sanitary conditions of milk handling equipment or that any differences were not measured by initial counts. It is of interest that in summer, geometric means of initial counts in each sanitary rank varied little.

The overall geometric means of P.I. counts increased as sanitation rank increased. However, there was considerable variation between seasons and within sanitation ranks. Growth ratios showed no consistent trend with sanitation rank or season on inspection of data grouped in the table. Analysis of variance also showed no significant relationships. However, growth ratios were lowest in summer for all sanitation ranks. This might result from the high initial counts obtained in summer since, as shown in previous tables, the growth ratios usually were lowest when initial counts were highest.

Relationship of sanitary rank of producing farms to P.I. coliform count

The data in table 5 are arranged on a seasonal basis. The overall arithmetic means for initial coliform counts indicate little difference among sanitation ranks. However, initial counts were highest in summer and lowest in winter within each sanitation rank, suggesting that prevailing seasonal temperatures affect initial coliform counts. Although the summary indicates a direct relationship of sanitation rank with P.I. count and growth ratio, the relationships on a seasonal basis are very inconsistent. Only the spring season shows consistency with initial counts, P.I. counts, and growth ratios increasing with sanitation rank. Consequently, seasonal variations involved

TABLE 4. RELATIONSHIP OF SANITARY RANK OF PRODUCING FARMS TO INCREASE IN STANDARD PLATE COUNTS DUE TO PRELIMINARY INCUBATION OF MILK SAMPLES EXAMINED DURING DIFFERENT SEASONS

	Sanitation rank I				Sanitation rank II				Sanitation rank III			
Season	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A
Fall	10	8,600	29,000	3.3	8	10,000	140,000	14.0	8	16,000	45,000	2.8
Winter	10	14,000	63,000	4.5	17	11,000	43,000	3.9	8	25,000	130,000	5.2
Spring	12	5,800	18,000	3.1	16	8,400	39,000	4.7	8	32,000	110,000	3.4
Summer	38	25,000	63,000	2.5	39	21,000	59,000	2.3	14	26,000	73,000	2.4
Summary	70	12,000[a]	38,000[a]	3.2	80	12,000[a]	61,000[a]	5.0	38	24,000[a]	83,000[a]	3.45

[a]Geom. avg. of seasonal means

TABLE 5. RELATIONSHIP OF SANITARY RANK OF PRODUCING FARMS TO INCREASE IN COLIFORM COUNTS DUE TO PRELIMINARY INCUBATION OF MILK SAMPLES EXAMINED DURING DIFFERENT SEASONS

	Sanitation rank I				Sanitation rank II				Sanitation rank III			
Season	No. of samples	Arith. mean of initial counts (A)	Arith. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Arith. mean of initial counts (A)	Arith. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Arith. mean of initial counts (A)	Arith. mean of P.I. counts (B)	Growth ratio B/A
Fall	10	200	1,400	7.0	8	190	4,800	25.3	7	670	600	0.9
Winter	8	8	120	15.0	17	67	1,200	17.9	7	84	760	9.0
Spring	12	25	56	2.2	16	170	2,600	15.3	9	600	40,000	66.7
Summer	38	1,700	3,400	2.0	39	1,300	4,600	3.5	14	2,000	4,400	2.2
Summary	68	500[a]	1,200[a]	2.4	80	400[a]	3,300[a]	8.3	37	800[a]	11,000[a]	13.8

[a]Arith. avg. of seasonal means

make it questionable that P.I. coliform counts would be a suitable index of sanitation.

Relationship of sanitary rank of producing farms to P. I. psychrophile counts

The initial geometric average of psychrophile counts in each season increased with sanitation rank, except for sanitation rank III during fall and summer (Table 6). In summer, averages remained generally similar in each rank. However, analysis of the grouped data indicated differences in initial count due to season and sanitation rank were not statistically significant.

Overall geometric means of P.I. counts were 26,000, 35,000 and 110,000/ml for sanitary ranks I, II, and III, respectively, showing that, for the year, P.I. counts increased as sanitary rank increased. However, the relationship was not so consistent in all seasons. P.I. counts were highest during winter.

Although the overall growth ratio was not consistently related to sanitation rank, it was highest (40.7) for rank III. Seasonal growth ratios were highest in winter and highest in sanitation rank III during each season.

The effects of season and sanitation rank on growth ratios were statistically significant ($P < 0.05$). It appears that growth ratios and P.I. counts of psychrophiles are indices of sanitation. However the time involved limits their value.

Relationships among level of initial standard plate count, sanitary ranking, and P.I. counts

Summarized data are grouped in Table 7 to indicate relationships of P.I. counts and growth ratios with levels of initial standard plate count and sanitation rank.

In ranks I, II and III, 48%, 41%, and 40%, respectively, of the samples in each rank had initial counts less than 10,000/ml. Geometric mean counts of these samples were 4,900, 4,600, and 5,400/ml in sanitation rank I, II and III, respectively. Obviously milk with relatively low initial standard plate counts (<10,000/ml) was not necessarily limited to that produced on farms with sanitation rank I. Moreover, 77%, 84%, and 62.5% of the samples in sanitation rank I, II and III, respectively, had counts of 50,000 or less. This suggests that the present standard of 200,000/ml for raw bulk milk is exceptionally lenient.

In all count ranges, the P.I. count increased as initial count increased, a relationship that occurred in all sanitation ranks. However, actual growth ratios did not show a uniform relationship. It is significant that in all ranges of initial counts up to 50,000/ml, the growth ratios increased as sanitation rank increased. Statistical analysis of the logs of individual counts by linear regression also showed that as sanitation became poorer, the growth ratios became greater.

In view of the relationships of P.I. counts and growth ratios with sanitary conditions of farms, it appears that the P.I. count would be a better measure of sanitary conditions of bulk milk producing farms than the standard plate count procedure. With milk of low initial count, the P.I. procedure helps to detect undesirable sanitary conditions. However, with high count milk (>50,000/ml) there is less relationship between growth ratio and sanitation rank. Hence with such milk the P.I. count would appear to have little advantage over the standard plate count as a measure of sanitation. Nevertheless, since the initial count level seldom can be predicted, a consistent P.I. program would seem desirable. A plate incubation temperature of 32 C for standard plate counts might have indicated different relationships among the various types of counts.

TABLE 6. RELATIONSHIP OF SANITARY RANK OF PRODUCING FARMS TO INCREASE IN INITIAL PSYCHROPHILIC COUNTS DUE TO PRELIMINARY INCUBATION OF RAW MILK SAMPLES IN DIFFERENT SEASONS

	Sanitation rank I				Sanitation rank II				Sanitation rank III			
Season	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A
Fall	5	700	14,000	20.0	7	5,000	10,000	2.0	5	1,000	21,000	21.0
Winter	10	3,000	160,000	53.3	18	3,600	140,000	38.9	8	6,000	500,000	83.3
Spring	12	700	12,000	16.1	16	1,600	36,000	22.5	9	6,600	410,000	62.1
Summer	37	1,300	16,000	12.3	39	1,900	30,000	15.8	14	1,300	29,000	22.3
Summary	64	1,200[a]	26,000[a]	21.7	80	2,700[a]	35,000[a]	12.9	36	2,700[a]	110,000[a]	40.7

[a]Geom. avg. of seasonal means

TABLE 7. RELATIONSHIP OF RANGE OF INITIAL STANDARD PLATE COUNT AND SANITARY RATING OF FARMS TO PRELIMINARY INCUBATION COUNTS

	Sanitation rank I				Sanitation rank II				Sanitation rank III			
Range of initial standard plate count	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A	No. of samples	Geom. mean of initial counts (A)	Geom. mean of P.I. counts (B)	Growth ratio B/A
<10,000	33	4,900	15,000	3.1	34	4,600	20,000	4.3	16	5,400	31,000	5.7
10,000 to 30,000	17	16,000	52,000	3.3	27	18,000	79,000	4.4	9	18,000	91,000	5.1
30,000 to 50,000	5	39,000	79,000	2.0	6	36,000	77,000	2.1	3	37,000	310,000	8.4
>50,000	14	150,000	290,000	1.9	12	120,000	430,000	3.6	12	160,000	460,000	2.9

CONCLUSIONS

The present initial standard plate count maximum of 200,000/ml seems exceptionally lenient for raw, bulk tank, grade-A milk.

With low count milk, the P.I. standard plate count procedure is a better index of sanitary conditions of producing farms than is the initial standard plate count. When the initial count range is >50,000/ml and during the summer season, the standard plate count and P.I. count are about equal as measures of sanitation. Under practical laboratory conditions, it appears that a routine P.I. standard plate program would have merit.

The variation among seasons and sanitation ranks makes it questionable that the P.I. coliform count would be a suitable index of bacteriological quality.

Growth ratios and P.I. counts of psychrophiles appear to be the best indices of sanitary conditions. However, the time involved in incubation of plates for psychrophile counts limits the value of such procedure.

ACKNOWLEDGMENTS

Acknowledgment is made to Riley County Sanitarian, W. P. Deam, for assistance in obtaining milk samples and to Dr. Stanley Wearden of the Department of Statistics, Kansas State University, for help with statistical analyses.

REFERENCES

1. American Public Health Association, Inc. Standard methods for examinaion of dairy products. 11th ed. Am. Pub. Health Assoc., New York, N. Y. 1960.

2. Davis, R., and Killmeier, J. T., Jr. A new method for determining the quality of bulk tank milk. Am. Milk Rev. and Milk Plant Mo., 21 (7):37-40. 1959.

3. Johns, C. K. Appraisal of methods for assessing the sanitary quality of milk. Canada Dept. Agr. Publ. 1084. 1960.

4. Johns, C. K. Preliminary incubation of raw milk samples. Jour. Milk Food Technol. 23:137-141. 1960.

5. Johns, C. K., and Berzins, I. The value of preliminary incubation in bacteriological tests for milk. 15th Intern. Dairy Congr., 3:1293-1302. 1959.

6. Orr, M. J., McLarty, R. M., and Baines, S. Further studies on bulk milk collection. Dairy Indus., 25:360-364. 1960.

7. Smillie, D. M., Orr, M. J., and McLarty, R. M. Bulk milk collection in Wigtownshire: Bacterial counts at 6 and 30 hours. Dairy Indus. 23:560-564. 1958.

8. U. S. Dept. of Health, Education and Welfare. Milk ordinance and code. P. H. S. publication No. 229. 1953. U. S. Govt. Printing Office, Washington 25, D. C.

STATEMENT OF OWNERSHIP, MANAGEMENT AND CIRCULATION

(act of October 23, 1962; Section 4369, Title 39, United States Code)

Of The Journal of Milk and Food Technology, published monthly at Blue Ridge Rd., R. R. 6, Shelbyville, Indiana, for November 14, 1964.

The names and addresses of the publisher, editor, and managing editor are:

Publisher, International Association of Milk, Food and Environmental Sanitarians, Inc.

Editor, Dr. J. C. Olson, University of Minnesota, St. Paul, Minn.

Managing editor, H. L. Thomasson, R. R. 6, Shelbyville, Ind.

The owners of International Association of Milk, Food and Environmental Sanitarians, Inc. are:

Non-profit Corp. – No stockholders.

The known bondholders, mortgagees, and other security holders owning or holding 1 per cent or more of total amount of bonds, mortgages, or other securities are:

Column one average No. copies each issue during preceeding 12 months — Column two single issue nearest to filing date.

A. Total No. Copies Printed (Net Press Run)	5000	4900
B. Paid Circulation		
1. To term subscribers by mail, carrier delivery or by other means	4681	4610
2. Sales through agents, new dealers or otherwise	35	23
C. Free Distribution (including samples) by mail, carrier delivery, or by other means	110	100
D. Total No. of Copies distributed. (Sum of lines B1, B2 and C)	4826	4733

I certify that the statements made by me above are correct and complete.

H. L. Thomasson, Managing Editor