

## Comparison of Incubation at 30 and 32 C for 48 and 72 Hours for Enumeration of Raw-Milk Bacteria<sup>1</sup>

C. N. HUHTANEN,<sup>2</sup> A. R. BRAZIS,<sup>3</sup> H. J. ANDERSON,<sup>4</sup> W. L. ARLEDGE,<sup>5</sup> C. B. DONNELLY,<sup>6</sup> R. E. GINN,<sup>7</sup>  
E. J. KOCH,<sup>8</sup> F. E. NELSON,<sup>9</sup> W. S. LaGRANGE,<sup>10</sup> D. E. PETERSON,<sup>11</sup> H. E. RANDOLPH,<sup>12</sup>  
E. L. SING,<sup>13</sup> D. I. THOMPSON,<sup>14</sup> and H. M. WEHR<sup>11</sup>

### ABSTRACT

Plates incubated for 48 h showed significantly higher ( $0.01 < P < 0.05$ ) counts at 30 than 32 C (arithmetic means were 5.4% higher; geometric means were 1.5% higher). These higher counts, however, were largely obtained by two of 15 analysts representing 12 of 135 samples. Seventy-two-hour incubation gave significantly higher ( $P < 0.01$ ) counts than 48 h (arithmetic means for 72 h were 4.53% higher than 48 h; geometric means for 72 h were 2.58% higher) at both temperatures. There were interaction effects indicating a geographical or personal bias in the results.

Some of the earliest work on comparing incubation temperatures for plate counts of bacteria from raw-milk and milk products was by Pederson and Yale (18) who indicated that a 32 C incubation gave higher and more reproducible counts than did 37 C. They also indicated that a  $\pm 2$  degree variation from 32 C decreased counts by 6% (30 C) and 13% (34 C). These results were obtained using the standard peptone agar then recommended by *Standard Methods for Milk Analysis* (2). Yale and Pederson (24) later showed that raw-milk plated in a tryptone-glucose skim milk agar gave higher counts at or slightly below 30 C than at 32 C. The fallacy in

translating plate counts from one medium to those obtained with was pointed out by Abele (1) in 1939.

More recent work has been with Standard Methods agar (tryptone-glucose-yeast extract) now recommended by *Standard Methods for the Examination of Dairy Products* (6). Pure cultures from milk were studied by Lawton and Nelson (16). They found that most of the psychrotrophic bacteria grew better at 21 or 25 C than at 5 or 10 C while some grew slower or not at all at 32 C. They did not study growth at 30 C. Nelson and Baker (17) showed that higher counts were obtained at 25 C for 3 days or 21 C for 4 days than at 32 C for 2 days. Greene and Jezeski (10) also studied several psychrotrophs from creamery water supplies and demonstrated a shorter lag period and more rapid growth at 30 C than at 25 C or lower. Thomas et al. (23) showed that thermotrophic bacteria from pasteurized milk (30 min at 62.5) were recovered in greater numbers when plates were incubated at 28 C for 4 days than when incubated for 32 C at 2 or more days. They indicated that incubation at 32 C gave increasingly higher counts with the increased duration of incubation.

Pedraja and Mengelis (19) also showed that for determining the Standard Plate Count of nonfat dry milk, 3 days incubation at 32 C gave higher counts than 2 days. The effect of incubation for 2 or 3 days was investigated by Babel et al. (7). They found no increase in counts for plates incubated at 32 C at 3 days among approximately 40 raw-milk samples tested although they indicated that incubation longer than 3 days at 26 C gave higher counts. Randolph et al. (20) however, found no significant advantage for a 27-C incubation temperature for 2 days over that presently recommended. Huhtanen (12) found higher (although not statistically significant) counts in 2 days at 30 C than at 32 C. Evidence was presented to show that 33-C incubation gave significantly lower counts than 30 C.

Hartley et al. (11) found higher, statistically significant, geometric mean counts at 28 than at 32 C while the effect of two types of agar media (standard methods and eugonagar) seemed to depend on the origin of the milk sample. Pasteurized milk and manufacturing-grade raw bulk tank samples did not show agar

<sup>1</sup>A contribution from the Subcommittee for the Examination of Milk and Milk Products, Applied Laboratory Methods Committee, International Association of Milk, Food, and Environmental Sanitarians, Inc.

<sup>2</sup>Eastern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, Philadelphia, Pennsylvania 19118 (Chairman of the Subcommittee).

<sup>3</sup>Division of Microbiology, FDA, 1090 Tusculum Avenue, Cincinnati, Ohio 45226 (Chairman, Applied Laboratory Methods Committee).

<sup>4</sup>Minnesota Department of Agriculture, St. Paul, Minnesota 55155

<sup>5</sup>Dairymen, Inc., 200 West Broadway, Louisville, Kentucky 40202

<sup>6</sup>Division of Microbiology, FDA, 1090 Tusculum Avenue, Cincinnati, Ohio 45226

<sup>7</sup>Dairy Quality Control Institute, Inc. 2353 N. Rice Street, St. Paul, Minnesota 55113

<sup>8</sup>Biometrician, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland 20705

<sup>9</sup>Department of Nutrition and Food Science, University of Arizona, Tucson, Arizona 85721

<sup>10</sup>Extension Service, Iowa State University, Ames, Iowa 50010.

<sup>11</sup>State Department of Agriculture, Salem, Oregon 97310

<sup>12</sup>Department of Animal Science, Texas A and M University, College Station Texas 77843

<sup>13</sup>Moseley Laboratories, 3862 E. Washington Street, Indianapolis, Indiana 46201

<sup>14</sup>State Board of Health, 437 Henry Mall, Madison, Wisconsin 53706

differences, while Grade A raw-milk counts varied with agar; higher counts were obtained on standard methods agar.

Recently Huhtanen et al. (13) showed that a 3-day incubation at 32 C gave significantly higher ( $P < 0.05$ ) counts than incubation for 2 days. Roughley et al. (22) and Johns and Smith (15) showed that incubation for 3 days at 30 and 32 C gave higher counts than 2 days; they also indicated that 30-C incubation gave higher counts than 32 C for raw but not for pasteurized milk.

The basic philosophy for the introduction of new methodology in the introduction to the 12th Edition of *Standard Methods*, edited by Walter (5), was that "no new method or modification of an old method should be introduced unless it has undergone careful cooperative testing in several laboratories, with the data available to the committee (on standard methods), and to any other interested parties, preferably by publication in a recognized scientific journal." Some consideration is being given to changing the Standard Plate Count incubation time and temperature from 2 days at 32 C to 3 days at 30 or 32 C. The study reported here was undertaken to determine the validity of any such change using the concepts advanced above.

#### MATERIALS AND METHODS

Fifteen analysts from the states of Ohio, Minnesota, Tennessee, Virginia, Kentucky, Iowa, Oregon, Arizona, Wisconsin, Texas, and Indiana participated in this study. Each selected his own raw-milk bulk tank samples (135 in all) and assayed them according to *Standard Methods for the Examination of Dairy Products* (4). Each analyst was asked to assay a minimum of 12 raw-milk samples. Previous cooperative testing (13) had indicated that six samples were adequate for satisfactory statistical results; however, many of the counts of the 12 samples were not used due to spreaders, too few colonies (less than 30), too many colonies (more than 300), or incompleteness due to laboratory accidents. Duplicate petri dishes were used for each experimental condition. Comparisons were made of 30 and 32 C incubation

temperatures for 48 and  $72 \pm 3$  h. Statistical analysis was by conventional analysis-of-variance with mixed classification of variables done on  $\ln$  transformed data.

The analysis of variance was, for convenience in computer programming, divided into two parts; one with the results for 48-h incubation at 30 and 32 C (all 15 analysts, 135 milk samples); the other with data from both temperatures and times of incubation (8 analysts, 80 milk samples). The statistical model for the analysis was a mixed-variable one with random samples, fixed temperatures of incubation, and random "analysts." Since each analyst assayed different lots of raw-milk and no samples were assayed by more than one analyst, the concept of analyst error was unavoidably confounded with samples. The actual error term used could be due to factors other than differences between analysts per se, such as source of milk samples, geographical locations, etc. With the mixed model, the interaction error for samples and temperatures was used for testing main effects while the residual (between petri dishes) error tested the sample-temperature interaction.

The raw data are not given in this report due to their voluminous nature (860 individual plate counts were made), but are available to interested parties.

#### RESULTS

##### Means of incubation temperatures

The arithmetic and geometric means are shown in Table 1. Fifteen analysts incubated plates for 48 h at 30 and 32 C while 8 analysts incubated plates for 48 and 72 h at both temperatures. Eight of the 15 analysts incubating plates for 48 h obtained higher arithmetic mean count at 30 than at 32 C (mostly due to analysts 8 and 13); seven obtained higher counts at 32 C. For all 15 analysts, however, the arithmetic means were 5% higher at 30 than at 32 C; the geometric means were 1% higher.

##### Means of incubation times

All eight analysts of those reporting results for both 48 and 72 h found higher arithmetic and geometric means at 72 h for both temperatures of incubation. For 30 and 32 C, 17% higher arithmetic and 6% higher geometric means were found at 72 than at 48 h.

TABLE 1. Means of raw-milk plate counts incubated at 30 and 32 C for 48 and 72 h

Analyst	Number samples tested (total = 135)	Arithmetic mean <sup>a</sup>				Geometric mean <sup>b</sup>			
		48 h		72 h		48 h		72 h	
		30	32	30	32	30	32	30	32
1	14	112	111	114	115	4.48 <sup>b</sup>	4.48	4.52	4.52
2	7	14.7	15.9	15.7	16.6	2.47	2.48	2.53	2.53
3	19	28.7	28.8	29.3	31.2	2.76	2.80	2.86	2.86
4	11	10.3	11.0	12.6	11.9	2.14	2.22	2.31	2.30
5	7	63.1	63.0	69.2	67.3	3.55	3.59	3.68	3.70
6	7	15.1	14.0	16.5	15.8	2.52	2.46	2.59	2.57
7	5	10.3	10.7	11.3	10.8	2.27	2.31	2.38	2.33
8	7	174	145	—	—	5.07	4.91	—	—
9	10	59.9	57.1	62.1	58.8	3.49	3.36	3.55	3.45
10	9	26.8	27.1	—	—	3.25	3.24	—	—
11	10	24.5	23.8	—	—	2.57	2.54	—	—
12	8	6.34	5.96	—	—	1.83	1.75	—	—
13	5	73.8	57.9	—	—	4.15	3.90	—	—
14	10	12.3	12.8	—	—	2.33	2.28	—	—
15	6	42.0	43.8	—	—	3.14	3.17	—	—
Average of 15 analysts		44.7	42.4	—	—	3.06	3.03	—	—
Average of 8 analysts		39.3	38.9	45.9	45.8	2.96	2.96	3.15	3.13

<sup>a</sup>Actual counts divided by 1000 and rounded off from 9 significant figures of computer data. Averages are based on computer data and are true overall means.

<sup>b</sup> $\ln$  of original counts divided by 1000.

TABLE 2. Analysis of variance of plate counts incubated for 48 h at 30 and 32 C

Line	Source	df	ms	F <sup>a</sup>	Significant with	
					P < 0.05	P < 0.01
A	"Analysts" <sup>b</sup> (a)	14	28.9347	10.1	yes	yes
B	Samples within analysts (s)	120	2.8636	145	yes	yes
C	Temperature (t)	1	0.0792	4.01	yes	no
D	a × t	14	0.0572	2.90	yes	yes
E	s × t	120	0.0198	1.02	no	no
F	Residual	270	0.0194			
	Total	539				

<sup>a</sup>Ratios from lines A/B, B/E, C/E, D/E, E/F.

<sup>b</sup>See text for explanation of analyst error. This table includes data from all 15 analysts with 72-h incubation omitted for analysts 1-7 and 9. A total of 135 milk samples was analyzed.

TABLE 3. Analysis of variance of plate counts incubated for 48 or 72 h at 30 and 32 C

Line	Source	df	ms	F <sup>a</sup>	Significant with	
					P < 0.05	P < 0.01
A	"Analysts" <sup>b</sup> (a)	7	54.8576	8.75	yes	yes
B	Samples within analysts (s)	72	6.2680	652.92	yes	yes
C	Temperature (t)	1	0.0044	0.46	no	no
D	Incubation times (d)	1	1.0010	104.37	yes	yes
E	t × d	1	0.0191	1.99	no	no
F	t × a	7	0.0456	4.75	yes	yes
G	a × d	7	0.0171	1.78	no	no
H	a × t × d	7	0.0105	1.09	no	no
I	s × t	72	0.0298	3.11	yes	yes
J	s × d	72	0.0190	1.98	yes	yes
K	s × t × d	72	0.0096	0.72	no	no
L	Residual	320	0.0133			
	Total	639	1.3228			

<sup>a</sup>Ratios from lines A/B, B through J/K, K/L.

<sup>b</sup>See text for explanation of analyst error. This table includes data from analysts 1-7 and 9 with a total of 80 milk samples.

### Analysis of variance for temperatures

The analysis of variance for 48 h is shown in Table 2. The samples, as expected, were highly significantly different. "Analysts" also were significantly different (P < 0.01). In this analysis, the plate counts at the two temperatures of incubation were significantly different at 5% but not at the 1% level. There was analyst-temperature interaction indicating some geographical or personal bias.

The second part of the analysis of variance with temperatures and both incubation times is shown in Table 3. There was no difference between 30 and 32 C incubation but there was a statistically significant difference between 48 and 72 h incubation. There were also significant analyst-temperature, sample-temperature and sample-days interactions.

## DISCUSSION

This study indicates that significantly higher Standard Plate Counts for raw-milk bacteria are obtained when the incubation time is extended to 72 from 48 h. The incorporation of such a change in *Standard Methods for the Examination of Dairy Products* would seem to be warranted if its aim is to determine the greatest possible numbers of total bacteria in raw-milk with the greatest possible precision. The early concept underlying the introduction of standardized methodology for plate counts seemed to be one of detecting potential human pathogens with plates being incubated at 37 C. This concept apparently underwent a metamorphosis with the

work of Pederson and Yale (18) and Yale and Pederson (24), who demonstrated increasing precision and higher counts at 32 C. The eighth edition of *Standard Methods*(3) recognized incubation at 32 or 37 C, while the ninth edition recommended only 32 C (4).

The rationale behind the Standard Plate Count was discussed by Reinbold (21), who indicated that there seemed to be no direct connection between public health and the Standard Plate Count as presently constituted. Barnum (8) and Blankenagel (9) suggested further that one could not equate poor farm sanitation practices with high bacterial counts in the raw-milk obtained using the Standard Plate Count.

Any change in the Standard Plate Count should be considered only after the inconvenience and cost to the industry have been assessed. A mandatory 72-h incubation would necessitate considerable restructuring of laboratory schedules. An optional 72- or 48-h incubation might be a reasonable compromise and would permit the greatest flexibility in the dairy laboratory.

A change from 32 to 30 C incubation temperature is not warranted solely by the small, questionably significant, differences obtained. A change to 30 C probably would not decrease the Standard Plate Count and would be the same as that adopted by the International Dairy Federation (14) for determination of mesophiles in milk. The present tolerance of temperatures for incubators is  $\pm 1$  C (6); a recent study (12) indicated that lower counts were obtained at 33 C than 32 C. A similar tolerance at 30 C would assure that

incubators did not reach a possibly deleterious temperature.

The highly significant "analyst" error observed in this study underscores the wisdom in the cooperative testing concept of the 12th edition of *Standard Methods for the Examination of Dairy Products* (5). The results obtained at any one location or by one analyst cannot be a satisfactory basis for changing present methodology.

### CONCLUSIONS

Incubation for  $72 \pm 3$  h gave a significantly higher Standard Plate Count than 48 h. There was no significant difference in counts obtained at 30 or 32 C incubation temperature.

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