

INCUBATION TEMPERATURES AND RAW MILK BACTERIAL COUNTS

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ABSTRACT

Raw milk samples were plated and incubated at seven temperatures (2, 10, 20, 27, 30, 33 and 37 C). Lowest counts were obtained at 37 C. Plates at 33 C showed higher counts than at 37 C but were significantly lower than the other temperatures. The 10, 20, 27 and 30 C incubated plates were not statistically different in counts. When 32 C was substituted for 33 C, there was a significant difference (lower) only in the 37 C count. Colonies from 2, 20, 27, 33 and 37 C plates were picked and stabbed into plates subsequently incubated at these temperatures. All colonies developed best at 20 and 27 C. Evidence is presented that psychrophilic counts can be indirectly determined as the difference between counts at 27 C and 37 C.

The effect of plating temperatures on recoverable numbers of raw milk bacteria has been studied by a number of workers (1, 3, 5, 6, 7). Nelson and Baker (5) studied the effect of incubation time and temperature on market milk. The ideal temperature should be one that permits the growth of the largest numbers of bacteria in a reasonable amount of time. Temperatures of 32 and 35 C were approved by the 11th Edition of *Standard Methods for the Examination of Dairy Products* (American Public Health Association, Inc., New York, N. Y. 10019, 1960).

MATERIALS AND METHODS

Procedures outlined in the 11th Edition of *Standard Methods for the Examination of Dairy Products* were followed except for the variable of incubation temperature. Incubation times were 10 days at 2 C, 7 days at 10 C, 5 days at 20 C and 2 days at 27, 30, 32, 33 and 37 C. Colonies picked for determination of temperature requirements for growth were representative of the types observed (pin point, surface, pigmented, lenticular, etc.). Each colony was picked once with a needle and stabbed into cooled petri dishes containing 25-30 ml Plate Count agar. Sixteen such stabs representing 16 different colonies were made per plate, the same needle being used to serially stab a total of 5 plates. Preliminary results indicated that up to 100 stabs could be made serially with the bacteria picked up on 1 needle. The stabs showing any signs of growth, as observed in a Quebec colony counter, were considered positive. Raw milk samples were obtained from the bulk tank delivered milk of a local dairy. Regular bacterial counts of colonies were made, also with a Quebec colony counter.

The incubators used were BOD types for 2 C and 27 C.

TABLE 1. EFFECT OF TEMPERATURE ON RECOVERIES OF RAW MILK BACTERIA¹

Sample Date	Incubation Temperature (C)						
	2	10	20	27	30	33	37
5/5/66	11.5	7.1	14.0	18.7	16.0	19.2	
	8.0	5.0	13.2	19.5	18.2	16.1	
5/9	60	130	250	210	210	190	
	120	150	260	280	200	150	
5/11	1880	2150	1500	1690	1370	1050	
	2000	2250	2000	2400	1650	1100	
5/17	115	135	155	135	140	25	
	122	117	143	175	145	32	
5/19	182	182	165	140	101	100	
	135	171	155	182	66	98	
5/25	31	23	27	68	32	26	10
	25	33	47	51	48	24	12
5/27	205	196	210	197	160	68	33
	195	220	260	220	180	69	33
6/10	440	330	460	480	280	340	100
	450	380	420	460	370	310	95
6/14	67	75	86	55	45	37	7
	70	63	80	66	43	40	15
6/20	39	87	360	320	330	300	320
	34	94	300	350	330	340	270
6/27	29	35	60	67	87	57	16
	33	32	70	70	77	75	16
7/6	1350	2240	2800	3000	2900	1800	210
	1150	2640	2600	3100	3000	1750	150
7/12	850	850	770	1030	830	750	33
	800	1050	900	870	900	830	25
7/18	2250	1750	1900	2050	2100	2000	95
		1820	1800	2060	2400	2100	125
8/15	59	77	101	90	106	60	13
	52	90	95	95	110	75	19
8/22	950	1500	1320	1500	1360	920	65
	1100	1400	1460	1500	1180	1100	90
8/29	324	310	290	310	330	150	35
	360	280	320	350	260	210	36
9/12	36	60	105	92	85	70	16
	33	75	86	95	70	75	29
9/19	60	130	130	180	178	130	53
	57	135	145	180	150	125	51
9/26	50	58	102	71	85	85	21
	60	76	85	90	100	80	27
10/11	72	140	135	152	115	105	65
	100	137	170	175	105	90	30
10/18	150	205	195	169	143	170	16
	240	210	205	197	170	140	14
11/2	2500	2900	2300	2500	2400	2450	58
	2100	2200	2500	2550	2200	2700	57
11/14	85	102	118	100	110	115	34
	88	96	110	115	105	100	23
12/1	1450	1150	1150	1200	1400	1250	26
	1250	1350	1750	1450	1300	1400	37
12/12 A	18	36	50	62	91	87	38
	17	28	52	82	79	97	45
B	15	72	78	65	90	70	12
	18	66	81	85	120	51	9
1/13/67	310	390	370	530	410	360	36
	300	260	480	360	320	380	40
1/17 A	2600	2500	2800	2850	2800	280	12
	2700	2700	2300	2900	3000	260	11
B	210	200	200	235	215	120	16.5
	175	200	245	260	225	120	17
1/30	104	81	125	140	137	90	18
	125	115	135	150	140	105	24
2/13	25	45	55	58	85	53	10
	40	54	70	72	66	61	10.5
2/28	2650	2400	2400	2140	2400	2720	9.2
	2400	2200	2500	2500	2300	2250	5.7
Averages ²	556 ^a	608 ^{ab}	629 ^b	668 ^b	629 ^b	479 ^c	46 ³

¹Total counts—Multiply above by 1000.

²Figures superscripted with like letters are not significantly different.

³The 37 C results were intuitively significantly lower and were not analyzed statistically.

¹Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

TABLE 2. ANALYSIS OF VARIANCE OF DATA IN TABLE 1

	Sum of Squares (ss)	Degrees of Freedom (df)	Mean Square (ms)	F	Significant Differences
Sample date (Rows)	272,440,474.25	33	1,254,559.82	161.28	+
Incubation temperature (Columns)	1,552,250.37	5	310,450.07	6.06	+
Error	18,936,523.96	370	51,179.79		
Total	292,889,248.58	408			

TABLE 4. ANALYSIS OF VARIANCE OF DATA IN TABLE 3

	ss	df	ms	F	Significant Differences
Sample date (Rows)	77,805,717.91	6	12,967,619.25	51.32	+
Incubation temperature (Columns)	6,445,126.89	6	1,074,187.81	4.25	+
Error	19,474,640.25	85	252,642.82		
Total	105,725,485.04	97			

These had temperature variations of ± 0.5 degree. The 30, 32 and 33 C incubators were forced draft type modified by adding a copper coil in front of the air stream. Cold tap water (10 C) circulated through these coils at 12 psi. No noticeable temperature variation occurred except under rare

conditions when the ambient temperature fluctuated greatly. Under these conditions, a maximum variation of ± 0.5 degree was observed. The 10 and 20 C incubators were controlled temperature storage rooms with variations less than ± 0.5 degree. The 37 C incubator was a forced draft type with ± 0.5 degree variation. Temperatures were recorded using a thermometer immersed in a sealed flask of water.

TABLE 3. EFFECT OF TEMPERATURE ON RECOVERIES OF RAW MILK BACTERIA¹

Sample Date	Incubation Temperature (C)						
	2	10	20	27	30	32	37
3/13/67	46	60	77	84	78	75	20
	38	66	84	74	88	80	20
3/21	2700	3800	4000	3300	2000	1950	16
	3500	4000	3600	3500	1900	1750	14
4/11	46	76	40	61	39	18	12.5
	64	71	32	52	39	25	12
4/28	11	16	49	37	31	37	2.2
	7	10	32	24	32	27	2.6
5/10	35	39	54	38	32	25	8.5
	24	39	64	25	28	32	10.1
5/17	68	69	92	62	65	43	6.5
	71	78	90	54	35	59	8.3
6/28	1200	1450	760	1520	810	740	26
	2150	1150	1100	1200	800	700	37
Averages ²	711 ^a	780 ^a	720 ^a	716 ^a	427 ^a	397 ^a	14 ^b

¹Total counts—Multiply above by 1000.

²Figures superscripted with like letters are not significantly different.

RESULTS

Table 1 shows the numbers of bacteria recovered from incubation at 2, 10, 20, 27, 30, 33 and 37 C. Analysis of variance showed very significant F values (for this analysis the data obtained at 37 C were not included since these were obviously lower than all the rest). The Duncan Multiple Range Test (2) showed the plates incubated at 33 C to have significantly fewer colonies than the others analyzed. Plates incubated at 2 and 10 C were not significantly different from each other; but those held at 2 C were significantly lower in numbers than plates at 10, 20, 27 or 30 C. Hence, plates held at 10, 20, 27 and 30 C could be considered to have the same numbers of colonies while the results from incubation at 2 and 33 C might be different.

Table 3 was compiled from data using 32 C instead of 33 C. In this instance only the plates at 37 C had fewer bacterial colonies while those at 2, 10, 20, 27, 30 and 32 C were similar. The counts at 30 and 32 were lower, but analysis by the Duncan

TABLE 5. RELATIONSHIP OF COUNTS OBTAINED AT 27 C INCUBATION TO THOSE FROM 2 AND 37 C COMBINED^{1 2}

Date	Average Counts From			2 + 37-27 = \bar{u}
	2 C	37 C	27 C	
5/25/66	28	11	59.5	-20.5
5/27	200	33	208.5	24.5
6/10	445	97.5	470	72.5
6/14	68.5	11	60.5	19.0
6/20	36.5	295	335	-3.5
6/27	31.0	16	68.5	-21.5
7/6	1250	180	3050	-1620.0
7/12	825	29	950	-96.0
8/15	55.5	16	92.5	-21.0
8/22	1025	77.5	1500	-397.5
8/29	342	35.5	330	47.5
9/12	34.5	22.5	93.5	-36.5
9/12	27.5	29.0	70.0	-13.5
repeat				
9/19	58.5	52.0	180	-69.5
9/26	57.5	24.0	80.5	+1.0
10/11	86.0	47.5	163.5	-30.0
10/18	195	15.0	183	27.0
11/2	2300	57.5	2525	-167.5
11/14	86.5	28.5	107.5	7.5
12/1	1350	31.5	1325	56.5
12/2 A	17.5	41.5	72	-13.0
B	16.5	10.5	75	-48.0
1/13/67	305	38.0	445	-102.0
1/17 A	2650	11.5	2875	-213.5
B	192.5	16.75	247.5	-38.25
1/30	114.5	21.0	145	-9.5
2/13	32.5	10.25	65	-22.25
2/28	2525	7.45	2320	+212.45
	14,355.5	1265.95	18,097.00	-2475.55
	15,622			

¹Student's t = 1.47 • critical t value = 1.703 at .10 level of significance.

²d² = 2,946,353.8775.

Multiple Range Test did not permit assigning significance to these differences. It is interesting to note in these 2 tables that the sum of the total counts of the plates grown at 2 and 37 C nearly equaled the count at 27 C. This suggests that the 27 C counts were composed of the high and low temperature bacteria. This sum also approached the counts at 10, 20 and 30 C.

These counts were analyzed more carefully with the results shown in Table 5. The hypothesis that the counts at 27 C were composed of the 37 C and 2 C counts was set up with the difference (d) being the sum of the 2 and 37 C counts minus the 27 C count. The true difference (μ_d) should be 0. The mean of the differences (\bar{d})

was calculated from the relationship $\bar{d} = \frac{\sum d}{n} = \frac{-2475.55}{28}$

= -88.41250. The $Sd^2 =$

$$\frac{n\sum d^2 - (\sum d)^2}{n(n-1)} = \frac{28(2,946,353.8775) - (2475.55)^2}{28(27)} =$$

101,793.753 and $S\bar{d} = \sqrt{\frac{Sd^2}{n}} = 60.29$. The Student

t value, $t = \frac{\bar{d} - u}{S\bar{d}} = \frac{-88.4125 - 0}{60.29} = -1.47$ while the

critical t value for 27 degrees of freedom at a 0.10 level of significance was ± 1.703 . In other words, the difference observed here was not great enough to negate the hypothesis that the counts at 27 C were composed of the combined counts at 2 and 37 C.

Bacteria usually grow over wide ranges of temperature with much overlapping. This is shown in Tables 6 and 8. When colonies picked from plates incubated at 2 C were stabbed into petri dishes, a few stabs developed at 37 C; a greater number at 33 C; but most at 2, 20 and 27 C (Table 6).

Significantly fewer colonies developed at 33 and 37 C. Colonies from plates held at 20 C showed equal numbers at 20 and 27 C which were statistically higher than those at the other temperatures. The stabbed plates incubated at 37 C showed lowest numbers of colonies developing. Statistically there were no differences between the numbers developing at 2 and 33 C. The results of the stabbed plates, inoculated with colonies from the plates incubated at 27 or 20 C were similar.

The stabbed colonies from the plates held at 33 C grew best at 20, 27 and 33 C with no significant differences. Fewer colonies developed at 2 and 37 C. Colonies picked from the plates held at 37 C showed equally good growth at 20, 27, 33 and 37 C but fewer colonies developed at 2 C.

Table 8 shows similar results when 32 C was substituted for 33 C. In all instances the 20 and 27 C incubation temperatures showed equal numbers of developing colonies and were as high as the plate from which the picked colony had been incubated. Tables 6 and 8 also show that the intermediate temperature counts (20, 27, 32 and 33 C) can be approximated by combining the counts of colonies developing at 2 and 37 C. The results obtained when colonies were picked from plates incubated at 27 C were also subjected to statistical evaluation by setting up the same hypothesis as previously discussed. These results are shown in Table 10. An extremely low Student's t value of 0.06 was obtained indicating that the combined colonies developing at 2 and 37 C were indeed very nearly equal to those developing at 27 C.

TABLE 6. GROWTH OF PICKED COLONIES¹

Incubation Temperature of plate from which colony was picked (C)																									
2					20					27					33					37					
Incubation temperature of picked colony (C)																									
Sample Date	2	20	27	33	37	2	20	27	33	37	2	20	27	33	37	2	20	27	33	37	2	20	27	33	37
6/10/66	16	16	16	14	2	16	16	16	14	4	16	16	16	12	7	13	14	14	15	6	11	16	16	16	16
6/20	14	16	16	5	2	2	16	15	13	12	0	15	15	15	13	6	15	15	15	15	5	16	16	16	16
7/6	16	15	15	8	8	16	16	16	13	0	10	16	16	15	4	6	16	15	15	7	6	16	16	16	16
8/15	16	16	16	7	0	10	16	15	13	6	7	16	16	16	9	9	16	16	16	10	1	16	16	16	16
8/22	16	16	16	12	0	16	16	16	10	1	15	16	16	12	5	14	16	16	16	3	4	16	16	16	16
8/29	16	16	16	14	0	15	16	16	12	1	12	16	16	9	2	14	16	16	16	4	0	16	16	16	16
9/12	9	16	15	11	0	5	16	16	14	7	7	16	16	14	4	6	16	16	16	10	2	16	16	16	16
9/19	16	16	16	14	1	13	16	16	13	6	3	16	16	13	11	8	16	16	16	11	3	16	16	16	16
9/26	14	15	16	14	1	6	15	15	15	10	12	16	16	14	4	5	14	14	14	8	2	16	16	16	16
10/11	16	16	16	7	2	7	16	16	10	5	2	16	16	10	6	7	16	16	16	6	2	16	16	16	16
10/18	16	16	15	14	0	11	16	16	12	1	14	16	16	16	1	13	16	16	16	3	1	16	16	16	16
11/2	16	16	16	14	2	15	16	16	14	2	16	16	16	16	1	15	16	16	16	0	1	16	16	16	16
11/14	16	16	16	16	0	12	16	16	16	4	15	16	16	14	4	8	16	16	16	9	6	16	16	16	16
12/1	16	16	16	14	0	16	16	16	16	0	16	16	16	15	0	16	16	16	16	0	4	13	14	15	16
12/12 A	16	16	16	16	0	11	16	16	15	4	9	13	15	14	6	9	14	16	16	8	1	16	16	16	15
12/12 B	16	16	16	14	2	15	16	16	12	2	13	16	16	13	3	12	16	16	16	7	6	16	16	16	14
1/13/67	16	16	16	16	0	13	16	16	15	4	16	16	16	16	0	13	16	16	16	3	1	16	16	16	16
1/17 A	16	16	16	3	0	16	16	16	3	2	16	16	16	0	0	16	16	16	16	0	2	15	16	16	16
1/17 B	16	16	16	7	2	14	16	16	8	3	14	16	16	10	2	15	16	16	16	1	2	12	14	16	16
1/30	16	16	16	12	0	14	16	16	16	4	14	16	16	14	4	11	16	16	16	5	1	14	15	15	15
2/13	16	14	15	14	0	13	16	16	13	3	11	16	16	15	4	12	16	16	16	4	0	15	16	16	13

Ave. ²	15.5	15.8	15.8	11.7	1.05	12.2	16.0	15.9	12.7	3.9	11.3	15.8	15.9	13.0	4.3	10.9	15.7	15.7	15.8	5.7	2.9	15.5	15.6	15.8	15.9

¹Figures in table represent numbers of colonies developing out of a possible maximum of sixteen.
²Figures connected by lines were not significantly different at .05 level of significance.

TABLE 7. ANALYSES OF VARIANCES OF DATA IN TABLE 6

Temperature of Picked Colonies		ss	df	ms	F	Significant Differences
2 C	Sample date (Rows)	74.51	20	3.72	0.18	-
	Inc. temp. (Columns)	3383.96	4	845.99	40.88	+
	Error	1655.44	80	20.69		
	Total	5113.91	104			
20 C	Rows	53.03	20	2.65	0.43	-
	Columns	2043.01	4	510.75	83.12	+
	Error	491.59	80	6.14		
	Total	2587.63	104			
27 C	Rows	96.93	20	4.85	0.42	-
	Columns	1904.53	4	476.13	41.72	+
	Error	913.07	80	11.41		
	Total	2914.53	104			
33 C	Rows	31.26	20	1.56	0.21	-
	Columns	1688.44	4	417.11	57.10	+
	Error	584.36	80	7.30		
	Total	2284.06	104			
37 C	Rows	53.26	20	2.66	1.54	-
	Columns	2753.52	4	688.38	398.80	+
	Error	138.08	80	1.72		
	Total	2944.86	104			

TABLE 8. GROWTH OF PICKED COLONIES¹

Incubation Temperature of plate from which colony was picked (C)																										
Incubation temperature of picked colony (C)																										
Sample Date	2					20					27					32					37					
	2	20	27	32	37	2	20	27	32	37	2	20	27	32	37	2	20	27	32	37	2	20	27	32	37	
3/13/67	16	16	16	15	0	11	16	16	16	5	11	16	16	16	5	8	15	16	16	8	0	11	16	16	16	
3/21	16	16	16	10	1	16	16	16	10	0	16	16	16	16	9	0	16	16	16	16	0	3	16	16	15	15
4/11	16	16	16	8	0	9	16	16	11	7	14	16	16	16	7	1	4	16	15	16	8	0	16	16	14	16
4/28	16	16	16	5	1	8	16	16	11	3	4	15	9	7	12	4	16	10	16	12	0	16	9	15	14	
5/10	16	16	13	5	0	14	16	16	9	4	9	16	16	13	6	10	16	16	16	5	0	16	16	16	16	
5/17	16	16	14	11	0	16	16	16	5	0	16	16	16	13	1	15	16	16	16	1	0	16	16	16	16	
6/28	16	16	16	8	0	16	16	16	13	1	15	16	16	15	0	13	16	16	16	2	0	16	16	16	16	
Ave. ²	16.0	16.0	15.3	8.8	0.3	12.8	16.0	16.0	10.7	2.9	12.2	15.8	15.0	11.4	3.6	10.0	15.9	15.0	16.0	5.2	0.4	15.3	15.0	15.4	15.6	

¹Figures in table represent numbers of colonies developing out of a possible maximum of sixteen.

²Figures connected by lines were not significantly different at .05 level of significance.

TABLE 9. ANALYSES OF VARIANCES OF DATA IN TABLE 8

Temperature of Picked Colonies		ss	df	ms	F	Significant Differences
2 C	Rows	19.54	6	3.26	1.18	-
	Columns	1311.43	4	327.86	118.90	+
	Error	66.17	24	2.76		
	Total	1397.14	34			
20 C	Rows	18.74	6	3.12	1.13	-
	Columns	822.40	4	205.60	74.31	+
	Error	66.40	24	2.77		
	Total	907.54	34			
27 C	Rows	42.00	6	7.00	1.75	-
	Columns	661.26	4	165.31	41.27	+
	Error	96.14	24	4.01		
	Total	799.40	34			
32 C	Rows	7.20	6	1.20	0.20	-
	Columns	630.68	4	157.67	26.55	+
	Error	142.51	24	5.94		
	Total	780.40	34			
37 C	Rows	18.68	6	3.11	1.47	-
	Columns	1243.31	4	310.83	146.60	+
	Error	50.88	24	2.12		
	Total	1312.88	34			

TABLE 10. RELATIONSHIP OF COLONIES DEVELOPING AT 27 C TO THOSE AT 2 AND 37 C COMBINED^{1, 2}

Date	No. of colonies developing at		27 C	$\frac{2 + 37 - 27}{= d}$
	2 C	37 C		
6/10/66	16	7	16	7
6/20	0	13	15	-2
7/6	10	4	16	-2
8/15	7	9	16	0
8/22	15	5	16	4
8/29	12	2	16	-2
9/12	7	4	16	-5
9/19	3	11	16	-2
9/26	12	4	16	0
10/11	2	6	16	-8
10/18	14	1	16	-1
11/2	16	1	16	1
11/14	15	4	16	3
12/1	16	0	16	0
12/12 A	9	6	15	0
12/12 B	13	3	16	0
1/13/67	16	0	16	0
1/17 A	16	0	16	0
1/17 B	14	2	16	0
1/30	14	4	16	2
2/13	11	4	16	-1
3/13	11	5	16	0
3/21	16	0	16	0
4/11	14	1	16	-1
4/28	4	12	9	7
5/10	9	6	16	-1
5/17	16	1	16	1
6/28	15	0	16	-1
	323	115	439	-1
	438			

¹Student's $t = 0.06$ critical $t 1.703$ at .10 level of significance
² $d^2 = 239$

DISCUSSION

The assessment of the bacteriological quality of raw milk destined to be pasteurized presents several difficulties. A milk high in bacterial count may be more desirable than one with relatively fewer numbers of organisms if the thermal resistance of the two be considered. Psychrophiles are unusually sensitive to pasteurization temperatures while the mesophiles and thermophiles often survive these conditions. The temperature of incubation of the plates will therefore determine to a very large extent the efficiency of pasteurization which in turn determines the safety of milk for public consumption. If one incubated the plates at 2 C, the pasteurization efficiency would, on the surface, be very commendable since these organisms have a low heat tolerance. However, the data reported here indicated that very

few of the mesophilic organisms, originally isolated from the higher temperature 37 C, could grow at 2 C; therefore the pasteurization efficiency would be misleading as far as the mesophilic organisms were concerned. Most of the organisms of public health significance grow well at 37 C (enterococci, *Enterobacteriaceae*, staphylococci, etc.).

The experiments reported here indicate that both the psychrophiles and mesophiles grow with ease in the temperature range 20-27 C. The 20 C temperature of incubation requires more time for colonial development than 27 C, and there were no significant differences in counts. The counts obtained at 33 C were lower than those noted at the lower temperatures while the counts at 32 C also may have been lower. It appears that these temperatures are perilously close to the maximum for the psychrophilic group. An incubation temperature of 27 C for 2 days should be in a safe range to permit all types to flourish. The results reported here show that this temperature permits growth of bacteria isolated from plates incubated at 2 and 37 C. Ingraham (4) showed that a mesophile (*Escherichia coli*) and a psychrophilic pseudomonad had nearly identical generation times at a temperature around 28 C, again indicative of the suitability of this temperature range for bacterial multiplication.

These results point up the possibility of determining numbers of psychrophilic bacteria in raw milk as the difference obtained from plates incubated at a moderate temperature (such as 27 C) from those at a more elevated temperature (37 C). These analyses could be made in 2 days instead of the present 7-10 days required for the standard psychrophilic counts. In addition the counts at 37 C would be of importance since this is a favorable temperature for human pathogens.

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