

# EFFECTS OF TIME OF HOLDING DILUTIONS ON COUNTS OF BACTERIA FROM RAW MILK<sup>1</sup>

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## ABSTRACT

Raw milk samples were diluted with buffered water and held at room temperature for periods up to 20 min before plating. There was an increase in counts at the 95% but not at the 99% level of significance. Most of this increase appeared at 10 min holding time. Interaction effects were highly significant ( $p < 0.01$ ) between holding times and investigators and also between treatments and samples within investigators. It is suggested that the holding time of dilutions to be used for the standard plate count be no longer than 5 min.

The antibacterial effects of sea water are well known (3, 11) and probably account for the rapid disappearance from it of bacteria such as the typhoid bacillus. Less work has been done on the survival of bacteria in demineralized water although Carlucci and Pramer (3) indicated that in their studies, *Escherichia coli* died more rapidly in demineralized water than in water containing 25% sea water. Butterfield (2) studied recovery of bacteria from river waters after 15 and 30 min in various dilution fluids and observed a diminution in counts. Better survival of bacteria was observed in dilute phosphate buffer or

phosphate buffer fortified with calcium chloride, magnesium sulfate, and ferric chloride. This work was probably the origin of the recommendation by *Standard Methods* for incorporating dilute phosphate buffer in the dilution fluid for the plate count. The amount of phosphate buffer recommended by Butterfield is the same as that suggested by *Standard Methods*.

The effect of the dilution fluid on bacteria of raw milk has not been investigated to any extent. *Standard Methods* (1) recommends that not more than 20 min elapse from the time that the milk sample is diluted to the time it is plated. Geldreich and Clark (5) devised a test for determining the suitability of distilled water for microbiological use based on the growth and survival of *Aerobacter aerogenes*. They found that some water samples supported growth of *A. aerogenes* after a 24 hr incubation period; others were either toxic (due to chlorine) or had no effect. Garvie (4) found that *E. coli* and *Pseudomonas fluorescens* would grow in distilled water when nutrients or buffer were added. Price and Gore (13) found certain distilled waters to cause erratic results in folic acid assays with *Streptococcus faecalis* R and postulated the existence of volatile inhibitors, other than chlorine, in certain distilled waters.

The growth rates at different temperatures of the predominant bacteria of raw milk, the psychrotrophs, have been studied by Jezeski and Olson (10), Huhtanen (8), Greene and Jezeski (6), Heather and Vanderzant (7), Lawton and Nelson (12), and others. In all instances, the psychrotrophs grew readily at near-room temperatures (20-30 C). The present study was undertaken to determine the effect of short periods of holding diluted raw milk on recovery of bacteria from it.

## MATERIALS AND METHODS

Methods advocated by *Standard Methods* (1) were followed for the plate counts except when counts of <30 per plate were encountered. In this instance statistical procedures forced us to use the actual numbers. The protocol for the experiment and the statistical analyses were in general similar to those of a previous study (9). Since there were significant interaction effects between treatments and investigators and between treatments and samples within investigators, the standard model for expected mean square E (MS) for the

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mixed model, as in this experiment, did not include a satisfactory denominator for determining significance of the F statistic. A quasi F ratio (Satterthwaite's correction) was derived using a denominator for mean square and degrees of freedom as outlined by Winer (14). The Hartley test for inhomogeneity of variances was used as outlined by Winer (14).

Results from ten investigators were included in the study. The results of investigator D were composed of two analysts, D<sub>1</sub> and D<sub>2</sub>, each of whom assayed different milk samples. Five dilution bottles containing 99 ml of phosphate buffered water (either demineralized or distilled depending on the normal supply) were inoculated with 1 ml of raw milk obtained either from farm bulk tanks or from holding tanks at processing plants. The dilution bottles were mixed by gently inverting and were held at room temperature at specified times. One bottle was shaken according to *Standard Methods* recommendations and plated in duplicate as soon as possible, using 1 or 0.1 ml of the dilution fluid. Plate counts used for the analyses were, for each milk sample, from either the 1-100 or 1-1000 dilution even though the colony counts might have been <30 or >300. Results were transformed into log<sub>10</sub> for the analysis-of-variance and were calculated using an IBM 1130 computer.

## RESULTS AND DISCUSSION

### Average plate counts

Table 1 shows the plate counts obtained by the different analysts at the five different holding times of 0, 5, 10, 15, and 20 min. These counts were from either the 1-100 or 1-1000 dilution but for convenience were transformed in the table to 1-1000 equivalent counts. The overall average counts were highest with a 10-min holding time and were about 28% more than the control which was shaken and plated immediately. Counts were higher than controls at all holding times; although the increase was only 4.1% at 5 min. Six investigators (B, C, D<sub>1</sub>, F, H and I) found increases in counts at all holding times; two (D<sub>2</sub> and G) found decreases at all holding times. Investigator A found decreases at all holding times except for a slight increase at 10 min. Investigator E found increases at all holding times except 5 min when there was a decrease.

### Statistical evaluation

The analysis-of-variance for this experiment is shown in Table 2. The degrees of freedom for the denominator for the F test of treatment effects were calculated to be 24 by the method of Winer (14). The denominator mean square was calculated to be 0.0151 using the Satterthwaite formula.

There were highly significant differences between investigators and, as expected, between samples. The holding time effects were of significance with  $p < 0.05$  but not with  $p < 0.01$ . Some of the reasons for this low level of significance were the highly significant interaction effects encountered (lines d and e). Such interactions were found before (9) and may represent investigator bias, differences in types of

bacterial flora, variable storage times of the bulk milk, etc.

Further analysis of the treatment effects showed a highly significant linear but an insignificant quadratic trend even though the means from Table 1 indicated that counts were highest at 10 min and then fell at 15 min rising again at 20 min.

The treatments times investigators interaction was highly significant. Part of this interaction resulted from differences in regression lines among the investigators (non-parallel lines); however, another substantial portion of this interaction was unexplained. The slopes of the regression lines for investigators varied from 0.000038 for investigator A to 0.187180 for investigator I; there were obviously great differences in the way the different milk samples behaved when held for different times in the dilution bottles. This could be a reflection of the types of bacteria present or their stage in the growth cycle.

### Tests for reproducibility

Single-degree-of-freedom variances were calculated for each pair of observations and were summarized as shown in Table 3. The Hartley test for inhomogeneity of variances [Winer (14)] was made using the statistic

$$F_{\max} = \frac{\text{largest of } k \text{ variances}}{\text{smallest of } k \text{ variances}} = \frac{0.006053}{0.000927} = 6.5$$

with  $k = 10$  (number of investigators) and  $M-1$  degrees of freedom (29) for each investigator. The  $F_{\max}$  of the Hartley test for inhomogeneity of variances exceeded the tabled  $F_{\max}$  statistic at the 99% level of significance of approximately 3.4. Although the hypothesis of equality of variances was, therefore, rejected, it was felt that the analysis-of-variance test was still robust enough to withstand these inhomogeneities. Alternatively, one could eliminate the "outlying" variances and calculate the analysis-of-variance with the more homogeneous deviations; this, however, would have required a *post facto* decision and its justifiability could be questioned.

Another test for determining inhomogeneity of variances was made using an analysis of the variances obtained from investigators and holding times (Table 4). The variances of Table 3 were transformed to log<sub>10</sub> and a two-way analysis of variance was done. There were no significant differences between the variances of holding times but there was a highly significant difference between investigators ( $p < 0.01$ ). The variance of investigator B was lower than the others—an effect also observed previously (9). A test of this variance against the others was made using an orthogonal contrast—the obtained F ratio was 22.0 and indicated a highly significant ( $p < 0.01$ ) decrease in variance (increased reproducibility between

TABLE 1. EFFECT OF DILUTION BOTTLE HOLDING TIME ON PLATE COUNT

Investigator	Milk sample no.	Holding time (min)									
		0		5		10		15		20	
A	1	4.5*	5.2	4.5	4.9	5.3	3.8	4.3	4.9	4.3	4.9
	2	4.2	6.2	6.2	5.1	6.9	7.8	6.6	8.8	6.6	5.8
	3	6.5	5.6	5.0	4.4	5.1	5.2	4.3	5.0	5.6	5.3
	4	9.9	10.0	6.9	8.6	10.5	7.8	7.6	9.8	9.0	10.0
	5	4.4	6.1	5.0	4.2	5.4	5.9	5.5	4.1	5.3	4.7
	6	22.7	24.9	26.5	21.4	21.9	25.8	22.1	20.4	22.9	21.6
Average		9.18		8.56(-)		9.28(+)		8.62(-)		8.83(-)	
B	7	7.0	7.2	7.7	7.0	7.5	7.8	7.9	7.6	8.1	8.7
	8	2.9	3.2	3.9	4.1	3.3	4.0	3.4	4.1	4.8	4.2
	9	110	120	108	121	116	127	118	134	115	129
	10	62.0	58.0	64.0	59.0	67.0	63.0	69.0	82.0	82.0	75.0
	11	4.5	4.8	4.7	5.3	5.8	5.5	6.1	6.4	6.1	6.0
	12	24.0	23.6	26.6	24.7	27.5	25.0	29.6	25.5	28.3	26.7
Average		35.6		36.3(+)		38.3(+)		41.1(+)		41.2(+)	
C	13	111	119	132	116	148	118	159	132	100	159
	14	980	1000	850	980	1200	1300	1200	1000	1300	1200
	15	228	286	366	388	490	586	414	534	516	416
	16	9.2	12.0	9.2	8.4	8.4	8.8	10.4	8.2	9.3	11.2
	17	340	380	350	370	780	520	390	444	350	464
Average		346.5		357.0(+)		515.9(+)		429.2(+)		452.6(+)	
D <sub>1</sub>	18	11.9	13.6	14.0	12.4	13.2	15.0	12.2	14.0	11.0	12.5
	19	9.0	9.0	7.6	8.5	8.3	12.7	10.4	9.0	8.4	13.0
	20	196	226	227	238	252	244	316	278	256	296
	21	7.8	7.1	7.9	7.5	8.0	5.5	7.4	9.5	6.1	12.0
	22	13.7	15.7	13.7	15.0	14.1	15.2	16.7	14.9	13.6	16.6
	23	9.7	9.5	9.0	7.8	10.0	9.7	7.7	7.3	10.7	8.6
	24	86.0	77.0	73.0	60.0	69.0	75.0	67.0	70.0	75.0	69.0
	Average		49.4		50.1(+)		53.7(+)		60.0(+)		57.8(+)
D <sub>2</sub>	25	5.6	6.0	7.3	7.1	6.4	8.5	5.9	9.5	7.3	7.2
	26	12.5	12.0	11.5	14.5	10.0	12.0	13.0	12.9	12.1	13.5
	27	8.2	6.7	7.7	9.0	7.2	9.8	8.8	8.9	7.7	6.4
	28	43.0	41.0	39.0	35.0	21.0	32.0	42.0	30.0	31.0	45.0
	29	23.5	21.5	18.5	18.5	22.6	21.4	21.5	19.6	24.5	23.0
	30	4.5	4.7	4.4	4.4	5.1	4.1	4.1	4.3	4.3	5.1
Average		15.8		14.7(-)		13.3(-)		15.0(-)		15.6(-)	
E	31	13.4	11.0	12.5	11.8	11.5	11.4	13.3	12.5	11.1	13.7
	32	6.0	5.4	7.4	6.7	8.1	6.8	8.7	10.2	10.2	12.1
	33	7.1	6.8	7.2	5.0	7.2	5.5	6.4	5.0	6.1	6.2
	34	85.0	96.0	77.0	90.0	90.0	103.0	94.0	97.0	95.0	90.0
	35	3.4	2.9	3.3	3.2	3.4	3.0	3.8	3.8	3.4	3.5
	36	133	147	124	142	137	146	136	146	147	160
Average		43.1		40.8(-)		44.4(+)		44.7(+)		46.5(+)	
F	37	22.7	22.5	21.6	23.0	21.8	22.1	23.5	25.6	22.9	23.5
	38	8.7	10.0	9.8	9.0	9.4	9.2	12.1	8.9	11.2	9.3
	39	7.9	9.4	8.1	7.9	9.4	8.8	10.4	9.6	8.1	6.9
	40	3.8	3.4	5.2	3.8	4.2	4.9	2.9	4.5	2.8	3.9
	41	1.3	1.6	2.5	1.7	1.8	1.7	1.6	1.2	1.3	1.6
	42	5.6	5.6	6.7	8.6	6.0	6.4	9.3	7.5	5.5	5.9
Average		8.54		8.99(+)		8.81(+)		9.76(+)		8.58(+)	
G	43	131	222	170	162	139	134	117	142	163	152
	44	13.1	11.2	10.0	13.1	13.6	11.5	12.3	11.3	11.8	11.9
	45	8.3	7.1	9.0	8.9	6.4	11.9	5.2	5.4	6.2	8.4
	46	1.9	1.8	3.2	2.0	0.9	0.8	1.0	1.0	1.9	1.3
	47	49.0	40.0	54.0	33.0	40.0	53.0	50.0	43.0	36.0	51.0
48	6.2	6.7	6.1	7.5	8.7	7.7	6.2	6.6	6.7	7.4	
Average		44.3		39.9(-)		35.6(-)		33.4(-)		38.1(-)	

H	49	5.9	3.3	4.5	4.3	4.4	4.9	5.6	6.5	5.8	5.3
	50	7.3	6.2	6.4	6.7	8.8	10.2	7.4	7.6	7.1	6.0
	51	6.7	6.9	5.4	7.4	6.3	5.0	5.1	5.0	5.4	6.9
	52	10.9	12.3	12.1	11.4	12.0	13.9	23.8	21.8	13.4	14.0
	53	4.0	4.0	4.2	6.2	2.5	4.1	3.2	2.4	5.7	4.5
	54	39.0	38.0	54.0	47.0	84.0	91.0	75.0	54.0	48.0	33.0
	55	10.1	10.4	13.4	14.0	11.3	14.4	14.2	15.1	16.0	18.2
	56	13.3	13.0	14.7	15.6	13.2	13.7	14.7	13.0	11.5	11.4
	Average	12.0		14.2(+)		18.7(+)		17.2(+)		13.3(+)	
I	57	65.0	35.0	77.0	68.0	126	98.0	123	150	118	152
	58	23.5	22.5	26.8	29.0	26.0	25.7	21.9	25.0	27.3	32.0
	59	125	123	165	128	165	190	155	160	160	163
	60	81.0	78.0	108.0	97.0	155	95.0	188	105	210	112
	61	87.0	86.0	95.0	140	110	102	117	100	108	105
	62	23.6	26.2	24.5	27.9	26.7	25.0	27.6	26.4	24.2	24.7
	63	21.0	22.2	20.6	22.3	20.1	21.9	25.6	23.5	21.7	24.9
	Average	58.5		73.5(+)		84.7(+)		89.1(+)		91.6(+)	
	Overall Average	56.05		58.36		71.60		65.69		68.19	
	Increase %	—		4.1		27.7		17.2		21.6	

\*For actual counts multiply by 1000. (+) is increase in counts from 0 time; (—) is a decrease.

TABLE 2. STATISTICAL EVALUATION OF PLATE COUNTS

Line	Source of variation	Degrees of freedom	Sum of squares	Mean	F ratio <sup>1</sup>	Significant with	
						p < 0.01	p < 0.05
a	Investigators	9	99.7044	11.0782	4.07	yes	yes
b	Samples within investigators	53	144.2401	2.7215	878	yes	yes
c	Treatments	4	0.1697	0.0424	2.81	no	yes
	linear trend	1	0.1466	0.1466	9.71	yes	yes
	quadratic trend	1	0.0208	0.0208	1.38	no	no
	cubic trend	1	0.0009	0.0009	<1.0	no	no
	quartic trend	1	0.0011	0.0011	<1.0	no	no
d	Treatments times						
	investigators	36	0.6535	0.0182	2.22	yes	yes
	non-parallel lines	9	0.2398	0.0266	3.25	yes	yes
	residue	27	0.4137	0.0153	1.87	yes	yes
e	Treatments times samples within investigators	212	1.7345	0.0082	2.64	yes	yes
f	Error (between duplicate plates)	315	0.9866	0.0031	—	—	—
	TOTAL	629	247.4888				

<sup>1</sup>F ratios were lines a/b, b/f, d/e, e/f. F ratio for treatment effects included a denominator mean square of 0.0151 derived from Satterthwaite's correction with 24 degrees of freedom.

TABLE 3. AVERAGE VARIANCE ESTIMATES

Investigator	Holding time (min)					Average variance
	0	5	10	15	20	
A	0.004867	0.002917	0.003900	0.004383	0.001017	0.003430
B	0.000417	0.000800	0.000967	0.001667	0.000750	0.000927
C	0.002620	0.000960	0.004820	0.003880	0.007200	0.003904
D <sub>1</sub>	0.001043	0.001328	0.004743	0.001828	0.010500	0.003897
D <sub>2</sub>	0.000917	0.001417	0.006850	0.005517	0.003417	0.003633
E	0.001600	0.002983	0.002217	0.001500	0.001317	0.001927
F	0.001650	0.005033	0.000567	0.006750	0.003433	0.003493
G	0.005917	0.009133	0.008200	0.001133	0.005850	0.006053
H	0.004500	0.003338	0.004938	0.002825	0.003662	0.003858
I	0.005400	0.003643	0.004571	0.005800	0.006800	0.005248
Average variance	0.002893	0.003155	0.004177	0.003528	0.004395	0.003637

TABLE 4. ANALYSIS OF VARIANCE SUMMARY OF VARIANCE ESTIMATES FOR INVESTIGATORS AND HOLDING TIMES

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F ratio	Significant with	
					p < 0.01	p < 0.05
Investigators	9	2.4169	0.2685	3.63	yes	yes
Treatments (holding times)	4	0.4243	0.1060	1.43	no	no
Investigators times treatments	36	2.6617	0.0739			
TOTAL	49	5.5029				

duplicate plates) as compared to the nine other investigators.

It is rather interesting that the counts increased so remarkably in only 10 min in the dilution bottles. The shock of dilution might have been expected to cause a decrease in counts. The increase may have been a result of growth of the organisms or may have been caused by a breaking-up of clumps of bacteria. It might be argued that if the clumps of bacteria were indeed broken up, resulting in a more homogeneous suspension, then the counts should have shown a greater reproducibility with time of holding. Tables 3 and 4 did not indicate such a difference.

The 12th edition of *Standard Methods* (1) specifies that not more than 20 min elapse between diluting and pouring of the plates. According to the work reported here such an indeterminate interval would lead to widely disparate results. For instance, the counts after 5 min would be 4% higher; after 10 min they would be 28% higher; and at 20 min they would be 22% higher. The closest approximation to the true counts would seem to be a 5-min holding time. We would suggest that this time interval be considered as the maximum time allowed between diluting and pouring of the plates.

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