COMPARATIVE DETECTION OF COLIFORM ORGANISMS IN MILK AND WATER BY THE PRESUMPTIVE COLIFORM TEST

WITH AN APPENDIX ON THE POSSIBLE BACTERICIDAL EFFECT OF BILE SALT

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Introduction

It has been shown (Barkworth & Irwin, 1938) that the distribution of coliform organisms in milk follows a Poisson series, and on this basis it should be possible to estimate the population from the results of replicate tests at several dilution levels by reference to tables. In later experiments with water (Barkworth & Irwin, 1941), it was found that the number of positive tubes did not always reach expectation, having regard to the known numbers of organisms inoculated. The peculiar physico-chemical constitution of milk naturally raises the question whether coliform organisms, though present, might fail to give a positive reaction in the presumptive test. It is well known that in favourable circumstances lactic acid bacteria will overgrow all other organisms in milk. In the presumptive test the greatest concentration of milk in routine technique is 1 ml. of milk in 5 ml. of medium. This dilution would not affect the ratio of coliform organisms to lactic acid bacteria. Even when the coliform contamination at the time of testing is 'present in 0·001 ml.' the agreement with the fermentation test is only about 50% (Barkworth, 1929). It will be argued that the gas tube would disclose the presence of gas in more cases than the fermentation test, nevertheless it is not perfect, for Chalmers (1928) recovered *Bact. coli* from plates of 1 : 10 dilution of samples of milk which had given a negative presumptive test. There is therefore some reason to expect a small proportion of false negative coliform results to occur with milk samples, and the object of the present experiment was to see if inoculation with a known number of organisms would produce similar numbers of positive tubes in milk and in water.

Milk

A small sample of milk can be drawn from the udder direct into a sample bottle with a minimum of external contamination, but in case the fat globules might play a part by entangling or occluding the bacteria, it was considered advisable to use whole milk,

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in order to obtain a representative sample. As the fat is largely contained in the latest drawn milk the sample would have to be taken from the complete milking of the udder. Also it would be inadvisable, owing to the physical and chemical alterations resulting, to rely on heat for sterilization of the milk. The problem was, therefore, to obtain a sample of whole milk free, in the absolute sense, from coliform organisms.

The udder was washed with water and a brush, and then wiped with a clean udder cloth (which had been wrung out) to remove excess water. This is normal cowshed routine. A minute or two was allowed so that the body heat would complete drying, and the udder and teats were then swabbed with rectified spirit. After another wait to allow evaporation, the cow was hand milked into a narrow-mouthed 1 gal. milk can, previously sterilized by the Tindall method.

Freedom from coliform organisms was checked by incubating about 100 ml. of the milk for 24 hr. at 72° F. and then applying the presumptive test (3 tubes of 1 ml.). Under these conditions coliform organisms will increase about 10,000-fold in 24 hr. (Barkworth, 1927), and therefore a negative result is adequate proof that the milk was free from coliform organisms.

It is obvious that the sample, though free from coliform organisms and collected with care, was not sterile. There would always be bacteria present even with the usual cowshed routine of 'discarding the fore milk' (three squirts from each teat) which was, of course, observed.

Out of forty-two samples milked as above, only six were positive to the incubation test. Results from these samples have not been included.

Lay-out of experiment

A suspension of coliform organisms was prepared as in previous experiments (Barkworth & Irwin, 1941), and amounts of 100 ml. water and 100 ml. milk inoculated with 1 ml. of a suspension diluted so as to give about 50 organisms per bottle. Test-tubes $6\frac{5}{8}$ in. in length containing 6 ml. of lactose-litmus-bile-salt peptone broth (Min. Agric. 1934) were inoculated with 1 ml. of milk or water and incubated 3 days at 37° C. The number of organisms inoculated was checked by plating 1 ml. of a 1/10 dilution of the penultimate dilution bottle. The desired population was 250 colonies per plate.

Preliminary experiments

In the first seven trials the milk was tested immediately after milking and the results are shown in Table 1.

Table 1. *Presumptive coliform test in water and in milk tested immediately after milking*

No. of sample	No. of positives out of 30		Plate counts of coliform suspension						
	Water	Milk	1	2	3	4	5	Total	Average
1	9	8	262	254	293	266	258	1333	267
2	14	7	194	212	228	246	229	1109	222
3	13	13	297	269	250	277	274	1367	273
4	14	12	219	218	185	225	207	1054	211
5	14	11* (13)	282	271	263	324	2286	3426	685
6	13	9 (10)	255	278	277	276	271	1357	271
7	17	7 (8)	269	252	290	223	322	1356	271

* The numbers of positives were the same after 72 hr. incubation as after 24 hr. except in Exps. 5, 6 and 7, where they were the numbers in brackets.

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Bactericidal action of milk

The lower number of positives in these trials might be due to the bactericidal action of milk. Hunziker (1901) states that the intensity of the phenomenon varies with individual cows and with each quarter of the udder, but is greatest at 21° C. Orla-Jensen & Jacobsen (1930) found the same and state that at 5° C. it reaches its highest point in 24 hr., while Klein (1917) states that the count of milk decreases for 8–10 hr. at 26–29° C. Rosenan & McCoy (1908) found that the count decreased for 6–8 hr. at 26–29° C. and for 14 hr. at 16–23° C.

Table 2. *Comparative detection of presence of coliform organisms in water and in milk after 24 hr. storage of milk samples*

No. of sample	Plate count at time of testing, of milk sample stored at 72° F.	No. of positives out of 30			Plate counts of coliform suspension						
			Milk stored at								
		Water	40° F.	72° F.	1	2	3	4	5	Total	Average
1	224,000	11	5	7	185	205	195	209	169	963	193
2	3,760,000	11	10	4	200	192	233	210	221	1056	211
3	2,570,000	9	7	9	174	199	160	186	183	902	180
4	1,070,000	4	5	8	182	188	202	183	190	945	189
5	970,000	5	9	5	169	222	180	201	170	942	188
6	6,540,000	4	7	10	124	117	120	123	113	597	119
7	11,260,000	8	8	3	146	183	149	172	152	802	160
8	1,950,000	5	2	6	180	163	168	163	165	839	168
9	3,360,000	9	7	6	149	173	184	169	191	866	173
10	12,560,000	11	11	2	160	202	181	218	202	963	193
11	4,040,000	11	6	5	211	197	201	235	198	1042	208
12	5,000,000	8	4	3	123	134	137	152	138	684	137
13	30,500	8	5	5	106	119	140	123	148	636	127
14	14,000,000	10	8	2	242	248	287	278	236	1291	258
15	1,250,000	8	3	5	181	183	187	207	172	930	186
16	12,000,000	9	7	3	199	199	205	209	215	1027	205
17	2,970,000	6	9	11	236	219	196	255	201	1107	221
18	8,000,000	13	9	5	234	245	235	192	253	1159	231
19	15,500,000	9	11	5	165	169	161	147	195	837	167
20	23,000	9	5	6	202	209	188	188	208	995	199
21	10,500,000	9	6	7	194	215	196	218	216	1039	208
22	5,830,000	6	8	3	184	150	162	176	166	838	168
23	2,500	7	4	9	182	132	189	135	161	799	160
24	5,200,000	9	7	5	187	187	209	194	203	980	196
25	10,300	5	6	3	232	211	195	201	174	1013	203
26	2,640,000	8	6	4	118	116	140	142	150	666	133
27	28,000	7	7	8	201	208	191	170	186	956	191
28	4,400	10	12	13	211	214	223	238	235	1121	224
29	4,800,000	7	6	7	164	142	153	166	168	793	159

It was decided to store 100 ml. samples of milk for 24 hr. at 40 and 72° F. and then to inoculate and prepare presumptive tests as before. In addition, a plate count of the sample stored at 72° F. was made immediately previous to inoculation. The results are shown in Table 2.

The statistical analysis of the results

The same methods were used as in former experiments (Barkworth & Irwin, 1941).

(1) *The first seven trials, in which the milk was tested immediately after milking*

Straightforward pooling of the seven results gives 33·8% of positive tubes for milk and 44·8% for water, the difference is 11·0 with a standard error of $\sqrt{(39{\cdot}3 \times 60{\cdot}7/105)} = 4{\cdot}77$. The percentage of positives is significantly lower for milk than for water.

The legitimacy of estimating the experimental error from the binomial distribution can be checked, as before, by analysis of variance of $\phi = \sin^{-1}\sqrt{p}$. This gives:

	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Experiments	152·22	6	25·37	1·1
Milk *v.* water	149·18	1	149·18	6·5
Error	137·31	6	22·88	
Total	438·71	13		
Theoretical error variance, 820·7/30			27·36	
5% values of variance ratio				4·3 for 6 and 6 D.F. 6·0 for 1 and 6 D.F.

The error is in good agreement with its theoretical value, and there is no significant difference between experiments, but the difference between milk and water is significant. The mean values of ϕ are 35·43 for milk and 41·96 for water with a standard error of 1·81. The corresponding percentages positive are 33·7 and 44·7.

Five plate counts were made in each experiment. The mean results are given in Table 1. The general mean is 256·3 with a standard error of 10·8.

The analysis of variance of plate counts between and within experiments is as follows:

	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between experiments	23,840	6	3973	8·4
Within experiments	12,840	27*	476	($P<0·001$)
Total	36,680	33*		

* One plate count, evidently anomalous, was omitted.

There are significant differences between experiments, but the mean is in agreement with the 250 expected. A plate count of 250 corresponds to 0·5 organism per tube, or to 39·35% of positive tubes on the bases of the Poisson distribution. The very close agreement of this with the percentage actually observed (39·3%) must be partly fortuitous, since the milk gave a percentage of positive tubes significantly lower than the water.

(2) *The twenty-nine trials in which the milk was tested after 24 hr. storage*

Here pooling all the twenty-nine results gives:

		Milk stored for 24 hr. at	
	Water	40° F.	72° F.
No. of positive tubes	236/870	200/870	169/870
% positive	27·1	23·0	19·4

The analysis of variance of $\phi = \sin^{-1}\sqrt{(p)}$ is as follows:

	Sum of squares		Degrees of freedom		Mean square	Variance ratio	
Experiments	964·45		28		34·44	1·0	
Treatments:							
Water *v.* milk	343·28	449·80	1	2	224·90	10·4	6·8
Milk 40 and 72° F.	106·52		1			3·2	
Error	1851·66		56		33·07		
Total	3265·91		86				
Theoretical error variance 820·7/30					27·36		
5% values of variance ratio						4·0 for 1 and 56 D.F. 3·1 for 2 and 56 D.F.	

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The error is again in good agreement with its theoretical value

$$(\chi^2 = 67{\cdot}7,\ \sqrt{(2\chi^2)} = 11{\cdot}6,\ \sqrt{(111)} = 10{\cdot}5),$$

and there is no significant difference between experiments. The difference between water and milk is significant, but temperature of storage has no significant effect.

The mean values of ϕ and the corresponding percentages are as follows:

		Milk stored for 24 hr. at		
	Water	40° F.	72° F.	
ϕ	31·17	28·31	25·60	S.E. 1·07
$100p$	26·8	22·4	18·6	

Five plate counts were again made in each experiment. The mean results are given in Table 2. The general mean is 184·7 with a standard error of 6·0.

The analysis of variance of plate counts within and between experiments is as follows:

	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between experiments	145,220	28	5186	19·7
Within experiments	30,495	116	263	($P < 0{\cdot}001$)
Total	175,715	144		

There are significant differences between experiments, and the mean is decidedly lower than the 250 expected. We have noted that a plate count of 250 corresponds to 39·35% of positive tubes on the basis of the Poisson distribution; 184·7 corresponds to 0·3694 organism per tube or to 30·9% of positives. Thus the percentage of positive tubes actually observed is below its theoretical expectation on the basis of the plate count.

The same phenomenon was observed to a still greater extent in our main 1941 experiment (Barkworth & Irwin, 1941, p. 191).

Comparing the effect of immediate testing and of storage for 24 hr. we have:

	% positive		
	Water	Milk	Difference
Milk tested immediately	44·7	33·7	11·0
Milk stored for 24 hr	26·8	20·5	6·3

Thus milk still gave a lower percentage of positives than water even when stored for 24 hr. before inoculation, and the *relative* difference is about the same as when it was inoculated immediately. If the difference between milk and water is due to bactericidal action of the milk, this must persist for longer than 24 hr.

DISCUSSION

Coliform organisms were inoculated into milk and water at the same rate, and whether the milk was stored or not, the inoculation was made immediately prior to testing. The lower percentage of positives in the milk must be due to failure of the organisms to grow or failure to develop gas. As the milk is diluted by adding 1 ml. of milk to 6 ml. of broth, it seems unlikely that any cream layer formed could be a factor. The initial contamination was insufficient to cause curdling during incubation of the tubes. This occasionally arises when 1 ml. quantities of milk of high bacterial content are inoculated into lactose tubes.

Plate counts of the milk after 24 hr. storage at 72° F. are high, but not abnormal for 'dirty' milk of the same age. Under ordinary conditions there is a correlation between

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plate count and coliform content; market milk of this plate count would be positive in 0·001 ml. (Barkworth, 1935).

The presumptive coliform test is an enrichment test, and if the inoculated organism (or organisms) did grow it would be expected that they would in 72 hr. attain sufficient numbers to give a positive result.

If the lower percentage of positives is due to failure to grow this must be due to competition or residual bactericidal action. The method of obtaining the samples would suggest that the balance of species in the incubated samples was probably not the same as would be found in market milk of the same count after similar storage. If the difference is due to bactericidal action, then such action persists for more than 24 hr. under conditions of storage such as we have used.

Whatever the cause, it is clear that presumptive coliform tests in *milk* will underestimate the coliform population.

Such under-estimation in no way invalidates conclusions concerning distribution of coliform organisms in milk (Barkworth & Irwin, 1938).

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APPENDIX

Has bile salt any bactericidal effect on coliform organisms in the presumptive coliform test?

The experiment which is described here arose out of our earlier work with water.

In our previous paper (Barkworth & Irwin, 1941) it was noted that in a series of twenty experiments with water, each involving 60–70 tests, the percentage of positives was only about half that to be expected from the plate counts. It was thought possible that the bile salt might have some inhibitory action, and accordingly parallel tubes were made using medium with and without bile salt.

Fifteen trials were made. 1 ml. quantities of water inoculated at the rate of 0·5 organisms per ml. of water were added to lactose-broth tubes. Each trial consisted of thirty tubes with the completed medium and thirty tubes of medium made up without bile salt.

Plate counts gave good agreement with expectation, and the density of coliform organisms is therefore comparable with previous experiments.

The statistical analysis which follows shows no difference due to the presence of bile salt.

At 0·5 organism per ml. we should expect 39% positives, whereas the percentage observed was 36–37. In this point the trials are at variance with the earlier experiments

but in agreement with trials reported in the appendix (Barkworth & Irwin, 1941), where the observed percentage of positives was 38·6% compared with an expectation of 42·9%.

This variation in the relation between the percentage of expected and observed positives does not invalidate the conclusion concerning the action of bile salt.

STATISTICAL ANALYSIS

The data are shown in Table 3.

Table 3. *Presumptive coliform tests in water; medium with and without bile salt*

No. of sample	No. of positives out of 30 tubes after 72 hr. With bile salt	Without bile salt	Plate counts of coliform suspension 1	2	3	4	5	Total	Average
1	8	9	276	255	238	269	278	1316	263
2	7	11	197	222	196	200	200	1015	203
3	6	9	269	271	286	260	260	1346	269
4	14	9	282	282	298	330	282	1474	295
5	14	11	328	317	335	299	300	1579	316
6	15	14	240	234	220	207	209	1110	222
7	14	9	254	215	210	202	186	1067	213
8	8	10	285	284	273	304	271	1417	283
9	12	8	248	254	298	259	235	1294	258
10	14	14	278	277	262	242	296	1355	271
11	13	15	273	270	268	285	263	1359	272
12	12	14	241	209	206	202	211	1069	214
13	9	9	259	294	290	245	207	1295	259
14	8	15	226	207	255	178	236	1102	220
15	11	11	203	184	182	135	195	899	180

(1) *Straightforward pooling of the fifteen results*

No. of positives:	With bile salt	165/450 = 36·7%	Diff. 0·6%	S.E. 3·22
	Without bile salt	168/450 = 37·3%		

(2) *Analysis of variance of* $\phi = sin^{-1}\sqrt{p}$

	Sum of squares	Degrees of freedom	Mean square
Experiments	550·47	14	39·32
Bile salt *v.* no bile salt	1·78	1	1·78
Error	327·18	14	23·37
Total	879·43	29	
Theoretical error variance 820·7/30			27·36

There are no significant differences between experiments or treatments, and the error variance is in good agreement with its theoretical value.

(3) *Plate counts. Analysis of variance*

	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between experiments	102,548	14	7325	17·3
Within experiments	25,378	60	423	($P < 0·001$)
Total	127,926	74		

There are significant differences between experiments. The general mean is 249·3 with a standard error of 9·9 in good agreement with the 250 expected.

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