THE REPRODUCIBILITY OF EXTINCTION TIME ESTIMATES

PART I.—VARIATIONS IN RESISTANCE OF TEST ORGANISMS AND VIABILITY OF TEST SUSPENSIONS

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THE most pertinent criticism which has been levelled at extinction methods of estimating bactericidal activity in general, as distinct from phenol coefficient determinations in particular, has been the alleged unreliability of the tests for sterility on samples of the bacteria-bactericide reaction mixture. Supposedly inconsistent and anomalous distributions of culture samples showing growth or absence of growth were observed soon after the introduction of the methods[1-4]; and it was generally recognised that the closer the sampling intervals, the greater were the anomalies. The type of result was often described as showing "skips" or "wild plusses". The explanation of these results is to be found in the survival of very small numbers of bacteria of greater than average resistance and which are to be found towards the end of the disinfecting period, as shown graphically by Chick[5], Withell[6], Jordan and Jacobs[7] and Berry and Michaels[8]. Where the number of survivors is very small—on the average one or no organism in each sample volume—there must be some negative samples in a number of replicate samples removed. This was realised by Thaysen[9] and by Cade[10], who approached the problem statistically, using the analysis of Halvorson and Ziegler[11] for the calculation of the most probable number of organisms in the parent reaction mixture from a knowledge of the sterility or fertility of the samples. This Cade was able to relate with counts of surviving organisms. He suggested that lack of reproducible results from extinction data could be ascribed largely to sampling variations and he advocated the employment of much more extensive replication than had been customary in obtaining such data.

The extinction method of Berry and Bean,[12] overcomes the errors inherent in previously described extinction methods, for it provides sufficiently extensive replication with adequately short sampling intervals for the determination of a highly reliable estimate. If the loglog analysis described by Mather[13] be used, the magnitude of the sampling variations and the standard error of an estimate may be computed. The reliability of the extinction times (expressed as mean single survivor times) so derived has been proved by Cook and Wills[14], who demonstrated a relation between the intervals of time required for different percentages survival and the mean single survivor times.

It is evident that estimates of extinction times are subject to two components of variation: that 'between estimates' and that lying 'within estimates' or between replicates. The reliability of an estimate is improved by narrowing the contact time intervals, i.e. increasing the number

of samples removed in a given time, which results in a progressively larger increase in the within-estimate variability; therefore it follows that the extinction method of choice will be that which gives the highest within-estimate variance with the provision that variations due to other sources are small compared with random chance variations. In terms of Mather's analysis, the method must yield a highly significant regression with a maximum of degrees of freedom. Berry and Bean[12] gave details of the "within-estimate' variability attached to their method, but were able to give little attention to the magnitude and sources of 'between-estimates' variability. It is the object of this communication to describe investigations into the reproducibility of estimates of extinction times. Bearing in mind that Berry's extinction method is the only method yet described which takes account of sampling variations, it follows that the investigations described here will provide probably the first attempt to adequately assess the sources of variability between extinction time estimates. Previous investigators must have attributed sampling variations to other sources.

Experimental Details

Bactericide. The bactericide solutions were prepared by diluting with sterile distilled water a 5 per cent. aqueous solution of phenol A.R., which, stored in well-closed containers, was found to be stable.

Organism, Bacterium coli, (*Escherichia coli*), type I, 44° C.-positive, laboratory strain, formerly N.C.T.C. No. 5933.

Media. The medium used for testing the sterility of samples of the reaction mixture was that described by Berry and Bean[12]. The solid medium used for cultivation of the test organisms contained 1 per cent. "Oxoid" peptone and 0·5 per cent. sodium chloride, was solidified with 2 per cent. Davis bacteriological agar and was adjusted to pH 7·2. The procedure adopted by Berry and Bean was as follows. At monthly intervals, a freeze-dried sample of culture of the organism was opened and transferred to a "master" slope of peptone agar, the 24 hour growth from which was used to propagate 4 "sub-master" agar cultures. Each day for 14 days, slopes were inoculated by successive subculture from one of the "sub-master" slopes. These daily slopes were used in experiments from the fourth to the fourteenth day, after which a fresh "sub-master" slope was introduced. When all four "sub-master" slopes had been used, a fresh "master" culture was generated from another specimen of freeze-dried culture.

Experimental Technique. The method adopted for the cultivation of the test organisms was similar to that described by Berry and Bean[12]. Test suspensions were adjusted to a density equivalent to 2×10^9 per ml. by the use of a clinical photoelectric colorimeter (Baird and Tatlock, model ZTA 7530). Variations in the viable counts of suspensions from day to day were estimated from two series of data: colony counts by the method of Miles and Misra[15], which gave an estimate from 39 counts of a mean count of $1{\cdot}948 \times 10^9 \pm 0{\cdot}099 \times 10^9$ per ml. ($P = 0{\cdot}95$);

and 10 counts by dilution series, as described by Cook and Wills[14], from which was obtained a mean estimate of $2{\cdot}211 \times 10^9 \pm 0{\cdot}237 \times 10^9$ per ml. ($P = 0{\cdot}95$). The design of the extinction method and the analysis of the results followed the scheme described by Cook and Wills[14]. All experiments consisted of between 15 and 20 replicate determinations, each with at least six different contact times.

A trial series of determinations was undertaken in which extinction times were measured on exposure of *Bact. coli* to solutions of phenol of concentration varying from 1·1 per cent. to 1·5 per cent. Of the eight determinations carried out, all estimates of 'mean single survivor time' obtained from the regression line best fitted by inspection easily fell within the limits of error of estimates derived from both first and second calculated approximations to the regressions lines. Hence, the reading of mean single survivor times from regression lines best fitted by inspection in subsequent experiments was considered to be justified. A highly statistically significant regression was demonstrated between logarithms of mean single survivor times and logarithms of phenol concentrations, this finding being in agreement with the observations of many other workers, see e.g. Berry and Bean[12], Phelps[16].

TABLE I

EXTINCTION TIMES OF *Bacterium coli* ON EXPOSURE TO SOLUTIONS OF PHENOL AT 20° C.

Concentration of phenol (per cent. w/v)	Mean single survivor times (minutes)	
	First series of determinations	Second series of determinations
1·10	39·0	50·2
1·15	24·0	32·7
1·20	16·4	20·2
1·25	10·6	13·8
1·30	6·39	9·64

Later, a further series of experiments was undertaken in which the mean single survivor times to five strengths of phenol were redetermined. The results shown in Table I indicate a considerable apparent increase in resistance of the test organism, the new values being about 20 per cent higher than those which were first determined. The components of the experiment which could have varied between the performance of the first and second series of determinations were:

(i) The organism, which had been generated from separate freeze-dried samples. The first series had been completed just before expiry of a monthly "master" culture.

(ii) The batch of medium used for reviving unkilled cells at the end of the reaction period. A new batch had been commenced for the second series of experiments.

It was decided to investigate both of these possible sources of variability, and in addition to investigate the viability of the test suspensions from day to day and within each day. This first communication will describe investigations into the resistance of test organisms and viability of test suspensions.

A Comparison of Methods of Maintaining Cultures

The method of maintaining cultures by limited series of subcultures from freeze-dried cultures of the organism had been adopted in these laboratories in view of the variations in resistance on repeated subculture reported by Chick and Martin[17] and Withell[18]. It was decided to compare variations in phenol resistance of organisms maintained by the monthly freeze-dried method of Berry and Bean[12] with those obtained by repeated daily subculture.

Procedure. One "submaster" slope of the then current freeze-dried culture was maintained in continuous daily subculture over a period of more than two years. This will be referred to as the "C" series of subcultures. A "submaster" slope of the succeeding culture was similarly maintained and was known as the "B" series. At varying intervals during the following two years, the mean single survivor times to 1·10 per cent. phenol were determined for both "B" and "C" series and for the corresponding culture in normal current use.

TABLE II

Mean Single Survivor Times of *Bact. coli* when Exposed to 1·10 per cent. Phenol, the Organism being Maintained in Culture by Different Methods

Extinction times of cultures maintained from monthly freeze-dried samples (minutes)		Culture "C"		Culture "B"	
		Serial number of subculture	Extinction time (minutes)	Serial number of subculture	Extinction time (minutes)
50·2	39·8	12	50·2	7	35·9
44·4	33·5	52	49·5	12	29·3
35·9	25·8	96	38·0	121	23·0
29·3	31·4	103	28·5	257	26·4
40·1	29·6	117	26·0	305	28·3
32·3	33·4	130	26·8	639	23·4
36·4	28·4	233	28·8		
41·4	29·0	370	22·3		
33·2	26·3	420	29·6		
		645	24·8		
		757	17·1		

Method of propagation of culture	Degrees of freedom	Variance	Standard error	Mean single survivor time (minutes)	95 per cent. Confidence limits of means.
1. Monthly series	17	43·35	1·552	34·47	± 3·27
2. Series "C"	10	112·29	3·195	31·05	± 7·12
3. Series "B"	5	22·49	1·936	27·72	± 4·97

Results and Analysis. The results, together with a summary of their statistical treatment, are shown in Table II. Comparison of variances 1 with 2 and 2 with 3 gave ratios of 2·59 and 4·99 respectively which, with the appropriate degrees of freedom, corresponds to a probability level slightly below 0·05 in each case. Comparison of variances 1 with 3 yields a ratio of 1·93 which is not statistically significant at the 5 per cent level. Similar comparisons of means give values for t of 1·077, 0·723 and 2·305 respectively, so that a significant difference exists only between means 1 and 3.

Inspection of the results for the ordinary monthly cultures reveals that one estimate, 50·2 minutes, lies well above the remainder. This culture was used for propagation of the "C" series of daily subcultures. On the other hand, the value 35·9 minutes, obtained for the culture used to propagate the "B" series, lies close to the mean of the monthly culture estimates. Both "B" and "C" series show a declining resistance during the course of subculture: with the "B" series the decline has been sufficient to reduce the mean result significantly from that of the ordinary series and the variance is smaller, but not significantly so. In the "C" series the decline from a high initial resistance to a steady resistance roughly the same as that of the "B" series has greatly increased the variance to an extent significantly greater than the variances of the other two series, and this large variance leads to a mean which is not significantly different from the means of the other series. If, however, the results from the "C" series up to and including the 52nd subculture are omitted, the remaining values have a variance not significantly greater than the "B" series ($F = 1{\cdot}44$ with 8 and 5 degrees of freedom) and a mean not significantly different from that of the "B" series ($t = 0{\cdot}298$ with 13 degrees of freedom) but which is significantly smaller than that of the monthly series ($t = 2{\cdot}95$ with 25 degrees of freedom).

Discussion. These findings indicate that cultures of *Bact. coli* derived from individual samples of a freeze-dried culture may vary considerably in phenol resistance, from limits of 50·2 minutes to 25·8 minutes extinction times on exposure to 1·10 per cent phenol. It appears that on continued daily subculture of a sample of the freeze-dried culture, using our medium, the resistance soon begins to decline to reach a reasonably steady resistance which is maintained for several hundred subcultures. Thereafter there may be a further decline. Over the period of reasonably constant resistance, estimates of extinction times may be more reproducible than with the use of the monthly method of propagation, although a statistically significant difference could not be detected. It is concluded that daily subculture has advantages over limited numbers of subcultures from freeze-dried specimens in experimentation lasting for little more than one year.

At six monthly intervals, samples of "B" and "C" series of subcultures were subjected to microscopical and biochemical examination. Normal characters were maintained with the exception of an increasing capacity of the organism to grow anærobically as demonstrated by reduction in catalase activity and enhanced methylene blue reduction. The tendency to anærobiosis was reversed by growth in peptone water containing 2 per cent. whole blood with 10 μg. per ml. riboflavine or 50 μg. per ml. nicotinamide. The change may therefore be associated with a metabolic disability after growth for long periods on poor media.

Examination of the data for the monthly series of cultures showed that apparently the distribution was predominantly single-tailed. However, a test for normality by computation of the four moments, employing correction for continuity, revealed that the distribution of resistances did not depart seriously from normality and was symmetrically platykurtic.

Variations Between and Within Monthly Cultures

The results for extinction times of the ordinary monthly cultures given in the previous section did not differentiate between variations between "master" (monthly) cultures and variations within "master" cultures (or between "submaster" cultures). A comparison of variances due to these sources was desirable, since it would show whether each monthly culture would give rise to a short series of subcultures of more constant resistance than would be encountered in a random series of observations spread over several monthly cultures.

TABLE III

Extinction Times of *Bacterium coli* when Exposed to 1·20 per cent. w/v Phenol at 20° C.

Values of t are mean single survivor times in minutes determined for subcultures of serial number n. A,B,C,D denote the four "submaster" cultures of the "master cultures" 1,2 and 3.

"MASTER" 1		"MASTER" 2				"MASTER" 3	
n	t	n	t	n	t	n	t
A 9	18·44	A 4	15·39	C 10	17·21	A 2	15·02
B 4	11·42	A 5	15·10	C 11	16·98	A 4	19·87
C 6	13·83	A 6	16·26	C 13	18·45	A 7	18·42
C 15	15·03	B 4	19·05	C 15	16·54	A 9	15·70
D 8	16·38	B 10	17·53	D 5	16·23	A 11	17·67
D 9	17·58	B 13	16·70	D 8	15·02		
D 10	18·04	C 5	16·75	D 10	14·13		
D 11	18·40	C 6	17·40	D 13	12·20		
		C 8	17·42				

Procedure. Data for the mean single survivor times of *Bact. coli* when exposed to 1·20 per cent. phenol were obtained over a short period. The determinations were made during the use of all four "submaster" cultures derived from each of two "master" cultures and during the introduction of the first "submaster" culture of a third "master" culture.

Results and Analysis. The results are recorded in Table III. Since the experimental structure was both unbalanced and incomplete, an analysis of variance could not be undertaken. Instead, variances were computed as follows:—

(i) Between "submaster" cultures; within "masters" and replicates. The variances for blocks 1, 2 and 3 were calculated.

$\bar{x}_1 = 16{\cdot}14$ $S(\bar{x}_1 - \bar{x}_1)^2 = 44{\cdot}9845$ $s_1^2 = 6{\cdot}427$ (7 degrees of freedom)
$\bar{x}_2 = 16{\cdot}37$ $S(\bar{x}_2 - \bar{x}_2)^2 = 43{\cdot}2114$ $s_2^2 = 2{\cdot}701$ (16 degrees of freedom)
$\bar{x}_3 = 17{\cdot}34$ $S(\bar{x}_3 - \bar{x}_3)^2 = 15{\cdot}7481$ $s_3^2 = 3{\cdot}937$ (4 degrees of freedom)

Comparison of the largest variance estimate, s_1^2, with the smallest, s_2^2, gives a ratio of $F = 2{\cdot}380$ ($N_1 = 7$, $N_2 = 16$), corresponding to a probability level between 0·2 and 0·1. Hence there is no evidence of significant heterogeneity among variances between "submaster" cultures.

(ii) Between "master" cultures; within "submasters" and replicates. The variances for groups A,B,C, and D were computed.

$\bar{x}_A = 16{\cdot}87$ $S(x_A - \bar{x}_A)^2 = 24{\cdot}9940$ $s^2_A = 3{\cdot}124$ (8 degrees of freedom)
$\bar{x}_B = 16{\cdot}18$ $S(x_B - \bar{x}_B)^2 = 32{\cdot}9873$ $s^2_B = 10{\cdot}996$ (3 degrees of freedom)
$\bar{x}_C = 16{\cdot}62$ $S(x_C - \bar{x}_C)^2 = 15{\cdot}4104$ $s^2_C = 1{\cdot}926$ (8 degrees of freedom)
$\bar{x}_D = 15{\cdot}99$ $S(x_D - \bar{x}_D)^2 = 31{\cdot}5125$ $s^2_D = 4{\cdot}502$ (7 degrees of freedom)

Comparing the highest variance, s^2_B, with the lowest variance, s^2_C, $F = 5{\cdot}709$ ($N_1 = 3$, $N_2 = 8$), corresponding to a probability level of between 0·05 and 0·01. The homogeneity of variance must therefore be tested, using the general form of Bartlett's test, which gives an estimate of $\chi^2 = 4{\cdot}82$ with 3 degrees of freedom. This corresponds to a probability of approximately 0·2. A significant heterogeneity therefore does not exist between these variances. An average variance of each of the two sets of variances (i) and (ii) was computed as 3·850 (27 degrees of freedom) and 4·035 (26 degrees of freedom) respectively. The variance ratio does not attain significance at the 5 per cent. level.

Conclusions. Definite conclusions are difficult to draw. Almost as much variability in extinction times is found on repetition of results for a single "submaster" culture and for other "submaster" cultures within the same monthly culture as would be found on performance of a few experiments on a "submaster" culture in one month and then passing on to the corresponding "submaster" culture on the following month.

Variations in Viability of Test Suspensions

The following factors could presumably affect the reproducibility of extinction time estimates:

(i) Variations in viability of suspensions of the test organism which had been adjusted to the same density using the photoelectric colorimeter.

(ii) Variations in viability of suspensions on storage for periods during a day on which extinction time determinations would be carried out.

(iii) Variations in viability and phenol resistance of slope cultures of the organism incubated over varying periods. Each of these factors was investigated, and a comparison made between variations in viability from day to day and variations within days.

Procedure. The counting method used was that described by Miles and Misra[15]. The method was selected to economise in apparatus and time and to reduce colony counting errors. The test suspension was serially diluted by addition of five drop samples to required volumes of sterilised water to give four final dilutions for plating which contained about 5, 10, 15 and 20 organisms per drop. Dilutions were made in screw-capped bottles, which were well shaken before removal of samples. The plates, which contained 20 ml. overdried peptone agar, were divided into eight sectors and one drop of each dilution placed on each sector. Each dilution was plated 20 times and the colonies were counted after incubation at 37° C., for 12–15 hours. Counts for each dilution were

subjected to χ^2 tests for homogeneity, and all results giving a value of χ^2 corresponding to a probability of less than 0·4 rejected. Counts were performed on suspensions prepared on 7 different days, performing them at varying times—from 1 hour to 15 hours after preparation. At least one dilution always gave a count of acceptable homogeneity; generally the results discarded were those with the lowest mean counts.

TABLE IV

VIABLE COUNTS (X 10^{-9} PER ML.) OF SUSPENSIONS OF *Bacterium coli* PREPARED ON SEVEN DIFFERENT DAYS, STORED FOR VARYING PERIODS AND DETERMINED FROM FOUR DIFFERENT DILUTIONS

Age after preparation	Dilution	Suspension 1	2	3	4	5	6	7	Means
A	1	1·5069	2·3933	2·6592	—	—	—	—	2·1865
less	2	1·8327	2·2350	2·3691	—	—	—	1·6092	2·0115
than	3	1·7957	2·1821	2·2275	1·7729	—	1·7502	1·5684	1·8828
2 hours	4	—	—	2·0662	1·4910	1·9488	1·6201	1·5849	1·7422
	Means	1·7118	2·2701	2·3305	1·6320	1·9488	1·6852	1·5875	1·9230
B	1			—		—	—		—
Between	2			2·5926		—	—		2·5962
2 and 4	3			2·1954		1·9093	—		2·0524
hours	4			2·0545		{1·8901 2·0310	{1·5732 1·4792		1·8056
	Means			2·2808		1·9435	1·5262		1·9657
C	1	2·2160					—		2·2160
Between	2	1·5645					—		1·5645
4 and 8	3	1·9321					1·8411		1·8866
hours	4	1·9019					1·9606		1·9313
	Means	1·9036					1·9009		1·9027
	1	—	—					2·3933	2·3933
D	2	—	—					1·7880	1·7880
Over	3	2·1594	1·7957					2·0230	1·9927
8 hours	4	2·0662	2·0075					—	2·0369
	Means	2·1128	1·9016					2·0681	2·0333
MEANS		1·8862	2·1227	2·3092	1·6320	1·9448	1·7041	1·8278	1·9484 ± 0·0986 (p=0·95)

Results and Analysis. Table IV shows the mean viable counts per ml. of suspensions as determined from four dilutions at different storage times on each of seven days. It is at once seen that there is a much greater variability between days than between storage times. However, these variations may be considerably influenced by variations between dilutions and the following tests were used to assess this influence.

(a) Variances between storage times for each dilution were calculated as $s_1^2 = 0{\cdot}0125$, $s_2^2 = 0{\cdot}197$, $s_3^2 = 0{\cdot}0069$, $s_4^2 = 0{\cdot}0173$, with 2,3,3 and 3 degrees of freedom respectively. Only one variance ratio, s_2^2/s_3^2, attains significance at the 0·05 level and a significant difference exists between only one pair of means, $\bar{x}_A$ and $\bar{x}_D$. The pooled estimate of variance is 0·0625 with 11 degrees of freedom.

(b) The variations between dilutions for each storage time were calculated as $s^2_A = 0{\cdot}0357$, $s^2_B = 0{\cdot}164$, $s^2_C = 0{\cdot}0712$, $s^2_D = 0{\cdot}0633$, with 3,2,3 and 3 degrees of freedom respectively. No significant difference exists

between any pairs of variances or means. The pooled variance estimate is 0·0944 with 11 degrees of freedom.

(c) Taking the mean counts over all dilutions within each storage time, the variances within each storage time were computed as $s^2_A = 0{\cdot}0955$, $s^2_B = 0{\cdot}143$, $s^2_C = 0{\cdot}000004$, $s^2_D = 0{\cdot}0124$, with 6,2,1 and 2 degrees of freedom respectively. A significant heterogeneity exists between these variances. The pooled variance estimate is 0·0803 with 11 degrees of freedom. The pooled variance estimates for (a), (b) and (c) are not significantly different.

Conclusions. It is concluded that variations between storage times of the suspensions up to 15 hours are certainly no greater than variations in viability of suspensions from day to day. Variations due to different dilutions are of similar magnitude. Generally, higher dilutions gave a larger estimate of viable density than the lower dilutions, a finding which might be explained either as an overcrowding effect when many organisms are exposed to a small surface area of medium or in terms of errors in counting confluent colonies where the numbers are considerable.

Variations in Viability and Extinction Times on Prolonged Incubation of Slope Cultures

Table V records the mean viable counts and the corresponding mean single survivor times to 1·10 per cent. phenol for suspensions prepared from slope cultures incubated for varying periods from 12 hours to 15 days. All the slopes had been inoculated at the same time from a

TABLE V

VIABLE COUNTS AND EXTINCTION TIMES TO 1·10 PER CENT. PHENOL OF *Bacterium coli* (SUBCULTURE C771) AFTER VARYING PERIODS OF INCUBATION

Age of slope culture (hours)	Viable count (x 10^{-9} per ml.)	Mean single survivor time (minutes).
12	1·540	18·8
25	1·588	17·4
50	1·738	18·6
75	0·973	16·4
170	0·281	11·7
195	0·294	11·1
335	0·287	10·3

serial subculture of the organism which had been previously maintained in successive daily subculture "C". Viability and extinction times remain reasonably constant for the first 50 hours. After 75 hours, the count has dropped by more than 30 per cent. and the extinction time has been reduced by about 10 per cent. But between 75 hours and 170 hours after inoculation, the count has fallen by more than 80 per cent. and the killing time has been reduced by about 40 per cent. Incubation for a further week is seen to result in no appreciable change in viability, although the extinction times continue to fall slightly.

In order to furnish confirmation of these results, two viable counts and extinction time determinations were carried out using suspensions

prepared from 24 hour and 170 hour slope cultures propagated from the culture in current use at that time and which was prepared from a monthly freeze-dried culture. The results were as follows:

24 hours. Viable count: $1{\cdot}632 \times 10^9$ per ml. Extinction time: 29·0 minutes

170 hours. Viable count: $0{\cdot}274 \times 10^9$ per ml. Extinction time: 15·8 minutes.

It is observed that, although the extinction times are higher than those obtained with the continuously subcultured organisms, the decrease in both extinction time and viable count are comparable to those determined in the first experiments. The steep fall in viability during the first week of incubation on a slope culture should be contrasted with the observations of Cook and Steel[19], who found that the viability of aqueous suspensions of the same organism remained remarkably constant for periods of over 6 weeks.

The results obtained here show that the extinction times of cultures are dependent upon their viability. But we conclude that viability is probably not related to phenol resistance since we have been unable to demonstrate any change in the phenol resistance of organisms with greater viability on an agar slope over those with less.

Summary

1. Attention has been drawn to the inadequacy of all extinction methods, except that of Berry and Bean[12], for the investigation of factors affecting the reproducibility of extinction times.

2. Reproducibility of extinction time estimates has been shown to be influenced by variations in resistance of freeze-dried samples of the test organism.

3. Methods of maintaining cultures of the test organism have been compared.

4. Variations in viability of test suspensions of the organisms on storage are probably smaller than day-to-day variations in the viability of suspensions.

5. Prolonged incubation of slope cultures of *Bacterium coli* results in decrease in both viability and extinction times.

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