SCIENCE PAPERS AND DISCUSSIONS

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART III. BACTERIA IN FIXED OILS AND FATS

SECTION 1. VIABLE COUNTS OF MICRO-ORGANISMS IN FIXED OILS AND FATS

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IN a subsequent paper the results of some experiments concerned with the survival of spores and bacteria in fixed oils and fats, and in such media containing bactericides will be described. Before this work could be undertaken it was necessary to elaborate a method for the enumeration of viable organisms in lipid media and to assess the accuracy of the process. In a previous communication[1] it has been shown that peptone powders, containing *Bacterium lactis aerogenes* or the spores of *Bacillus subtilis* in even distribution, can be obtained by the technique of spray-drying. Methods were there described for the carrying out of viable counts of organisms in the powders, and for determining the errors involved. It has now been confirmed that, as suggested at the end of the paper[1a], *Streptococcus fæcalis* is a more suitable organism than *Bact. lactis aerogenes* for the purpose of this type of work. It survives spray-drying much better. It is obtained in even distribution in the resultant powders. The errors involved in viable counts on such powders have been assessed below. In all subsequent work on lipid media therefore *S. fæcalis* and the spores of *B. subtilis* have been used as representing resistant vegetative cells and spores respectively.

The advantage of using a powder of this type is that the number of viable organisms per g. of powder can be ascertained before it is triturated with the oil, so that, using known proportions of powder and oil, the initial concentration of the viable organisms in the oil can be calculated. Techniques for viable counts can be tested on such a suspension and their errors ascertained. It is hoped that in a later paper it will be possible to describe how such suspensions in great dilution can be used in judging the efficiency of proposed sterility tests. A viable count technique accurate down to zero concentration constitutes an ideal sterility test. The counting method described below involves the dilution of a weighed quantity of the oily suspension with a volatile solvent, decantation after centrifuging, and washing the deposit several times with fresh portions of solvent. Finally the deposit is freed from residual solvent under reduced pressure, taken up in a suitable aqueous diluent, and the organisms present counted by the roll tube technique. Volatile

solvents are not without effects on micro-organisms so that these effects had first to be assessed.

Early in the course of these experiments it was realised that in spray-dried powders the spores or bacteria may be surrounded by a layer of dried peptone which might conceivably protect them from the oil and possibly from the action of bactericides dissolved in the oil. Attempts to overcome this objection have so far been successful only in the case of the spores of *B. subtilis*. An agar slope of this organism was incubated as previously described[1b] so as to give a culture consisting largely of well-developed spores. The surface growth was dehydrated by suspension in previously purified and dried acetone. The acetone suspension was added to a much larger volume of a 2 per cent. solution of stearin dissolved in acetone, and the resultant mixture was "spray-dried" in a current of air at room temperature. A fine powder of stearin containing the *B. subtilis* spores was obtained. It was established using the same technique as with the peptone powders[1c], that the spores in this powder were evenly distributed and that viable counts on the powder could be carried out. The stearin powder was then added in known proportions to the fixed oils. The stearin dissolved, leaving the spores in suspension in the oil without any peptone coating. So far no non-sporing organism has been found to be capable of resisting the action of the acetone in this process. Having obtained suitable suspensions of the organisms in the oils and fats, the rest of the work described in this paper was concerned with evaluating the errors involved in performing viable counts on such suspensions.

Materials

The *test organisms* used were *B. subtilis*, National type Culture Marburg 3610 and *S. fæcalis*, National type Culture No. 370.

The *peptone* used was of high commercial quality stated by the makers to be suitable for use in bacteriological work.

The *aqueous diluents* referred to in the text were glass-distilled sterile water for the spores of *B. subtilis* and quarter strength Ringer's solution for use with *S. fæcalis*.

The quarter strength Ringer's solution contained in 100 ml., sodium chloride 0·225 g., potassium chloride 0·0105 g., calcium chloride, anhydrous, 0·012 g., sodium bicarbonate 0·005 g.

The *stearin* was H.P.K. grade kindly supplied by the Universal Oil Company.

Arachis oil, liquid paraffin, oil of theobroma, cod-liver oil and *anæsthetic ether* were all of B.P. quality while the *acetone* was of B.P. 1932 quality, *Light petroleum* b.pt. 40° to 60°C. was of commercial quality, purified by shaking successively with N sodium hydroxide, 0·1 N potassium permanganate and water. It was then dried over anhydrous potassium carbonate and redistilled.

Iodatol is stated by the manufacturers to be a vegetable oil containing 10 per cent. of iodine in organic combination.

THE USE OF *STREPTOCOCCUS FÆCALIS* AS A TEST ORGANISM

This section should be read in conjunction with the paper published by Bullock, Keepe and Rawlins [1], since the tests carried out on *S. fæcalis* are almost identical with those previously described for *Bact. lactis aerogenes*. References in brackets refer to Tables in the earlier paper.

That *S. fæcalis* suspended in 4 per cent. peptone solution survives spray-drying very much better than *Bact. lactis aerogenes* is shown in Table I (*cf.* Table XIX).

TABLE I

THE MORTALITY OF *S. fæcalis* SUSPENDED IN 4 PER CENT. PEPTONE WATER DURING SPRAY-DRYING AT VARIOUS TEMPERATURES

Temperature of air inlet °C	Percentage mortality
65 to 80	5 to 15
85 to 100	35 to 60
110 to 175	70 to 94

The standard error of the mean of three counts on the powder was found to be 1·507 (*cf.* Table XI).

Of the three types of medium formerly employed (*cf.* Table XII, but now using 2·5 per cent. agar in place of 3 per cent. agar), medium C gave very poor results. A comparison of the results obtained using medium A and medium B at different temperatures is given in Table II.

TABLE II

THE EFFECT OF TEMPERATURE AND COMPOSITION OF THE MEDIUM ON THE VIABLE COUNT

	Medium A				Medium B			
Incubation temperature ...	26°C	32°C	37°C	40°C	26°C	32°C	37°C	40°C
	323	361	523	546	308	372	541	581
	320	366	549	569	327	351	520	527
	311	366	489	569	319	364	555	510
	338	360	550	560	329	380	499	513
	322	366	536	501	328	342	579	504
Mean	325	363	529	548	322	362	539	527

TABLE III

EFFECT OF THE DURATION OF INCUBATION AT 37°C. ON THE VIABLE COUNT OF *S. fæcalis*

Period of incubation	Count	Mean	Variance
24 hours	533 : 558 : 534 : 516 : 551	538	219
48 „	523 : 489 : 549 : 550 : 536	529	505
96 „	519 : 499 : 570 : 543 : 539	534	570
168 „	520 : 536 : 532 : 501 : 560	530	376

The effect of the duration of incubation is shown in Table III (*cf.* Table XIV).

Although there is no significant difference in the counts between 24 to 168 hours yet 48 hours was the period chosen for future experiments.

TABLE IV

THE REPRODUCIBILITY OF VIABLE COUNTS ON SUCCESSIVE BATCHES OF MEDIA

Batch I (Old)		Batch II (New)			
Counts	Mean	Counts	Mean	T	P
136 : 159 : 147 : 135 : 143	144	170 : 140 : 128 : 133 : 136	141	·348	0·7 to 0·8
99 : 88 : 90 : 86 : 81	89	102 : 82 : 84 : 79 : 73	84	·875	0·4 to 0·5
341 : 366 : 380 : 363 : 366	363	373 : 340 : 381 : 375 : 360	366	·312	0·7 to 0·8
251 : 282 : 268 : 276 : 253	266	262 : 284 : 279 : 273 : 259	271	·640	0·5 to 0·6
127 : 139 : 113 : 122 : 123	126	118 : 129 : 133 : 117 : 108	121	·810	0·4 to 0·5

TABLE V

SUMMARY OF VALUES OF x^2 OBTAINED FROM SETS OF 20 ROLL TUBES OF *S. fæcalis*

Batch No.	No. of tubes	N	x^2	P
1	20	19	11·05	0·95 to 0·90
2	20	19	12·65	0·90 to 0·80
3	20	19	10·40	0·95 to 0·90
4	20	19	14·94	0·80 to 0·70
5	20	19	10·74	0·95 to 0·90
6	20	19	15·26	0·8 to 0·7

TABLE VI

GOODNESS OF FIT OF VALUES OF x^2 OBTAINED FROM COUNTS ON SETS OF 5 ROLL TUBES, USING *S. fæcalis*

Values of χ^2	Expected Frequency (m)	Observed frequency (m+x)	Difference (x)	$\frac{\chi^2}{m}$
Under 1	19·01	23	+3·99	0·837
1 - 2	36·31	34	−2·31	0·147
2 - 3	37·51	43	+5·48	0·854
3 - 4	32·03	33	+0·97	0·029
4 - 5	25·04	22	−3·04	0·368
5 - 6	18·61	18	−0·61	0·020
6 - 7	13·35	12	−1·35	0·136
7 - 9	15·79	17	+1·21	0·092
Over 9	12·89	9	−3·89	1·251
N=8 χ^2=3·734 P=0·90- 0·80				

TABLE VII

THE PROTECTIVE ACTION OF QUARTER-STRENGTH RINGER'S SOLUTION ON *S. fæcalis*

	Time of exposure to diluent (minutes)					
	0	10	20	40	80	120
Replicate counts	265	232	229	250	262	246
	247	230	220	240	225	227
	280	240	245	229	224	244
	257	242	245	228	227	250
	224	227	250	208	209	248
Mean count ...	250·6	235	237·8	231	233·4	243

TABLE VIII

QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER CONTAINING *S. fæcalis*

Sample	1	2	3	4	5	6	7	8	9	10
Weight (g.)	·0646	·0512	·0758	·0806	·0622	·0595	·0730	·0564	·0693	·0834
Volume (ml.)	12·9	10·2	15·2	16·1	12·6	11·3	14·6	11·3	13·9	16·7
Counts	449	443	418	430	464	453	480	440	420	470
	421	386	450	462	445	454	431	452	469	419
	448	406	476	445	469	445	452	439	426	423
	431	453	443	446	476	433	417	441	440	459
	478	452	449	453	408	452	451	448	478	419
Total counts	2227	2140	2236	2236	2262	2237	2231	2220	2233	2190

TABLE IX

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER CONTAINING *S. fæcalis*

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples ...	3840	9	427	1·02	>0·2
Difference between individuals ...	16619	40	415		
Total	20459	49			

This was because at 24 hours the colonies were small and indeterminate—being thus more difficult to count and no useful purpose was served by prolonging the incubation beyond 48 hours.

Tables IV, V, VI, VII, VIII and IX, correspond exactly with Tables VI, VIII, XVI, XXVI and XXVII of the previous paper.

It will be recalled that the uneven distribution of *Bact. lactis aerogenes* in spray-dried peptone was attributed to the high death rate on spray-drying[1d]. This was confirmed by spray-drying *S. fæcalis* with an air inlet temperature of 180°C. The figures given in Tables X to XIII show that the organisms in the resultant powder were unevenly distributed, but

that exactly as with *Bact. lactis aerogenes* (Tables XXIV to XXVII) an even distribution could be obtained by milling. With an air inlet temperature of 180°C. the mortality of *S. fæcalis* is over 98 per cent., but with an air inlet temperature of 70°C. when the mortality is less than 15 per cent. the organisms were evenly distributed in the powder as shown in Tables VIII and IX.

TABLE X

QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER CONTAINING *S. fæcalis* DRIED AT 180°C.

Sample	1	2	3	4	5	6	7	8	9	10
Weight (g.)	·0405	·0388	·0287	·0720	·0336	·0282	·0376	·0275	·0341	·0327
Volume (ml.)	16·2	15·3	11·5	28·8	13·45	11·25	15·0	11·0	12·6	12·8
Counts	118	70	71	90	60	69	104	66	108	67
	112	72	71	97	59	70	113	78	125	75
	121	78	76	116	51	70	93	59	105	86
	105	76	56	101	51	48	111	69	94	89
	114	68	77	97	51	51	80	68	108	64
Total count	560	364	351	501	272	308	501	340	540	381

TABLE XI

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER CONTAINING *S. fæcalis*

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples ...	17694	9	1966	3·3	<0·01
Difference between individuals ...	23341	40	583·5		
Total	41035	49			

TABLE XII

QUINTUPLICATE PLATINGS OF 10 SAMPLES OF SPRAY-DRIED POWDER CONTAINING *S. fæcalis* DRIED AT 180° C. AND THOROUGHLY MIXED

Sample	1	2	3	4	5	6	7	8	9	10
Weight (g.)	·0577	·0607	·0615	·0534	·0814	·0545	·0578	·0551	·0722	·0617
Volume (ml.)	11·55	12·15	12·3	10·7	16·3	10·9	10·6	11·0	14·45	12·3
Counts	106	120	96	89	110	87	105	90	101	104
	104	118	85	99	104	76	115	92	120	96
	106	100	106	108	98	100	103	86	110	88
	118	95	118	100	84	108	96	87	103	95
	98	100	109	92	103	97	101	98	107	92
Total count	532	533	514	482	499	478	520	453	521	491

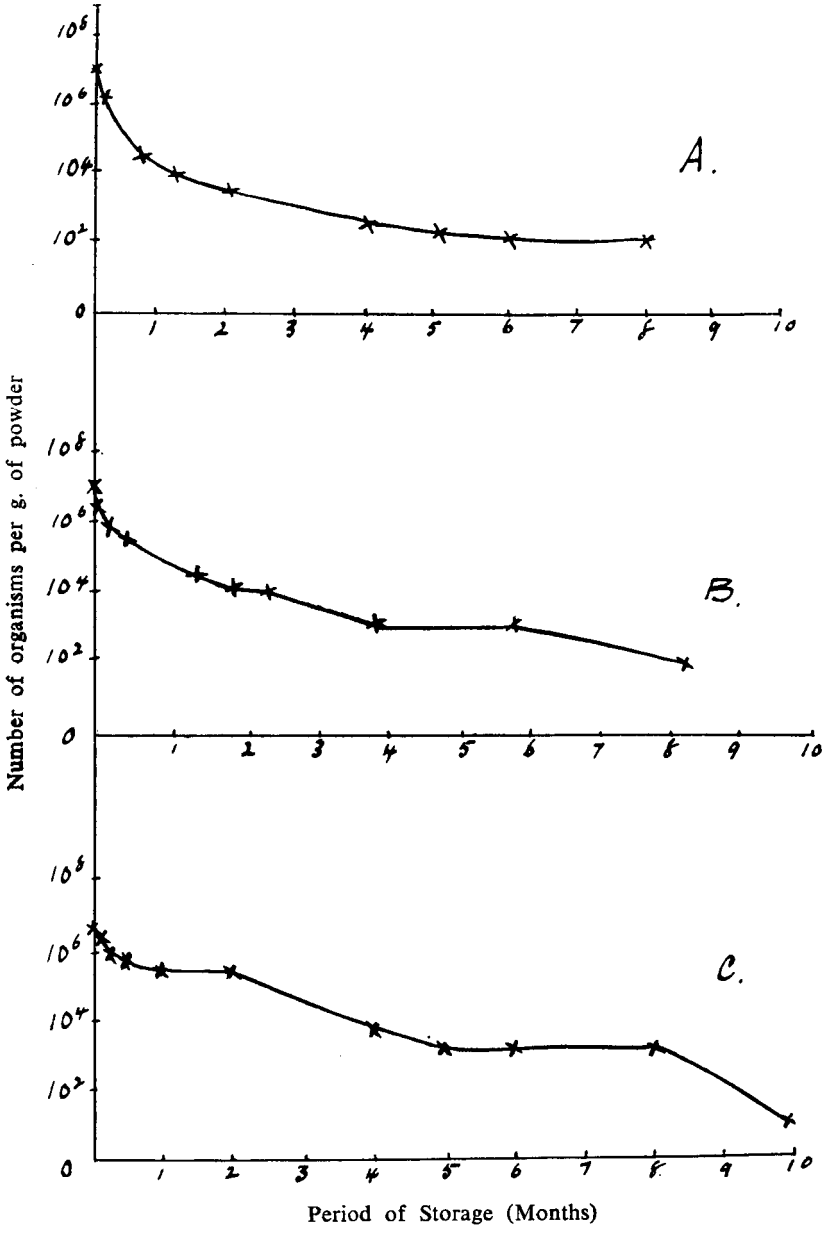

FIG. 1. Effect of storage in atmospheres of different humidities, upon the count of *Streptococcus fæcalis* in spray-dried peptone powders.

A. Stored over hydrated calcium chloride.
B. „ „ anhydrous calcium chloride.
C. „ „ phosphorus pentoxide.

TABLE XIII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER CONTAINING *S. fæcalis*

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Differences between samples ...	1239	9	137·7	1·112	<0·2
Differences between individuals	4923	40	123·8		
Total	6162	49			

S. fæcalis has another advantage over *Bact. lactis aerogenes*. It survives storage in dry peptone powders very much better. The results obtained by storing the peptone powder containing *S. fæcalis* at three different degrees of humidity are shown in Figure 1 (*cf.* Table XXVIII).

THE EFFECTS OF LIPID SOLVENTS ON THE VIABILITY OF *S. FÆCALIS* AND SPORES OF *B. SUBTILIS* IN PEPTONE POWDERS

0·05 to 0·1 g. quantities of the appropriate powders were thoroughly mixed with approximately 10 ml. of the lipid solvent. After 10 minutes in the case of *S. fæcalis* and after 90 minutes in the case of spores of *B. subtilis* the mixtures were centrifuged at 1500 times gravity and the supernatant solvent decanted, residual solvent being removed entirely from the deposited powder as rapidly as possible under reduced pressure. 10 minutes was chosen as being approximately the time during which the organisms are in contact with the lipid solvent during the performance of a viable count on a sample of oil by the method described below. The time was increased to 90 minutes with spores of *B. subtilis* because they were much more resistant than the vegetative cells. Samples of the powder before and after treatment with the volatile solvent were taken up in suitable volumes of the appropriate aqueous diluent and viable counts performed in quintuplicate on the dilutions.

The results are shown in Tables XIV and XV.

TABLE XIV

RESULTS OF ACTING ON SPORES OF *B. subtilis* IN SPRAY-DRIED POWDER WITH LIPID SOLVENTS FOR 90 MINUTES

All counts $\times 10^8$

Solvent	Ether	Absolute ethanol	70 per cent. ethanol	Acetone	Chloroform	Light petroleum b.pt. 40° to 60°C
Count on untreated powder ...	206	206	211	230	211	199
Count on treated powder ...	108	197	182	181	109	198
Percentage mortality	48	7·0	12·9	21·0	48	0·7

It is clear that the most suitable solvents are light petroleum (b.pt. 40° to 60°C.) for spores of *B. subtilis* and anæsthetic ether for *S. fæcalis*. The observation that the spores of *B. subtilis* are unaffected by light

TABLE XV

THE EFFECT OF ACTING UPON *S. fæcalis* IN SPRAY-DRIED POWDERS, WITH LIPID SOLVENTS FOR 10 MINUTES

All counts $\times 10^2$

Solvent	Light petroleum b.pt. 40° to 60°C.	Toluene	Ethyl ether (anæsthetic)	Aceto-phenone	Ethyl benzoate
Viable counts per g. of untreated powder	5	24	24	24	24
Viable counts per g. of treated powder	0·5	0	15	0	0
Percentage mortality	99	100	40	100	100

TABLE XVI

THE EFFECT OF LIGHT PETROLEUM (B.PT. 40° TO 60°C.) ON SPORES OF *B. subtilis* IN SPRAY-DRIED PEPTONE POWDER

All counts $\times 10^2$

Sample	1	2	3	4	5
Viable counts per g. of untreated powder ...	161	167	166	158	162
Viable counts per g. of treated powder ...	156	161	171	174	173

TABLE XVII

THE EFFECT OF 10 MINUTES CONTACT BETWEEN ANÆSTHETIC ETHER AND SPRAY-DRIED PEPTONE POWDER CONTAINING *S. fæcalis* CELLS

Sample (control)	1	2	3	4	5
Weight of powder (g.)	0·0568	0·0678	0·0489	0·0562	0·0518
Volume of diluent (ml.)	14·1	16·9	12·2	14·0	12·9
Counts	163	148	158	149	163
	159	155	147	159	142
	162	139	139	160	165
	165	146	167	167	168
	161	149	163	167	159
Total counts	810	737	774	802	797
Sample (test)	**1**	**2**	**3**	**4**	**5**
Weight of powder (g.)	0·0661	0·0563	0·0495	0·0450	0·0664
Volume of diluent (ml.)	16·5	14·1	12·4	11·2	16·6
Counts	83	87	93	110	102
	99	95	90	91	98
	90	89	110	85	93
	91	94	93	89	97
	90	104	102	99	104
Total counts	453	469	488	464	494

petroleum (b.pt. 40° to 60°C.) was confirmed by repeating the experiment using 5 different samples of powder. The viable count of each powder after treatment with the solvent for 90 minutes was not significantly different from that of untreated powder. The figures obtained are shown in Table XVI.

In the case of *S. fæcalis* there is an obvious fall in the viable count of the powder after treatment for only 10 minutes with anæsthetic ether. To assess this fall and to ascertain whether it is constant, 5 different samples of powder were divided into 2 parts, 1 serving as control, the

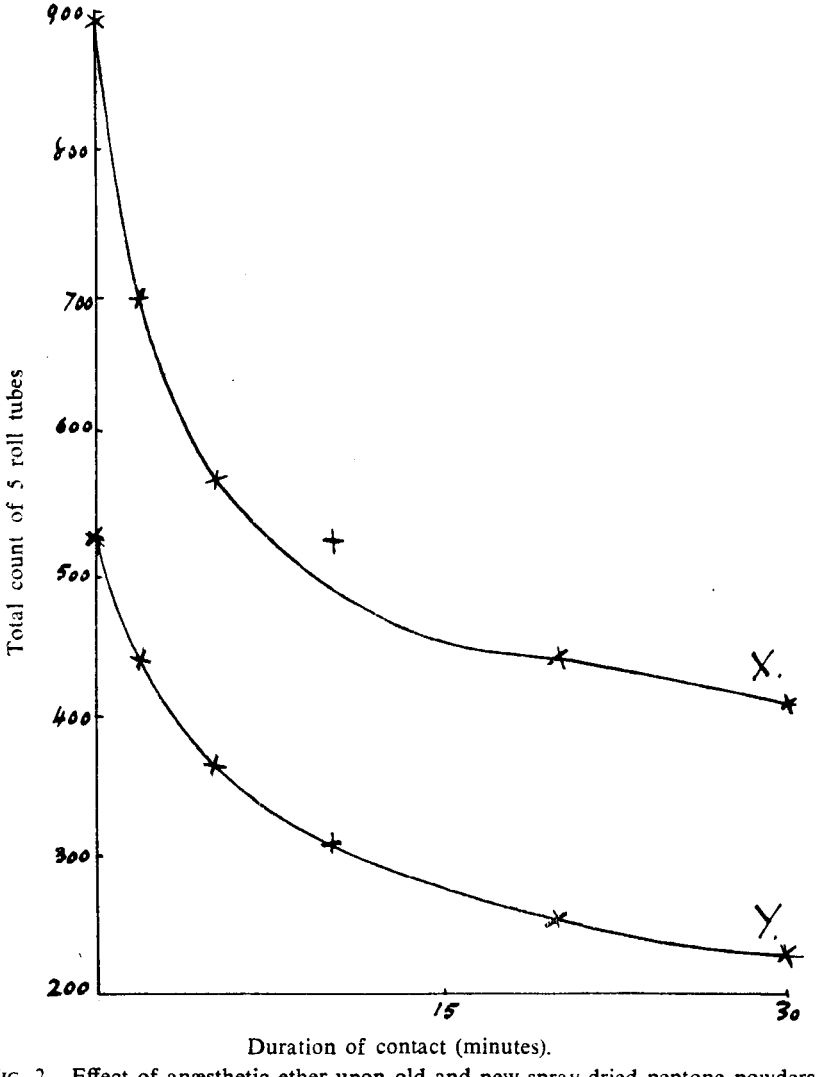

FIG. 2. Effect of anæsthetic ether upon old and new spray-dried peptone powders containing *Streptococcus fæcalis*. X, 1 day old. Y, 2 months old.

other being treated with ether for 10 minutes as described above. Approximately 0·05 g. quantities of the resultant 10 portions of powder were weighed out accurately and diluted with such quantities of quarter-strength Ringer's solution as to give 10 solutions of peptone all of equal concentration. Five 1-ml. quantities of each solution were then transferred to roll tubes and the resultant colonies counted in the usual way.

From these figures by the customary mathematical calculations the confidence limits for the percentage mortality can be calculated. They were found to be 41·9 per cent. to 37·2 per cent.

To ascertain to what extent the action of anæsthetic ether on *S. fæcalis* in dry peptone powders is progressive, further experiments were carried out in which the time of contact between the powder and the anæsthetic ether was increased. 2 samples of powder were used, 1 of which had been kept for only 1 day, the other for 2 months after having been obtained by spray-drying.

The results are shown graphically in Figure 2.

Method of Infecting the Fixed Oils or Fats

In the majority of cases the oil or melted fat, after having been sterilised at 150°C. for 1 hour and cooled, was mixed by light trituration in a sterile mortar with 5 per cent. of dry peptone powder containing a known concentration of either *S. fæcalis* cells or spores of *B. subtilis*. These suspensions were cloudy, since they contained approximately 5 per cent. of peptone. In some experiments the sterile oil or liquified fat was triturated with stearin powder containing a known concentration of spores of *B. subtilis*. The stearin dissolved leaving an almost clear oil or melted fat. The suspensions were transferred to sterile glass-stoppered bottles which were sealed by dipping the stopper into melted hard paraffin. The contents were agitated by continuously repeated inversion of the bottles. The infected melted fats were agitated in wide-mouthed glass stoppered bottles until the fat solidified.

Proposed Technique for Viable Counts on Fixed Oils and Fats

All apparatus coming into contact with the oils and fats was previously sterilised, usually by dry heat for one hour at 150°C. The fats were treated in exactly the same manner as the oils except that, where necessary, they were gently warmed until melted. If the oil already contained peptone powder, approximately 5 g. was accurately weighed directly into a 20 ml. centrifuge tube and was then mixed thoroughly with 10 ml. of volatile solvent light petroleum (b.pt. 40° to 60°C.) for spores of *B. subtilis* or anæsthetic ether for *S. fæcalis*. If the oil did not already contain peptone powder, 5 g. was first mixed with approximately 0.25 g. of spray-dried sterile peptone powder by light trituration in a mortar and washed into a centrifuge tube with 10 ml. of light petroleum. The tube was centrifuged at 1500 time gravity for 2 minutes, the supernatant fluid was decanted and the deposit twice washed, by stirring with 15 ml. of

solvent, centrifuging and decanting the supernatant fluid. The total period of contact between *S. fæcalis* and the anæsthetic ether up to this point was carefully timed to be exactly 10 minutes. The centrifuge tube was then placed in a vacuum desiccator and the last traces of solvent removed under reduced pressure (approximately 2 cm. of Hg. for approximately 15 minutes). The residue was taken up with such a quantity of aqueous diluent as to give a suitable concentration of organisms. Viable counts were performed on 5 × 1 ml. quantities of the aqueous suspension by the usual roll tube technique.

The Accuracy of the Viable Count on Oils

The accuracy of the above technique for viable counts was tested on 4 types of infected lipid material.

In each case 10 samples of the same powder were submitted to the viable count technique, and an analysis of variance was carried out. The results obtained were as follows:—i. oil containing a suspension of peptone powder infected with the spores of *B. subtilis.* (Tables XVIII and XIX); ii. oil containing a suspension of peptone powder infected

TABLE XVIII

QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME POWDER-OIL SUSPENSION OF SPORES OF *B. subtilis*

Sample	1	2	3	4	5	6	7	8	9	10
Weight of suspension (g.) ...	2·1751	2·8331	2·8207	2·2157	2·9771	2·0973	2·3329	2·1536	2·8838	2·1827
Volume (ml.) ...	15·8	14·20	14·10	11·10	14·9	10·5	11·65	10·75	14·40	10·90
	209	198	194	206	204	198	170	200	200	250
	202	213	222	211	217	219	192	225	225	215
Counts	203	200	218	201	227	194	247	190	214	210
	207	192	200	200	190	229	234	227	220	201
	228	182	201	216	215	178	200	198	194	204
Total counts ...	1049	985	1035	1034	1053	1018	1053	1040	1053	1090

TABLE XIX

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME POWDER-OIL SUSPENSION OF SPORES OF *B. subtilis*

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples	1329	9	148	1·72	0·1 to 0·2
Difference between individuals (error) ...	3536	40	83·9		
Total	4865	49			

with *S. fæcalis.* (Tables XX and XXI); iii. cocoa butter infected with peptone powder containing spores of *B. subtilis.* (Tables XXII and XXIII); iv. oil infected with stearin, powder containing spores of *B. subtilis.* (Tables XXIV and XXV).

TABLE XX

QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME POWDER-OIL SUSPENSION CONTAINING *S. fæcalis*

Sample	1	2	3	4	5	6	7	8	9	10
Weight of suspension (g.) ...	1·3712	1·4926	1·8400	1·3921	1·7046	1·2017	1·3056	1·0027	1·1840	1·8216
Volume (ml.) ...	13·70	14·90	18·40	13·90	17·05	12·00	13·05	10·00	11·85	18·20
	104	109	105	106	125	108	120	122	108	114
	113	108	120	108	105	118	121	119	102	107
Counts	110	103	118	115	110	117	117	122	117	107
	115	118	127	115	112	109	113	123	116	117
	108	117	118	113	107	117	120	111	109	121
Total counts ...	550	555	588	557	559	569	591	597	552	566

TABLE XXI

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME POWDER-OIL SUSPENSION CONTAINING *S. fæcalis*

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples ...	545	9	60·55	1·28	>0·2
Difference between individuals (error)	1891	40	47·27		
Total	2436	49			

TABLE XXII

QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME POWDER-OIL OF THEOBROMA SUSPENSION CONTAINING SPORES OF *B. subtilis*

Sample	1	2	3	4	5	6	7	8	9	10
Weight of suspension (g.) ...	2·3176	2·4210	2·1379	2·8255	3·0012	2·3652	2·4948	2·9883	2·5220	2·3824
Volume (ml.) ...	11·55	12·10	10·7	14·10	15·10	12·8	12·45	14·9	12·60	11·90
	251	260	275	289	249	291	232	285	261	280
	238	250	249	253	267	264	254	272	254	273
Counts	272	265	257	272	278	259	248	243	233	269
	256	255	269	269	236	230	239	258	279	252
	260	270	261	281	238	265	245	279	221	261
Total counts ...	1277	1300	1311	1364	1268	1309	1218	1337	1247	1335

TABLE XXIII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF THE SAME POWDER-OIL OF THEOBROMA SUSPENSION CONTAINING SPORES OF *B. subtilis*

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples ...	3572	9	396·9	1·19	>0·2
Difference between individuals (error)	13338	40	333·4		
Total	16910	49			

TABLE XXIV

QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME STEARIN-ARACHIS OIL SUSPENSION CONTAINING SPORES OF *B. subtilis*

Sample	1	2	3	4	5	6	7	8	9	10
Weight of suspension (g.) ...	2·1731	2·0016	2·7901	2·5216	2·2416	3·1627	2·8726	3·1810	2·7299	3·6122
Volume (ml.) ...	10·85	10·0	13·95	12·60	11·20	15·80	14·35	15·90	13·65	18·05
Counts	123	110	113	125	130	134	129	113	100	103
	126	115	125	109	107	118	104	128	92	118
	139	104	123	127	118	131	118	109	128	124
	108	127	117	120	120	126	119	115	120	125
	118	128	115	106	115	119	125	103	123	115
Total counts ...	614	584	593	587	563	628	595	568	563	585

TABLE XXV

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 10 SAMPLES OF THE SAME STEARIN OIL SUSPENSION CONTAINING SPORES OF *B. subtilis*

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples ...	805	9	89·4	1·00	>0·2
Difference between individuals (error)	3583	40	89·0		
Total	4388	49			

It is possible to conclude from these figures that the proposed technique gives reasonably concordant results and that it may be assumed that the viable organisms are evenly distributed in the oils. It does not prove that all the viable organisms present are counted; a fixed proportion may in all cases be killed or not recovered from the oil. To investigate

TABLE XXVI

RECOVERY OF SPORES OF *B. subtilis* FROM OIL SUSPENSIONS—ALL COUNTS $\times 10^2$

Spores added per g. of oil	Spores recovered per g. of oil	Percentage recovery	Spores added per g. of oil	Spores recovered per g. of oil	Percentage recovery
74	74	100	242	197	82
48	45	93	172	161	94
45	47	104	208	215	103
53	46	87	75	69	90
89	86	97	102	89	88
133	119	90	156	147	94
132	119	91	119	113	95
147	120	90	54	41	76
152	111	73	79	78	99
135	115	86			

Mean percentage recovery = 91 per cent.

the goodness of recovery of the viable organisms a comparison must be made of the number of viable organisms known to have been added to each g. of the oils or fats with the average of the viable counts expressed as organisms per g. of oil. This is done in Tables XXVI and XXVII.

TABLE XXVII

RECOVERY OF *S. fæcalis* FROM OIL SUSPENSION—ALL COUNTS $\times 10^2$

Organisms added per g. of oil	Organisms recovered per g. of oil	Percentage recovery
2013	1344	66
591	331	56
852	508	59
280	189	67
654	374	57
32	17	52

Mean percentage = 59 per cent.

DISCUSSION

Many pharmacists seem to have formed the opinion that oils are self-sterilising, at least as regards vegetative bacteria. But experience has shown that some bacteria die off very rapidly when dry, even though they are not suspended in oil. For example, the viable count of *Bact. lactis aerogenes* in peptone powder stored over phosphorus pentoxide fell in 37 days from over 5,000 to 64 per g. of powder[1d]. To study the effects of oils on bacteria it is essential that the survival rate in the oils should be compared with the survival rate in a control powder. *Bact. lactis aerogenes* was found not to be a suitable test organism owing to its relatively feeble resistance to the drying process and to the desiccated state.

On the contrary, *S. fæcalis* has been found to be a very suitable non-sporing organism for the purpose of these experiments. The percentage mortality on spray-drying can, as shown in Table I, be kept down to not more than 15 per cent. provided that the air inlet temperature is below 80°C. As a result of this low mortality the organisms are evenly distributed in the resultant powder (Tables VIII and IX). It is of interest to note that, if the air inlet temperature is allowed to rise to over 175°C. resulting in a mortality of over 98 per cent. then, as in the case of *Bact. lactis aerogenes,* where the mortality on spray-drying was always over 98 per cent., the viable cells become unevenly distributed in the powder. Even so, as with *Bact. lactis* aerogenes, in such powders evenness of distribution can be attained by milling (Tables X to XIII).

The low mortality of *S. fæcalis* during the drying process has a further advantage. The surviving cells are not a selected population, and there is less chance that they may have been damaged by the drying process itself. Although, as shown in Figure 1, the initial rate of mortality of *S. fæcalis* in peptone powders is fairly high, the rate falls off and a con-

siderable proportion of the cells do survive for 7 to 8 months. This is sufficiently long for satisfactory storage experiments, and is very much better than the month in the case of *Bact. lactis aerogenes*[16].

At first sight it appeared questionable whether consistency of viable counts could be obtained with a streptococcus, also the colonies of *S. fæcalis* are not very large on some nutrient media. It has been found, however, that, provided that optimum conditions are employed, namely, medium B (Table II), an incubation temperature of 37°C. (Table II), and 48 hours incubation (Table III), well-defined colonies can be obtained and reliable viable counts performed (Tables IV, V, VI).

At one time it appears to have been believed that apparatus and even suitably insoluble powders could be sterilised by washing with volatile solvents such as ether and ethanol. How far short of the truth this belief was is illustrated by the results shown in Table XIV. Absolute ethanol and 70 per cent. ethanol were included in this table out of general interest. Only 13 per cent. of the spores of *B. subtilis* were killed in 90 minutes by 70 per cent. ethanol; it is interesting that ether was more effective. On the other hand, Table XV shows that ether was the least harmful solvent in the case of *S. fæcalis*.

The real object of the work summarised in Tables XIV and XV was to ascertain the least harmful lipid solvents. Spores of *B. subtilis* are very resistant to light petroleum, and from Table XVI it is obvious that 10 minutes' treatment with this solvent can have no appreciable effect on the viable counts.

As was expected, more difficulty was experienced with the *S. fæcalis*. It is quite possible that all vegetative bacterial cells are damaged by exposure to lipid solvents. With *S. fæcalis* the least harmful solvent (anæsthetic ether) renders about 40 per cent. of the organisms non-viable in 10 minutes. Figure 2 shows that this toxic action is progressive with time. However, Table XVII shows that the 40 per cent. mortality for 10 minutes exposure is reasonably constant in replicate experiments. It was decided, therefore, to proceed with storage experiments on oils containing *S. fæcalis* even though the viable count technique to be used would give results low by about 40 per cent. This appeared to be reasonable since in the storage experiments to be described later the emphasis is on the number which survive. A method giving low results can, under the circumstances, be used; with a perfect process even more organisms would be shown to survive.

The above observations form the basis of the proposed technique for viable counts on oils and fats described on page 701 of this paper. The technique has been in use in this department for over 3 years and has proved a very useful research tool. In most experiments it has been carried out on oils or fats containing peptone powders. It can, however, be applied to clear oils by initially stirring in 5 per cent. of sterile spray-dried peptone powder. Experiments are in progress with a view to elaborating a sterility test for oils and fats along these lines.

The limits of accuracy of the technique have been ascertained in a number of experiments. Tables XVIII to XXVII illustrate some of these. The analyses of variance shown in Tables XVIII to XXV indicate the concordance of the results obtained, while Tables XXVI and XXVII show that the average percentage recoveries are 91 per cent. for the spores of *B. subtilis* and 59 per cent. for *S. fæcalis*. The low value for *S. fæcalis* is, of course, due to the destructive action of the ether used in the recovery process. In 10 minutes ether kills 40 per cent. of the organisms. If we allowed for this, then the percentage recovery of *S. fæcalis* would be 98 per cent., i.e., higher than that for *B. subtilis*, which is unlikely. It is probable that mixtures of ether and oil are not so toxic to the cells as ether alone, which would mean that less than 40 per cent. would be killed in the 10-minute period, leading to a lower figure for the percentage recovery.

Summary

1. It has been shown that *S. fæcalis*, a non-sporing bacterium, survives spray-drying well and very much better than *Bact. lactis aerogenes*. The resultant powder contains the bacteria in even distribution and is suitable for use in storage and other experiments designed to determine the survival rate of the bacteria in systems of low moisture content.

2. *S. fæcalis* cells and spores of *B. subtilis* in peptone power can be obtained in even distribution in oils and fats.

3. Spores of *B. subtilis*, dehydrated with acetone and mixed with a solution of stearin in acetone can be spray-dried so as to give a stearin powder containing the spores in even distribution.

4. The stearin powders can be dissolved in oils and fats so as to give a suspension of the spores, unprotected, and in even distribution in oils and fats.

5. A technique is described for the performance of viable counts on oils and fats.

6. Statistical analyses and recovery experiments show the counting technique to be accurate usually within the limits of approximately ± 10 per cent.

References

1. Bullock, Keepe and Rawlins, *J. Pharm. Pharmacol.*, 1949, **1**, 878. (a) 899, (b) 879, (c) 895, (d) 898, (e) 897.