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Conditions affecting the Growth of *Bacterium coli* on Bile Salts Media. Enumeration of this Organism in Polluted Waters

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SUMMARY: The growth of *Bacterium coli* on agar media containing bile salts is conditioned by a number of factors. The proportion of total cells able to grow on a medium not containing bile salts rapidly declines at temperatures of incubation above 43°. On a medium containing bile salts and lactose a distinctly inhibitory effect is observed at 37°; with most strains this effect is still more pronounced at 44°. Some brands of bile salts are appreciably more inhibitory than others. Inclusion of phosphate in a bile salts medium introduces a markedly inhibitory factor, the severity of which varies with the strain of organism; some strains are virtually unable to grow on such a medium.

When a culture of *Bact. coli* is suspended in water containing only small concentrations of inorganic salts an increasing proportion of the population becomes attenuated so that the cells are unable to grow on bile salts lactose agar at 44°. An occasional strain may also exhibit sensitiveness to the presence of neutral red. This attenuating effect may be largely decreased if, before the inoculum is mixed with the bile salts and agar, it is subjected to a short period of incubation with lactose broth. This treatment has been made the basis of a technique for obtaining a colony count of *Bact. coli* which is applicable to polluted waters.

Several workers (cf. Wilson, Twigg, Wright, Hendry, Cowell & Mair, 1935; Clegg & Sherwood, 1939; Sherwood & Clegg, 1942; Batty-Smith, 1942; Allen, Brooks & Williams, 1949), have found ability to ferment bile salts lactose broth at 44° a reasonably specific test for *Bacterium coli* type I, and a method of enumeration based on this test was outlined by the Ministry of Health (1939). Disadvantages of the method are the large quantity of medium required and the large error involved in the computation of the most probable number. Thus Halvorson & Ziegler (1933) calculated that, with five tubes of medium to each dilution, the count obtained will be between 70 % below and 260 % above the true value.

Enumeration of this organism provides a simple method of following the effect of different treatments of sewage on the numbers of faecal bacteria discharged into a river and of the rate at which these numbers decrease as the organisms are carried downstream. A colony count of *Bact. coli* would introduce into sanitary surveys of this character a welcome improvement in accuracy. Clegg & Sherwood (1947) introduced a roll-tube method of counting faecal coli in shellfish in which inocula were mixed with a modified MacConkey agar and the tubes were incubated at 44°. Preliminary tests showed, however, that the ability of cells of *Bact. coli* to form colonies on bile salts lactose agar

was profoundly affected by a number of conditions. These conditions were investigated before the results were embodied in the method of enumeration finally adopted.

EXPERIMENTAL

After various methods of preparing films of agar media in tubes and bottles had been tried a technique similar to that described by Tai & van Heyningen (1950) was adopted. A chuck, rotating horizontally, was mounted on the shaft of a variable speed motor so that a bottle containing molten nutrient medium and an inoculum could be rotated at a speed of several hundred revolutions/min. For inocula of 1 ml. small glass bottles of the type described by Proom & Hemmons (1949) with a capacity of 30 ml. were used. For a larger size of inoculum bottles measuring about 4 cm. diameter and 15 cm. in length from base to shoulder were found suitable. Details as to the kind and quantity of medium and size of inoculum are given later but, in general, the inoculum was added to a bottle containing the liquid medium at a temperature of about 48°. The bottle was gently shaken to mix the contents and immediately placed in the chuck and rotated. The chuck and bottle were arranged to project over a sink so that, while rotating, the bottle could be cooled with a jet of cold water. Four similar machines were arranged side by side. Water cooling was found to be preferable to air cooling in that it ensured a firm adherent film after the bottle had been rotated for a comparatively short time (for the small bottles about 30 sec. sufficed). With air cooling several minutes are required on a warm day and if the bottles are removed from the chuck a little too soon the agar film develops slight wrinkles and usually collapses before or during incubation.

Comparative counts in bottles and Petri dishes and with different brands of agar

Comparative trials with Petri dishes and bottles, and with New Zealand and Japanese agars, showed that neither the kind of agar nor the type of container made any significant difference to the counts. Since, owing to the firmer gel it forms, there are advantages in using New Zealand agar for bottle counts, this material was used in the subsequent experiments. In most experiments bottle counts were averages of three or four determinations.

Effect of temperature of incubation on the count

The effect of temperature of incubation on the proportion of the total population of cells able to proliferate was tested on nutrient agar (without bile salts). Six strains of *Bact. coli* were grown in nutrient broth at 37°. After incubation for 6 hr., and again after 3 days, replicate counts were made in bottles of nutrient agar at 37, 39, 40, 41, 42, 43, 44 and 45°. Counts, expressed as percentages of the corresponding counts at 37°, are shown graphically in Fig. 1 (only three of the strains were tested at temperatures up to 41°).

It is clear that a temperature of 44° is critical for this organism (cf. Clegg &

Sherwood, 1939; Taylor, 1945), and it is important that the temperature of the water-bath should be accurately controlled. The average of the twelve counts at 44° was 74 %, and of the eleven counts at 43°, 90 % of the count at 37°.

Fig. 1. Colony counts of broth cultures (when 6 hr. and when 3 days old) of six strains of *Bact. coli* at different temperatures of incubation (each count expressed as percentage of count of the same culture at 37°).

Proportion of cells able to grow on bile salts-lactose agar at 37° and at 44°

Colony counts, at both 37 and 44°, on nutrient agar and on bile salts lactose agar prepared from two different recipes (given below) were made of broth cultures of six strains of *Bact. coli* (Table 1). The inhibiting action of the bile salts media, which was appreciable at 37°, was still more marked at 44°. Medium D, containing phosphate as well as bile salts, was inferior to medium A and at the higher temperature two strains were scarcely able to grow at all. Further tests were made with this medium in which different concentrations of bile salts were tried (0.5, 1.0, 1.5 g./l.), but no difference was observed in the inhibitory effect.

Comparison of different bile salts media

It is evident from the literature that the count of *Bact. coli* will be affected by the kind and concentration of bile salts, though it is doubtful whether this is generally appreciated. Leifson (1935) found that of the bile acids he tested deoxycholic acid exerted the strongest effect on bacterial growth, and that a number of substances exerted a strongly inhibitory action on coli-aerogenes bacteria when incorporated in a sodium deoxycholate medium. These substances included sodium chloride, acetates, propionates, butyrates and citrates,

Table 1. *Comparative colony counts of Bact. coli on nutrient agar at 37° and on bile salts lactose agar at 37° and at 44°*

Culture		Nutrient agar at 37°	Bile salts lactose agar			
			At 37°		At 44°	
			Medium A	Medium D	Medium A	Medium D
Strain	Age		Count (millions/ml.)			
H 7	6 hr.	332	287	259	215	210
	5 days	91	51	37	30	20
H 1	6 hr.	250	165	197	156	104
	5 days	92	75	67	73	20
H 8	6 hr.	241	163	165	118	*
	5 days	44	35	19	38	<0.1
H 6	6 hr.	235	175	145	69	50
	5 days	43	36	30	32	18
H 0	6 hr.	224	184	200	157	151
	5 days	35	28	26	22	11
H 13	6 hr.	253	185	153	102	44
	5 days	6.0	4.8	0.2	2.9	0.5

Medium A=Ministry of Health recipe. Medium D=Hajna & Perry's 'EC' recipe (plus agar and neutral red).

* Counts in triplicate tubes highly inconsistent.

and their inhibitory action was enhanced by the presence of unknown substances in meat infusion and in certain brands of peptone.

Cultures of two strains of *Bact. coli* in nutrient broth were incubated at 37°, and at intervals comparative counts in bottles at 44° were made in the following four media:

A: MacConkey agar (Ministry of Health, 1939) of the following composition (g./l.): peptone, 20 g.; sodium taurocholate, 5 g.; lactose, 10 g.; neutral red, 10 ml. of 1 % solution.

B: similar to A but containing 5 g./l. of a proprietary brand of specially pure bile salts.

C: similar to A but containing 20 g./l. of ox-gall instead of sodium taurocholate.

D: the 'EC' medium prescribed by Hajna & Perry (1943) made up with agar and containing neutral red as indicator and the same brand of bile salts as B. The composition (g./l.) was as follows: Bacto tryptose, 20 g.; NaCl, 5 g.; K₂HPO₄, 4 g.; KH₂PO₄, 1.5 g.; bile salts 'X', 1.5 g.; neutral red, 5 ml. 1 % solution.

In all media 1.5 % New Zealand agar was incorporated.

Results (Table 2) showed that medium A gave higher counts than B or C. On medium D counts were much lower than on the other three media and with strain H8 the effect was so marked that virtually no growth occurred. The concentration of bile salts was less in medium D (0.15 %) than in medium B (0.5 %). This substance by itself could not, therefore, have been the main inhibitory agent.

Inhibitory effect of phosphate in bile salts media

Counts of broth cultures of six strains of *Bact. coli* were determined when 6 hr. old, and again after 6 days, on media A and D, and on medium D from which the phosphate was omitted. Results (Table 3) show that omission of phosphate removed a strongly inhibitory factor from medium D which now gave higher counts than medium A.

Table 2. *Comparative counts of Bact. coli in bottles at 44° on different bile salts media*

Age of culture	Strain H 1				Strain H 8			
	Bile salts media							
	A	B	C	D	A	B	C	D
	Count (millions/ml.)							
0 hr.	0.70	0.44	0.69	0.44	0.28	0.072	0.126	0*
4 hr.	151	122	119	48	61	16.2	32.2	0
24 hr.	382	204	276	208	140	15.0	22.8	0
48 hr.	306	156	164	78	250	34.2	22.0	0
72 hr.	—	—	—	—	92	7.8	10.4	0
4 days	90	44	76	3.6	—	—	—	—
5 days	—	—	—	—	52	18.4	20.4	0
6 days	18.8	12.0	14.6	2.9	41	2.4	18.6	0
7 days	20.4	16.4	12.8	10.0	—	—	—	—

A=MacConkey agar (Ministry of Health, 1939).

B=Similar to A but 5 g. proprietary brand of specially pure bile salts/l. instead of Na taurocholate.

C=Similar to A but 20 g. ox-gall/l. instead of Na taurocholate.

D=Hajna & Perry's (1943) 'EC' medium.

* less than 10/ml.

Table 3. *Effect of phosphate on counts of Bact. coli on bile salts lactose agar*

Culture		Bile salts lactose agar at 44°		
		Medium A	Medium D (containing phosphate)	Medium D (without phosphate)
			Count (millions/ml.)	
Strain	Age			
H 8	6 hr.	530	11	700
	6 days	1.52	< 0.01	1.69
H 0	6 hr.	430	380	490
	6 days	5.8	0.5	7.3
H 7	6 hr.	180	99	316
	6 days	56	10	71
H 1	6 hr.	153	62	219
	6 days	60	9.3	92
H 73	6 hr.	169	80	196
	6 days	22	1.0	25
H 6	6 hr.	154	c. 3	172
	6 days	40	c. 3.5	38

*Effect of suspension in water on proportion of total cells of Bact. coli
able to grow on bile salts lactose agar*

A dilute suspension of cells was obtained by adding a small quantity of a broth culture of *Bact. coli* to $\frac{1}{4}$ -strength Ringer's solution (0.0015 ml. culture/l.); this was incubated at 20° and at intervals counts were made on nutrient agar at 37°, and on bile salts lactose agar (medium A) at 37° and at 44°. This procedure was repeated with broth cultures of different ages. Counts on the bile salts medium were expressed as percentages of the corresponding counts on nutrient agar, it being assumed that this medium exerted no appreciable inhibitory effect at 37°. Results for broth cultures of different ages (Table 4) indicate that in general the longer the period of immersion in Ringer's solution the smaller was the proportion of the cell population able to grow on the bile salts medium. With a young culture immersed for 3 hr. or more the inhibitory effect was much more marked at 44° than at 37°.

Table 4. *Percentage of total population of Bact. coli able to grow on bile salts lactose agar at 37° and at 44° after different periods of immersion in $\frac{1}{4}$ -strength Ringer's solution*

Period of immersion in Ringer's solution at 20° (hr.)	Age of broth culture (hr.)							
	17		41		65		89	
	Bile salts lactose agar at		Bile salts lactose agar at		Bile salts lactose agar at		Bile salts lactose agar at	
	37°	44°	37°	44°	37°	44°	37°	44°
	Counts as percentage of those on nutrient agar at 37°							
0	77.5	66.3	82.4	97.3	52.4	50.5	57.3	48.3
1	78.4	63.7	72.5	62.5	32.6	48.4	49.0	40.6
2	63.8	40.6	50.0	54.8	17.4	8.8	53.5	53.5
3	46.3	16.3	51.9	23.3	13.8	7.5	46.9	46.0
6	15.8	5.7	13.0	5.4	38.0	25.4	27.1	21.1
24	—	—	11.0	7.8	—	—	8.9	7.8

In a similar experiment (Table 5) the effects of immersion in full-strength and in $\frac{1}{4}$ -strength Ringer's solution were compared. By making counts on bile salts lactose agar and on a similar medium from which the bile salts had been omitted the relative effect of the bile salts and of the high temperature of incubation (44°) was assessed. The broth culture was 41 hr. old at the time of immersion. With cells immersed in the full-strength solution bile salts exerted no very marked effect, and 60 % or more of the total population was able to grow at 44°. With cells immersed in the $\frac{1}{4}$ -strength solution, on the other hand, only 25–45 % of the total population of cells was able to proliferate at 44°, and of these on the average only half could tolerate bile salts. The relatively severe action of the more dilute liquid on the bacterial cells was also reflected in the rates of death in the two solutions, counts on nutrient agar at 37° showing that at the end of the 24 hr. period the percentages of the original cells surviving were 1.6 in $\frac{1}{4}$ -strength and 46.7 in full-strength Ringer solution.

Experiments with a strain of *Bact. coli* in 0.003M phosphate buffer at pH 6.0 showed that before suspension in this solution 90–100 % of cells were able to grow at 44°. After immersion for 7–12 days, however, only about one-half, and after 24 days only about one-third, of the cells could proliferate at this temperature. The temperature of incubation (44°) and the presence of bile salts thus both tend to inhibit growth of cells of *Bact. coli* into colonies, and the effects are more pronounced with cells attenuated by prolonged immersion in water.

Table 5. *Percentage of total population of Bact. coli able to grow on bile salts lactose agar at 44°, and on a similar medium without bile salts, after different periods of immersion in Ringer's solution of two different strengths*

Period of immersion in Ringer's solution (hr.)	Ringer's solution used for immersion			
	Full-strength		$\frac{1}{4}$ -strength	
	Lactose agar at 44°			
	With bile salts	Without bile salts	With bile salts	Without bile salts
	Counts as percentage of those on nutrient agar at 37°			
0	72.0	72.0	80.7	79.0
1	82.0	77.4	28.3	45.1
2	69.4	83.9	10.0	25.5
3	51.6	69.4	16.2	30.4
6	62.3	68.8	38.6	45.5
12	60.3	58.6	12.7	24.2
24	91.4	94.3	11.1	33.3

Differences in inhibitory action of different brands of bile salts

Two batches (N and O) of bile salts lactose agar were prepared, both being made with brands of bile salts of high quality but marketed by two different firms. A suspension of *Bact. coli* was made in 0.003M phosphate buffer at pH 6.0, and colony counts in triplicate were determined on each of the two media at intervals over a period of 52 hr. In sixteen such tests medium O invariably yielded a higher count than medium N, the increases ranging from 25 to 100, with an average of 66 %.

Effect of 'resuscitation' on proportion of cells able to grow on bile salts lactose agar at 44°

Tests were made to see whether cells attenuated by a period of suspension in water could be so resuscitated by preliminary incubation in a nutrient medium that they were subsequently more resistant to the inhibitory action of bile salts and to a high temperature of incubation. The general principle in these experiments was to prepare the bile salts medium in two portions: (i) a broth containing the peptone and the sugar, and (ii) an agar portion containing the bile salts, neutral red and agar. Each portion contained constituents at twice the final concentration required so that mixing resulted in a medium of normal constitution. The inoculum was added to the broth,

which was incubated for a period at 37° before adding to the agar portion of the medium and preparing the film in the bottle.

For preliminary resuscitation the following two sugar broths were compared (constituents as g./l.) broth E: peptone, 40; sodium chloride, 10; lactose, 10; broth F: peptone, 15; Yeastrel, 6; glucose, 10. A 45 hr. broth culture of *Bact. coli* was suspended in 0.003M-phosphate buffer at pH 6.0 (4 parts culture to 1 million parts buffer). This suspension was incubated at 20°, and at intervals samples were removed and treated as follows: 20 ml. volumes of suspension were added to 50 ml. broths E and F, and the mixtures were incubated at 37°

Fig. 2. Colony counts on corresponding bile salt agar medium at 45° of *Bact. coli* after period of suspension in 0.003M-phosphate buffer followed by different periods of resuscitation in (a) lactose broth, (b) glucose Yeastrel broth. (After suspension in buffer: I, for 24 hr.; II, for 48 hr.; III, for 96 hr.; IV, for 125 hr.)

in a water-bath. At intervals of 30 min., 3.5 ml. of each mixture were added separately to small culture bottles containing 2.5 ml. of the agar portion of the medium (consisting of 3.0% New Zealand agar, 0.3% bile salts 'X', and 0.02% neutral red) and colony counts were made in the usual way.

Counts in the two bile salts media after different periods of resuscitation are shown in Fig. 2. In each case it appears that two effects are superimposed, resulting in a sigmoid curve. It seems likely that these two effects comprise (a) a preliminary period in which increasing numbers of attenuated cells become 'rejuvenated' and are able to grow on the bile salts medium, and (b) a period when cells which have been incubating in lactose broth or glucose Yeastrel broth come to the end of the lag period and start to proliferate. Tests showed that no appreciable growth occurred in the glucose Yeastrel broth during the first 1½–2 hr. of incubation. The two effects—resuscitation and proliferation—are, however, more distinctly separated in the glucose Yeastrel medium than in the lactose medium, suggesting that in the latter growth of some cells takes place at a somewhat earlier stage, and before all the cells have been resuscitated.

It appears from Fig. 2 that, for organisms immersed in dilute buffer for periods up to 96 hr., a count approximating to the count on glucose Yeastrel agar at 37° may be obtained by incubating the organisms for 1 hr. in lactose broth before introducing the bile salt, neutral red and agar. For organisms immersed for 125 hr. the counts so obtained would be somewhat lower. The curves suggest, in fact, that with increasing periods of immersion in buffer a decreasing proportion of total cells is capable of being resuscitated before proliferation of some of the cells begins.

Effect of neutral red on count

It was found by trial that a concentration of 0.006 % neutral red in the medium was sufficient to give easily visible red colonies after incubation. This concentration was therefore adopted in subsequent work. Tests with two samples of sewage and six samples of polluted river water, in which colony counts on bile salts lactose agar containing neutral red were compared with counts on a similar medium containing no neutral red, showed that the dye had no significant effect either on the direct count or on the count following resuscitation. Similar results were obtained with a mixture of five strains of *Bact. coli* suspended in dilute buffer solution and incubated at 20° in the laboratory. With one of these strains, tested singly, however, there proved to be a marked effect when the culture had been attenuated by a prolonged period of immersion. In the presence of neutral red the direct colony count of this strain was sometimes only a fraction of the corresponding count in the absence of the dye. The process of resuscitation appeared to remove this sensitiveness for in the subsequent colony counts, the presence of neutral red made no significant difference.

RESULTS OF APPLYING METHODS TO SAMPLES OF RIVER WATER AND SEWAGE

Direct colony counts and counts after preliminary resuscitation were determined (by the methods given in the Appendix) on sixty-five samples of polluted river water and sixteen samples of settled sewage. The Most Probable Numbers (M.P.N.) of *Bact. coli* were determined on the same samples by the usual dilution technique (primary incubation in bile salts lactose broth at 37° being followed by subculture of positive tubes into a similar medium at 44°).

Analysis of the results for river water showed that the direct colony counts (average of duplicates) ranged from 9 to 402 % with an average of 90 % of the M.P.N. Colony counts after preliminary resuscitation ranged from 37 to 676 % with an average of 177 % of the M.P.N. The wide range in percentages of colony counts is largely explained by the error of the M.P.N.

Results for the sixteen samples of sewage showed that direct colony counts ranged from 2.5 to 344 % with an average of 109 % of the M.P.N. After preliminary resuscitation the counts ranged from 80 to 2700 % with an average of 572 % of the M.P.N. The greatly increased count after resuscitation suggests that a large proportion of the organisms in sewage are in a moribund condition incapable of growing directly on the bile salts medium.

The increases in counts of water and sewage following resuscitation may be compared with the results of experiments with pure cultures. From Tables 4 and 5 it appears that if, after immersion in water, all cells could have been successfully resuscitated, the counts on bile salts lactose agar at 44° would have been increased by amounts ranging from 118 to 1233 %. In the experiments referred to in Fig. 2, resuscitation increased the counts of cells immersed for 24–96 hr. by 900–2040 %; none of the cells immersed for 125 hr. was capable of growth on the bile salts medium at 44° without previous resuscitation.

Specificity of colony count for faecal coli

Fifty-four colonies (sixteen from 'direct' counts and thirty-eight from counts after resuscitation) were isolated from fourteen samples of water and two samples of sewage, and the resulting cultures were submitted, without purification, to the indol, MR, VP, and citrate tests. Forty-seven strains gave the correct reactions for *Bact. coli* type I, six further strains conformed to type except for slight growth in citrate medium, and one strain appeared to be a 44°-positive variant of *Bact. aerogenes*. The colony counts therefore appeared to be reasonably specific for faecal coli.

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APPENDIX

Methods of making colony counts

Direct colony counts

(1) Agar medium: peptone, 20 g.; NaCl, 5 g.; bile salts,* 1.5 g.; New Zealand agar, 15 g.; distilled water, 900 ml.; pH value 7.0. (2) Lactose neutral red solution: lactose, 5 g.; 0.6 % neutral red, 10 ml.; distilled water, 90 ml. Sterilization by autoclaving at 15 lb. for 20 min.

Just before use 10 ml. of solution 2 are added to 90 ml. of the melted agar medium, and the mixture is maintained at about 48°. This is distributed in the culture bottles in quantities of 5 ml. and the bottles are maintained at the same temperature for a short time before adding the inoculum,† which consists of 1 ml. of a suitable dilution of the sample being examined. The bottles are rotated until the medium has set and are then incubated in a water-bath at 44° for 24 hr.

Colony counts after preliminary resuscitation

(1) Double-strength broth: peptone, 40 g.; NaCl, 10 g.; distilled water, 900 ml.; pH value, 7.0. Fill into bottles in 90 ml. quantities and just before use add 10 ml. of 10 % lactose solution which has been separately sterilized. (2) Double-strength agar medium: bile salts, 3.0 g.; New Zealand agar, 30 g.;

* The concentration of bile salts required depends on the purity and strength of the commercial article. Guidance can usually be obtained from the manufacturers.

† In larger bottles inocula up to 5 ml. were used. Quantities of agar medium and of lactose neutral red solution were increased proportionately.

distilled water, 1 l.; pH value, 7.0. Fill into bottles in 90 ml. quantities and just before use add 10 ml. of 0.12% neutral red solution which has been separately sterilized.

Solution 1 is dispensed in 7.5 ml. quantities in $6 \times \frac{3}{4}$ in. test-tubes which are incubated in a water-bath at 37°. Inocula of 3 ml. of suitable dilutions of the sample being tested are added to separate tubes and the mixtures are allowed to remain at 37° for 1 hr. Quantities of 3.5 ml. of this mixture (equivalent to 1 ml. of inoculum*) are then added to the small culture bottles to which 2.5 ml. of the agar medium have been added several minutes previously and which have been maintained at 48–50°. After rotating in the usual way the bottles are incubated in the 44° water-bath for 24 hr.

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* In larger bottles inocula up to 5 ml. were used. Quantities of broth and of agar medium were increased proportionately.

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