ENUMERATION OF *STREPTOCOCCUS FAECALIS*, WITH PARTICULAR REFERENCE TO POLLUTED WATERS

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(With 2 Figures in the Text)

A faecal type of streptococcus and its variants, a salivary type, and a few haemolytic streptococci of groups B, C, F and G, are normal inhabitants of the faeces of man and other animals. Dible (1921) gave the specific name of *Enterococcus* or *Streptococcus faecalis* to that group of streptococci found in the faeces which was heat-resistant, did not liquefy gelatin, and was able to ferment mannite, but not raffinose or inulin. Several other streptococci, including *Str. liquefaciens* (Orla-Jensen, 1919), *Str. zymogenes* (MacCallum & Hastings, 1899), and *Str. durans* (Sherman & Wing, 1937) have been found to possess an antigen and certain other properties in common with enterococci and are now usually regarded as varieties of these organisms. Among the more important characteristics of the group are the ability to grow in the temperature range of 10–45° C., in broth containing as much as 6·5 % sodium chloride, and in broth as alkaline as pH 9·6.

Observations by a number of workers in different fields (cf. Savage & Read, 1916; Houston, 1910, 1931, 1932; Ostrolenk, Kramer & Cleverdon, 1947; Ritter & Treece, 1948; Ingram, 1952; Allen, Grindley & Brooks, 1953) support the view that counts of *Bacterium coli* and *Str. faecalis* together probably constitute a better index of faecal pollution than examination for either organism alone. The fact that *Str. faecalis* has been used much less than *Bact. coli* as an indicator of faecal pollution has been due largely to the lack of suitable methods for detecting and enumerating it.

The tendency of sodium azide to block the assimilatory process more readily in Gram-negative than in Gram-positive bacteria was taken advantage of by Snyder & Lichstein (1940), who tested the use of this substance in a selective medium. They found that a concentration of 0·01 % allowed growth of *Proteus* species, of *Bact. coli*, and of streptococci, but that at a concentration of 0·05 % growth of the Gram-negative organisms was inhibited while *Str. faecalis*, *Str. viridans*, and some haemolytic streptococci were able to grow.

Hajna & Perry (1943) used a glucose-phosphate-peptone broth, containing 0·05 % sodium azide, and a temperature of incubation of 45·5° C. for detection and enumeration of faecal streptococci. Mallmann & Seligmann (1950) claimed that a broth containing 0·02 % sodium azide and incubated at 37° C. gave much higher counts of *Str. faecalis* in samples of river water than did Hajna & Perry's medium at 45° C. It should be remembered, however, that at this temperature the medium is not specific for *Str. faecalis* nor even for streptococci (cf. France & Fuller, 1940; Ritter & Treece, 1948). Hannay & Norton (1947) used a glucose broth containing

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0·025 % sodium azide and 0·3 % yeast extract, and a temperature of incubation of 45° C. for enumerating streptococci in river water. Allen, Brooks & Williams (1949) found that this technique permitted only *Str. faecalis* to be grown from sewage and sewage effluents, and that higher counts were obtained and a smaller number of doubtfully positive results occurred than when Hajna and Perry's medium was used. The beneficial effect of yeast extract on the growth of *Str. faecalis* in a glucose-azide medium has been confirmed by Brown & Gibbons (1950).

These observations suggested that a selective medium, capable of yielding reasonably accurate counts of *Str. faecalis* in a mixed flora, might be based on glucose azide agar if various factors affecting the ability of streptococci to form colonies on such a medium could be elucidated by investigation.

EXPERIMENTAL

The apparatus described by Allen, Pasley & Pierce (1952) for determining the viable count of *Bact. coli* was adapted for counting *Str. faecalis*. In this apparatus a bottle, containing molten nutrient medium to which an inoculum has been added, is inserted in a chuck mounted on the shaft of a motor. The bottle is rotated on its horizontal axis at several hundred revolutions per minute under a jet of cold water. The medium solidifies as a thin film on the inside periphery of the bottle, and any organisms in the inoculum will develop into colonies on incubation. In order to facilitate counting, the bottle is placed in a cylindrical holder mounted in a sloping position at the top of a chamber containing two small electric light bulbs. The bottle is illuminated through two slits on the under side of the cylindrical holder and the colonies are viewed through a 'window' on the upper side.

Glucose agar was prepared as a basal medium; it had the following composition: peptone, 10 g.; NaCl, 5 g.; glucose, 5 g.; Yeastrel, 3 g.; K_2HPO_4, 5 g.; KH_2PO_4, 2 g.; New Zealand agar, 15 g.; distilled water, 1000 ml. Different concentrations of sodium azide were incorporated in this medium as required, and in the earlier experiments 0·004 % neutral red was added as an indicator. When conducting experiments to find the proportion of viable cells which were able to grow on the azide medium under different conditions pure cultures were used and the total viable count was determined by inoculating the basal medium and counting the colonies after incubation at 37° C. Counts were usually made in triplicate.

Effect of temperature of incubation and of presence of azide on the colony count

Broth cultures of six strains of *Str. faecalis* were incubated at 37° C. After 6 hr., and again after 3 days, viable counts of these cultures were made on the basal medium and on the same medium containing 0·005, 0·010, or 0·015 % of azide. Counts of colonies which developed after incubation of the media at 37° C. were compared with the counts on the same media incubated at 45° C.

In the absence of azide, temperature had little, if any, effect on the count of the 6 hr. cultures, but a considerable effect on the count of the 3-day cultures. Less than 50 % of the cells of four of the six strains tested were able to grow at 45° C., and with one strain the proportion was less than 5 %. The inhibitory effect of azide was also much more marked with the older cultures.

Effect of suspension in dilute mineral salts solutions on proportion of cells able to grow on glucose-azide agar

Preliminary tests with dilute suspensions of *Str. faecalis* in ¼-strength Ringer's solution showed that the proportion of cells able to grow on glucose agar containing 0·2 % sodium azide and incubated at 45° C. depended on the age of the culture from which the suspension was prepared and on the period for which the cells had been immersed in the liquid. With a comparatively young culture most of the cells immersed for periods up to 24 hr. were able to form colonies; with older cultures the proportion was much lower, ranging from 13 to 52 % after different periods of immersion.

Table 1. *Percentage of total viable population of* Streptococcus faecalis, *as estimated by the colony count on glucose agar at* 37° C., *which was able to form colonies on glucose-azide agar at* 45° C. *after varying periods of suspension in* 0·003 M-*phosphate buffer containing different concentrations of organic matter*

Period of suspension (hr.)	Concentration of organic matter (p.p.m. glucose broth)			
	0	2·5	25	250
	Strain W 17			
0	93	95	95	90
4½	83	95	101	94
24	51	48	72	95
48	11	13	33	100
72	14	8	19	80
120	—	—	6	76
	Strain W 30			
0	77	91	94	80
4½	58	74	57	84
24	3·7	49	38	87
48	—	14	<4	16
72	—	16	—	4·7

Results of tests made with suspensions of *Str. faecalis* in 0·003M-phosphate buffer are shown in Table 1. A suspension free from organic matter was prepared from a suitable dilution of a 24 hr. broth culture by repeated centrifuging and re-suspension of the deposit in buffer solution. Colony counts of this suspension were made at intervals during a prolonged period of incubation at 20° C. The proportion of viable cells which were able to form colonies on the azide medium at 45° C. decreased very considerably as the period of immersion in the liquid was prolonged.

The effect on this process of attenuation of immersed cells of the presence of organic matter in the suspending fluid was studied by adding to similar suspensions in dilute buffer solution concentrations of glucose broth ranging from 2·5 to 250 p.p.m. With these additions a larger proportion of cells was able to grow on glucose-azide agar at 45° C. than was the case with the suspension to which no organic matter had been added, the effect being particularly marked with the

highest concentration, 250 p.p.m. of glucose broth tested. This was equivalent to a concentration of 10 p.p.m. of the combined organic constituents—glucose, peptone, yeast extract, and Lemco.

Effect of neutral red on the colony count

Tests with 24 hr. broth cultures of pure strains of *Str. faecalis* suggested that the inhibitory effect of concentrations of neutral red in the neighbourhood of 0·002–0·004 % was very slight. In the course of a large number of examinations of sewage and river water, however, it was apparent that with most samples the inhibitory action was appreciable. Colony counts on glucose azide agar containing neutral red were nearly always much lower than the 'Most Probable Number' (M.P.N.) in glucose azide broth containing brom-cresol purple as indicator.

With three samples of sewage and twelve samples of river water counts of colonies on glucose azide agar with and without neutral red were compared (Table 2). Omission of the indicator multiplied the count several fold, the factor ranging from 2·4 to 62, with an average of 12.

Table 2. *Effect of neutral red* (0·0025 %) *on colony counts* (*per ml.*) *of* Streptococcus faecalis *in samples of sewage and of river water. Colonies were counted on glucose azide agar after incubation of the medium at* 45° C.

		Colony counts				Colony counts	
Sample no.	Nature of sample	With neutral red	Without neutral red	Sample no.	Nature of sample	With neutral red	Without neutral red
1	Sewage	210	4500	9	River water	13	59
2	Sewage	600	4540	10	River water	47	170
3	Sewage	210	1640	11	River water	2	93
4	River water	18	150	12	River water	32	85
5	River water	6	154	13	River water	35	84
6	River water	23	110	14	River water	3	30
7	River water	26	75	15	River water	2	19
8	River water	9	65	—	—	—	—

Effect of 'resuscitation' on proportion of cells able to grow on azide medium at 45° C.

Allen *et al.* (1952) found that when a culture of *Bact. coli* was suspended in very dilute mineral salts solution the proportion of cells which was able to form colonies on bile salts-lactose agar at 44° C. decreased as the period of suspension was prolonged. The colony count therefore often indicated only a fraction of the total number of cells of *Bact. coli* present. If, however, the bile salts medium was divided into two portions, and the inoculum was incubated with lactose broth for 1 hr. at 37° C. before the bile salts and agar were added, the cells weakened by prolonged immersion were largely resuscitated and a larger proportion was now able to form colonies when the agar medium was incubated at 44° C. With the strain of *Bact. coli* tested this preliminary resuscitation also largely removed the sensitiveness of the organism to neutral red in the medium.

A similar process of resuscitation was applied to *Str. faecalis*. The glucose-azide agar was divided into two portions, each of double strength; the first portion was a broth containing the glucose, yeast extract, and peptone, and the second portion contained the agar and the azide. Preliminary tests showed that the inhibitory effect of neutral red was so pronounced, even after the inoculum had been resuscitated, that it would be advisable to omit this indicator from the medium. Brom-cresol purple, litmus, and brom-thymol blue proved to be unsuitable indicators for demonstrating acid-forming colonies in a thin layer of a solid medium. A large area of the medium around the colony or, if the colonies were at all numerous, the whole medium in the bottle, changed colour. The experiments were therefore continued with a medium containing no indicator.

A thin suspension of *Str. faecalis* in 0·003 M-phosphate buffer (pH 6·0) was prepared by adding 10 parts of a 48 hr. broth culture to 1 million parts of buffer; the suspension was incubated at 20° C. At intervals, during a period of incubation of 5 days, the total viable count of the suspension was determined by counting the colonies which developed on glucose agar incubated at 37° C. On each occasion 60 ml. of an appropriate dilution of the suspension were then added to 150 ml. of double-strength glucose broth and the mixture was incubated at 37° C. Volumes of 3·5 ml. of this mixture were abstracted immediately, after 1 hr., and after 2 hr., and were added to volumes of 2·5 ml. of double-strength azide agar in the small bottles; colonies were counted after spinning the bottles and incubating them at 45° C. The concentration of sodium azide in the mixed medium after addition of the inoculum was 0·02 %, and each bottle contained the equivalent of 1 ml. of inoculum mixed with 5 ml. of medium of normal strength.

Table 3. *Effect of preliminary resuscitation in glucose broth on the proportion of viable cells in a suspension of* Streptococcus faecalis *in dilute phosphate buffer which were able to form colonies on glucose azide agar incubated at* 45° C. *The total viable count was made on glucose agar incubated at* 37° C. *Each figure in the table shows the colony count on the azide medium at* 45° C. *expressed as a percentage of the total viable count*

Period of resuscitation (hr.)	Period of suspension in dilute buffer (hr.)						Average for all periods of suspension
	0	24	48	72	96	120	
0	75	81	30	54	17	17	47
1	80	81	59	103	57	85	78
2	117	89	83	103	62	108	94

Results (Table 3) show that with this strain of *Str. faecalis* an average colony count on glucose-azide agar incubated at 45° C., which approximated to the average total viable count, was obtained by first subjecting the inoculum to a period of resuscitation of 2 hr.

Before recommending the method for general use tests were made to see how generally this finding applied to other strains of *Str. faecalis* and to what extent the medium would inhibit the growth of other organisms when used for samples containing a mixed flora. Tests with five other strains of *Str. faecalis* showed that

resuscitation for 2 hr. of a broth culture which had not been suspended in water usually resulted in appreciable growth and that proliferation occurred with some strains after they had been suspended in water for as long as 24 hr.

Samples such as river water and sewage effluent contain organisms in varying conditions of viability; these organisms will vary in their response to resuscitation. Comparative tests were therefore made with twenty-six samples of river water and thirty-two samples of sewage and sewage effluent to see whether, on the average,

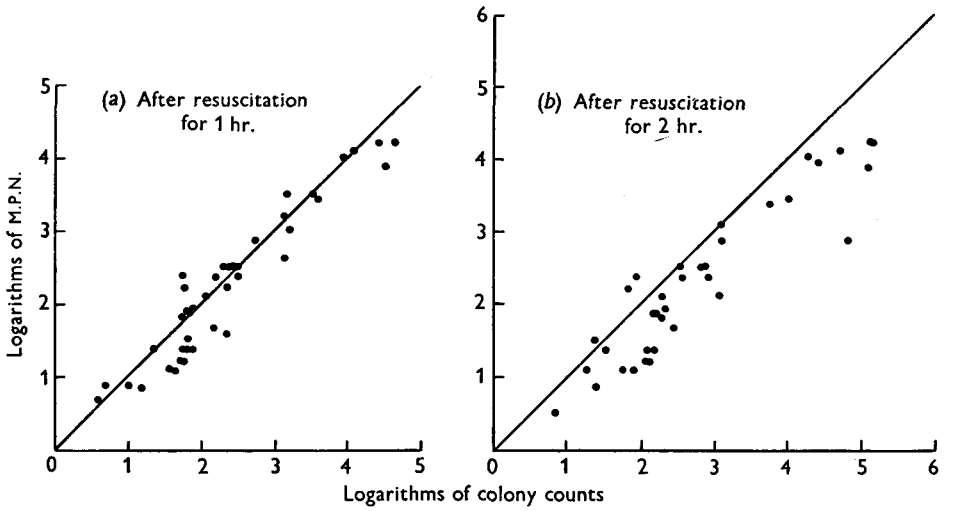

Fig. 1. Colony counts of *Str. faecalis* after resuscitation for 1 and 2 hr. compared with M.P.N. obtained by primary incubation of inoculated tubes of glucose azide broth at 37° C. followed by subculturing positive tubes to tubes of the same medium incubated at 45° C.

a period of resuscitation of 1 or 2 hr. gave the more accurate count. In such samples, which contain a mixed flora, the total viable count of *Str. faecalis* cannot be determined by counting the colonies on glucose agar incubated at 37° C. A measure of the total count was, however, obtained from the M.P.N., as estimated in glucose azide broth by each of the following two methods: (1) The 'subculture' method. Tubes of glucose-azide broth are inoculated and incubated at 37° C. for 24–48 hr.; positive tubes are then subcultured to fresh tubes of the same medium incubated at 45° C. (2) The 'resuscitation' method described by Childs & Allen (1953). The inocula are added to double-strength glucose broth and the mixture is held at 37° C. for 2 hr.; the calculated quantity of a solution of sodium azide is then added and the tubes are incubated at 45° C.

Results were compared by plotting the logarithms of the colony counts against the logarithms of the corresponding M.P.N. (Figs. 1 and 2). Colony counts, after resuscitation for 1 hr., were comparable with the M.P.N. obtained either by the subculture or by the resuscitation method. Colony counts, after resuscitation for 2 hr., were almost always higher, and often much higher, than the M.P.N. obtained by either method. The evidence therefore suggests that resuscitation for 2 hr. may, with many samples, allow appreciable growth to take place so that the resulting

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colony counts are too high. For this reason, and because the counts are then more comparable with the M.P.N., preliminary resuscitation for only 1 hr. is recommended.

Effect of phosphate on the colony count

Colony counts of *Bact. coli* on bile salts-lactose agar have been found by Allen *et al.* (1952) to be much reduced by the presence of phosphate in the medium, growth of some strains being virtually inhibited. Tests were therefore made to determine the effect of phosphate on the count of *Str. faecalis* on glucose-azide agar.

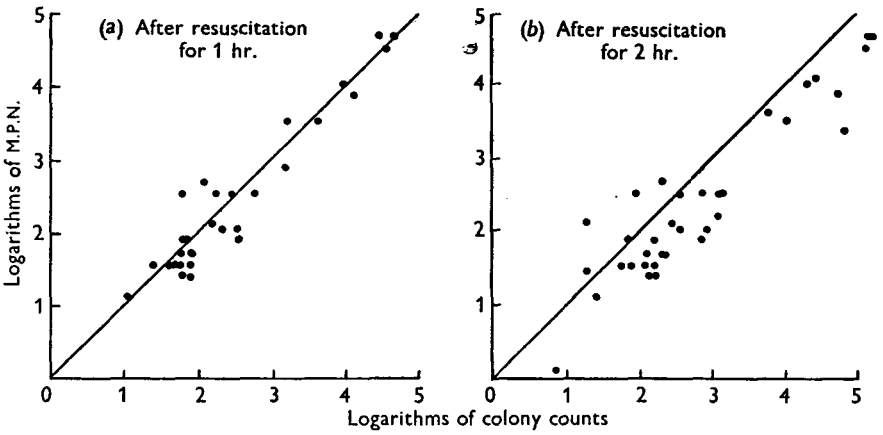

Fig. 2. Colony counts of *Str. faecalis* after resuscitation for 1 and 2 hr. compared with M.P.N. obtained after preliminary resuscitation for 2 hr.

Colony counts of nine samples of sewage and sewage effluent were made, using this medium incubated at 45° C. after preliminary resuscitation of the inoculum for 1 hr. at 37° C. The phosphate was added both to the broth portion of the medium, containing peptone, yeast extract, and sodium chloride, and to the agar portion, containing agar and sodium chloride, and these portions were sterilized in the autoclave; solutions of glucose and of azide were sterilized and added separately. Colony counts of the same samples were also made on a similar medium from which the phosphate had been omitted. Colonies on the latter medium were found to be smaller than those growing in the presence of phosphate, some being at the limit of vision and making accurate counting difficult. Results (Table 4) indicated, however, that the presence of phosphate prevented a number of cells from growing into colonies, although subsequently it encouraged those which did grow to form larger colonies.

If phosphate was omitted from both portions of the medium before being autoclaved and if a suitable amount of a separately sterilized solution was added subsequently, counts were much higher than corresponding counts on the medium in which phosphate was included before sterilization (Table 5). Calculation from the figures in Table 4 shows that on the average omission of the phosphate increased the count by 75 %. Similarly, results in Table 5 show that when the phosphate was added separately the count, on the average, was 72 % higher than when the

Table 4. *Effect of including phosphate in the medium on the colony counts (per ml.) of* Streptococcus faecalis *in samples of sewage and sewage effluent. Colonies were counted on glucose azide agar after incubation at* 45° C.

Sample no.	With phosphate	Without phosphate
1	36,000	93,000
2	4,500	7,800
3	296	580
4	42,000	77,000
5	12,900	16,600
6	11	15
7	50,000	80,000
8	8,300	13,600
9	28	56

Table 5. *Effect of phosphate in the medium (a) when autoclaved with other constituents, and (b) when added separately, on the colony counts (per ml.) of* Streptococcus faecalis *in samples of sewage and sewage effluent. Colonies were counted on glucose azide agar incubated at* 45° C.

Sample no.	Phosphate included before autoclaving	Phosphate added separately
1	6,500	8,200
2	8,700	14,300
3	6,600	16,300
4	6,600	15,200
5	88	146
6	47,000	75,000
7	6,100	9,800
8	174	221

phosphate was included before autoclaving. When added separately, therefore, phosphate exerts no depressing effect on the colony count. By increasing the size of the colonies the phosphate makes counting much easier than when it is omitted from the medium.

The phosphate solution was sterilized and added separately in the method finally adopted for obtaining a colony count. The details of this method are given in the Appendix.

Selectivity of method for Streptococcus faecalis

From time to time throughout the investigation pure strains were isolated from the colonies being counted and were examined for their morphology, for their action on litmus milk, and for their reaction to the four tolerance tests, namely growth at 45° C., growth in broth containing 6·5 % sodium chloride, growth in broth at pH 9·6, and survival after heating at 60° C. for 30 min.; the technique of these tests has been described by Shattock & Mattick (1943) and by Shattock & Hirsch (1947). All fifty-five strains tested were Gram-positive cocci in pairs and short chains, which clotted litmus milk. With the exception of one strain, which failed to grow at pH 9·6, all strains passed the four tolerance tests. The method therefore appeared to permit the growth only of *Str. faecalis* when used for samples containing a mixed flora.

SUMMARY

Factors affecting the growth of *Streptococcus faecalis* on glucose-yeast extract-sodium azide agar have been studied. Both the high temperature of incubation used (45° C.) and the presence of azide reduced the proportion of cells able to form colonies, the inhibitory action being much more marked with cultures which had become attenuated, either through age or through prolonged immersion in water, than with comparatively young and vigorous cultures. This inhibitory action was found to be largely overcome if the inoculum was subjected to a preliminary period of 'resuscitation', by incubating it with double-strength glucose broth before adding the azide-agar portion of the medium and allowing the mixture to set.

Neutral red was so inhibitory to some strains of *Str. faecalis* that it could not be included in the medium. Phosphate, as the potassium salt at a concentration of 0·7 %, if autoclaved with the remaining constituents of the medium, exerted a depressing effect on the counts. Added separately it showed no inhibitory action.

The spinning-bottle technique (Allen *et al.* 1952) was adapted for *Str. faecalis*. When used for samples containing a mixed flora the method, described in the Appendix, permitted the growth only of *Str. faecalis*.

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APPENDIX

Methods of determining colony counts after preliminary resuscitation

(1) *Double-strength broth*

Peptone, 20 g.; Yeastrel, 6 g.; NaCl, 5 g.; distilled water, 733 ml. Sterilize at 15 lb. for 20 min.

Fill into bottles in 160 ml. quantities. Just before use add 20 ml. of 10 % glucose solution and 13·6 ml. of 10 % phosphate solution (containing 2·86 g. KH_2PO_4 and 7·14 g. K_2HPO_4 per 100 ml.) which have been separately sterilized.

(2) *Double-strength agar*

NaCl, 5 g.; New Zealand agar, 30 g.; distilled water, 733 ml. Sterilize at 15 lb. for 20 min.

Fill into bottles in 80 ml. quantities. Just before use add 6 ml. 0·72 % sodium azide solution and 6·5 ml. 10 % phosphate solution, which have been separately sterilized.

Procedure

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° C. 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° C. 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

* In larger bottles inocula up to 5 ml. were used. Quantities of broth and of agar media were increased proportionately and the agar portion of the medium was made up with 45 g. instead of 30 g. agar. This was found to be necessary to ensure firm adherence of the film to the surface of the larger bottle.

agar medium have been added several minutes previously and which have been maintained at 48–50° C. After rotating, the bottles are incubated in a 45° C. (± 0·2°) water-bath for 40–48 hr.

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