FURTHER OBSERVATIONS ON THE DIFFERENTIATION OF LACTOSE-FERMENTING BACILLI, WITH SPECIAL REFERENCE TO THOSE OF INTESTINAL ORIGIN.

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In a previous paper (1906) I entered a plea for the use of certain tests in the bacteriological examination of waters and food stuffs as it seemed we should thus be enabled to better differentiate the lactose-fermenting bacilli. Such differentiation seems necessary because of the importance attached to *B. coli* as an indicator of faecal contamination, and because of the differences of opinion which exist as to the characters which justify us in identifying a bacillus as *B. coli.*

Thus according to Savage (1906, p. 77) the English Committee appointed to consider the Standardisation of Methods for the Bacterioscopic Examination of Water defined *B. coli* as:

> "A small, motile, non-sporing bacillus, growing at 37° C. as well as at room temperature. The motility is well observed in a young culture in a fluid glucose medium. It is decolorised by Gram's method of staining. It never liquefies gelatine, and the gelatine cultures should be kept at least 10 days in order to exclude a liquefying bacillus. It forms smooth thin surface growths and colonies on gelatine, non-corrugated, growing well to the bottom of the stab (facultative anaerobe). It produces permanent acidity in milk, which is clotted within 7 days at 37° C. It ferments glucose and lactose, with the production of both, acid and gas. The typical bacillus coli must conform to the above description and tests. It generally also forms indole, gives a thick yellowish brown growth on potato (greatly dependent on the character of the potato), sometimes ferments saccharose (about 50 %), changes neutral red (Grübler's) and reduces nitrates, and half the gas produced by it from glucose is absorbable by KOH; and these tests, if time and opportunity permit, may be performed in addition to the foregoing";

and according to Prescott and Winslow (1908, p. 103) the Committee on Standard Methods of Water Analysis of the American Public Health

Association in 1905 drew the following set of diagnostic characters for *B. coli*:

"(1) Typical morphology—non-sporing bacillus, relatively small and often quite thick.

(2) Motility—when a young broth or gelatine culture is examined.

(3) Fermentation of dextrose broth, with the formation of about 50 % of gas, of which about one-third (CO_2) is absorbed by a 2 % solution of sodium hydrate.

(4) Coagulation of milk, with the production of acid, in 48 hours or more at 37° C., either spontaneously or upon boiling.

(5) Non-liquefaction of gelatine.

(6) Production of indole in peptone solution.

(7) Reduction of nitrates."

These two descriptions differ in the importance they attach to the various characters: the American Committee insisting upon the indole and nitrate tests, while the English Committee consider that it is only necessary to perform these tests "if time and opportunity permit." The characters about which they appear to be in accord are: Morphology, motility, the fermentation of dextrose and lactose, the production of acid and clot in milk and the non-liquefaction of gelatine. But even these few have not all been accepted as absolutely necessary; for Prescott and Winslow (1906, p. 104) and Savage (1906, pp. 154 and 157) think that motility is not always present and that its absence is of no significance. There are also differences of opinion with regard to the value of the appearance of the growth on gelatine and on agar.

It seems therefore advisable to go into the question of the weight which each character should be allowed to carry.

Tests in common use for differentiation purposes.

Morphology.

B. coli is described as a short bacillus with rounded ends but all sizes may be seen in the same culture.

Buxton (1902, p. 201), speaking of the group of organisms intermediate between *B. coli* and *B. typhosus*, says "Morphologically the intermediates cannot be distinguished among themselves nor with any degree of certainty from *B. coli communis* or *B. typhosus*." Durham (1900—1901, p. 354) says "Speaking generally, morphological characters are not of much value for subdivision of these bacteria" (*B. typhosus*, *B. coli*, etc.). Horrocks (1903, p. 369) describes the morphology of his six groups in the same terms. As his groups include *B. coli*, *B. lactis aerogenes* and

others it is obvious he did not find morphology of much differential value. Barber's (1907) studies "On Heredity in certain micro-organisms" also show how much *B. coli* can vary in its morphology. I have several times examined carefully 50—100 film preparations, one after the other, and tried to classify them according to the size and shape of the bacilli, but I have never yet succeeded in separating even one variety from all the others. I am consequently of opinion that no one would reject an organism as *B. coli* simply on account of an "atypical" morphology.

Motility.

It is very difficult to arrive at a conclusion with regard to this character. There is no doubt that an organism may be actively motile in a young (say a 6 hours' broth) culture and yet be quite immobile when examined in a drop of the same culture 12—18 hours later. Sometimes dilution has the effect of starting movements in an apparently non-motile culture. An organism may also be motile when grown at 37° C. or vice versa. One would then be inclined to say that this character varies so much that it can carry no weight one way or the other; and yet no one would accept a motile bacillus as *B. lactis aerogenes* or *B. pneumoniae* (Friedländer). If we allow a value to this character in one case can we consistently deny it a value in a closely allied case. May it not be simply want of knowledge which prevents us forming a just appreciation of it, and would it not be as well to observe this character in each case until we can form a definite opinion about it. A drop of a 6 hours' broth culture placed on an ordinary slide without coverslip and examined, under dark ground illumination, with a $\frac{1}{2}$ inch objective and a × 8 eyepiece will give an excellent idea of the power of movement possessed by an organism. The drop can then be spread, dried and stained in the ordinary way. It seems a pity not to make this observation when it entails so little labour and may eventually prove a necessity.

Broth, bloodserum, agar, potato.

The general opinion is that these media have no value for differentiation purposes.

Litmus milk.

Almost every lactose-fermenter I have tested has produced acid and clot in milk when grown for a sufficient time. All have produced

enough acid to cause coagulation on boiling the milk. This medium is used in routine work not for the investigation of enzyme action but merely to observe the production of acid from lactose. Therefore when a pure lactose medium is used it is unnecessary to use milk as well. If milk be used it must according to Biffi (1906) be sterilised always at the same temperature as many bacteria coagulate it when sterilised at 100° C. which will not coagulate it when sterilised at a higher temperature.

Production of acid in litmus whey.

This test has not come into general use, and rightly so in my opinion, as the small amount of information obtained from it is far from being commensurate with the trouble entailed in the production of the medium and the performance of the test.

Production of fluorescence in neutral red media.

So many organisms give this reaction that the tendency is not to lay any stress upon it.

Reduction of nitrites.

This also appears to be a property common to a large number of organisms and no differential value is attached to it. (Savage, 1906, p. 80—Horrocks, 1903, p. 364—Gruber, Th. 1906, p. 656.) I tested some 70 lactose fermenters and non-lactose fermenters and found that all produced nitrites from nitrates as evidenced by Ilosvay's reagent.

Nutrient gelatine.

The appearance of slope or stab cultures depends so much upon the quantity and kind of material used for inoculation that it is difficult to understand how these can be preferred to plate cultures. In the case of the latter the presumption is that each colony starts from a single organism and therefore if there is plenty of room for development any characteristic appearance should be more evident in a colony than in a streak or a stab culture. But the colonies of an organism may vary, even on the same plate, and organisms with different characters may grow alike in colony form (Savage, 1904, p. 358, 1906, p. 81—Horrocks, 1903, p. 369—Radzievsky, 1900, p. 369—von Freudenreich, 1904, p. 408—Leichmann, 1899—Löhnis, 1907, pp. 114 and 115—Gruber, 1906, pp. 655

and 719). Klein (1899—1900, p. 373) says "it is not safe from mere appearances on gelatine to regard particular colonies as those of *B. coli* or its varieties. Such colonies cannot without animal experiment be declared not to be the bacillus of pseudo tuberculosis. Moreover they may be neither *B. coli*, nor its varieties, nor the bacillus of pseudo tuberculosis."

Zlatogoroff (1904, p. 520) thinks that the colonies of *B. pestis* on gelatine resemble those of *B. coli*.

Klein and Houston (1899—1900, p. 601) describe a bacillus which gave typical coli-like colonies on gelatine and appeared in every way typical of *B. coli* except that it did not clot milk and liquefied gelatine after some weeks.

Con and Esten (1904 a) after describing the appearance of the colonies of the *B. lactis aerogenes* group on milk-whey-peptone-gelatine (reaction +15 to phenolphthalein) say that this group includes several different species. There are at least four different types. Some of the colonies prove to be *B. coli communis*; some to be cocci instead of rods; others to be *B. lactis aerogenes* except that they do not ferment milk sugar; and in a few cases these colonies prove to be different from any of the foregoing. The same writers in another communication (1904 b) state that *B. lactis aerogenes* and *B. coli communis* produce colonies very similar to each other.

Houston (1904, p. 105) says "The picking out of the coli-like colonies for study in pure culture is after all, even to the expert, a speculative venture."

Longley and Baton (1907) in a paper on the determination of *B. coli* in water say "On account of the insignificant value of the test for liquefaction of gelatine in the examination of the Potomac water.....it may hereafter be omitted from our routine examinations without introducing any appreciable error."

My own experience is that if a pure culture of an organism be plated it is not at all uncommon to find colonies of more than one kind on the same plate, and that it is useless to attach to "typical" gelatine growths more than a confirmatory value. The real value of a gelatine culture lies in the information it affords us of the power of the organism to liquefy gelatine. Even this value is discounted by the fact that some of these organisms liquefy very slowly—too slowly for this character to help us in routine work.

Production of indole.

Opinions seem very much divided regarding the value of this test. On going through the literature one very frequently meets with the statement that the power of producing indole is a variable character. Most bacteriologists would agree that a typical *B. coli* should produce indole in peptone water and yet few would state definitely that they would reject a bacillus which differed from a typical *B. coli* only in failing to produce indole. It seems to me that this conflict of opinion in part arises from the use of the sulphuric acid and nitrite test, which without doubt does give varying results. On the other hand, if Ehrlich's test (Böhme, 1905, Marshall, 1907, Steensma, 1906) be used it is rare to find a second test yield a result different to the first. When using this test it is best to use cultures which have been grown for 6—7 days (2 or 3 days is not long enough). Then unless there is a distinct red colour produced it is advisable to shake up the culture with amyl alcohol and extract the colouring matter. On more than one occasion I have noticed a faint pink colour appear after the addition of the test reagents, and I have put down the reaction as positive, but on shaking up with amyl alcohol the pink has entirely disappeared—neither the culture nor the alcohol showing the least trace of it. And on the other hand, cultures which did not show any colour after the addition of the test reagents yielded a pale pink to the amyl alcohol.

If these precautions are taken this test seems to be reliable. One cubic centimetre of each solution is sufficient for a test, and if a red colour appears on the addition of the benzaldehyde it is unnecessary to add the potash.

Cultures in which a deep red colour has appeared often show a peculiar result when treated with amyl alcohol. If such a red culture be shaken up with a small quantity of amyl alcohol some of the red colour will be extracted by the spirit. If the coloured spirit be pipetted off, more alcohol added and the shaking up repeated, more of the colour will be removed, and if this process be repeated several times it will be found that the culture has lost its distinct red and become bluish or violet and sometimes almost black.

Fermentation tests.

Glucose and lactose are the only fermentable substances in common use for the differentiation purposes we have in view. *B. coli* is

acknowledged by all to decompose both these sugars with the production of acid and gas. Further tests of this kind seem to be considered unnecessary, and Savage (1906, p. 83) may be said to voice the general opinion when after giving a definition of *B. coli* he says: "Organisms with all the above characters whether they ferment saccharose, dulcit, etc. or not can all be spoken of as *B. coli.*"

We have now considered all the tests which are usually employed for the purpose of differentiating *B. coli* from other organisms. It remains to see how far these tests will help us in connection with organisms isolated in the present research.

Examination of 497 *lactose fermenting bacilli isolated from various sources.*

In all 76 samples have been studied, the origin of which was as follows:

Human faeces	20 samples	Rain water	1 samples
Human sputum	9 „	Roof washings	2 „
Human pus	1 „	Oats	1 „
Horse faeces	11 „	Crushed oats	1 „
Calf faeces	7 „	Beans	1 „
Goat faeces	2 „	Ear of corn	1 „
Goose faeces	6 „	Bran	1 „
Pig faeces	1 „	Old hay	1 „
Cesspool sewage	1 „	Malt	1 „
Soil	2 „	Baker's yeast	1 „
Pond water	4 „	Cheese (Coulommier)	1 „

The

"Human pus" was the purulent discharge from the operation wound in a case of appendicitis.

"Pond water" was turbid water taken from a pond in the Institute grounds. It receives the washing of a heavily manured flower border and contains hundreds of gold fish.

"Rain water" was rain water caught in a sterile funnel placed in a sterile flask and left out all one night in a field during rainy weather.

"Roof washings" were rain water collected as it flowed from the rain water pipe of a cow shed in an open field.

"Crushed oats"—"Bran." These were samples taken from the bin in which they were stored for feeding horses.

"Oats"—"Beans" were samples of whole oats and beans collected with little

risk of faecal contamination, and for which I desire to thank Prof. John Percival, Director, Agricultural College, Reading.

"Old hay" was from the interior of a truss cut from a stack two years old.

"Malt" had been kept in a tin box in the laboratory for quite three years.

"Ear of corn" was picked from a hedge bordering a narrow country lane. It hung about 6 feet from the ground. There had been no rain for about 3 weeks, the dust in the lane was about 1 inch deep and the hedges were covered with it. The ear was dropped into a tube of ordinary nutrient bouillon, incubated over night at 37° C. and plated on bile salt lactose agar.

The method of examination was to plate out either direct from the sample or after preliminary incubation in a liquid medium. Where possible bile salt media (MacConkey, 1908) have been employed because of their inhibiting effect upon many organisms of the air, soil and water, the presence of which would have materially increased the amount of labour necessary for the prosecution of the work.

Cultures were made from single colonies, and these presumably pure cultures were subjected to the tests named. That it is necessary to remember that colonies may be composed of more than one variety of organism was emphasized by the fact that some of these cultures were not pure and had to be replated. The results are given in Tables I and II (pp. 94, 95).

A word of explanation is necessary with regard to the numbers attached to the bacilli in the tables. In previous papers the lactose fermenting bacilli were divided into 4 groups according to the fermentative action on saccharose and dulcit. Thus:

Group	I	contained	bacilli	which	were	saccharose	−	dulcit	−
„	II	„	„	„	„	„	−	„	+
„	III	„	„	„	„	„	+	„	+
„	IV	„	„	„	„	„	+	„	−

If we add, as further tests, the action on adonit and inulin the presence or absence of motility, of indole and of Vosges and Proskauer's reaction, it is possible to form 128 combinations, or to put it another way, we might isolate 128 varieties of lactose fermenting bacilli. But only about $\frac{1}{4}$ of this number have been met with, and so in order to allow for the correct placing of other bacilli 32 numbers have been assigned to each group.

If we study Table I in the light of what has gone before we note that

(1) all these bacilli ferment lactose with the production of acid and gas,

TABLE I. *Characters of the bacilli isolated.*

	No.		Lactose	Litmus milk	Gelatine	Gram's stain	Motility	Indole	Reduction of nitrates	Acidity in litmus whey	Saccharose	Dulcit	Adonit	Inulin	Inosit	Voges and Proskauer's reaction	Remarks
Group I, Nos. 1–32	1	1	+	+	−	−	+	+	+	23 %	−	−	−	−	−	−	+ = acid and gas, acid and clot, liquefaction of gelatine etc. as the case may be.
	2	1	+	+	+	−	−	+	+	14 %	−	−	+	−	−	+	⊥ = acid without gas.
	3	1	+	+	−	−	+	−	+	20 %	−	−	−	−	−	−	− = no production of either acid or gas.
	4	1	+	+	−	−	+	+	+	25 %	−	−	−	−	+	−	*Inosit.* This substance was only used in certain special cases. Nos. 104, 108, 109 gave only slight acid in most cases.
	5	1	+	+	−	−	−	+	−		−	−	−	−	−	−	
	6		+	+	−	−	−	+	−		−	−	−	−	−	−	
	7		+	+	−	−	+	−	+		−	−	−	−	−	+	
	8	1	+	+	−	−	−	−	−		−	+	+	−	−	−	*Gelatine.* In most cases the liquefaction of gelatine took place very slowly. It is possible that some of those put down as non-liquefiers might have proved themselves liquefiers if they had been kept long enough. All were kept for 2—3 months. Some developed a yellow tint. These were always white at first.
Group II, Nos. 33–64	33	1	+	+	−	−	+	+	+	28 %	−	+	+	−	−	−	
	34	1	+	+	−	−	−	+	+	22 %	−	+	+	−	−	−	
	35	1	+	+	−	−	−	+	+		−	+	+	−	−	−	
	36		+	+	+	−	+	−	+		+	+	+	−	−	−	
	65	1	+	+	+	−	−	+	+	26 %	+	+	+	−	−	+	
	66		+	+	+	−	−	+	+		+	+	+	−	−	+	
	67		+	+	+	−	−	−	+		+	+	+	−	−	−	
	68	1	+	+	−	−	−	−	+	14 %	+	+	+	−	−	−	
	69		+	+	+	−	+	−	+		+	+	+	−	−	−	
	70		+	+	+	−	+	−	+		+	+	+	−	−	−	
	71		+	+	−	−	+	+	−		+	+	+	−	−	−	
	72	1	+	+	−	−	−	+	+	22 %	+	+	+	−	−	−	
	73		+	+	−	−	+	−	+		+	+	+	−	−	−	
	74		+	+	−	−	+	−	−		+	+	+	−	−	−	
	75		+	+	−	−	−	−	−		+	+	+	−	−	−	
Group III, Nos. 65–96	97		+	+	+	−	−	+	+		+	−	+	+	−	+	*Litmus whey.* The cultures were grown for 3 days at 37° C. and were then tested with $\frac{n}{10}$ NaOH. The percentages are given in terms of $\frac{n}{10}$ NaOH, and are meant to be merely average percentages. * = Both *B. Schafferi* and *B. gasoformans non-liquefaciens* produce usually only a small amount of gas.
	98		+	+	−	−	−	+	+		+	−	+	+	−	+	
	99		+	+	−	−	−	−	+		+	−	+	+	−	+	
	100		+	+	−	−	+	+	−		+	−	+	+	−	−	
	101		+	+	−	−	−	+	+		+	−	+	+	−	−	
	102		+	+	+	−	+	−	−		+	−	+	+	−	−	
	103	1	+	+	−	−	−	−	+	30 %	+	−	+	−	+	−	
Group IV, Nos. 97–128	104	1	+	+	−	−	−	−	+	12 %	+	−	−	−	⊥	±	
	105		+	+	+	−	−	+	+		+	−	−	+	−	+	
	106		+	+	−	−	+	+	−		+	−	−	−	−	−	
	107	1	+	+	−	−	−	+	+	25 %	+	−	−	−	−	−	
	108	1	+	+	+	−	+	−	+	20 %	+	−	−	−	⊥	+	
	109		+	+	−	−	−	−	−		+	−	−	−	⊥	−	

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TABLE II. *Distribution of the bacilli isolated.*

	No.		Human faeces	Human pus from appendicitis abscess	Human sputum	Horse faeces	Calf faeces	Goat faeces	Pig faeces	Goose faeces	Cesspool sewage	Soil	Pond water	Rain water	Roof washings	Crushed oats	Oats	Beans	Bran	Cheese	Bakers' yeast	Malt	Ear of corn	
Group I, Nos. 1–32	No. 1		9	—	—	3	2	2	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	21
	,, 2	*B. acidi lactici*	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 3	*B. levans*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0
	,, 4	*B. Grünthal, B. sulcatus gasoformans, B. castellus*	8	—	—	1	—	—	—	5	4	—	—	—	—	—	—	—	—	—	—	—	—	18
	,, 5	*B. vesiculosus*	33	5	1	—	—	—	3	1	—	1	—	—	—	—	—	—	—	3	—	—	1	48
	,, 6		—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 7		1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	2
	,, 8	*B. coli mutabilis* (Massini)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0
Group II, Nos. 33–64	,, 33		—	—	—	5	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	9
	,, 34	*B. coli communis, B. cavicida*	37	—	3	15	3	13	1	3	1	—	—	2†	—	4	—	—	—	—	—	—	—	82
	,, 35	*B. Schafferi*	11	—	—	1	—	—	3	—	—	—	—	—	—	—	—	—	—	1	—	—	—	16
	,, 36		1	—	—	1*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	3*	—	8
Group III, Nos. 65–96	,, 65	*B. oxytocus perniciosus*	1	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	3	—	—	—	8
	,, 66		—	—	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4
	,, 67		1	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
	,, 68	*B. rhinoscleroma, B. Friedländer*	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 69		—	—	—	3*	—	—	—	—	—	—	—	—	6*	—	1	—	—	—	—	—	—	10
	,, 70		—	—	—	—	—	—	—	—	—	—	—	1*	—	—	—	—	3	—	—	—	—	4
	,, 71		42	5	—	32	30	—	—	9	—	6	3	1	5	1	—	—	—	—	4	—	5	143
	,, 72	*B. neapolitanus*	15	—	1	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	17
	,, 73		—	—	—	1*	—	—	—	—	—	—	1*	—	7*	—	1*	3*	—	—	—	6*	5*	24
	,, 74		1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 75		—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	1
Group IV, Nos. 97–128	,, 97		1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 98		—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
	,, 99		—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
	,, 100		1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 101		1	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	5
	,, 102		—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	2
	,, 103	*B. lactis aerogenes, B. dysenteriae vitulorum, B. capsulatus*	8	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	15
	,, 104	*B. gasoformans non-liquefaciens*	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	2
	,, 105		—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	7	—	—	—	9
	,, 106		2	—	1	1	—	1	—	4	—	—	—	—	—	—	—	—	—	—	—	—	—	9
	,, 107	*B. coscoroba*	1	—	—	—	1	—	—	1	—	3	—	—	—	—	—	—	—	—	—	—	—	6
	,, 108	*B. cloacae*	—	—	—	2	—	—	—	—	—	1	5	3	3	—	—	—	—	—	—	—	—	14
	,, 109		2	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	2	3	—	—	9
			178	10	13	67	40	17	7	23	6	16	11	7	31	5	4	3	3	26	10	9	11	497

* Yellow liquefier. † Yellowish.

(2) they produce acid and clotting in milk,

(3) the majority do not liquefy gelatine, and those which do so liquefy so slowly that they would in routine work be classed as non-liquefiers,

(4) all are Gram-negative,

(5) the motility and indole tests are positive in some cases and negative in others,

(6) in all cases which have been tested nitrates were reduced to nitrites.

The morphology is not noted in the Table, because it proved not to be of any differential value.

Now, according to the tests usually employed, all these bacilli would be classed as *B. coli.* But if we kept them long enough we should find that in several cases gelatine was liquefied and we should be in the position of having classified a liquefying organism as *B. coli*, though all bacteriologists are agreed that *B. coli* is not a liquefying organism.

It has been suggested that the error from this cause is so small that it may be neglected, but an enumeration of the liquefiers in Table II shows that the error would be more than 10 % and therefore cannot be ignored. It follows that the present methods of differentiation are not adequate and that a change is necessary.

In a former paper I proposed that we should omit the observation of the (1) characters of the growth on gelatine, (2) action on milk, (3) action on glucose, (4) action on neutral red, and (5) the indole test, and that we should substitute for them (1) the action on dulcit, (2) the action on adonit, (3) the action on inulin and (4) Vosges and Proskauer's reaction.

Further experience has only confirmed me in this opinion except as regards the indole test and Vosges and Proskauer's reaction. With regard to the fermentation reactions it is necessary to remember that some bacilli act slowly and that, in consequence, we may be premature if we draw conclusions after the cultures have been incubated for only 24, 48 or even 72 hours. I have changed my opinion of the indole test because an extensive use of Ehrlich's reaction has shown me that this test cannot be omitted with safety.

Vosges and Proskauer's reaction.

I have used this reaction for some years, and it seemed to be reliable and valuable until at the end of 1907 I isolated organisms which gave sometimes a negative or doubtful reaction and sometimes a positive one.

Such results have weakened my faith in this test and yet I cannot say it is of no value as it certainly is not given by every organism. For instance, in 1905 I isolated from an ear of corn 11 lactose fermenting bacilli of which 10 agreed as regards motility and fermentation reactions. These differed in that 5 liquefied gelatine, gave Vosges and Proskauer's reaction and did not produce indole, while the other 5 did not liquefy gelatine, did not give Vosges and Proskauer's reaction but did produce indole. These cultures were kept and tested from time to time and always gave identical results until September 1908 when one of the non-indole-producers gave a positive indole reaction. This culture was at once plated and 6 colonies were subcultured and worked through the various tests. All were motile and all gave the same fermentation reactions as before, but 4 were indole negative and 2 were indole positive. The indole-negative culture gave Vosges and Proskauer's reaction, while the indole-positive ones did not. In this instance Vosges and Proskauer's reaction was of distinct value in confirming the presence of contamination.

No one of these tests is of much value by itself. They must be considered together, each one merely forming one link in the chain of evidence. An example may perhaps make my meaning clearer.

The *B. oxytocus perniciosus*, No. 65, Table I, would according to the usual tests be classed as a non-motile *B. coli*. But it liquefies gelatine, taking perhaps 8—12 months to liquefy ½ inch of the medium. So unless we use further tests we should classify this bacillus incorrectly. When however we study the series of tests given in Table I we observe that it

(1) gives a positive reactive with *all* the fermentable substances used,

(2) produces indole,

(3) is non-motile,

(4) gives Vosges and Proskauer's reaction.

If any reliance is to be placed upon these tests we ought to find that bacilli having these characters liquefy gelatine. This has in my experience proved to be the case.

I isolated from soil several organisms which were *B. oxytocus perniciosus* so far as was evidenced by fermentation reactions, indole and motility. But they appeared to be non-liquefiers. This led me to try what may be termed the "massive inoculation" test for liquefaction (MacConkey, 1906 a). A positive result proved that these bacilli were in truth *B. oxytocus perniciosus* and at the same time afforded evidence in favour of the value of this series of reactions.

One might multiply examples of this kind, but there seems to be no real advantage to be gained thereby. It will suffice to say that as regards *B. cloacae* these results have been confirmed by Ferreira, Horta and Paredes (1908).

Coming now to Table II we are struck by the distribution of the bacilli isolated. If all these organisms were the same bacillus we might reasonably expect that they should be distributed fairly evenly throughout the samples. But such is not the case.

The *B. levans* for instance is so rare that it has not been met with once in 497 bacilli, and yet *B. levans* has been stated to be identical with *B. coli* (Papasotiriu, 1902).

On the other hand 7 of the varieties given in Table I claim 87 % and 3 varieties 62 % of the 178 bacilli obtained from human faeces, etc., and of the 154 organisms with an origin in animal faeces 68 % belong to 2 varieties and 46 % to a single variety. If we consider the 497 bacilli as one whole we find that 54·9 % of them belong to one or other of 3 varieties. If all these bacilli are *B. coli* we must allow that certain varieties of this organism are very common in faeces and material exposed to faecal contamination, while certain other varieties are rare in faeces and more common in other materials.

This suggests that the term *B. coli* as at present used is not a happy one, it is too comprehensive, and it would be better to avoid using it. There can be little doubt that all these bacilli have already been isolated and partially described under some name, but I have found it impossible to obtain more than a few named cultures. For the others we must be content to use numbers until we find out the names by which they should properly be known.

I think I have now brought forward sufficient evidence to justify me in once more urging the adoption in routine work of the series of tests recommended in this paper.

Only two further points need be touched upon. Firstly objection may be taken to my classification on the ground that the fermentation reactions are not stable. Some workers, Klotz (1906), Reevis (1908), Twort (1907), seem to consider that these reactions are too inconstant to be of use in classification, but the work of Savage (1906), Horrocks (1903), p. 370, MacConkey (1905, p. 356, 1906 b, p. 399), Villinger (1894), and Benczur (1908), is evidence in favour of the reliability of these tests.

Nor must it be forgotten that this very kind of reaction is considered of great value in the differentiation of *B. coli*, *B. typhosus* and *B. enteritidis* (Gaertner). If they are looked upon as reliable enough for this purpose why should they not be equally of value in separating *B.*

coli from *B. lactis aerogenes* and similar organisms. To say that an organism which does not decompose lactose is not a *B. coli* and then to say that the decomposition or not of some other substance, say dulcit, is of no value in separating *B. coli* from another lactose fermenter is, to say the least, inconsistent.

But there is no need to labour this point. From a practical standpoint it matters not whether these organisms are different bacilli or one bacillus in various guises. If organisms isolated from a certain material (*A*) give in the majority of cases a certain series of reactions (a_1, a_2, a_3), and if organisms isolated from some other material (*B*) give in the majority of instances a different series of reactions (b_1, b_2, b_3) then we would be justified in associating the series (a_1, a_2, a_3) with the material (*A*) and the series (b_1, b_2, b_3) with the material (*B*); and if we found both the (a_1, a_2, a_3) series and the (b_1, b_2, b_3) series given by organisms isolated from (*B*) we could presume that some of (*A*) had become mixed with (*B*). It might be that both series of reactions were given by the same organism and that the difference was simply the effect of environment. This would not affect the conclusion. It would only show that the organism had not been long enough in (*B*) to have its (a_1, a_2, a_3) series altered by the changed environment. Secondly it may be asked: "Why limit oneself to these few reactions? Why not use more fermentable substances and have still better differentiation?" I can only answer that I have not found any real advantage in so doing.

We all of us always wish to identify organisms as accurately as possible, in as short a time as possible, and with as little trouble as possible. This desire with regard to time and trouble is intensified when it is a question of routine work. The exigencies of this class of work must always be borne in mind. With this end in view I have given a thorough trial to the following substances:

Glucose—laevulose—galactose—lactose—maltose—mannose—arabinose—raffinose—saccharose—mannit—dulcit—adonit—quercit—erythrit—inosit—sorbit—glycerine—arbutin—salicin—amygdalin—α-methyl-glucoside—inulin—dextrin—and starch;

and I have come to the conclusion that for the purpose of differentiating the lactose fermenters we can be content to use lactose, saccharose, dulcit, adonit, inulin, inosit, and may be mannit[1]. It does not seem worth while using any of the other substances mentioned: for quercit

[1] It may become necessary to use mannit because there is a bacillus which ferments glucose and lactose but not mannit.

and erythrit remained unaffected when tested with about 100 organisms taken at random; and the others did not afford any more information than is to be gained by using the substances mentioned. These together with the indole test, the observation of motility and perhaps Vosges and Proskauer's reaction will enable us to travel a fair distance on the road to our goal.

The method of procedure suggested is that a sloped agar tube should be inoculated from a single colony on a plate, the growth being rubbed all over the surface of the medium and in the water of condensation. After 4—6 hours' growth at 37° C. a drop of the condensation water can be examined to ascertain the presence or absence of motility. After 24 hours' incubation at 37° C. a good loopful of the growth is put into tubes of gelatine, lactose, saccharose, dulcit, adonit and inulin. The agar tube is returned to the incubator, together with the rest of the tubes, and is used later for the indol test. An inosit tube and a glucose tube (for Vosges and Proskauer's reaction) may be inoculated at the same time as the others, or these two may be used as confirmatory tests. Vosges and Proskauer's reaction may be tested for at the end of 4 days. The other tubes should be kept under observation as long as there is no change in the reaction of the medium.

In the course of this work several bacilli have been isolated which have not been included in the results given above. All these bacilli will be found in the subjoined Table III which gives their characters.

Conclusions.

It has been shown that the tests at present in general use do not allow us to differentiate adequately the lactose fermenting bacilli from each other. It has also been shown that by the substitution and addition of certain other tests we shall gain in accuracy with little increase in labour, and that we shall thus have a fair prospect of being able to pick out those organisms which are most closely associated with faeces and put the bacteriological examination of water supplies upon a firmer basis than that upon which it stands at present.

Note.

While this communication was in the press my attention was directed to a paper by Bergey and Deehan on "The Colon-aerogenes

TABLE III.

Source	Lactose	Saccharose	Dulcit	Adonit	Inulin	Indole	Motility	Milk	Gelatine	Glucose	Mannite	
Human faeces	–	–	–	–	–	+	+	⊥	–	...	...	
,, ,,	–	–	⊥	–	–	–	+	⊥	–	⊥	⊥	
,, ,,	⊥	⊥	⊥	–	–	–	+	...	–	...	...	
,, ,,	–	–	–	–	–	+	+	alk.	–	+	–	Case of typhoid.
,, ,,	–	–	–	–	–	–	+	alk.	–	–	–	
,, ,,	⊥	–	⊥	–	–	+	–	+	–	...	...	
Human sputum	–	–	–	–	–	+	+	sol. of casein	+ green	...	...	Only organism found in the sputum of the same person on two occasions at two weeks' interval.
,, ,,	–	–	–	–	–	–	–	–	–	...	...	
,, ,,	–	–	–	–	–	+	+	+	+ yellow	+	–	
,, ,,	–	–	–	+	–	–	–	+	–	⊥	⊥	
Horse faeces	–	+	+	–	–	+	+	⊥ alk.	–	...	...	1000 per gramme.
,, ,,	⊥	–	⊥	–	–	+	–	+	–	...	...	100,000 per gramme.
,, ,,	–	–	–	–	–	+	–	...	–	+	–	
Cesspool sewage	–	+	+	–	–	+	+	–	–	...	...	
Pond water	–	⊥	–	–	–	+	+	+	+	+	⊥	
Rain water	–	–	–	–	–	–	–	⊥ sol. of casein	–	–	...	
,, ,,	–	⊥	–	–	–	–	+	⊥	+ yellow	...	...	
Soil	–	–	–	–	–	+	–	alk.	–	+	...	
Old hay	⊥	–	⊥	–	–	–	+	+	+ yellow	...	...	
,,	⊥	–	–	–	–	+	+	+	+ yellow	⊥	...	
Cheese	–	⊥	–	–	+	–	+	+ alk.	–	...	...	
,,	–	–	–	–	–	+	+	alk.	–	...	...	
Faeces & tap water	–	–	–	–	–	–	+	alk.	–	–	...	
,, ,,	–	–	–	–	–	–	+	+	+	–	–	
,, ,,	⊥	⊥	–	–	–	+	+	+	+ yellow	...	...	
,, ,,	⊥	+	–	–	–	+	+	+	+	...	...	

+ = production of acid and gas, acid and clot, or liquefaction of gelatine as the case may be.
⊥ = production of acid only, no gas. – = neither acid nor gas produced.

* It has been my experience that in cases of this kind (where an organism produces acid and gas in one medium and apparently only acid in another medium) under proper subcultivation the organism will produce gas also in the second medium. The last organism in the Table could I think have been made to produce gas in lactose. But where an organism produces only acid in several media, *e.g.* the 3rd organism in Table III, then I have never succeeded in inducing it to produce gas in any one of them.

Group of Bacteria" in the *Journal of Medical Research*, Vol. XIX. No. 1 (July, 1908).

These workers examined 50 samples of milk, 1 sample of Kefir and 8 samples of sewage. They made use of the tests advocated above and their results cause them to express opinions which are in complete agreement with mine as to the value of these tests. They attach importance to the gas amount and gas ratio. I have not referred to these two tests because they have been shown to be unreliable by Longley and Baton (1907).

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