THE BACTERIOLOGICAL EXAMINATION OF MUSSELS

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Introduction

In September, 1932, under the auspices of the Conseil International pour l'Exploration de la Mer, a Conference was held at Middelburg, Holland, which had, as its main object, the recommendation of a standard method of bacteriological examination of edible molluscs.

This Conference was attended by representatives of the governments of seven countries—Belgium, England, France, Germany, Holland, Irish Free State and Portugal. The majority of the representatives were experts who were engaged in the bacteriological examination of shell-fish in their own countries.

From the Conference emerged the facts that completely different methods were in use in different countries, and that no one method was official in any country.

It was resolved that members of the Conference should test Salle's medium, which was strongly recommended by two delegates, in parallel experiments with their usual methods. In order to obtain comparable results certain rules were adopted relating to the methods of examination and the criteria to be observed for the identification of *B. coli*.

The writer, who represented the Irish Free State at the Conference, is convinced, after prolonged trial, that Salle's medium is unsuitable for the bacteriological examination of shell-fish. The testing of it and of other solid media, however, supplied a suitable opportunity for testing fully the method used by the writer for some years, a method somewhat modified from that formerly described by him (1929). In using his method, the modifications suggested by the Middelburg Conference were adopted, and all organisms isolated were investigated in considerable detail for purposes of identification. The results, which are here recorded, are believed to be of some interest and importance to those engaged in the bacteriological examination of shell-fish.

Method of examination

(1) Ten mussels of average size are selected.

(2) These are washed with running tap water, using a boiled nail brush.

(3) One (A) is grasped with a sterile ovum forceps, rinsed under the tap, and then with sterile water.

(4) It is placed on a piece of sterile parchment paper in which it is grasped with the left hand. The shell is held with the flat edge towards the body, the anterior (pointed) end to the left, and the left valve of the shell upwards.

(5) A small portion of the shell at the broad (posterior) end is nibbled away with a sterile nibbling forceps and, through the opening, the blade of a sterile scalpel is inserted. With this the posterior adductor muscle and the other attachments of the mussel to the left valve are cut, and, holding them with the paper interposed between them and the hands, the two valves of the shell are separated, and the left one removed.

(6) All the fluid in the shell is poured off and, with the help of the scalpel, the body is transferred to a small beaker provided with a graduation at the 25 c.c. level.

(7) The body of the mussel in the beaker is thoroughly minced with a sterile pair of scissors. Sterile saline is added up to the 25 c.c. mark, and mixed thoroughly with the minced body, the scissors being used for this purpose. This is the reconstituted mussel (R.M.).

(8) The whole contents of the beaker are transferred to a larger sterile beaker, and 25 c.c. of sterile saline are added. This is a 1/2 dilution of R.M.

(9) With a sterile 1 c.c. pipette, 1 c.c. of 1/2 R.M. is added, after mixing with the pipette, to each of two tubes of lactose bile broth (L.B.B.). These are labelled respectively A/·5/1 and A/·5/2.

(10) With the same pipette, 2 c.c. of 1/2 R.M. are added to 8 c.c. of sterile saline in a test-tube, making a 1/10 dilution of R.M.

(11) With a fresh sterile 1 c.c. pipette, 1 c.c. of 1/10 R.M. is added to each of two tubes of L.B.B. These are labelled respectively A/·1/1 and A/·1/2.

(12) With the same pipette 0·2 c.c. of 1/10 R.M. is added to each of two tubes of L.B.B. These are labelled respectively A/·02/1 and A/·02/2.

(13) Operations 3–11 are repeated with the next mussel (B), the cultures being labelled B/·5/1, B/·5/2, B/·1/1, B/·1/2, B/·02/1 and B/·02/2. The same procedure is carried out with the other eight mussels C to J.

(14) The cultures are incubated at 37° C. for 24 hours when the results are read. (In the investigation here described an additional reading was made after a further 24 hours' incubation.)

NOTES ON METHOD OF EXAMINATION

(1) In practice it is more convenient to complete steps 3–8 for the ten mussels before steps 9–13 are commenced.

(2) Steps 6–8 may be condensed by transferring the body of the mussel directly from the shell to the large beaker, mincing in it, and making up the volume with sterile saline to 50 c.c.

(3) The instruments are sterilised by boiling in a pie-dish over a Bunsen burner. After a few minutes they are transferred with a flamed forceps to another pie-dish containing cold sterile water in order to cool them. From this they are removed for use with flamed forceps.

(4) The L.B.B. used is prepared in the usual way, and contains 2 per cent. of peptone, 0·5 per cent. of sodium taurocholate, 1 per cent. of lactose and 1 per cent. of Andrade's indicator. So that as much gas as possible may be collected the inner tube almost fills the bore of the outer. It is kept in position high up in the outer tube by a dimple in the wall of the latter made by heating a small area of the glass with a blow-pipe flame and pushing in the softened portion with a carbon rod. This device, which was suggested by my laboratory attendant, Mr W. Kampff, is superior to the usual one of supporting the inner tube on a piece of glass rod.

To avoid the error observed by Dodgson (1928), the production of acid in the medium due to fermentation of glucose derived from the mussel body, a large volume of L.B.B. should be used in each tube. For the tubes containing 0·5 c.c. of R.M., 30 c.c. of medium are used, and 15 c.c. for the tubes containing 0·1 c.c. and 0·02 c.c. of R.M. With these amounts of L.B.B. sufficient acid cannot be produced from the glucose present in the mussel to alter the colour of the indicator to red.

(5) One of the innovations requiring mention is the rejection of shell fluid. This step, which was recommended by the Middelburg Conference, was adopted for two reasons. The first is that this fluid is little more than a sample of the last water in which the mussel opened and is, therefore, little indication of the bacterial content of the mussel body. The second is that its volume is very variable. In freshly examined mussels a large amount may be present. How much of this is included in the sample cultivated depends largely on the operator's skill. It is practically impossible to open a mussel without losing some of the fluid, especially when a fat mussel is present in a shell with a large amount of fluid. The usual procedure of mincing the body in the shell is impossible without allowing some fluid to overflow. In mussels which have been, for some days, out of water little or no shell fluid may be present. Under these circumstances it is impossible to measure a volume of minced body with a graduated pipette unless fluid, such as saline, is added. This is commonly done, but the amount added is not standardised in any way, and such addition introduces a factor of uncertainty. For these reasons it was decided to discard shell fluid, and to replace it by sterile saline.

(6) In the method used by Prof. Eyre (1924) 0·1 c.c. of mussel mince (composed of body plus a variable amount of shell fluid) is the quantity tested. This bears no constant relationship to the volume of the shell contents, and therefore the results obtained cannot be translated into number of *B. coli* per mussel. In the method here described, the reconstituted mussel has a constant volume of 25 c.c., and results obtained by cultivating from various volumes can be reported in terms of bacteria per mussel.

The body volume of an average mussel is about 15 c.c. and 20 c.c. is very rarely attained. The mixture of minced body and saline up to 25 c.c. is generally capable of being manipulated with a pipette. Where the body volume is very large, however, it will be found more convenient to transfer the body to the

large beaker, and to make up the volume to 50 c.c. with saline, so giving directly a 1/2 dilution of R.M.

(7) The method of mincing the whole body, in preference to Dodgson's method (1928) of opening the alimentary tract by scraping away part of the body and "stroking" the gills, was adopted, as it was hoped to devise a method which might be used successfully by any bacteriologist called upon to examine shell-fish. Dodgson's method requires more knowledge of the anatomy of the mussel than is likely to be possessed by many bacteriologists, and also a considerable amount of skill and experience.

THE INVESTIGATION

Ten samples, each consisting of ten mussels, were examined during the year 1933. These were derived from six separate localities in the Irish Free State. Six cultures—two of 0·5 c.c., two of 0·1 c.c. and two of 0·02 c.c. of R.M.—were made in L.B.B. from each mussel. Six hundred cultures in all were, therefore, considered. Readings were made after 24 and 48 hours' incubation at 37° C. Any tube showing acid and/or gas at either period was considered positive. In reading the results the following symbols were used:

A = full acid as shown by deep red colour of medium.

a = slight acid, medium varying in colour from orange-pink to light red.

G = gas collected in inner tube more than sufficient to fill rounded part at top of tube.

g = gas collected in inner tube but not sufficient to fill rounded part at top of tube, or gas only evolved when tube was shaken.

At both the 24- and 48-hour readings tubes showing the following positive results were noted:

Full acid and gas	= *AG*
Gas with deficiency in either acid or gas	= *Ag*, *aG*, *ag*, *G*, *g*
Acid only	= *A*, *a*

A loopful from each positive culture was spread on a plate of MacConkey's medium, which was incubated for 24 hours at 37° C. Where red colonies, more or less closely resembling those of typical *B. coli*, were seen, one at least was subcultured on agar. Where no such colonies were observed, but pale colonies resembling non-lactose-fermenting bacilli were seen, one at least of these was similarly subcultured. In the absence of either of these types of colony, such colonies as were present were examined in Gram-stained films. If the colonies were not found to consist of Gram-negative bacilli, the culture was recorded as containing only "not Gram-negative bacilli." The great majority of these organisms were Gram-positive cocci. In a few cases, where positive L.B.B. cultures were obtained, the MacConkey plates showed no growth, and films from the L.B.B. tubes showed anaerobic bacilli. Gram-stained films were prepared from the subcultures on agar. Where organisms other than non-sporing, Gram-negative bacilli were found, they were relegated to the class of "not Gram-negative bacilli." Organisms of correct morphology and staining were

inoculated into fluid media containing lactose and glucose with Andradi's indicator, into broth, buffered glucose broth (two tubes) and Koser's medium.

At the Middelburg Conference it was agreed that an organism possessing the following characteristics should be regarded as a typical *B. coli*.

(1) Gram-negative, non-sporing bacillus.
(2) Producing acid and gas from lactose.
(3) Producing acid and gas from glucose.
(4) Producing indol.
(5) Giving a positive methyl-red test.
(6) Giving a negative Voges-Proskauer test.
(7) Failing to grow in Koser's medium.

It was also agreed to regard the first three characteristics as essential, and to include as *B. coli* those atypical bacilli which satisfied the first three requirements, and three of the four requirements 4, 5, 6, 7.

The results in lactose and glucose media were read after 24 and 48 hours' incubation. Broth culture was tested for indol by Ehrlich's Rosindol test after 24 hours' incubation. The buffered glucose broth cultures were used for the Voges-Proskauer reaction after 24 and 72 hours' incubation, and for the methyl-red test after 96 hours' incubation. Koser's medium was examined for naked eye evidence of growth after 96 hours' incubation. The loose term "coliform bacillus" is here used in a restricted sense as implying a non-sporing, Gram-negative bacillus which produces acid and gas from both lactose and glucose. Non-sporing, Gram-negative bacilli which failed to ferment both carbohydrates are included in the group of "not coliform bacilli." It may be stated here that it seems doubtful if the use of both sugars is necessary, as out of the thirty-seven "not coliform bacilli" isolated there was only one which fermented lactose but failed to ferment glucose.

Bacilli which fermented both sugars are regarded as coliform bacilli. This group is subdivided, on the basis of indol production, methyl-red test, Voges-Proskauer reaction and growth in Koser's medium. From these four tests, sixteen types should theoretically emerge. Actually only ten types were encountered. These are distinguished by the numbers I–X as shown in Table I.

Table I. *Types of coliform bacilli.*

Type	Indol	M.R.	V.P.	Koser
I	+	+	+	+
II	+	+	−	+
III	−	+	+	+
IV	+	+	−	−
V	−	+	−	+
VI	−	−	+	+
VII	−	+	+	−
VIII	−	+	−	−
IX	−	−	+	−
X	−	−	−	+

Type IV is the typical *B. coli* and type VI the typical *B. lactis aerogenes*. Simplification is achieved if atypical *B. coli* (types II and VIII) are included

with type IV as *B. coli*, and if atypical *B. lactis aerogenes* (types I and III) are included with type VI as *B. lactis aerogenes*. The remaining organisms (V, VII, IX, X) can be grouped as intermediates.

In many cases two or three colonies were investigated. Frequently the organisms were found to be of the same type, despite differences in the appearance of their colonies. Where more than one organism was isolated from a L.B.B. culture, only one is included here for consideration. In all cases the organism retained is the one judged to approach most closely in its characteristics to the typical *B. coli*. So, when type IV and type II organisms were isolated from a tube, or type V and type VI, or type VI and "not coliform," or "not coliform" and "not Gram-negative bacillus," in each case the former organism is retained, and the latter rejected. Since there were in all 371 positive L.B.B. tubes, there are 371 organisms for consideration.

Table II shows, for each of the ten batches of mussels examined, the number of positive cultures obtained from the tubes (twenty in each case) inoculated with each of the three volumes of R.M., and the number of each of the twelve types of organisms isolated.

Table II

Batch	Mussels giving positive cultures	Positive cultures from tubes inoculated with R.M. (c.c.) 0·5	0·1	0·02	No. of types of bacteria isolated	Not Gram-negative bacilli	Not coliform bacilli	Types of coliform bacilli I	II	III	IV	V	VI	VII	VIII	IX	X
1	10	20	13	8	6	18	3	0	0	0	17	1	0	1	1	0	0
2	8	11	8	5	6	15	3	0	0	0	3	1	1	0	0	1	0
3	10	18	6	1	5	14	1	0	0	0	7	0	0	0	2	0	1
4	10	20	15	4	7	3	1	0	0	1	1	29	2	0	2	0	0
5	10	19	14	11	6	28	8	0	0	0	4	1	0	1	2	0	0
6	10	20	20	16	10	11	3	1	2	3	18	8	4	0	5	0	1
7	10	14	3	1	5	7	6	0	0	0	2	1	0	2	0	0	0
8	8	10	5	2	7	3	2	1	0	4	1	3	0	0	3	0	0
9	10	20	18	10	9	15	2	0	4	3	13	7	1	1	2	0	0
10	10	20	20	19	5	0	8	0	1	0	6	10	0	0	34	0	0
Total	96	172	122	77	12	114	37	2	7	11	72	61	8	5	51	1	2

When we group the organisms into a smaller number of types, as described above, we get the distribution of the 371 cultures shown in Table III.

Table III

Group	Number	%
Not Gram-negative bacilli	114	30·7
Not coliform bacilli	37	10·0
B. coli (II, IV, VIII)	130	35·0
Intermediates (V, VII, IX, X)	69	18·6
B. lactis aerogenes (I, III, VI)	21	5·7
	371	100·0

The figures show that *B. coli*, as defined above, is the most commonly occurring organism in positive mussel cultures in L.B.B. This statement must, however, be taken in conjunction with the fact that its finding was favoured at the expense of other bacteria, *i.e.* when it was present other organisms were ignored. A consideration of the details of all the cultures shows, however,

that if this had not been done, and if all organisms isolated had been included in the results, the position of *B. coli* would not have been very much altered. It would probably have come second in frequency to the heterogeneous group of "not Gram-negative bacilli." *B. coli*, in the figures given, represents 59·1 per cent. of all coliform bacilli isolated. The inclusion of all organisms would not have lowered the percentage to a large extent, and *B. coli* would still have remained the commonest coliform bacillus.

One of the most important points which it was hoped that this investigation would clear up was how and when the results in L.B.B. tubes should be read. The combined results obtained with the ten batches of mussels, according to the reading, are given in the Table IV, in which reading no. 3 is a combination of nos. 1 and 2, and reading no. 6 a combination of nos. 4 and 5.

The time and the method of reading selected as the optimum should be the one which reveals the presence of the largest possible proportion of the *B. coli* present in the mussels examined and, at the same time, includes the smallest possible proportion of organisms other than *B. coli*. In other words it should combine completeness and selectivity.

Table IV

No.	Reading	Time of reading hours	Total number	Not Gram-negative bacilli	Not coli-form bacilli	Types of coliform bacilli									
						I	II	III	IV	V	VI	VII	VIII	IX	X
1	Full acid and gas	24	178	1	4	2	5	4	67	51	5	0	37	0	2
2	Gas, with deficiency in acid or gas	24	39	12	8	0	2	0	3	4	2	0	7	1	0
3	Gas (independent of amount or acidity)	24	217	13	12	2	7	4	70	55	7	0	44	1	2
4	Full acid and gas	48	187	5	10	2	6	6	62	49	6	1	38	0	2
5	Gas with deficiency in acid or gas	48	94	40	14	0	1	3	10	9	2	2	12	1	0
6	Gas (independent of amount or acidity)	48	281	45	24	2	7	9	72	58	8	3	50	1	2
7	Acid only	24	56	34	12	0	0	2	0	1	1	2	4	0	0
8	Acid only	48	57	45	8	0	0	1	0	1	0	1	1	0	0
	Total isolated		371	114	37	2	7	11	72	61	8	5	51	1	2
	Percentage distribution of all isolated		100	30·7	10·0	0·5	1·9	3·0	19·4	16·4	2·2	1·3	13·8	0·3	0·5

A glance at Table IV shows at once that readings nos. 7 and 8 (acid only at 24 and 48 hours) may be rejected as being far removed from the optimum. Reading no. 7 gives only four *B. coli* (types II, IV, VIII) as against thirty-four "not Gram-negative bacilli" (7·1 and 60·7 per cent. respectively). With reading no. 8 the figures are still worse, one (1·8 per cent.) *B. coli* and forty-five (79·0 per cent.) "not Gram-negative bacilli." These two readings need not, therefore, be considered further. The thirty-nine tubes which gave reading no. 2 yielded twelve *B. coli* (II, IV, VIII) and twelve "not Gram-negative bacilli" (30·8 per cent. for each). The ninety-four tubes which gave reading no. 5 yielded twenty-three *B. coli* (II, IV, VIII) and forty "not Gram-negative bacilli" (24·5 and 42·6 per cent.). When these are contrasted with the percentages of all organisms isolated (35·1 *B. coli* (II, IV, VIII) and 30·7 "not

Gram-negative bacilli") it is seen that both readings are inferior to the sum of all readings, and should therefore be rejected.

We have now to consider readings nos. 1, 3, 4 and 6. The good and bad features of each are most easily compared in Table V, in which columns A and B deal with the completeness with which the method of reading reveals *B. coli* (typical and atypical), and columns C, D, E and F deal with specificity for typical *B. coli*, typical and atypical *B. coli*, coliform bacilli and Gram-negative bacilli. In the table percentages only are given, as this method of presentation facilitates comparison. The data from which the percentage figures were calculated are given in Table IV.

Table V

		A	B	C	D	E	F
No.	Reading	% of all typical *B. coli* (IV) which give reading	% of all typical and atypical *B. coli* (II, IV, VIII) which give reading	% of organisms giving reading which are typical *B. coli* (IV)	% of organisms giving reading which are typical or atypical *B. coli* (II, IV, VIII)	% of organisms giving reading which are coliform bacilli (I–X)	% of organisms giving reading which are Gram-negative bacilli
1	Full acid and gas (24 hours)	93·0	83·8	37·6	61·1	97·2	99·4
3	Gas (independent of amount or acidity) (24 hours)	97·2	93·1	32·3	55·8	88·5	94·0
4	Full acid and gas (48 hours)	86·1	81·5	33·2	56·7	92·0	97·3
6	Gas (independent of amount or acidity) (48 hours)	100·0	99·2	25·6	45·9	75·4	84·0

In the case of each criterion, the higher the percentage figure, the better is the method to be considered. It may first be observed that for each criterion the figure for reading no. 4 is lower than that for reading no. 1. Reading no. 4 may, therefore, be eliminated. The remaining three readings as regards completeness (A and B) are to be placed in the following order—6, 3, 1. As regards selectivity (C, D, E, F) the order is reversed—1, 3, 6. No. 1 is the most selective and the least complete, no. 6 is the least selective and the most complete, while no. 3 is midway between the extremes, both as regards selectivity and completeness.

So long as the degree of completeness with which the method reveals the presence of *B. coli* is high, in our opinion the preference should go to the method which is most selective. We therefore believe that L.B.B. tubes, inoculated with mussel mince, should be read after 24 hours' incubation, and that results should be regarded as positive only when a strongly acid reaction has developed, and when the gas collected in the inner tube more than fills the rounded top of the tube. Subsequently, in this paper, the term "positive" will be used in this sense. 93·0 per cent. of all typical *B. coli* found to be present in the quantities of mussel mince examined in the present investigation gave this result, as did 83·8 per cent. of all typical and atypical *B. coli*. 37·6 per cent. of the tubes giving positive results by this method contained typical *B. coli*; 61·1 per cent. contained either typical or atypical *B. coli*; 97·2 per cent. contained coliform bacilli.

A practical method of examining shell-fish bacteriologically must give results quickly, must not necessitate the isolation and testing of large numbers of bacteria, must reveal the presence of a high proportion of *B. coli* present, and must not give a large proportion of false positive results.

It is claimed that the inoculation of measured volumes of mussel mince into tubes of L.B.B. (using the methods and precautions already described) is satisfactory in all these respects. The chief criticism that might be made is that 62·4 per cent. of positive reactions, as defined above, are due to organisms which are not typical *B. coli*. It should be recollected, however, that it is not *B. coli* itself which is of importance. This bacillus is used as an indicator because it is known to be a common intestinal organism. All the coliform bacilli isolated grew freely in the presence of bile salts and are probably, therefore, at least facultatively intestinal. The only one known to have considerable powers of multiplication under natural conditions outside the intestine is *B. lactis aerogenes*, which was found in only five of the 178 positive tubes (2·8 per cent.). We believe, therefore, that the method of reading here adopted is safe, in that it will not allow contaminated mussels to pass and, at the same time, is not unduly severe, as it does not condemn mussels as contaminated without sufficient reason. Apart from the present investigation it has been used for the examination of many batches of oysters and mussels taken from natural beds, and many of these have passed the test without difficulty.

The next matter to be considered is the amount of mussel mince which should be inoculated into tubes of L.B.B. In this investigation three volumes were used—0·5, 0·1 and 0·02 c.c. of R.M.—and two tubes of L.B.B. were inoculated with each volume. Table VI shows the positive results, *i.e.* the tubes in which full acid and gas were produced in 24 hours.

It will be observed that considerable differences occur between the number of positive tubes out of ten inoculated in the first and second series. In each case the tubes were inoculated one immediately after the other and, apart from chance, there should be no difference between them. The belief that the differences are due merely to chance is supported by the fact that, in all, there were eighty-eight positive tubes in the first series and ninety in the second. In batch no. 6, the first series of tubes inoculated with 0·1 c.c. of R.M. showed nine out of the ten positive, a result which would lead to the condemnation of the batch, while in the second series only four out of ten inoculated with the same amount were positive, and therefore the batch would pass the standards of the Fishmongers' Company. When the basis adopted is agreement between the two tubes inoculated with the same volume of R.M., different results are again obtained. On this basis the results are much more trustworthy as the operations of chance are greatly minimised. In the examination of water for the presence of *B. coli* and of milk for the presence of lactose-fermenting bacilli, the principle of inoculating duplicate tubes with the same volume of fluid is almost universally adopted. It seems only logical, therefore,

that duplicate inoculations should be made in the case of shell-fish, and that a given volume of R.M. should be considered as giving a positive result only when both tubes inoculated with that volume show such a result.

Table VI

Batch No.	Volume of R.M. c.c.	Number of positive tubes out of ten inoculated		
		First tubes	Second tubes	Both positive
1	0·5	3	6	2
	0·1	3	2	1
	0·02	1	1	0
2	0·5	1	1	1
	0·1	0	1	0
	0·02	0	0	0
3	0·5	4	3	0
	0·1	1	0	0
	0·02	0	0	0
4	0·5	10	9	9
	0·1	7	6	4
	0·02	1	1	0
5	0·5	2	5	1
	0·1	0	0	0
	0·02	0	0	0
6	0·5	10	10	10
	0·1	9	4	4
	0·02	0	1	0
7	0·5	0	2	0
	0·1	0	0	0
	0·02	0	0	0
8	0·5	3	2	2
	0·1	1	1	1
	0·02	0	0	0
9	0·5	8	7	7
	0·1	1	3	0
	0·02	3	0	0
10	0·5	8	10	8
	0·1	7	9	7
	0·02	5	6	3

In order to test this a large number of mussels were obtained from the same bed. These were thoroughly mixed together and divided, by random sampling, into four batches of ten each. The mussels were dealt with in the manner described and R.M. prepared. The same volume of 1/2 R.M. (0·2 c.c.) of each mussel was inoculated into two tubes (first and second) of L.B.B. The results, which were read after 24 hours' incubation, are shown in Table VII.

Table VII

Batch No.	Number of mussels positive out of ten		
	First tubes	Second tubes	Both tubes
1	5	6	3
2	8	6	4
3	5	3	3
4	6	5	4

The number of positive tubes out of ten, in the eight groups, varies from three to eight, but, where positive means positive in both tubes inoculated with the same volume of R.M. of the same mussel, the number is either three or four.

Both from the results of this special investigation and from the routine use of the method, the writer is convinced that duplicate inoculation of tubes of L.B.B. is essential if reliable results are to be obtained.

The next point is the use of three different volumes of R.M. In the method originally described by the writer (1929), the mussel was minced in the shell with the shell fluid, and three volumes of the mince were used to inoculate tubes of L.B.B.—0·2, 0·1 and 0·02 c.c. The method, as here described, which has now been used for some time, differs in that the shell fluid is rejected, the body is made up to a volume of 25 c.c. with saline, and the volumes of the minced body (R.M.) used for inoculating into L.B.B. in duplicate are 0·5, 0·1 and 0·02 c.c. Reasons for the rejection of shell fluid and for standardising the volume of R.M. at 25 c.c. have already been given. The largest inoculum (0·5 c.c. in place of 0·2 c.c.) was adopted, as it forms with the other amounts (0·1 and 0·02 c.c.) a geometric series which is more logical, and also furnishes a more strict basis for ascertaining the degree of contamination of the mussels examined.

In the method of examination used by Prof. Eyre, working for the Fishmonger's Company (1924), the result can only be stated in some modification of the form "so many mussels out of 10 examined contained *B. coli* in excessive numbers." With the method now described a more explicit form of statement may be used. 0·5, 0·1 and 0·02 c.c. of R.M. represent respectively 1/50, 1/250 and 1/1250 of the mussel body. Where both tubes inoculated with 0·5 c.c. of R.M. are positive this means the presence of at least fifty *B. coli* in the mussel; where both the 0·1 c.c. of R.M. tubes are positive, at least 250 *B. coli* are present in the mussel; where both the 0·02 c.c. of R.M. tubes are positive, at least 1250 *B. coli* are present.

In the case of batch no. 10 (Table VI), the result might be reported, "Out of ten mussels examined, eight showed the presence of more than fifty *B. coli*, seven the presence of more than 250 *B. coli*, and three the presence of more than 1250 *B. coli*." By this method the examination becomes a quantitative rather than a qualitative one.

The use of three inoculated volumes enables one to differentiate two batches of mussels, one of which has a small number of mussels heavily contaminated from one which has a large number contaminated to a lesser degree. If only one volume (0·5 c.c. of R.M.) had been used in examining batches nos. 9 and 10, the two batches would have been thought to be of similar quality. The use of the other two volumes shows, however, that while, in the case of batch no. 9, a large proportion of the mussels were contaminated, but only to a slight degree, in the case of batch no. 10 the degree of contamination was enormously greater.

Prolonged experience of the method will be required before we are able to fix definite standards, but it is tentatively suggested that a batch of mussels should be considered to show an undesirable degree of contamination if, out of ten examined, more than one shows the presence of 1250 *B. coli*, or more

than three show the presence of 250 *B. coli*, or more than seven show the presence of 50 *B. coli*. It should be noted that, here and above, *B. coli* is used as synonymous with an organism producing full acid and gas in L.B.B. after 24 hours' incubation.

That the standard suggested is not unduly severe is shown by the fact that seven out of the ten batches investigated pass it. These seven batches came from five different localities. Of the three batches which fail to pass the test, two come from a bed the sale of mussels from which has been prohibited for some years. Mussels derived from beds situated in safe localities or mussels correctly treated in a tank should have no difficulty in passing the test, but mussels coming from contaminated waters will, almost inevitably, fail to pass.

No bacteriologist believes that a single good result should be taken as implying that all shell-fish from the same locality should be unquestionably accepted as satisfactorily free from contamination. Topographical investigation of the bed and its surroundings, and repeated examinations of samples are both essential to prevent risk to consumers of shell-fish. Once a bed has been accepted as satisfactory, its products should be examined bacteriologically several times a year as, frequently, the result of a test may show the advent of some new source of contamination which might otherwise escape recognition for a considerable time.

The same locality may, at different times, supply mussels of very different bacteriological qualities. Batches nos. 3, 5 and 10 all came from the same bed. This is situated in a small tidal river, about half a mile from its mouth. Batch no. 3 was collected in February, batch no. 5 in April and batch no. 10 in November. For a number of years highly discrepant results have been obtained from this bed. On some occasions the mussels have been singularly free from contamination while, at other times, they have been highly contaminated. It appears probable that this bed is liable, on occasions, to gross contamination from some source not yet ascertained.

Two batches from the mouth of a large river (nos. 4 and 6) are of interest. Batch no. 4 was collected on February 21st, 1933, and batch no. 6 on May 1st, 1933. Batch no. 4 gave thirty-four positive cultures (full acid and gas in 24 hours), and batch no. 6 exactly the same number. The types of organism present were, however, quite different.

Table VIII

	No. 4	No. 6
Not Gram-negative bacilli	0	1
Type I	0	1
,, II	0	2
,, III	1	2
,, IV	1	18
,, V	28	6
,, VI	2	2
,, VIII	2	1
,, X	0	1

Batch no. 4 gave 2·9 per cent. of typical *B. coli* (IV), 8·8 per cent. of typical and atypical *B. coli* (II, IV, VIII), and 82·4 per cent. of one of the intermediates (V). Batch no. 6 gave 52·9 per cent. of typical *B. coli* (IV), 61·8 per cent. of typical and atypical *B. coli* (II, IV, VIII) and 17·4 per cent. of type V. If we were to assess the contamination by the number of *B. coli* in the strict sense batch no. 4 would pass, which would certainly be quite wrong, as the river from which the mussels came is highly contaminated. This comparison demonstrates that the basis used, the production of full acid and gas in 24 hours, gives better practical results than would the finding of *B. coli*.

Experiments performed to test the method

These experiments were performed to test the method.

Exp. 1. Ten mussels from a clean bed were opened in the usual way and the shell fluid was rejected. They were minced with the usual precautions, and the volume made up to 250 c.c. with sterile saline. This mixture represented R.M. Of five tubes of L.B.B., each inoculated with 0·5 c.c. volume of R.M., one showed acid and gas after 24 hours' incubation. Of five tubes each inoculated with 0·1 c.c. R.M., and of five tubes, each inoculated with 0·02 c.c. R.M., none showed acid and gas. It is probable, therefore, that the R.M. did not contain more than 1/3 *B. coli* per c.c., a number so small that, in this experiment, it may be ignored.

Eight mixtures, each consisting of equal volumes of R.M. and of saline plus a suspension of *B. coli*, were prepared. The number of living *B. coli* in the suspension was accurately estimated by plating. Six cultures in L.B.B. were made from each mixture (equivalent to 1/2 R.M.)—two with 1·0 c.c., two with 0·2 c.c., and two with 0·04 c.c. The amount of mussel mince in each corresponded to the amount in the tubes in the routine test. The eight mixtures may, therefore, be regarded as eight mussels. In Table IX the results are shown for each tube, and also the number of *B. coli* per "mussel" as deduced from the results and as calculated from the amount of suspension present in each of the eight "mussels."

Table IX

	Results in L.B.B. inoculated with following volumes of 1/2 R.M. (c.c.)						*B. coli* per "mussel"	
Mixture	1·0	1·0	0·2	0·2	0·04	0·04	Deduced	Calculated
A	+	+	+	+	+	+	>1250	2750
B	+	+	+	+	+	−	> 250<1250	1375
C	+	+	+	+	−	−	> 250<1250	687
D	+	+	+	−	−	−	> 50< 250	275
E	+	+	+	−	−	−	> 50< 250	137
F	+	+	−	−	+	−	> 50< 250	55
G	+	+	−	−	−	−	> 50< 250	27
H	−	+	−	−	−	−	< 50	11

The numbers of *B. coli* present, as deduced from the results in L.B.B., agree reasonably closely with the calculated figures.

https://doi.org/10.1017/S0022172400034513 Published online by Cambridge University Press

Exp. 2. Twelve mussels were opened in the usual manner, the shell fluid rejected, the bodies minced, and the volume made up to 300 c.c. with sterile saline. This (R.M.) was heated to 60° C. for half an hour to kill any *B. coli* present. Eighteen mixtures were prepared, one-half of each consisting of R.M., and the other half of suspensions of *B. coli* (accurately counted by plating), and saline. These may be regarded as 1 in 2 dilutions of R.M. of eighteen mussels. 1, 0·2 and 0·04 c.c. of these mixtures were inoculated in duplicate into tubes of L.B.B., and the results read after 24 hours' incubation at 37° C. In Table X the number of *B. coli* per "mussel" is given as calculated from the number added, and as deduced from the results in the L.B.B. tubes.

Table X

Mixture	Results in L.B.B. inoculated with following volumes of 1/2 R.M. (c.c.)						*B. coli* per "mussel"	
	1·0	1·0	0·2	0·2	0·04	0·04	Deduced	Calculated
A	+	+	+	+	+	+	>1250	2850
B	+	+	+	+	+	+	>1250	2137
C	+	+	+	+	+	+	>1250	1425
D	+	+	+	+	+	+	>1250	1140
E	+	+	+	+	−	+	> 250<1250	835
F	+	+	+	+	+	+	>1250	570
G	+	+	+	+	+	−	> 250<1250	427
H	+	+	+	+	+	−	> 250<1250	285
I	+	+	+	+	+	−	> 250<1250	213
J	+	+	+	+	−	−	> 250<1250	142
K	+	+	+	−	−	−	> 50< 250	114
L	+	+	−	+	−	+	> 50< 250	83
M	+	+	−	+	−	−	> 50< 250	57
N	−	+	+	−	−	−	< 50	42
O	+	−	−	−	−	−	< 50	27
P	+	+	−	−	−	−	> 50< 250	21
Q	+	−	−	−	−	−	< 50	14
R	−	+	−	−	−	−	< 50	11

Again the results show a satisfactory degree of agreement between the deduced and calculated numbers of *B. coli* present.

Exp. 3. The next experiment was carried out with the same mussel mince as used in Exp. 1. Ten mixtures were prepared from R.M., a suspension of *B. coli*, and a suspension of *B. lactis aerogenes* and saline. In each mixture R.M. constituted one-half of the total volume. The number of living *B. coli* and *B. lactis aerogenes* in the suspensions was accurately counted by plating. A volume (0·2 or 1 c.c.) of each mixture was inoculated into a tube of L.B.B. which was incubated at 37° C. for 24 hours. Each tube then showed acid and gas. A loopful of each was plated on a MacConkey plate, which was incubated at 37° C. for 24 hours. The proportion of *B. coli* to *B. lactis aerogenes* colonies (which were easily identified by examination of the plates) was noted.

These results demonstrate that, in cultures made from mixtures of *B. coli* and *B. lactis aerogenes* in the presence of mussel mince, there is no tendency for *B. coli* to be overgrown by *B. lactis aerogenes*. Colonies of *B. coli* were found on the plates even when this organism was greatly outnumbered by *B. lactis aerogenes* in the inoculum. It is improbable, therefore, in the investi-

gation, that the presence of *B. coli* in the mussels examined was masked by *B. lactis aerogenes*.

Table XI

Mixture	Amount inoculated into L.B.B. c.c.	Number of organisms in inoculum		Colonies on MacConkey plate
		B. coli	*B. lactis aerogenes*	
I	0·2	5·5	312·5	One *B. coli*; many *B. lactis aerogenes*
J	0·2	5·5	62·5	Few *B. coli*; many *B. lactis aerogenes*
K	0·2	5·5	12·5	Many *B. coli*; few *B. lactis aerogenes*
L	0·2	5·5	2·5	*B. coli* only
M	0·2	5·5	0·5	*B. coli* only
N	1·0	2·75	312·5	Few *B. coli*; many *B. lactis aerogenes*
O	1·0	2·75	62·5	Few *B. coli*; many *B. lactis aerogenes*
P	1·0	2·75	12·5	*B. coli* and *B. lactis aerogenes*, the latter in slight excess
Q	1·0	2·75	2·5	*B. coli* only
R	1·0	2·75	0·5	*B. coli* only

Consideration of solid media

During the course of this investigation three solid media were tested to determine their suitability for use in the examination of shell-fish. These were the media of Salle, MacConkey and Burke-Gaffney.

Salle's medium (1930) was described by its author as "a solid streaking medium." According to him its chief use was to distinguish *B. coli* from *B. lactis aerogenes*. In his words, "The combination of erythrosine and methylene blue gives a bright metallic appearance to the colonies of *B. coli*, but not to those of *B. aerogenes*. *B. coli* changes the colour of the brom-cresol purple from purple to orange, while *B. aerogenes* is unable to affect the colour of the indicator."

It is obvious that, if this medium is used for deep plating, one of its advantages is lost—the production of a bright metallic appearance in the colonies of *B. coli*—since this only occurs with surface colonies. We must, therefore, rely on the alteration of colour from purple to orange around the colonies of *B. coli*, and the absence of this colour change in the neighbourhood of colonies of *B. lactis aerogenes*.

Further, a consideration of the organisms obtained from mussels in this investigation shows that *B. lactis aerogenes* is by no means common, and suggests that, in the examination of shell-fish, we should do as we do in the case of milk, that is, examine for the presence of lactose-fermenting organisms, rather than for *B. coli* as is done when water is examined.

Despite these inherent disadvantages, many trials of Salle's medium were made. In some cases the occurrences of orange zones around colonies was well marked, but on testing the colonies producing such zones, we found that sometimes they were of *B. coli*, but quite frequently of other organisms. In one experiment identical orange zones were present around colonies of coliform bacilli of types II, III, IV, V and VI. When working with cultures of known *B. coli* it was sometimes found that no orange zones were present. It appeared to us that the development of the zones depended, to a considerable extent,

on the depth of medium in the plate, and the distance the colonies were from the surface of the medium. It is recommended that the minimum quantity of medium should be used. This is a disadvantage as, with minimal amounts of medium, the glucose derived from the shell-fish body attains its maximum concentration and, if fermented by non-lactose fermenters, may cause errors in reading of results.

On some occasions the orange zones were large and fused together, turning the whole plate orange, and so preventing the identification of possible *B. coli* colonies. In our experience gas is more freely evolved in Salle's than in MacConkey's medium. The gas blisters crack the medium, and lead to spreading of colonies in the medium, rendering the counting of colonies difficult or even impossible. Finally, the medium is somewhat difficult to prepare, and different batches vary considerably. We have given Salle's medium a fair trial, and are convinced that it is quite useless for the bacteriological examination of shell-fish.

MacConkey's medium has been extensively used by Dodgson (1928), who recommends it for the bacteriological examination of shell-fish. It differentiates bacteria which ferment lactose, producing red colonies, from those which do not. On the surface it is usually possible to differentiate, by inspection, colonies of typical *B. coli* from those of *B. lactis aerogenes* and of cocci, but, in deep culture, such differentiation is more difficult. Dodgson relies, for the recognition of colonies of members of the *B. coli* group, on the more rapid appearance of their colonies (24–30 hours as against 48 hours or longer for most cocci), the greater size attained by the colonies, and the appearance around the colony of an "opalescent halo, due to the precipitation of bile salts." He confesses that these differences are not absolutely constant, and that colonies of members of the *B. coli* group may appear late, remain small or fail to produce a halo.

Dodgson's use of MacConkey's medium for the examination of purified mussels appears legitimate, as he wished to use a method which would test as severely as possible treated shell-fish. If all red colonies are regarded as contaminations the necessary severity is secured. For the routine testing of mussels from beds or layings, however, he states "the practice of making confirmatory tests of a large number of colonies has always been followed before a verdict has been given." In our opinion, if this is necessary, it renders the use of MacConkey's medium impracticable. As regards the subculturing of all colonies, Dodgson himself says "in routine practice, it is obviously impossible to adopt such a course."

The chief objection, and we believe that it is a fundamental one, to the use of MacConkey's medium is that it differentiates bacteria merely by their ability to produce acid from lactose, and ignores the production of gas. The typical *B. coli* produces both acid and gas from lactose, as do the majority of organisms which are characteristically intestinal in habitat, and we do not think that any method which omits consideration of the gas-producing power

of bacteria can be recommended for the examination of shell-fish. From L.B.B. cultures made in this investigation a considerable number of bacteria were isolated which produced acid without gas from lactose. In our opinion it would be quite wrong to base any opinion on the quality of the mussels examined on the presence or absence of these bacteria.

Dodgson's method, using MacConkey's agar, was tested, in parallel with our method, on a number of batches of mussels. Plates prepared with 1·0 c.c. of R.M. of each mussel in batch no. 1, after 24 hours' incubation, yielded such large numbers of colonies that even the distinguishing of red zones around the colonies was difficult. From each of the ten plates six colonies were picked and tested and, from these, only four organisms which produced acid and gas from lactose were obtained. All were typical *B. coli*.

In the case of batch no. 2, 0·5 c.c. of R.M. of each mussel was plated in MacConkey's medium. The number of red colonies (which were indistinguishable from those of *B. coli*) visible after 24 hours' incubation in each of the ten plates was as follows: 29, 20, 15, 11, 3, 12, 0, 19, 14, 5. Four of each (except in the case of E and G, where less than four colonies were present) were isolated and tested. Only one produced acid and gas from lactose. This was a coliform bacillus (type IX). After a further 24 hours' incubation (when more colonies had appeared in the E plate, and one colony in the G plate), one further colony from each plate was picked out and tested. Of these ten organisms only two produced acid and gas from lactose. Both of these were atypical *B. coli* (type VIII).

With batch no. 3, where 0·5 c.c. of R.M. of each mussel was inoculated in MacConkey's medium, the number of red colonies visible after 24 hours varied from fourteen to 356. Five of each were picked and tested. The fifty organisms yielded two which produced acid and gas from lactose. One was a typical *B. coli* (type IV), the other was an atypical *B. coli* (type VIII).

It would be wearisome to give further results. It is sufficient to say that, in our experience, MacConkey's medium does not enable *B. coli*, or even coliform bacilli, to be recognised by inspection. The isolation and testing of red colonies is impracticable, and if red colonies in excessive numbers are to be regarded as indicative of dangerous contamination, practically no batch of shell-fish, direct from beds, will be considered satisfactory.

We regret being obliged to condemn Dodgson's method so completely as, from reading his account of its use, we started with a definite prejudice in favour of it. After a long and fair trial we feel obliged to state that we consider it quite unreliable.

Burke-Gaffney's medium (1932) was given a less prolonged trial than either Salle's or MacConkey's. On *prima facie* grounds the use of glucose instead of lactose is an objection. Its author states that practically all glucose-fermenting organisms found in water also ferment lactose; but this is not the case with the bacteria of mussels. Batch no. 3 was tested with this medium, 0·5 c.c. of R.M. of each mussel being used. The number of yellow-zoned colonies in

the ten plates were as follows: 55, uncountable, 68, 42, 68, 45, 54, 74, 26, 14. The bacteria of two colonies from each plate were isolated and tested. None produced acid and gas in lactose. Another batch of mussels was tested with the medium and similar results were obtained. Further work with Burke-Gaffney's medium was not carried out. We were disappointed that better results were not obtained, especially as we had had the benefit of seeing how this medium acted in the hands of its author when he was testing it in our laboratory.

CONSIDERATION OF THE USE OF FLUID AND SOLID MEDIA

For the estimation of the number of bacteria in a fluid a solid medium is, other things being equal, always to be preferred to a fluid one. No bacteriologist would, for example, estimate the total number of bacteria in milk by making decimal dilutions in sterile water, and inoculating equal volumes of the dilutions into broth, judging the presence or absence of bacteria in the various dilutions by the evidence or lack of evidence of growth in the broth tubes after incubation. No elaborate mathematical consideration is needed to prove that it is more satisfactory to state the result as "100 colonies grew from 0·1 c.c. of the sample of milk" than as "Bacteria were present in 0·001 c.c. of the sample of milk, but not in 0·0001 c.c." The two results are often reported as "The sample of milk contained 1000 bacteria per c.c." In so translating the result we must be careful to avoid, so far as practicable, the operations of chance. In the case of solid media, where the number of colonies counted is small (*e.g.* 1 to 10), chance plays a large part; where large, a much smaller part. The growth of three colonies in a plate inoculated with 0·001 c.c. of milk may be taken as equivalent to 3000 bacteria per c.c. of milk, but there is quite a reasonable probability of milks ranging in bacterial content from 1000 or less to 6000 or more per c.c. giving the same result. When, however, the number of colonies in a plate is large (*e.g.* 300 in a plate inoculated with 0·1 c.c. of milk) the statement of the result as 3000 per c.c. is very much more likely to approximate closely to the facts. Where fluid media are used the "hit or miss" method must be employed. The organism is found either to be present or absent in a culture inoculated with a given volume of the material. Reasonable certainty can only be reached by the inoculation of a large number of tubes of medium with the same volume of the material. Where all of these give a positive result we can conclude that at least one organism is present in each quantum. The use of two tubes in place of one, and regarding as positive for the organism such quanta as give a positive result in both tubes greatly reduces the operation of chance, but is very far from eliminating it completely.

For these reasons, in the examination of shell-fish, solid media would be preferable to fluid media if they could give the same indication of the presence of the organisms for which search is made. As regards this we are in a difficulty, for there is no agreement as to whether the correct indicator

organism is the typical *B. coli*, *B. coli* whether typical or to a greater or less extent atypical, some coliform bacilli or all coliform bacilli. Considerable experience of the bacteriological examination of shell-fish has brought us to the belief that shell-fish, in which excessive numbers of bacteria which produce full acid and considerable gas within 24 hours in lactose bile broth are found, should be looked upon with suspicion. This investigation shows that this criterion will include almost all *B. coli* and only very few non-coliform bacilli. In our opinion gas production is at least as important as acid production, and the presence of both should be considered essential for a positive reading. If this belief be accepted it follows that no solid media can be used since, in such media, the production of gas cannot be satisfactorily observed. Such a medium as that of MacConkey which indicates only acid production must therefore be regarded as unsatisfactory. Media, such as Salle's and Burke-Gaffney's, which attempt to differentiate *B. coli* from *B. lactis aerogenes*, suffer from the same disadvantage plus another. The second, which only operates if the media, when used for the examination of shell-fish, actually do what they were devised to do, is that they must place in one class or other (*B. coli* or *B. lactis aerogenes*) all coliform bacilli occurring in shell-fish. We are not yet in a position to state, and it will probably be a long time before we can state, even for the ten types of coliform bacilli isolated in this investigation, which of these may safely be regarded as primarily of intestinal habitat, and which can grow reasonably freely outside the animal body under natural conditions. Even then the media might be legitimately used only if all, or the great majority, of intestinal coliform bacilli gave the same reaction as *B. coli*, while all, or the great majority, of non-intestinal coliform bacilli gave the reaction of *B. lactis aerogenes*. None of the media tested, and none known to the writer, satisfy these requirements, and therefore we are apparently forced to use fluid media.

In any comparison of fluid and solid media for the bacteriological examination of shell-fish it is impossible to ignore Dodgson's observation (1928) that the body of the shell-fish may yield some substance (probably glucose derived from glycogen) which may be fermented by bacteria other than lactose fermenters, and so cause the appearance of red colonies in MacConkey's solid medium, and acid and gas in lactose bile salt broth. Since we know that glucose-fermenting but non-lactose-fermenting bacilli do occur in shell-fish, Dodgson's observation of a possible fallacy in the usual methods of examination is of considerable importance.

We may consider first the production of acid in fluid and in solid media due to this substance which, for convenience, we can call glucose. If a given weight of this glucose is fermented by an organism, a certain amount of acid will be produced. If the volume of medium is small, the reaction of the whole will become acid, and the indicator will change colour but, if the volume of the medium is large, the acid produced will be unable to overcome its buffering effect, and the indicator will not change colour.

Dodgson reports the production of acid in tubes of "peptone-water litmus medium," to which were added various amounts of mussel juice (fluid obtained by mixing shell fluid and minced mussel). Acid was produced when the amount of juice added was as small as 0·1 c.c. If we assume that 0·1 c.c. of juice corresponds to 0·06 g. of mussel body, and if we assume that mussel body contains 5 per cent. of glucose, the amount of glucose present in the inoculum was 0·003 g. Dodgson gives no information as regards the amount of medium present in each tube but, if we suppose the tubes used were $6 \times \frac{5}{8}$ in. or $6 \times \frac{3}{4}$ in., so far as can be judged from the photographs the amount of medium could not have exceeded 10 c.c., and was probably less. 0·003 g. of glucose in 10 c.c. is equivalent to 0·03 per cent. Had Dodgson used double the volume of medium, or the same volume of a medium containing sodium taurocholate (which has considerable buffering properties), it is unlikely that the indicator present would have changed colour.

The effect of altering the volume of the medium, where the volume of inoculum remains constant, is shown in an experiment in which 0·5 c.c. amounts of sterile 1 per cent. glucose solution were added to a series of tubes containing various volumes of peptone-sodium taurocholate broth with Andrade's indicator. This medium is exactly the same as L.B.B. save that it contains no lactose. The tubes were inoculated with a glucose-fermenting bacillus (*B. paratyphosus* B), incubated at 37° C. for 24 hours and examined.

No.	Volume of 1 % glucose solution	Volume of medium	% of glucose	Colour of medium after incubation
1	0·5	1·0	0·33	Red
2	0·5	2·0	0·20	Red
3	0·5	5·0	0·09	Red (less intense)
4	0·5	10·0	0·047	Orange-brown
5	0·5	20·0	0·024	Yellow-brown

Tubes nos. 1 and 2 would, if these were routine tests, have been described as giving full acid reactions. No. 3 would probably have been described as full acid, although the colour was a little less intense. Nos. 4 and 5 would have been reported as "no acid." (No. 5 had the same colour as the uninoculated medium.)

In the next experiment 8·0 c.c. amounts of peptone-sodium taurocholate-Andrade broth were measured into a series of tubes. Various amounts of sterile 1 per cent. glucose solution were added to these and sufficient sterile water to bring the volume of each to 10·0 c.c. The tubes were inoculated with *B. paratyphosus* B and incubated at 37° C. for 24 hours, when readings were made.

No.	% of glucose present	Colour of medium after incubation
1	0·20	Red
2	0·15	Red
3	0·125	Red
4	0·10	Red
5	0·075	Pinkish red
6	0·060	Very faint orange-pink
7	0·050	Orange brown

Tubes nos. 1–4 would have been read as "full acid," and tube no. 5 as slight acid. No acid would have been recorded in the case of tubes nos. 6 and 7.

With medium as used by us for the examination of shell-fish less than 0·075 per cent. of glucose cannot give rise to any fallacy through the fermentation of that sugar. For the tube to which 0·5 c.c. of R.M. is added, 30 c.c. of medium are employed. About 60 per cent. of R.M. is derived from the mussel body, say therefore that we add the equivalent of 0·3 g. of mussel body to 30 c.c. of medium. The mussel would have to contain or produce 7·5 per cent. of glucose in order to supply enough of the sugar to cause a slight acid reaction in the medium when it was fermented. It is improbable that R.M. ever contains sufficient glucose to introduce this fallacy when our method is used. In order to cause this fallacy in the tube to which is added 0·1 c.c. of R.M., the mussel would have to contain the impossibly large amount of 18 per cent. of glucose in order to produce a slight acid reaction.

With fluid media the fallacy in relation to acid-production can therefore be avoided by using a suitably buffered medium in sufficient quantity. When solid media are employed the volume cannot be increased indefinitely. Dodgson used 15·0 c.c. of MacConkey's medium in a 3½ in. Petri dish for the cultivation of the bacteria in 1·0 c.c. of mussel mince. If the mussel examined contained 5 per cent. of glucose, the concentration of glucose in the medium would be 0·2 per cent., more than sufficient to give to colonies of glucose-fermenting bacilli a bright red colour. To reduce the glucose to 0·05 per cent., which would probably eliminate the fallacy, the amount of medium would have to be increased to about 60 c.c., which is quite impracticable. The same objection holds with regard to Salle's medium, which must be poured in very thin layers and, in fact, to all solid media which rely on the fermentation of lactose for the recognition of *B. coli*. The fallacy due to acidity caused by fermentation of mussel carbohydrate can therefore be eliminated by the use of fluid media, but cannot be avoided if solid media are employed.

As regards gas production from glucose the problem is more difficult. It does not arise with solid media, but does with fluid media. If glucose is fermented gas will be produced independent of the volume of medium. If the volume of medium is large some will probably go into solution, and more will thus be got rid of with large volumes of medium than with small. If the reaction of the medium remains slightly alkaline it is probable that some, at least, of the carbon dioxide evolved will be dissolved, and that the gas collected will consist chiefly of hydrogen. Unless, therefore, the amount of glucose in the mussels examined is unusually high, it is doubtful if the amount of gas collected will lead to false positive results. Even if quite large amounts of gas are produced by the action of glucose fermenters on mussel glucose no fallacy can arise in our method if the correct medium, in adequate amounts, is used, and if for positive results, in addition to gas production, a full acid reaction be insisted upon.

DISCUSSION

It is believed that the method here described for the bacteriological examination of mussels is superior to any in common use. The standardisation of the volume of mussel and the elimination of shell fluid together with the examination of three volumes from each mussel make the test a quantitative rather than a qualitative one, and the duplication of tubes greatly reduces inaccurate results due to chance in sampling.

The investigation has shown the great variety of bacteria which are present in mussels, and which ferment lactose with the production of acid and gas, or acid only. Eyre (1924) states: "The value of the presumptive evidence as to the presence of *B. coli* can be readily tested by plating out "positive" tube cultivations, isolating separate colonies, and working them through in accordance with well-known bacteriological methods. Having done so in hundreds of instances, I can fully endorse Klein's dictum: 'If material taken from an oyster...and implanted in this fluid turns it in and within twenty-four hours at 37° C. red and full of gas, we may safely conclude that this material contains the *B. coli*.'" (Klein in his dictum mentioned the mussel as well as the oyster.) If by "full of gas" Klein and Eyre mean literally that the inner tube is completely filled with gas, the statement may be true. If, however, this is a necessary requirement, it is certain that many tubes containing *B. coli* will be counted as negative. There is reason to believe that "full of gas" is interpreted as meaning that a reasonable volume of gas has collected in the inner tube. If this is so, it is not correct to state that all tubes showing such a change contain *B. coli*. In this investigation acid and gas in L.B.B. tubes were produced by eight types of coliform bacilli, by other Gram-negative bacilli, and by one other organism.

Despite the variable bacterial flora of positive tubes, it is believed that the test furnishes a very satisfactory method of determining the degree of contamination of mussels. With the method of reading adopted (full acid and a reasonable amount of gas in 24 hours), the method is positive with 93·1 per cent. of the typical *B. coli* found to be present. In 97·2 per cent. of the positive tubes coliform bacilli were present, and in 61·2 per cent. of positive tubes *B. coli* (typical or only slightly atypical) were found.

The standards suggested are believed to be reasonable in avoiding both excessive stringency and leniency. They will not pass mussels likely to cause illness in consumers, nor will they prohibit the use of mussels from safe beds. Further experience may suggest the advisability of modifications in the standards, but it is hoped that the method will secure wide approval.

The only objection to the method is that it takes more time and more materials than some in common use. We believe that the advantages gained in greater accuracy and fuller information outweigh these disadvantages.

SUMMARY

1. This paper reports the results obtained by the cultivation of the bacteria of ten batches each of ten mussels, 100 mussels in all, in lactose bile broth.

2. From these mussels 371 cultures of bacteria were obtained. 30·7 per cent. of these were not Gram-negative bacilli, 10·0 per cent. were Gram-negative bacilli which did not ferment lactose and glucose, 59·3 per cent. were coliform bacilli.

3. By reading the results in lactose bile broth after 24 hours' incubation, and by regarding as positive those tubes which show strong acid and good gas production the most reliable results were obtained. 93·0 per cent. of all typical, and 83·8 per cent. of all typical and atypical *B. coli* found gave this reaction. 37·6 per cent. of the organisms giving this reaction were typical *B. coli*, 61·1 per cent. of them were typical or atypical *B. coli*, and 97·2 per cent. of them were coliform bacilli.

4. The writer's method is described in detail. Its essentials are the rejection of shell fluid, the standardisation of the volume of each mussel at 25 c.c. by the addition of saline, the inoculation of three volumes of mussel mince into lactose bile broth—0·5, 0·1 and 0·02 cc., each in duplicate, and the regarding as positive each of these amounts only when acid and gas are produced in 24 hours in both tubes.

5. It is suggested that a batch of mussels showing more than seven positives in the tubes inoculated with 0·5 cc.. of mince, or more than three in the tubes inoculated with 0·1 c.c. or more than one in the tubes inoculated with 0·02 c.c. should be considered as contaminated to an undesirable extent.

6. Three types of solid media—Salle's, MacConkey's and Burke-Gaffney's—were tested. None were considered suitable for the bacteriological examination of mussels.

7. Reasons are given for considering any solid medium inferior to fluid medium for the bacteriological examination of mussels.

ACKNOWLEDGMENTS. The writer acknowledges with gratitude the help afforded him by Mr G. H. Farran of the Department of Lands and Fisheries, who kindly arranged for the collection of the batches of mussels. He is also grateful to his assistants, Dr L. L. Griffiths and Dr C. H. Adderley, for their help in the investigation.

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(*MS. received for publication* 30. I. 1934.—Ed.)