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The Presumptive Enumeration of Lactose Negative as well as Lactose Positive *Enterobacteriaceae* in Foods

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Seeliger (1952) and Henriksen (1955) have rightly pointed out that in the current methods of detection or enumeration of coliform bacteria in foods, that is, with the help of solid or liquid lactose-base media, an important class of *Enterobacteriaceae* of public health significance, namely, the *Paracolobacteriaceae* (Stuart *et al.*, 1943; Hobbs *et al.*, 1949; Murphy and Morris, 1950; Edwards and Ewing, 1952; Heyl, 1954; is missed. Henriksen (1955) has suggested that this situation be corrected by substituting in current formulae mannitol for lactose because mannitol is a polyol that is fermented with gas formation by many *Enterobacteriaceae* including most *Paracolobacteriaceae*. So far, Henriksen's excellent suggestion has neither been applied in food microbiology, nor even received proper attention. We have investigated therefore, the possibilities for applying mannitol base media in the hygienic evaluation of foods.

MATERIALS AND METHODS

Preliminary Experiments

As a first step, 65 strains of *Enterobacteriaceae* present in the culture collection of the Central Institute for Nutrition Research were seeded in a medium containing brilliant green bile, 1 per cent mannitol, peptone and water. This was done to check their expected ability to ferment mannitol in the presence of these current inhibitors. Among the cultures investigated were 16 strains of the genus *Escherichia*, 13 strains of *Klebsiella*, 6 strains of Paracolobacter, 2 cultures of *Proteus*, 27 strains of the genus *Salmonella* and one species of *Serratia*. All strains, except those belonging to *Proteus*, *Serratia*, Group *Providencia*, and two of the five anaerogenic *Salmonella* species tested (Mossel *et al.*, 1957) formed copious amounts of gas in this medium, and mostly so within 24 hr at 37C.

Use of a Solid Medium

It is preferred generally in diagnostic bacteriology to use solid instead of liquid media because, in the former, competition phenomena play virtually no role, while the result that isolation of primary cultures is a far more reliable procedure. This consideration led to using the well-established crystal violet neutral red bile agar (violet red bile agar of Bartram and Black, (1936)) for the present purpose. Though in this formula lactose could have been replaced by mannitol, the complete medium supplemented with mannitol is preferred to allow laboratories, who so far have used the dehydrated culture medium for the enumeration of coliform bacteria, to continue to do so. The new medium is prepared by adding 1 per cent of *d*-mannitol to the dehydrated medium and dissolving it together with the other ingredients.

The formula of the medium, for which we propose the name CNBLM agar, is therefore: Yeast extract, dehydrated, 3 g; peptone, 7 g; sodium chloride, 5 g; lactose (optional), 10 g; *d*-mannitol, 10 g; bile salts, dehydrated, 1.5 g; crystal violet, 2 mg; neutral red, 30 mg; agar, 15 g; water, 1 L (pH = 7.4 $\pm$ 0.1).

The medium is not sterilized but, rather, as is usual with the classical violet red bile agar, pasteurized by heating the ingredients suspended in water to about 100 C immediately before use. This heat treatment is

TABLE 1

Comparative counts of the four main groups of Enterobacteriaceae

Species	TGY Agar*	CNBL Agar†	CNBLM Agar‡
Escherichia coli B	9.1×10^7	—	9.6×10^7
E. coli C	3.0×10^8	2.3×10^8	2.4×10^8
E. coli D	2.2×10^9	2.0×10^9	2.1×10^9
E. coli J	1.4×10^8	1.4×10^8	1.5×10^8
E. coli R	1.4×10^8	1.0×10^8	1.0×10^8
E. coli RCI, 1601	3.0×10^8	1.3×10^8	1.5×10^8
E. coli V	2.4×10^8	2.2×10^8	2.2×10^8
E. freundii	6.5×10^8	4.9×10^8	5.3×10^8
Klebsiella aerogenes 598 (*Aerobacter*)	5.6×10^8	5.9×10^8	5.3×10^8
K. aerogenes 599 (*Aerobacter*)	2.5×10^7	1.7×10^7	2.9×10^7
K. aerogenes 959 (*Aerobacter*)	1.6×10^8	1.8×10^8	1.9×10^8
K. cloacae (*Aerobacter*)	3.0×10^8	3.1×10^8	3.0×10^8
Arizona Group	1.9×10^8	1.3×10^8§	1.5×10^8
Bethesda-Ballerup Group	4.4×10^8	5.6×10^8	7.0×10^8
Hafnia Group	4.6×10^9	4.7×10^9§	4.2×10^9
Providencia Group	1.9×10^8	1.5×10^8§	1.7×10^8§
Paracolobactrum aerogenoides	1.3×10^9	1.2×10^9§	1.2×10^9
P. coliforme	2.3×10^8	1.8×10^8§	1.8×10^8
Proteus B	7.8×10^6	4.2×10^6¶	3.7×10^6§
Proteus W	4.3×10^9	4.5×10^8¶	4.4×10^8§
Salmonella anatum (*S. anatis*)	1.8×10^8	—	1.9×10^8
S. sp. (Type Bareilly)	5.1×10^8	—	3.9×10^8
S. sp. (Type Berta)	7.9×10^7	—	8.0×10^7
S. bovis-morbificans (*morbificans*)	1.1×10^9	—	0.7×10^9
S. sp. (Type Braenderup)	7.0×10^7	—	6.0×10^7
S. choleraesuis, H_2S − 22	1.0×10^8	—	0.7×10^8
S. choleraesuis, H_2S + 22	1.3×10^9	—	1.2×10^9
S. sp. (Type Dublin) 3	1.0×10^9	—	1.0×10^9
S. sp. (Type Dublin) 19	2.4×10^8	—	1.9×10^8
S. enteritidis	1.1×10^8	—	0.8×10^8
S. sp. (Type Kaapstad)	3.3×10^9	—	3.1×10^9
S. sp. (Type Newington)	5.5×10^8	—	3.5×10^8
S. sp. (Type Newport)	4.6×10^8	—	4.5×10^8
S. sp. (Type Oranienburg)	1.0×10^9	—	1.0×10^9
S. sp. (Type Oregon)	2.4×10^9	—	3.5×10^9
S. paratyphi A	4.4×10^8	—	3.7×10^8
S. paratyphi B	7.2×10^8	—	6.0×10^8
S. pullorum	1.7×10^9	—	1.0×10^9§
S. pullorum R	1.3×10^8	—	1.2×10^8
S. sp. (Type Sendai)	2.8×10^8	—	3.1×10^8
S. sp. (Type Senftenberg)	4.2×10^8	—	4.0×10^8
S. suipestifer (*S. choleraesuis*)	1.0×10^9	—	0.8×10^9
S. typhimurium	3.4×10^9	—	3.0×10^9
S. typhisuis	7.0×10^6	—	$<10^6$

TABLE 2

Comparative counts of bacteria potentially interfering with the enumeration of Enterobacteriaceae in mannitol media

Species	TGY Agar	CNBL Agar	CNBLM Agar
Bacillus cereus, Smith 232	2.1×10^6	—	<10
B. cereus, Smith 233	3.8×10^5	—	<10
B. polymyxa	3.4×10^6	—	<10
B. polymyxa 7575	2.0×10^7	—	<10
B. subtilis, Marburg strain	2.0×10^5	—	<10
B. subtilis B 91	6.3×10^6	—	<10
Staphylococcus aureus 1829 (*Micrococcus pyogenes* var. *aureus*)	3.6×10^8	<10	<10
S. aureus 1850	8.9×10^8	<10	<10
S. aureus 1878	4.6×10^8	<10	<10
S. aureus 1910	2.0×10^8	<10	<10
S. aureus 1973	2.3×10^8	<10	<10
S. aureus 1989	1.6×10^8	<10	<10
Streptococcus faecalis Reading N 83	6.2×10^7	<10	<10
S. faecium P 6	1.9×10^7	<10	<10
S. zymogenes	1.8×10^7	<10	<10
S. lactis G 16	3.8×10^6	<10	<10
S. lactis 30	3.1×10^6	<10	<10
S. lactis 3132	2.4×10^6	<10	<10

sufficient, because, like count plates of the original lactose base agar, plates of the new medium are incubated for about 18 hr at 36 ± 1 C.

RESULTS

Tests with Enterobacteriaceae. A total of 44 strains of *Enterobacteriaceae* were counted in tryptone glucose yeast extract agar (TGY agar; Buchbinder *et al.*, 1953) after 3 days incubation at 32 C, and in crystal violet neutral red bile lactose mannitol agar (CNBLM) after about 18 hr at 37 C. The strains were 8 cultures of *Escherichia*, 4 of *Klebsiella*, 6 of paracolobacter, 2 of *Proteus*, and 24 of *Salmonella*. The coliform and paracolobacter strains were also counted in standard violet red bile agar (CNBL) after 18 hr at 37 C to obtain a comparison of the two media.

The results obtained are shown in table 1. It appears that the new mannitol agar (CNBLM) is as suitable for counting coliforms as the old one (CNBL), but moreover permits almost quantitative recovery of many paracolobacters and salmonellae, which grow barely or not at all in violet red bile agar. The new

* Tryptone glucose yeast extract agar.

† Violet red bile agar.

‡ Crystal violet neutral red bile lactose mannitol agar.

§ Colonies of diameter < 1 mm.

¶ Pin point colonies.

The species designations included in the table have been modified by the editor to conform with Bergey's Manual of Determinative Bacteriology, 6th edition, The Williams & Wilkins Company, Baltimore, 1957.

CNBLM agar therefore fulfills the requirement that many lactose negative *Enterobacteriaceae* are detected that are ordinarily missed when foods are tested for faecal contamination.

It should be realized, however, that many *Proteus* bacteria as well as one type of *Paracolobacteriaceae*, the group *Providencia* (Kauffmann, 1954), could still possibly be missed even when using this improved medium.

Tests with potentially interfering strains. It might be anticipated that mannitol positive bacteria not belonging to the *Enterobacteriaceae* could interfere with counts carried out with the new CNBLM medium.

To investigate this possibility, 6 strains of the genus *Bacillus*, 6 cultures of *Staphylococcus* (*Micrococcus*) and 6 strains of *Streptococcus* were counted comparatively in TGY, CNBL, and CNBLM agar. The data obtained from these counts are summarized in table 2. These results show that growth of mannitol positive non-*Enterobacteriaceae* is negligible, and for this reason the new mannitol agar is also acceptable.

These results do not preclude, however, that other food bacteria form large violet colonies, resembling those of *Enterobacteriaceae* in CNBLM agar, just as they sometimes do in violet red bile agar (Druce *et al.*, 1957). Therefore, the new medium, like violet red bile agar, may only be considered as yielding presumptive counts, which may need confirmation.

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Summary

Violet red bile agar to which one per cent of *d*-mannitol is added immediately before use (CNBLM agar) appeared to secure quantitative recovery of lactose positive as well as many lactose negative *Enterobacteriaceae*. Other mannitol positive bacteria do not grow in the medium.

CNBLM agar might therefore be used for the hygienic evaluation of foods to detect many lactose negative *Enterobacteriaceae* together with coliforms.

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