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# Influence of Carbon Source, Bile Salts and Incubation Temperature on Recovery of *Enterobacteriaceae* from Foods Using MacConkey-type Agars

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**ABSTRACT**

The plea made many years ago (*27*) to replace the ill-defined coli-aerogenes ('coliform') bacteria as indicator organisms in foods processed for safety with the *Enterobacteriaceae* which are taxonomically accurately defined and as a rule more abundant has of late been more generally accepted. This called for development of a rigorously standardized formula for violet red bile glucose agar and for assessment of the optimal incubation temperature. Four reference strains of *Enterobacteriaceae*, 120 samples of minced meat and 100 samples of frozen broiler chickens were used in these studies. Considerable differences in the performance of commercially available dried formulae, when used as poured plates were observed. These applied both to productivity and to the type of colony produced by a given pure culture. As expected, replacement of lactose plus glucose by an equimolar amount of glucose did not influence the performance of the medium. Intrinsic toxicity of some batches of medium to non-stressed *Enterobacteriaceae* appeared to be mainly responsible for substandard performance. It could be overcome by careful selection of the preparations of crystal violet and particularly the bile salts (*39*) used in the formulae. Incubation at 30 C led to higher confirmed colony counts in minced meat than at 37 C. However, incubation temperature did not greatly influence similar counts in broiler drip. This observation could be substantiated by identification of the types of *Enterobacteriaceae* isolated from the two commodities. Psychrotrophic species predominated in minced meats, which are often made from raw materials stored for some time under refrigeration, whereas mesophilic species were in the majority on frozen broilers, which are generally frozen shortly after slaughter.

In monitoring foods processed for safety (*11,33*), it is advantageous to replace the coli-aerogenes bacteria, indicator organisms used in the past, by the taxonomically better defined and more abundant *Enterobacteriaceae* (*38*). Subsequently it has been suggested to apply *Enterobacteriaceae* counts also to unprocessed foods (*4,8,19,25,44*). Extreme care should be exercised in doing so. Particularly, any conclusions about lack of safety or mishandling drawn from high counts should be based on studies on the mesophilic or psychrotrophic character of the *Enterobacteriaceae* isolated (*28,38,40*).

For enumeration of the entire *Enterobacteriaceae* group MacConkey's agar with glucose added was originally recommended (*29*). For various reasons it seemed better to replace lactose in MacConkey's medium with glucose. The effect of this substitution had to be assessed experimentally. In addition, we had repeatedly observed, that the properties of the bile salts, and to a lesser extent of the triphenyl methane dyes, used in this type of medium could significantly influence its productivity (*32,34,36,37,39*). This investigation was designed to study this effect in more detail.

Furthermore, the temperature of incubation of MacConkey-type agar plates is worthy of consideration. Occurrence of psychrotrophic *Enterobacteriaceae* in foods has repeatedly been demonstrated (*6,10,12,15,21, 24,28,41,42,45,46,47,56*). Incubation at 35-37 C leads to suppression of many of these strains (*14*) and hence to an underestimation of the degree of recontamination of products processed for safety. On the other hand, we observed that incubation at lower temperatures occasionally led to the growth of non-fermentative bacteria in MacConkey-type agars, even when these were overlayered with sterile agar (Harrewijn and Mossel, 1971, unpublished). In this investigation the influence of the incubation temperature in the range 30 to 37 C on gross and confirmed counts of typical colonies in MacConkey type agars was therefore also studied.

## MATERIALS AND METHODS

### *Pure cultures*

The pure cultures studied in this investigation originated from the collection of the Laboratory of Microbiology, Department of the Science of Food of Animal Origin. They consisted of *Escherichia coli*, *Klebsiella* sp., *Salmonella typhimurium* and *Shigella sonnei* cultures. Before use they were streaked on lactose lauryl sulfate agar (*11*) and incubated overnight at 37 C. After their mode of attack on lactose had been verified, their key properties were determined by deep stabbing into gram negative diagnostic tubes (*36*).

### *Minced meat samples*

One hundred and twenty samples of minced meat were obtained from 10 different butcher's shops within 11 months. The minced meat was composed of approximately equal parts of pork and beef.

### *Samples of drip from frozen broilers*

Ten samples from each of 10 different consignments of frozen broilers were defrosted for about 24 h at 4 ± 2 C. Thereupon the packs were hand-pressed (*33*) and about 5 ml of drip were withdrawn using a sterile syringe.

### *Culture media*

In the pure culture studies two non-selective media, tryptone glucose yeast extract agar (Difco[1]) and tryptone soya peptone agar (Oxoid[1]) were used. The media were prepared following the manufacturers' instructions, and after sterilization, tempered at 47-51 C to prepare poured plates.

[1]*Brand names are presented for the purpose of identification of materials used in this investigation. Their mentioning does not imply recommendation.*

The violet red bile agars used in this investigation originated from four different manufacturers. They will be indicated as A, B, C and D. Media A and B contained 10 g of lactose/l (VRBL), and a further 10 g of glucose/l was added, as done previously (VRBLG). Medium C contained 10 g of glucose/l only (VRBG). From brand D two different formulae were tested, $D_1$ the lactose-containing formula with glucose added (VRBLG) and $D_2$ the medium containing glucose only (VRBG). For the remainder, the media were identical in composition, containing per 1 l of distilled water: peptone, 7 g; yeast extract, 3 g; NaCl, 5 g; bile salts, 1.5 g; neutral red, 30 mg; crystal violet, 2 mg; and agar 15 g.

All media were prepared by bringing to a boil to dissolve the ingredients, and were not further sterilized. One brand, chosen at random (A), was also tested after sterilization for 15 min at 121 C. This medium is indicated as A(S).

*Plating procedure*

Pure cultures were incubated overnight at 37 C on nutrient agar (Oxoid). From these a series of decimal dilutions in peptone-saline solution was prepared in such a way, that at least one could be expected to contain 150 ± 50 cfu/ml. As a rule, three successive decimal dilutions had to be examined to attain this objective.

Minced meat samples were homogenized in 25-g portions with 250 ml of peptone-saline solution in a "stomacher" and, after allowing resuscitation of stressed cells for 2 h at room temperature (*20,30,49,50,51,52,59*), further decimal dilutions up to $10^{-4}$ were prepared. A series of three dilutions was examined in every experiment, again with the purpose of obtaining at least one series of duplicate plates containing approximately 150 colonies.

Broiler drip was kept for 2 h at room temperature before dilutions were made, once more to allow resuscitation of cells injured by freezing.

One-ml portions of dilutions of pure cultures, minced meat and drip were transferred to 9-cm petri dishes, thoroughly mixed with 15 ml of tempered agar, and allowed to solidify. Subsequently the plates were overlayered with 10 ml of sterile medium, tempered at 47-51 C. Incubation was done at 30 or 37 C for 18-20 h.

## RESULTS

### *Pure culture studies*

The colony counts obtained with the various media have been summarized in Table 1. An assessment of the types of colonies is presented in Table 2. This criterion is of some relevance since at one time it was thought that the diameter of the colony and of the precipitation zone around it could help in recognition of particular *Enterobacteriaceae* types (*19*).

It is quite obvious that considerable differences in performance of the various media occurred. The differences in the numbers of colonies remained within reasonable limits, although the recovery was usually maximal in $D_1$ and $D_2$. Considerable differences in colony size, variation in colony size and in particular the character of the precipitation zones were observed. The media $D_1$ and $D_2$ were markedly better than the other brands. The type of carbon source, lactose + glucose or only glucose did not influence the counts or colony types.

TABLE 1. *Recovery of pure cultures of* Enterobacteriaceae *in a variety of MacConkey-type selective agar media.*

| Strain | Number of replicates | Recovery, in vertical order min., max., mean, as fraction of colonies obtained from same inoculum in the non selective reference agar medium | | | | | |
|---|---|---|---|---|---|---|---|
| | | Medium and type of sugar | | | | | |
| | | A[a] L + G[b] | A (S) L + G | B L + G | C G | $D_1$ L + G | $D_2$ G |
| *Escherichia coli* | 6 | | | 0.54 | 0.59 | 0.83 | 0.65 |
| | | NT | NT | 0.83 | 0.90 | 1.02 | 0.92 |
| | | | | 0.68 | 0.78 | 0.93 | 0.76 |
| *Klebsiella* | 4 | 0.42 | — | 0.30 | 0.24 | 0.49 | 0.43 |
| | | 0.42 | — | 0.64 | 0.57 | 0.50 | 0.50 |
| | | 0.42 | 0.31 | 0.47 | 0.41 | 0.50 | 0.46 |
| *Salmonella typhimurium* | 6 | | | 0.48 | 0.41 | 0.88 | 0.81 |
| | | NT | NT | 0.84 | 0.90 | 1.15 | 0.91 |
| | | | | 0.75 | 0.65 | 0.99 | 0.86 |
| *Shigella sonnei* | 4 | 0.85 | — | 1.09 | 0.83 | 1.02 | 0.87 |
| | | 0.95 | — | 1.16 | 0.96 | 1.12 | 1.11 |
| | | 0.89 | 0.98 | 1.13 | 0.90 | 1.07 | 0.99 |

[a] A-D = different manufacturers (cf. text), (S) = medium sterilized instead of pasteurized, as is customary.
[b] L = lactose, G = glucose, NT = not tested.

TABLE 2. *Character of colonies obtained from pure cultures in a variety of MacConkey-type selective agar media.*[a]

| Strain | Medium and type of sugar | | | | | |
|---|---|---|---|---|---|---|
| | A L + G | A (S) L + G | B L + G | C G | $D_1$ L + G | $D_2$ G |
| *Escherichia coli* | | | 0.60 | 0.70 | 0.85 | 1.00 |
| | NT | NT | 0.50 | 0.50 | 1.0 | 0.87 |
| | | | 2 | 1 | 3 | 3 |
| *Klebsiella* | 0.50 | 0.50 | 0.75 | 0.50 | 1.0 | 0.75 |
| | 0 | 0 | 0.50 | 0 | 0.50 | 0.62 |
| | 0 | 0 | 2 | 0 | 3 | 2 |
| *Salmonella typhimurium* | | | 0.95 | 0.65 | 1.10 | 1.12 |
| | NT | NT | 1.2 | 0.50 | 1.0 | 1.50 |
| | | | 2 | 0 | 3 | 3 |
| *Shigella sonnei* | 0.75 | 0.75 | 1.0 | 1.0 | 1.3 | 1.12 |
| | 0 | 0.50 | 0.60 | 0.50 | 0.80 | 0.50 |
| | 0 | 0.50 | 2 | 1 | 3 | 2 |

[a] Parameter combination: colony size, average, mm;/ precipitation zone, *do.*;/quality of precipitation zone, expressed in 3 = excellent, 2 = good, 1 = fair, 0 = absent.

Consequently another medium component, most probably the bile preparation (*5,7,37,39*) influenced performance of the MacConkey-type media.

*Lactose versus glucose at 30 and 37 C*

To study the effect of lactose in comparison to that of glucose, agars purchased from manufacturer D were used in all experiments, because the pure culture studies reported in the previous section indicated that much more easily countable colonies were obtained from these agars. In Tables 3 and 4 the data obtained with VRBG and VRBL are recorded. Minced meat was used as the inoculum. Colony counts were confirmed by testing the square root of the total number of colonies obtained, chosen at random, for the criteria of *Enterobacteriaceae*: negative oxidase reaction and fermentative attack on glucose. Three-layer gram negative diagnostic tubes, as described elsewhere (*36*) were used for this purpose.

TABLE 3. *Effect of glucose on the recovery of* Enterobacteriaceae *from minced meat using VRB agars*[a] *incubated at 37 C.*

| Percentage of the samples | $\log_{10}cfu_{VRBG} - \log_{10}cfu_{VRBL}$ |
|---|---|
| 14 | 0.00 |
| 22 | 0.00-0.10 |
| 53 | 0.11-0.50 |
| 11 | > 0.50 |

Av. $\log_{10}cfu_{VRBG\ net} = 3.61$; av. $\log_{10}cfu_{VRBL\ net} = 3.53$.

[a]VRBG = violet red bile glucose agar; VRBL = violet red bile lactose agar; net = confirmed as *Enterobacteriaceae*.

TABLE 4. *Effect of glucose on the recovery of* Enterobacteriaceae *from minced meat using VRB-agars incubated at 30 C.*

| Percentage of the samples | $\log_{10}cfu_{VRBG} - \log_{10}cfu_{VRBL}$ |
|---|---|
| 6 | 0.00 |
| 19 | 0.00-0.10 |
| 41 | 0.11-0.50 |
| 34 | > 0.50 |

Av. $\log_{10}cfu_{VRBG\ net} = 4.26$; av. $\log_{10}cfu_{VRBL\ net} = 3.92$. Difference 0.34.

In analyzing the data, the results of earlier studies on reproducibility of counts in MacConkey type agars (*27,38*) were used as the base line. These had indicated that the confidence interval of such counts was of the order of a factor 2; therefore differences below 0.3 $\log_{10}$ were not considered as significant. Applying this approach to the data in Tables 3 and 4 it appears that net counts at 37 C did not depend on the carbon source, but those at 30 C did.

The figures in Tables 3 and 4 also indicate that net recoveries at the lower temperature are considerably higher than at 37 C. This is further analyzed in Tables 5 and 6. The differences between confirmed counts at 30 and 37 C were greater in VRBG than in VRBL.

As indicated earlier, we had observed in preliminary studies that the rate of confirmation as *Enterobacteriaceae* of typical colonies in MacConkey-type agars increased with increasing temperature of incubation. The data obtained in this investigation were analyzed for this effect in Tables 7-10. Such a difference was not

TABLE 5. *Influence of the incubation temperature on the recovery of* Enterobacteriaceae *from minced meat using VRBG-agar.*

| Percentage of the samples | $\log_{10}cfu_{30\ C} - \log_{10}cfu_{37\ C}$ |
|---|---|
| 5 | 0.00 |
| 9 | 0.00-0.10 |
| 41 | 0.10-0.50 |
| 45 | > 0.50 |

Av. $\log_{10}cfu_{30\ C\ net} = 4.26$; av. $\log_{10}cfu_{37\ C\ net} = 3.61$. Difference 0.65.

TABLE 6. *Influence of the incubation temperature on the recovery of* Enterobacteriaceae *from minced meat using VRBL-agar.*

| Percentage of the samples | $\log_{10}cfu_{30\ C} - \log_{10}cfu_{37\ C}$ |
|---|---|
| 15 | 0.00 |
| 23 | 0.00-0.10 |
| 36 | 0.10-0.50 |
| 26 | > 0.50 |

Av. $\log_{10}cfu_{30\ C\ net} = 3.92$; av. $\log_{10}cfu_{37\ C\ net} = 3.53$. Difference 0.39

TABLE 7. *Confirmation rate of typical colonies obtained from minced meat using VRBG-agar incubated at 37 C.*

| Percentage of the samples | $\log_{10}cfu_{gross} - \log_{10}cfu_{net}$ |
|---|---|
| 71 | 0.00 |
| 5 | 0.01-0.05 |
| 5 | 0.06-0.10 |
| 19 | 0.10 max. |

Av. $\log_{10}cfu_{net} = 3.61$; av. $\log_{10}cfu_{gross} = 3.76$. Difference 0.15

TABLE 8. *Confirmation rate of typical colonies obtained from minced meat using VRBG-agar incubated at 30 C.*

| Percentage of the samples | $\log_{10}cfu_{gross} - \log_{10}cfu_{net}$ |
|---|---|
| 75 | 0.00 |
| 6 | 0.01-0.05 |
| 6 | 0.06-0.10 |
| 13 | 0.10 max. |

Av. $\log_{10}cfu_{net} = 4.26$; av. $\log_{10}cfu_{gross} = 4.31$.

TABLE 9. *Confirmation rate of typical colonies obtained from minced meat using VRBL-agar incubated at 37 C.*

| Percentage of the samples | $\log_{10}cfu_{gross} - \log_{10}cfu_{net}$ |
|---|---|
| 86 | 0.00 |
| 2 | 0.01-0.05 |
| 7 | 0.06-0.10 |
| 5 | > 0.10 |

Av. $\log_{10}cfu_{net} = 3.53$; av. $\log_{10}cfu_{gross} = 3.55$.

TABLE 10. *Confirmation rate of typical colonies obtained from minced meat using VRBL-agar incubated at 30 C.*

| Percentage of the samples | $\log_{10}cfu_{gross} - \log_{10}cfu_{net}$ |
|---|---|
| 79 | 0.00 |
| 2 | 0.01-0.05 |
| 12 | 0.06-0.10 |
| 7 | > 0.10 |

AV. $\log_{10}cfu_{net} = 3.91$; av. $\log_{10}cfu_{gross} = 3.94$.

observed in any of the data, the confirmation rates of typical colonies being very high in all instances. This disagreement with the results of previous studies may be caused by two factors. First, the earlier investigations had been carried out with quite different MacConkey-type agar formulae; as we have observed that some of such media lead to poorly discernible colonies, our previous results may have suffered from this shortcoming of the media used. In addition, minced meat might contain less interfering types of organisms than the commodities studied earlier, which were mainly dried foods.

*Lactose plus glucose versus glucose at 30 and 37 C*

The results of the experiments with pure cultures recorded in Tables 1 and 2, point to the absence of any influence of the use of glucose alone or in combination with lactose on the productivity of MacConkey-type agars as expected. This required yet further substantiation by the use of the two types of media for examination of foods containing considerable numbers of *Enterobacteriaceae.* Minced meat and chicken drip were chosen as such substrates.

The data obtained with minced meat are summarized in Table 11. Once more, considering differences below $\log_{10} = 0.30$ as non-significant because within the confidence interval of the mode of examination, the results for glucose and glucose plus lactose were identical Again, the differences between net and gross counts were negligible. And, once more the counts at 30 C were higher than those at 37 C.

TABLE 11. *The influence of carbon source and incubation temperature on the recovery of* Enterobacteriaceae *from minced meats.*

| Incubation temperature | Gross ($\log_{10}$ cfu/g) | | Confirmed ($\log_{10}$ cfu/g) | |
|---|---|---|---|---|
| (C) | VRBLG | VRBG | VRBLG | VRBG |
| 30 | 4.46 | 4.42 | 4.37 | 4.40 |
| 37 | 4.14 | 4.11 | 4.14 | 3.90 |

The results of the examination of the samples of broiler drip are summarized in Table 12. In agreement with the observations made on minced meat, gross and confirmed counts did not differ, and the carbon source did not exert any influence. However, with broiler drip the temperature of incubation did not influence the colony counts. We had observed earlier that the types of *Enterobacteriaceae* isolated from minced meat were quite different from those present in chicken drip. Among the latter *Escherichia coli* seemed to predominate, whereas from minced meat mostly psychrotrophic types were isolated. To investigate this matter in more detail, 72 colonies taken at random from plates of minced meat, and 68 picked similarly from plates of chicken drip were identified. The following procedure was used for this purpose: (*a*) the three layer tubes referred to earlier, allowing assessment of oxidase reaction, mode of attack on glucose, motility and formation of $H_2S$ and indole; (*b*) slants of Simmons citrate agar, DNA agar, urea agar and gelatin agar. The

TABLE 12. *The effects of carbon source and incubation temperature on the recovery of* Enterobacteriaceae *from broiler drip.*

| Incubation temperature | Gross ($\log_{10}$ cfu/g) | | Confirmed ($\log_{10}$ cfu/g) | |
|---|---|---|---|---|
| (C) | VRBLG | VRBG | VRBLG | VRBG |
| 30 | 1.97 | 2.03 | 1.97 | 2.03 |
| 37 | 1.96 | 1.94 | 1.96 | 1.94 |

results obtained are presented in Table 13. Over 75% of the isolates obtained from minced meat were typical psychrotrophic types. These are mostly considered to be primarily of aqueous or vegetable origin. Psychrotrophic types occurred only to a total of 30% in chicken drip, with *E. coli* predominating there. It is therefore not surprising that *Enterobacteriaceae* counts in chicken were almost equal at 37 C and 30 C, but higher at the lower temperature when minced meat was examined.

TABLE 13. *Types of* Enterobacteriaceae *isolated from minced meat and drip from frozen broilers in voilet red bile media at 30 C.*

| Species or genus[a] | Percentage of isolates from | |
|---|---|---|
| | Minced meat | Broiler drip |
| *Serratia liquefaciens*[b] | 47 | 12 |
| *Escherichia coli*[b] | 9 | 35 |
| *Enterobacter* | 21 | 6 |
| *Erwinia* | 10 | 12 |
| *Hafnia* | 7 | 13 |
| *Citrobacter* | 4 | 13 |
| *Klebsiella* | < 1 | 6 |
| *Proteus* | < 1 | 3 |

[a]The order is that of decreasing summated frequency.
[b]Including a minority of irregular types.

## DISCUSSION

The investigations reported in this paper substantiate observations made repeatedly in the past.

First, we have demonstrated once more that indiscriminate use of dried formulae of MacConkey-type agars may lead to drastically reduced recoveries of *Enterobacteriaceae* and hence to serious underestimation of health or spoilage risks in foods. This confirms observations on similar and related media by numerous authors (*3,13,22,23,26,34,43,53,54,55,57,58*). These results call for constant monitoring of purchased dried culture media. Such testing should include (*a*) pure culture studies to assess productivity, and (*b*) practical challenge tests to estimate selectivity (*31*).

Furthermore, the possible influence of the incubation temperature on the results of testing for *Enterobacteriaceae* has been demonstrated once more. Incubation at ca. 30 C may lead to consistently higher results than at 37 C, as could be anticipated from physiological studies and ecological surveys carried out many years ago. The consequence of these observations for ecological studies are obvious. However, for practical monitoring the inference is less strict. Results of the bacteriological examination of foods depend strongly on the method by which they were obtained. Consequently Reference Values (*35*) carry little weight, unless accompanied by prescribed methods. Hence, any of the temperatures customarily used so far for enumeration of *Entero-*

*bacteriaceae* may be applied, provided Reference Values are adapted accordingly.

One new, although barely surprising, element emerged from our investigation. Using glucose or glucose plus lactose in MacConkey-type media does not in the least influence confirmed counts of *Enterobacteriaceae* at a given temperature. However, the composition of the media in other respects is of great significance. Particularly characteristics of the bile preparations may result in dramatic differences in recoveries (*5,7,37,39*). For practical purposes the conclusion is obvious: glucose-base media can be used in confidence, provided the bile preparation to be incorporated has been carefully selected by the manufacturer. Nevertheless it remains an obligation of the bacteriologist to check every new batch purchased from suppliers for productivity and selectivity, even when a manufacturer has previously been found to be offering satisfactory media.

With regard to the fundamental concept of replacing lactose by glucose, the data in Table 4 substantiate earlier findings (*11*) that this may lead to higher counts. However, this is not always the case, as the data in Table 3 show - for the simple reason that lactose-negative types of *Enterobacteriaceae* do not always occur in large numbers. Actually the types of *Enterobacteriaceae* colonizing proteinaceous staples may vary greatly, as the data in Table 13 and those of other research teams (*9,47*) clearly indicate. But in no instance will glucose media lead to lower results, as this study and other investigations (*8,16*) demonstrated beyond any doubt. Therefore, use of glucose-containing MacConkey-type media will result in data of considerably better consistency than those reported for similar media based on lactose (*2,48*).

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