SYNTHETIC CULTURE MEDIA FOR REFERENCE USE IN DAIRY BACTERIOLOGY

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Having noted that different lots of the same brand of milk plating agar showed considerable irregularity in colony counts of the same samples of milk, the authors devised a reproducible synthetic medium in dry form which might serve as a Reference Standard against which different lots of peptone media could be evaluated. Milk samples plated in this synthetic medium yielded colony counts comparable to those obtained in samples of the TGEM medium of two different manufacturers provided only that plates containing the synthetic medium were incubated longer to allow for the somewhat slower development of the colonies.

The standardization of any bacteriological medium, from the standpoint of its chemical composition and hence its nutritive characteristics, in the strict sense of the word presents a difficult problem when such variable ingredients as peptone, meat extract, yeast extract, etc. are employed. This is particularly true when the medium is used for the cultivation of numerous species of bacteria from samples which may vary considerably both as to total number and types present. Such is the case with the present standard Aryptose-glucose-beef extract—milk (1 percent of skimmed milk to be added) agar (TGEM) medium used in bacteriology.

Numerous individual studies and surveys have been conducted in the past in an attempt to arrive at a formula which would be superior to the tryptone glucose beef-extract agar (TGEM) since this medium possesses certain undesirable features (Black).

In such studies and under the present circumstances it is usual to evaluate all candidate media in terms of the corresponding performance of TGEM agar. The assumption must be made that all factory batches of media prepared according to the performance when tested with milk TGEM formula will give identical samples. That this assumption is correct, is improbable.

A more rational and scientific approach to this problem would be to assemble a medium of synthetic composition, consisting of pure chemical compounds, except agar, to serve as a "Reference Medium," against which candidate peptone media or all lots of the standard medium could be judged for suitability as milk count media. Such a "Reference Medium" would have the obvious advantage of being readily and exactly reproducible. To arrive at the formula of such a "Reference Medium" might well be a formidable task, but the tremendous accumulation of knowledge on bacterial nutrition during the past ten years does not make it seem impossible.

The experimental data which follow constitute preliminary attempts toward the development of such a "Reference Medium."

EXPERIMENTAL

In an attempt to formulate a medium of synthetic composition which might support the growth of most of the bacteria present in milk, we have elected to prepare what might be referred to as "shot gun" mixtures of amino acids, vitamins, purines, pyrimidines, salts, carbohydrates and agar. Selection of compounds, the amounts, and combinations, were based upon results reported for the growth of various microorganisms in chemically defined media. Generally, liter quantities of a medium were prepared employing conventional techniques common to preparation of synthetic media for bacteriological studies. The media were sterilized by autoclaving for 10 minutes at 15 lbs pressure.

Evaluation of media prepared in the above manner was attempted by plating samples of raw and pasteurized milk in multiple sets of duplicates. One set of the duplicates would receive TGEM agar (1 percent sterile skimmed milk added) and the remaining sets would receive the experimental synthetic media. Plates were incubated at 35° C for 48 hours, at which time colony counts were made and the size and countability of colonies in various media were compared. The plates were reincubated at the same temperature for an additional 24 or 48 hours (sometimes longer) and the colony count and colony size again determined. After several such trials it was decided to eliminate the use of raw milk samples as the did not provide a severe enough criterion for judging the performance of the synthetic mixes.

One of the first formulae which seemed to warrant more detailed study was the medium designated as S4, the ingredients of which are presented in table 1. Additional quantities of this medium were prepared and inoculated in duplicate sets, using S4 and TGEM agar containing 1 percent sterile skimmed milk. Incubations and recording of results were carried out as described earlier. Generally, four or ten pasteurized milk samples were plated on any one day. Approximately 100 samples have been examined to date, at various times of incubation. It is felt that the number of milk samples counted in the two media is not large enough to permit a valid statistical analysis. Therefore, a series of counts, typical of results obtained with the S4 medium is presented in table 2.

Growth in the S4 medium was extremely slow. Generally only a small percentage of the number of colonies appearing in the TGEM agar at 48 hours were countable in
the S₄ medium at the end of the same incubation time. In addition, the colonies in this medium were extremely small and consequently difficult to count. Upon incubation of the S₄ agar plates for an additional 24 hours, the colony count increased with a corresponding increase in colony size. In many instances the count at this point did not equal the 48-hour count obtained in TGEM agar. After another 24-hour incubation (a total of 96 hours) there appeared to be more general agreement between the 48-hour count in TGEM agar and the 96-hour S₄ agar count. However, some exceptions are to be noted.

A disconcerting feature in these experiments is the extremely variable pattern obtained with various milk samples, which in turn requires preparation of extremely large quantities of the synthetic medium. (Perhaps an alternative method of comparing the performance of the experimental medium might be feasible, i.e. use of pooled milk samples or a selected set of pure cultures used as test organisms). Subsequent experiments were performed in an attempt to improve the performance of the S₄ medium, i.e. to obtain more and larger colonies during an incubation period of less than 96 hours. The following supplements, individually or in various combinations were added to the S₄ formula:

- Amino acids—concentration of each doubled
- Vitamins—
- Purines and pyrimidines—

Addition of:
- Inorganic salts (mgs. per liter): NaCl (10), Fe₃(PO₄)₂ (10), MnSO₄·H₂O (10), CuSO₄·H₂O (trace), MgSO₄·H₂O (200).
- Fumaric acid (0.05 percent) and Sodium ethyl oxalacetate (0.05 percent).
- Sodium citrate (0.05 percent).
- Sodium acetate (5.0 percent).
- Tween 80 (0.2 percent).
- Ascorbic acid (0.1 percent).

**TABLE 2**

<table>
<thead>
<tr>
<th>TGEM</th>
<th>S₄</th>
<th>TGEM</th>
<th>S₄</th>
</tr>
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<tr>
<td>48 hrs.</td>
<td>48</td>
<td>72</td>
<td>96 hrs.</td>
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<tr>
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**TABLE 3**

**TABLE 1**

<table>
<thead>
<tr>
<th>Amino Acids:</th>
<th>Grams per liter</th>
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<tr>
<td>DL-Isoleucine</td>
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<tr>
<td>DL-Valine</td>
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</tr>
<tr>
<td>L-Valine</td>
<td>0.2</td>
</tr>
<tr>
<td>DL-Aspartic acid</td>
<td>0.2</td>
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<tr>
<td>DL-Norleucine</td>
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<tr>
<td>L-tyrosine</td>
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<tr>
<td>DL-Methionine</td>
<td>0.2</td>
</tr>
<tr>
<td>DL-Glutamic acid</td>
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</tr>
<tr>
<td>DL-Threonine</td>
<td>0.2</td>
</tr>
<tr>
<td>DL-Serine</td>
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<tr>
<td>DL-Phenylalanine</td>
<td>0.2</td>
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<tr>
<td>DL-Asparagine</td>
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<tr>
<td>DL-Leucine</td>
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<td>L-Histidine</td>
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<td>DL-tryptophan</td>
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<td>L-Arginine</td>
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<td>L-Lysine</td>
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<tr>
<td>Glycine</td>
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<td>K,HPO₄</td>
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</tr>
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<td>MgSO₄</td>
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<td>L-glucose</td>
<td>5.0</td>
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<tr>
<td>Agar</td>
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</table>

**Vitamins**

- Thiamine: 0.5 mg/liter
- Pyridoxamine: 0.5 mg/liter
- Nicotinamide: 1.0 mg/liter
- Riboflavin: 0.5 mg/liter
- Ca-pantothenate: 0.5 mg/liter
- Folie acid: 0.01 mg/liter

**Purines & Pyrimidines**

- Xanthine: 5.0 mg/liter
- Thymine: 5.0 mg/liter
- Uracil: 5.0 mg/liter
- Adenine: 5.0 mg/liter
- Guanine: 5.0 mg/liter

Obviously, with such an array of media to test, it was not practical to perform a large series of milk counts in each. In most instances, a pooled milk sample was employed, representing five or ten individual samples of pasteurized milk. Several of such pooled samples were used. Plate counts were conducted simultaneously using TGEM agar and the unsupplemented S₄ agar. The usual comparisons were made. A significant and consistent response noted was noted only in the S₄ medium containing sodium ethyl oxalacetate. Colony development, both in total number and size, was superior to that obtained with the S₄ agar. Although the 48-hour count was considerably less than the 48-hour count on TGEM agar, the count after 72 hours incubation in most instances equalled or surpassed the 48-hour TGEM agar count. A series of counts made on pasteurized milk samples using these two media is presented in table 3.

Finally, the S₄ agar supplemented with 0.05 percent sodium ethyl oxalacetate has been compared with TGEM agar with respect to its ability to support the growth of some 50 pure cultures of bacteria. These cultures, selected from a stock culture collection, were streaked (or stabbed) on an agar slant of each medium and incubated at 35°C. Cultures used represented species of the following genera: Micrococcus, Bacillus, Aerobacter, Mycobacterium, Achromobacter, Alcaligenes, Proteus, Lactobacillus, Streptococcus, Pseudomonas, Serratia, Escherichia, and Microbacterium. All organisms grew on both media. Only in a few instances was the growth on the TGEM agar more profuse than on the synthetic agar medium.

**DISCUSSION AND SUMMARY**

Bacterial counts of pasteurized milk samples, as determined in synthetic agar media after a 48-hour incubation period, were considerably lower than those determined by using TGEM agar. However, by prolonging the incubation period (96 hours for S₄ agar and 72 hours for S₄ agar-sodium ethyl oxalacetate) counts approaching those of the 48-hour TGEM agar count could be obtained. Attempts to obtain early colony development on the synthetic agars have thus far failed. The de
using the standard soil as the soilng media. It was found (table 6) that considerable variation, dependent upon the many minor factors in the operation of the washer, was evident in the case of the china dishes when such large numbers of soiling cycles were employed. On the other hand, regardless of the number of cycles, the film remained more or less constant on the used and unused plastic dishes.

It was found also that insofar as an adherence of soil films was concerned, the length of the time which plastic plates were used is not a factor in film retention. In other words, films will adhere as quickly and readily to new as to used plastic dishes.

**Conclusions**

1) Soil films build up faster on surfaces with low (plastic) wetting properties than on surfaces with high wetting properties (china).

2) When soiled with milk or beef and mutton fats, plastic does not clean as readily as china.

3) Soiled plastic after a series of washes cleans easier than cleaned plastic. The soiling is more easily removed from the collected films on the dirty plastic surface than the plastic itself.

4) Continued use and checking (other than appearance) has little effect on relative cleaning possibilities of plastic.

5) China varies greater (but at a lower level) in its film retention than plastic.

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**Synthetic Culture Media**

(Continued from page 91)

It is conceivable that some of the first organisms to grow out might elaborately substances into the medium when they permit growth of the more fastidious types present which develop into colonies after the 72- or 96-hour incubation period.

In a study such as this it is apparent that many combinations of ingredients must be evaluated. It is not practical to prepare these experimental media in large quantities or to perform a large series of counts to determine the effect of each of these changes in the basic formula. At the same time, examination of only a few milk samples can result in erroneous interpretations, depending upon the particular bacterial flora represented in the samples selected. An alternative scheme for assessing the performance of an experimental formula might be the use of single pooled milk samples or the use of a selected group of bacteria, in pure cultures, which might be representative of pasteurized milk flora. This approach is under investigation.

**Conclusions**

The conclusion seems unescapable that such a Reference Medium is needed if the irregular results inherent in peptone media are to be avoided.

**Reference**