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# METHODS FOR THE QUANTITATIVE STUDY OF THE AEROBIC AND ANAEROBIC INTESTINAL BACTERIAL FLORA OF MAN‡

The methods employed for the quantitative isolation of stool bacteria in man have not been standardized. Schaedler, Dubos, and Costello have outlined in detail their methods for isolation of aerobic and anaerobic bacteria in specimens obtained from mice.[1] However, *in vitro* growth factors for the isolation of the bacterial flora of mice are not applicable at all times to man.

While undertaking a study of variations of intestinal bacteria in human disease states, it became apparent that considerable effort was required to establish and standardize reproducible bacteriologic techniques. The present report is a detailed account of the methods we found most satisfactory along with data obtained in normal subjects.

## METHODS

*Stool collection.* Patients were given a sterile disposable petri dish with an applicator stick and asked to deposit a small amount of freshly passed fecal material in the dish. The specimen was cultured within 15 to 30 minutes. (Present data indicate that cold or room temperature storage does not preserve the quantitative relationships of the flora.)[2,3]

A small No. 2 bone currette (Sklar Instruments) (Fig. 1) was found convenient for measuring approximately 0.1 gm. of stool. The amount measured by this instrument varies between 0.09 and 0.11 gm. regardless of whether the stool is firm, soft, or almost liquid. This small variation in weight is negligible in view of the dilution factor necessary for quantitative counting. Liquid feces can be measured by volume. (0.1 ml. approximating 0.1 gm.)

*Dilution of specimen.* One tenth gram or 0.1 ml. of the specimen to be cultured was diluted with 9.9 ml. of norite A charcoal water. Charcoal water was prepared by filtering distilled water over norite A (Nutritional Biochemicals Co., Cleveland) which has marked absorptive properties to remove fine particles. This first tube con-

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taining a 1:100 ($10^{-2}$) dilution was placed on a vigorous shaker (General Purpose Variable Speed Eberbach Shaker) for 15 minutes to emulsify the specimen. The contents were then serially diluted by adding 1 ml. from the first tube to 9 ml. of charcoal water, and so on, until seven tubes were prepared. The first tube prior to serial dilution is a 1:100 ($10^{-2}$) and the last tube represents a 1:100,000,000 ($10^{-8}$) dilution.

*Procedure for streaking.* A loopful from the tube dilutions selected for analysis was then streaked on appropriate solid media. The platinum-rhodium loop has a 4 mm. inside diameter and delivers .01 ml. of fluid (Fig. 1). Therefore, growth from the streak taken from the first tube represents the number of colonies at a $10^{-4}$ ($10^{-2} \times 10^{-2}$) dilution and from the last tube at a $10^{-10}$ ($10^{-2} \times 10^{-8}$) dilution. To determine total bacterial growth, the third, fifth, and seventh tubes are streaked, so that final counting would be $10^{6}$, $10^{7}$, and $10^{9}$ (Fig. 2). If five colonies are detected on the plate representing $10^{-9}$ dilution, the total count on this specimen would be $5 \times 10^{9}$ colonies. If desired, this can be converted into a common logarithmic number of 9.699 which represents the log number of colonies of bacteria per gram of fecal material.

*Media.* Eight selective media were employed for routine isolations. These are listed in Table 1 along with some alternate useful media. (The formula for these media are given in the Appendix.) Additional selective media may be chosen depending upon the organisms to be studied.

Medium A was employed for total counts and two plates were streaked; one was incubated aerobically and the other anaerobically.[1] Medium C was used for the isolation of *Bacteroides spp.* and *Clostridia spp.*,[1] medium G for *Lactobacilleae spp.*,[1] and Rogosa medium for *Veillonellae spp.*[4] Enterococcus M medium was used to isolate gram-positive cocci.[5] All of these media were streaked and incubated anaerobically. Medium G also was incubated microaerophilically in a candle jar, and the E (Tergitol-7),[1,6,7] PEA,[7,8] and XLD,[9] media were incubated aerobically. The XLD medium was streaked with undiluted material for isolation of pathogens since the first streaking at $10^{-4}$ may miss these organisms. Figure 2 demonstrates the method of streaking and results obtained. Table 1 illustrates the dilutions streaked and predominant organisms isolated from the various plates as well as some alternative media.

In separate experiments, A medium was compared to 5% sheep red blood cell agar,[10,11] C medium to a chocolate blood-neomycin agar, and G medium to Rogosa SL agar[8,12,13] and tomato juice agar.[7] Specimens were streaked concomitantly on both media.

*Incubation techniques.* A standard incubator at 37°C. was employed for aerobic incubation. Different anaerobic techniques were tested. A candle jar was used for microaerophilic growth of lactobacilli in order to compare with strict anaerobic methods. For obligate anaerobes we employed an anaerobic incubator (National Instrument Co.) which was maintained at anaerobic conditions by bringing it to a negative pressure of 22 inches of mercury with a standard vacuum pump and then flushed three times with equal parts of $CO_2$ and $N_2$ (Fig. 3). The incubator was maintained at a negative pressure of 9 inches of mercury for incubation at 37 degrees C. for 48 hours. The anaerobic incubator is more expensive than simple anaerobic jars but more efficient and reliable. Some laboratories employ milk cans instead of an anaerobic jar.[1] An essential factor in monitoring the anaerobic conditions is that a container can be used that can be predictably maintained at negative pressure.

Many texts advise the use of oxygen absorber within the container. We found this helpful and the method we employed, suggested to us by Dr. S. Gorbach, was the use of iron pads coated with a copper sulphate solution.[14]

TABLE 1. MEDIA USED FOR QUANTITATIVE ISOLATION OF FECAL BACTERIAL FLORA

| *Medium (substitute*)* | *Final dilutions*** | *Incubation* | *Organisms* | *Confirmation* |
|---|---|---|---|---|
| A (Blood agar) | 4, 6, 8<br>5, 7, 9 | Aer. +An. | Total Aer. and total An. | 0 |
| C (Blood-Neomycin agar) | 5, 7, 9 | An. | Bacteroides, Clostridia | Gram stain, subculture to blood agar for An. & Aer. incubation. |
| G (Rogosa-SL agar) | 5, 7, 9 | An. | Total + | Gram stain, subculture to blood agar if morphology is uncertain. |
| | 5, 7, 9 | Microaerophilic | Aer. Lactobacilli<br>Total Aerobic Lactobacilli | |
| PEA | 4, 6, 8 | Aer. | Streptococci, Diphtheroids<br>Staphylococci | Gram stain, subculture to blood agar for suspected diphtheroids. |
| Enterococcus MG | 4, 6, 8 | An. | Streptococci | Gram stain, subculture to blood agar if necessary. |
| Rogosa's Veillonella | 4, 6, 8 | An. | Veillonellae | Gram stain. |
| E (EMB) | 4, 6, 8 | Aer. | Coliforms, Shigella, Salmonella | If suspicious colonies, subculture to TSI (serotype if desired). |
| XLD (S S, MacConkey's, etc.) | None | Aer. | Shigella, Salmonella | If suspicious colonies, subculture to TSI (serotype if desired). |

* See appendix for formulas and sources.
** Expressed in logarithms.
An. = Anaerobic.
Aer. = Aerobic.

*Recording of data and confirmation of growth of selective media* (Table 1). Total colony counts of aerobic and anaerobic growth were made from medium A. The total anaerobic count often includes aerobic organisms that grew under anaerobic conditions. The aerobic growth was usually significantly less, so that it did not interfere with the final total anaerobic count( see RESULTS). When the anaerobic and aerobic total counts were similar, the significance of the total anaerobic count on the A medium was evaluated further by correlation with counts on the selective anaerobic media, C, G, Enterococcus M and Rogosa's Veillonella. Specific counts were made from C medium and typical colonies stained by the gram method and transferred to two blood agar plates which were then incubated aerobically and anaerobically. *Clostridia spp.* were identified as gram-positive rods, with and without spores, or by polar staining, depending on the species; their thick colony growth was confirmed on an anaerobic blood agar subculture. The *Bacteroides spp.* appear as thin, pleomorphic, fusiform, or small gram-negative rods which grow only anaerobically as small clear or gray colonies on blood agar media.

Gram stains from the G medium revealed the large, pleomorphic or thick, short gram-positive lactobacilli. Occasionally streptococci were seen on this medium, and they were identified by the gram stain. Occasionally it was difficult to identify short gram-positive organisms on G medium and in these instances characteristic morphology was usually observed after blood agar subculture.

Colonies from the PEA and Enterococcus-M media were stained by the gram method to identify streptococci, larger gram-positive cocci of the *Micrococcus spp.*, and diphtheroid organisms. Colonies from PEA medium were transferred to blood agar and incubated aerobically to confirm the presence of streptococci or staphylococci. Characteristic streptococcal colonies from the Enterococcus-M medium were transferred to two blood agar plates and incubated aerobically and anaerobically to determine the presence of anaerobic forms.

Veillonella organisms usually appeared as small gray or pink colonies on the Rogosa Veillonella medium. On gram stain they have the appearance of very small gram-negative cocci in masses.

Total coliform counts were recorded from the E medium. On occasion Pseudomonas, Proteus, Alcaligenes and paracolon organisms were isolated from this medium.

When colonies suggestive of enteric pathogens were observed on XLD medium, the organisms were transferred to TSI slants for further identification. Standard methods were used for biochemical diagnosis of gram-negative pathogens.[7]

## RESULTS

A series of 15 stool cultures were obtained from eight volunteers ranging in age from 21-40 years. The data are recorded in Table 2 and expressed in logarithms to the base 10. (This logarithm number was employed in all statistical analyses.) Total anaerobic counts, 9.4 $\pm$ 0.58 organism/gram feces, were always more common than aerobic counts, 7.2 $\pm$ 0.75 organisms/gram of feces. *Bacteroides spp.* were the most common (9.4 $\pm$ 0.6) of the anaerobic organisms. Lactobacilli were the next most frequent anaerobes but varied greatly among normal subjects ranging from 5.9 to 10/gm. Lactobacilli were always more numerous after anaerobic culture as compared with cultures incubated microaerophilically. Coliform

bacteria and streptococci were next in number; and strict anaerobic streptococci were only occasionally encountered. The counts ranged between 4 and 8/gm. Clostridia, Veillonellae, and diphtheroids were recovered sporadically in significant numbers.

Comparison of strict anaerobic lactobacillus growth on G medium, tomato juice agar, and Rogosa's SL medium in seven specimens revealed similar counts with G and tomato juice medium. Rogosa's SL medium was satisfactory to support microaerophilic lactobacilli but always contained fewer colonies under anaerobic conditions. Medium G was superior to tomato juice agar in that there was less growth of other organisms after 48 to 72 hours of incubation. Although colony counts from C medium were equal to blood agar, C medium was considered preferable since contaminants (i.e., lactobacilleae, streptococci) seemed to be suppressed while isolation of Clostridia was enhanced. A blood agar-neomycin medium gave excellent gram-negative anaerobic growth but poor gram-positive isolation.

TABLE 2. QUANTITATIVE FECAL BACTERIAL COUNTS OF HEALTHY AMBULATORY SUBJECTS

| *Controls* | *A* | *B* | *C* | *D* | *E* | *F* | *G* | *H* | *Mean ± 1 S.D.* |
|---|---|---|---|---|---|---|---|---|---|
| Total aerobes | 7.4 | 6.9 | 5.8 | 7.3 | 7.0 | 8.0 | 8.5 | 7.0 | 7.2 ± 0.75 |
| Total anaerobes | 9.8 | 9.7 | 9.0 | 9.7 | 9.0 | 9.0 | 8.6 | 10.2 | 9.4 ± 0.51 |
| Coliforms | 5.7 | 6.9 | 5.7 | 7.5 | 7.5 | 7.9 | 8.0 | 7.3 | 7.1 ± 0.84 |
| Streptococci | 7.7 | 6.1 | 4.0 | 7.2 | 6.0 | 6.9 | 6.8 | 6.0 | 6.4 ± 1.06 |
| Lactobacilli | 7.7 | 10.0 | 6.0 | 6.0 | 8.9 | 9.4 | 8.5 | 9.2 | 8.2 ± 1.43 |
| Bacteroides | 9.8 | 10.3 | 8.9 | 9.0 | 9.6 | 9.0 | 8.7 | 10.2 | 9.4 ± 0.60 |
| Clostridia | 8.7 | 6.3 | 0 | 0 | 0 | 0 | 6.6 | 0 | — |
| Diphtheroides | 0 | 0 | 6.0 | 7.5 | 0 | 7.3 | 0 | 0 | — |
| Veillonella | + | 0 | 0 | 0 | 0 | 8.0 | 0 | 0 | — |

Enterococcus-M medium was specific and reliable for isolation of streptococci. Lactobacilli were recovered occasionally and diphtheroids rarely appeared on this medium. Comparison with PEA revealed excellent correlation for total counts of streptococci. Therefore, Enterococcus medium was incubated to isolate all gram-positive organisms (staphylococci, diphtheroids, and streptococci).

Serial stool studies were performed on two subjects (Table 3). The results were remarkably reproducible. Geatest variation *in a given individual* occurred in the number of coliform organisms. When this occurred it also appeared to be reflected in the total count of aerobes.

## DISCUSSION

Rosebury[15] has characterized the bacterial flora indigenous to man and Donaldson[16] has recently reviewed and tabulated the great number of species of bacteria isolated from stool. However, considerably more informa-

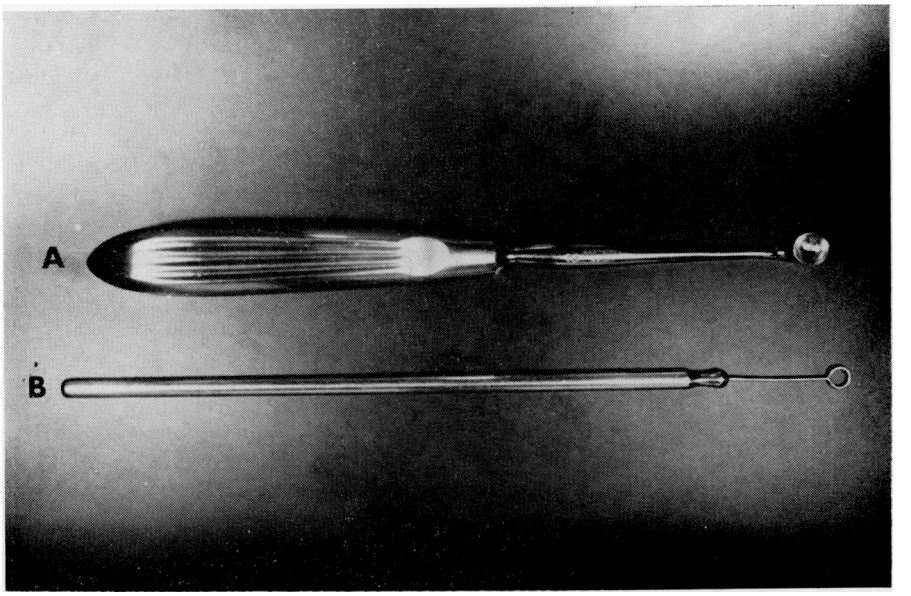


FIG. 1. A. Bone curette (No. 2). Stool specimen is picked up in round bowl at tip of curette.
B. Platinum-rhodium loop which delivers 0.01 ml. of fluid.

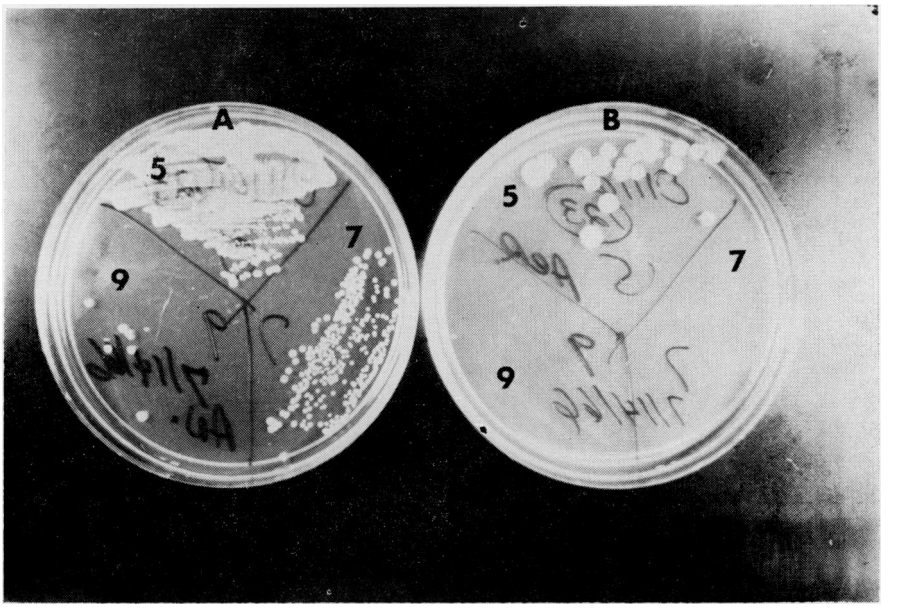


FIG. 2. Photograph of Schaedler, *et al.* A medium which was incubated anaerobically (A) and aerobically (B) streaked at dilutions of 5, 7, and 9. Note that the anaerobic plate (A) had maximal growth on dilution 9 of nine colonies. This would then represent $9 \times 10^9$ of anaerobic bacteria per gram of feces. Note that on the aerobic plate (B), the maximal growth was at dilution 5. There were 27 colonies representing $27 \times 10^5$ aerobes per gram of fecal material.

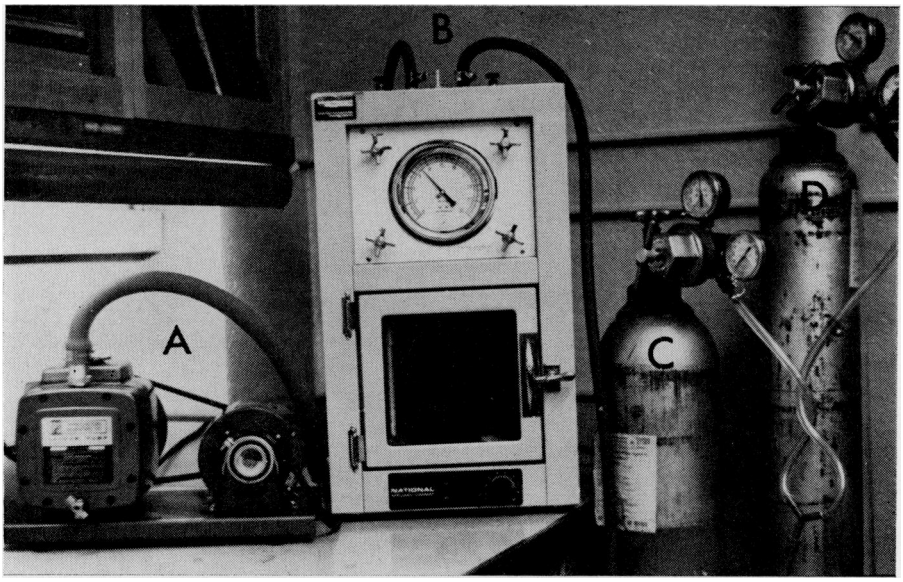


FIG. 3. This is a photograph of the anaerobic incubation equipment system employed. A is a standard vacuum pump which is connected to B, the anaerobic incubator, which in turn is connected to tank C ($CO_2$) and tank D (N). The gauge in the center records the vacuum created by the suction pump within the incubator, and is helpful in facilitating constant observation that a negative vacuum is maintained during the period of incubation.

TABLE 3. BACTERIAL COUNTS FROM SERIAL FECAL SPECIMENS

| *Sub. A* | *1* | *2* | *3* | | | |
|---|---|---|---|---|---|---|
| Total aerobes | 7.4 | 7.9 | 7.2 | | | 7.5 ± 0.3 |
| Total anaerobes | 9.8 | 10.3 | 9.3 | | | 9.8 ± 0.4 |
| Coliforms | 5.7 | 8.0 | 7.6 | | | 7.1 ± 1.0 |
| Streptococci | 7.7 | 7.0 | 7.7 | | | 7.5 ± 0.3 |
| Lactobacilli | 7.7 | 7.7 | 8.3 | | | 7.9 ± 0.3 |
| Bacteroides | 9.8 | 9.3 | 9.3 | | | 9.4 ± 0.2 |
| *Sub. B* | *1* | *2* | *3* | *4* | *5* | |
| Total aerobes | 6.9 | 9.0 | 7.0 | 9.6 | 6.6 | 7.8 ± 1.2 |
| Total anaerobes | 9.7 | 10.1 | 10.1 | 10.2 | 10.5 | 10.1 ± 0.3 |
| Coliforms | 6.9 | 8.0 | 7.3 | 8.8 | 6.2 | 7.4 ± 0.9 |
| Streptococci | 6.1 | 6.2 | 6.0 | 7.0 | 6.3 | 6.3 ± 0.4 |
| Lactobacilli | 10.0 | 9.7 | 9.6 | 10.0 | 9.2 | 9.7 ± 0.3 |
| Bacteroides | 10.3 | 10.4 | 8.9 | 10.3 | 10.5 | 10.1 ± 0.6 |

tion is necessary to understand the variations that occur in intestinal bacterial populations under normal conditions. In his quantitative and qualitative study of animal flora, Smith demonstrated that there is great variation in the predominant organisms amongst different species.[21] For instance, Bacteroides are the most common organisms recovered from the stool of mice, yet oxen have a very poor anaerobic flora.[21]

This paper has described in detail methods for culturing enteric specimens from man and has considered the effect of different media on the quantitative and qualitative recoverability of bacteria. The results obtained are comparable to those described in recent studies.[17-25]

The predominant recovered bacteria from the stool of man are *Bacteroides spp.* The next most common are lactobacilleae and the coliforms, and streptococcae are next with some variation in the frequency with which they are found. Other organisms are not regularly recovered but may be as common as $10^8$/gm. when isolated (e.g. Veillonellae and Clostridia). Of note also is the variability of coliform counts in the two subjects studied. Recovery of the other groups of organisms appeared to be reproducible. The variation in coliforms is interesting and warrants further investigation.

When studying the flora of one host species, it would be desirable to employ standard inoculation and isolation techniques. Quantitative counts obtained in one host species are difficult to compare with similar studies in the same host species when different media are used for isolation of bacteria, since growth varies according to the media and techniques employed. Furthermore, studies in man cannot simply be compared to similar studies in other animal species because it is necessary to employ different media for maximal growth of bacterial flora from other species. The investigations

of Dubos and colleagues[26-28] in mice and our experience in other animals[29,30] and in man indicate that the media necessary to support maximal growth in vitro will vary depending upon the host under study. An example of this is our experience with the "F" medium of Schaedler which results in excellent recoveries of streptococci from mice, but only fair to moderate recoveries from man.

We tried several types of media recommended in the literature in an attempt to establish those which give the greatest reproducibility and highest recovery of bacteria in man. The enriched A medium of Schaedler, *et al.*, has given the most satisfactory total counts for both aerobic and anaerobic organisms. Although blood media used in recent reports gave similar counts,[2,22,31] our experience has been that the A medium is more consistent. Similarly, medium G of Schaedler, *et al.* is more consistent than Rogosa's SL[12,13] for the isolation and quantitation of the anaerobic lactobacilleae. Tomato juice agar[6] gives total counts as high as the G medium; however, it permits heavier growth of other organisms. Medium C of Schaedler, *et al.* proved satisfactory for isolation of Bacteroides and Clostridia species. It did not result in a higher yield than a blood-neomycin medium for total counts of *Bacteroides spp.*; however it did increase the recoverability of Clostridia organisms. The Enterococcus medium developed by Slanetz was effective in permitting the isolation and counting of enterococci.[5] Excellent yields of Veillonella organisms were obtained on the medium of Rogosa[4] although the antibiotic used in it appears to influence recovery.[32] In our experiences in man, streptomycin is superior to vancomycin and reproducibility appears to depend on the strain of Veillonella isolated. We are presently extending our studies on recoverability of these organisms from man.[32] It should be noted that other authors recommend similar media and methods they have found successful in their laboratories.[11,24] Our recommendations for the use of these media are based on our data as well as inevitable random selection determined by our laboratory conditions.

Changes in bacterial isolation techniques and in identification criteria have made interpretation of older data difficult. For example, studies early in the century of Rettger and Cheplin[33] and Cruickshank[34] in which they demonstrated the enhancement of *Lactobacillus acidophilus* growth in the intestine by forced increased lactose diets have not been confirmed by recent investigations.[30,35] The experiments of Sanborn[36] and Torrey and Monta[37] demonstrating the influence of diet on human stool flora did not include a consideration of the anaerobic intestinal flora. The latter authors showed a decrease in stool bacterial counts in a subject on a strict protein diet. This contrasts with recent results demonstrating an increase in anaerobic organisms in subjects on a similar high protein diet.[23]

There has been little uniformity in media or techniques employed in any of the modern quantitative bacteriological studies. The pioneering work of Cregan and Hayward demonstrated the flora in jejunal juice, but the limited use of selective media resulted in no classification of the anaerobes.[38] Later Dellipiani and Girdwood employed five media that gave a good spectrum of growth revealing numerous species of aerobic gram-negative rods in jejunal aspirates from pernicious anemia patients.[39] Bornside, *et al.* used surface inoculation for their extensive small bowel studies in man.[25,31] In their initial work they made no attempt to isolate lactobacilli but later did use Rogosa's SL medium which demonstrated recoverable lactobacilli in less than 20% of their patients. Kalser, *et al.* used seven media in their studies but no selective media for Bacteroides.[2] In addition, they used Case anaerobic jars without an oxygen absorber.[2] Their data failed to reveal the predominance of anaerobes in stool, contrary to the results of others. We have found Case anaerobic jars to be variable in performance. Some jars leak and are not readily repaired by the manufacturer. Gorbach, *et al.* used an oxygen absorber in their anaerobic incubation with which they obtained excellent anaerobic growth on non-selective blood agar, but they used pour plates which do not permit easy identification of other bacterial species.[22] Zubrzycki and Spaulding used many media and streaking techniques in their experiments demonstrating the stability of human fecal flora.[24] Their methods appear to be most similar to those we have employed. When the European literature is reviewed, similar technical variations can be listed.[17-21] These variations have hampered correlation of data from different studies. It is hoped that in the future, investigators will attempt to reproduce or evaluate their data in the light of methodological differences.

SUMMARY

Methods for the isolation, identification and quantitation of the fecal bacterial flora are described and studies on eight healthy adults recorded. Anaerobic organisms were recovered in higher numbers with these techniques than were aerobic organisms. *Bacteroides spp.* were the most common bacteria in the stool and coliforms and lactobacilli showed the greatest individual and host variations.

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APPENDIX

I. *Recipe for A Medium* (modified from Schaedler, *et al.*[1])

1. Make up hemin solution by dissolving 100 mg. of recrystallized hemin (Nutritional Biochemicals Co.) in 99 ml. of distilled HOH and 1 ml. of 5N NaOH. Store in the refrigerator.
2. Make up cystine solution just before using by dissolving 4 gm. of L-Cystine (Pfanstiehl) in 99 ml. of distilled water and 8 ml. of 5N NaOH. (Prepare fresh each time.)
3. Measure out the following ingredients and pour through a funnel into a 2,000 cc. flask:

| | |
|---|---|
| Pancreatic digest of Casein, U.S.P. (Trypticase) (Fisher Scientific) | 10 gm. |
| Proteose peptone No. 3 (Difco) | 5 gm. |
| Dextrose | 5 gm. |
| Yeast extract (Difco) | 5 gm. |
| Tris buffer 7 to 9 (Sigma) | 3 gm. |
| Hemin solution (1.) | 10 ml. |
| L-Cystine solution (2.) | 10 ml. |
| Agar agar #3 (Oxoid) | 15 gm. |
| Distilled HOH | 1,000 ml. |

4. Bring solution in the flask to a full boil, swirling constantly.
5. Plug flask, and autoclave media for 15 minutes at 15 lbs. pressure.
6. Pour immediately into plates and flame each plate as pouring.
7. Allow to harden, and store in refrigerator upside down.

II. *Recipe for C medium* (modified from Schaedler, *et al.*[1])

1. Steps 1 and 2 as in "A" medium (I).
2. Reconstitute neomycin sulfate powder by adding 5 cc. of sterile saline to an ampoule containing 0.5 gm. of the antibiotic. Add the saline with a sterile syringe. Shake the ampoule until powder is dissolved and store in the refrigerator until needed. Reconstitute a new ampoule of the antibiotic each time the media is made, and discard the ampoule after use.
3. Measure out the ingredients as listed in step 3 in "A" media (I). Add 2 gm. of placenta powder (Nutritional Biochemical Co.)
4. Cool flask to approximately 50°C. by swirling the flask in a basin filled with cold tap HOH.
5. Add 0.2 cc. of the reconstituted neomycin with a sterile 1 cc. stylex disposable tuberculin syringe.
6. Mix thoroughly.
7. Pour media into plates, flame plates, and when hard, store upside down in the refrigerator.

III. *Recipe for E medium* (Modified from Schaedler, *et al.*[1])

1. Prepare triphenyl tetrazolium chloride (Nutritional Biochemicals Co.) by bringing 0.4 gm. up to 100 cc. with distilled HOH in a 100 cc. volumetric flask and mix to dissolve.
2. Filter the 4% tetrazolium chloride through a millipore filter, which has been previously sterilized by autoclaving.
3. Rehydrate Tergitol 7 Agar (Difco).
4. Bring to full boil, shaking continuously and then autoclave.
5. Cool, as in step 4 under "C" medium (II).
6. Add 10 ml. of the tetrazolium chloride, with a sterile pipet, to the cooled medium.
7. Swirl thoroughly.
8. Pour, flame plates, and when hard, store plate in refrigerator upside down.

IV. *Recipe for G Medium* (Modified from Schaedler, *et al.*[1])

1. Make up 1% oleic acid in 10% triton by mixing 10 ml. of triton (WR-1339, Rugar Chem. Co., Irvington-on-Hudson, N.Y.) with 90 ml. of distilled HOH and shake continuously until these ingredients are dissolved. Mix 99 cc. of this solution with 1 ml. of oleic acid (Nutritional Biochemicals Co.) in a beaker. Heat and let cool until solution clears.
2. Make up cystine solution by dissolving 4 gm. of L-Cystine (Pfanstiehl) in 100 ml. of HOH and in 7.5 ml. HCl (Approximately 37%). (Prepare fresh solution each time.)
3. Prepare 1% sodium azide (prepare fresh) by bringing 1 gm. up to 100 cc. with distilled HOH in a 100 cc. volumetric flask.
4. Measure out the following ingredients and pour through a funnel into a 2,000 cc. flask:

| | |
|---|---|
| Pancreatic digest of Casein, U.S.P. (Trypticase) (Fisher Scientific) | 10.0 gm. |
| Yeast extract (Difco) | 5.0 gm. |
| Dextrose | 10.0 gm. |
| $KH_2PO_4$, anhydrous (Mallinckrodt) | 2.0 gm. |

| | |
|---|---|
| $K_2HPO_4$, anhydrous (Mallinckrodt) | 1.5 gm. |
| $(NH_4)_2SO_4$ (Mallinckrodt) | 1.0 gm. |
| Cystine solution | 2.5 gm. |
| 1% oleic solution (1.) | 40.0 ml. |
| 1 per cent sodium azide (Difco) | 10.0 ml. |
| Agar agar #3 (Oxoid) | 1,000.0 ml. |
| Distilled HOH | |

5. Bring to boil, shaking and then autoclave.
6. Make up 1% $K_2Cr_4O_7$ by bringing 1 gm. up to 100 cc. with distilled HOH in a 100 cc. volumetric flask. Pour this solution through a millipore filter to sterilize. (Store in refrigerator in a brown bottle.)
7. After autoclave medium, add 8 ml. of the 1% $K_2Cr_4O_7$ to the medium with a sterile pipet.
8. Swirl medium, pour, flame plates, allow plates to harden, store plates in refrigerator upside down.

VI. *Veillonella-Streptomycin Medium* (or any other antibiotic desired)
1. Rehydrate Veillonella agar (Difco).
2. Bring to boil, shaking continuously and then autoclave.
3. Cool to 50°C. (approx. 1 hr.) in water bath.
4. Add 10 micrograms of streptomycin per ml. of media.
5. Mix thoroughly, pour media, flame plates, allow to harden, store plates in refrigerator upside down.

VII. *PEA, and Enterococcus MG media* available from Difco.[7,8]

VIII. *XLD medium* obtainable from Baltimore Biological Laboratory.[9]