

# STUDIES ON THE STABILITY OF THE NORMAL HUMAN FECAL FLORA

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## ABSTRACT

ZUBRZYCKI, LEONARD (Temple University, Philadelphia, Pa.) AND EARLE H. SPAULDING. Studies on the stability of the normal human fecal flora. *J. Bacteriol.* **83**:968-974. 1962.—The results of two series of stool cultures show that members of the genus *Bacteroides* constitute the most numerous group of bacteria in the normal human adult fecal flora. Together with the enterococci, coliform bacilli, diphtheroids, and lactobacilli, these major components account for more than 99% of the total counts. Wide fluctuations in the number and types of minor organisms observed suggest the probability that they are held in check by these major components which may also possess mechanisms for preventing pathogens from establishing themselves in the large bowel.

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Cultures of normal stool specimens and rectal swabs from about 150 hospitalized adults without clinical enteric infection were examined in a semi-quantitative manner (Spaulding et al., 1957) during studies on intestinal antisepsis extending over a period of 5 years. Analysis of the results shows that the *Bacteroides* outnumbered all other types of microorganisms. This finding agrees with those of Eggerth and Gagnon (1933), Lewis and Rettger (1940), and, more recently, of Hehre and Sery (1952) and Riddell, Morton, and Murray (1953). Nevertheless, Halbert (1948) indicated that he understood coliform bacilli to "make up the bulk of bacteria in normal human feces." In the field of intestinal antisepsis, the majority of investigators have ignored the *Bacteroides* (Gaylor et al., 1961; Seneca and Henderson, 1950; Shidlovsky, Marmell, and Prigot, 1957; Winkelstein, 1955). Others either acknowledge their presence, without any attempt to culture them, or they use inadequate methods (Hartman et al., 1961).

Some standard textbooks of microbiology mention the presence of *Bacteroides* in the intestine and suggest that they may outnumber the coli-

forms (Burrows, 1959; Dubos, 1958). Smith and Conant (1960) make the same suggestion in one place and then contradict it in another with the comment that the majority of fecal bacteria are members of the coliform group. Wilson and Miles (1955) on two occasions point with some emphasis to the fact that non-sporulating anaerobes outnumber aerobes, but they do so without ever using the term *Bacteroides*. And Burdon (1958) makes the unequivocal assertion that coliform bacilli predominate in the lower intestine. Thus, it appears that the numerical preponderance of *Bacteroides* in human feces is a fact not yet generally recognized and, therefore, needs further emphasis.

Misconceptions about the adult fecal flora appear to be based on results of studies in which inadequate identification methods such as Gram stains and broth cultures or too few selective culture media were employed. Consequently, the wide variation in the findings of different investigators, and especially those indicating that the flora changes markedly with fluctuations in diet, seem to be responsible for the notion that the flora varies widely in composition not only among different persons but also from day to day in the same person.

Because of the lack of conformity of published opinions on this point, and because we believe the stability of the adult fecal flora is an important factor in the natural resistance of the host, we are reporting further cultural studies on normal stool specimens and on serial specimens from four healthy adults. The latter series were subjected to careful cultural examination, the results of which support the view that the fecal flora is remarkably stable.

## MATERIALS AND METHODS

*Stool specimens.* These were cultured promptly or stored at 4 C for no longer than 6 hr.

*Culture methods.* A 4-mm biconvex loopful of formed stool weighs approximately 20 mg. Two loopfuls were suspended in 4 ml of tryptone broth

(Difco) to make a  $10^{-2}$  dilution. Tenfold dilutions were made in broth using a separate pipette per dilution.

Two culture methods were used to obtain viable counts. The first consisted of one 4-mm biconvex loopful of inoculum (0.02 ml) spread thoroughly over the surfaces of selected types of agar media in petri plates. Preliminary work confirmed the reliability of this method when compared with the classical pour plate method. Trypticase soy agar (Baltimore Biological Laboratory, BBL), with the addition of 7% human blood, was used for a total count, and duplicate plates were incubated aerobically and anaerobically (Kolmer, Spaulding, and Robinson, 1951). EMB agar (Difco) was similarly inoculated for coliform bacilli, SS agar (Difco) for other enteric bacilli and pathogens, Littman's medium (BBL) for yeastlike fungi, LBS medium (BBL) for lactobacilli, and Chapman-Stone medium (Difco) for staphylococci. Viable counts for enterococci were determined, however, by the pour plate method. Samples of 1 ml were added to SF medium (BBL) to which agar had been added.

Each dilution sample was plated in duplicate or, in the case of blood agar, in quadruplicate. All plates were incubated at 37 C, and LBS plates in an atmosphere of 10%  $CO_2$ .

Colony counts were made after 48 hr incubation except for LBS and Littman plates which

received an additional 48 hr at 37 C and room temperature, respectively.

*Identification procedure.* Coliform colonies were recognized by the typical appearance on EMB agar. Colorless colonies on EMB and SS agars were designated as "NLF" (nonlactose fermenters). Chapman-Stone plates served both for staphylococcal counts and aerobic spore-formers (labeled *Bacillus subtilis*). Hemolytic colonies seen only on anaerobic blood agar and consisting of gram-positive bacilli (with or without spores) were labeled as clostridia.

Other strictly anaerobic colonies were considered *Bacteroides* if they were sporeless, gram-negative bacilli, and produced the penetrating foul odor typical of this group. Another characteristic of *Bacteroides* is difficulty in obtaining pure cultures and, indeed, in getting any growth on subculture if surface colonies are exposed to air for more than a few minutes. Subcultures to both aerobic and anaerobic blood agar, followed by examination of stained smears, was often necessary to identify various colony types. The pleomorphism implied in the generic term *Bacteroides* was observed most often as branching and filamentous forms, and as coccoid and swollen bodies. Some fusiform-shaped bacilli were also seen. They were probably fusobacteria, but no attempt was made to count them separately since they occurred sporadically and usually in small

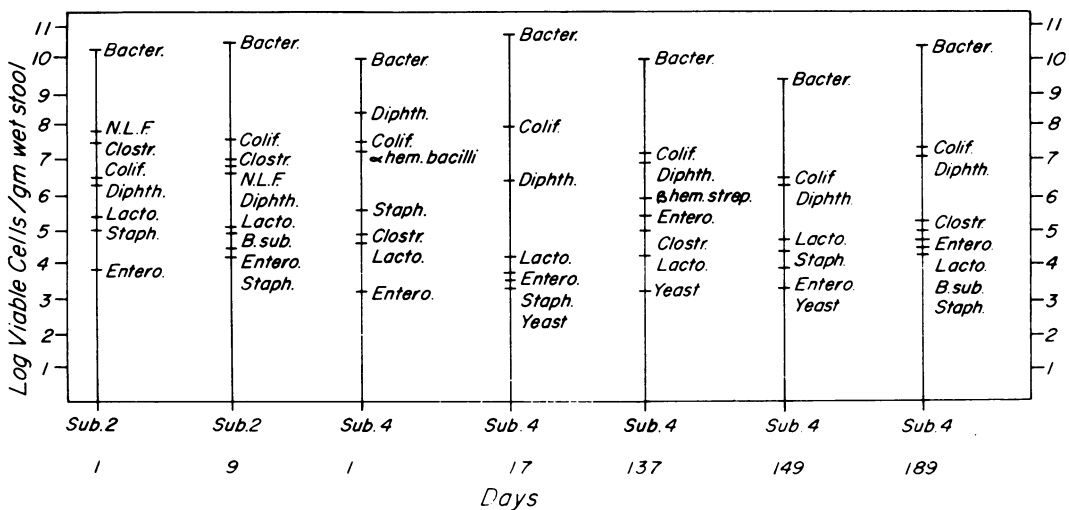


FIG. 1. Fecal flora of subjects 2 and 4, adult males. Coliforms were *Escherichia coli* except for a few *Aerobacter* in subject 4 on day 189; "α hem. bacilli" represent gram-negative bacilli producing α hemolysis on aerobic blood agar plate; "β hem. strep." represents a β-hemolytic streptococcus, probably not an enterococcus; "NLF" represents enteric bacilli producing colorless colonies on EMB or SS agar.

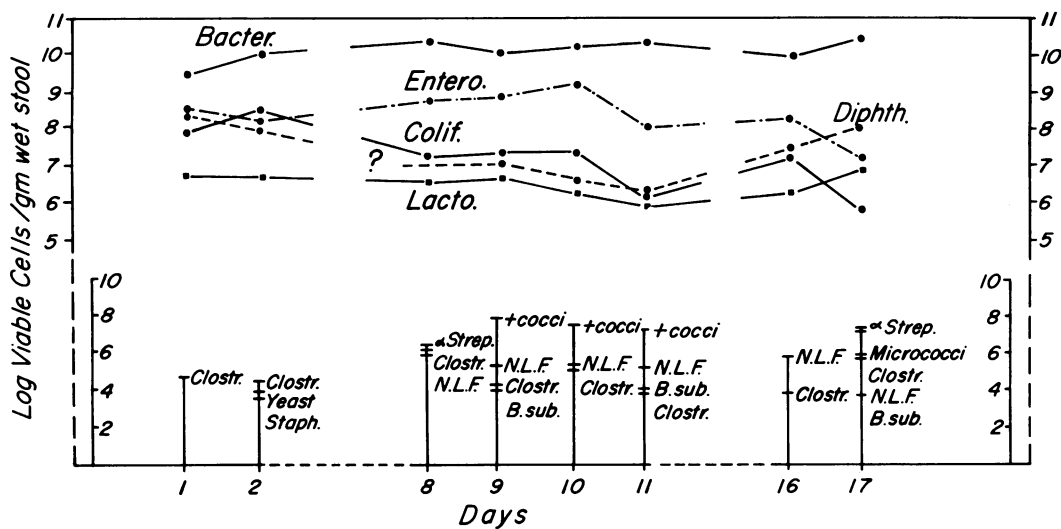


FIG. 2. Fecal flora of subject 1, an adult male. *Bacteroides melaninogenicus* was present on days 2, 9, 10, and 16. Coliforms were *Escherichia coli* except for a few *Aerobacter* on days 8 and 9. The designation, +cocci, represents gram-positive cocci growing on aerobic blood agar plates; "α-strep." represents gram-positive cocci producing α hemolysis on aerobic blood agar plates.

numbers. Although the great majority of "Bacteroides" colonies were composed of gram-negative bacilli, gram variability was frequent, and we even included some frankly gram-positive bacilli since the limits of this heterogeneous group are not well defined.

Other colony types that grew on both aerobic and anaerobic blood agar, and often on Chapman-Stone medium, were identified as diphtheroids if they were gram-positive bacilli or pleomorphic forms without spores.

The lactobacilli, enterococci, and yeastlike fungi were counted on the media used for their selection. The Gram stain reaction and colonial appearance were used to identify bacteria which occasionally appeared in large numbers on some of our plates. Final identification often required the inoculation of 1% tryptone broth (Difco) for indole production, MR-VP broth (BBL) for the methyl red and Voges-Proskauer reactions, a motility medium, triple sugar iron agar (BBL), urea broth (Difco), lactose broth (Difco), and brom-cresol purple milk.

#### RESULTS

Over a period of 5 years, several hundred rectal swab or stool specimens were collected before administration of chemotherapeutic drugs from hospitalized adult patients free of known intesti-

nal abnormalities. Although our methods were changed from time to time, 181 of these specimens were cultured in essentially the same manner. Total aerobic and anaerobic (blood agar) counts were obtained, as well as the number of coliform bacilli and spores. Most of the specimens were also cultured on selective media for enterococci, yeastlike fungi, and staphylococci. The presence of *Pseudomonas*, *Proteus*, nonlactose-fermenting gram-negative bacilli or clostridial species was recorded as "many", "few", etc. No attempt was made to culture lactobacilli.

Analysis of the results shows, in confirmation of our earlier impression, that the anaerobic exceeded the aerobic count in 80 to 90% of the specimens. Frequent gram-stained smears of colonies on high-dilution plates revealed a predominance of gram-negative bacilli morphologically consistent with the identification of *Bacteroides*, the presence of which was further suggested by the characteristic penetrating putrefactive odor produced by these cultures.

The total anaerobic counts of stool specimens averaged about  $1 \times 10^9$  g; those of rectal swabs were about one log smaller. The same difference was seen between the two aerobic counts; and, in each instance, the average anaerobic count was approximately three times as large as the aerobic count. The number of coliform bacilli in stool

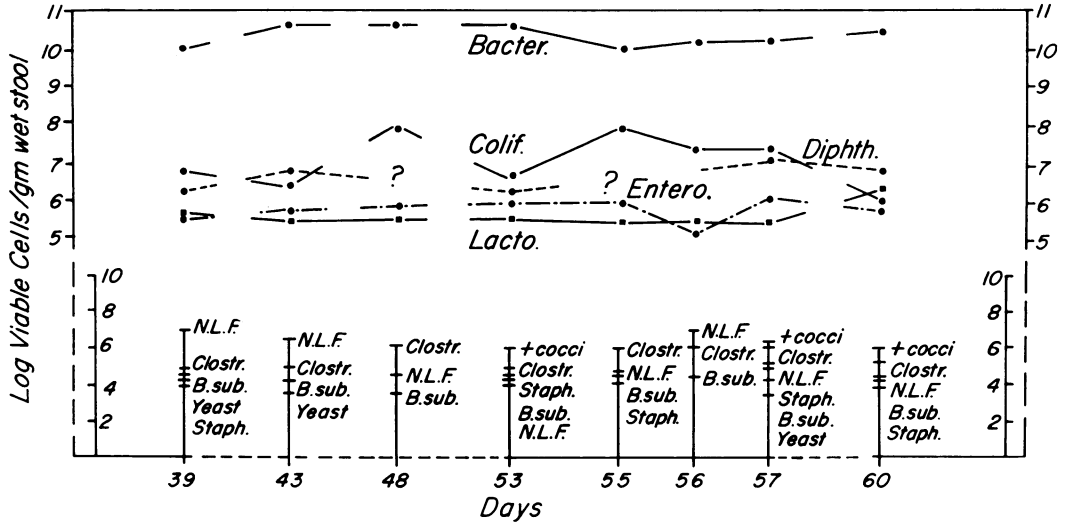


FIG. 3. Fecal flora of subject 2, an adult male. *Bacteroides melaninogenicus* was present on days 43 and 57. Coliforms were *Escherichia coli* except for a few *Aerobacter* on day 53.

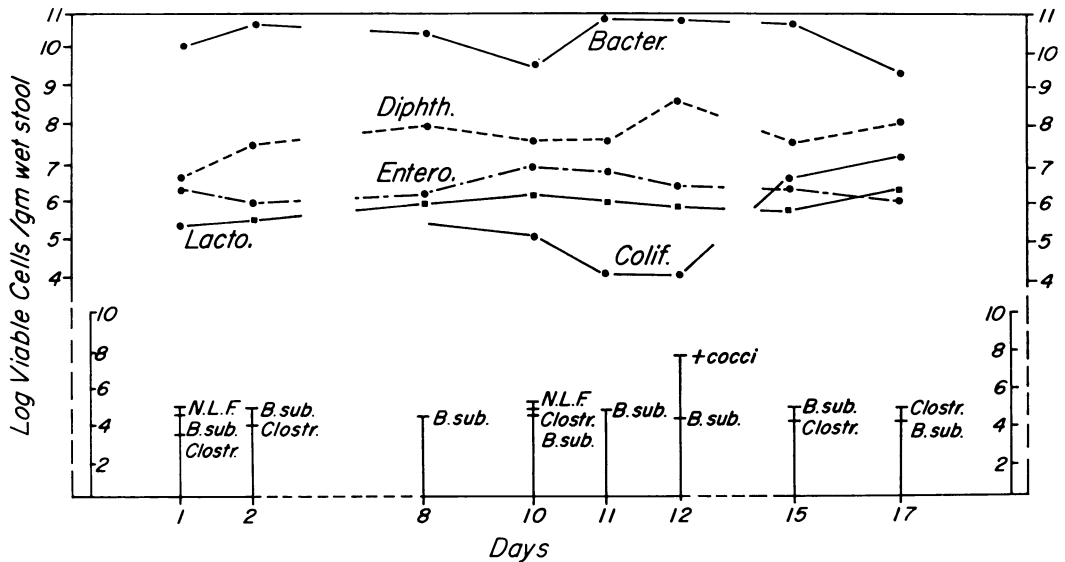


FIG. 4. Fecal flora of subject 3, an adult female. Coliforms were *Escherichia coli* on days 10, 11, 12, and 17 except for a few *Aerobacter* on day 10, whereas they were *Aerobacter* on days 8 and 15 except for a few *E. coli* on day 15.

specimens averaged about  $1 \times 10^8$ , as did the enterococci.

In contrast, the average number of spores was less than  $1 \times 10^4$ ; the corresponding figures for yeasts and staphylococci were about  $1 \times 10^2$ . *Pseudomonas*, *Proteus*, clostridia, and nonlactose-fermenting organisms (probably *Enterobacteriaceae*) collectively were recorded as present in 20% of the specimens.

*Serial stool specimens.* Some time after the

conclusion of the intestinal antiseptics studies, a series of specimens was collected over a period of several months from each of four healthy adults and subjected to thorough cultural examination. None of the subjects changed his diet, received antimicrobial drugs, or experienced any untoward intestinal disturbance during the periods of observation. The microbiological methods used were those described above.

Specimens recorded in Fig. 1 predate those on

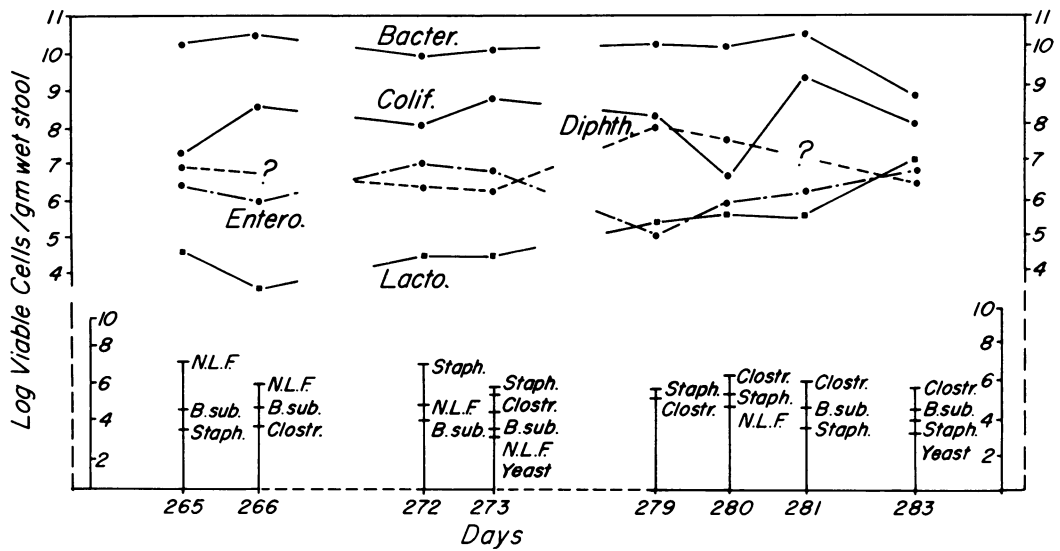


FIG. 5. Fecal flora of subject 4, an adult male. *Bacteroides melaninogenicus* was present on day 283. All coliforms were *Escherichia coli*.

Fig. 3 and 5. The results of successive specimens collected over a shorter period of time (17 to 22 days) are shown in Fig. 2 to 5. The major components of the fecal flora are connected by lines; to avoid overcrowding the graphs, the other organisms are drawn on a different log scale.

*Bacteroides*, using our definition, was the most numerous type found, constituting from 78% (on one occasion) to, in most cases, 88 to 99% of the total viable count. Four other bacterial types (enterococci, coliform bacilli, diphtheroids, and lactobacilli) were also quite consistently present in numbers ranging from  $10^4$  to  $10^9$  per g. The exceptions were: the apparent absence of diphtheroids in a few specimens (indicated by question marks on the graphs), which was attributed to crowding by the large coliform colonies on the blood agar plates, and the absence of coliforms in three specimens from subject 3. (The same observation has also been reported by Olsen, 1945; and Young et al., 1960.) If these results are representative, they collectively account for more than 99% of the total viable counts. The relative constancy with which these five types occurred from day to day, week to week, and month to month leads us to believe they constitute the major components of the normal flora.

Among the ten miscellaneous types recognized by us, the most persistent were the clostridia (subjects 1 and 2), nonlactose-fermenting or-

ganisms (subject 2), and *B. subtilis* (subjects 2 and 3); but these organisms were detected only sporadically in other subjects. Occasionally, a single type was present in large numbers, such as the gram-positive coccus in subject 1. The erratic presence of these miscellaneous types, usually in small numbers, marks them as minor components of the normal adult fecal flora.

#### DISCUSSION

Comprehensive culturing methods are needed to reveal the total fecal flora. Diphtheroids had been frequently found in feces by Sanborn (1931a, b). Our observation, that they are numerically present in large numbers, was possible because we streaked high dilutions of fecal suspensions on aerobic and anaerobic blood agar plates, thereby avoiding the overgrowth by large coliform colonies on the lower dilution plates. Crowding out of smaller groups of organisms and the lack of selective media for many groups of bacteria make it likely that all the viable organisms in the feces have not been accounted for. Vibrios, spirochetes, and protozoa are also a part of the flora.

Although we seldom employed the proper tests to identify them, some of our nonlactose-fermenting organisms were undoubtedly *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, and even coliforms.

We made no attempt to serotype the *Enterobacteriaceae*.

The most numerous group of organisms in the fecal flora is our most poorly defined one. Some of the gram-positive bacilli included in our *Bacteroides* counts could have been anaerobic diphtheroids. If so, however, the numerical error was slight because the great majority of our *Bacteroides* were gram-negative. The one species of *Bacteroides* readily identified colonially was *Bacteroides melaninogenicus*, and it occurred frequently.

One is tempted to suspect, as did Weiss and Rettger (1937), that *Bacteroides*, because of their numerical dominance, must play a leading role in determining the nature of the bacterial population of stool specimens, and, presumably, that of the large bowel. Miller's observations (1959) lend support to this possibility.

We believe our results indicate the existence of an equilibrium in the intestinal flora, as postulated by Wilson and Miles (1955) and found, by Kraus and Gaston (1956), to be true for another complex microbial flora (the oral one). The state of stability produced by this equilibrium may be important in protecting the host against overt infection. For example, a *Shigella* strain, reaching the lower intestinal tract, may gain a foothold, as did the nonlactose-fermenting organism in subject 3 on day 1, but then be suppressed as promptly as that organism. The staphylococcus in subject 2 on day 39 was not phage typed, but pathogenic strains might similarly be kept in check.

It is difficult to see how the factors responsible for this stability can be studied satisfactorily by in vivo methods. One might try to extrapolate results obtained in animals. Another way is to devise an in vitro continuous culture system in which the inoculation of a fecal sample gives rise to a mixed population, closely approximating the in vivo flora. The results obtained with such an apparatus will be described in a forthcoming publication.

#### LITERATURE CITED

- BURDON, K. L. 1958. Textbook of microbiology, p. 409. 4th ed. MacMillan Co., New York.
- BURROWS, W. 1959. Textbook of microbiology, p. 562. 17th ed. W. B. Saunders Co., Philadelphia.
- DUBOS, R. 1958. Bacterial and mycotic infections of man, p. 632. 3rd ed. J. B. Lippincott Co., Philadelphia.
- EGGERTH, A. H., AND B. H. GAGNON. 1933. The bacteroides of human feces. *J. Bacteriol.* **25**:389-413.
- GAYLOR, D. W., J. S. CLARKE, Z. KUDINOFF, AND S. M. FINEGOLD. 1961. Postoperative bowel "sterilization"—a double-blind study comparing kanamycin, neomycin, and placebo. *Antimicrobial Agents Ann.* 1960, p. 392-403.
- HALBERT, S. P. 1948. The relation of antagonistic coliform organisms to *Shigella* infections. II. Observations in acute infections. *J. Immunol.* **60**:359-381.
- HARTMAN, C. R., J. H. EPSTEIN, W. G. McCARTEN, M. H. GAFFEY, AND M. J. ROMANSKY. 1961. A controlled study of the effects of antibiotics on the gastrointestinal tract. *Antimicrobial Agents Ann.* 1960, p. 404-408.
- HEHRE, E. J., AND T. W. SERY. 1952. Dextran splitting anaerobic bacteria from the human intestine. *J. Bacteriol.* **63**:424-426.
- KOLMER, J. A., E. H. SPAULDING, AND H. W. ROBINSON. 1951. Approved laboratory techniques, p. 415. 5th ed. Appleton-Century-Crofts Inc., New York.
- KRAUS, F. W., AND C. GASTON. 1956. Individual constancy of numbers among the oral flora. *J. Bacteriol.* **71**:703-707.
- LEWIS, K. H., AND L. F. RETTGER. 1940. Non-sporulating anaerobic bacteria of the intestinal tract. I. Occurrence and taxonomic relationships. *J. Bacteriol.* **40**:287-307.
- MILLER, C. P. 1959. Protective action of the normal microflora against enteric infection: An experimental study in the mouse. *Univ. of Mich. Med. Bull.* **25**:272-279.
- OLSEN, E. 1945. On the coliform bacteria of human feces. *Acta Pathol. Microbiol. Scand.* **22**:108-118.
- RIDDELL, M. I., H. S. MORTON, AND E. C. D. MURRAY. 1953. The value of dihydrostreptomycin in preoperative preparation of the gut. *Am. J. Med. Sci.* **225**:535-546.
- SANBORN, A. G. 1931a. The fecal flora of adults, with particular attention to individual differences and their relationship to the effects of various diets. I. Individual differences on normal diets. *J. Infectious Diseases* **48**:541-569.
- SANBORN, A. G. 1931b. The fecal flora of adults, with particular attention to individual differences and their relationship to the effects of various diets. II. Individual differences in response to special diets. *J. Infectious Diseases* **49**:37-89.
- SENECA, H., AND E. HENDERSON. 1950. Normal

- intestinal bacteria in ulcerative colitis. *Gastroenterology* **15**:34-39.
- SHIDLOVSKY, B. A., M. MARMELL, AND A. PRIGOT. 1957. Novobiocin used alone and in combination with neomycin for bowel sterilization. *Antibiotics Ann.* 1956-1957. p. 232-235.
- SMITH, D. T., AND N. F. CONANT. 1960. *Zinsser Microbiology*, p. 116, 985. 12th ed. Appleton-Century-Crofts Inc., New York.
- SPAULDING, E. H., R. R. TYSON, M. J. HARRIS, B. JACOBS, L. WILDRICK, AND K. O. JOHNSON. 1957. Further studies on intestinal antiseptics: neomycin-nystatin. *Antibiotics Ann.* 1956-1957. p. 236-243.
- WEISS, J. E., AND L. F. RETTGER. 1937. The gram-negative bacteroides of the intestine. *J. Bacteriol.* **33**:423-434.
- WILSON, G. S., AND A. A. MILES. 1955. *Topley and Wilson principles of bacteriology and immunity*, p. 468, 2250, 2253. 4th ed. Williams and Wilkins Co., Baltimore.
- WINKELSTEIN, A. 1955. Lactobacillus acidophilus tablets in the therapy of various intestinal disorders; A preliminary report. *Am. Practitioner and Dig. Treatment* **6**:1022-1025.
- YOUNG, V. M., H. C. GILLEM, E. D. MASSEY, AND W. C. BRANCHE, JR. 1960. Observations on *Escherichia coli* as a component of the normal intestinal flora: Aberrant findings in certain individuals. *Bacteriol. Proc.*, p. 134.