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Fecal Microflora in Healthy Persons in a Preindustrial Region

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Procedures for quantitating the fecal microflora of man were described. Special attention was given to criteria for characterizing the culturable aerobic, microaerophilic, and anaerobic bacteria. Three groups of healthy persons were studied: wholly breast-fed infants (2 to 4 month-olds), weanlings (1 to 2 year-olds), and adults. In breast-fed children, bifidobacteria predominate and outnumber by one or several logs all other culturable bacteria. The fecal flora of wholly breast-fed infants is "simpler" and more numerous [10^{11} to 10^{12} per g (wet weight) of feces than that of the adult 10^{2} to 10^{11} per g]. In the adult, gram-negative anaerobic bacilli (bacteroides) outnumber all other groups by a factor of 1 log or more. Weanlings receiving an adult-type diet, but still breast-fed, showed a flora intermediate between that of the wholly breast-fed infant and that of the adult, but more similar to the latter. Anaerobes always constitute the predominant component of the culturable flora of children and adults and are always found in large numbers under conditions of health. The aerobes are significantly less numerous, and vary widely in their number and in the frequency with which they appear in feces.

The development and characteristics of the intestinal microflora of healthy persons are not well known. Although there are some recent studies of the microflora of persons from industrialized countries (9, 13, 19, 20), no data on the microflora of healthy persons is available from preindustrial regions where sanitary conditions and diet are poor, leading to malnutrition, intestinal infection, and diarrheal disease, particularly in children (1, 8).

We deal with the fecal microflora of healthy persons from Guatemala, mainly children in an Indian village. Because of the scarce information on techniques and diagnostic criteria available to characterize intestinal anaerobes and the greater difficulty that this type of work presents, special emphasis is made here of the methods employed to quantitate anaerobic bacteria.

MATERIALS AND METHODS

Study groups. Nineteen children, 2 to 4 months old and wholly breast-fed, comprised the first of three study groups. They were from the Indian village of Santa Maria Cauqué in the Guatemalan highlands (11). All were growing adequately according to the accepted standard (7). None of the individuals in the study had gastrointestinal symptoms or any other clinical manifestation of disease the week preceding the examinations, and no medicines were given during the study period.

Twelve children, 2 to 3 years old, from the same village formed the second study group. They were receiving solid and semisolid foods in addition to breast milk, which was by then scarce; complete weaning in this village usually is accomplished in the third year of life. Food supplements consisted mainly of tortilla (a cooked, flat cake of lime-treated corn meal), black beans, and small amounts of diluted coffee and other fluids (11). These children were all underweight for their age, but their weight to height ratio was adequate. None had gastrointestinal symptoms or any other symptomatology in the week preceding the examinations, and no medicines were given during the study period.

A third group consisted of twelve healthy Guatemalan laboratory workers. Their ages ranged from 23 to 37 years. None of them had had diarrheal disease or medical treatment of any sort in the previous 4 weeks, nor did they present overt gastrointestinal disturbances.

Feces. Morning stool specimens were collected in half-pint cartons. Village mothers were instructed to collect the specimens; in many instances the nurses working in the village supervised and helped in the collection of samples. All specimens were processed within 15 to 30 min of evacuation. The specimens were processed by standardized laboratory procedures (2, 14), described in detail.

Although several samples from each person were employed in the various trials to standardize and

evaluate the technique, the data presented in Results were calculated from one fecal specimen from each person. These single samples were examined after the methodology had been carefully evaluated. Either one of two technicians processed and examined the samples. Microscopic examination of smears and colony counts were made independently by two workers on each sample. Discrepancies required examination by a third worker to reach agreement.

Dilution of feces. With an alcohol-sterilized plastic spoon, approximately 0.9 g of feces was diluted 1:10 in a two-oz (60 ml) glass bottle, with tap water which had been previously adsorbed with charcoal, filtered, and sterilized (CW; 14). Other diluents, such as CW with resazurine and cysteine (17) or nutrient broth also were tested. With a 1 ml pipette, 1- and 0.1-ml amounts of the 10^{-1} dilution were transferred to tubes containing 9 and 10 ml of diluent to make 10^{-2} and 10^{-3} dilutions, respectively. Thereafter, dilutions up to 10^{-8} were prepared. To favor an even suspension, dilutions were mechanically agitated in a Vortex-genie agitator for not more than 10 sec. Before inoculation, dilutions were agitated again for 5 sec.

To determine the reproducibility of the method, duplicate cultures of fecal specimens from five adult subjects were prepared immediately after evacuation by two technicians working simultaneously. Cultures were incubated in the same anaerobic chamber. No significant variations were observed between duplicate tests in the same individual. Tests were also made with CW, CW with resazurine and cysteine, and nutrient broth. There were no differences when the various diluents were compared. CW was used as diluent for all experiments that followed.

Culture media. Selected dilutions were streaked on agar media as outlined in Table 1. Several media were prepared from the base medium of Schaedler et al. (14). This medium was modified by adding 1% Trypticase Soy Broth (BBL) instead of Trypticase (BC-I agar). It was used for total counts of all groups of anaerobic bacteria. BC-I with added neomycin and placenta powder (BC agar) gave excellent growth of bacteroides, clostridia, and other anaerobes. BC-I with added NaCl and neomycin (LS agar) was used mainly for anaerobic lactobacilli and bifidobacteria, although other anaerobes also grew. Growth on BC and LS agars, however, was often 1 log less than that obtained on BC-I. The cultivation of microaerophilic lactobacilli and streptococci, and of enterococci, was on media described by Schaedler et al. (14), here referred to as L agar (lactobacilli and streptococci), and E agar (enterococci). In addition, BBL Tergitol 7 Agar, with 0.4% tetrazolium chloride (T7T), BBL Mannitol Salt Agar (MS), and Levine Eosin Methylene Blue Agar (Difco) with 1% chlortetracycline (LC) were used for enumeration of other microorganisms (Table 1).

Incubation of media. The period of incubation is shown in Table 1. Plates to be incubated anaerobically were streaked first and immediately placed in anaerobic chambers (14) that were exhausted to reach a vacuum of 61 to 64 cm (field laboratory altitude, 6,100 ft), and then filled with N_2, leaving a negative pressure of approximately 12.7 cm. The operation

TABLE 1. *Quantitation of the fecal microflora of man*

Agar medium	Group of microorganism	Dilution streaked (log 10)	Percentage of area of 100-mm plate streaked per dilution	Period of incubation at 37 C[a]	Range of bacterial counts
				hr	
BC-I, BC, and LS	Bacteroides, clostridia, bifidobacteria, lactobacilli, streptococci, and veillonellae	6, 7, 8	33	48[b]	10^8–10^{12}
L	Microaerophilic lactobacilli, and streptococci	5, 6, 7, 8	25	48[c]	10^7–10^{12}
E	Enterococci	4, 5, 6, 7	25	24	10^6–10^{11}
MS	Micrococci, staphylococci, and bacillus	2, 3, 4, 5	25	24	10^4–10^9
T7T	Enterobacteriaceae	4, 5, 6, 7	25	18	10^6–10^{11}
SS and MacConkey	Enterobacteriaceae	Undiluted	100	24	10^2–10^4
LC	Yeasts and molds	2, 3, 4, 5	25	72[c]	10^4–10^9

[a] Incubation was aerobic, except where indicated otherwise.
[b] Incubation was anaerobic.
[c] Incubation in candle jar.

was repeated five times, the entire process usually taking 8 to 10 min. A vacuum of 38 cm was produced with the last filling with N_2; CO_2 then was added to reduce it to 25.4 cm. CO_2 and N_2 are produced locally and are at least 99.8% pure.

After incubation at 37 C, the resulting vacuum was of the order of 19 to 20 cm, which increased to 24 cm after the jars remained at room temperature for 30 min. The losses were accounted for by water vapor in the chamber, by changes in temperature induced by the expansion of CO_2 during filling of the jar, and by expansion of the gases upon incubation.

Anaerobic conditions were checked by an indicator of methylene blue and glucose in an alkaline medium. Germinated soy beans or steel wool, or both, in a saturated solution of $CuSO_4$ did not significantly improve the conditions for growth of anaerobes. Consequently, they were not utilized in the experiments from which the following data were derived. Correct sealing of the vacuum-proof container and adequate purity of the gases were the crucial factors in obtaining anaerobiosis.

Viability of anaerobes. Serial dilutions of fecal specimens from five adult subjects were prepared as indicated before, at intervals of 30 min during 6 hr after evacuation of the specimen. Bacterial counts of anaerobes decreased 1 to 3 logs after 2 hr. Within the first two hr, however, bacteroides and anaerobic lactobacilli were as numerous as in freshly evacuated specimens, providing that the material examined was an inner portion of the specimen. After 2 hr, gram-negative aerobic bacilli and micrococci often increased by 1 log.

Once the specimen was diluted and the dilutions were streaked, counts of anaerobes decreased progressively if they were not incubated promptly. The highest counts of culturable anaerobic bacteria were obtained when less than 15 min elapsed from the time the specimen was manipulated to the time plates were placed in the anaerobic chamber. An interval of 20 min or longer reduced the counts significantly. The usual time required for the operation was 10 min.

Identification and enumeration of bacteria. Colony counts were made directly from the areas streaked and were approximated to the nearest log. Representative colonies (10 to 25) were examined by Gram stain. Colonies were picked from plates of all dilutions, but emphasis was placed on those found in the highest dilutions. When doubt arose in examining the Gram-stained smears, other differential stains were used. Organisms isolated under anaerobiosis were subcultured aerobically to determine whether they were obligate anaerobes. Based on growth characteristics in the various media, macroscopic and microscopic morphology, staining properties, behavior upon aerobic subcultivation, and other criteria, several groups of microorganisms were identified as follows.

Bacteroides. On BC, BC-I, and LS media, bacteroides formed small- to medium-size colonies. These colonies were smooth to finely granular, circular, convex with regular and translucent edges and dense centers, and often produced a foul odor. Subculture under aerobic or microaerophilic conditions was not possible. Their morphology was that of pale staining, nonsporulated, highly pleomorphic, gram-negative rods. The rods were of all sizes, isolated or in filaments, globous, fusiform, or like cocci. More than one morphological type was often cultured from the same person on the same day.

Clostridia. Clostridia produced colonies of variable size, usually large, opaque, curled or circular, commonly irregular, and sometimes with undulate borders. Colonies ranged from translucent to cream colored. Growth on BC-I, BC, and LS media anaerobically produced a foul odor. No growth was obtained aerobically.

The clostridia were gram-positive, straight rods of variable length with well-defined ends; frequently they destain and appear gram-negative. Spores were found occasionally.

Bifidobacteria. The bifidobacteria produced small or medium sized, opaque, whitish to cream colored, circular, convex colonies, with regular edges. On BC-I, BC, and LS media incubated anaerobically, an aromatic odor was produced. These organisms were strongly gram-positive rods, in palisades and "cord-like" arrangements. Also, irregularly stained Y, L, and branching forms were seen.

Veillonellae. These formed colonies of small or medium size on BC-I, BC, and LS media incubated anaerobically. Colonies were translucent at the edge with a yellowish to brown center, or whitish to cream colored. No growth could be obtained under aerobiosis. Microscopically, they were small gram-negative cocci, in masses or discrete.

Lactobacilli. These produced small or medium-sized colonies of at least four types: (i) opaque, circular, raised, with regular edges; (ii) translucent, irregular, raised and flat with undulate edges; (iii) wavy interlaced, irregular, raised, and flat, with fimbriate edges; and (iv) as in (iii) but granular. Some grew on L medium microaerophilically; others grew on BC-I, BC, and LS agar anaerobically. Cultures were aromatic. Microscopically, they were gram-positive rods, some with irregular staining, sometimes pleomorphic and of variable size, in chains, single, or in filaments. At least four morphological types occurred: rhizoid (long, thin filaments); slender (long rods, straight); rough (thick rods, small or long, straight or curled); and catenulate (chains of short, thick rods). Other varieties occur.

Enterococci. Enterococci grew aerobically, producing small- or medium-sized colonies on E medium, white, or with blue centers, circular, raised, with regular edges. Staining showed gram-positive cocci in chains, diplococci, or single organisms.

Micrococci and staphylococci. These were small- to medium-sized colonies, white, yellow, or orange, circular, raised, or convex, with regular edges. They were grown on MS aerobically and appeared as gram-positive cocci of variable size, in pairs, tetrads, or masses.

Gram-positive aerobic bacilli. These were small- to medium-sized colonies, whitish, circular, raised, convex, or flat, with regular edges. They often grew on MS aerobically. They stained gram-positive (sometimes appearing gram-negative), and were bacilli of various sizes, sometimes pleomorphic.

Enterobacteriaceae. Colonies were picked from T7T agar, transferred to Triple Sugar Iron Agar (TSI; BBL), and identified and grouped by the methods of Edwards and Ewing (3). They were also found in anaerobic plates of BC-I, BC, and LS agar, where growth was often 1 log greater than that in Tergitol.

Yeasts. These appeared as white or cream colored colonies on LC agar in a candle jar. Yeasts were also isolated from E agar and other media. *Candida* species were identified by the following criteria: chlamydospore formation, production of germ-tubes in serum, auxanogram, and zymogram.

TABLE 2. *Fecal anaerobic and aerobic microflora in healthy persons*[a]

Bacterial group	Samples tested from[b]		
	Breast-fed children	Weanlings	Adults
Anaerobes	11.5 ± 0.5 (11–12)	11.0 ± 0.4 (10–12)	10.5 ± 0.7 (9–11)
Aerobes	8.3 ± 1.1 (6–10)	8.0 ± 1.0 (6–9)	8.8 ± 0.6 (7–9)

[a] Values are expressed as log 10 of average counts ± SD. Values in parentheses are limits of ranges.

[b] Nineteen breast-fed children, 12 weanlings, and 12 healthy adults were tested.

RESULTS

Tables 1, 3 and 4 show arithmetic averages, standard deviations, and ranges of the various bacterial groups investigated. In general, anaerobes outnumbered the aerobic flora by 1 to 3 logs, anaerobes being consistently of the order of 10^{10} to 10^{12} bacteria per g (wet weight) of feces and aerobes 10^8 to 10^9 per g (Table 2). Slightly higher counts of anaerobic bacteria were observed in infants wholly breast-fed than in weanlings, and counts were definitely higher than in adults. The counts of total aerobes were similar for the three groups.

In the first group, consisting of breast-fed children, the predominant organisms of the fecal flora were bifidobacteria (Table 3). Veillonellae were the next most common, but were not found in as high numbers or as regularly. Other anaerobes were found, but the incidence was much more erratic. For example, 16 of 19 children did not have bacteroides. Only three children had detectable numbers of clostridia. However, it is of major interest that when either of these two groups of organisms was detected they were present in large numbers. Similarly, anaerobic lactobacilli and streptococci were found in large number in only a few of the children. Thus, the only two groups of organisms consistently present in large numbers in this group of children

TABLE 3. *Fecal anaerobic and microaerophilic microflora in healthy persons*[a]

Bacterial group	Samples tested from		
	Breast-fed children[b]	Weanlings	Adults
Bacteroides	10.7 ± 0.6 (<8–11) 16	9.2 ± 0.8 (<8–10) 7	10.3 ± 0.6 (9–11) 0
Clostridia	10.0 ± 0 (<8–10) 17	10.0 ± 0 (<8–10) 10	9.3 ± 0.9 (<8–10) 5
Bifidobacteria	11.4 ± 0.5 (11–12) 0	10.6 ± 0.5 (10–11) 0	9.4 ± 0.9 (<8–10) 3
Anaerobic lactobacilli	9.7 ± 0.5 (<8–10) 13	9.6 ± 0.5 (<8–10) 4	9.0 ± 0.9 (8–10) 4
Anaerobic streptococci	9.6 ± 0.9 (<8–11) 10	9.8 ± 0.6 (<8–11) 2	10.1 ± 0.6 (<8–11) 3
Veillonellae	9.8 ± 0.5 (<8–11) 3	9.6 ± 0.5 (<8–10) 5	9.2 ± 0.8 (<8–10) 6
Microaerophilic lactobacilli	8.2 ± 1.3 (<7–10) 14	9.3 ± 0.8 (<7–10) 5	8.6 ± 0.5 (<7–9) 5
Microaerophilic streptococci	7.9 ± 1.0 (<7–9) 4	9.6 ± 0.7 (<7–11) 1	8.7 ± 0.8 (<7–10) 1
Total anaerobes	11.5 ± 0.5 (11–12) 0	11.0 ± 0.4 (10–12) 0	10.5 ± 0.7 (9–11) 0

[a] Arithmetic average for a particular bacterial group was calculated excluding those cases where that group could not be demonstrated at lowest dilution tested. Values are expressed as log 10 ± SD. Figures in parentheses are limits of range. Figures after parentheses indicate numbers of persons from whose samples the bacterial groups were not identified at the lowest dilution tested.

[b] The same number of persons per group as in Table 2 were tested.

TABLE 4. *Fecal aerobic microflora in healthy persons*[a]

Bacterial group	Samples tested from[b]		
	Breast-fed children	Weanlings	Adults
Enterococci	7.2 ± 1.0 (<6–9) 4	7.8 ± 0.9 (6–9) 0	7.9 ± 1.0 (<6–9) 1
Micrococci and staphylococci	5.9 ± 1.1 (4–7) 0	5.2 ± 0.8 (<4–6) 6	5.0 ± 1.1 (<4–7) 6
Enterobacteriaceae (total)	8.3 ± 1.0 (<6–10) 2	8.0 ± 1.0 (6–9) 0	8.7 ± 0.7 (6–9) 0
Escherichia and *Klebsiella-Aerobacter*	8.3 ± 1.0 (<6–10) 2	7.9 ± 1.0 (6–9) 0	8.6 ± 0.7 (7–9) 0
SLF	8.3 ± 1.2 (<6–10) 13	8.0 ± 1.0 (<6–9) 0	7.6 ± 1.1 (<6–9) 4
Candida yeasts	5.2 ± 1.2 (<4–7) 10	4[c]	4.0 ± 1.0 (<4–5) 9
Total aerobes	8.3 ± 1.0 (6–10) 0	8.0 ± 1.0 (6–9) 0	8.8 ± 0.6 (7–9) 0

[a] Arithmetic average for a particular bacterial group was calculated excluding those cases where that group could not be demonstrated at lowest dilution tested. Values are expressed as log 10 ± SD. Figures in parentheses are limits of range. Figures outside parentheses indicate number of persons from whose samples the bacterial group was not identified at lowest dilution tested.

[b] Number of persons in each group is the same as in Tables 2 and 3.

[c] *Candida* was found in only one person.

were the bifidobacteria and veillonellae. The bulk of the aerobic flora consisted of the enterobacteriaceae, mainly lactose-fermenters (*Escherichia coli* and *Klebsiella-Aerobacter*; Table 4). It should be pointed out that enterobacteriaceae were not demonstrated in two of the 19 children in this group.

In the second group, consisting of weanling children, the bacterial flora differed from that of wholly breast-fed children and adults. Bifidobacteria were less abundant (1 log less) than in infants wholly breast-fed, and anaerobic lactobacilli and streptococci were found more frequently (Table 3). The level of microaerophilic streptococci and lactobacilli was 1 log higher than the level observed in breast-fed babies. All children showed lactose-fermenting enterobacteriaceae in concentrations of 10^6 to 10^9 bacteria per g. Slow lactose fermenters (SLF) were rare as in wholly breast-fed infants (Table 4).

In the third group studied, normal adults, bacteroides were the predominant flora and were always present in high numbers, 10^9 to 10^{11} bacteria per g. Bifidobacteria were always less and could not be demonstrated in 3 of the 12 persons studied. On the other hand, clostridia were more frequent in adults than in breast-fed children. Again, when these organisms were detected, they were present in large numbers. The frequency and concentration of other anaerobic bacteria in adults were similar to those of weanlings (Table 3). The gram-negative aerobic bacilli behaved in adults as they did in weanlings, except that SLF were more common in the latter group (Table 4).

Enterococci were common in the three groups and were found in similar concentrations in all. Average concentration of aerobic micrococci (nonpigmented, sporadically yellow, mannitol-negative or -positive, and usually coagulase-negative) were also similar in the three groups (Table 4), but they were found in only 50% of the weanlings and adults, whereas all wholly breast-fed infants had micrococci ranging from 10^4 to 10^7 per g. *Bacillus* occurred occasionally in low numbers (10^4 to 10^7). Most yeasts were identified as *Candida albicans*, and were in low numbers, 10^4 to 10^7 per g (Table 4).

In general, the culturable flora of the breast-fed child was more abundant and "simpler" than that of weanlings and adults.

DISCUSSION

Since the work of Tissier (*see* 13) bifidobacteria have been recognized as the main bacterial component in feces of breast-fed children. In adults, bacteroides was found as the commonest organism in feces by Eggerth and Gagnon (4) and by Lewis and Rettger (10). Despite these observations and many others (20), textbooks and other publications frequently note that coliforms are predominant microorganisms of the fecal flora. Bifidobacteria and bacteroides may not even be mentioned, or their preponderance is not recognized.

Part of the problem resulted from lack of appropriate techniques to quantitate the anaerobic microflora in a reproducible way in the past. Satisfactory methods, however, recently became available (12, 14–17). We have described procedures (2, 14) modified and proved adequate for work on human microflora in laboratory and field studies. Additionally, some of the general characteristics of the culturable groups of bacteria were described. The observations are of the fecal microflora of healthy adults from a preindustrial country and of children from a Mayan Indian village.

The study showed that the anaerobes outnumber the aerobes by 1 to 3 logs under conditions of good health. In general, breast-fed children had higher counts of total anaerobes than did older children and adults. Bifidobacteria was the dominant component of the culturable fecal microflora, outnumbering by a considerable margin all other anaerobic and aerobic components. They were present in lesser numbers during and after weaning, and still less among adults. Bacteroides were rare in breast-fed children, however, but present in larger numbers in about 50% of the weanlings. In adults, the bacterioides constituted the predominant bacteria surpassing all other culturable bacteria by 1 or 2 logs. Clostridia were found only occasionally in children or adults. Anaerobic lactobacilli and streptococci tended to be more common in older children and adults.

Enterobacteriaceae almost always were demonstrable in numbers of 10^6 to 10^{10} per g, lactose fermenters were well established in weanlings and in adults, and SLF were less frequent.

Bifidobacteria in breast-fed infants and bacteroides in adults were the stable groups of the microflora. All other culturable groups varied widely, to the extent that some decreased in numbers by several logs or were not demonstrable at the dilutions cultured. This behavior suggests that certain kinds of bacteria (enterobacteriaceae, enterococci, microaerophilic lactobacilli, and some anaerobes) are more dependent than others on the pabulum ingested and on other environmental factors affecting the intestinal milieu. The stable components of the flora (i.e., bifidobacteria or bacteroides) seem to be in closer association with the host, demonstrating greater constancy and stability under conditions of adequate health.

The fecal flora of the adult was more abundant than that reported by Kalser et al. (9) and Gorbach et al. (5), although similar to that described by Zubrzycki and Spaulding (20) and Weijers and van de Kamer (18). Methodological procedures likely account for these differences. The fecal flora of breast-fed children was similar to that described by Gyllenberg and Roine (6) and Smith and Crabb (15) in children from two industrialized countries.

The flora of the weanlings seems to be a transitional one, between those of infants and adults, with bifidobacteria still predominating and bacteroides appearing with greater frequency and in large numbers. The fecal flora of healthy weanlings varied from that described for other weanlings of the same region recovering from malnutrition and suffering *Shigella* infections (2). In the latter, coliforms were fewer, as were lactobacilli, streptococci, and the strict anaerobes.

These observations are being extended by a long-term field study of rural children from birth through breast-feeding and the weaning process, as they encounter the frequent infectious diseases (primarily diarrheas and dysenteries) and the prevailing malnutrition.

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