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Bacterial succession in the colon during childhood and adolescence: molecular studies in a southern Indian village[1–3]

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ABSTRACT

Background: The colonic bacterial flora, largely anaerobic, is believed to establish and stabilize in the first 2 y of life.

Objective: This study was undertaken to determine whether the bacterial flora of the colon undergoes further changes (succession) during childhood and adolescence.

Design: This cross-sectional study examined fecal samples from 130 healthy children and adolescents in the age group 2–17 y and from 30 healthy adults (median age: 42 y) residing in a single village in southern India. DNA was extracted and subjected to 16S rDNA-targeted real-time polymerase chain reaction to determine the relative predominance of *Bifidobacterium* genus, *Bacteroides-Prevotella-Porphyromonas* group, *Lactobacillus acidophilus* group, *Eubacterium rectale*, and *Faecalibacterium prausnitzii*.

Results: *Bifidobacterium* species and *Bacteroides-Prevotella* group were dominant fecal bacteria overall. *E. rectale* and *Lactobacillus* species were considerably less abundant. Clear age-related differences emerged, with a steep decline in *Bifidobacterium* species in adults ($P < 0.0001$), a steep decline of *Lactobacillus* species >5 y of age ($P < 0.0001$), an increase in *Bacteroides* during late adolescence and in adults ($P = 0.0040$), an increase in *E. rectale* during childhood and adolescence followed by a steep decline in adults ($P < 0.0001$), and a late childhood peak of *F. prausnitzii* with decline in adolescents and adults ($P < 0.0001$).

Conclusions: Changes in the bacterial flora occur during childhood and adolescence characterized by reduction in *Lactobacillus* and *Bifidobacterium* species and an increase in *Bacteroides, E rectale*, and *F. prausnitzii* peaked during late childhood in this population. *Am J Clin Nutr* 2008;88:1643–7.

INTRODUCTION

The human gastrointestinal tract harbors numerous species of bacteria throughout its extent. They are particularly abundant in the colon, where the flora is largely anaerobic (1). The mutuality established between the host and the resident bacteria plays important functional roles such as protection from pathogenic organisms, production of short-chain fatty acids that serve roles in colonic epithelial and mucosal physiology, and production of vitamins such as vitamin K and biotin (2). The basis of the relation between the colonic flora and human health continues to be the object of much study. To clearly understand the basis of this relation, and before introducing interventions such as probiotics to promote health, it is important to characterize the bacterial composition of the gut in a population of healthy persons.

The gut of the newborn is sterile at birth. The bacterial flora is established at or shortly after birth by bacteria in the immediate environment. The first 2 y after birth are characterized by a succession of bacteria within the intestine that reflects changes in the diet of the neonate and the infant (3). It is believed that once the flora is established in the early years, it generally continues to exist unchanged through life (3).

Until recently, methods used to determine the composition of bacterial flora were based on culture techniques, with inherent limitations related to the growth fastidiousness of these organisms. The availability of molecular techniques, particularly those based on examination of the 16S ribosomal RNA genes, provide an excellent opportunity to reexamine our earlier understanding of the bacterial flora of the human colon (4). The present study was designed to examine whether the bacterial flora of the colon remain stable after early childhood and to determine whether there was a pattern of succession of bacteria in the intestine during adolescence and adulthood. To this end, real-time polymerase chain reaction targeting 16S rDNA of bacteria was used to provide a quantitative determination of several major classes of fecal anaerobic bacteria. To minimize the variables that would be introduced by variations in diet and socioeconomic status, residents of a single village, comprising a relatively homogeneous population with a relatively homogeneous dietary pattern, were used to provide this information.

SUBJECTS AND METHODS

Subjects

Volunteers were recruited from a single village of ≈2000 residents located 30 km from Vellore, India. Initial survey visits to the village with focus group discussions preceded the study. Informed consent was obtained from the participants, and the

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study was approved by the Research Committee of the Christian Medical College, Vellore, India.

Volunteers were randomly selected from those attending the focus group discussion that preceded the study. The volunteers included 130 children and adolescents 2–17 y of age, attending the local school. The age-wise distribution of children and adolescents was as follows: 2–3 y ($n = 16$), 4–5 y ($n = 17$), 6–7 y ($n = 23$), 8–9 y ($n = 19$), 10–11 y ($n = 9$), 12–13 y ($n = 12$), 14–15 y ($n = 16$), and 16–17 y ($n = 18$). Thirty adults in the age group 28–50 y (median age: 42 y) were also included. Volunteers were each given a stool collection kit that included a specimen container, tissues, and a wooden spatula and were requested to collect stool the next morning. Volunteers were excluded from the study if they gave a history of antibiotic intake within the past month.

The socioeconomic status of the family was assessed with the use of a method that was standardized for the Indian rural population (5). It included 10 variables including occupation of the family head, literacy of parents, land owned, type of housing, number of household articles, farm animals possessed, and social participation. On the basis of the scores obtained, the residents of this village fell into the lower-middle class and lower class groups of the scale. An intensive diet survey was conducted by a trained dietitian on 20 randomly selected subjects comprising 2 adult women and 2 children in each of the age groups 1–3, 4–6, 7–9, 10–12, 13–15, and 16–18 y, with ages <10 y having separate representation by sex. A 24-h recall food-frequency questionnaire and a set of standardized cups, glasses, and spoons were used. Dietary intake of nutrients was calculated from food composition tables for Indian diets and expressed as a percentage of age-specific recommended daily allowances (RDAs) for Indians (6).

Sample collection and processing

Fresh fecal samples were collected in the morning and transported on ice within an hour to the laboratory, where samples were labeled and stored in aliquots at −80 °C until processing. Samples were processed in batches. DNA was extracted from ≈250 mg (wet weight) of fecal sample with the use of the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). The DNA was eluted in a final volume of 200 μL and stored at −20 °C.

Polymerase chain reaction quantification of anaerobic bacteria

Real-time polymerase chain reaction (PCR) was done with the use of primers and PCR conditions that were described earlier (7, 8). Primers were directed at amplifying 16S rDNA from the following groups or genera: *Bacteroides-Prevotella-Porphyromonas* group, *Bifidobacterium* genus (detecting all *Bifidobacterium* species), *Bifidobacterium longum* group (detecting *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium pseudolongum*, and *Bifidobacterium suis*), *Lactobacillus acidophilus* group (detecting *Lactobacillus acidophilus acidophilus*, *Lactobacillus acidophilus amylovorus, Lactobacillus acidophilus amylolyticus, Lactobacillus acidophilus crispatus, Lactobacillus acidophilus gasser*, and *Lactobacillus acidophilus johnsonii*), and the following species: *Bacteroides fragilis*, *Eubacterium rectale*, and *Faecalibacterium prauznitzii.* Primers were also used to amplify a conserved 16S rDNA sequence present in all bacteria, the universal primer set, the amplification of which served as the denominator against which amplification of other bacterial nucleic acid was compared. Real-time PCR was done in a Chromo4 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with the use of the SYBR Green master mix (Eurogentec, Liege, Belgium).

The PCR conditions were standardized initially with gradient PCR. (See Figure S1 under "Supplemental Data" in the online issue.) The OPTICON 3.1 software (Bio-Rad) plots the rate of change of the relative fluorescence unit (RFU) with reference to time (T) [$-d$ (RFU)/dT]) on the y-axis compared with the temperature on the x-axis, with the curve peaking at the melting temperature, and melting curve analysis was always done to check the specificity of the amplification. Quantification was based on the fluorescence intensity obtained from the intercalated SYBR Green dye. The cycle number at which the signal was first detected [the threshold cycle (Ct)] correlated with the original concentration of DNA template. DNA copy was not expressed as an absolute number, but it was expressed by the Ct at which DNA for each target was detected relative to the Ct at which universal bacterial DNA was detected after amplification. This relative quantification was done automatically by the OPTICON 3.1 software by using the formula $2^{-\Delta Ct}$ and expressed as relative fold difference compared with the reference (universal) amplicon. (See Figure S2 under "Supplemental Data" in the online issue.) The relative difference of the target bacterial population is thus the ratio of the target bacterial rDNA compared with universal rDNA, providing a quantitative comparison between different samples. The ratio calculated for each of the duplicate samples always correlated well with the other, with an adjusted R^2 in the region of 0.80.

Statistics

Values are presented as median (range). The significance of differences was assessed with the use of Kruskal-Wallis analysis of variance for nonparametric data because the variances were different between different age groups for the same bacterial group. Posttest comparisons between individual age groups were done with the use of the Dunn test. A 2-sided P value < 0.05 was considered significant. Statistical analyses and graphing were done with the use of PRISM 4.0 (GraphPad Software Inc, San Diego, CA).

RESULTS

The study village population was fairly homogeneous, and included farmers and manual laborers who belonged to the lower or lower-middle class of the socioeconomic scale. Their diet was predominantly lactovegetarian, consisting primarily of cereals and some milk and milk products, with meat intake on average once a week. Cereal intake ranged from 33% to 106% (median: 73.2%) of RDA, the most commonly used cereals being rice and ragi (finger millet, *Eleusine coracana*). Other food groups that were eaten (shown as percentage of age-specific RDA) were lentils (42–72.8%; median: 54.3%), milk (13.8–60%; median: 38%), roots and tubers (2.6–72.6%; median: 17.6%), green leafy vegetables (3.3–50%; median: 11%), other vegetables (13.3–54.7%; median: 33.4%), fruit (36–195.6%; median: 100%), sugar (35.5–83.3%; median: 55.5%), and fats and oils (30–98.6%; median, 80%). Overall, energy and carbohydrate intakes in all selected subjects were slightly less than the RDAs, the protein intake was significantly less than RDAs, and the fat intake was near normal.

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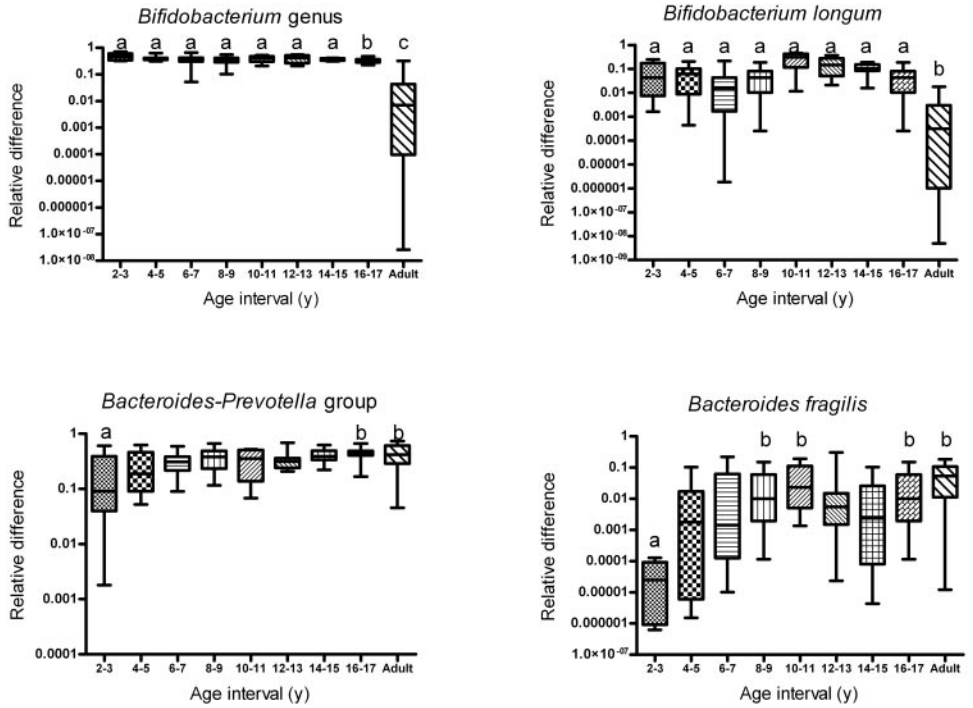

FIGURE 1. Quantitative estimate of the specified fecal bacteria. Bacterial rDNA was amplified by real-time polymerase chain reaction with specific primers targeted at 16S rDNA sequences and expressed as the difference in amplification relative to amplification of a universal bacterial 16S rDNA sequence. Results are depicted as relative difference on a logarithmic *y*-axis, and the scale is different in each panel. Healthy volunteers of different age groups from 2-y-olds to adults are represented. Statistical analyses were done by using the Kruskal-Wallis test with post hoc comparisons using the Dunn test. Kruskal-Wallis $P < 0.0001$ for *Bifidobacterium* genus, *Bifidobacterium longum* group, and *Bacteroides fragilis*, and $P = 0.0040$ for the *Bacteroides-Prevotella-Porphyromonas* group. Box plots of bacteria depict the median (middle line), interquartile range (box), and range (whiskers). Boxes not sharing a common identifying letter are statistically significantly different ($P < 0.05$) from one another.

Quantitative studies showed variations in the fecal bacteria with age. The *Bifidobacterium* genus was prominent at 2–3 y of age and showed a steep decline in adults (**Figure 1**). *Bifidobacterium longum*, which was separately estimated, paralleled changes in *Bifidobacterium* genus, declining steeply in adults (Figure 1).

The *Bacteroides-Prevotella-Porphyromonas* group of bacteria was a main constituent of the fecal bacterial flora particularly in older children and adults. They were relatively lower in number at 2–3 y of age and gradually increased in relative numbers to peak to adult amounts by the age of 17 y (Figure 1). *B. fragilis*, one of the constituents of this group of bacteria, was quite low in young children but mirrored changes in *Bacteroides-Prevotella*, increasing in adults (Figure 1).

Bacteria belonging to the *E. rectale* were a quantitatively significant component of the fecal bacterial flora ≥6 y and reached maximal amounts in late adolescence (**Figure 2**). They were low in relative number in preschool children <5 y of age, as well as in adults.

L. acidophilus bacteria were found in relatively higher numbers in early childhood in children aged 2–3 y but then declined exponentially (3–4 log reduction) at >5 y of age to reach low amounts in the feces (Figure 2). *F. prausnitzii* were low in early childhood, suddenly increased to reach a peak around the ages of 6–9 y, and then declined rapidly with a second small peak in adolescence (Figure 2).

DISCUSSION

The importance of the normal gut microflora to human health is well recognized. Our understanding of the gut flora is that it is established at or shortly after birth, changes during weaning and the first 2 y of life, and remains more or less constant thereafter (3). Although it is now appreciated that there may be significant alterations in the gut flora in the elderly (9), the general perception remains that the composition of the gut flora is constant from early childhood into adulthood. The findings in the present study challenge this perception.

Anaerobic bacteria constitute >99% of the fecal bacteria (1). This study examined main classes of fecal anaerobic bacteria, including *Bacteroides* and *Bifidobacteria*. The studies reported here show that there is a continuing and gradual change in fecal bacterial flora beyond early childhood and this change continues into adolescence and adulthood. This change with age was reflected by a rapid decline in *Lactobacillus* in the preschool children so that by the time a child reaches school age, the *Lactobacillus* group has become extremely small in number. Changes during adolescence included an increase in *E. rectale* and *F. prauznitzii*. Both these, along with *Bacteroides*, are main producers of short-chain fatty acids from fermentation of unabsorbed carbohydrates and thus important to colonic physiology.

Comparison of the present study with other available information is limited because much of our prior knowledge derives from culture-based studies of the flora and because molecular

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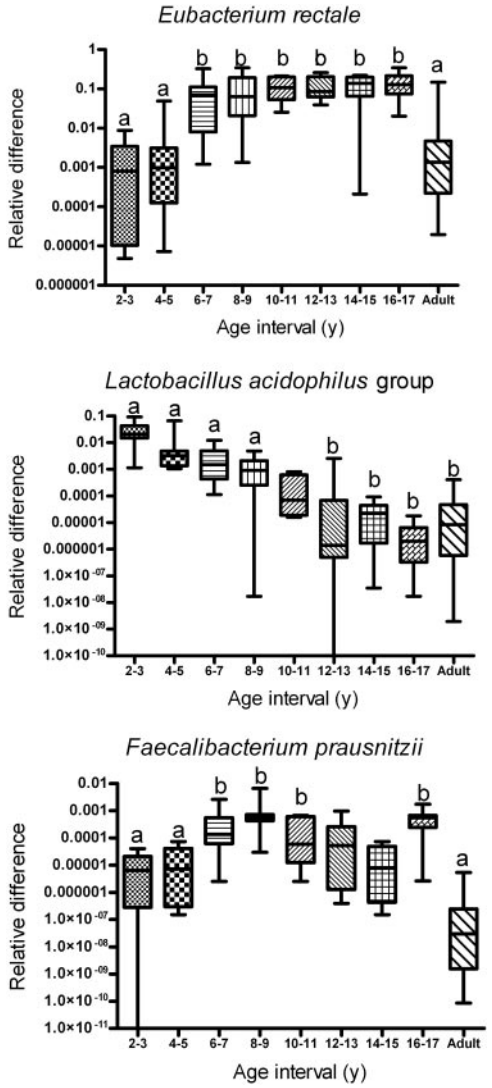

FIGURE 2. Quantitative estimate of specified fecal bacteria. Bacterial rDNA was amplified by real-time polymerase chain reaction with specific primers targeted at 16S rDNA sequences and expressed as the difference in amplification relative to amplification of a universal bacterial 16S rDNA sequence (relative difference). The *y*-axis is logarithmic, and the scale is different in each panel. Healthy volunteers of different age groups from 2-y-olds to adults are represented. Box plots of bacteria depict the median (middle line), interquartile range (box), and range (whiskers). Statistical analyses were done by using the Kruskal-Wallis test with post hoc comparisons using the Dunn test. Kruskal-Wallis $P < 0.0001$ for *Eubacterium rectale*, *Faecalibacterium prausnitzii*, and *Lactobacillus acidophilus* group. Boxes not sharing a common identifying letter are statistically significantly different ($P < 0.05$) from one another.

studies have focused on infants (<1 y) and adults, without specifically examining changes in children and adolescents. A large study that used fluorescence in situ hybridization with probes targeting 16S rDNA showed that the fecal flora in a European population (mainly adults) was composed largely of the *Clostridium coccoides-E. rectale* group and the *Clostridium leptum* group (the predominant constituent of which was *F. prausnitzii*) followed by *Bacteroides* (10). Examination of select bacterial groups in infants by real-time PCR indicated that fecal amounts of *Bacteroides*, *C. coccoides*, and *F. prausnitzii* increased markedly after the age of 6 mo (11). However, real-time PCR and fluorescence in situ hybridization study of the fecal flora in infants found that *Bifidobacterium* genus was the predominant bacterial group in stool, and that *Bacteroides*, *Lactobacillus*, and *Clostridium* were all ≥1 log less in number in children <2 y of age (12).

Although many factors are known to influence the nature of the colonic bacterial flora, diet is likely to be the single most important factor leading to variation in a heterogeneous population. For these studies, a single village population was chosen to minimize dietary variations. The study population was homogeneous belonging to the lower or lower-middle class, and their dietary practices were fairly homogeneous because of restrictions on availability of foods. The children were all from a single public school. Preliminary dietary surveys and focus group discussion identified the predominant dietary constituents and indicated that there was little variation among participants in the various age groups with respect to diet. The diet was predominantly cereal based (rice, ragi) with vegetables and lentils and milk and the occasional use of meat. Meat intake was less frequent than once a week and was predominantly during a particular month ("Aadi") of the year. No main differences were observed in diet between the participants, and, although formal dietary survey was only done in 20 persons, it substantiated the relatively homogeneous dietary practices in the group studied. No association of floral pattern with dietary preference was noted. Meat intake was infrequent in all age groups and did not appear to be a main factor in the change in bowel flora with age.

The fecal amounts of total *Bifidobacterium* species varied in this group with the maximum numbers seen in the 2–3 y age group, declining steeply in adulthood. *B. longum* formed a small subset that mirrored changes in *Bifidobacterium* species generally. Dietary nonabsorbable carbohydrates, such as fructooligosaccharides, oligosaccharides, and digestion-resistant starch, increase bifidobacterial populations in the gut (13). The variation in bifidobacterial numbers with age could potentially be related to differences in dietary habit and preferences as children grow older and turn into adults. Because bifidobacteria are often considered for use as probiotics and may be advantageous in the maintenance of good health (14), the decline in this fecal population in adults could be a matter of concern and a target for nutritional strategies to preserve them. Bifidobacteria were shown to inhibit the growth of a number of enteric bacterial pathogens and may prevent gastroenteritis in humans (15).

Bacteroides is generally considered a group of bacteria that may have adverse effects on the host, even though individual components of this group may have beneficial health effects (16). *Bacteroides* bacteria were relatively less common than bifidobacteria in young children. *Bacteroides* proportions increased in older children, reached their highest amounts in the adolescent population, and remained steady thereafter. *Bacteroides* bacteria are nutritionally versatile, are able to use a wide variety of different polymerized carbon sources, and are therefore responsible for digesting most polysaccharides. *Bacteroides* species, along with *E. rectale* and *F. prausnitzii*, is the main producer of short-chain fatty acids from carbohydrates (17). An increase in bacteroidetes was originally reported in obese children compared with lean children (18), although a more recent study suggests that higher *Bifidobacterium* amounts in the feces in early childhood are associated with a lesser likelihood of developing obesity later in childhood (12). A nutritional anthropometry survey in the school preceding the study indicated that

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>80% of the children were between 80% and 100% of expected weight for age and weight for height. However, anthropometric variables were not recorded for individual participants, and a possible relation between fecal bacteria and nutritional status was not examined in this study.

The fecal amounts of *L. acidophilus* were found to be relatively high in young children and then precipitously declined. Lactobacilli are well known to have beneficial effects on health and immunity and are widely used as probiotics (19–21). This decline in their numbers during childhood and adolescence may reflect changes in dietary intake of nutrients that support growth of lactobacilli or simply reflect the growing prominence of other bacteria.

The fecal amounts of *E. rectale* were low to undetectable in the young children, but increased in late childhood and adolescence, declining again in adults. *Eubacterium*, the second most prominent group of bacteria normally found in feces, have complex nutritional requirements, and their increase in total numbers in this group may have health consequences because of the greater potential for bile acid transformations, creating potentially harmful metabolites in the gut (22–24). *Eubacterium* have positive influences on health as well, and they are main producers of butyrate, which is important to colonocyte metabolism and health (25). In the present study *E. rectale* were the third most numerous organisms after *Bifidobacterium* and *Bacteroides*.

In conclusion, the present study shows ongoing changes in the composition of the fecal anaerobic bacterial flora through childhood and adolescence, and it suggests that the bacterial flora of the gut is engaged in an ongoing process of change and bacterial succession with aging. Further examination of this phenomenon and its environmental and health correlates are warranted.

The author's responsibilities were as follows—BSR and RB: were responsible for conception, analysis of data, and writing the manuscript; HPJ, SG, and SPC: were responsible for sample and data collection; SPC and RB: were responsible for the PCR analyses. None of the authors had a personal or financial conflict of interest.

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