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Molecular Analysis of the Composition of the Bifidobacterial and Lactobacillus Microflora of Humans

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The bifidobacterial and lactobacillus populations of fecal samples collected from two human subjects during a 12-month period were studied. The total numbers of bifidobacteria were stable throughout the study period in both subjects, but lactobacillus numbers were less constant. Analysis of the composition of the bifidobacterial populations by using ribotyping or pulsed-field gel electrophoresis to differentiate between bacterial strains demonstrated major differences between the subjects. Subject 1 harbored five strains of bifidobacteria throughout the 12-month period, and one strain was numerically predominant. In contrast, subject 2 harbored a more complex bifidobacterial population (five to six strains per sample) whose composition fluctuated throughout the 12 months. One lactobacillus strain was numerically predominant throughout the study in both subjects. Strains of bifidobacteria and lactobacilli common to both subjects were not detected.

The large intestine of humans is inhabited by a complex collection of microbes, mostly bacterial species, known collectively as the normal microflora (17). The numerically predominant species of bacteria are obligately anaerobic and are represented by both gram-positive and gram-negative genera (17). Members of the genus *Bifidobacterium* colonize the intestinal tract soon after birth and are present at high population levels in both infants and adults (16). Lactobacilli are also common inhabitants of the large intestine of humans, although they are outnumbered by the cells of obligately anaerobic species and, according to Finegold and colleagues, cannot be detected in fecal samples collected from approximately 27% of Americans (4). Knowledge of the composition of the intestinal (fecal) microflora of humans comes largely from comparisons of the microbial contents of fecal samples collected from subjects ingesting contrasting diets and following different lifestyles (3). From the results of these studies, it has been concluded that, in the absence of major stressors, the intestinal microflora is relatively stable from the perspective of bacterial species (10) and that it is possible to predict the bacterial species likely to constitute 99% of the fecal microflora of an individual (2). Identification of bacterial inhabitants at the species level can, however, be considered to be a rather crude measure of the microbial content of an ecosystem, especially one suspected of dynamism on the basis of evidence obtained from other mammalian hosts. Biological successions of biotypes of *Enterococcus faecium* and of strains differentiated by plasmid profiling of *Lactobacillus acidophilus* and *Lactobacillus fermentum* have been described in mice and in piglets, respectively (15, 18). Furthermore, fluctuations in the composition of populations of *Escherichia coli*, detected through the use of serotyping, in human fecal samples have been reported (5). While stability in species composition may be a feature of the normal microflora, stability at the level of bacterial strains may be less common. If this is the case, the acquisition of the normal microflora may be never ending as new strains of endogenous or exogenous origin proliferate and attain dominance in the intestinal ecosystem under the influence of allogenic or autogenic factors (16). We have monitored the fecal microfloras of two humans with respect to the bacterial strain composition of the bifidobacterial and lactobacillus populations over a 12-month period. These two genera were chosen for investigation because they are often proposed as being suitable components for health food yogurts and other probiotics (direct fed microbials).

MATERIALS AND METHODS

Subjects. Two healthy human subjects maintained their usual lifestyles and dietary intakes throughout the study period, which had a duration of 12 months. Subject 1 (50 years) was male; subject 2 (25 years) was female. Subject 2 was administered amoxicillin trihydrate (1 g/day) for 7 days during weeks 21 and 22 of the study to treat a respiratory tract infection. Neither subject included yogurt or other milk products containing Acidophilus-Bifidus cultures in his or her diet.

Sampling. A fecal sample was collected at least once every month from each individual for 12 months. The samples were collected in sterile specimen jars and immediately taken to the laboratory, where they were introduced into an anaerobic chamber for processing (gas mixture: 5% CO_2, 10% H_2, and 85% N_2). On average, processing began within 5 min of collection of the fecal sample. A 0.5- to 1-g portion of feces was removed from the middle of the sample and used to make a fecal homogenate in 4.5 ml of prereduced brain heart infusion broth (Difco, Detroit, Mich.) supplemented with yeast extract, cysteine hydrochloride, and vitamin K-hemin (BHIS [6]). A dilution series (10^{-1} to 10^{-7}) was then made in the same medium, and 100-μl aliquots of each dilution were used to inoculate selective media by spread plating. Beerens medium (1), Rogosa agar (Difco), *Bacteroides* bile esculin agar (14), and MacConkey agar (Difco) were used to isolate *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and *Enterobacteriaceae* strains, respectively. The plates were incubated anaerobically for 2 to 3 days at 37°C with the exception of the MacConkey plates, which were incubated aerobically overnight at 37°C.

Enumeration and selection of bacterial isolates. Following incubation, colony counts were made from dilution plates of the various selective media that contained discrete colonies (30 to 300), and the total population of each genus was calculated as CFU per gram (wet weight) of feces. A preliminary experiment, in which three homogenates and dilution series were prepared from a single fecal sample, showed that bacterial counts were within a 0.5-log range (data not shown). Only one enumeration per fecal sample for each of the bacterial groups was therefore made thereafter. For each fecal sample, 10 colonies of bifidobacteria and 10 colonies of lactobacilli were randomly selected and subcultured onto the appropriate agar medium for future genetic fingerprinting. The number of randomly collected isolates examined per sample was chosen on the basis of the publication of Hartley et al. (5) that reported that 10 colonies gave an adequate representation of the major bacterial strains cultured on a selective medium. To ensure that the subculture of 10 colonies was adequate, however, two additional fecal samples were collected from subject 2. Ten colonies of bifidobacteria were randomly selected from an appropriate dilution plate for ribotyping, and then all of the colonies remaining on that plate (about 30) were also subcultured for ribotyping. Subcultures were made only from primary dilution plates that gave discrete colonies. Therefore, only the composition of the bacterial populations with regard to numerically predominant strains was obtained. Once pure cultures (i.e., a single colony morphology) were obtained, the bacterial isolates were

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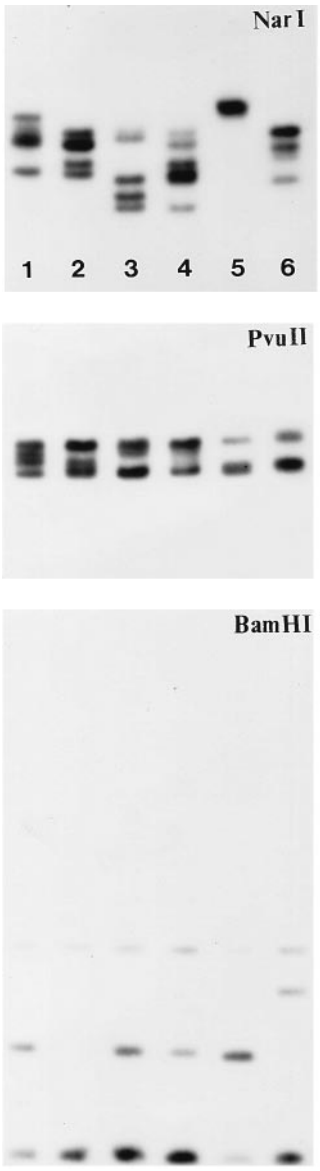

FIG. 1. Autoradiographs showing ribotypes (A24, A24a to A28) of six *Bifidobacterium* isolates. Panels: top, DNA digested by *Nar*I; middle, DNA digested by *Pvu*II; bottom, DNA digested by *Bam*HI. Lanes (all three rows): 1, A24; 2, A24a; 3, A25; 4, A26; 5, A27; 6, A28.

cultured in liquid media—trypticase-phytone-yeast extract (TPY [11]) for *Bifidobacterium* species and Lactobacilli MRS medium (Difco) for *Lactobacillus* species—and stored with the addition of skim milk at −70°C.

Provisional identification of genera was made on the basis of Gram stain reactions and cell morphology, in conjunction with the ability of the isolate to grow on the appropriate selective medium. Lactobacilli were examined by the catalase test. Detection of the major fermentation products of strains grown in peptone-yeast extract-glucose medium (PYG [6]) by gas-liquid chromatography permitted identification of the bacteria to the genus level (6). The identification of *Bifidobacterium* strains was further confirmed by demonstration of the enzyme fructose-6-phosphate phosphoketolase in cell extracts (11). *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium angulatum* DSM 20098, *Bifidobacterium bifidum* DSM 20456, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium catenulatum* DSM 20103, *Bifidobacterium infantis* ATCC 15697, *Bifidobacterium longum* ATCC 15707, *Bifidobacterium pseudocatenulatum* DSM 20438, and *L. acidophilus* ATCC 4356 were used as reference strains or sources of 16S rRNA genes (16S rDNA) for use as DNA probes.

Differentiation of bifidobacterial and lactobacillus strains by ribotyping and pulsed-field gel electrophoresis (PFGE). The ribotyping method of McCartney and Tannock (7) was used to differentiate the strains among randomly selected bifidobacterial isolates collected from each monthly sample. Briefly, cells were embedded in low-melting-point agarose prior to DNA extraction. Three restriction endonucleases (*Nar*I, *Pvu*II, and *Bam*HI) were used separately to digest the bifidobacterial DNA. The DNA fragments were separated by agarose gel electrophoresis and transferred to nylon membranes by the alkali blot method. A radioactively labelled 16S rDNA probe obtained by PCR from *B. infantis* ATCC 15697 DNA was used to detect restriction fragments in the digests that contained 16S rRNA sequences. Hybridization was detected by autoradiography. Only distinctive hybridization patterns detected by autoradiography were considered to signify unique ribotypes. Subtle differences in hybridization patterns, such as the addition of an extra fragment or change in the migration of a fragment compared with that of an established ribotype, were not considered to represent a new strain (13). A pattern that demonstrated such subtle differences for any one of the enzymes used to prepare digests, when compared with an established ribotype, was designated a subribotype. Examples of ribotypes obtained during the study are shown in Fig. 1.

PFGE (12) was used to confirm the differentiation of bifidobacterial strains obtained by ribotyping. Bifidobacterial DNA, extracted in the same manner as that used for ribotyping, was digested with *Xba*I, and restriction fragments were separated by PFGE over 17 h. An initial pulse time (the time the field of current remains in one orientation before changing) of 1 s and a final pulse time of 12 s were used. The included angle (change in orientation of the field) was 120°, and the gel was run at 5 V/cm with the buffer maintained at 14°C. Gels were stained with ethidium bromide (5 μg/ml) and examined by UV transillumination.

Ribotyping and PFGE were also used to differentiate lactobacillus strains. The DNA extraction method differed from that used for bifidobacterial strains in that the lactobacilli were cultured in Lactobacillui MRS medium and lower concentrations of lysozyme (20 mg/ml) and mutanolysin (40 μg/ml) were used for cell lysis (9). *Hin*dIII and *Eco*RI were used in ribotyping of lactobacilli (9), while *Apa*I was used for PFGE. A 16S rDNA probe (9) obtained by PCR of *L. acidophilus* ATCC 4356 DNA was used in the ribotyping of lactobacillus strains.

Species identification of representative strains. Representative isolates of each *Bifidobacterium* ribotype were speciated following the approaches of Scardovi (11), Holdeman et al. (6), and Mitsuoka and Kaneuchi (8). Bifidobacteria

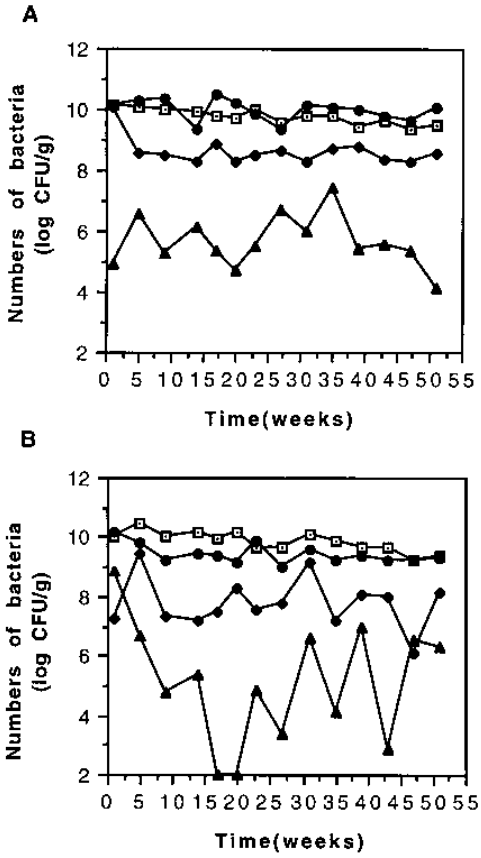

FIG. 2. Total populations of *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and *Enterobacteriaceae* strains in the feces of two human subjects. (A) Subject 1; (B) subject 2. Symbols: ⊡, *Bifidobacterium* strains; ◆, *Lactobacillus* strains; ●, *Bacteroides* strains; ▲, *Enterobacteriaceae* strains.

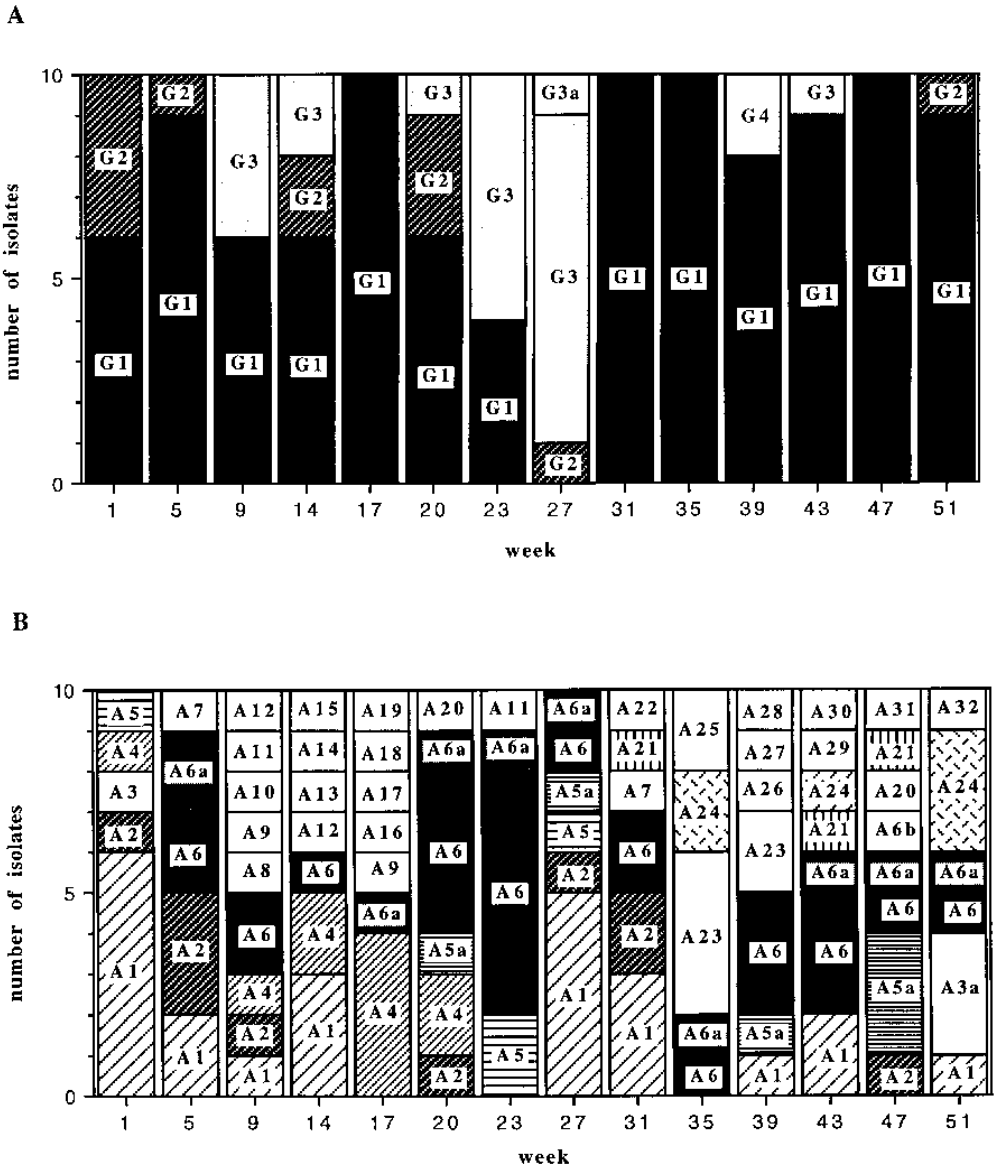

FIG. 3. *Bifidobacterium* strains detected in fecal samples collected from two human subjects over a 12-month period. (A) Subject 1; (B) subject 2.

were identified by their cell morphologies on TPY agar stabs and by their ability to ferment amygdalin, arabinose, cellobiose, glycogen, lactose, melezitose, raffinose, ribose, salicin, sorbitol, starch, trehalose, and xylose. Fermentation was defined as a reduction in the pH of the fermentation media by at least 0.9 pH unit relative to that of an uninoculated control after 48 h of incubation. Species identification of the *Lactobacillus* strains was obtained by using API 50 CH strips (bioMerieux S.A., Marcy l'Etoile, France) and then referring to the manufacturer's databank.

RESULTS

Enumeration of total bacterial populations. Fourteen fecal samples were collected from each individual over the 12-month period. The total populations of bifidobacteria and bacteroides remained constant (about 10^{10}/g [wet weight] of feces) throughout the 12-month period for both individuals (Fig. 2). Lactobacillus numbers were relatively constant (about 10^9/g) in fecal samples from subject 1 but fluctuated in the fecal samples from subject 2 (10^7 to 10^9/g). The enterobacterial numbers in the feces of both individuals fluctuated, to varying degrees, throughout the study period, with numbers below the level of detection (10^2/g) in the case of subject 2 in weeks 17 and 20 of the study. The administration of amoxicillin to subject 2 did not affect bacterial numbers to a detectable extent.

Composition of the bifidobacterial population. Five strains of bifidobacteria were detected in the feces of subject 1 by ribotyping during the 12-month period. Figure 3A shows each of the strains as a proportion of the total bifidobacterial population detected each month in subject 1. Strain G1 was the numerically predominant member of the bifidobacterial population of subject 1 throughout the 12-month period, being absent from only one sample (week 27). The strain constituted between about 40 and 100% of the population at each sampling. We considered G1 to be a resident strain of subject 1 since it was detected regularly. Strains G2 and G3 were considered to be sporadic because they appeared, disappeared, and reappeared during the study. Subject 2 harbored a more complex bifidobacterial population than subject 1, with 36

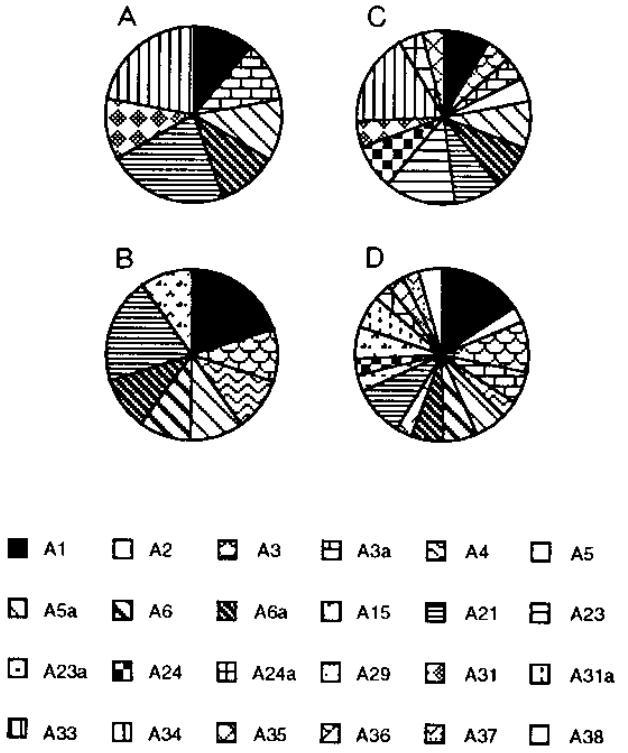

FIG. 4. Bifidobacterial strains detected in additional fecal samples collected from subject 2. (A and B) Proportions of strains obtained by ribotyping 10 randomly selected colonies from samples 1 and 2, respectively; (C and D) proportions obtained by ribotyping all colonies on selective agar plates from samples 1 and 2, respectively.

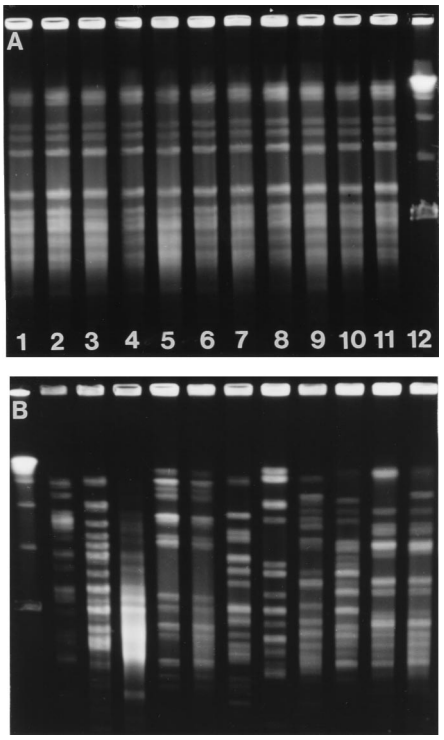

FIG. 5. PFGE of *Bifidobacterium* DNA digested by *Xba*I. (A) Lanes 1 to 11, multiple isolates of strain G1; lane 12, λ ladder standards (see Fig. 7). (B) Lanes: 1, λ ladder standards; 2, *B. infantis* ATCC 15697; 3, *B. adolescentis* ATCC 15703; 4, *B. longum* ATCC 15707; 5, *B. angulatum* DSM 20098; 6, *B. catenulatum* DSM 20103; 7, *B. pseudocatenulatum* DSM 20438; 8, *B. breve* ATCC 15700; 9, A1; 10, A2; 11, A3; 12, A3a.

strains detected during the 12-month study. Figure 3B shows each of the strains as a proportion of the total bifidobacterial population in subject 2 at each sampling time. Strains A6 and A6a were regularly detected members of the bifidobacterial population. None of the strains became numerically dominant in an obvious way as was the case with subject 1. The composition of the bifidobacterial population was always complex, with an average of five to six strains detected per sample. The majority of these strains were sporadic or transient (detected on a single occasion) members of the population. Each subject harbored a distinctive collection of bifidobacterial strains in that none of the strains was common to both individuals.

Ribotyping larger numbers of isolates of bifidobacteria obtained from additional samples collected from subject 2 demonstrated that the random selection of 10 colonies from the appropriate dilution plate afforded good coverage of the numerically predominant strains constituting the bifidobacterial population (Fig. 4). The examination of additional isolates detected even more strains of bifidobacteria but did not alter the overall interpretation as to which strains constituted the largest proportion in the feces.

PFGE was useful for confirming the ribotyping results for the bifidobacteria in some cases. For example, the characteristic PFGE pattern for bifidobacterial isolates identified as strain G1 by ribotyping is shown in Fig. 5A. PFGE of bifidobacterial strains did not, however, demonstrate the same level of discrimination as ribotyping. Twenty-six distinctive DNA patterns and 10 subpatterns were demonstrated by PFGE, compared with 42 distinctive ribotypes and eight subribotypes. Strains A3 and A3a, detected by ribotyping, displayed similar but distinguishable PFGE patterns (Fig. 5B, lanes 11 and 12). Not all subribotypes, however, demonstrated PFGE patterns similar to those of the strain(s) they resembled on the basis of ribotyping. Some strains differentiated by ribotyping had identical PFGE patterns (data not shown).

The identification of representative isolates of each strain was attempted on the basis of cell morphology in Gram-stained smears and the results of carbohydrate fermentations. The species to which all of the strains were tentatively assigned are shown in Table 1. Strains with similar ribotypes (e.g., A3 and A3a) were not always assigned to the same species, but since they differed in only one fermentation result, this disparity may be of little significance. For each subject, the same collection of

TABLE 1. Species identification of *Bifidobacterium* strains

Species	Bacterial strain(s) (ribotypes)
B. adolescentis	A2, A5a, A8, A9, A11, A14, A20, A27, G3, G3a, G4
B. angulatum	A6a, A6b, A31a
B. bifidum	A1, A13
B. breve	A10
B. catenulatum	A3a, A4, A7, A17, A18, A19, A21, A22, A23, A23a, A24, A24a, A25, A26, A30, A31, A32
B. infantis	A6, A16
B. longum	A12, G2
B. angulatum or *B. breve*	A5, A15, A29
B. angulatum or *B. longum*	A35, A36, A37, G1
B. breve or *B. longum*	A28, A34, A38
B. breve or *B. pseudocatenulatum*	A3

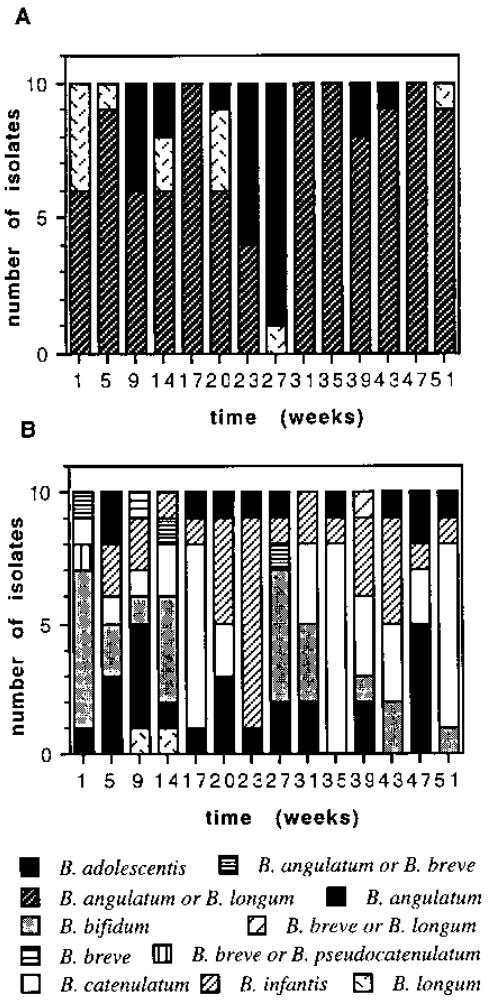

FIG. 6. *Bifidobacterium* species detected in fecal samples from two human subjects. (A) Subject 1; (B) subject 2.

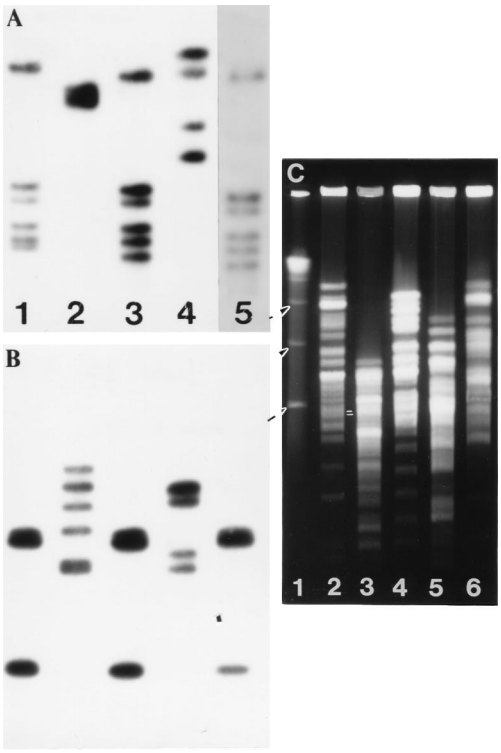

FIG. 7. (A and B) Autoradiographs showing ribotypes of *Lactobacillus* isolates. (A) DNA digested by *Eco*RI; (B) DNA digested by *Hin*dIII. Lanes: 1, LB1; 2, LB2; 3, LB3; 4, LB4; 5, LB5. (C) PFGE of *Lactobacillus* DNA digested by *Apa*I. Lambda ladder PFG marker 340 (New England Biolabs) standards were used (arrows; from the top: 145.5, 97.0, and 48.5 kb). Lanes: 1, λ ladder standards; 2, LB1; 3, LB2; 4, LB3; 5, LB4; 6, LB5.

Bifidobacterium species could be detected regularly during the 12-month period (Fig. 6). The bifidobacterial microflora of subject 1 consisted mainly of *B. angulatum* or *B. longum*, while *B. infantis*, *B. adolescentis*, *B. catenulatum*, *B. angulatum*, and *B. bifidum* were commonly present in the feces of subject 2.

Composition of the lactobacillus population. The strain composition of the *Lactobacillus* population was determined for fecal samples collected at weeks 1 to 23 inclusive. Lactobacilli of the same ribotype, usually constituting 100% of the lactobacillus population, were detected from both subjects during this period (Fig. 7A and B, lanes 1 and 3). A second strain (LB2, 10% of population) was identified for subject 1 (Fig. 7A and B, lane 2) in the sample collected at week 14. PFGE of lactobacilli with the same ribotype obtained from both subjects 1 and 2 showed, however, that the two individuals harbored distinctly different strains (LB1 and LB3) (Fig. 7C, lanes 2 and 4). In other words, PFGE was more discriminatory than ribotyping. This observation demonstrated the importance of using more than one genetic fingerprinting method in an epidemiological or microecological investigation of this kind. Strain LB2, by PFGE, had a characteristic pattern (Fig. 7C, lane 3) in agreement with the results of ribotyping. The strain compositions of the fecal samples collected during weeks 43 and 51 of the study were investigated, for both individuals, by PFGE. The continued persistence of strains LB1 and LB3 was demonstrated for subjects 1 and 2, respectively, and two additional strains were identified for subject 2 (LB4 [10% of the population at weeks 43 and 51] and LB5 [40% at week 51]) (Fig. 7C, lanes 5 and 6). Subsequent ribotyping of these strains confirmed that PFGE gave greater discrimination of lactobacillus strains because strain LB5 displayed the same ribotype as strain LB3 (Fig. 7A and B; compare lanes 3 and 5). Ribotyping of strain LB4 demonstrated a distinctive ribotype (Fig. 7A and B, lanes 4). Identification of representative isolates of each strain by using API 50 CH strips indicated that LB1 was *L. acidophilus* (66.8% confidence), LB2 was *L. fermentum* (98.3%), LB3 was *Lactobacillus crispatus* (85.7%), LB4 was *Lactobacillus rhamnosus* (98.7%), and LB5 was *Lactobacillus plantarum* (98.5%).

DISCUSSION

The observations recorded during this investigation of the fecal microfloras of two adult subjects during a 12-month period have implications for interpretation of past and future studies of the microecology of the human digestive tract. The study supports the concept that the fecal microflora of adult humans shows considerable stability in composition when considered in terms of the total populations of obligate anaerobes (*Bifidobacterium* and *Bacteroides* strains). These populations were maintained at a constant size throughout the 12-month period in both subjects. For a given individual, the species composition of the bifidobacterial population was also predictable, although the species that were commonly detected dif-

fered between the two subjects. The difference in individual microfloras became more evident, however, when the strain composition of the bifidobacterial populations was determined by either ribotyping or PFGE of DNA from the bacterial isolates. The bifidobacterial microflora of subject 1 was remarkably stable, with one strain predominating throughout the study. In contrast, subject 2 harbored a complex collection of strains throughout the 12-month period, and the bifidobacterial population appeared to be more dynamic with regard to strain composition. The administration of amoxicillin did not alter this pattern to a discernable degree. We have concluded from these results that while the dramatic biological succession that occurs in the digestive tract following birth (16) may indeed terminate before adulthood, the intestinal ecosystem continues to undergo fluctuations in composition in some humans, even within populations of obligate anaerobes. It would be interesting to investigate the colonization resistance of these two subjects in relation to the consumption of a probiotic food. Perhaps implantation of a new bifidobacterial strain in the intestine would be impossible in subject 1 but more readily achieved in the case of subject 2. Or would the converse be true? What is the proportion of the human population that harbors a stable, simple bifidobacterial microflora compared with that which harbors a complex, dynamic microflora? Clearly, much experimentation is required in order to extend the fundamental observations made in this study.

The total lactobacillus population of subject 2 showed considerable variation between samples, and the enterobacterial populations of both subjects fluctuated unpredictably. The results of monitoring these populations over a 12-month period demonstrated the importance of conducting lengthy baseline studies in experiments aimed at evaluating the effect of dietary modification, such as the consumption of a probiotic food, on intestinal microecology. Unfortunately, all such experiments to date that we are aware of have been of relatively short duration (a few weeks or a few months). Short-term experiments could give misleading information as to the significance of probiotic products with regard to the intestinal microecology. If, for example, in the case of subject 2 (Fig. 2B), samples collected at weeks 9 and 14 and then at weeks 23 and 27 had constituted the unmodified-diet periods during a trial and samples collected at weeks 17 and 20 constituted the probiotic period, a dramatic but misleading effect of dietary modification would have been recorded. Our study highlights the requirement for fundamental knowledge of the normal intestinal microflora in order for the impact of dietary components on the intestinal ecosystem to be investigated reliably.

We are also concerned, as a result of this study, with the inadequacy of identification methods for determining *Bifidobacterium* species. Carbohydrate fermentation profiles are the mainstay of identification procedures for this genus (6, 8, 11), yet there is considerable variation in substrate utilization even within a species and considerable overlap between species. Long-chain fatty acid analysis of bifidobacteria has not been particularly helpful in identification of these bacteria in our past experience (7). Alignment (clustal method with weighted residue; DNA Star Inc., Madison, Wis.) of GenBank (National Institutes of Health, Bethesda, Md.) data relating to the sequences of 16S rDNAs of type cultures of bifidobacterial species of intestinal origin showed that the genes had 90 to 98% similarity. Although we have followed the conventional approach and allocated our isolates to species, it is our opinion that the results of carbohydrate fermentation tests provide little more than a means of recognizing biotypes of intestinal bifidobacteria.

Genetic fingerprinting of bifidobacterial and lactobacillus strains by either ribotyping or PFGE has provided the means of detecting the numerically predominant strains of bacteria harbored by a particular individual. Bifidobacterial strain G1 and lactobacillus strains LB1 and LB3 are of particular interest because they were long-term members of the fecal microflora. Perhaps the colonization attributes of these persistent strains are superior to those of the transient or sporadic strains and so may be of considerable interest for use in studies that seek to define host-microbe relationships at the molecular level.

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