Molecular assessment of intestinal microflora[1–3]

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ABSTRACT The application of molecular methodologies to intestinal microflora analysis should enable the development of a detailed knowledge of the microbial ecology of the human colon. This knowledge is essential to derive scientifically valid probiotics. Molecular typing (genetic fingerprinting) methods, eg, ribotyping and pulsed field gel electrophoresis of DNA digests, provide a means of distinguishing bacterial strains inhabiting the intestinal tract. Analysis of lactobacillus, bifidobacterial, and enterobacterial populations with the use of these methods has shown that human and porcine subjects harbor a characteristic collection of bacterial strains. Additionally, perturbations and transitions that occur in these populations and are caused by antibiotic administration or by autogenic or allogenic factors can be detected by molecular analysis of the intestinal microflora. In future studies, molecular typing methods could be used to analyze the composition of bacterial populations before, during, and after the administration of the probiotic product. This experimental approach would provide information on the effect of the probiotic on indigenous strains inhabiting the intestinal tract of humans and other animals. *Am J Clin Nutr* 2001;73(suppl):410S–4S.

KEY WORDS Probiotics, intestinal microflora, molecular typing, lactobacillus, bifidobacteria, enterobacteria

INTESTINAL MICROFLORA OF HUMANS

The intestinal microflora comprises a diverse collection of microbial species that are mostly bacterial and are commonly detected in human feces. Such bacterial genera are as follows (1): *Acidaminococcus, Bacteroides, Bifidobacterium, Clostridium, Coprococcus, Enterobacter, Enterococcus Escherichia, Eubacterium, Fusobacterium, Klebsiella, Lactobacillus, Megamonas, Megasphaera, Peptostreptococcus, Proteus, Ruminococcus*, and *Veillonella*. Some of these microbial groups attain high population levels ($\approx(1 \times 10^{10}$ colony-forming units per gram wet weight of intestinal contents or feces). Besides the use of electron microscopy in studies of the gastrointestinal microflora of experimental animals, the study of intestinal microflora composition has relied almost exclusively on the quantitative culture of microbes from fecal samples. Culture results obtained in these studies compose between 50% and 80% of the total microscopic count. Enumeration of particular microbial genera or species relies on the use of selective media. The inability to culture all of the microbes present in samples and the use of a limited range of reliable selective media doubtless introduces bias into analyses of the composition of the normal microflora (2). Even when cultivable, the identification of isolates to species level can be difficult because of the considerable variation in biochemical attributes (fermentation profiles) that seem to occur among strains currently considered to represent the same species. Differentiation of isolates into species, especially in the case of the obligate anaerobes, is always logistically difficult and is subject to intuitive interpretations.

The microflora has marked influences on the animal host, which has been observed in experiments in which the characteristics of germfree (absence of a microflora) and conventional (presence of a microflora) animals were made. These comparisons showed that many biochemical, physiologic, and immunologic characteristics of the animal host are strongly influenced by the presence of the normal microflora (**Table 1**, **Table 2**, and **Table 3**). Because of this, although sometimes overlooked, biochemical assays of microflora-associated activities provide a suitable method of analyzing the overall functioning of the intestinal microflora (Table 2).

MOLECULAR METHODS AND THE ANALYSIS OF ECOSYSTEMS

Analysis of terrestrial and aquatic ecosystems in more recent years has benefited from the use of molecular methods with which community profiles have been established (17). The molecular methods involve the amplification by polymerase chain reactions (PCR) of 16S ribosomal RNA genes (16S rDNA) from microbial DNA extracted from samples collected from particular habitats. The amplified 16S rDNA sequences are cloned and should contain copies of the gene from all of the species represented in the sample. The 16S rDNA clones are screened (some sequences are cloned more than once) and representative clones are sequenced. Because 16S rDNA sequences are one of the cornerstones of microbial taxonomy, alignment of the sequences with those stored in databanks permits the recognition of which species are represented in the habitat, including those that cannot be cultivated by conventional techniques. Interestingly,

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TABLE 1
Comparison of selected properties of germfree (absence of microflora) and conventional (presence of microflora) animals[1]

Biochemical, physiologic, and immunologic host characteristics	Conventional	Germfree
Bile acid metabolism	Deconjugation, dehydrogenation, and dehydroxylation	Absence of deconjugation, dehydrogenation, and dehydroxylation
Bilirubin metabolism	Deconjugation and reduction	Little deconjugation; absence of reduction
Cholesterol	Reduction to coprostanol	Absence of coprostanol
β-Aspartylglycine	Absent	Present
Intestinal gases	Hydrogen, methane, and carbon dioxide	Absence of hydrogen and methane; less carbon dioxide
Short-chain fatty acids	Large amounts of several acids	Small amounts of a few acids
Tryptic activity	Little activity	High activity
Urease	Present	Absent
β-Glucuronidase (pH 6.5)	Present	Absent
Organ weights (heart, lung, and liver)	High	Low
Cardiac output and oxygen utilization	High	Low
Mucin content of intestinal mucus	High	Low
Extent of degradation of mucins	More	Less
Cecal size (rodents)	Small	Large
Enzyme activities associated with duodenal enterocytes	Low	High
Intestinal wall	Thick	Thin
Intestinal mucosal surface area	Great	Small
Rate of enterocyte replacement	Fast	Slow
Peristaltic movement of contents through small bowel	Fast	Slow
Body temperature	High	Low
Serum cholesterol concentration	Low	High
Lymph nodes	Large	Small
γ-Globulin fraction in blood	More	Less

[1] Data from references 3–5.

Wilson and Blitchington (18) compared culture and 16S rDNA sequence analysis as methods of analysis of human fecal samples. They found that, overall, there was good agreement in the biodiversity of the samples analyzed by the 2 methods.

Temperature-gradient gel electrophoresis and denaturing-gradient gel electrophoresis are being developed as an additional means of intestinal microflora analysis. In this technique, 16S rDNA is amplified by PCR from the DNA of the bacterial cells in a sample. The various molecular forms (from different bacterial species) of 16S rDNA in the sample can be separated from one another by use of temperature-gradient or denaturing-gradient gel electrophoresis. A temperature or chemical gradient is established in a polyacrylamide gel (6% acrylamide, 0.1% bis-acrylamide, urea, formamide, and glycerol) in parallel to the electric field. The DNA samples migrate through the gradient from low to high temperature (or low to high chemical gradient). At the point in the gradient where partial denaturation of the double-stranded DNA occurs, the migration of the DNA fragment is drastically retarded and sequences of the same size, but of different thermal or chemical stability, can be separated (19). 16S rDNAs from different species have different nucleotide base sequences in the variable regions and thus have differing stability. Sequences differing in only one base substitution can, theoretically, be separated by this method. A pattern of DNA fragments in the gel that is characteristic of the bacterial content of the sample can be observed after the application of an appropriate detection system (eg, silver staining). 16S rDNA fragments can be eluted from the gel for further amplification by PCR and then sequenced to provide identification or characterization of the microbe from which it was amplified.

The rapid and reliable identification of bacterial isolates is now greatly aided by the availability of PCR and automated nucleotide base sequencing of amplified DNA. 16S rDNA gene sequences (partial or complete) can be obtained easily using universal primers and PCR, and the sequences can then be compared with those in databanks. Shorter regions of genomic DNA may prove even more useful for identification purposes. For example, species of lactic acid–producing bacteria can be differentiated on the basis of the ≈500–base pair spacer region located between the 16S and 23S rRNA genes. The nucleotide base sequence of this region can be obtained by direct sequencing of the amplified DNA (20). Species can be enumerated directly in samples by means of oligonucleotide probes based on the 16S rDNA sequences (in situ hybridization). These probe molecules are labeled with a fluorescent dye and the procedure is termed fluorescent in situ hybridization (21).

It is important to note that the PCR-based and oligonucleotide probe methods outlined above cannot differentiate between strains belonging to the same bacterial species. Differentiation between strains can, however, be achieved by the use of molecular typing methods that have contributed importantly to epidemiologic studies of pathogenic microbes. Summaries of useful molecular typing (genetic fingerprinting) methods are provided in Table 3.

ANALYSIS OF BACTERIAL POPULATIONS BY USE OF MOLECULAR TYPING TECHNIQUES

Molecular typing methods, eg, ribotyping and pulsed-field gel electrophoresis of DNA digests prepared from bacterial isolates, have permitted the analysis of the human fecal microflora at the level of strains. In one study, examination of bifidobacterial and lactobacillus populations in monthly fecal samples collected over a

TABLE 2
Examples of biochemical assays used in analyzing the normal microflora of the intestinal tract

Assay	Reference
Azoreductase activity	6
β-Glucosidase activity	7
β-Glucuronidase activity	8
Short-chain fatty acids	7
Phenolic products (skatole, indole)	9
Bile salt hydrolase activity	10
Ratio of conjugated and unconjugated bile salts	11
pH	7
Production of methane, carbon dioxide, and hydrogen	12
Urease activity	13
Mucin degradation	14
Proteolytic activity	7
Urobilinogen	7
Coprostanol	15
β-Aspartyl glycine	16

12-mo period showed that there can be marked variation in the complexity and stability of these bacterial populations in humans (22). In this study, one subject harbored a relatively simple (5 strains detected during the 12-mo period) and stable collection of bifidobacteria but the other subject harbored 32 strains, some of which appeared, disappeared, and sometimes reappeared during the course of the study. The collection of strains detected in each subject was unique to the individual in that a strain common to both subjects was not detected. Similarly, each subject harbored a characteristic lactobacillus strain that predominated throughout the 12-mo period (22).

The aforementioned study was extended by analysis of the bifidobacterial and lactobacillus populations of an additional 10 healthy humans with use of molecular typing. Two fecal samples were obtained from each subject. About half of the subjects harbored a relatively simple bifidobacterial population whereas the other subjects harbored a more complex collection of these bacteria. Most subjects harbored a simple lactobacillus population, often composed of a single numerically predominant strain. Unique collections of bifidobacteria and lactobacilli that persisted throughout the study were detected in each subject (23). Given the highly specific nature of individual microfloras, it is not surprising that a probiotic strain is eliminated relatively quickly from the intestinal ecosystem once regular consumption of the product has ceased (24). Perhaps the differences in the complexity and dynamics of the intestinal populations of lactic acid-producing bacteria is reflected, too, in the great variation in numbers at which a probiotic strain can be detected in different subjects consuming the same product. For example, *Lactobacillus* GG was detected in fecal samples of different individuals at concentrations between <0.1% and 98% of the total lactobacillus population (24). Such variations in probiotic populations among subjects must surely influence the efficacy of the product in the community.

MOLECULAR ANALYSIS CAN DETECT PERTURBATIONS OF THE MICROFLORA

In a recent study of fecal microflora with the use of ribotyping, 27 strains of enterobacteria were detected in samples obtained from a human subject during a 12-mo period (22, 25). During weeks 21 and 22 of the study, the subject was administered amoxicillin trihydrate (1g/d) perorally for 7 d to treat a respiratory tract infection. It was of particular interest to observe whether the administration of amoxicillin would affect the composition of the enterobacterial microflora of this subject in light of growing concern regarding the selection of antimicrobial-resistant strains of bacteria of medical importance. Members of *Enterobacteriaceae* are opportunistic pathogens of particular significance in nosocomial and urinary tract infections. Strains of enterobacteria (*Escherichia coli*) isolated before the administration of amoxicillin were susceptible to all of the antibiotics tested (ampicillin, doxycycline, nalidixic acid, and trimethoprim). *E. coli* strains isolated after the amoxicillin administration

TABLE 3
Molecular typing methods commonly used to differentiate bacterial strains

Method	Description
Multilocus enzyme electrophoresis	Characterization of isolates by the relative electrophoretic mobilities of a large number of water-soluble enzymes. Net electrostatic charge and hence the rate of migration of a protein during electrophoresis are determined by its amino acid sequence. Variations in the sequences of the genetic determinants of the enzymes are reflected in the mobilities of the proteins.
Pulsed-field gel electrophoresis	Characterization of isolates based on restriction fragment length polymorphisms of chromosomal DNA. Bacteria are embedded in agarose and lysed in situ and the chromosomal DNA is digested with a restriction endonuclease that cuts the DNA infrequently. Slices of agarose are added to an agarose gel and the restriction fragments are resolved into a pattern of discrete bands in the gel by an electrophoretic apparatus that switches the direction of the current according to a predetermined program.
Ribotyping	DNA extracted from bacterial isolates is digested with an appropriate restriction endonuclease; the resulting fragments are separated in an agarose electrophoretic gel, transferred to a hybridization membrane, and probed with a radiolabeled ribosomal RNA gene sequence. Because bacteria have multiple copies of rRNA operons in their chromosome, several fragments in the restriction digest hybridize the probe. The patterns produced provide a means of differentiating between bacterial strains.
Random amplification of polymorphic DNA	A primer of ≈10 nucleotides in length is arbitrarily selected and allowed to anneal to bacterial DNA under conditions of low stringency. The short primer molecules hybridize at random sites to initiate DNA polymerization in the polymerase chain reaction (PCR). The proximity, number, and location of these priming sites varies between strains and the electrophoretic pattern of DNA fragments amplified by PCR provides a fingerprint characteristic of each bacterial strain.

(weeks 23–31) were resistant to ampicillin, doxycycline, and trimethoprim (ie, multiple drug resistant). *Klebsiella pneumoniae* strains predominated in the fecal samples at weeks 35, 39, and 43 of the study and were resistant only to ampicillin. Approximately 6 mo (weeks 47 and 51) after the administration of amoxicillin, the fecal microflora of the subject contained only antibiotic-susceptible enterobacteria, a single strain of *E. coli* being predominant. Therefore, the microbial ecology of the intestinal tract was apparently severely altered after amoxicillin administration. This perturbation was not obvious from examination of total enterobacterial numbers because they had always fluctuated widely in this individual (22), but was apparent when the strain composition and antibiotic sensitivity of the enterobacterial population was determined. *E. coli* strains that exhibited multiple resistances predominated in the fecal samples for a period of ≈13 wk after administration of the antibiotic. The multiple-resistant strains were then no longer detectable but were replaced by a complex community containing ampicillin-resistant klebsiella and antibiotic-susceptible enterobacter and serratia strains. It was striking that antibiotic-resistant strains continued to predominate in the enterobacterial microflora for almost 6 mo, even though amoxicillin was administered for a standard treatment period of 7 d. These phenomena would not have been observed without detailed analysis of the composition of the enterobacterial microflora with the use of genetic fingerprinting techniques. The results of this study showed that molecular typing of bacterial isolates can be a valuable aid in the detection of perturbations to the intestinal microflora by allogenic influences (25).

MOLECULAR ANALYSIS OF THE MICROFLORA CAN DEMONSTRATE TRANSITION

Molecular typing of isolates of lactobacilli obtained from stomach and cecal contents of pigs aged 6–150 d showed that a succession of lactobacillus strains occurred in the gastrointestinal tract of the pigs throughout their lives (26). Periods of uniformity and stability in relation to the composition of the lactobacillus microflora were observed in which relatively few strains were numerically predominant (preweaning, 18 d; finisher, 85 d; and baconer, 150 d) in all of the pigs sampled. Only one strain, designated R20, was detected consistently in samples from pigs after weaning. This was particularly evident in cecal samples, in which R20 became the numerically predominant strain in all animals aged 85 and 150 d. In contrast, lactobacillus populations detected in gastrointestinal samples from pigs aged 24 (postweaning) or 60 d (grower accommodation) were heterogeneous in regard to strain composition and varied between animals. The large number of strains that were detected during this study (103 strains) belonged mostly to 2 species: *Lactobacillus acidophilus* and *Lactobacillus fermentum*.

The reasons for the fluctuations in the composition of the lactobacillus populations of pigs of different ages are not readily apparent. Physiologic changes occurring in the digestive tract of the pigs may influence the composition of the microflora. Dietary changes may be important, especially in the case of lactobacilli present in the stomach contents. The periods of greatest heterogeneity in the composition of the lactobacillus populations occurred in postweaning animals (24 d; stomach and cecal contents) and in grower accommodation animals (60 d; cecal contents). These are particularly stressful times for pigs because at weaning they are deprived of contact with the sow and are moved to new accommodations. At the grower phase, the animals are redistributed in pens and must reestablish a social order. Stress is known to influence the gastrointestinal microflora, and total lactobacillus populations are among those that are shown to be affected (27). The results of this study showed that molecular analysis of a bacterial population inhabiting the gastrointestinal tract can detect transitions in the composition of the microflora associated with age and animal husbandry practices.

IMPLICATIONS FOR PROBIOTIC RESEARCH AND DEVELOPMENT

Molecular analyses of populations inhabiting the intestinal tract will enable investigators to adequately assess the effect of consumption of a probiotic on the intestinal microflora. Populations of interest (eg, lactobacilli and bifidobacteria) detected in fecal samples collected over several months could be analyzed to obtain baseline knowledge of the normal microflora of individual humans. These subjects could then consume the probiotic for several more months. Molecular typing of isolates would permit the determination of the proportion of the specific population constituted by the probiotic strain and the fate of the indigenous strains. Additional fecal samples could be examined after consumption of the probiotic has ceased to establish the long-term effects of the probiotic on the intestinal microflora. This would include observations on the loss of the probiotic strain from the ecosystem and the colonization or recolonization of the intestinal tract by autochthonous or allochthonous strains. In conjunction with enumeration of total bacterial populations composing the intestinal microflora and the use of biochemical assays of bacterial metabolic activity, molecular typing methods will facilitate a detailed analysis of the intestinal microflora to be made. Doubtless, in the future, PCR-based techniques will contribute to analysis of the intestinal ecosystem by lightening the logistical burden that studies of the microflora currently incur, as well as enabling subdominant populations of microbes that are currently not able to be analyzed to be detected and quantitated. A probiotic is defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (28). Knowledge of the composition of the intestinal microflora is therefore critical to the development and marketing of these products.

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