

The Bifidobacterial and Lactobacillus Microflora of Humans

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A Plethora of Prokaryotes

The human body, even in health, is colonized by microbial communities that together constitute the "normal microflora." These communities can be detected in the upper respiratory tract, oral cavity, distal intestinal tract and vagina, and on the skin. Each of these regions of the body is biochemically and histologically distinct because of their characteristic secretions, epithelia, and glands. The prevailing physicochemical factors in each of the body regions provide conditions that select for appropriately adapted microbes. Thus, the skin microflora has a different composition than that of the oral cavity, which in turn is different from that of the other regions of the body that support a microbial community, and so on. Therefore, each colonized body site represents an ecosystem defined by unique physicochemical and microbial constituents (1). Although the microbial (largely bacterial) communities of the skin, respiratory tract, oral cavity, and vagina have been researched, particularly from the perspective of their disease-producing capability, much of the interest in the normal microflora has focused on the bacterial community of the human colon (2). The observation of patient's stools has long been a diagnostic aid for physicians, reflecting the belief that a healthy gut was essential to the general well-being of the human organism. This was extended in the early twentieth century to an interest in the microbial composition of the feces, and speculation as to the appropriate "balance" of microbes (fermentative vs putrefactive) that should be maintained in the colon in order to promote longevity. Lactic-acid-producing bacteria, such as lactobacilli, were central to this research, because it had been observed that milk fermented by lactic-acid bacteria did not support the growth of pro-

teolytic (putrefactive) bacteria. Additionally, consumption of fermented milk as a food staple in Eastern Europe was apparently related to longevity (3,4). In the 1970s, interest in the microflora of the colon was fueled by the statistical inference that dietary factors were associated with the pathogenesis of colon cancer. Using improved anaerobic culture methods (derived from rumen microbiology), approx 10^{10} bacteria per g could be cultured from human feces. These bacteria—mostly obligate anaerobes—belonged to at least 113 bacterial species, and on a statistical basis, it seemed that perhaps 400 to 500 species might be recovered through extensive bacteriological studies. It also seemed likely that chemical transformations of food components in the colon by at least some of these bacterial species could generate carcinogens or co-carcinogens. Large-scale investigations of bacterial communities in human feces were conducted in the hope that comparison of the microfloras of subjects following different dietary patterns (low and high incidence of colon cancer) would implicate the bacterial felons (5–9). Although these were never found, a great deal of important information about the composition of the fecal (colonic) microflora was gathered. This information provided a perspective on the composition of the microflora that has only recently been challenged.

The Shock of the Viable But Non-Cultivable Bacteria

Even in the 1970s, researchers had observed that the total microscopic count of bacterial cells in human fecal smears was always higher than the total viable count, or colony-forming units (CFUs) obtained by culture on a nonselective agar medium. However, it was claimed that good bacteriological methods would permit the culture of 88% of the total microscopic count (8). This comparison was obtained by using total microscopic “clump” counts (aggregates of bacterial cells) rather than by counting individual bacterial cells in smears. Although valid from the perspective that CFUs on agar plates do not always arise from a single bacterial cell, the comparisons gave a false sense of confidence with regard to analytical results at that time. Total fluorescent *in situ* hybridization (FISH) (Table 1) microscopic counts, utilizing the 4', 6-diamidino-2-phenylindole (DAPI) stain and computer imaging, has revealed average total bacterial cell counts in human feces approaching 1×10^{11} per g (wet wt) (11). State-of-the-art bacteriological methodologies still only permit about 40% of this bacterial community to be cultivated on nonselective agar medium in the laboratory (11). Thus, a large proportion of the bacterial cells observed in microscope smears have never been investigated. Although some of these cells may be nonviable, it is likely that many are viable but noncultivable because

Table 1
Molecular Methods for Analysis of the Composition
of Bacterial Communities

Method	Description
Fluorescent <i>in situ</i> hybridization (FISH)	Oligonucleotide probes labeled with a fluorescent dye are used in hybridizations with smears to detect and enumerate specific bacterial groups comprising the bacterial community. The probes usually target 16S rRNA sequences in the bacterial cells (10).
Phylogenetic analysis	16S rRNA genes are amplified by PCR from DNA extracted from samples. The genes are randomly cloned in a plasmid vector in <i>Escherichia coli</i> . This gene library is screened for redundancy, usually by restriction endonuclease analysis, and selected cloned inserts are sequenced. The sequences are aligned with those in a databank such as GenBank in order to determine the biodiversity of the bacterial community (12).
DNA/RNA hybridization	Total bacterial RNA (mostly rRNA) is extracted from the sample and dot-blotted to hybridization membrane. A radioactively labeled oligonucleotide probe that targets a universal 16SrRNA sequence in bacteria is used as a standard against which the degree of hybridization of group-specific probes can be compared and measured. This yields a measurement of the proportions in which the various bacterial groups were present in the sample (15).
PCR/Denaturing gradient gel electrophoresis (DGGE)	DNA (or RNA) is extracted from samples and a 16S rRNA gene sequence no more than 400 bp, which is hypervariable, is amplified using universal primers and PCR (reverse transcription-PCR in the case of RNA as a template). The mixture of 16S rDNA fragments thus generated represents all of the bacteria in the sample. The various 16S rDNA sequences, each characteristic of a particular member of the community, are separated by denaturing gradient gel electrophoresis (a polyacrylamide gel with an increasing concentration of denaturing chemicals in the direction of electrophoresis). A profile of 16S rDNA fragments is produced when the DNA in the gel is stained. The profile is generated because of the

Table 1
(continued)

Method	Description
	different chemical stabilities of the DNA fragments. Migration of a fragment almost ceases when the polynucleotide strands separate. The chemical stability is dictated by the nucleotide-base composition of the DNA sequence. Complete denaturation (the profile would be too complex if single-stranded DNA was present) of the 16S rDNA fragments is prevented by the incorporation of a "GC clamp" at one end of the fragment during PCR (86).

of their fastidious requirements for anaerobiosis—or, more likely, because of the complex nutritional interactions that can occur between the inhabitants of bacterial communities. These nutritional complexities may be difficult, if not impossible, to achieve in laboratory culture media. Molecular analysis of fecal communities (phylogenetic analysis; *see* Table 1), shows that many of the bacteria inhabiting the colon are "unknown" (12). The realization that many bacterial species cannot be cultivated extends beyond the gut ecosystem even to terrestrial and aquatic environments where perhaps as few as 1% of the bacterial inhabitants have been cultivated in the laboratory (13).

16S Ribosomal RNA to the Rescue

Carl Woese revealed that small ribosomal subunit (16S rRNA in the case of bacteria) contained regions of nucleotide base sequence that were highly conserved, and that these were interspersed with hypervariable regions. These hypervariable regions contained the signatures of phylogenetic groups, and sometimes, even species (14). Modern analysis of the colonic microflora of humans depends on the 16S rRNA sequences as a cornerstone of bacterial classification. To date, three molecular analytical approaches based on 16S rRNA sequences have proven to have the capacity to provide reliable knowledge about the richness (diversity of species) and evenness (population sizes) of the colonic microflora of humans. These approaches are: phylogenetic analysis by polymerase chain reaction (PCR), cloning, and sequencing; FISH analysis using oligonucleotide probes that target specific 16S rRNA sequences; and oligonucleotide/total bacterial RNA hybridizations on membranes (10,12,15; Table 1). All three methods can

Table 2
Cultivated Bacterial Species
within the *Bacteroides-Prevotella* Group

<i>Porphyromonas salivosa</i>	<i>Bacteroides caccae</i>
<i>Porphyromonas catoniae</i>	<i>Bacteroides ovatus</i>
<i>Porphyromonas gingivalis</i>	<i>Bacteroides thetaiotaomicron</i>
<i>Porphyromonas cangingivalis</i>	<i>Bacteroides forsythus</i>
<i>Porphyromonas endodontalis</i>	<i>Bacteroides distasonis</i>
<i>Rikenella microfus</i>	<i>Bacteroides merdae</i>
<i>Cytophaga fermentans</i>	<i>Prevotella heparinolytica</i>
<i>Bacteroides putredinis</i>	<i>Prevotella pallens</i>
<i>Bacteroides splanchnicus</i>	<i>Prevotella veroralis</i>
<i>Bacteroides vulgatus</i>	<i>Prevotella denticola</i>
<i>Bacteroides fragilis</i>	<i>Prevotella oulora</i>
<i>Bacteroides stercoris</i>	<i>Prevotella oris</i>
<i>Bacteroides uniformis</i>	<i>Prevotella oralis</i>
<i>Bacteroides eggerthii</i>	<i>Prevotella ruminicola</i>

detect noncultivable as well as cultivable bacteria. Cloning, FISH, and DNA/RNA hybridization methods are logistically demanding. Thus, a limited number of oligonucleotide probes have been used routinely in studies, or a very limited number of subjects have been examined in the case of analysis of cloned 16S rRNA sequences. The probes recognize phylogenetic groups of bacteria rather than enumerating individual species. Nevertheless, it is clear from molecular methods of analysis that three groups of bacteria predominate in human feces: the *Bacteroides-Prevotella* group, the *Clostridium coccoides* group, and the *Clostridium leptum* group (10,12,15,16). Cultivated species of bacteria within these groups are listed in Tables 2–4.

Getting Bifidobacteria and Lactobacilli into Perspective

Members of the genus *Bifidobacterium* are nonsporing, nonmotile, gram-positive, rod-shaped bacteria. Some species, under particular conditions of culture, may show cells with a branching morphology. They are obligately anaerobic bacteria, producing major amounts of acetic and lactic acid from the fermentation of glucose (17). They represent no more than 10% of the fecal microflora of adult humans when analysis is by culture, and 1–5% when analysis is by molecular methodologies (10,15,18). Population sizes, according to some studies, vary from person to person, although levels of 10^9 to 10^{10} per g of feces are often reported (19). Japanese authors have reported that the numbers of bifidobacteria in feces decline in elderly subjects (20). There is considerable commercial interest in boosting bifidobacterial numbers in the gut through the consumption of yogurts that contain bifidobacteria (“bifidus”

Table 3
Cultivated Bacterial Species
within the *Clostridium Coccoides* Group

<i>Clostridium polysaccharolyticum</i>	<i>Eubacterium xylanophilum</i>
<i>Clostridium herbivorans</i>	<i>Eubacterium ventriosum</i>
<i>Clostridium populeti</i>	<i>Eubacterium eligens</i>
<i>Clostridium coccoides</i>	<i>Eubacterium formicigenerans</i>
<i>Clostridium nexile</i>	<i>Eubacterium contortum</i>
<i>Clostridium oroticum</i>	<i>Eubacterium rectale</i>
<i>Clostridium clostridiiforme</i>	<i>Eubacterium hadrum</i>
<i>Clostridium celerecrescens</i>	<i>Eubacterium halii</i>
<i>Clostridium xylanolyticum</i>	<i>Eubacterium ramulus</i>
<i>Clostridium symbiosum</i>	<i>Lachnospira pectinoschiza</i>
<i>Clostridium aminovalericum</i>	<i>Ruminococcus obeum</i>
<i>Clostridium aminophilum</i>	<i>Ruminococcus hansenii</i>
<i>Coprococcus eutactus</i>	<i>Ruminococcus productus</i>
<i>Butyrivibrio crossotus</i>	<i>Ruminococcus torques</i>
<i>Butyrivibrio fibrisolvens</i>	<i>Ruminococcus gnavus</i>
	<i>Roseburia cecicola</i>

Table 4
Cultivated Bacterial Species
within the *Clostridium Leptum* Group

<i>Fusobacterium prausnitzii</i>	<i>Ruminococcus bromii</i>
<i>Eubacterium siraeum</i>	<i>Eubacterium plautii</i>
<i>Clostridium sporosphaeroides</i>	<i>Clostridium viride</i>
<i>Clostridium leptum</i>	<i>Eubacterium desmolans</i>
<i>Ruminococcus flavefaciens</i>	<i>Termitobacter aceticus</i>

yogurts) or by the addition to foods of "prebiotics" (nondigestible carbohydrates which pass to the colon, where they become fermentable substrates for some members of the microflora, including bifidobacteria). This commercial activity is aimed at maintaining, or even improving, the health of the consumer, because bifidobacteria are putatively beneficial to the intestinal environment (21–23). The population size of bifidobacteria required to produce a "healthy" effect has not yet been elucidated.

Interest in bifidobacteria is derived mainly from the observation of Tissier that these branching bacteria were commonly present in the feces of infants (24). Indeed, they appeared to dominate the bacterial community present in the feces of infants who were suckled at the breast. A more varied microflora was evident in the feces of infants fed with cow's-milk formula. Since pediatricians have recognized, and still recognize, that breast-fed infants experience less intestinal disturbances than formula-fed children, the inference that bifidobacteria were asso-

Table 5
Variation in the Sizes of Bacterial Populations
in the Feces of Infants During the First Week of Life

Bacterial group	Range of log ₁₀ CFU per g of feces ¹	
	Breast milk	Formula
Enterobacteria	4–10	6–10
Enterococci/streptococci	3–9	1–10
Bifidobacteria	1–10	2–7
Bacteroides	2–8	1–8

¹Values taken from graphical data presented by Moreau et al. (25)

Table 6
Comparison of Bacterial Populations Detected
in the Feces of Infants Approx 4 wk of Age

Bacterial group	Range of log ₁₀ CFU per g of feces	
	Breast milk	Formula
Clostridia (Stark and Lee) ¹	<3.0–<3.0	<3.0–7.1
Clostridia (Benno et al.) ¹	5.3 (1.5) ²	8.1 (1.2)
Enterobacteria (Stark and Lee)	3.3–8.3	8.6–10.2
Enterobacteria (Benno et al.)	8.2 (1.1)	9.3 (0.7)
Enterococci (Stark and Lee)	5.4–8.3	9.1–10.2
Enterococci/streptococci (Benno et al.)	6.8 (1.5)	9.1 (0.7)

¹Data from Stark and Lee (26) and Benno et al. (27)

²Mean and standard deviation

ciated with health was made. Currently, infant formulas resemble human milk much more closely. Probably for this reason, it is now more difficult to recognize different microfloras associated with breast-fed or formula-fed infants on the basis of bifidobacterial numbers. There is considerable infant-to-infant variation in the population size of particular bacterial genera during the first week of life in both breast milk- and formula-fed infants (Table 5), which may have contributed to the somewhat variable interpretations of the composition of the infant microflora reported in the literature (25). More consistent values have been obtained from babies older than 1 wk, however, and realistic comparisons between infant groups are possible. The critical test as to whether biologically significant differences exist between the microfloras of breast milk- and formula-fed infants is whether it is possible to determine, based on the bacteriological results of fecal examination, what the diet of the infant has been. Population sizes of certain bacterial

Table 7
Examples of Probiotic Effects

Probiotic effect	Reference
Increased resistance to infectious diseases particularly of the intestine	(87)
Decreased duration of diarrhea	(88)
Reduction in blood pressure	(89)
Reduction in serum cholesterol concentration	(90)
Reduction in allergy	(74)
Stimulation of phagocytosis by peripheral blood leukocytes	(91)
Modulation of cytokine gene expression	(92)
Adjuvant effect	(93)
Regression of tumors	(94)
Reduction in carcinogen or co-carcinogen production	(57)

groups appear to be characteristic of the infant's diet when the data of Stark and Lee (26) and of Benno et al. (27) are compared (Table 6). In the case of infants about 4 wk of age, if clostridial counts are greater than 10^7 , and enterococcal/streptococcal and enterobacterial counts are greater than 10^9 per g, the feces are likely to have originated from formula-fed babies. In other, less complete studies, the prediction regarding clostridia holds true: the clostridial population is always lower in breast milk-fed babies (28–34).

Members of the genus *Lactobacillus* are non-sporing, gram-positive, rod-shaped bacteria. Intestinal isolates grow best under anaerobic conditions, but many can be cultivated microaerophilically. Lactobacilli produce a large amount of lactic acid when they ferment glucose (35). There is a long history of interest in the lactobacilli because they are responsible for the properties of several foods: fermented dairy products and fermented meats provide good examples. Lactobacilli are important in the acidification of plant materials to make silage. Under some conditions, their growth on food causes spoilage (36). Additionally, the lactobacilli are common components of "probiotics" (37). Generally defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance," the use of probiotics is aimed at donating bacteria to the intestinal tract (38). The popularity of probiotics has shown a cyclical pattern since Metchnikoff's time, with peaks of interest during the 1930s, including the introduction of "acidophilus" milk in the United States, and during the 1970s in animal husbandry circles (39,40). Since the 1970s, they have generated astounding interest as adjuncts of the dairy industry worldwide (41). The consumption of probiotics is believed to promote "wellness" and the literature is filled with claims of this nature (Table

Table 8
Bifidobacterial Species

<i>B. adolescentis</i>	<i>B. angulatum</i>
<i>B. animalis</i>	<i>B. asteroides</i>
<i>B. bifidum</i>	<i>B. boum</i>
<i>B. breve</i>	<i>B. catenulatum</i>
<i>B. choerinum</i>	<i>B. coryneforme</i>
<i>B. cuniculi</i>	<i>B. dentium</i>
<i>B. gallicum</i>	<i>B. gallinarum</i>
<i>B. globosum</i>	<i>B. indicum</i>
<i>B. infantis</i>	<i>B. inopinatum</i>
<i>B. lactis</i>	<i>B. longum</i>
<i>B. magnum</i>	<i>B. merycicum</i>
<i>B. minimum</i>	<i>B. pseudocatenulatum</i>
<i>B. pseudolongum</i> subsp. <i>globosum</i>	<i>B. pseudolongum</i> subsp. <i>pseudolongum</i>
<i>B. pullorum</i>	<i>B. ruminantium</i>
<i>B. saeculare</i>	<i>B. subtile</i>
<i>B. suis</i>	<i>B. thermophilum</i>

7). Unfortunately, many of the published studies are inadequate, and scientific validation of claims has been impossible (42). Despite the intense interest in lactobacilli as probiotics, these bacteria make up no more than 1% of the total bacterial community of human feces (15). Finegold et al. detected lactobacilli in 73% of 62 healthy Americans when a single fecal sample from each was examined (19).

What Types of Bifidobacteria Live in the Human Gut?

There are at least 32 species of bifidobacteria (Table 8) that are almost impossible to distinguish reliably by traditional phenotypic observations, such as the results of fermentation tests, cell morphology, and electrophoretic mobilities of enzymes (for example, transaldolase, 14 types; 6-phosphogluconate dehydrogenase, 19 types) (17). Even 16S rRNA sequences offer only minimal support in the identification of bifidobacterial species because of the high level of relatedness (43). DNA-DNA reassociation (Table 9) was, until recently, the only reliable method for delineation of bifidobacterial species (44). PCR primers that distinguish between bifidobacterial species encountered in feces have been described by Matsuki et al., and these hold some hope for the practical identification of—at the least—groups of bifidobacterial species (45).

Perhaps because of the difficulty in identifying bifidobacterial species, comprehensive studies to define the bifidobacterial microflora of humans have rarely been attempted. Probably the best comparisons available are those reported by Mitsuoka, Biavati et al., and Matsuki et

Table 9
Molecular Methods for Identification of Bacterial Species
and Differentiation of Strains

Method	Description
DNA-DNA reassociation	Sheared DNA extracted from two bacterial cultures is prepared. The DNA of one of the cultures has been radioactively labeled by growing the bacteria in the presence of a radioactive nucleotide. DNA from the unlabeled culture is bound to a membrane and the radioactive DNA from the other culture is added. Hybridization is allowed to occur under appropriate conditions of stringency. Non-hybridized DNA is removed by washing, and then the amount of hybridized DNA is measured in a scintillation counter. The amount of hybridisation infers the degree of relatedness of the two bacterial types. Bacteria of the same species yield hybridization results of >60–70% (95).
Species-specific PCR primers	A unique nucleotide base sequence in the bacterial DNA or RNA of a specific bacterial type is amplified using PCR (45).
Ribotyping	DNA extracted from bacterial isolates is digested with an appropriate restriction endonuclease, and the resulting fragments are separated in an agarose electrophoretic gel, transferred to a hybridization membrane, and probed with a radiolabeled rRNA gene sequence. Because bacteria have multiple copies of rRNA operons in their chromosomes, several fragments in the restriction digest hybridize the probe. The patterns thus produced provide a means of differentiating between bacterial strains (96).
Pulsed-field gel electrophoresis of DNA digests (PFGE)	Characterization of strains based on restriction-fragment-length polymorphisms of chromosomal DNA. The bacteria are embedded in agarose and lysed <i>in situ</i> , and the DNA is digested with a restriction endonuclease that cuts the DNA infrequently. Slices of agarose are added to an agarose gel,

Table 9
(continued)

Method	Description
Plasmid profiling	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 programme (97). Plasmid DNA is partially purified from a bacterial culture, then detected in an agarose electrophoretic gel. Isolates of some bacteria genera, such as <i>Lactobacillus</i> , frequently contain several plasmids of different sizes. Electrophoresis of a plasmid DNA preparation generates a plasmid profile characteristic of the bacterial isolate (strain) (84).

al. (20,45–47). Mitsuoka listed *Bifidobacterium longum* as a predominant species in the feces of adults, with occasional detection of *Bifidobacterium bifidum*. In contrast, *Bifidobacterium infantis* and *Bifidobacterium breve* were the prevalent species in infant feces, with *B. longum* and *B. bifidum* occasionally detected. Biavati et al. listed *Bifidobacterium pseudocatenulatum*, *B. longum*, *Bifidobacterium adolescentis*, *B. bifidum*, and *Bifidobacterium catenulatum* as common species in adult feces. The feces of infants usually contained *B. infantis*, *B. breve*, *B. pseudocatenulatum*, and *B. longum*. *B. bifidum*, *Bifidobacterium dentium*, and *B. catenulatum* were detected less frequently. Matsuki et al. detected *B. catenulatum*/*B. pseudocatenulatum* in 92% of 48 fecal samples from adults. *B. longum* and *B. adolescentis* were also found. *B. breve*, *B. infantis*, and *B. longum* were frequently detected in the feces of infants.

What Types of Lactobacilli Can Be Detected in Human Feces?

The taxonomy of the lactobacilli has been the subject of flux during recent years, but at least 70 species have been described (Table 10). Mitsuoka listed *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* as the most commonly detected lactobacilli in human feces. *Lactobacillus johnsonii*, *Lactobacillus ruminis*, *Lactobacillus casei*, and *L. brevis* were occasionally detected (20). Analysis of the human fecal microflora using molecular methodologies for differentiation and identification has shown, in more recent studies,

Table 10
Lactobacillus Species

<i>L. acetotolerans</i>	<i>L. acidophilus</i>
<i>L. agilis</i>	<i>L. alimentarius</i>
<i>L. amylolyticus</i>	<i>L. amylophilus</i>
<i>L. amylovorus</i>	<i>L. animalis</i>
<i>L. aviarius</i> subsp. <i>araffinosus</i>	<i>L. aviarius</i> subsp. <i>aviarius</i>
<i>L. bifermentans</i>	<i>L. brevis</i>
<i>L. buchneri</i>	<i>L. carnis</i>
<i>L. casei</i>	<i>L. catenaforme</i>
<i>L. cellobiosus</i>	<i>L. collinoides</i>
<i>L. coryniformis</i> subsp. <i>coryniformis</i>	<i>L. coryniformis</i> subsp. <i>torquens</i>
<i>L. crispatus</i>	<i>L. curvatus</i> subsp. <i>curvatus</i>
<i>L. curvatus</i> subsp. <i>melibiosus</i>	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>	<i>L. delbrueckii</i> subsp. <i>lactis</i>
<i>L. farciminis</i>	<i>L. fermentum</i>
<i>L. fructivorans</i>	<i>L. fructosus</i>
<i>L. gallinarum</i>	<i>L. gasseri</i>
<i>L. graminis</i>	<i>L. hamsteri</i>
<i>L. helveticus</i>	<i>L. hilgardii</i>
<i>L. homohiochii</i>	<i>L. iners</i>
<i>L. intestinalis</i>	<i>L. jensenii</i>
<i>L. johnsonii</i>	<i>L. kandleri</i>
<i>L. kefiranofaciens</i>	<i>L. kefirgranum</i>
<i>L. kefiri</i>	<i>L. kunkeei</i>
<i>L. lindneri</i>	<i>L. malefermentans</i>
<i>L. mali</i>	<i>L. maltaromicus</i>
<i>L. manihotivorans</i>	<i>L. murinus</i>
<i>L. oris</i>	<i>L. panis</i>
<i>L. parabuchneri</i>	<i>L. paracasei</i> subsp. <i>paracasei</i>
<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>L. parakefiri</i>
<i>L. paraplantarum</i>	<i>L. pentosus</i>
<i>L. plantarum</i>	<i>L. pontis</i>
<i>L. reuteri</i>	<i>L. rhamnosus</i>
<i>L. rogosae</i>	<i>L. ruminis</i>
<i>L. sakei</i> subsp. <i>carnosus</i>	<i>L. sake</i> subsp. <i>sakei</i>
<i>L. salivarius</i> subsp. <i>salicinius</i>	<i>L. salivarius</i> subsp. <i>salivarius</i>
<i>L. sanfranciscensis</i>	<i>L. sharpeae</i>
<i>L. suebicus</i>	<i>L. uli</i>
<i>L. vaccिनostercus</i>	<i>L. vaginalis</i>
<i>L. vitulinus</i>	<i>L. zeae</i>

that members of the *Lactobacillus casei* group were the most commonly detected lactobacilli. *Lactobacillus plantarum*, *L. brevis*, *L. salivarius* subsp. *salivarius*, *L. ruminis*, *L. crispatus*, *L. acidophilus*, and *L. gasseri* were also detected (11,48). A long-term study of the fecal microflora of 10 human subjects showed that those strains of lactobacilli that persisted as members of the fecal microflora were members of only two species: *L. ruminis* and *L. salivarius* subsp. *salivarius*. The remaining species fluctuated

greatly in numbers and frequency during the study, suggesting that they were not residents of the intestinal tract, but rather transients that were introduced in food (11).

Something Special

Studies using genetic fingerprinting methods such as ribotyping and pulsed-field gel electrophoresis (PFGE) (Table 9) of DNA digests prepared from bacterial isolates have permitted the analysis of the human fecal microflora at the level of strains (49,50). Examination of bifidobacterial and *Lactobacillus* populations in monthly fecal samples collected over a 12-mo period has shown that there can be marked variation in the complexity and stability of these bacterial populations between human subjects. These studies clearly indicate that individual humans harbor unique microfloras when considered at the level of bacterial strains. Bifidobacterial populations were consistently large in all samples that were examined, and strains that persisted in the feces for at least 12 mo could be recognized. *Lactobacillus* populations fluctuated in size in most subjects, but unique and persisting strains could often be detected (49,50).

Research carried out at the Rockefeller Institute in the 1960s demonstrated that certain bacterial populations inhabiting the gastrointestinal tract of rodents were acquired soon after birth and persisted throughout the life of the animal (51). All members of the rodent colony harbored these bacteria in similar numbers. Other bacterial populations were present in only some colonies of animals of the same species. These observations led Rene Dubos and colleagues to postulate that microbial populations detected in the digestive tract could be divided into three categories.

1. "Autochthonous microbiota" composed of persistent populations that were represented in every individual.
2. "Normal microbiota" composed of populations that were frequently (but not always) detected in the ecosystem, but of variable population size.
3. "True pathogens" that were accidentally acquired and produced disease.

The terminology was not adopted at the time, but the concept of "autochthony" is a valid one in the light of recent ecological observations of the intestinal ecosystem. The persistent strains of bifidobacteria or lactobacilli detected in humans could be described as "autochthonous" (inhabiting a place or region from earliest times) to the intestinal tract of the particular individual. Strains that were detected consistently but unpredictably could be termed "opportunistic" strains because they

may represent autochthonous, but numerically subdominant populations whose numbers vary over time so that their detection is intermittent among the numerically dominant strains. They could be said to be opportunistic because their population density may be determined by changes in the intestinal milieu produced by endogenous or exogenous factors. For example, the amount of nondigestible oligosaccharides and polysaccharides in the diet may influence the availability of certain substrates to bacteria inhabiting the intestinal ecosystem. When appropriate substrates became available, the subpopulations of opportunistic strains would increase and become detectable by the molecular typing strategy.

The contrasting term to "autochthonous" is "allochthonous" (found in a place other than where they were formed). Strains of bacteria incorporated in probiotic products can usually only be detected in the feces of human subjects while they continue to consume the product (52). Therefore, their presence in the intestinal tract is dependent on environmental circumstances (purchase and consumption of the probiotic). These strains could be referred to as "allochthonous" to the intestinal tract. They are unlikely to become true components of the intestinal ecosystem of the majority of consumers because they originated elsewhere and because of the homeostatic reactions (competitive exclusion) that they face when introduced into the intestinal ecosystem (1).

The introduction of this terminology is important because it is necessary to rationalize the effects of probiotic consumption on the consumer, relative to that of strains which already reside in the intestinal tract. Autochthonous strains must be highly compatible with the intestinal environment of their host, because they persist as members of the particular bacterial population. Probiotic strains, in contrast, do not belong to the ecosystem of the consumer at all. At best, they will be opportunistic in their relationship with the consumer, but are usually allochthonous and will therefore affect the consumer differently than the autochthonous strains. However, much research effort is required to delineate the effects on the human host of these various relationships.

Where do the autochthonous strains of lactobacilli and bifidobacteria originate? The source of the lactobacilli that initially colonize the intestinal tract has usually been assumed to be the maternal vagina (26,53–55). Until about ten years ago, such an assumption had not been supported or refuted by scientific data because sufficiently discriminatory tests permitting the comparison of bacterial strains isolated from maternal and infant sources were not available. In one study, plasmid profiling of bacterial isolates (Table 9), supplemented with biochemical profiling of bifidobacteria, permitted an assessment of the contribution

made by the maternal microflora to that of the infant (56). The vaginal microflora of the mother was not represented among the numerically predominant strains of lactobacilli in the infant feces. Although lactobacilli were present in vaginal swabs collected from five mothers, only two of the infants harbored lactobacilli in their feces. These isolates had different plasmid profiles or were different species from those detected in the respective mothers. Bifidobacteria and enterobacteria detected in vaginal swabs were different strains from those isolated from the corresponding infant. Evidence for the transmission of fecal bacteria from mother to infant was obtained in four out of five mother-infant pairs when observations concerning enterobacterial and bifidobacterial isolates were combined. Unfortunately, long-term studies to follow the fate of bacterial strains acquired early in life have not been made.

Tracking Changes in the *Lactobacillus* Microflora

Genetic fingerprinting of bacterial isolates has been used to analyze the composition of the *Lactobacillus* populations present in the feces of humans during a study aimed at measuring the impact of probiotic consumption on the composition of the fecal microflora (11). The composition of the fecal microflora of ten healthy subjects was monitored before (control period of 6 mo), during (test period of 6 mo) and after (post-test period of 3 mo) the administration of a milk product containing *Lactobacillus rhamnosus* DR20 (daily dose of 1.6×10^9 lactobacilli). Monthly fecal samples were examined by a variety of methods. These included bacteriological culture, fluorescent *in situ* hybridization with group-specific DNA probes, DGGE (Table 1) of the V2-V3 region of 16S rRNA genes amplified by PCR, gas liquid chromatography to measure short-chain fatty-acid concentrations resulting from bacterial fermentations, and bacterial enzyme activities (azoreductase, β -glucuronidase). The composition of the *Lactobacillus* population of each subject was analyzed by PFGE of bacterial DNA digests in order to differentiate between DR20 and other strains present in the samples. Consumption of the probiotic altered the composition of the *Lactobacillus* populations of the subjects, but to varying degrees. The presence of DR20 among the numerically predominant strains was related to the presence or absence of a stable autochthonous population of lactobacilli during the control period. A previous study revealed that about one-half of the subjects that were investigated harbored a *Lactobacillus* population of relatively simple composition in which one or two strains persisted throughout the study as numerically predominant isolates (Kimura et al.). The same phenomenon was observed in the DR20 study in which four of the ten subjects were host to a single dominant strain. These four subjects consistently harboured *Lactobacillus* populations of

between 10^4 and 10^9 throughout the study. In contrast, other subjects had widely fluctuating numbers of lactobacilli, which were undetectable in some fecal samples. Strain DR20 predominated among the strains of lactobacilli during the test period in subjects with fluctuating (unstable) *Lactobacillus* populations in terms of size or composition in their control-period samples. In these subjects, different strains of lactobacilli were present in the feces during the post-test period compared to the control period, emphasizing the general instability of the *Lactobacillus* population in these individuals. The probiotic strain did not predominate in samples collected from subjects with *Lactobacillus* populations of stable composition. Thus, the presence of lactobacilli capable of persisting long-term (autochthonous strains) in the feces of a subject appeared to preclude the establishment of DR20 (allochthonous) as the numerically dominant strain. Therefore, in the future, the results of long-term control studies may permit predictions of modifications to the fecal microflora to be made prior to consumption of a probiotic.

Strain DR20 did not persist in numbers $>10^2$ per g in the feces of any of the subjects after consumption of the product ceased, except for one subject in which it could be detected for 2 mo during the post-test period. Overall, it was concluded that consumption of the DR20-containing milk product transiently altered the *Lactobacillus* content of the feces of the majority of consumers without markedly affecting biochemical or other bacteriological factors.

Scoring a Bull's-Eye

How can the consumption of a probiotic containing bifidobacteria and/or lactobacilli promote the well-being of the consumer? Traditionally, the impact of probiotic consumption has been presumed to involve modification of the composition of the microflora and the creation of a "healthy" balance between lactic-acid-producing bacteria and proteolytic bacteria. Yet healthy humans have no more than a few per cent of the total microflora represented by bifidobacteria, and no more than 1% by lactobacilli (15). Probiotic consumption does not boost *Lactobacillus* numbers beyond this level. Bacterial enzyme activities (azoreductase and β -glucuronidase) have been reported to decrease when lactobacilli were consumed as a probiotic in some short-term studies (57). But longer periods of observation have shown that these activities fluctuate greatly, regardless of whether or not probiotics were consumed (11). Moreover, molecular analysis has demonstrated the enormous homeostatic capacity of the colonic ecosystem (58). Therefore, the impact of bifidobacteria and lactobacilli on the adult consumer is unlikely to occur by altering the composition of the microflora. It is much more likely to be felt in the upper-to-middle small bowel that, although

devoid of bacterial colonization, is lined by a mucosa rich in immunological cells, including Peyer's patches. For example, in mice some of the largest Peyer's patches are located in the proximal duodenum. The impact of the consumption of relatively large doses (10^9) of gram-positive, allochthonous bacterial cells on the mucosal and systemic immune system of the consumer is surely worthy of study. This may be the realistic target for probiotics containing bifidobacteria and/or lactobacilli.

Biological Freudianism

"Biological Freudianism" was a term used by Dubos and colleagues to denote the lasting effects of early environmental influences on the physiology of animals (59). They reported, as an example, a lasting influence that depended on the composition of the intestinal microflora of mice. A specific-pathogen-free colony of Swiss mice (the NCS colony) was derived at the Rockefeller Institute in New York by sterile caesarean delivery of the initial breeding stock of animals. The mice were maintained under conditions of high sanitation, and unlike the mice from which they were derived (the SS colony), did not harbor gram-negative bacteria such as *Escherichia coli*, *Proteus vulgaris*, or *Pseudomonas* species as members of the intestinal microflora. The two types of mice were found to differ markedly in their response to the parenteral administration of endotoxin. NCS mice were tolerant to doses that were lethal when administered to SS mice. Exposure to SS mice during early life, or prior injection with small doses of heat-killed gram-negative bacteria that therefore gave exposure to minor amounts of endotoxin, increased the susceptibility of the NCS mice. Their sensitivity to endotoxin now matched that of the SS mice. Thus, it was concluded that the SS microflora containing enterobacteria (bacteria that are usually numerous in the murine intestinal tract early in life) sensitized the animals so that, as adults, they were highly susceptible to endotoxin.

Comparison of the immunological characteristics of germ-free (raised in the absence of microbes) and conventional (raised in the presence of microbes) animals has demonstrated that the presence of the normal microflora stimulates the reticuloendothelial tissues of the animal, particularly those tissues associated with the intestinal tract. Peyer's patches and mesenteric lymph nodes are more developed, the lamina propria of the intestinal wall contains more neutrophils and lymphocytes, and the serum contains a higher concentration of immunoglobulins in conventional animals compared to germ-free animals (60). Other striking differences between germ-free and conventional animals concern intestinal intra-epithelial lymphocytes that in conven-

tional mice are about equally divided into $\alpha\beta+$ and $\gamma\delta+$ subpopulations. In germ-free mice, $\gamma\delta+$ lymphocytes are predominant in the intestinal epithelium. The $\alpha\beta+$ subpopulation quickly increases in size when ex-germ-free mice acquire an intestinal microflora (conventionalization) (61,62).

The influence of particular bacterial groups on the development of the immune system during early life has not been investigated systematically. Studies with germ-free mice, monoassociated with bacterial species or injected with heat-killed bacteria, have been reported. These studies, reviewed by McCracken and Gaskins, are open to several criticisms, and firm conclusions about the significance of specific bacteria in "programming" the immune system is lacking (63). A recent report investigating the diversification of the primary antibody repertoires in the rabbit shows how scientifically profitable microflora-immune system studies could be (64). Somatic diversification (rearrangement of lymphocyte V, D, J, and C genes to generate a diversity of receptors) occurs in the rabbit after birth in the gut-associated-lymphoid tissue. In experiments reported by Lanning et al., the sacculus rotundus was removed from rabbits within 24 h of birth and the appendix was ligated and severed from the caecum while retaining the appendix vasculature. This prevented bacterial colonization of the appendix. Peyer's patches were removed when they became visible at 5 wk of age. About 90% of the Ig VDJ genes of peripheral-blood leukocytes from these animals were undiversified when examined at 12 wk of age (compared to 66% diversified in control animals). In other experiments, hand-raised rabbits, obtained by sterile delivery, acquired a different microflora than rabbits in the conventional colony. These hand-raised rabbits had an underdeveloped gut-associated lymphoid tissue (GALT), and 70% of the Ig VDJ genes in peripheral-blood leukocytes were undiversified.

The influence of the intestinal microflora on the development of the immune system needs to be studied intensively. A practical application of such studies would be to understand the increasing incidence of atopic disorders, such as asthma of children in affluent countries (65–67). Atopic disorders are characterized by dominant T-helper 2 (Th2) mechanisms and the production of immunoglobulin E (IgE) to common environmental antigens. Asthma is an atopic disorder characterized by activation and recruitment of eosinophils to the lung, resulting in chronic swelling and inflammation of the airways. The reasons for the increase in atopic disorders in developed countries are unknown, but several factors in addition to hereditary predisposition that may be of significance—all concerning microbial exposure in childhood—have recently been identified in retrospective studies.

1. A steady decline in the extent to which the human population is exposed to major human diseases such as tuberculosis, measles, and influenza (68). These infections characteristically induce a T-helper 1 (Th1) type immune response that produces an immunological environment rich in interferon-gamma. This, in turn, is viewed as a potent suppressor of Th 2 activity. The less frequent exposure of children to these infections, at an age when allergen sensitization is occurring, has been theorized to increase the risk of developing atopy (65,69).
2. Immunization with whole-cell pertussis vaccine has been reported as a predictor of atopic disorders (70). *Bordetella pertussis* is well-known for its Th2-promoting potential, and has been used experimentally as an adjuvant to stimulate antibody production. *Bordetella pertussis* is a strong promoter of IgE synthesis with an effect on primary and secondary antibody responses to heterologous antigens (71).
3. Treatment with oral antibiotics during the first 2 yr of life has been identified as a predictor of subsequent atopic disease (70,72). Three categories of broad-spectrum antibiotics were identified in this role in a study conducted in the United Kingdom: penicillins, cephalosporins, and macrolides. The mechanism by which antibiotics influence the programming and development of the immunological system most likely involves alterations to the collection of bacterial species inhabiting the intestinal tract. Treatment of young children with broad-spectrum oral antibiotics may produce disturbances in the composition of the intestinal microflora, so that bacteria important in promoting Th1 mechanisms are depleted at a crucial age. This could result in Th2 dominance over Th1 immune responses to environmental antigens and an increased incidence of atopic disorders (70).

The etiology of atopic disorders is surely complex, but their molecular origins must be determined in order to develop effective countermeasures. One such countermeasure may be the administration of lactobacilli or bifidobacteria to children if these bacterial types can be shown to program the immune system toward a Th1 response as has been demonstrated with avirulent *Mycobacterium bovis* (73). Promising ameliorating results have been obtained in the management of atopic eczema through the administration of lactobacilli or bifidobacteria to children, and in influencing the Th1 vs Th2 response in mice (74–76).

It Just Gets More Complicated

Modern knowledge of the intestinal microflora reveals it to be an even more complex collection of microbes than previously imagined,

in which as many as 76% of the inhabitants are only known by partial sequences of their 16S rDNA (12). The marked homeostasis of the adult intestinal microflora is evident from modern investigations of this bacterial community (58). The consumption of foods or other products containing lactobacilli or bifidobacteria will therefore produce only transient modifications to the microbial content of the intestinal tract. More realistic opportunities for modification of the intestinal microflora may be found in early life, when a biological succession occurs in the intestinal tract and probably lasts for perhaps about 2 yr (77,78). During this period, extensive changes occur in the proportions of the bacterial community that particular bacteria constitute and in their metabolic activity (29,77,79,80). The administration of lactobacilli or bifidobacteria during these early years may have lasting influences on the child, largely because of the impact of the bacterial community on the developing immune system. Studies to date, however, show that it is not an easy matter to influence the acquisition of the intestinal microflora by oral administration of bacteria. The *Lactobacillus* population of piglets, for example, undergoes extensive modification with regard to component strains as the animals age and are subjected to various farm husbandry practices (81–84). The microflora of the human infant will surely be no less difficult to manipulate, and elegant investigations are needed. These studies will require the talents of microbiologists, immunologists, and pediatricians. Fortunately, molecular methods of analysis of the intestinal microflora mean that collaborative studies can be mounted on an international basis (85). This offers bright prospects for future research concerning lactobacilli and bifidobacteria.

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