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The Microbiome and Human Biology

Rob Knight,[1,2,3] Chris Callewaert,[1,4] Clarisse Marotz,[1] Embriette R. Hyde,[1] Justine W. Debelius,[1] Daniel McDonald,[1] and Mitchell L. Sogin[5]

[1]Department of Pediatrics, University of California, San Diego, La Jolla, California 92093; email: robknight@ucsd.edu

[2]Department of Computer Science and Engineering, University of California, San Diego, La Jolla, California 92093

[3]Center for Microbiome Innovation, University of California, San Diego, La Jolla, California 92093

[4]Center for Microbial Ecology and Technology, Ghent University, 9000 Ghent, Belgium

[5]Josephine Bay Paul Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

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Abstract

Over the past few years, microbiome research has dramatically reshaped our understanding of human biology. New insights range from an enhanced understanding of how microbes mediate digestion and disease processes (e.g., in inflammatory bowel disease) to surprising associations with Parkinson's disease, autism, and depression. In this review, we describe how new generations of sequencing technology, analytical advances coupled to new software capabilities, and the integration of animal model data have led to these new discoveries. We also discuss the prospects for integrating studies of the microbiome, metabolome, and immune system, with the goal of elucidating mechanisms that govern their interactions. This systems-level understanding will change how we think about ourselves as organisms.

INTRODUCTION

When we think about what defines us as a species, our thoughts naturally turn to the human genome. The Human Genome Project was a remarkable success in government-funded "big science": In President Obama's 2013 State of the Union address, he estimated the cost of the Human Genome Project at US$3.8 billion, with an incredible 140:1 return on investment. Yet the human genome, which is essentially fixed at birth, represents only a small fraction of the genetic diversity associated with the human body. Estimates of the gene content of the microbiome from either back-of-the-envelope calculations (155) or empirical observations (64, 127) place the number of microbial genes associated with the human body at 2–20 million, exceeding the ~20,000 human genes by at least a factor of 100. Microbial cells even outnumber human cells, although the early and widely reproduced estimates of a 10:1 ratio of microbial cells to human cells are overstated; the most detailed report to date suggests that we are only approximately 47% human on average by cell count (139) (of course, because microbial cells are much smaller, this corresponds to only a couple of kilograms of microbial biomass in a typical adult). The impact of this enormous number of microbial genes and cells on human biology must be profound. Furthermore, unlike the fixed human genomes, these microbial gene repertoires are highly malleable, offering exciting prospects for novel therapies.

Our ability to read out these complex microbial communities and understand their impact on human biology has been transformed by advances in technology, especially DNA sequencing and computational methods. In just a decade, a typical study has advanced from collecting a few dozen sequences for each sample to collecting a few hundred million. These advances have opened up a panoramic vista of how microbes change over space and time and how they relate to processes ranging from the physiological to the psychological.

THE EARLY DEVELOPMENT OF THE MICROBIOME FIELD

The term microbiome has been used to refer to the collection of genes contained within a community of microbes; today, it is also used to refer to the organisms themselves. (The latter are also often called the microbiota; in this article, however, we generally defer to the common use of microbiome to refer to the organisms.) However, the first appearance of the term microbiome in the literature did not include a definition of relatedness according to genomic criteria. Instead, the microbiome represented the collection of all taxa that comprise microbial communities, including bacteria, fungi, and protists in the intestinal tract and their relationship to microbes in the oral cavity (24) and protozoan populations in sewage-contaminated environmental waters (113). At that time, microbiology systematics lacked a compelling phylogenetic context. Efforts by Stanier & Van Niel (147) to transform the morphology-based microbial systematics of Bergey et al.'s (15) manual into a phylogenetic framework had reached an impasse (148). Morphology, cell staining characteristics, physiological properties, capacity for biochemical reactions, and other anecdotal features could not identify membership or evolutionary relatedness between phylogenetic assemblages.

The resolution of this problem and the foundation for today's microbiome studies emerged from the physicist Carl Woese's quest for the origin of life. Influenced by Zuckerkandl & Pauling's "Molecules as Documents of Evolutionary History" (172), Woese predicted that molecular analysis of the RNA components of the ribosome would reveal primordial branching patterns in the tree of life. He reasoned that the earliest life forms must have invented protein synthesis machinery with RNA components that would then be locked inflexibly with their binding partners and therefore would evolve slowly. Specifically, interactions with the multiprotein complexity

of the ribosome and with all other cellular proteins during their synthesis would impose strong evolutionary constraints on the ribosomal RNAs.

Even before the development of DNA and RNA sequencing technology, DNA and RNA competition hybridization experiments confirmed Woese's hypothesis by demonstrating that ribosomal RNAs evolved 100-fold more slowly than protein-coding regions in bacterial genomes (122). Comparisons of the fragmentary rRNA sequence information captured by the then state-of-the-art two-dimensional oligonucleotide fingerprinting technology, initially for 5S rRNAs (145) but ultimately and more comprehensively for 16S rRNAs, provided a phylogenetic framework that still underpins contemporary microbial ecology and systematics (145, 160). These early oligonucleotide cataloging efforts redefined microbial systematics through the discovery of 11 major microbial phyla but also, more profoundly, led to the discovery of a third domain of life, the Archaea (160, 161). When coupled with the first report of a full-length 16S rRNA sequence (21), the comparisons of oligonucleotide catalogs from hundreds of cultured organisms revealed a series of interspersed slow- and fast-evolving regions that would eventually provide important technical advantages for contemporary microbiome investigations. This interleaved conservation pattern of slow- and fast-evolving regions allows rRNAs to serve as multihanded molecular chronometers over disparate evolutionary timescales (159). The slow-evolving regions allow the construction of amplification primers, and differences between the highly conserved regions identify phylogenetic relationships that span the longest evolutionary timescales. By contrast, the fast-evolving regions provide fine-scale resolution often suitable for human microbiome investigations. Because the rRNA evolves at different rates in different taxa, even the full-length rRNA sequence does not permit resolution of all taxa; in general, however, the faster-evolving regions provide better resolution, which is often important for answering clinical or forensic questions.

The introduction of labor-intensive Maxam-Gilbert DNA sequencing (102) and Sanger dideoxy chain termination sequencing (135) led to a rapid expansion of cultivar rRNA databases that included sequences 100–300 nucleotides long. The conserved regions served as primer sites for reverse-transcriptase-mediated direct sequencing (85) of purified 16S rRNAs or chain elongation sequencing of cloned rRNA genes (40). The molecular biologist Norman Pace (123) realized that the revolution in microbial systematics enabled by molecular phylogenetics would have a profound impact on microbial ecology. The initial molecular surveys of microbial diversity from environmental samples required the isolation and DNA sequencing of cloned rRNA gene inserts from recombinant libraries. Each sequence served as a proxy for the occurrence of a microbial genome in an environmental sample. Comparisons of environmental rRNA gene sequences with the rapidly growing rRNA database from cultivars revealed microbial diversity to be at least two orders of magnitude greater than previously appreciated. New technologies, including the adaptation of polymerase chain reactions (PCRs) for amplifying and cloning rRNA gene sequences (106) and automated DNA sequencing machines, accelerated the rate of discovery (142). Woese's 11 major bacterial phyla grew to more than 1,000 (164). Finally, the development of next-generation sequencing technologies capable of generating millions of reads for less than one cent per read once again pushed back the known limits of microbial diversity, much of which represents the very-low-abundance taxa that make up the rare biosphere (144).

THE MOVE TO NEXT-GENERATION SEQUENCING

Early studies of the microbiome were limited to relatively small numbers of sequences because of the costs and time required for cloning and Sanger sequencing (typically several dollars per sequence). These studies traditionally focused first on rRNA gene amplification by PCR and then on relating the individual sequences to one another via multiple sequence alignment and subsequent

phylogenetic reconstruction (e.g., 18). One critical aspect in these phylogenetic analyses was the assessment of the taxonomic composition of a community or set of communities; new sequences were often placed in a characterized reference such as the Ribosomal Database Project (121) or into a phylogenetic tree, with new sequences added to those with existing taxonomy and/or environment annotation in GenBank. These workflows, going from sequence to phylogeny, became commonplace, and tools such as ARB (96)—which allow users to align sequences, insert them into a phylogeny, and visualize the tree—became widespread.

Because cost limited the number of sequences available per sample, obtaining abundance estimates for the organisms corresponding to the sequences was problematic, especially because many investigators used cheaper fingerprinting methods to choose only unique and diverse representative molecules for sequencing. Additionally, it was clear that the diversity was very high, with many new unknown sequences in each new data set (including new sequences that were artifacts of sequencing error), exceeding the capacity of phylogenetic reconstruction software and the computers of the time. Exacerbating the issue was the considerable controversy about which phylogenetic methods to use, especially because theoretically more powerful methods, such as maximum likelihood, were limited to a few hundred sequences at most.

To address these issues, researchers began to cluster sequences into operational taxonomic units (OTUs), often using a level of 97% sequence identity as a proxy for species; the number of sequences present in an OTU acted as a proxy for its abundance and allowed the computation of ecological diversity estimates (104). Naively, the clustering could be performed using the Basic Local Alignment Search Tool (BLAST) (4); however, this approach was slow even with small numbers of sequences, because early methods relied on computing each pair of distances between sequences. Optimized methods for such calculations, notably DOTUR (136), which leverages the Phylogeny Inference Package (PHYLIP) (13) to compute the distances, greatly improved the ability to generate OTU tables and pick representative sequences for phylogenetic reconstruction. DOTUR made the important contribution of refining the concept of an OTU beyond the idea of an identity threshold by introducing different methods of clustering (essentially asking whether the threshold defined the maximum difference between any two sequences in an OTU, the average difference between any two sequences, or the maximum difference to the nearest sequence).

The adoption of the OTU concept paved the way for classically trained ecologists to become involved in microbiome research, bringing with them decades of ecological theory and methods developed for the qualitative and quantitative study of macroscopic environments. In particular, they brought approaches that were well suited for assessing the relationships between environmental factors and the organisms in a community, such as ordination techniques (20). One extremely powerful concept was the notion of beta diversity, which represents the dissimilarity between a pair of samples. These distances can be computed pairwise across a large number of samples to produce a matrix that describes how similar every sample is to every other sample. These distance matrices can then be assessed for the presence of systematic structure through techniques such as permutational multivariate analysis of variance (PERMANOVA) (7) and principal coordinates analysis.

There are many ways to compute beta diversity, but not all are equally informative for studying microbial communities (82). In particular, methods that do not explicitly take phylogeny into account tend to underperform in expressing differences between samples and can lead to inaccurate or implausible biological conclusions. In the mid-2000s, Lozupone & Knight (94) asked whether including phylogeny could improve comparisons of microbial communities and developed UniFrac to perform such comparisons. Applying UniFrac to meta-analysis of more than 100 existing 16S rRNA studies spanning every conceivable natural environment on the planet rapidly yielded a remarkable pattern: The structure of a microbial community was influenced more by whether it came from a saline or nonsaline environment than by any other factor recorded (95).

However, once samples from vertebrate guts were included, the split between host-associated and non-host-associated samples proved to be even more profound than the saline-nonsaline split (89), suggesting that microbial communities residing within hosts are uniquely specialized relative to other communities.

In parallel with analytic improvements such as UniFrac, the price of DNA sequencing dropped precipitously because of the huge investment in sequencing technology from the Human Genome Project, particularly the advent of pyrosequencing, which performs sequencing by synthesis and detects the incorporation of a nucleotide by monitoring the release of pyrophosphate (100). In addition to the cost reduction, a single sequencing run could produce hundreds of thousands of sequences without requiring the laborious clone libraries used by Sanger sequencing, albeit with increased sequencing error. These errors are particularly problematic when assessing the presence of rare members of a community, because an erroneous sequence may appear to be real. Relatively simple criteria could be applied to remove many of the low-quality reads, yielding error rates that were better than those of Sanger sequencing (65, 128). The first application of pyrosequencing to 16S rRNA data yielded an unprecedented view into the so-called rare biosphere (144), revealing an extremely long tail of rare taxa in marine ecosystems that was rapidly extended to other environments.

Although pyrosequencing was cheaper than traditional capillary-based methods, it was not cost effective to perform a whole sequencing run per sample. For example, in 2007, the 454 platform typically yielded approximately 500,000 reads for US$12,000; although this reduced a multiyear, multimillion-dollar sequencing exercise to an eight-hour project, the cost per sample was still prohibitive for most applications. Simply combining libraries would have made it impossible to track each sequence back to the sample it came from. This challenge led to methods for multiplexing samples together, such as ligating nucleotide tags unique to each sample during PCR (17) or including these tags on the PCR primers themselves, allowing readouts of multiple communities simultaneously (62). Including formal error-correcting codes in the construction of these barcodes made it possible to tolerate errors within the barcode itself and allowed confident multiplexing of hundreds of samples simultaneously on a run (56).

Through projects such as the Human Microbiome Project (HMP) (64) and Metagenomics of the Human Intestinal Tract (MetaHIT) (127), these advances in sequencing quickly moved the microbiome field from a data-poor science to a data-rich one. This wealth of data quickly resulted in computational bottlenecks and prompted the development of tools such as mothur (137), Visualization and Analysis of Microbial Population Structures (VAMPS) (65a), and Quantitative Insights into Microbial Ecology (QIIME) (26). The latter is an accessible and modular microbiome-focused analysis framework that can handle large volumes of data and can operate in environments ranging from laptops to supercomputers.

The modularity of the QIIME platform greatly simplified subsequent technological transitions. For example, at the same time as the HMP was under way, Illumina sequencing technology began to support tens of millions of sequences per run. The HMP used QIIME to process 454 amplicon data, as described in Reference 64. In the same issue of *Nature* in 2012, Yatsunenko et al. (166) also published a paper describing a study in which they used QIIME with data from the Illumina platform that spanned more than 1 billion amplicon reads, at a depth of coverage of more than 1 million reads per sample.

A secondary benefit of the modularity of QIIME was the ability to easily add novel analysis methods. This approach allowed methods development in concert with study design, such as the exploration of the biogeography of microbes on a person's hands (54). Similarly, the flexibility of the platform made it possible to utilize the statistical and visualization components to analyze different types of data beyond 16S rRNA (for example, shotgun metagenomic data and

metabolomics) in order to study the distribution of small molecules across the body in relation to the microbial inhabitants (19). Critically, this approach standardizes a large portion of the bioinformatics pipeline and reduces technical bias, which can often outweigh the biological differences among samples (91).

Most amplicon-based microbiome studies still perform OTU-level analyses. However, in the last few years, improved methods have provided a strong motivation to move to the sub-OTU level. One such method, oligotyping (41), allowed researchers to partition OTUs into finer groups in a supervised fashion based on the Shannon entropy of the variation within nucleotide positions and to then assess whether these partitions were significantly correlated with study covariates. A related method, minimum entropy decomposition (42), operates in an unsupervised manner but is similar in concept. More recently, DADA2 (23) and Deblur (6) leverage the error profiles of the sequencing instruments themselves to determine the most probable molecules that were presented to the sequencing instrument. Excitingly, although these approaches do not offer assured species- or strain-level resolution because the necessary variation does not always exist in the target amplicon, they do offer maximal precision and specificity from the data obtained.

Next-generation sequencing also enabled shotgun metagenomics, which essentially isolates the total DNA from a sample, fragments it, and sequences all the fragments. Overall, shotgun metagenomics has been adopted less than amplicon sequencing in human microbiome studies because it requires far greater depth of coverage and is therefore more expensive, although it has been critically important for obtaining functional as well as taxonomic insight into the human microbiome. A particularly exciting emerging area is metagenomic assembly, in which complete genomes can be assembled from metagenomic data, allowing detailed tracking of the colonization of individuals by specific strains of bacteria during development (120). However, earlier gene-based approaches [such as those used in the HMP (64) and MetaHIT (127)] have been very useful for functional investigations, especially when correlations among reads across multiple samples are used to generate coabundance groups corresponding to genomes or large genome fragments (90).

PLACING THE HUMAN MICROBIOME IN CONTEXT

Next-generation sequencing techniques and the tools developed to effectively handle the data produced from these sequencing efforts have enabled a deep exploration of the microbiome that was not possible even a decade ago. In the early 2000s, several groups were studying the human microbiome, particularly the skin microbiome, and the first shotgun metagenomic analysis of the gut was performed in 2006 using Sanger sequencing (52). Many patterns, such as the high level of diversity among individuals in the human gut (39) and the profound differences among human body sites (31), were clear from the first amplicon studies to systematically explore these topics. However, microbiome research exploded after the release of data collected from the HMP, which was the first large-scale effort to characterize the healthy human microbiome across the body.

The HMP was a National Institutes of Health–funded, multimillion-dollar project with many components, among the largest of which was a description of the microbiomes of up to 18 body sites in 242 healthy humans; the findings were published in two companion papers in *Nature* in 2012 (63, 64). Unsurprisingly, different body sites in the HMP cohort harbored drastically different microbial communities in terms of both diversity and composition, which was consistent with earlier results from amplicon sequencing alone (31). Stool and oral communities were the most diverse microbial communities in terms of number of different organisms present, and the microbial communities of vaginal samples proved to be the least diverse, comprising mainly *Lactobacillus* spp. (63). Interestingly, sublocations of the body site classes (i.e., skin, mouth, vagina, or

gut) harbor specific microbial communities; for example, although all locations in the oral cavity harbor *Streptococcus* as a major taxonomic group, the second-most-dominant group is different in the buccal mucosa (*Haemophilus*), supragingival plaque (*Actinomyces*), and subgingival plaque (*Prevotella*) (64). Individual differences in the microbiome are sufficiently large and reproducible that they may be useful for forensic purposes (43, 48) and can even match up family members (146) and sexual partners (171), although courtroom applications of such technologies remain for the future.

Cross-individual differences in the HMP cohort were specific to body site; for example, although an individual can harbor a variety of species in his or her oral cavity, all individuals in the HMP cohort appeared to carry the same or similar types of organisms (*Streptococcus*, *Neisseria*, *Haemophilus*, and *Veillonella*), and although the skin microbiome in general is not highly diverse for a single individual (dominated by *Propionibacterium*, *Staphylococcus*, or *Corynebacterium*), individuals tended to harbor markedly different communities from one another (64). Stool samples collected from individuals in the cohort exhibited tremendous variability, ranging from complete dominance by Firmicutes to complete dominance by Bacteroidetes (the two major bacterial phyla in the gut). Despite this wide variety in taxonomic composition both within and between body sites, metabolic and functional pathways in the metagenomes were much more constant and evenly diverse, with several core pathways, including ribosome and translational machinery, ATP synthesis, and glycolysis, ubiquitous across body sites and individuals (64), consistent with previous work that had identified the same patterns in the gut alone (154). This observation lends strong support to a multi-omics approach to characterizing healthy (and diseased) microbiomes, because determining the microbial composition of a community alone will not be sufficient.

Although humans have explored a remarkably wide ecological niche relative to other vertebrate species, with an unprecedented geographic range and diversity of diets, placing the human microbiome in the context of those of other mammals suggests that humans are relatively unremarkable. At the level of both taxonomy and function, humans closely resemble other omnivorous primates, such as chimpanzees (88, 115). In general, mammals with the same diet and gut physiology harbor similar microbiomes, although there is substantial phylogenetic inertia; bears, for example, have diversified over a span of ~5 million years into obligate carnivores (polar bears), herbivores (pandas), and omnivores, yet they all harbor similar microbiomes (88). Comparative studies of the microbiomes of great apes (including humans) show a considerable level of cophylogeny, such that the gut microbiome tracks the overall pattern of host evolution (111, 118). Unfortunately, very few data are available for other body sites, such as the oral and skin communities, in part because of the difficulty of collection. Intriguingly, the gut microbiomes of different species of apes can converge when the animals are living in the same habitat, suggesting the possibility of interspecies transfer (112).

The mouse microbiome is notably different from the human microbiome, and although the dominant phyla (Firmicutes and Bacteroidetes) are the same, the genus-level composition is notably different. In general, techniques such as principal coordinates analysis can perfectly separate mouse samples from human samples (92). This has important implications for the use of mouse models to make inferences about human biology. In general, because the background microbiomes are so different, mouse studies are more effective for demonstrating possible mechanisms than for identifying specific taxa associated with human biological processes. Gnotobiotic mice (i.e., mice colonized with known microbial communities, such as those derived from human samples) are extremely useful in understanding the impact of human-associated bacteria on conserved mechanisms in the host, and, for example, individual human microbiomes transferred to mice can confer phenotypes ranging from adiposity (131) to features resembling Parkinson's disease (134). Perhaps most excitingly, *Christensenella*, a genus of microbe that is associated with low-body-mass

indices in human twin studies, causes mice inoculated with an obesogenic microbiome from humans to remain lean (55), demonstrating the ability to move from population-level observations to mechanistic work in an individual microbe. However, following exposure to mouse-derived microbes, human-derived microbes are displaced from gnotobiotic mice within a few days (138), underscoring the need to keep such mice completely isolated environmentally.

In terms of the environmental context, the microbiology of the human-built environment consists mainly of human-derived microbes, with the dominant input being from the skin, and the individual-specific nature of this input can be tracked longitudinally to demonstrate transmission of microbes from individual people to surfaces they touch and spaces they inhabit (87). After death, the microbiome undergoes a specific suite of changes that mix endogenous community members with those derived from the soil, in a pattern so systematic that it can be used to estimate the postmortem interval (107). Intriguingly, the skin acts as a taxonomic bridge between other human body sites and environmental microbiomes, being dominated by Firmicutes (common in other human body sites), Actinobacteria (common both in human body sites and in the environment), and Proteobacteria (more common in environmental samples) (89). However, the profound differences between host-associated and free-living microbial communities also apply specifically to the human microbiome, and this factor dominates large-scale comparisons of microbiomes.

ASSOCIATIONS BETWEEN THE MICROBIOME AND HUMAN DEVELOPMENT

Although most studies of the human microbiome have focused on adults, there is immense interest in the microbiome during development both because of the profound changes that it undergoes during this process and because of the exciting prospects for early-life interventions that could promote health over a lifetime. Additionally, the microbiome appears to have far-reaching effects on reproductive and developmental biology that were unanticipated until recently.

The vaginal microbiomes of pregnant women differ from those of the general population (2, 132), although the degree of stability within the community depends on its composition (98, 132). Overall, pregnancy is associated with a loss of microbial diversity within the vaginal community (2) and a transition toward community structures dominated by *Lactobacillus* (2, 98, 132). These shifts may be hormonally driven, with the bacterial community responding to increased estrogen during gestation (98). The elevated *Lactobacillus* may also be protective for the developing infant. *Lactobacillus*-dominated communities protect against bacterial vaginosis (130), a defect in the vaginal microbiome that is associated with elevated risk of preterm birth (60).

The mother's gut microbiome is radically remodeled between the first and third trimesters (79), with third-trimester microbiomes differing markedly from those of nonpregnant women. Late-term pregnancy has been associated with increases in the relative abundances of the phyla Proteobacteria and Actinobacteria and a loss of community diversity, creating an atypical community relative to those of healthy, nonpregnant women (79).

The changes in the maternal microbiome seed the infant's first microbial communities, but there is some controversy regarding whether this seeding occurs prenatally or during birth (1, 75, 86). Recent papers have argued for a placental microbiome in the absence of amniotic infection (126, 169). However, evidence suggests that the observed placental microbiome may be a result of contamination in low-biomass samples: One study was unable to detect differences between a placental microbiome and negative controls (86). Regardless of whether the microbial community is seeded prenatally, the mode of delivery has a strong role in shaping the neonatal microbiome. The microbial communities across multiple body sites of vaginally born infants more closely resemble an adult vaginal community, whereas infants born by cesarean section have communities that

more closely resemble the skin microbiome (11, 38). Additionally, the birth method modulates the vertical transmission of gut microbes from mother to child (11), and the effect of vaginal birth is detectable in the microbiome through one year of age (11). Studies in adults have not described a significant difference associated with delivery method, although it remains unclear whether the early-life changes in the microbiome can affect later phenotype by acting at a critical period in development. In mice, the time of colonization of initially germ-free mice with microbes determines whether the microbes cause permanent alterations in behavior and in gene expression at distal tissues, including the brain (36), and the same may be true in humans.

The microbiome undergoes rapid changes during the first three years of life, which is followed by a more gradual maturation (11, 78, 166). The development of the infant microbiome correlates with changes in the breast milk composition and microbiome over the first year of development (11, 22, 30, 99). Human breast milk contains unique oligosaccharides not found in any other mammalian milk (109). Many of these sugars are recalcitrant to host digestion but can be directly utilized as a primary carbon source by *Bifidobacterium* and, to a lesser extent, some *Bacteroides* species. This is directly reflected in the distinct infant gut microbiota, which is dominated by *Bifidobacterium* (84). Over the first four months of breastfeeding, the amount of lactose increases and the concentrations of both monosaccharides and oligosaccharides decrease (30). The carbohydrate metabolism capacity of vaginally delivered, breastfed infants responds to this change in breast milk composition: At four months of age, infant metagenomes show an increase in the abundance of lactose-specific transport genes (11).

Weaning forces a maturation of the microbiome (11, 78). The introduction of solid food diversifies the microbiome and shifts carbohydrate metabolism to complex carbohydrates and starch (11). Chronologically older infants who were exclusively breast fed appeared microbially younger than their peers who had been introduced to solid food. However, nutritional disruption can alter this maturation: Children with persistent malnutrition were microbially delayed compared with their healthy peers (11, 78).

The widespread use of antibiotics, which began in the 1940s with large-scale production of penicillin [discovered in 1928 (5)], ushered in a new era of human medicine. Diseases and infections that had taken the lives of thousands were no longer considered dangerous, leading to the designation of antibiotics as "wonder" or "miracle" drugs. Inappropriate use of antibiotics, however, has led to a swath of serious health problems. In particular, overuse of antibiotics in children is a major public health problem. Most of the common illnesses experienced during childhood, such as diarrhea and upper-respiratory-tract infections, are caused by viruses, not bacteria; therefore, the prescription of antibiotics in these cases is ineffective at best (83). The detrimental effects of antibiotic usage on the gut microbiome have been described in both children and adults (35, 46). Given that the first three years of life are a crucial time for gut microbiome maturation, antibiotic treatments during this period have the potential to inflict detrimental alterations on normal gut microbiome maturation, with potentially serious long-term effects. In mice, early-life treatment with antibiotics increases weight gain and adiposity, delays microbiome maturation, and alters the metabolic activity of the fecal microbiome even into adulthood, long after antibiotic exposure (117). The negative effects on microbiome maturation are also cumulative, becoming more pronounced with additional antibiotic courses—a significant observation, given that the average child in the United States receives ten courses of antibiotics by age ten (117). Similarly, children exposed to multiple antibiotic treatments in the first three years of life have less diverse and more unstable gut microbiomes compared with their untreated counterparts (165). Antibiotic resistance genes in the gut microbiome also rise sharply in this group after antibiotic administration (165). Antibiotic treatment in children has also been associated with an increased risk for obesity (10), asthma and

allergies (8, 108), diabetes (72), and inflammatory bowel disease (66), all of which are associated with dysbiosis and, like antibiotic usage, have increased in prevalence over recent decades.

Intriguingly, the rate of approach to the adult microbiome state is consistent in different populations, although the states that are reached differ markedly (166). Efforts to understand the specific factors that lead to these cross-population differences in the adult microbiome are a major area of interest. Far less information is available about the maturation of the microbiome at other body sites, although characterizing these patterns of change will clearly be of great importance.

LARGE AND SMALL EFFECTS ON THE MICROBIOME: WHAT REALLY MATTERS?

As noted above, the human gut microbiome matures during the first three years of life and remains relatively stable throughout adulthood (166). Therefore, all factors that influence the microbiome likely have the greatest impact in this early developmental time window. Nevertheless, some factors have a dramatic impact on microbial composition at all life stages, and some factors have more subtle influences (34).

One of the most dramatic ways to influence the microbiome is with antibiotics. Even short-term doses of antibiotics prescribed for acute infections can perturb the gut microbial composition for years (68). Resistance genes selected for during antibiotic treatment can persist in the microbial community long after the therapy has ended. Although the degree and direction of dysbiosis in response to antibiotic treatment vary by individual, there are some overarching trends. For example, bacterial diversity decreases during the week immediately following antibiotic exposure and then begins to recover, although often the original state does not fully return. In addition to changes in alpha diversity, antibiotic treatment can also have a significant effect on microbial gene expression, protein activity, and overall microbial metabolic function (47).

Perhaps unsurprisingly, long-term diet is the primary determinant of the taxonomic and functional structure of the human gut microbiota. The nutritional composition of food affects which microbial species flourish and, in some cases, selectively depletes certain taxa. All mammalian gut microbiomes share a core set of genes covering essential metabolic functions, but carnivores, omnivores, and herbivores clearly differ in their relative abundances of these genes and the specific taxa that carry them (115). This leads to the observation that diet drives the convergence of gut microbiomes across mammalian species.

The differences in the microbiomes of western and nonwestern populations are profound and likely driven at least partly by diet (53, 166), although neither controlled experiments to confirm this nor detailed studies in immigrant populations have been performed. Such studies that compare large numbers of populations, rather than individuals, will be essential for understanding the plasticity of function in the human microbiome in response to the diverse diets in human populations worldwide.

Within the US population, long-term dietary patterns shape stable microbial communities, and small dietary changes are often not enough to disrupt the community factors that make an individual's microbiome unique (150, 162). In two studies of short-term dietary interventions lasting less than a week, the microbiome reverted to its original state within days, and the magnitude of the change was smaller than the baseline differences among individuals (33, 162). In children with severe malnutrition, antibiotic treatment followed by a dietary intervention was not sufficient to alter microbial development in the long term, and the improvements in the microbial community structure associated with therapy required continuing dietary intervention (143, 150).

Many examples of the effects of individual dietary items are emerging. For example, dietary emulsifiers can disrupt the gut mucosal barrier, inducing low-grade inflammation and changes in

microbial composition (28). Similar effects have been observed with artificial sweeteners in both human and mouse studies, with individual differences in response explained largely by the microbiome (151). The microbiome may explain the large individual-level differences in response to specific dietary items that have long confounded weight-loss studies. One recent groundbreaking study used continuous glucose monitors to explore postprandial glucose response in a population of 800 people who were fed a controlled sequence of meals, allowing the effect of individual food items to be determined and related to various parameters, including the microbiome (167). Although the population's average results recaptured the glycemic index for each food almost perfectly, individual variation in response was very high, and in a validation cohort of 100 individuals who were not involved in the initial study, it was possible to design "good" and "bad" isocaloric, macronutrient-balanced diets for a single individual based on his or her microbiome, with markedly different impacts on glycemic response. These studies have tremendous potential to stratify patients effectively for dietary treatments of diabetes and obesity.

Host genetics also contribute to the structure of the microbiome, albeit with a far smaller effect size. The best evidence for a genetic influence on human microbiomes comes from twin studies comparing monozygotic and dizygotic twins. Initial 16S rRNA gene sequencing studies of dozens of people revealed no significant differences between mono- and dizygotic twins in terms of the similarity of their gut microbiomes, although both types of twins were more similar to each other than to their mother or an unrelated adult (154). However, a more targeted approach using a much larger cohort of hundreds of twin pairs revealed that community membership (alpha diversity) rather than community structure (relative abundances) is responsible for driving the similarities in monozygotic twins. Although host genetics seem to play a relatively minor role in shaping the microbiome, certain taxa (for example, Christensenellaceae) are indeed heritable.

The microbiome has been associated with many other processes, including exercise (25), infections (32), stress (93), and sleep cycles (14), although the effect sizes are typically small. Despite much interest in the possibility that probiotics modify the microbiome, the effects tend to be very small and may occur more at the level of transcription than at the level of community change (105). Identifying probiotics that have large, permanent effects on the microbiome remains an important goal for future research.

MICROBIOMES AND DISEASE

Bacteria in and on the human body have a significant impact on health and the development of disease states. Microbiome alterations at different body sites have been associated with many diseases, including perhaps obvious examples, such as dental caries and bacterial vaginosis; chronic conditions, such as obesity, cardiovascular disease, inflammatory bowel disease, and malnutrition; and even diseases not traditionally suspected to be linked to the microbiome, such as Parkinson's disease, autism, and depression. **Table 1** provides a partial list of these diseases and their links to the microbiome; it is impossible to be comprehensive, given the rapid discovery of new links between diseases and the microbiome.

Any study of the microbiome and disease should address the question of correlation versus causation. For example, psoriasis vulgaris patients have a different skin microbiome than healthy controls (3), but is this altered microbiome the cause of the disease state or a consequence of the altered skin texture? Recent discoveries have suggested that an altered microbiome may cause atopic dermatitis (77), whereas the altered microbiome in psoriasis patients appears to be a side effect or an effect of the physiological changes of the skin (116). Even if the microbiome does not directly cause a condition, the subsequent shift in the community may increase the risk of

Table 1 A partial list of diseases and their links to the microbiome

Disease	Description and microbiome link	Reference(s)
Acne vulgaris	This skin disorder is mediated by specific *Propionibacterium acnes* strains, together with the vitamin B_{12} pathway in addition to other pathways.	44
Acute anorexia	Anorexia patients have lower gut alpha diversity. Molecular mimicry of microbial metabolites may contribute to autoantibody production.	74
Addiction	In a mouse model of addiction, antibiotic treatment increased addictive behavior in animals receiving low-dose opioids.	73
Alcoholic liver disease	Alcoholic liver disease is characterized by intestinal dysbiosis, bacterial overgrowth, and increased gut permeability.	58
Asthma and allergies	Dust on traditional farms stimulates the immune response and protects against asthma and allergies.	149
Atherosclerosis	There are suggested links with the translocation of oral microbes into atherosclerotic plaques. Microbially mediated lipid metabolism in the gut may affect the formation of plaques as well.	70
Atopic dermatitis	Skin inflammation is driven by *Staphylococcus aureus* dominance (with genetic predisposition).	77
Autism	Differences in gut microbial communities have been observed between children with autism and neurotypical controls; however, there are some inconsistencies. Maternally produced microbial metabolites lead to an autism phenotype in mice.	37, 61
Bacterial vaginosis	Bacterial vaginosis is characterized by deviation from a low-pH, *Lactobacillus*-dominated community to a higher-pH, more diverse microbial community.	49
Cardiovascular disease	Diet and the gut microbiome are linked with trimethylamine-*N*-oxide levels in plasma and cardiovascular disease risk (with genetic predisposition).	152
Chronic skin wounds	*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and other bacteria play a role in pathogenesis in chronic wounds.	16
Clostridium difficile–associated diarrhea	*C. difficile*–associated diarrhea is a typical example of a change in the gut microbiome leading to an enduring disease state.	12
Colorectal cancer	Pathogenic microorganisms can potentially initiate and facilitate the development of colorectal cancer.	50
Cystic fibrosis	Cystic fibrosis is characterized by chronic lung infections, commonly with hypermutable *Pseudomonas aeruginosa* strains.	97, 119
Dental caries	Dental caries are associated with increased phylogenetic diversity and overabundance of *Prevotella* taxa.	125, 163
Depression	Transplantation of microbiota from individuals suffering from major depressive disorder into germ-free mice induced depression symptoms in the mice. These symptoms are associated with alterations in carbohydrate metabolism in the microbiome and hippocampus.	170
Diabetes, type 1	In mouse models, the microbiome is required for the development of diabetes, although low-dose antibiotics increase susceptibility. Changes in microbial development mark the progression to disease but predate the clinical presentation.	80, 124
Diabetes, type 2	The blood of type 2 diabetes patients has reduced levels of bacterial lipopolysaccharide.	157
Inflammatory bowel disease	Gut inflammation disease is driven by genetic, environmental, and altered microbial factors. Adherent enterobacteria may promote initial ulceration events.	81, 110
Irritable bowel syndrome	Patients with irritable bowel syndrome show mucosal and luminal gut microbial changes, although the causal effect is unproven.	141

(*Continued*)

Table 1 (*Continued*)

Disease	Description and microbiome link	Reference(s)
Malnutrition	An altered gut microbiome is strongly linked with childhood malnutrition.	71
Multiple sclerosis	Gut microbiota changes may be related to autoimmunity and the pathology of multiple sclerosis.	69
Obesity (metabolic disease)	The gut microbiomes of obese individuals show an increased capacity to harvest energy from the diet.	156
Osteoporosis	The gut microbiome has both direct and indirect effects on deregulated bone remodeling.	59
Parkinson's disease	The microbiome can promote Parkinson's disease progression in genetically susceptible individuals.	134
Rheumatoid arthritis	Rheumatoid arthritis patients show altered gut and oral microbiomes. They also have increased translocation of oral bacteria in the gut, which treatment partially corrects.	168

other diseases; furthermore, the microbiome may still be useful for assaying specific diseases or subtypes, particularly in difficult-to-diagnose conditions such as Crohn's disease.

Interestingly, direct and indirect microbial links have been discovered for osteoporosis (59), colorectal cancer (50), irritable bowel syndrome (141), and mineral deficiency diseases, although whether the altered microbiome is a cause or a consequence of these conditions remains uncertain. An especially important method of determining causality has been demonstrating the ability to transfer human phenotypes to germ-free mice (wild type or otherwise). This has now been done successfully for multiple diseases and conditions, including obesity (131), malnutrition (143), insulin resistance resulting from artificial sweeteners (151), depression (170), and even jet lag (153). This technique has tremendous potential for establishing that microbes can cause a specific phenotype and for untangling molecular mechanisms, especially when combined with metabolomics, although negative results can be difficult to interpret in light of the many genetic and physiological differences between humans and mice. Gnotobiotic pigs, which provide a better physiological model, have recently been of intense interest in this respect, especially in studies of nutrition (27).

Like all other mammals, humans coevolved with their microbiota and developed a wide range of innate immune responses to protect the body against infection while still sustaining bacterial presence. The gut microbiome is in constant and intimate interaction with the host immune system and influences both innate and adaptive immune function (133). The innate immune response involves dendritic cells, neutrophils, and natural killer cells; the adaptive immune response involves the activation of T and B cells. Specific microbiomes are associated with the development of particular T cell subtypes (103). A shift in the gut microbiome can cause beneficial as well as detrimental outcomes mediated by $CD4^+$ T cell subtype regulation (9). This relationship between the gut microbiome and the immune system is strongly implicated in a range of inflammatory disorders. In particular, dysbiosis has been associated with increased oxidative stress, which can drive a chronic inflammatory response (114) that is exacerbated by a decrease in community members known to produce short-chain fatty acids (51). A lack of these fatty acids, such as butyrate, has the potential to promote an inflammatory response in the gut epithelium (67). Given the association between inflammation and autoimmune diseases, it is no surprise that many of them have been linked with the gut microbiome.

However, the microbiome is only a piece of the puzzle in the development and progression of disease. The microbiome, genetic susceptibility, epigenetic regulation, and environmental factors create a complex interactome. Genetic susceptibility is often required as an underlying etiology for conditions. Although cystic fibrosis has long been considered a disease with classic Mendelian

inheritance, the lung microbiome plays an important role in long-term prognosis (129). In more complex genetic diseases, such as inflammatory bowel disease (76) and Parkinson's disease (134, 140), the microbiome plays an important role in the etiology but is not causative alone. Crosstalk between the microbiome and epigenetic regulation may also modulate disease susceptibility, although the directionality of this interaction is unclear (57).

The role of diet and chemical exposure in disease management is also an important consideration. For instance, both diet and medication are used in the treatment of type 2 diabetes. Conventional wisdom suggests that type 2 diabetics should avoid simple carbohydrates to control their blood sugar. However, there is large variation in interindividual glycemic response to foods, some of which can be explained by the individual microbiomes. Indeed, the microbiome can be a far better predictor of a diet that limits postprandial glucose spikes than a nutritionist (167).

The role of drug treatment in disease management and the role of the microbiome in disease response are also under active investigation. A large cross-sectional study of patients with type 2 diabetes found that treatment with metformin was a better predictor of their microbiomes than their diagnosis was (45). Treatment played a role in microbiome remediation in a study of rheumatoid arthritis in which treatment with disease-modifying antirheumatic drugs partially restored microbial balance (168). However, the microbiome can regulate the way in which drugs are metabolized: Detrimental side effects of both metformin (45) and acetaminophen (29, 101) are related to microbial metabolism.

One challenge in therapeutic applications of the microbiome, however, is that microbiomes are unique to each individual and change over time. These properties complicate both diagnostic use of the microbiome and its viability as a therapeutic target. In diseases such as *Clostridium difficile* infection, the shift in the microbial community is remarkable (158), but the picture is less clear cut for many other associations of the microbiome with disease. As in human genetics work, it is necessary to understand the scope of diversity associated with different human populations, as well as the context of lifestyle and diet, so that we can understand deviations from the norm and specific aspects of concern for a particular disease state relative to a particular background.

CONCLUSIONS

The microbiome is complex, dynamic, and spatially structured. It is clearly important for many physiological and disease processes in which its involvement was completely unsuspected until recently. With all this complexity, especially the emerging links to metabolism, host genetics, epigenetics, and immunology, one might easily give up hope of being able to untangle the complex relationships, let alone exploit them for therapeutic benefit. However, other complex fields of biology offer hope. For example, although mass spectrometry analysis of an orange yields many compounds that have not yet been identified, we know that the vitamin C it contains is essential for preventing scurvy. Similarly, we can expect that some effects on the microbiome will be so large that they can be identified and exploited in a systematic way for any individual (for example, fecal transplant for recurrent *C. difficile*), whereas other dysbioses will be far more subtle and require individual-specific approaches, perhaps aided by model systems such as gnotobiotic mice, organoids, or in vitro models.

Although our capability to analyze the microbiome has expanded rapidly, it is clear that the ability to collect more sequences and time points will continue to revolutionize microbiome studies. Even a depth of coverage of 1 million amplicon reads per sample merely scratches the surface of a 39-trillion-strong gut microbiota, and it is entirely possible that rare species that we cannot yet detect play important roles in many ecosystems and disease processes. Better methods of metagenome assembly and interpretation, especially from shallow-coverage samples, are

urgently needed to provide statistical power for studies that need to cover hundreds to hundreds of thousands of individuals to reveal subtle effects. Computational methods, especially to integrate newly collected samples with large-scale resources such as the HMP, also need to be made far more accessible to a broad audience.

However, we can imagine a day, perhaps soon, when easy tracking of the microbiome together with continuous analytics in the cloud, perhaps even at the consumer level, will let individuals control their own microbiomes. Consumer products such as the TweetPee already allow a soiled diaper to alert the parents by Twitter or text message based on moisture alone; the potential for high-resolution tracking of additional microbiome variables is tremendous. Given the highly personal and personalized nature of the microbiome, the only questions will be how to protect privacy and how to best place control of the microbiome in the hands of each individual.

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