Chapter 4

The Human Microbiome

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INTRODUCTION

The human microbiome refers to the communities of microorganisms living in association with our bodies. These topographically diverse and temporally complex microbial populations are largely commensal, providing us with genetic variation and gene functions that human cells have not had to evolve on their own. Development of culture-independent isolation techniques and next-generation DNA sequencing technologies has enabled high-throughput surveys of human microbiota. These studies have linked alterations of both microbial community composition and diversity to various disease states. Although the microbiome has been shown to play an important role in shaping the host immune response, influencing metabolism, and modulating drug interactions, many important questions must be answered before we can fully utilize its prognostic and predictive potential. This chapter highlights the progress of the genomic technologies that drive microbiome research, examines how the microbiome modulates health and contributes to disease, and discusses the future challenges facing this emerging field of study.

16S RIBOSOMAL RNA GENE SEQUENCING

In the late 1800s, Robert Koch developed techniques to cultivate and isolate bacteria cells, which were then identified and characterized by biochemical

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staining, microscopic observation of their morphology, and the use of enrichment cultures. For over a hundred years, these culture-based techniques were the gold standard for classifying microbes. However, these approaches are restricted to the small subset of microbes that are able to survive in isolation and under specific laboratory conditions.

Genomic classification approaches offered a solution to biases of culture-based practices. In the late 1970s, Carl Woese and colleagues [1] generated the first bacterial phylogeny based on the small subunit 16S ribosomal RNA (rRNA). Unique to prokaryotic organisms, the 16S rRNA gene is highly conserved, but contains nine hypervariable regions with species-specific signatures. Soon after bacterial phylogeny was established, Norman Pace and colleagues [2] developed a technique to isolate the 16S rRNA gene from genomic DNA using PCR amplification. Sequences of the 16S rRNA gene could then be compared to the phylogenetic "reference" tree for taxonomic classification. While the 16S rRNA gene is ideal for profiling bacteria, the 18S rRNA and internal transcribed spacer (ITS) regions are similarly used to classify fungal species.

Standard human microbiome studies involve the extraction and sequencing of DNA from a sample containing a heterogeneous mixture of microbes, followed by computational analysis to examine those populations (Fig. 4.1). Rapid advances in DNA sequencing technology have been a key impetus for culture-independent microbiome studies. Early human microbial surveys relied upon fingerprinting techniques or Sanger sequencing of the amplified and cloned 16S rRNA gene. Today, next-generation sequencing platforms offer faster sequencing and vastly increased sampling depths at much lower costs.

The type of sequencing platform used is ultimately determined by the question being asked. In general, shorter reads are sufficient for most microbial community characterization studies, but decrease taxonomic precision. Longer read lengths are beneficial for studies attempting to distinguish between strains or species. Paired-end sequencing is often used to mitigate the problems associated with shorter read lengths by sequencing reads bidirectionally and merging the resulting pairs into a single, longer read.

Upon its introduction, many researchers in the field relied upon the Roche/454 pyrosequencing platform, which produced reads approximately 400−500 bp long. Currently, the Illumina MiSeq benchtop sequencer, which produces reads up to 300 bp, is a popular tool used in 16S rRNA characterization studies. A single run on a MiSeq can generate up to 50 million paired-end 300 bp reads in less than three days. Hundreds of samples can be sequenced on a single run by incorporating sample-specific barcodes into the 5′ primer sequence, in a process known as multiplexing.

A number of open-source software packages exist for computational analysis once microbial samples are sequenced. Two commonly used programs are QIIME [3] and mothur [4], which provide automated scripts for each

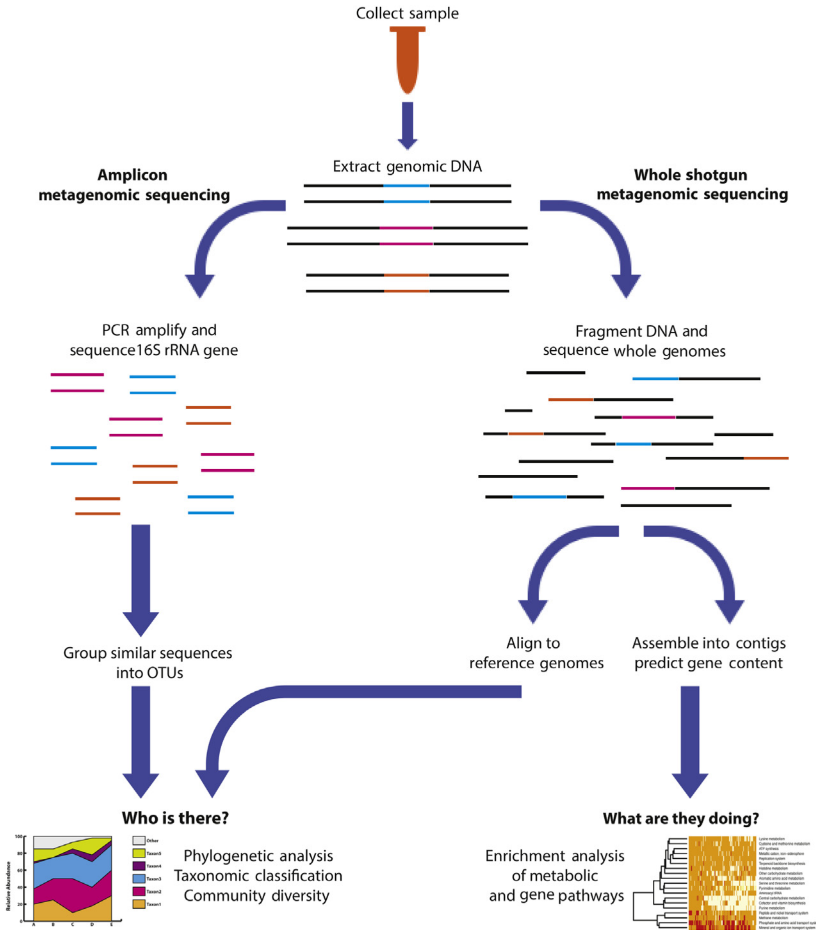

FIGURE 4.1 Microbiome study workflow for sample collection, sequencing, and analysis.

step of their bioinformatics pipelines. Raw DNA sequencing data is first demultiplexed into sample-specific sequences and filtered to remove low-quality sequences that may inflate diversity estimations or falsely suggest the presence of novel organisms. Highly similar sequences are grouped into operational taxonomic units (OTUs), which are compared to reference databases for taxonomic classification and used to calculate within-sample (alpha) and between-sample (beta) diversity. Statistical tests are used to identify significant associations between microbiome components and factors of interest.

General sequencing error, amplification bias introduced by selection of PCR primers or conditions, and the formation of hybrid sequences known as chimeras are just a few potential sources of inaccuracy in amplicon-based

sequencing approaches. Much research has been dedicated to the development of computational approaches aimed at reducing or eliminating these errors. Sequencing of a mock community sample, which contains genomic DNA from known microorganisms in specified quantities, alongside experimental samples is one way of estimating sequencing error rates.

WHOLE-GENOME SHOTGUN METAGENOMIC SEQUENCING

Whole-genome shotgun metagenomic analysis of microbial communities circumvents PCR bias by sequencing all DNA associated with an experimental sample and enables assessment of the full genomic coding potential of bacterial, fungal, and viral community members (Fig. 4.1). In this type of approach, paired-end libraries are constructed from extracted DNA, multiplexed, and sequenced on a highly parallelized platform, like the Illumina HiSeq. Prior to analysis, low-quality sequences and contaminant human DNA sequences are removed from the dataset. The power of metagenomic datasets lies in their ability to not only determine what microbes make up a community, but also to delve into the functional potential of these microorganisms. Furthermore, metagenomic sequencing allows for reconstruction of genomes that may not currently have a reference genome and are thereby not classified by culturing or 16S rRNA gene sequencing approaches.

There are many different tools available for identifying the taxonomic makeup of shotgun metagenomic datasets. MetaPhlAn [5] uses clade-specific marker genes to estimate relative abundances of different taxa, while MEGAN [6] relies on BLAST searches of sequences against microbial reference databases and employs a lowest common ancestor algorithm for classification. Although unassembled reads are required to calculate frequencies necessary for sample comparisons, overlapping sequence reads can also be assembled into contigs that provide more accurate gene annotation and phylogeny prediction. Assembly of the various genomes in complex metagenomic datasets is challenging. Toolkits, like IDBA-UD [7] and Ray Meta [8], utilize algorithms to assemble longer contigs with high accuracy. The functional capacity of the metagenome can be determined by comparing predicted protein-coding genes, identified by a BLASTX search, to databases such as the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database [9] and/or COG (Clusters of Orthologous Groups of proteins) functional categories database [10].

Metagenomic studies are a computationally intensive undertaking, generating an extremely large volume of sequence data. Subsequent analysis relies on incomplete reference databases that are highly biased toward cultivable organisms and genes with known functions. Thus, development of new methods for cultivating and isolating different organisms is crucial for construction of robust references. Once reference genome sequences are available,

additional obstacles to metagenomic sequencing analysis include the annotation of putative open reading frames and functional classification of hypothetical proteins.

CHARACTERIZING THE HEALTHY HUMAN MICROBIOME

In 2007, the NIH funded the Human Microbiome Project (HMP) and one of its key objectives was to define the "normal" human adult microbiome and investigate its role in various diseases [11]. Sampling a cohort of 242 volunteers at 18 diverse sites from five body areas, the HMP found that relative abundances of metabolic and functional pathways identified from the metagenomic data were much more stable than organismal abundances measured by 16S rRNA sequences (Fig. 4.2). Pathogenic organisms were rarely present in these microbial populations, and, as seen in previous microbiome studies, intrapersonal variation between body sites of the same subject was more significant than interpersonal variation between the same body sites of different subjects [12–16]. Because the communities found at each body site are highly specialized, the human microbiome can be considered as a composite of many different microbiomes. In the following sections, we highlight significant findings from individual studies of the gut, oral cavity, lung, urogenital tract, and skin, focusing on the contributions of the microbiota to human health.

Gastrointestinal Tract Microbiome

The gut is one of the first and most well-studied human body habitats regarding microbial communities. Fecal samples are commonly collected and used in microbiome analyses. The MetaHIT (Metagenomics of the Human Intestinal Tract) Consortium has been a key leader in gut microbiome and metagenomics research. Their study of 124 Europeans described a "core" gut metagenome containing genes essential for host–microbe interactions [14]. Analysis of this dataset, in conjunction with others, introduced the idea of "enterotypes", or groups of individuals defined by the composition of their gut microbiota [17]. Three enterotypes were identified, which could not be explained by nationality, body mass index (BMI), age, or gender. The notion that the composition of the human gut microbiota may be stratified, and not continuous, has sparked much debate in the field [18].

Analysis of the human gut virome has drawn attention to the prominence of bacteriophages, viruses that infect bacteria. Metagenomic sequencing of viruses colonizing a single adult gut found that almost 80% of the viral community persisted throughout the 2½-year study [19]. In addition to viral temporal stability, the study also identified high nucleotide substitution rates in certain bacteriophage families. The authors suggest that rapid evolution of

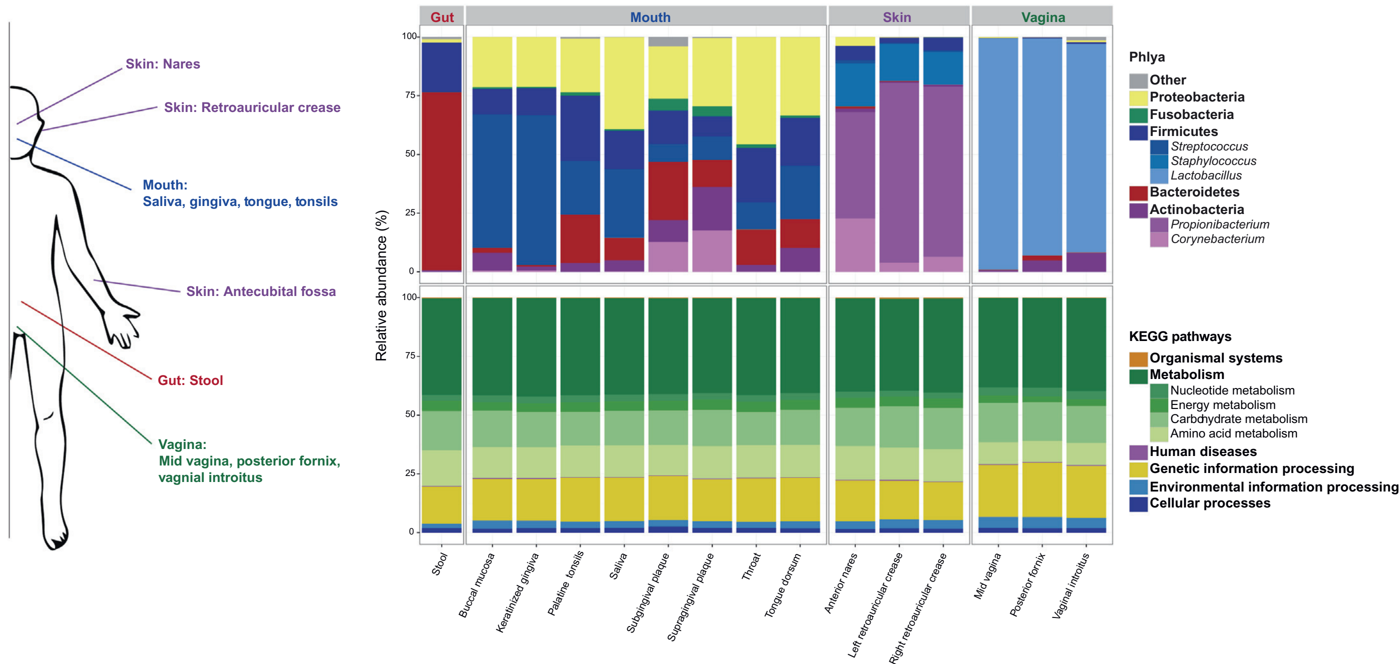

FIGURE 4.2 Taxonomic and functional relative abundance profiles of healthy individuals obtained via shotgun metagenomic sequencing as part of the HMP (MetaPhlAn taxonomic data file downloaded from http://www.hmpdacc.org/HMSMCP/ and KEGG pathway coverage calls downloaded from http://www.hmpdacc.org/HMMRC/).

long-term gut residents could give rise to new viral species, which may contribute, in part, to the gut's high interpersonal variability.

The gut microbiome is known to contribute to a variety of human diseases, including cancer and obesity. Colorectal carcinoma is associated with increased abundances of *Fusobacterium*, which is rarely found in the healthy gut [20,21]. Recent work suggests that this correlation is causal. One study found that introducing *Fusobacterium* into mice that develop intestinal tumors accelerated tumor development and induced a proinflammatory response [22]. These findings are supported by a second study that identified a highly conserved *Fusobacterium nucleatum* virulence factor, adhesion FadA, as an inducer of oncogenic and inflammatory responses that promote cancer cell growth [23].

While we do not fully understand human genetic variation associated with obesity, it has been established that the gut microbiota of obese individuals is significantly different from microbiota of lean individuals and carries with it a greater capacity for energy harvest [24]. Born and reared in sterile environments, germ-free mice are not colonized by microorganisms and are often utilized to determine the effects of microbial changes. One such study transplanted gut microbes from twins discordant for obesity into germ-free mice in order to elucidate how interactions between diet and the gut microbiome influence the human host [25]. Ridaura and colleagues saw that mice colonized with bacteria from the obese twin had significantly greater body mass and adiposity than mice colonized with bacteria from the lean twin. These differences in body composition were correlated with metabolic differences. Cohousing the mice not only prevented weight gain in mice colonized with bacteria from obese twins, but also caused their metabolic profiles to shift towards the profile of their lean cage mates. These results were dependent on the diet fed to the mice.

Other studies have similarly shown that diet has a strong influence on gut microbial communities. Wu and colleagues [26] demonstrated that gut enterotypes are strongly correlated with long-term dietary patterns. Gut enterotype identity was not affected by short-term dietary changes. Rapid shifts in both gut microbial community structure and gene expression were observed in volunteers who consumed either an animal- or a plant-based diet for five consecutive days [27]. The animal-based diet had a greater impact on the gut microbiome than the plant-based diet and was associated with decreased levels of *Firmicutes*, which metabolize plant polysaccharides, and increased expression of genes for the degradation of polycyclic aromatic hydrocarbons, compounds produced during the charring of meat.

Significant changes in the gut virome were also observed when the host was placed on a defined diet and these diet-induced changes co-varied with changes in the gut bacterial community [28]. Furthermore, the gut virus populations converged in individuals placed on similar diets. In

contrast, another study found the gut virome to be stable over time [29]. The source of the differences observed between these two studies is unknown, however these conflicting results emphasize the need for experimental standardization.

Host genetics has also been shown to influence gut microbial composition and function [30]. Analysis of fecal samples from monozygotic and dizygotic twin pairs identified *Christensenellaceae* as heritable taxa associated with low BMI. Furthermore, the addition of a *Christensenellaceae* species to an obese-associated microbiome reduced weight gain in germ-free mice. The authors suggest that the species not found to be heritable are more heavily influenced by environmental factors, such as diet.

Oral Microbiome

Streptococcus dominates the oral cavity, but other abundant genera include *Veillonella*, *Gamella*, *Rothia*, *Fusobacterium*, and *Neisseria* [31,32]. A recent study that used statistical models to partition human microbiome data into body-site specific community types identified a significant association between gut and oral community types, despite their strong taxonomic differences [33]. One potential explanation for this connection is that oral bacterial populations seed the gut, thereby giving rise to distinct gut community types.

The majority of human oral viruses are bacteriophage, individual-specific, and persist over time [34]. Genome-encoded clustered regularly interspaced short palindromic repeats (CRISPRs) are a form bacterial defense mechanism against mobile genetic elements like bacteriophage and provide a genomic record of phage–bacteria interactions. Streptococcal CRISPR sequences in the oral cavity revealed great diversity within individuals, suggesting that each individual was exposed to unique viral populations [35].

The oral microbiome has been linked to both dental caries (cavities) and periodontitis (gum disease). The complex microbial communities of caries are taxonomically and functionally different from those colonizing healthy oral cavities [36]. In periodontitis, *Porphyromonas gingivalis* is the suspected etiological agent. Small quantities of this bacterium were shown to induce changes in the oral microbiota by exploiting the complement cascade to cause periodontal bone loss [37]. Epidemiological studies have suggested a correlation between periodontitis and atherosclerosis. These two seemingly unrelated diseases may be linked by microbiota, as the types and abundance of bacteria in atherosclerotic plaques correlated with the abundance of those same bacteria in the oral cavity [38]. These studies indicate the potential utility of the microbiome as a clinical biomarker.

Lung Microbiome

Although healthy lungs were once thought to be a sterile environment, recent studies have characterized the lung microbiome and its associations with diseases such as asthma, chronic obstructive pulmonary diseases, and cystic fibrosis (CF). The lung microbiome is especially difficult to study because of its low biomass and the difficulty of sampling only microbiota from the lower respiratory tract without also picking up carryover microbes from the upper respiratory tract. Analysis of six healthy human lungs found that although the lung bacteria were much lower in biomass, they were compositionally similar to bacteria in the upper airways [39].

Infection and bronchiolitis obliterans syndrome are common causes of death after a lung transplant and can be partially attributed to microbial factors. Amplicon-based studies of bacterial and fungal communities have shown that the lungs of transplant subjects are significantly different from healthy subjects in both composition and diversity [40]. Furthermore, the lung microbiome of transplant recipients was less similar to their upper respiratory tract microbial communities and contained lung-enriched bacteria. Longitudinal analysis of lung samples after transplantation also identified significant differences between healthy and transplanted lungs and found that a majority of microbes present were transient colonizers [41].

Lung infection and inflammation is the primary cause of death in patients with CF. As a result, CF lung microbiota have been described at various stages of the disease. One group studied the lung microbiome of three stable and three progressing CF patients for over a decade [42]. They found that the lungs of patients with the progressing disease had decreased microbial diversity, and that antibiotic treatment is a stronger driver of this decrease in diversity than both age and lung function. A more recent study analyzing the daily lung microbiome of four subjects over 25 total days found that bacterial communities remained constant during periods of clinical stability, and microbial shifts were sometimes observed with the onset of CF respiratory exacerbations [43].

Fungal species have also been detected as important players in CF lungs, with *Candida* dominating the relatively stable mycobiome [44]. A metagenomic pilot study analyzing sputum samples from CF lungs identified differences in metabolic profiles of three patients with different responses to antibiotic treatment [45]. Additionally, they identified a reservoir of antibiotic resistance genes that may provide insight into microbial response to treatment.

Urogenital Tract Microbiome

Multiple urogenital diseases, including bacterial vaginosis (BV), yeast infections, sexually transmitted diseases, urinary tract infections, and human

immunodeficiency virus (HIV), have been associated with vaginal microbiota. In reproductive-aged women, vaginal bacterial communities generally fall into one of five groups, four of which are dominated by *Lactobacillus* species. Associated with a greater abundance of anaerobic species and increased bacterial diversity, the fifth group is also linked to higher vaginal pH and Nugent scores, both of which are indicators of BV [15]. A longitudinal study indicated that in cases of recurring BV, antibiotic treatments successfully depleted BV-associated bacteria, but the bacteria returned after the treatment ended [46]. The paper also noted the dynamic nature of vaginal microbial communities, finding that *Gardnerella vaginalis* and *Lactobacillus iners* increase in abundance during menstruation, possibly due to the increased availability of iron from menstrual blood. Another study collected daily samples from 135 women over 10 weeks [47]. Initial analysis revealed that vaginal microbiota associated with asymptomatic BV lacked *Lactobacillus* species and was comprised of strict anaerobes prior to symptomatic BV.

While some vaginal communities frequently fluctuate between several of the five different bacterial profiles, others are more stable [48]. During pregnancy, vaginal communities change as a function of gestational age, increasing in *Lactobacillus* species and decreasing in anaerobic species as pregnancy progresses [49]. No differences in microbiota were observed between women who had spontaneous preterm birth and those who delivered at full term [50].

Microbiota colonizing the male genitourinary tract are not as well studied, however they are known to play an important role in sexually transmitted infections. In a longitudinal study of the coronal sulcus microbiome of 77 uncircumcised compared to 79 circumcised African males, circumcision was shown to decrease both bacterial load and overall diversity [51]. In particular, anaerobic bacteria levels decreased, which the authors hypothesize may contribute to the reduced risk of HIV acquisition in circumcised males.

Skin Microbiome

The skin is home to a variety of microorganisms, including bacteria, fungi, viruses, and mites. Studies utilizing 16S rRNA gene sequencing to characterize skin microbial communities have found that microenvironment has the strongest influence on bacterial community composition. Oily microenvironments (such as the back and face) tend to be less diverse and are predominantly populated by *Actinobacteria*, whereas dry sites (arms and legs) harbor *Proteobacteria* and are typically more diverse [12,13]. Alterations in the composition and diversity of skin bacterial communities have been linked to multiple dermatological conditions. Acne is associated with a particularly virulent strain of *Propionibacterium acnes* [52], and atopic dermatitis is

characterized by increased colonization of *Staphylococcus aureus* and decreased bacterial diversity [53].

Fungi are known to thrive on the skin and have been implicated in disorders such as toenail infections and athlete's foot. Fungi colonizing healthy human skin have been characterized by amplification and sequencing of the 18S rRNA gene and ITS regions. *Malassezia* species are the predominant community members of most sampled sites, except for sites on the feet that were much more fungally diverse [54–56]. *Demodex* mites, which reside in facial sebaceous glands and hair follicles, are known to increase in abundance as we age and may play a role in disorders such as rosacea [57–59].

Until recently, whole-metagenome shotgun sequencing of skin microbiota was impeded by low microbial burden, preventing collection of the large amounts of DNA required for sequencing, and high quantities of human contamination. Advances in technology have enabled whole-metagenome studies of the skin, which emphasize the importance of biogeography in both taxonomic composition and functional potential [60]. This first metagenomic examination of healthy skin also allowed for identification of strain-level variation in the commensals, *Propionibacterium acnes* and *Staphylococcus epidermidis*, as well as reference-independent analysis of previously uncharacterized species.

CONCLUSIONS

Despite the major advances made over the last decade, human microbiome research is still in its infancy and faces many challenges on the road ahead. One of these challenges will be dealing with the massive volume of sequencing data. While increasingly inexpensive DNA sequencing makes generating data relatively easy, the bioinformatics expertise and computational resources required to store, process, and analyze this data are expensive and hard to come by.

Well-designed studies will produce the greatest advances in understanding the human microbiome. Because the human microbiome is an ecosystem, an important step forward will be integrating strategies and findings from ecology and environmental microbiology into human studies. Furthermore, researchers must take care to collect biologically relevant samples with well-annotated metadata to generate meaningful microbiome datasets.

There are still many unanswered questions regarding the role of the microbiome in human health. How are commensal microbiota regulated and maintained? How does the microbiome educate the immune system to distinguish between threatening pathogens and nonthreatening commensals? Can we manipulate the microbiota or the host response to microbiota to treat, or even prevent, disease? New approaches will be crucial in addressing the questions above, and functional studies will be required to move beyond associations of the microbiome with disease to causation.

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