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Genome Medicine

REVIEW

Functional profiling of the gut microbiome in disease-associated inflammation

Daniela Börnigen[1,2], Xochitl C Morgan[1,2], Eric A Franzosa[1,2], Boyu Ren[1], Ramnik J Xavier[2,3], Wendy S Garrett[2,4,5,6] and Curtis Huttenhower[1,2]*

Abstract

The microbial residents of the human gut are a major factor in the development and lifelong maintenance of health. The gut microbiota differs to a large degree from person to person and has an important influence on health and disease due to its interaction with the human immune system. Its overall composition and microbial ecology have been implicated in many autoimmune diseases, and it represents a particularly important area for translational research as a new target for diagnostics and therapeutics in complex inflammatory conditions. Determining the biomolecular mechanisms by which altered microbial communities contribute to human disease will be an important outcome of current functional studies of the human microbiome. In this review, we discuss functional profiling of the human microbiome using metagenomic and metatranscriptomic approaches, focusing on the implications for inflammatory conditions such as inflammatory bowel disease and rheumatoid arthritis. Common themes in gut microbial ecology have emerged among these diverse diseases, but they have not yet been linked to targetable mechanisms such as microbial gene and genome composition, pathway and transcript activity, and metabolism. Combining these microbial activities with host gene, transcript and metabolic information will be necessary to understand how and why these complex interacting systems are altered in disease-associated inflammation.

*Correspondence: chuttenh@hsph.harvard.edu
[1]Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115, USA
Full list of author information is available at the end of the article

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Human microbiome structure and function

The human gut is colonized by a large variety of microbial species that differ among healthy people [1,2]. Owing to the direct links between the human microbiome and the immune system, disruptions of the microbial ecology of the microbiome (dysbioses) have been implicated in many diseases, particularly those involving systemic or localized inflammation (Figure 1) [3-6]. This raises two exciting possibilities for the translation of basic research to clinical practice. The first is the use of the human microbiome as a diagnostic tool to predict disease risk, patient outcomes or response to treatment. The second is the eventual use of the microbiome as a therapeutic target, since microbial composition and metabolic activity are modifiable with relative ease by factors such as diet [7-9], the environment [10] and pharmaceuticals [11]. To realize this potential, however, a deeper understanding of biomolecular activity in these microbial communities will need to be developed by means of functional profiling of the human microbiome.

The gut microbiome has both the greatest microbial density in the human body and is the site at which microbes are most exposed to the immune system. This has led to its implication in a range of autoimmune diseases affecting the gastrointestinal tract [12], such as inflammatory bowel disease [13], colorectal cancer [4], type 1 diabetes [5] and metabolic syndromes [14]. Owing to its extensive interaction with the systemic immune system, however, the gut microbiome also contributes to the activity of the enteric nervous system (neurogastroenterological disorders [15]), extra-intestinal tissues (rheumatoid arthritis [16], allergy and atopy [17]), and the skin (atopic dermatitis [18]). In many of these diseases, genetic and environmental factors are known to play a role, but the biomolecular mechanisms linking microbial communities to disease are still unknown. Further functional profiling by metagenomics, metatranscriptomics and additional modalities will thus be required to understand how and why microbial genes and genome compositions, pathway and transcript activities, and metabolic processes are altered in inflammatory conditions, health and disease.

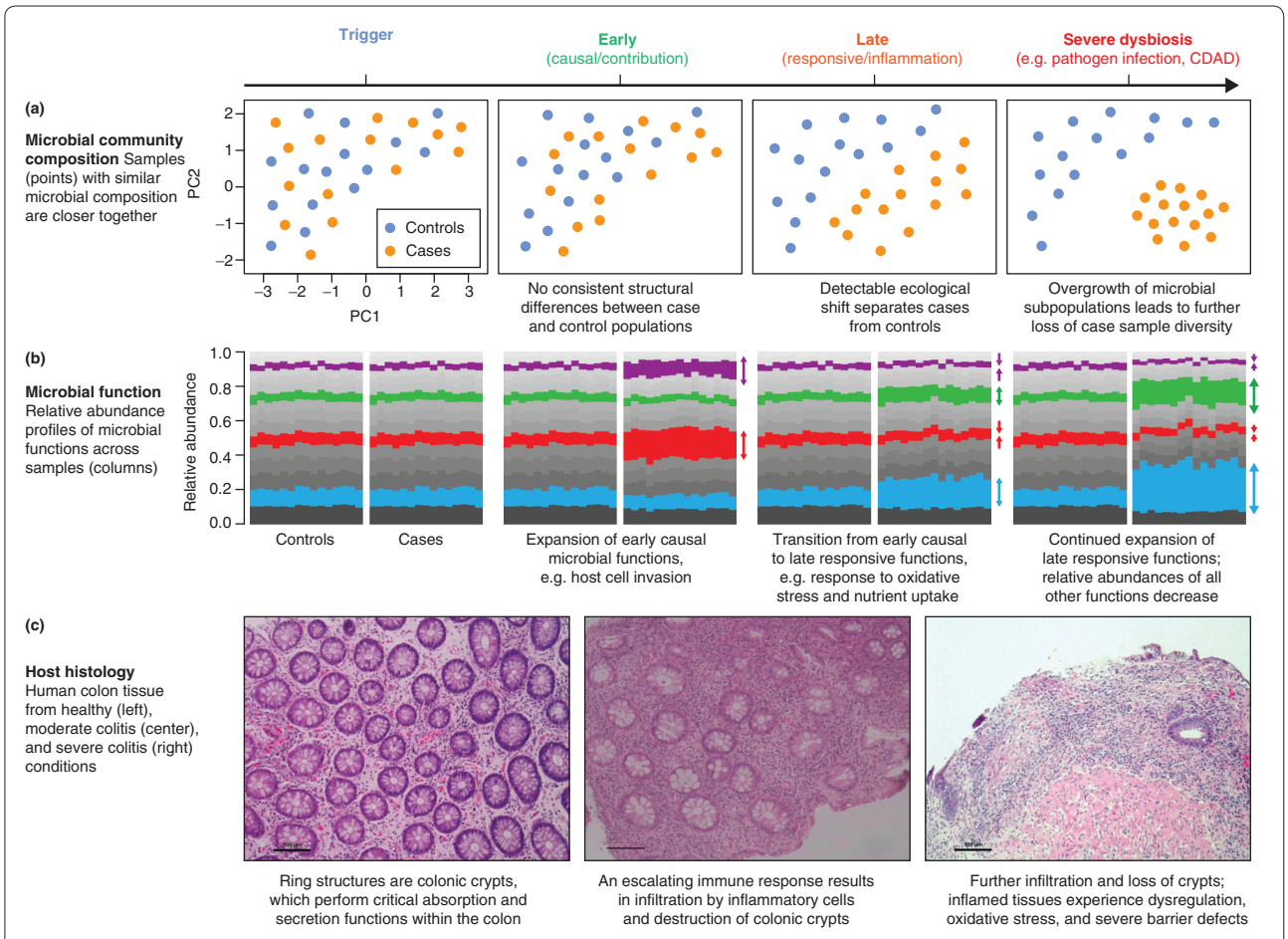

Figure 1. A model of functional dysbiosis in the human gut microbiome during initiation and progression of complex disease. Although many current studies focus on microbial composition shifts that occur subsequent to disease establishment, it is critical to differentiate functional from structural changes in the microbiome and their distinct patterns in early versus late disease. **(a)** An illustration of microbial community structural changes during complex disease progression. Ordinations such as principle coordinate analysis and multidimensional scaling are commonly used to qualitatively visualize microbial community structure among multiple samples (for example, cases and controls). Ordinations project distance measures such as beta diversity among samples into fewer dimensions in such a way that the patterns of greatest change occur on the primary axes (here, *x* and *y*). However, particularly in early disease, case/control status is frequently not among the factors with most influence on inter-subject microbial variation. Conversely, later-stage inflammation can have a very large effect on microbial structure, causing other sources of variation to become visually less apparent. **(b)** Functional profiles of gut microbial communities remain more stable among individuals in health than do microbial profiles, and they can likewise show more concerted differential responses in early and late disease stages. In this illustration, 'case' subject samples exhibit expansion of specific metagenomically encoded functions in their microbial communities during progressive phases of inflammation, as reported in [32]. **(c)** Representative host histology in different phases of the inflammatory response in Crohn's colitis. Colonic crypts (ring structures) are gradually destroyed by immune infiltration as colitis progresses. Images show transverse sections of human colonic mucosa stained with hematoxylin and eosin; 100 μm scale bars are included for reference (images provided by WSG). CDAC, *Clostridium difficile*-associated diarrhea; PC, principal coordinate.

As in single-species systems biology, various meta'omic tools can provide insight into multiple levels of biological regulation in the microbiome, including the detection of microbial organisms, genes, variants, pathways or metabolic functions characterizing the microbial community in an uncultured sample, such as fecal samples or mouth rinses. Microbial ecology has most extensively been studied using targeted 16S rRNA gene sequencing, but this provides only indirect information on molecular activities and will not be the focus of this review. Instead, we will focus on approaches that provide more direct information on biomolecular function within a microbial community, such as metagenomic shotgun sequencing of whole-community DNA to provide a survey of the overall genetic potential of a microbiome. Transcriptional activity can likewise be assayed by metatranscriptomic cDNA sequencing to identify regulatory activity occurring rapidly in response to changes in environment. Whole-community metaproteomics and metabolomics are currently less common, but each again captures

further downstream aspects of both microbial and host molecular activity [19]. In this review, we discuss functional profiling of the human gut microbiome using metagenomics and metatranscriptomics in inflammatory diseases to gain insight into the microbial species, pathways and metabolites, as well as host genes, transcripts and pathways that are altered during chronic inflammatory conditions.

The gut microbiome

Humans are born almost sterile, but during birth and early development they are rapidly and dynamically colonized by microbes throughout the body [20]. These reside primarily in the gut and include bacteria, viruses and, to a lesser degree, archaea and eukaryotic microbes [1,21]. The number of microbial genes involved in establishing and maintaining the community's ecology is immense, totaling 5,000,000 or more [1,21]. This genetic repertoire interacts with that of the host and with environmental factors to create and maintain a cellular system with a metabolic and regulatory capacity comparable to that of complex human tissues [22]. Indeed, in the absence of microbes, neither host gut physiology nor the immune system develop normally [23]. The distribution of microbes throughout the gut is highly structured and dedicated to a variety of biological functions (Box 1).

Inflammation seems to exert effects to which the gut microbiota is particularly sensitive, and studies with the mucosal disruptant dextran sodium sulfate, which elicits colonic inflammation in wild-type mice, have demonstrated that inflammation affects the microbiota [24]. Inflammation results in a cascade of cellular and molecular effectors that can be directly bactericidal or generate substantial environmental stress for a microbial community. In retrospect, it is intuitive that inflammatory bowel disease, celiac disease, rheumatoid arthritis and other chronic inflammatory conditions represent one of the largest families of known microbiome-perturbing human diseases. The additional roles of symbiotic microbial stimulation of innate and adaptive immunity in the gut and training of systemic immunity are much less well understood, but they undoubtedly function in the triggering, maintenance and remission of inflammatory conditions.

Gut microbes in chronic inflammatory and autoimmune disease

Inflammatory bowel diseases

It has long been accepted that the inflammatory bowel diseases - Crohn's disease and ulcerative colitis - occur in conjunction with a dysregulated host immune response to the normal gut microbiome, and include strong genetic components [25]. Recent genome-wide association studies (GWAS) have been very successful in revealing the responsible human genes [3]. However, disease-causing functional defects have only been explained for a few genes (for example, *NOD2*, *IL23R*), which are also intimately tied to the microbiome by crucial roles in controlling microbial infiltration in the gut [26].

Assessing microbial functional responses in tandem with additional human genetic risk variants may help to better identify their functional consequences *in vivo*. For example, low plasma levels of vitamin D (which inhibit pro-inflammatory p38 kinase signaling [27], affect innate immune function [28] and may promote development of T regulatory cells [29]) are associated with an increased risk of Crohn's disease [25]. The gut microbiome can alter both the distribution and expression of vitamin D receptors in the gut [30], suggesting that natural microbial variation is a contributing influence on vitamin D metabolism. Dietary fiber, which is metabolized by the gut microbiota to anti-inflammatory short-chain fatty acids (SCFAs), has been found to be protective against inflammatory bowel disease in some studies [25]. Both low vitamin D levels and dietary fiber intake represent a host-microbe metabolic interaction that potentially affects inflammatory bowel disease onset or activity.

The widely observed reduction in diversity of gut microbial ecology in inflammatory bowel disease [31,32] may be a consequence of more specific functional changes. For example, increased levels of Enterobacteriaceae may be the result of differences in this taxon's ability to tolerate inflammation-associated redox stress [33], and SCFA-producing Clostridia may be outcompeted by more generalist or opportunistic Enterobacteriaceae, resulting in decreased microbial SCFA production and contributing to a self-reinforcing pro-inflammatory state incorporating both host immune and microbial metabolic components [32]. Such host-microbe and microbe-microbe regulatory feedback loops provide novel potential targets for pharmaceutical and probiotic development, since both the introduction of specific microbes [34] and the disruption of individual microbial processes such as redox metabolism [35] have the potential to mitigate inflammatory processes in the gut.

Rheumatoid arthritis

Rheumatoid arthritis is a systemic inflammatory disorder that manifests as an inflammatory response to synovial tissues. Recent studies have associated the oral microbial community with the disease, with rheumatoid arthritis patients having a higher prevalence of periodontitis and tooth loss [36]. In the gut, several studies have shown that diet can have a therapeutic effect on rheumatoid arthritis in conjunction with decreased inflammation [37]. Some initial studies have been performed to gain

Box 1. Influences on gut microbiota structure and function

Overall, the gut microbiota comprises residents of the stomach, small intestine and large intestine [98]. However, owing to pH stress and bile salt toxicity, microbial biomass is very low before the ileum. The vast majority (more than 99%) of the gut microbiome is found in the colon, where (among other activities) it breaks down indigestible fibers and ferments them into SCFAs. These are an essential fuel for colonocytes, maintain colon health, and provide approximately 10% of dietary energy from a Western diet. The colon contains by far the most microbial cells in a typical human body, dominated by the Bacteroidetes and Firmicutes phyla, with lesser but still important consortia of Proteobacteria, Actinobacteria, other bacterial clades, and Archaea. Both stool samples and biopsies have been extensively investigated as representatives of the colonic mucosal and luminal communities; comparable taxa are detected regardless of sample origin but in different relative abundances [32], reflecting microbial dispersion and niche specialization.

The composition of the gut microbiome is influenced by both genetics and environmental factors such as diet [6] and age [32]. For example, monozygotic twins were found to be concordant for carriage of *Methanobrevibacter smithii* at a much higher rate than dizygotic twins (74% versus 14%) [99], although it is difficult to distinguish this effect from that of co-habitation [100]. The dynamics of microbial responses to perturbations are particularly critical to consider during early life and beyond [101-103], and longitudinal sampling of complex communities is an active area of research [104].

The gut microbiota seems to be resilient to short-term dietary change, as even profound shifts in diet (such as from a high-fat/high-protein to a low-fat/low-protein diet) tend to quickly change the relative abundance of microbial taxa but not their presence or absence [105,106]. However, humans from different environments (with correspondingly different long-term diets) do maintain distinct microbiomes. For instance, a recent study compared healthy children from Italy and Burkina Faso - the latter of whom consumed a much higher-fiber diet and very little meat. The microbiota of the children from Burkina Faso was much more phylogenetically diverse and had approximately fourfold higher fecal butyrate concentrations, indicating microbial communities more efficient at extracting nutrients from fiber than those of the Italian children [9]. Interestingly, abundant Enterobacteriaceae, decreased intestinal biodiversity and decreased intestinal levels of butyrate are all associated with inflammatory bowel disease, which is much less common in non-Western countries [32,107].

Non-dietary perturbations, such as antibiotics and other pharmaceuticals, also profoundly affect both host and microbiome. A study of mice given long-term, sub-therapeutic doses of antibiotics found large shifts in the microbial community that led to an increase in SCFAs. These in turn contributed to a corresponding increase in host adiposity, although the mice did not eat more [11]. Higher doses of antibiotics disrupt even more of a host's endogenous microbial community, potentially leaving human patients susceptible to opportunistic infections such as *Clostridium difficile*, which can precipitate a vicious cycle of microbial community disruption [108].

more insight into the functional consequences of changes in the intestinal microbiome and their impact on inflammation and immune responses [38]. For example, *Lactobacillus bifidus* was shown to trigger arthritis in a mouse model (IL-1-receptor-antagonist-deficient mice), which was specifically driven by an imbalance in T-cell homeostasis and mediated through Toll-like receptor (TLR2 and TLR4) signaling [39]. In this mouse model, which is known to spontaneously develop an autoimmune T-cell-mediated arthritis due to excessive interleukin (IL)-1 signaling [40], TLR2 and TLR4 were involved in the expression of autoimmune arthritis. Specifically, TLR2 slowed the progression of arthritis by controlling the function of T regulatory cells and regulating interferon (IFN)-γ-producing T helper 1 (Th1) cells, and TLR4 increased the severity of the disease by modulating the T helper 17 (Th17)-cell population and IL-17 production. Another study found that autoimmune arthritis was strongly attenuated in a K/BxN mouse model under germ-free conditions, accompanied by reductions in serum autoantibody titers, splenic autoantibody-secreting cells, germinal centers, and the splenic Th17 cell population [16]. The authors observed that their mouse model had a dearth of IL-17-producing T cells, which could be reversed by introducing segmented filamentous bacteria into the gut of germ-free-housed mice, provoking rapid onset of the disease. Taken together, these studies suggest that both the oral and gut microbiome may trigger rheumatoid arthritis by inciting local inflammatory responses in the host, but do not elucidate what mechanism might be at play in systematizing this response or targeting it to the synovium.

Allergy and atopy

The role of the microbiome in allergy and asthma is the foundation of the widely recognized 'hygiene hypothesis', which states that a combination of improved hygiene, frequent use of antibiotics, or vaccinations may lead to reduced bacterial and viral infections, and to an altered immune system that responds inappropriately to innocuous substances [41]. Recent functional studies of symbiotic microbes in these conditions have been primarily epidemiological, and have targeted environmental risk and preventive factors such as lifestyle, infections and diet [42]. Perhaps the strongest results have arisen from investigations of early life exposures to environmental microbes, establishing a link between home allergen levels, lymphocyte proliferation and wheeze in children at high risk for asthma [43]. In several such studies, early life 'urban' allergen exposures have been associated with later asthma and allergy risk, whereas environmental microbial exposures have generally been protective.

Although the skin microbiome has been the main habitat investigated for atopic skin diseases [44], the gut microbiome's extensive interaction with the immune system has also led to it being indirectly linked with atopic manifestations and sensitization [17], and directly with atopic dermatitis in infants [18]. These studies revealed several microbes, such as *Bifidobacterium, Staphylococcus, Escherichia coli* and *Clostridium difficile*, that were associated with a higher risk of atopic dermatitis in children, albeit not yet with a functional explanation. Interestingly, maternal intestinal and vaginal *Bifidobacteria*, one of the most important groups of early life microbes, have an incompletely characterized influence on the establishment of *Bifidobacteria* during infant gut colonization [45,46]. A recent cohort study investigating the influence of maternal gut microbiota on wheezing in early childhood found an association between higher total maternal aerobes and Enterococci with increased risk of infant wheeze. A core concept in the hygiene hypothesis is that microbial exposures in early life may 'tune' immune responses and ensure host-immune homeostasis over the human lifetime. $CD4^+$ T-helper cell and innate lymphoid cell populations and their effectors may be one component of this [41], and early life responses to specific microbial clades may participate in or trigger activation of these immune responses.

Disorders of the brain-gut axis

Bidirectional communication between the brain and the gut has long been recognized [47], and has become the focus of increasing research on the 'microbiome-gut-brain axis' [15]. Just as the microbiome affects the physical development of the gut, it can also influence mammalian brain development [48]. During adult life in rodents and insects, the composition of the gut microbiome has been found to influence a variety of complex behavioral traits, including anxiety [49] and mating preferences [50]. Potential mechanisms have been identified for associations between stress-related disorders (such as anxiety and depression) and the gut microbiome in laboratory mice [51]. In this study, for example, GABA transcriptional activity was found to be stimulated via the vagus nerve by *Lactobacillus rhamnosus.* Preliminary results in other systems suggest that early life stress may result in persistent changes to the gut microbiome, which in turn can contribute to symptoms resembling those seen in human psychiatric disorders [52]. Combining this with microbial metabolic responses to host hormones, as discussed earlier, and ongoing studies of the microbiome in weight loss [53], it seems likely that microbial products will be found to have a role in hunger signaling and host metabolic regulation as well.

One of the clearest links between the gut microbiota and neural disorders is in multiple sclerosis, by way of an autoimmune reaction. Multiple sclerosis is a chronic inflammatory disease of the nervous system notable for its T-cell responses to components of nerve fiber myelin sheaths [54]. Several loci associated with multiple sclerosis by GWAS are at or near genes with roles in T-cell-mediated immunity, and gut-resident viruses have been suggested as initial triggers of this autoimmune response [55]. *Mycobacteria* and their cell extracts have been implicated in a surprisingly wide range of immunoregulatory processes, and in particular are capable of suppressing central nervous system autoimmunity in the encephalomyelitis mouse model by altering T-cell migration, suppressing the IL-17 response, and inducing apoptosis of activated T cells [56]. The Bacillus Calmette-Guérin vaccination, which is prepared from an attenuated *Mycobacterium bovis* strain, was associated with decreased multiple sclerosis flare severity [57], and bacterial lipopolysaccharide was also shown to protect mice from central nervous system inflammation, by promoting the growth of neuroprotective T regulatory cells [58]. These findings are suggestive of host responses that may be triggered by metabolic or cellular components of the endogenous microbiota, but to date no specific microbial molecules have been identified as causative.

Functional profiling of the microbiome

The roles of the gut microbiota in inflammatory conditions have begun to be unraveled by functional profiling, or the assessment of host and microbial biomolecular activity in tandem with microbial community structure. Assessment using nucleotide sequencing is typically a two-step process. First, genes, proteins, or protein families in the community (and sometimes in the host) are quantified; second, individual gene families are merged into higher-level pathways, such as metabolic pathways and functional modules. There are several experimental assays and computational methods designed to accomplish these steps, and the choice of method depends on the nature of the microbial community under investigation, as well as the sequencing data available to describe it. Considerations in the choice and application of analysis methods are briefly summarized here and reviewed in depth elsewhere [59].

Functional information can be gleaned from almost any whole-community experimental data type; broadly, 16S rRNA gene sequencing [60], metagenomic or metatranscriptomic shotgun sequencing [61], metaproteomics [62] and/or metabolomics [63]. Host genetics and/or gene expression can also be considered, and host products are typically included in metabolite, protein, and sometimes RNA datasets. Most initial data acquisition and informatics are the same for whole-community studies as for single-organism studies, except that first, samples must be handled with care in order to preserve, lyse and

extract a wide range of microbial organisms without bias [64,65], and second, computational interpretation in the presence of multiple underlying genomes can be challenging. Metagenomics and metatranscriptomics (together meta'omics) currently represent the most cost-effective balance between functional and structural data.

Meta'omic data are typically interpreted by first assigning sequences to gene families [59]. This can be done by assembling short reads into contigs and identifying protein-coding sequences (CDSs, using approaches comparable to annotating single genomes), or reads can be assigned directly to gene or protein families. The latter approach may either map reads to annotated CDSs in microbial reference genomes, or they may be searched against databases of characterized protein families. In either case, the result is a profile of microbial gene families present in a community and their relative metagenomic or metatranscriptomic abundances. Gene family identification systems amenable to this process include the KEGG Orthology, COG [66], NOG [67], Pfam [68] and UniRef [69]. Each of these satisfy the necessary criterion of a database of systematically identified protein sequence groups, with each individual sequence representing a family member within an individual organism. For communities described by 16S sequencing data rather than shotgun data, direct inferences cannot be made about the CDSs present in the community, and instead one must rely on inferring the presence of particular functions by associating 16S sequences with gene content from annotated reference genomes [70].

Individual gene families profiled in any of these ways can then be hierarchically organized for ease of interpretation, just as individual microbes are organized taxonomically or phylogenetically. This is a critical step, as catalogs typically describe anywhere from tens of thousands to millions of gene families in the gut microbiome, but no pathway catalogs exist so far that are specifically appropriate to microbial communities. Databases developed for single organisms do help this effort, such as KEGG [71], MetaCyc [72] and SEED [73]. Integrated bioinformatics pipelines have been developed to streamline the multi-step processes described above, including IMG/M [74], MG-RAST [75], MEGAN [76] and HUMAnN [77]. Each of these procedures for functional sequence analysis provides researchers with an option for translating raw meta'omic sequence data into a more easily interpreted profile of the functional potential of a microbial community.

Functional profiling of the microbiome can be a time-consuming process for samples characterized by a large amount of sequence data, as mapping these sequences to a gene family or reference genome databases is computationally intensive. However, once this mapping step is completed, subsequent analyses (such as merging gene families into pathways) proceed quickly, and can rapidly produce clinically relevant results. For example, screening an individual's gut microbiome profile or the microbiome of an infection for known antibiotic-resistance genes [78] can illuminate the resistance potential of a microbial community, informing treatment options. In addition, profiling the enzymatic composition of a patient's gut microbiome may indicate how the cells in that community will interact with pharmaceutical interventions; for example, whether they will metabolize them to inactive or potentially hazardous forms [79,80]. Last but not least, the early stages of diseases with microbial involvement are often not associated with dramatic changes in microbial community composition. However, the community's functional profile may reveal disease-linked perturbations at a much earlier stage of disease progression, leading to the possibility of using functional profiling to generate biomarkers for disease diagnosis (Figure 1).

Functional profiling case studies in health and disease

A comprehensive example of functional interpretation of the human microbiome can be found in the Human Microbiome Project (HMP), which provides both experimental protocols [81] and computational pipelines [1] for assessing the gut and other body sites. The results of the HMP provide a useful reference for gut microbiome function in health, providing a variety of public data from a cohort of 242 individuals, including both 16S rRNA gene and metagenomic shotgun sequencing [82] for the analysis of microbial communities and functional profiles. All subjects were clinically screened to ensure a high level of health [83], and these data represent a powerful set of tools for meta-analysis alongside new disease-focused studies [8]. Within the study itself, it was shown that metagenomic carriage of metabolic pathways was stable among individuals even when microbial composition was not, and, of the recorded metadata, racial/ethnic background showed one of the strongest associations between clinical metadata and either pathways or microbes. The magnitude of this effect was larger than that of age in this cohort, in which diet was not deeply characterized; these two factors have been associated independently with microbiome composition in other studies [6,32]. On the basis of these data [1], 118 stool samples from healthy individuals were profiled, highlighting a core gut microbiome that consists of stable pathways that are present despite variation in microbial abundances (Figure 2). These findings thus specify the range of normal structural and functional configurations in the microbial communities of a healthy Western population, and they provide a framework for future studies of human microbiome function.

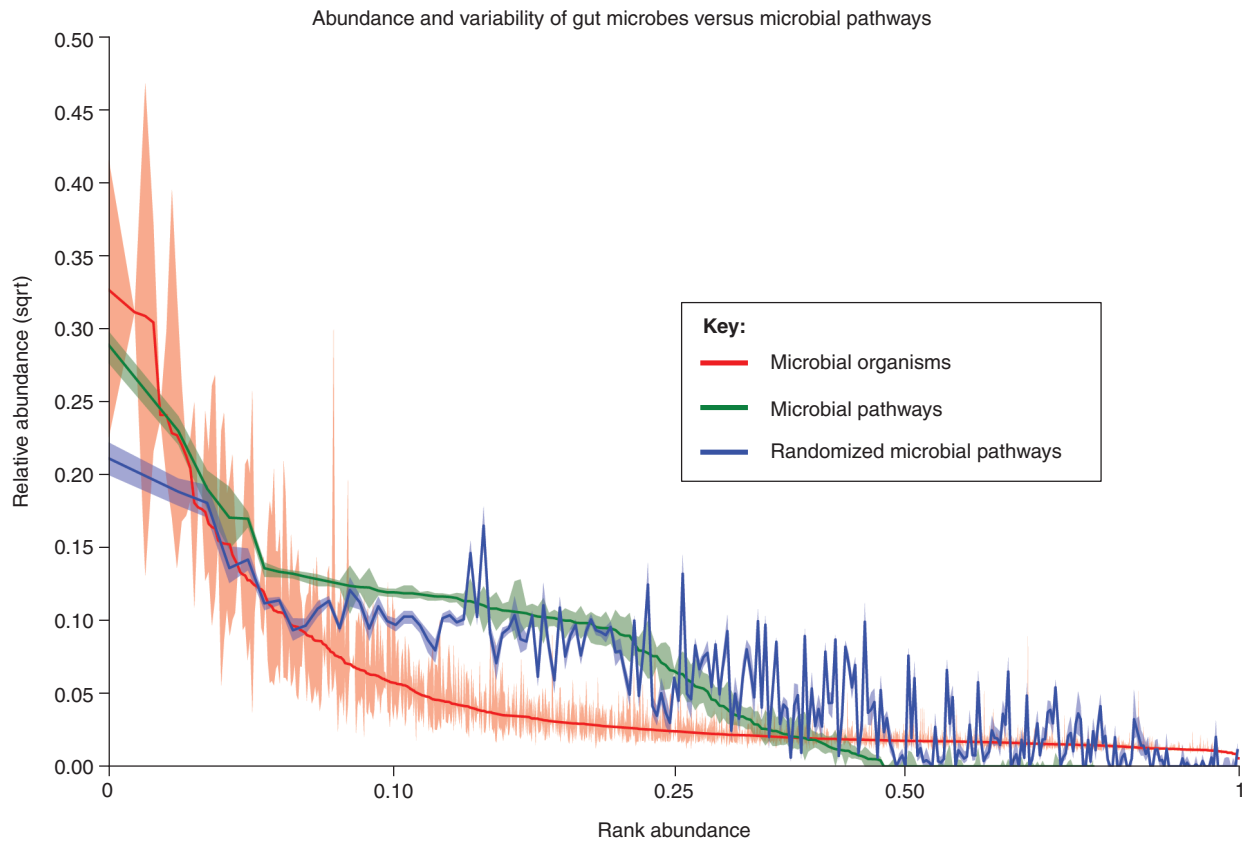

Figure 2. The core gut microbiome consists of stable pathways present despite variation in microbial abundances. Profiles of 118 stool samples from healthy individuals, showing the relative abundances of microbial organisms (red), inferred microbial pathways [70] (green), and microbial pathways after randomization (blue, all data from [1]). All relative abundances are shown as median and interquartile range across all samples (*y*-axis) ranked by median (*x*-axis) and square-root (sqrt) scaled for visualization. As illustrated by several studies (for example, [1,89]), a stable distribution of habitat-adapted microbial pathways is maintained on a functional level (green) rather than on a phylogenetic level (red). Random assignment of microbes to samples followed by re-inference of functional potential (blue) results in a metagenome that is more variable, more skewed, and of distinct composition from that in the observed 'core' of gut microbiome functions.

Maintaining community function in health

A companion project within the HMP characterized the function and composition of the digestive tract sites assayed by the project, comprising ten distinct body habitats (in the mouth, oropharynx and colon [84]). These microbial habitats formed four related areas of microbial community configurations: tooth hard surfaces; two distinct types of oral soft tissues and environments (cheek/gingiva/palate versus throat/tonsils/tongue/saliva); and the gut, as represented by stool samples. Metabolic profiling revealed a set of 'core' digestive tract pathways enriched in abundance throughout these communities, including pathways involved in the acquisition and export of metals, and cytochrome c heme lyase, an enzyme involved in porphyrin and chlorophyll metabolism. These pathways were unique in that most genes encoding exporters needed for heme tolerance (such as MtrCDE and HrtAB) were not significantly associated with specific organisms in the study, and the gene encoding hemerythrin (responsible for oxygen transport in specific organisms) was detected at multiple body sites but was highly enriched in stool. Conversely, each of the four habitats was also enriched in more niche-specific metabolism, such as the β-glucosidase pathway in stool (involved in cellulose breakdown to β-D-glucose), glycolysis and pyruvate generation by glucose metabolism, and several pathways for ammonia utilization (such as the urea cycle and ornithine biosynthesis), as well as methane production. The oral cavity, conversely, showed enrichment for energy harvest pathways reliant on simple sugars (mannose, fructose, trehalose, and so on) and in many cases oxidative metabolism (especially when contrasting, for instance, supra- versus sub-gingival plaques). While in many cases these pathways were broadly phylogenetically distributed among diverse clades, others were tightly tied to just a few microbes (for example, hydrogen sulfide production by the *Veillonella*, *Selenomonas* and *Prevotella* genera).

Perturbations of gut microbiome function in disease

Both protective immune responses and dysregulation during autoimmunity are activated by signals initiated by innate immunity and driven by microbial stimuli [85]. Many studies have thus investigated microbial function in the gut microbiome in these diverse autoimmune diseases, with several recent examples including inflammatory bowel disease [31,32,86,87], rheumatoid arthritis [36], and allergy and atopy [18,42,88] (as described earlier), as well as metabolic syndrome [89,90] and neurological disorders [15,47-49]. As a T-cell-mediated metabolic disease, type 1 diabetes is another prime candidate for involvement of the gut microbiota [5,10,91]. Much current work on the function of the gut microbiome in type 1 diabetes relies on the non-obese diabetic (NOD) mouse model [92,93], a well-known system in which immune-mediated pancreatic β-cell destruction is triggered by gut microbial colonization [93]. Table 1 summarizes these and additional relationships among microbial organisms and pathways, as well as human genes and pathways, that are known to be involved in these inflammatory conditions.

A recent study investigated the human gut microbiome in malnourished children, specifically in kwashiorkor, a childhood protein-deficiency disease [6]. The authors first identified nine well-nourished twin pairs and 13 twin pairs who became discordant for kwashiorkor over the study period of 18 months. Fecal metagenomics showed age to be the greatest determining factor in gut microbial variation in healthy children, along with family membership and diet. Healthy children showed a steady progression toward a consistent microbiome common to older children, which did not take place in subjects suffering from kwashiorkor. Surprisingly, though, no significant changes in the functional composition of the gut microbiome occurred after treatment. Instead, several metabolic pathways were already significantly different in discordant twin pairs at the time of diagnosis, such as α-mannosidase, an enzyme involved in glycan biosynthetic reactions and catabolism, and protein-N(PI)-phosphohistidine-sugar phosphotransferase, an enzyme involved in sugar catalysis. Microbial pathways including β-glucosidase and β-galactosidase activity remained significantly different in discordant twin pairs a month after cessation of treatment, suggesting substantial stability of changes induced in the microbiome by extreme environmental effects.

The authors subsequently transplanted fecal microbial communities from discordant twin pairs into gnotobiotic mice to identify features of the microbial community structure, metabolism, and host-microbial co-metabolism associated with donor health status and diet. In this mouse model, they found increased levels of the majority of SCFAs, carbohydrates, amino acids, nucleotides and lipid metabolism in cecal and fecal samples in mice receiving dietary treatment, whereas levels of several di- and monosaccharides (maltose, gentibiose and tagatose) were decreased. When the mice (both healthy and with kwashiorkor) started treatment, the levels of nine amino acids (valine, leucine, isoleucine, methionine, phenylalanine, threonine, alanine, tyrosine and serine) rapidly increased. After returning to a normal diet, most of these amino acids remained higher in healthy mice than before therapy, but in the kwashiorkor group, these values fell to pre-treatment levels. This suggests that the stable alteration of the microbiome specifically influences its future ability to maintain healthy host-microbe metabolic interactions. Additionally, the authors found that the urinary excretion of the tricarboxylic acid (TCA) cycle intermediates 2-oxoglutarate, citrate, succinate and fumarate were closely coupled in healthy mice but decoupled in kwashiorkor. This disruption of the TCA cycle resulted in an increased succinate-to-fumarate ratio, possibly from inhibition or depletion of succinate. The authors suggested that this might be the result of kwashiorkor-specific generation of chemical products selectively inhibiting TCA cycle enzymes, making energy metabolism an even more extreme challenge for children with kwashiorkor exposed to a micro- and macronutrient-deficient, low-calorie diet.

This result provides an informative case study in that it traces a microbiome-linked human disease from population-level epidemiology through a validated molecular mechanism to potential diet-driven treatment. Although the resulting human health recommendations remain to be validated, it provides an example of a case in which the three major elements of functional gut microbiome profiling were used to derive an actionable result: broad sequencing-based surveys of the gut microbiome in a human population, deep sequencing and functional assays in a gnotobiotic mouse model to detail metabolic mechanisms, and subsequent follow-up profiling of a potential treatment in humans. Even in this relatively straightforward example, interplay between environmental factors, diet, variable microbial composition and age must all be taken into account to understand host-microbiome interactions in human disease.

Functional profiling in the future: a perspective

The past five years have seen an explosion of human microbiome studies, most of which have associated changes in microbial ecology with human health or the environment [1,7,8,81,89,94]. In almost no cases, though, do we yet know the causality, mechanism or relevance of these microbial shifts. In the few instances where specific biomolecular interactions have been addressed [95,96], they have begun to effectively indicate routes by which

Table 1. Published relationships among microbial clades, pathways, and human genes and pathways involved in autoimmune diseases

Disease	Microbes	Microbial pathways	Host pathways	Representative host genes	References
Inflammatory bowel disease	Enterobacteriaceae, *Roseburia*, Ruminococcaceae	Glutathione metabolism and transport, riboflavin metabolism, short-chain fatty acid metabolism	Autophagy, Th17, T-cell responses and cytokines, JAK-STAT, NF-kB, microbial sensing	*ATG16L1*, *CARD9*, *DUOX2*, *IL10*, *IL23R*, *IRGM*, *FUT2*, *MHC*, *NCF4*, *NOD2*	[3,25,31,32, 86,87, 107,109]
Type 1 diabetes	*Akkermansia*, Bacteroidales, Lactobacillaceae	Amino acid metabolism, secondary metabolite biosynthesis, butyrate production, carbohydrate metabolism, glycan biosynthesis and metabolism, lactate production, lipid metabolism, nucleotide metabolism	Innate immune signaling, mucin, MyD88, Toll-like receptors	*CTLA4*, *IL2RA*, *IFIH1*, *INS*, *MYD88*, *MHC*, *PTPN22*, *TLR*	[5,91, 110-116]
Rheumatoid arthritis	*Bacteroides fragilis*, *Bacteroides vulgatus*, *Clostridium coccoides*, *Eubacterium rectale*, *Klebsiella*, *Lactobacillus*, *Porphyromonas*, *Prevotella*, SFB	-	CD40, IL-2, NF-kB activation, SAA or CCL5 signaling, T-cell activation and response	*CD40*, *CCL21*, *HLA-DRB1*, *IL2*, *IL17*, *IFNG*, *KIF5A*, *MHC*, *TLR2*, *TLR4*, *TNF*, *TNFAIP3*, *PRKCQ*	[38,117, 118]
Multiple sclerosis	Epstein-Barr virus, *Mycobacteria*	Vitamin D metabolism	Vitamin D, $CD4^+$ T cells	*DRB1*, *IL2*, *IL7*, *HLA*, *MHC*	[56,57, 119-121]
Allergy, atopy	Aerobes, *Bifidobacteria*, Enterococci, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium difficile*	-	IgE antibody regulation, vitamin D	*ADAM33*, *ADRB2*, *CD14TNF*, *IL10*, *IL4*, *IL13*, *IL4RA*, *IFNG*, *FLG*, *FCER1B*, *HLA-DRB1*, *HLA-DQB1*, *MHC*	[17,18,45, 46,122-128]

SFV, segmented filamentous bacteria.

microbiome shifts can be diagnostically interpreted or therapeutically targeted.

The recent history of cancer genomics suggests an important parallel for the next steps in translating human microbiome studies to the clinic. Early descriptive work in cancer functional profiling proved difficult to interpret or act on, and only a detailed understanding of molecular activities within the complex, mixed cellular population of a tumor allowed the creation of effective targeted therapies. The same necessity for deep biomolecular characterization is likely to hold true in the complex, mixed cellular population of a microbial community.

To this end, microbiome studies now have experimental design options that allow the integration of both descriptive and functional assays, as well as more convenient and holistic computational interpretation. Researchers must take advantage of these to test specific, well-controlled hypotheses in human subjects, model systems (mouse, zebrafish and others [97]), and *in vitro* (for example, cell culture and functional screens). Epithelial cell lines and synthetic systems (such as co-culture, microfluidics and organoids) represent an intriguing untapped resource. Conversely, large population surveys relating microbial structure to function (transcripts and proteins) have also not yet been performed and will establish an important baseline, building on references such as the HMP and MetaHIT.

Analytical limitations remain to be overcome in the translation of functional microbiome surveys to human health, both in our understanding of basic biological mechanisms and in our ability to leverage these data for clinical use. The former will require substantially more comprehensive integrative models of multi-microbe and host-microbe signaling, metabolic interaction, immunology and ecology than are available today. The latter, again not unlike personalized cancer therapies, in many cases still needs high-reliability, large-effect-size predictors of disease risk and outcome in humans to be clinically actionable. To address these challenges, carefully designed pre-clinical experimental systems are needed, particularly longitudinal prospective and outcome-based studies in human populations to detail the dynamics of microbial function during disease onset, treatment and resolution. In the future, in combination with novel computational models and the continued incorporation of sequencing technologies into the clinic, such investigations will lead us towards a deeper understanding of microbial communities and their functional roles in health, inflammation and disease.

Abbreviations
CDS, coding sequence; GWAS, genome-wide association studies; HMP, Human Microbiome Project; IFN, interferon; IL, interleukin; NOD, non-obese diabetic; SCFAs, short-chain fatty acids; TCA, tricarboxylic acid.

Competing interests
The authors declare that they have no competing interests.

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Author details
[1]Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115, USA. [2]The Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA. [3]Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02115, USA. [4]Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA. [5]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA. [6]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA.

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