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Review

# The Human Gut Microbiome: From Association to Modulation

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**Our understanding of the human gut microbiome continues to evolve at a rapid pace, but practical application of thisknowledge is still in its infancy. This review discusses the type of studies that will be essential for translating microbiome research into targeted modulations with dedicated benefits for the human host.**

The human microbiota is the focus of one of the most dynamic research fields of our time, and most efforts are directed at the gastrointestinal tract, which harbors most of our microbes. In the past decade, our understanding of the organisms inhabiting our gut, their functionality, and their roles in human health and disease has advanced greatly, facilitated by fast technological development. Research on the gut microbiome is progressing through several steps that mirror those of other fields on other biological systems: (1) compilation of parts lists, (2) association of the system or its components to external factors, (3) establishment of functional knowledge, and (4) translation of that knowledge into applications. For the gut microbiome, this is reflected in the following developments.

(1) The compilation of gut microbiome ''parts lists'' has been in full swing for more than a decade and is now almost complete, for the dominating prokaryotic domains, and at the resolution of genera and species. Several studies established the baseline structure and function of the microbiome—that is, lists of species and their genes—with major contributions from two large collaborative efforts of the MetaHIT (Li et al., 2014; Qin et al., 2010) and Human Microbiome Project (HMP) (Nelson et al., 2010; Human Microbiome Project Consortium, 2012) consortia. Although novel diversity continues to be discovered, in particular at subspecies and strain level, and although a large fraction of microbial genes remains functionally uncharacterized, the census of the most dominant lineages in industrialized populations is arguably approaching completion (e.g., Zhou et al., 2018).

(2) Using these parts list, a wealth of studies has probed for associations of the gut microbiome to disease, host factors, or the wider environment. As coverage and scope increase, these have been collectively referred to as metagenome-wide association studies (MWASs) (Wang and Jia, 2016), in analogy to genome-wide association studies (GWASs). Recently, MWASs have reached population level, as large-scale cross-sectional studies (Falony et al., 2016; Zhernakova et al., 2016) started to provide an integrated view of the relative impact of various host and environmental factors on microbiome composition (see Box 1).

(3) Associations identified by MWASs are observational, can be indirect or confounded by underlying factors, and do not easily translate into causal links. However, for a functional understanding of a complex system such as the gut microbiome, it is necessary to connect parts lists (1D) to networks (2D) in a spatial (3D) and temporal (4D) context (Raes and Bork, 2008), and this requires adapted concepts (see below) and methodological approaches (see Box 2). Although the study of the microbiome's taxa interaction networks (2D), i.e., the interactions between its parts (1D), is ongoing, the inference of species interactions from cross-sectional data remains challenging (Weiss et al., 2016). This is in part because current readouts (fecal samples) are still mostly non-quantitative (Vandeputte et al., 2017c) and poorly reflect the spatial organization of the intestinal tract (3D). Moreover, interactions and microbiome function are dynamic, and in consequence, individual gut microbes and entire communities need to be studied in the context of time (4D), though longitudinal studies so far remain scarce. Perturbation experiments, in particular, enable the study of a system's dynamics, both at the level of individual parts and the entire system. An increasing number of intervention studies adds to our functional understanding of the gut microbiome, but it remains unclear whether observed responses are generic, stratified, or indeed personal (see Box 3).

(4) Finally, knowledge on the microbiome begins to be translated into applications, and this entails a move from perturbation to modulation. Perturbations may trigger microbiome shifts, but most of these are unforeseeable or not intended. Targeted microbiome modulation, preferably with predictable outcome in terms of response and without side effects, will require a functional understanding of the system, but also an accepted operational definition of desired ''healthy'' endpoints, both intrinsically and in relation to the host. Given these, we expect microbiome modulation to become a major translational asset in the near future, establishing the microbiome as a versatile therapeutic target.

In this review, we focus on active and emerging areas in the context of the above (see Figure 1), and especially on studies

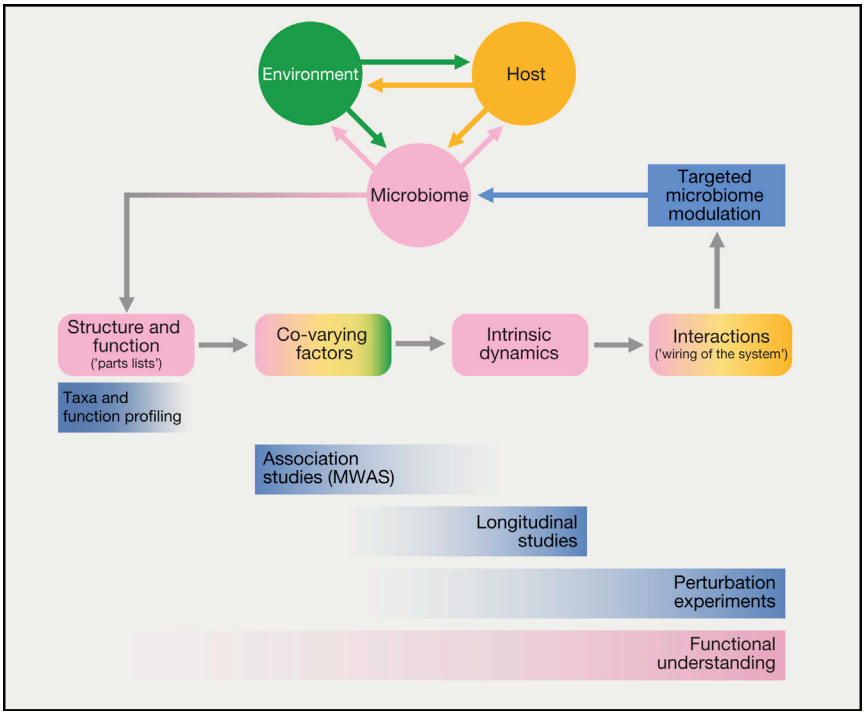


**Figure 1. The Route toward Targeted Microbiome Modulation Entails Three Consecutive and Mutually Dependent Lines of Investigation**

A "parts list" of the microbiome's structure and function has now been mostly established, and metagenome-wide association studies (MWASs) have identified important co-variates of microbiome composition (see Figure 2). At the same time, longitudinal studies have started to provide important insights into the microbiome's intrinsic dynamics. Taken together, these provide first cues toward a functional understanding of the gut microbiome. Perturbation experiments can significantly extend this, while also providing insights into the microbiome's ecological dynamics—the "wiring" of the system in terms of interactions between its parts. An integrated functional understanding will be essential toward translating microbiome research into targeted modulations, with dedicated benefits for the human host.

of the human gut microbiome *in natura*, with less emphasis on *in vivo* work in animal models. Specifically, we highlight recent findings on co-variates associated to microbiome composition, discuss the strengths and limitations of MWASs, and argue that a strong push toward longitudinal and perturbation-based study designs is essential for a deeper functional understanding of the gut microbiome, as well as for the development of microbiome modulation strategies toward improved health and well-being.

## Co-variates Associated to Human Gut Microbiome Composition

Taxonomic composition of the gut microbiome varies greatly between individuals, due to both microbiome-intrinsic and microbiome-extrinsic factors (see Figure 2). The former depend on the microbiome's state, e.g., following maturation during lifetime, which feeds back on itself, e.g., via taxa interactions. The latter (microbiome-extrinsic) factors refer to the various environmental layers that impact on or interact with the gut microbiome. These can explain part of the observed variation within a population and can be classified empirically into three overlapping categories: host-extrinsic factors (i.e., factors influenced by host lifestyle to some extent, such as dietary habits), host-intrinsic factors (e.g., host genetics), and environmental factors (e.g., the vertical transmission of maternal strains to neonates, or neocolonization constraints by regional strain pools; Figure 2).

Many small- to medium-scale MWASs have linked gut microbiome composition to such factors (see e.g., Lynch and Pedersen, 2016; Wang and Jia, 2016 for reviews). The majority of these studies have probed associations of taxonomic composition, usually of genera or species, whereas functional composition, i.e., gene and functional repertoire, has received less attention, mostly due to technical and economical constraints. Moreover, only recently have increasing cohort sizes and comprehensive phenotyping enabled the identification of associations to a wide range of co-variates with sufficient statistical power (Falony et al., 2016; Goodrich et al., 2016; Turpin et al., 2016; Wang et al., 2016a; Zhernakova et al., 2016). For the first time, such studies have allowed to quantify the relative contributions of relevant co-variates to microbiome composition. A key finding has been that even the strongest co-varying factors explain only a surprisingly small fraction of inter-individual gut microbiome variation, at an estimated combined effect size in the range of 10%–15% (see Box 1). This is, nevertheless, considerably larger than technical variation (Costea et al., 2017b) and known co-variates should therefore be taken into account as potential confounders of MWASs (see below). Here, we summarize previous findings on co-variates of human gut microbiome composition, with a focus on recent work.

### *Microbiome State, Including Disease Association and Host Age*

Microbiome compositional state is associated with microbiome-extrinsic factors and shaped by stochastic or ecological effects (e.g., founder effects when re-seeding from the environment) but also potentially self-reinforcing. Differences in microbiome state may underlie differential associations to extrinsic factors, and it is necessary to stratify analyses accordingly (see Box 3). One such intrinsic stratifying factor is probably the gut enterotype, although it is not clear whether such community types follow external co-variates such as diet, transit time, or inflammation or represent intrinsically different compositional optima with similar functionality or both (Costea et al., 2018). Importantly, microbiome associations are often complex and seldom unidirectional: an external influence may trigger a compositional shift that then becomes entrenched in an adapted microbiome state, but microbiome state also feeds back to the host in various ways (e.g., via the production of certain metabolites).

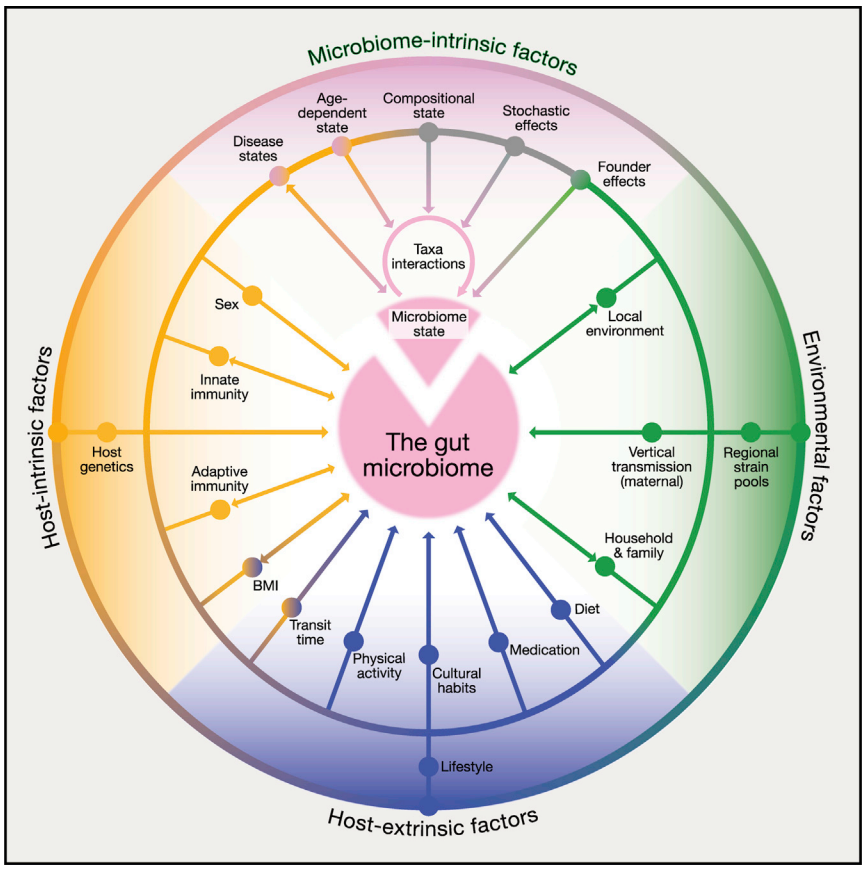


**Figure 2. Microbiome Composition Is Associated to Several Known Co-variates**

Microbiome-extrinsic factors can be empirically classified into three categories: host-intrinsic, host-extrinsic, and environmental. Moreover, microbiome state feeds back upon itself and thereby contributes to compositional variation between individuals. Clearly, these categories overlap, and many factors are also associated to each other. For example, diet contains microbes from environmental strain pools which may colonize the gut or even, in the case of food poisoning, trigger a shift into a diseased microbiome state that subsequently becomes entrenched intrinsically, but also prompts medication. In practice, it is therefore challenging to disentangle the effect size of individual factors, and it is often necessary to stratify for other co-variates, in particular also for microbiome state (see Box 3). Indeed, the overall effect of known co-variates on human gut microbiome variation is surprisingly small (Box 1).

An example of this is the complex association between microbiome state and diseases from various medical indication areas (Gilbert et al., 2016; Lynch and Pedersen, 2016; Wang and Jia, 2016). In some, such as in the case of colorectal cancer (Zeller et al., 2014) or arthritis (Scher et al., 2013; Tito et al., 2017; Zhang et al., 2015b), individual marker taxa are associated to the disease, whereas effects on overall composition are mild. Other disease states, in contrast, are associated with marked shifts in overall compositional features, such as reduced diversity or richness, as is, for example, the case for obesity (Le Chatelier et al., 2013; Turnbaugh et al., 2009) or inflammatory bowel disease (IBD) (Manichanh et al., 2006; Ott et al., 2004). However, for any detected association, it is not clear *a priori* whether microbiome shifts cause the disease or vice versa, or whether both the disease state and observed microbiome effects are caused by a third factor. Indeed, a recent meta-study of 28 MWAS datasets found an overlap of microbiome signatures between different diseases, implying that several reported disease-microbiome links might be non-specific (Duvallet et al., 2017) and possibly linked to other factors such as transit time or inflammation (see also Falony et al., 2016). Hence, disease specificity of reported microbiome markers needs to be established and preferably tested post hoc, e.g., if comorbidities or shared symptoms are known, as is the case for colorectal cancer and IBD (Zeller et al., 2014).

Other well-established differences in microbiome state follow host age (reviewed recently by Kundu et al., 2017; Lynch and Pedersen, 2016). Some age-related transitions are gradual, while others are more clearly defined, e.g., between neonates and older infants, and can correlate with lifestyle changes, such as the cessation of breastfeeding. After birth, infants are colonized by species present in the environment and the mother (Tamburini et al., 2016). Strain-level analyses have recently confirmed that a significant fraction of the developing microbiome is indeed of maternal origin but that seeding is selective, as strains from certain phyla are acquired from the environment (Korpela et al., 2018). Neonate and early-life microbiome composition has been linked to several childhood diseases, including atopy and asthma (e.g., by Fujimura et al., 2016; Stokholm et al., 2018). It has been suggested that this may be due to early life disturbances of the microbiome, e.g., as a side effect of antibiotic treatment (reviewed by Langdon et al., 2016). Other early life events such as birth mode (Caesarean section versus vaginal birth) or feeding (breastfeeding versus formula) have been associated with developing or adult microbiome composition (recently reviewed by Tamburini et al., 2016), but more recent evidence with regard to longer-term effects is mixed (Chu et al., 2017; Falony et al., 2016). Diversity increases after infancy and compositional shifts continue more gradually during late childhood, adolescence, and adulthood (Kundu et al., 2017; Odamaki et al., 2016). Elderly people show signatures of diversity loss, decreased temporal compositional stability, and compositional shifts, all of which are associated to general health but also to confounders like diet and housing environment, a more constrained lifestyle (O'Toole and Jeffery, 2015), or medication (Ticinesi et al., 2017).

### *Extrinsic Host Factors Including Medication, Diet, Lifestyle, BMI, and Stool Consistency*

A wealth of studies tested associations of the adult gut microbiome to factors that are host extrinsic (i.e., influenced by host lifestyle at least to some extent). For instance, medication is emerging as a major co-variate. It is commonly accepted that

**Box 1. Why Can We Explain So Little of Observed Microbiome Variation?**

It has been a sobering observation that the combined effect size of different microbiome co-variates (both technical and biological) appears to be intriguingly low: in the Flemish Gut Flora Project and LifeLines-DEEP cohorts, the total non-redundant compositional variation explained was in the single digit percent range (Falony et al., 2016; Zhernakova et al., 2016), the influence of host genetics has been reported in a similar range (Bonder et al., 2016; Turpin et al., 2016; Wang et al., 2016a) or below (Rothschild et al., 2018), as have disease associations (Duvallet et al., 2017). This could be due to the fact that (1) there are further important uncharacterized co-variates or the current ones are not measured accurately enough, that (2) associations of individual taxa are more relevant than global compositional shifts, that (3) intrinsic compositional constellations or stable states are resilient, that (4) true effects can only be detected at higher taxonomic resolution (Costea et al., 2017a), or that (5) neutral or stochastic processes (drift) have a stronger impact than previously appreciated. Moreover, (6) the gut microbiome's intrinsic ecological dynamics and interactions, ecological succession, and ecosystem maturation (G. Falony, S. Viera-Silva, and J.R., unpublished data) are possible factors that have so far remained understudied, in part due to a lack of longitudinal data.

Nevertheless, the current total quantification of external factors to microbiome variation is probably in the range of 10%–15%, and thus of significant enough effect size to be considered in clinical studies, as even some individual factors can confound associations. This likely remains true even if one extends the definition of MWAS to "microbiome-wide association studies" by also taking into account other data types, such as metatranscriptomic or metabolomic readouts, as recently suggested (Gilbert et al., 2016). Therefore, the proper consideration of and stratification for known microbiome covariates as potential confounders will greatly improve the accuracy of MWASs but can also inform the interpretation of longitudinal and interventional datasets.

broad-spectrum antibiotics—administered to diminish pathogens—impact the gut microbiota as a side effect, both on immediate and longer timescales (Becattini et al., 2016; Langdon et al., 2016). Perhaps more surprisingly, an increasing number of reports also link non-antibiotic drugs to microbiome modulation (reviewed by Le Bastard et al., 2018 and Maier and Typas, 2017). For example, the type 2 diabetes drug metformin has been shown to have a stronger impact on microbiome composition than the disease condition itself (Forslund et al., 2015), an effect that has recently been corroborated in a randomized crossover study (Wu et al., 2017). Similarly, proton pump inhibitors (Freedberg et al., 2015; Imhann et al., 2016; Jackson et al., 2016), atypical antipsychotics (Bahr et al., 2015; Flowers et al., 2017; Mäkivuokko et al., 2010), and non-steroidal anti-inflammatory drugs (Rogers and Aronoff, 2016), among others, have been reported to impact the gut microbiome. In the Flemish Gut Flora Project (FGFP) study, medication (including antibiotics, but also anti-histamines and hormones, for example) was found to be the most important co-variate of microbiome composition (Falony et al., 2016). In a recent large-scale *in vitro* screen testing 1,200 marketed drugs, around half of non-bacterial anti-infectives and a quarter of all human-targeted drugs were found to inhibit at least one gut commensal (Maier et al., 2018), implying that the effect of medication on the gut microbiome remains massively underexplored.

Most drugs are defined chemical compounds, but the gut microbiome is regularly confronted with a complex mix of millions of compounds of dietary origin. As gut commensals contribute to food digestion, links between diet and the microbiome have been studied for years, at different levels of resolution (reviewed e.g., by Flint et al., 2012; Sonnenburg and Bäckhed, 2016). These include microbiome signatures of broad nutritional categories, such as plant- and animal-based diets (David et al., 2014; Muegge et al., 2011), and longer-term dietary patterns (Smits et al., 2017; Wu et al., 2011). However, although diet-microbiome associations were confirmed in cross-sectional studies (Falony et al., 2016; Zeevi et al., 2015; Zhernakova et al., 2016), diet explained only a low single digit percentage of observed microbiome variation after adjusting for covariates. This range likely represents a lower limit, as most cross-sectional studies rely on self-reported dietary data, which has various issues (Ioannidis, 2013).

Several lifestyle factors such as cigarette smoking (Biedermann et al., 2013), alcohol usage (Dubinkina et al., 2017), and physical exercise (Barton et al., 2017; Clarke et al., 2014; Petersen et al., 2017) have been linked to microbiome composition but were not among the top-ranking covariates in recent population studies. Microbiome associations to body mass index (BMI) and obesity have received considerable attention, with links reported to decreased taxonomic (Turnbaugh et al., 2009) and functional (Le Chatelier et al., 2013) diversity. More recently, this observation was extended to subspecies resolution (Costea et al., 2017a). A significant but mild BMI-microbiome link was found in the FGFP (Falony et al., 2016), in line with recent meta-analyses (Finucane et al., 2014; Sze and Schloss, 2016; Walters et al., 2014).

Stool consistency, as assessed by the Bristol stool scale, was the factor with the overall largest effect size in the FGFP study, accounting for ~5% of observed compositional variation (Falony et al., 2016). First quantified in a small-scale cohort (Vandeputte et al., 2016), this factor was recently confirmed in independent cohorts (Tigchelaar et al., 2016; Vandeputte et al., 2017c; Zhernakova et al., 2016), shown to be independent of water activity (Vandeputte et al., 2017a) but driven by transit time (Roager et al., 2016).

Clearly, many of these host-extrinsic factors are not independent of each other (e.g., diet and transit time, BMI and drug usage) and may moreover be linked to host-intrinsic or environmental factors. It is therefore important to note that many observed microbiome signatures may be driven by mixed effects.

### *Intrinsic Host Factors such as Genetics*

Some of the above factors (e.g., BMI) can be partially attributed to genetics. For other factors, a host genetic component is more tangible: for example, the microbiome is intricately and reciprocally linked to both the innate and adaptive immune system (reviewed by Belkaid and Hand, 2014; Hooper et al., 2012;

**Box 2. Methodological Advances to Boost Microbiome Research**

Microbiomics, as a research field, evolves at a breakneck pace, and this is certainly true with regard to methodological advances (see Mallick et al., 2017 for a recent review). Here we highlight recent developments that we expect to make a strong impact in the near future, enabling us to tackle new questions and further complementing the transition from observational to interventional study designs.

### MULTI-OMICS

High-throughput 16S rRNA amplicon and whole-genome shotgun (WGS) metagenomic sequencing have boosted microbiome research for more than a decade, and these technologies continue to dominate the field. More recently, however, the taxonomic and functional census provided by metagenomics is increasingly complemented by readouts on *activity*, provided by metatranscriptomics, metaproteomics, and metabolomics (reviewed by Franzosa et al., 2015; Mallick et al., 2017). Metabolomic analyses, in particular, have served as independent lines of evidence to confirm hypotheses generated in MWASs, for example confirming a link of microbial metabolism to cardiovascular disease (Wang et al., 2011) or the impact of gut microbiome metabolism on insulin sensitivity (Pedersen et al., 2016).

Metatranscriptomic analyses provide a more direct readout on microbial gene expression profiles, and relating this information to baseline microbiome functional potential can reveal novel insights (see Abu-Ali et al., 2018; Schirmer et al., 2018 for recent examples). The gut metaproteome, in contrast, has not been analyzed on a large scale, although a few pilot-sized studies exist (Erickson et al., 2012; Heintz-Buschart et al., 2016; Kolmeder and de Vos, 2014).

An important challenge to multi-omic microbiome research is integration: the different data types provide intermingled layers of evidence and need to be interpreted in light of each other, and integrated analysis concepts (Heintz-Buschart et al., 2016; Mallick et al., 2017) start challenging common conceptions on the microbiome, e.g., on the relative importance of functional plasticity (Heintz-Buschart and Wilmes, 2017).

### QUANTITATIVE MICROBIOME PROFILING (QMP)

Most microbiome studies rely on compositional data—relative abundances of taxa or genes are scaled by non-informative total library sizes, and compositionality effects may introduce false positive taxa-taxa or taxa-covariate associations (Faust and Raes, 2012; Friedman and Alm, 2012; Weiss et al., 2017). The use of spiked-in standards (Satinsky et al., 2013), known cell numbers (Stämmler et al., 2016), or flow cytometry (Props et al., 2017; Vandeputte et al., 2017c) can enable absolute microbial quantification. Indeed, total microbial load showed large inter-individual variation, was linked to community composition, and was decreased in Crohn's disease (Vandeputte et al., 2017c). Thus, QMP can increase sensitivity and specificity in MWASs.

### *IN VITRO* MICROBIOMICS AND MICROFLUIDICS

While *in vitro* approaches have long been used to probe the microbiome in classical reductionist setups, they are currently experiencing a renaissance in high-throughput, explorative analyses. Several microfluidics-based "gut on a chip" systems provide increasingly better approximations of the human intestinal environment (Kim et al., 2012; Marzorati et al., 2014; Shah et al., 2016). At the same time, high-throughput cultivation now encompasses fastidious, anaerobic organisms (Rettedal et al., 2014), even in defined media (Tramontano et al., 2018).

### EXTENDED TAXONOMIC BREADTH AND RESOLUTION

As bacteria account for the vast majority of gut flora biomass and are most accessible to cultivation, microbiome research has mostly focused on the bacterial domain. Eukaryal (Parfrey et al., 2011; Wlodarska et al., 2015), archaeal (Gaci et al., 2014), and viral (Hurwitz et al., 2016; Ogilvie and Jones, 2015; Yutin et al., 2018) members of the gut flora have been studied in the past, and now are receiving renewed attention (Conceição-Neto et al., 2017; Sokol et al., 2017). At the same time, reference genomic representation of the archaeal and bacterial domain have increased greatly, in part due to coordinated efforts to sequence type strains (Mukherjee et al., 2017). This illustrates the dynamics of the field: just over a decade ago, early human fecal metagenomes contained mostly unclassifiable reads (Eckburg et al., 2005), and even in 2013, only around half the reads in a gut metagenome mapped to reference genomes (Sunagawa et al., 2013). Only a few years later, this gap may soon be closed, at least for the major prokaryotic lineages (e.g., Zhou et al., 2018).

This increase in taxonomic coverage is complemented by a similar increase in taxonomic resolution. Following a first mapping of the landscape of microbial single-nucleotide variants (SNVs) in the microbiome (Schloissnig et al., 2013), several tools to call microbial SNVs and to profile subspecies to strain-level variation have been developed (Costea et al., 2017c; Nayfach et al., 2016; Quince et al., 2017; Scholz et al., 2016; Truong et al., 2017) and applied to the human gut microbiome. Several species-level observations of the Human Microbiome Project were recently extended to strain level (Lloyd-Price et al., 2017), and associations of subspecies to co-variates were reported that were not apparent at lower taxonomic resolution (Costea et al., 2017a). This indicates that a resolution subordinate to species may help uncover novel and previously overlooked microbiome features and links.

Thaiss et al., 2016), though it has remained challenging to quantify the immune system's impact in shaping the gut microbiome independently of other factors. Similarly, there is increasing evidence for a reciprocal brain-gut-microbiota axis (reviewed e.g., by Carabotti et al., 2015).

Several studies have probed for more direct associations of the microbiome with individual host genetic loci (reviewed by Hall et al., 2017; Kurilshikov et al., 2017). In a large cross-sectional study of British twins, relative abundances of several genera were found to be heritable (Goodrich et al., 2016,

2014); this observation was later corroborated at species level and extended to function (gene content) on a smaller sub-cohort (Xie et al., 2016). A study of 1,561 North Americans likewise reported taxa heritability, as well as an association of 6 human SNPs to taxa abundance (Turpin et al., 2016), which has the same order of magnitude as the 9 and 33 loci associated with microbial taxa and pathways, respectively, reported in the Dutch LL-DEEP cohort (Bonder et al., 2016). A study on a large Northern German cohort reported that 42 human SNPs accounted for ~10% of observed microbiome compositional variation (Wang et al., 2016a). In contrast, a recent re-analysis of the above datasets, extended by 696 Israeli individuals, estimated that host genetics account for less than 2% of microbiome variation (Rothschild et al., 2018). Overall, the impact of host genetics on the gut microbiota appears significant, but with very low effect size. Potential discrepancies, such as with subject sex (reported among the highest-ranking co-variates in the FGFP and LL-DEEP studies), may be due to indirect effects, e.g., to culturally influenced behavioral, dietary, or proteotypic differences that cannot be pinpointed to the genome, such as hormone levels.

#### *Environmental Factors*

Environmental factors beyond the control of the human host have so far remained understudied, although geographical patterns in community composition have been reported, possibly connected to lifestyle (e.g., Suzuki and Worobey, 2014; Yatsunenko et al., 2012). When extending the taxonomic resolution to subspecies level or to a loose operational definition of strains, much more defined geographical patterns become obvious (Costea et al., 2017a; Truong et al., 2017), implying the existence of regional strain pools that harbor different functionality. Indeed, this can be further refined to the level of household and family where replacement of gut strains can happen in adulthood (Korpela et al., 2018), which may be part of the reason why family members show a more similar taxonomic composition than non-family members (Song et al., 2013). The study of effects of household in a broader context, the (built) environment (Hoisington et al., 2015; Lax et al., 2014), and close contact with nature (Obregon-Tito et al., 2015) will likely reveal further environmental factors influencing the individual gut microbiome.

## Limitations to Studying Microbiome Associations

Increased cohort sizes, improved study designs, and comprehensive metadata surveys have greatly enhanced the statistical power of MWASs. However, they cannot overcome inherent limitations to association studies, which are amplified by the complexity and variation of the underlying data, and which need to be accounted for when interpreting and comparing MWAS results.

#### *Technical Variation*

Like other omics-driven research fields, MWASs are prone to within-study and between-study batch effects. Two recent meta-analyses of microbiome-disease association studies found that between-study variation required explicit or implicit batch effect correction (Duvallet et al., 2017; Pasolli et al., 2016). Almost every step in a typical microbiomics study, including sample collection and storage (Hang et al., 2014; Song et al., 2016; Vandeputte et al., 2017d; Voigt et al., 2015), DNA extraction and processing (Costea et al., 2017b; Sinha et al., 2017), and bioinformatic analyses (Mallick et al., 2017), has been identified as an important source of technical variation. Indeed, two recent large-scale studies on technical limits to reproducibility have reported large variation between different workflows as well as between replications of the same workflow in the same and in different laboratories (Costea et al., 2017b; Sinha et al., 2017). This calls for refined standards, at least in comparison to reference standard operating procedures (Costea et al., 2017b).

#### *Specificity and Indirect Associations*

Even if technical variation can be reduced, there are several limitations common to association studies in general. First, the specificity of any link cannot be proven within such a study. For instance, discovery of a disease association does not necessarily imply that observed differences can serve as specific markers without independent replication and comparison with other phenotypes. Second, any association can be indirect. A case in point are the repeatedly reported microbiome associations to HIV that have recently been called into question, as most of the observed signal comes from one of the risk groups, men having sex with men (Noguera-Julian et al., 2016). Even this more direct association is probably confounded by further untested factors, such as sexual practices, social status, or lifestyle. Similarly, confounders are likely due to question several previously reported disease associations. For example, usage of the drug metformin caused the majority of the signal underlying earlier reports on a strong microbiome association with type 2 diabetes (Forslund et al., 2015). A comprehensive survey indicated that indeed, a wide range of previously reported associations are at least in part confounded by secondary factors (Falony et al., 2016).

#### *Taxonomic Resolution and Lack of Functional Characterization*

The majority of MWASs to date have relied on amplicon sequencing of the 16S rRNA gene. This approach is comparatively cost effective and has enabled a dramatic scale-up in cohort sizes. However, reliable taxonomic classification of current 16S amplicon sequences is generally limited to genus level (Matias Rodrigues et al., 2017), and several recent analyses indicate that many taxonomic associations might emerge only at levels subordinate to species (e.g., Costea et al., 2017a; Lloyd-Price et al., 2017). Moreover, amplicon approaches often limit the taxonomic scope to bacteria and archaea, thereby missing potentially informative signals on eukaryal and viral members of the gut flora. However, these limits to taxonomic resolution and scope may soon be overcome as whole-genome shotgun metagenomic sequencing becomes more affordable (see Box 2). This approach also provides readouts on the microbiome's gene and functional repertoires, but this valuable information often remains untapped, partially due to a blatant lack in functional annotation: a large fraction of gut microbial genes, both from cultured isolates and metagenomes, is uncharacterized to date.

#### *Correlation Does Not Imply Causation*

It has become a scientific truism in microbiome research that correlation does not imply causation: while causal directionality is trivial for some associations (e.g., antibiotics treatment impacts the microbiome, and not vice versa), it is difficult or impossible to infer for others, based on observational data only.

Several mathematical approaches for causality inference that have been applied successfully in other fields start to be adopted for microbiome data, such as structural equation modeling or Bayesian network inference. However, their wider utilization has been hampered by constraints on data size and complexity, and many inference frameworks require repeated (longitudinal) observations (see below).

The gold standard for assessing causality of individual associations are classical, reductionist approaches, often relying on mouse models. For example, a potentially protective role for *Clostridium immunis* was recently discovered in a murine colitis model, using a framework dubbed microbe-phenotype triangulation (Surana and Kasper, 2017) that satisfies a "commensal" version of Koch's postulates (Neville et al., 2017). However, such workflows require the successful isolation and cultivation of targeted taxa, which often remains challenging in practice. In some cases, MWAS findings are validated experimentally by transplanting human fecal microbiota into mouse models (reviewed by Wang and Jia, 2016). However, while murine models allow for controlled experimental setups, they suffer from several limitations, including anatomical and physiological differences between the human and murine digestive tract, cage effects due to coprophagy, fundamentally different microbiome composition with little species overlap, and different host immune pressures affecting transplanted microbiotas (Hugenholtz and de Vos, 2018; Nguyen et al., 2015). In consequence, the translation of *in vitro* or *in vivo* findings to human context often remains difficult.

## Understanding Microbiome Dynamics via Longitudinal Studies

Despite the discussed caveats, metagenome-wide association studies have identified important microbiome-disease links that can be followed up for diagnostic purposes and revealed major co-variates of gut microbiome composition. However, most of these studies were cross-sectional and hence mechanistic insights remain limited. Large-scale generation of longitudinal data, covering (1) baseline dynamics of the unperturbed gut microbiome and (2) the response to various perturbations (see next section), is crucial to understand the "wiring" of the gut ecosystem—temporal resolution of stimulus and response can help disentangle cause-effect directionality of microbiome associations *in natura* (i.e., directly in the human host).

Many studies have concluded that the gut microbiome is remarkably stable over time at baseline, in the absence of intervention, both in terms of taxonomic and functional composition. For example, intra-individual genus- and species-level compositional variation over time is lower than inter-individual differences (see e.g., Faith et al., 2013; Human Microbiome Project Consortium, 2012, among others), an observation that has since been extended to strain-level resolution (Costea et al., 2017a; Lloyd-Price et al., 2017; Schloissnig et al., 2013). More recently, the fecal microbiome has been reported to be transcriptomically stable over time as well, albeit to a lesser extent (Abu-Ali et al., 2018). In contrast to this general temporal stability of the adult unperturbed microflora, clear successional dynamics have been described for the developing microbiome of infants (Bäckhed et al., 2015; Koenig et al., 2011; La Rosa et al., 2014), and elderly people can show a marked loss of microbiome stability depending on further lifestyle factors (Jeffery et al., 2016).

All in all, however, the temporal variation of the human gut microbiota remains understudied and most of the currently published studies are statistically underpowered, either in number of individuals, in number of time points, or in temporal resolution. High-resolution studies with sufficient cohort sizes are essential to build predictive models of gut microbiome dynamics, which can then be challenged to model perturbation response (Bucci and Xavier, 2014; Faust et al., 2015). This will not be a trivial task: even the relatively defined community succession in neonates has proven elusive to predictive modeling, probably due to the relative importance of both maternally and environmentally contributed strains (Asnicar et al., 2017; Korpela et al., 2018).

## Disentangling the Microbiome's "Wiring" via Perturbations

Perturbation experiments have long been a framework of choice in both systems biology (Jansen, 2003) and community ecology (Bender et al., 1984), as community-level responses to a perturbation allow inferences about interactions between its members. Although the blind application of classical ecological theory to the microbiome is not without risk (Koskella et al., 2017), the value of perturbation designs in microbial ecology has been demonstrated repeatedly (Faust et al., 2015; Shade et al., 2012). Indeed, perturbation experiments are much more informative toward the development of (dynamic) predictive models for microbial community ecology than cross-sectional studies, in particular when complemented with *in vitro* and *in vivo* approaches (see Box 2). Such a perturb-to-predict paradigm can provide testable hypotheses and will be essential toward a targeted modulation of the gut microbiome, which in turn is at the heart of translational work (see next section).

Here, we review examples of how interventional studies can advance our understanding of the gut microbiome and highlight emerging trends. We use a broad definition of perturbation, including stimuli such as medication or dietary intervention.

### *Perturbation Response as a Window into Microbiome Community Structure and Dynamics*

Whereas longitudinal analyses are essential to understand baseline microbiome dynamics, perturbation of a microbial system allows much deeper insights into its ecological makeup (Faust et al., 2015; Shade et al., 2012; Sommer et al., 2017). Arguably, the longest-lasting perturbation experiment on the human gut microbiome is diet intake, as this natural process has evolved over millions of years. After adopting a more sedentary lifestyle, humans have adapted to an omnivore diet with high variety, and the impact of moderate dietary shifts should therefore be limited and transient. Indeed, several studies have shown that dietary interventions often seem to elicit only specific effects (see Zmora et al., 2016 for a recent review), although more extreme shifts can show more pronounced signatures. For example, radical switches to all-plant- or animal-based diets on the microbiome have a differential impact, and specific groups of taxa respond similarly across individuals (David et al., 2014). Another study found a consistent ecosystem-wide increase in gene richness in response to an energy-restricted high-protein diet in

**Box 3. The Microbiome Stratifies and Personalizes Host Response to Perturbations**

It is becoming increasingly clear that inter-individual microbiome variation is associated with differential response to perturbations. The human gut microbiome stratifies into distinct compositional types, termed *enterotypes* (Arumugam et al., 2011; Costea et al., 2018). First studies suggest that enterotypes are stable over time (Costea et al., 2018; Ding and Schloss, 2014), perhaps even upon short-term dietary intervention (Roager et al., 2014; Wu et al., 2011). Enterotypes may contribute to several microbiome-disease associations and have been linked to differential pharmacokinetics and drug metabolism (see Costea et al., 2018 for a recent review). For example, it was shown that *Prevotella copri* and *Bacteroides vulgatus*, two hallmark species underlying enterotype splits, mediate insulin resistance (Pedersen et al., 2016). The *Prevotella*/*Bacteroides* ratio was also found to predict improved glucose metabolism upon a dietary intervention (Kovatcheva-Datchary et al., 2015), and enterotype was found to be predictive of the response to treatment with the antibiotic cefprozil (Raymond et al., 2016), reinforcing the idea that enterotypes may underlie stratified responses to perturbation.

Several studies have demonstrated stratification of drug responses by specific microbiome features (recently reviewed by Vázquez-Baeza et al., 2018). For example, specific strains of *Eggerthella lenta* have been shown to metabolize the cardiac drug digoxin, rendering it inefficient in some patients (Haiser et al., 2013). The efficacy of anti-PD1 and anti-CTLA4 chemotherapy in melanoma patients has been shown to depend on the gut microbiome, with predictive compositional differences between treatment responders and non-responders (Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018; Sivan et al., 2015; Vétizou et al., 2015). Similarly, recent work in *C. elegans* demonstrated how gut bacteria differentially modulate the metabolism of fluoropyrimidine chemotherapeutics (García-González et al., 2017; Scott et al., 2017).

The microbiome is also thought to mediate host response to dietary intervention (Sonnenburg and Bäckhed, 2016), although in this case, even more complex and personalized patterns have emerged (Zmora et al., 2016). It was reported that complex models (including lifestyle and blood parameters beyond microbiome features) could successfully predict response to dietary intervention, as validated in a randomized control study (Zeevi et al., 2015). Similarly, microbiota-wide metabolic models could successfully predict differential effects of a dietary intervention (Shoaie et al., 2015). Such studies illustrate how the microbiome may mediate and thereby stratify and personalize host-level response to intervention, and that microbiome stratification is a relevant factor to account for in practice.

obese patients (Cotillard et al., 2013). In general, most studies to date have investigated rather broadly defined dietary shifts, e.g., to overall varying levels of non-specific nutrient classes such as proteins or carbohydrates, but the effects of defined, specific dietary interventions are only beginning to be explored.

In contrast to dietary shifts, clinical interventions can be expected to elicit more drastic responses, as they can dramatically change environmental conditions in the intestine. Bowel cleansing, often performed in preparation of other treatments, may be followed by a rapid recovery of overall microbiome composition (Voigt et al., 2015), though it may trigger the persistent loss of individual taxa (Jalanka et al., 2015). Other clinical interventions with long-term microbiome effects include bariatric surgery (Tremaroli et al., 2015) or induced iso-osmotic diarrhea. The latter has been reported to induce marked but transient effects, with post-perturbation recovery following a consistent succession across subjects (Fukuyama et al., 2017). Treatment with broad-spectrum antibiotics can have pronounced, persistent, and often non-specific effects, and recovery of compositional state post perturbation is sometimes incomplete, due to a loss of taxa from the community (Dethlefsen and Relman, 2011; Dethlefsen et al., 2008; Jakobsson et al., 2010; Jernberg et al., 2007; Voigt et al., 2015). Similarly, treatment with the narrow-spectrum antibiotic cefprozil triggered consistent responses of individual taxa, while community-level response was stratified (Raymond et al., 2016).

In general, one must note that most controlled interventional studies focus on a putative role of the microbiome in host response to perturbation, rather than on the microbiome's response itself. Host and microbiota effects are often difficult to disentangle: while antibiotics treatment, for example, clearly affects the microbiome (which may then mediate indirect effects on the host), the independent host and microbiome responses to dietary intervention are more difficult to unravel. In consequence, many perturbation studies have been conducted in mouse models which allow control of host effects to some extent, in spite of other limitations (Nguyen et al., 2015). Moreover, *in vitro* approaches are gaining renewed attention (see Box 2), as these allow fairly straightforward probing of the response of communities or individual strains to specific perturbations, independently of the host (Maier and Typas, 2017). *In vitro* screens are scalable and can go down to the resolution of individual genes in individual strains (e.g., Galardini et al., 2017), while at the same time allowing for very broad designs, a recent example being a screen of 1,200 drugs screened against 40 gut microbial strains (Maier et al., 2018). Thus, *in vitro* screens can serve as massive hypothesis generators to guide the study of microbiome perturbation responses *in vivo*, either in animal models or directly in humans, as shown in a recent study on the impact of salt on the microbiome (Wilck et al., 2017).

Nevertheless, systematic perturbation studies in humans with the sole purpose of understanding the microbial ecology of the gut microbiota will be needed as well. Larger and more controlled prospective and interventional study designs are increasingly adopted, metadata acquisition becomes more and more comprehensive and sophisticated, and data generation gets more affordable. This will enable us to probe taxonomic and functional interactions among the microbiome and to understand the factors underlying differential perturbation response. Given the complexity of the human-microbiome symbiosis, only "real life" data will yield the necessary information for building realistic predictive models.

### *From Perturbation to Prediction*

So far, predictive modeling of perturbation responses has proven extremely challenging (Bucci and Xavier, 2014; Faust et al., 2015), both because of complexity and variation and also because of our limited functional understanding of the wiring of the gut microbiome (see above). Moreover, it has been argued that the microbiome's response to many perturbations is inherently stochastic (Zaneveld et al., 2017) and therefore not fully predictable.

Yet, a number of predictive models of microbiome dynamics at the level of individual taxa or taxa groups exist (Bucci and Xavier, 2014). For example, Lotka-Volterra models were used to predict community dynamics in response to *Clostridium difficile* infection in mice (Stein et al., 2013). The resulting models could subsequently predict the success of a *C. difficile*-protective probiotic treatment (Buffie et al., 2015). Moreover, using complex models trained on both microbiome composition and non-microbiome features, the impact of personalized dietary interventions on select microbiome features could be predicted to some extent (Shoaie et al., 2015; Zeevi et al., 2015).

Despite such progress, even higher-level perturbation responses are often difficult to predict, such as the gain or loss of taxonomic and functional diversity, or the overall strength (let alone direction) of compositional shifts. This is also true for microbiome resilience—the extent to which a perturbed system recovers to a pre-perturbation state (Shade et al., 2012). As discussed above, the microbiome has been reported to be generally resilient to smaller perturbations, though more pronounced disturbances can have lasting effects. It has been argued that the differential resilience between individuals could be indicative of health and disease (Lloyd-Price et al., 2016; Sommer et al., 2017), even though the factors and mechanisms underlying microbiome resilience remain poorly understood, and though it remains challenging to predict how resilient to perturbation a given microbiome will be.

## From Perturbation toward Modulation

Empirical therapeutic modulation of the gut flora has been performed for thousands of years, for example implicitly in the use of traditional herbal medication (Xu et al., 2015) or consciously by fecal microbiota transplantation (de Groot et al., 2017). Despite a wealth of reports over the last decade, links between the gut microbiota and diseases continue to be discovered (Lynch and Pedersen, 2016), and in consequence the human gut microbiome continues to gain attention as a therapeutic target (Langdon et al., 2016; Walsh et al., 2014).

Here, we review recent progress on attempts at both untargeted and targeted microbiome modulation. In the context of this review, we broadly define modulation as an intervention with the intent of pushing the gut microbiome toward a desired state. This includes, among others, fecal microbiota transplantation, probiotic and prebiotic treatment, and directed dietary interventions.

### *Fecal Microbiota Transplantation (FMT)*

An FMT is the prime example of an untargeted microbiome modulation: stool from a (healthy) donor is transferred into the gastrointestinal tract of a recipient, with the aim of improving their health or an undesired microbiome state. FMTs have been shown to be highly efficient in the treatment of recurrent *Clostridium difficile* infection (RCDI), and indeed seem more suited than antibiotics for this disease (van Nood et al., 2013). Although success is less pronounced in other areas, such as for ulcerative colitis (Narula et al., 2017) or metabolic syndrome (Vrieze et al., 2012), FMTs are explored as a treatment option for a growing list of indications, with close to 200 registered clinical trials at the time of writing (clinicaltrials.gov, accessed January 2018). An obvious long-term goal is the replacement of rather undefined donor stool samples with formulated, recipient-tailored mixes of defined microbial strains.

FMTs are often preceded by preparatory antibiotics treatment or bowel cleansing in the clinical practice, and effects can be difficult to disentangle. Several studies have investigated microbiome-level effects of FMT and reported that the treatment is followed by an increase of alpha diversity in the recipient's microbiome, and a shift in community structure toward donor composition in RCDI patients (Fuentes et al., 2014; Seekatz et al., 2014), a trend that was also observed in IBD (Vermeire et al., 2016). In contrast, post-FMT community composition was only mildly associated with recipient pre-FMT composition in trials on metabolic syndrome (Kootte et al., 2017) and ulcerative colitis (Fuentes et al., 2017), calling for higher taxonomic resolution. Indeed, at the level of strain populations, engraftment of donor strains could be demonstrated, although successful colonization was more likely if strains of the same species were present in the recipient prior to the transplant (Li et al., 2016). Moreover, donor and recipient strains were found to co-exist in the recipient for prolonged periods of at least several months post FMT (Li et al., 2016), a finding that has since been corroborated on independent cohorts for different indications (Kumar et al., 2017; Lee et al., 2017; Moss et al., 2017).

While this is encouraging toward future adapted treatment options, our mechanistic understanding of the microbiome's response to FMT remains so far insufficient. Indeed, from a microbial ecology point of view, FMTs provide a unique setup to study microbiome colonization resistance, succession, and overall resilience.

### *Probiotics*

Probiotics, defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014), have been shown to be clinically efficient treatment options in some indications (Ford et al., 2014). In contrast to FMTs, probiotic treatment is an attempt at targeted modulation of the gut microbiota, notably by adding the probiotic to the community. However, microbiome-level effects of probiotics treatment may be mild: a recent systematic review of seven randomized clinical trials found no effects of different probiotics on microbiota composition and no evidence for persistent probiotic engraftment (Kristensen et al., 2016). This reaffirms the notion of gut microbiota colonization resistance, both to probiotics and pathogens. Studies in mice, in contrast, have concluded that engraftment success may depend on how complementary the probiotic is to the recipient's baseline microbiome composition. For example, administration of *Clostridium scindens* was found to metabolically complement the recipient's microbiota and to enhance colonization resistance to *Clostridium difficile* (Buffie et al., 2015). This outcome was based on clinical data, mouse models, and mathematical modeling, and illustrates that an ecology-inspired approach can enable successful microbiome modulation. The future of next-generation probiotics thus lies not only in supplementing beneficial functionalities, but also in providing the necessary ecological context to sustain them. Moreover, the shift of microbiome composition as a whole by supplementation of more complex mixtures of organisms will arguably soon be within reach.

### Prebiotics and Dietary Intervention

Prebiotics, defined as "substrate[s] that [are] selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017), are another means of targeted microbiome modulation. In contrast to the direct administration of probiotics, prebiotics treatment aims to confer a selective advantage to beneficial members of the microbiota. While several studies suggest a therapeutic potential of prebiotics for different indications (Beserra et al., 2015; Ford et al., 2014), surprisingly little is known about their effect on whole microbiome composition. Increased *Prevotella*/*Bacteroides* ratios and improved glucose metabolism have been reported to follow a transient shift to a fiber-rich diet (Kovatcheva-Datchary et al., 2015). Similarly, a fiber-rich diet, supplemented by other prebiotics, shifted gut microbiome functional composition and contributed to weight loss in obese children (Zhang et al., 2015a). Treatment with inulin-type fructans was reported to trigger an increase in *Bifidobacterium* and *Anaerostipes* with hardly any community-level effects (Vandeputte et al., 2017b).

Beyond the supplementation of usually defined prebiotics, diet represents a vast pool of chemical and biomolecular compounds, often implicitly amended with microbes. As such, it is an important factor in shaping microbiome composition, as discussed above (reviewed by Flint et al., 2017). In consequence, directed dietary interventions can not only provide informative perturbation experiments but also are explored as mild, microbiome-mediated therapy options (Suez and Elinav, 2017). Microbiome-wide metabolic models have been used to successfully predict microbiome metabolic responses to a dietary intervention in obese and overweight individuals, stratified by baseline microbial gene richness (Shoaie et al., 2015). Similarly, in using microbiome, clinical, and dietary data to train complex models, personalized dietary interventions toward improved glycemic responses were suggested and validated in a blinded randomized trial (Zeevi et al., 2015). Although both these studies optimized for host effects, the authors were also able to predict microbiome responses to intervention, to some extent. Importantly, both studies found that the microbiome stratified intervention effects and that the response to diet might be truly individual (see Box 3). Moreover, it remains to be determined how much of these inter-individual differences in response to intervention can be attributed to microbiome-intrinsic or host factors (see Figure 2).

### Toward Targeted and Predictable Modulation of the Gut Microbiome

The potential of targeted microbiome modulation has been demonstrated in several recent studies, albeit in mouse models. For example, it was found that *Clostridium sporogenes* metabolizes aromatic amino acids into several compounds that accumulate in the host's blood serum and that the replacement of wild-type *C. sporogenes* with a genetically engineered strain in gnotobiotic mice decreased serum levels of these metabolites and affected gut permeability and host immune response (Dodd et al., 2017). More recently, it was reported that tungstate treatment selectively inhibited overgrowth of certain *Enterobacteriaceae* and ameliorated symptoms in a murine colitis model (Zhu et al., 2018). The authors had previously found that molybdenum-dependent enzymes (that are inhibited by tungsten) were implied in *Enterobacteriaceae* blooms during induced colitis in mice (Hughes et al., 2017), and this ecological and functional insight enabled a successful gut microbiome modulation.

Such studies reaffirm the notion that targeted, hypothesis-driven modulation requires an understanding of the taxonomic and functional composition, the mutual interaction structure, and the relevant ecological dynamics of the microbiome. As this functional understanding is only beginning to emerge, current models have limited power to predict the outcome of microbiome modulations, and for many clinically effective interventions it is unclear how the microbiome mediates host-level effects. There are numerous macro-ecological examples of unexpected or catastrophic effects of human intervention on incompletely understood ecosystems. For instance, the invasive toxic cane toad (*Bufo marinus*) in Australia, originally introduced as a biological pest control in the 1930s, has since developed into a major burden on the local ecosystem (Phillips and Shine, 2004). In analogy, (rare) adverse effects have been reported for microbiome modulatory interventions, most prominently for FMT (Wang et al., 2016b), and microbiome-related causes of these remain poorly understood.

The majority of studies to investigate microbiome-level effects of modulation did so at genus or species level. However, for several probiotics, only specific strains of a given species were found to be clinically effective (Kristensen et al., 2016), and the efficacy of a given strain probably depends on the recipient's microbiome. Indeed, some strains of *Escherichia coli* are highly beneficial probiotics (Wassenaar, 2016), whereas others are potent pathogens (Kaper et al., 2004). This illustrates the importance of an appropriate taxonomic resolution to successful microbiome modulation (see Figure 3): precise intervention requires a precise understanding of the target system.

### Defining a Healthy Microbiome in a Healthy Individual

The definition of appropriate target endpoints remains a central challenge to microbiome modulation, as a consensus on microbiome "health" so far remains elusive (see Lloyd-Price et al., 2016 for a recent review). Recently, a microbiome "Global Positioning System" was proposed, in which healthy and diseased states are distinguished based on multi-omic readouts (Gilbert et al., 2016). However, while some disease states may be associated with specific microbiome signatures, microbiome states that are unequivocally "healthy" across cohorts are yet to be established (Lloyd-Price et al., 2016). Others have suggested distinctly time-resolved definitions of microbiome health, e.g., with regard to distinct and characteristic patterns of temporal variability to distinguish healthy and diseased states (Martí et al., 2017). Similarly, it has been proposed that microbiome health manifests itself in the response to perturbations, and that an "Anna Karenina" principle applies to the microbiome—that, in variation of Tolstoy, "healthy microbiomes are all alike; each unhealthy microbiome is unhealthy in its own way" (Zaneveld et al., 2017). Moreover, it has been repeatedly suggested that it is less the response to perturbation, but rather post-perturbation resilience, that is a hallmark of health (Sommer et al., 2017).

Certainly, any definition of microbiome health will depend on the frame of reference. From a clinical perspective, health is determined with a view of the human host—any microbiome state associated with a healthy host state could be considered "healthy." But

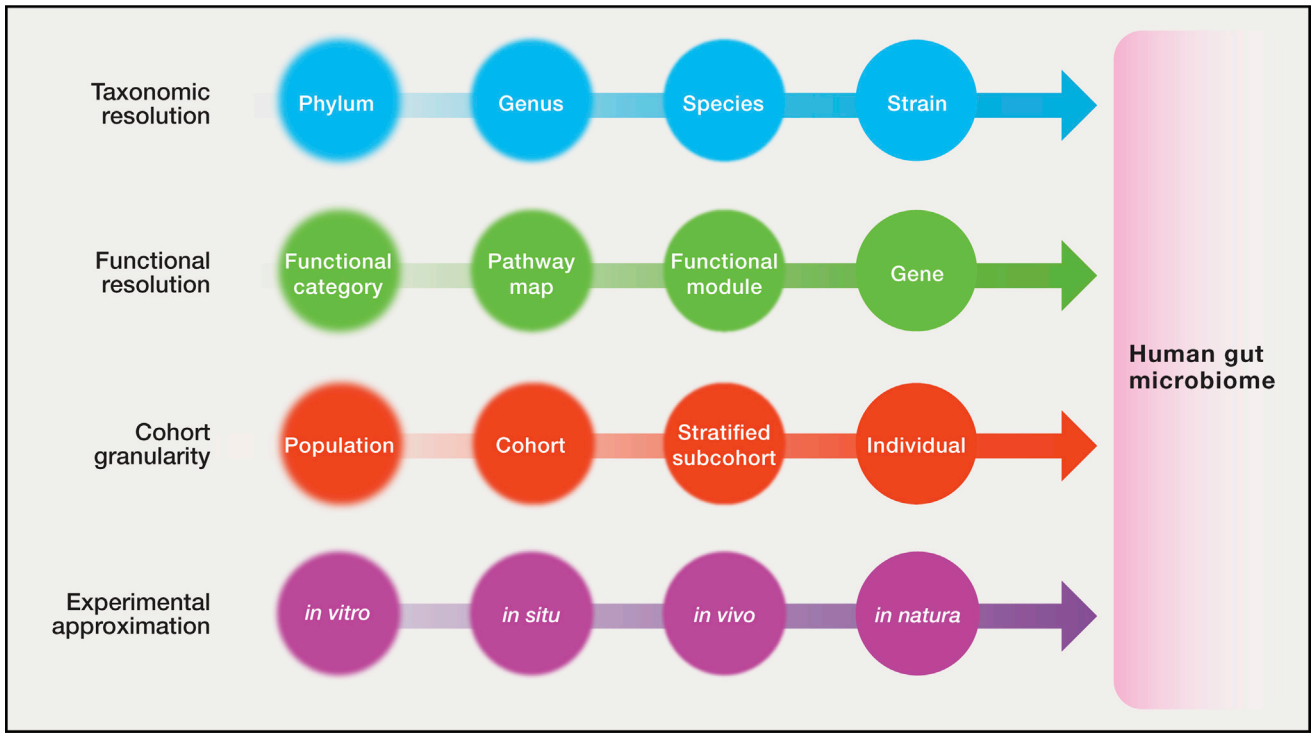


**Figure 3. Microbiome Research Advances Rapidly, but Current Approaches Abstract the Gut Microbiome via Gradual Approximations from Different Angles**

A few of these access routes are depicted and categorized here, and the required level of abstraction may vary between scientific questions or study designs.
(A) Microbial composition is usually determined at genus level based on 16S rRNA amplicon data, although many features in association studies emerge at higher resolution. More recently, the focus shifts further to reach the level of strains, the preferred taxonomic unit in microbiology.
(B) Functional associations are often determined for entire functional classes or more fine-grained functional units, although even individual genes can be informative in some contexts.
(C) Microbiome associations have been tested at the level of entire populations or of certain cohorts, though it is becoming increasingly clear that stratification is often necessary to increase observed signals. In some instances, associations are specific even at the level of individuals.
(D) For experimental access, simpler systems allow for higher throughput, but they are also less representative of the microbiome *in natura*, i.e., in humans with an individual environment.

such a host-centric definition is arguably incomplete and problematic for several reasons. As discussed above, links between host and microbiome are multivariate and complex, so that many diseases of the host do not necessarily carry clear and specific microbiome signatures, while even for well-described associations, the direction of causality is usually unclear. And while disease-associated microbiome imbalances are thus difficult to define, this has proven even more challenging for unequivocally health-associated microbiome states. Although microbiome and host health are clearly linked, multiple healthy microbiome states can probably exist within the healthy host space.

## Conclusion and Perspective

Our understanding of the human gut microbiome continues to evolve at a rapid pace. The census of the microbiome—the establishment of its "parts lists"—is arguably approaching completion for the major prokaryotic lineages, although a surprising amount of novel diversity continues to be discovered at sub-species and strain level, implying that the identification of novel genes in the gut is ongoing. Although prokaryotic lineages contribute the vast majority of the gut microbiome by abundance, important players may still be missed as the eukaryal and viral microbiome remain incompletely charted. Metagenome-wide association studies have identified major drivers of microbiome composition and linked individual microbial taxa and genes to diseases, host lifestyle, and physiology. However, they have also revealed that known factors can account only for a surprisingly small fraction of total microbiome variation, at least without stratification for microbiome state. Longitudinal studies have begun to establish a baseline on the gut microbiome's temporal dynamics and found it to be remarkably stable over time. The study of perturbations has further advanced our functional understanding of the microbiome, both with regard to its intrinsic interaction structure—the "wiring" of its parts—and to cause-effect relationships with external factors. Moreover, it is becoming increasingly clear that the microbiome mediates, stratifies, and possibly personalizes host-level responses to intervention.

The increasing functional understanding of the microbiome begins to be translated into practice, in the form of targeted microbiome modulation. Most attempts at *in vivo* microbiome modulation are of therapeutic intent: researchers aim to improve the wellbeing of patients, by proxy of the microbiome. However, a consensus on desired microbial endpoints—on what a "healthy" microbiome actually is—has yet to emerge.

Currently, understanding lags behind application: the underlying reasons why an untargeted intervention like FMT is effective in some cases but not others are mostly unclear, and effective informed, precise microbiome modulation is still in its infancy. This argues for a push toward more and larger-scale longitudinal and interventional studies, with an updated methodological toolkit, including multi-omic techniques and novel *in vitro* approaches, and with a focus less on the host, but on the microbiome in its own right. Such studies will further advance our understanding of the microbiome, have the power to elucidate missing links, and will enable us to better predict responses to intervention. The integrated study of perturbations will thereby allow us to truly advance research on the human gut microbiome, moving from association to modulation.

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