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More Than the Sum of Its Parts: Unlocking the Power of Network Structure for Understanding Organization and Function in Microbiomes

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Abstract

Plant and soil microbiomes are integral to the health and productivity of plants and ecosystems, yet researchers struggle to identify microbiome characteristics important for providing beneficial outcomes. Network analysis offers a shift in analytical framework beyond "who is present" to the organization or patterns of coexistence between microbes within the microbiome. Because microbial phenotypes are often significantly impacted by coexisting populations, patterns of coexistence within microbiomes are likely to be especially important in predicting functional outcomes. Here, we provide an overview of the how and why of network analysis in microbiome research, highlighting the ways in which network analyses have provided novel insights into microbiome organization and functional capacities, the diverse network roles of different microbial populations, and the eco-evolutionary dynamics of plant and soil microbiomes.

INTRODUCTION

We live in a world shaped by microbes. Soil, plant, animal, and water microbiomes are integral to the health, fitness, and functionality of all higher organisms and ecosystems (10, 13, 14, 48, 74, 93, 119, 131). Complex microbiomes colonize the interior and exterior of all higher organisms. The ubiquity of plant microbiome–host symbioses has forced us to rethink our conceptions of the role of microbes in plant health and disease in fundamental ways. Although disease-centric approaches have traditionally emphasized a two-way interaction between a plant and a pathogen, mediated by the physical environment, this approach obscures the diverse, significant, and often beneficial roles that microbes play in plant health (129). Moreover, the simple framework of a single microbe's influence on a host plant neglects the profoundly interactive nature of plant microbiomes. The phenotypes of individual microbial strains as well as the functional capacities of the aggregate microbiome reflect complex and often highly specific intra- and interspecies interactions, including resource competition, antibiosis, quorum sensing, cellular transduction signaling, biofilm formation, and others. Collectively, the recognition of the microbiome as a complex unit comprising interacting organisms that influence plant and crop productivity in diverse ways has led to a foundational restructuring of approaches to studying plants and microbes and their interactions (6, 8, 25, 29, 30, 47).

Rather than focusing predominantly on individual microbes, or on disease or dysbiosis, researchers over the past decade have shifted efforts to identifying features of the collective microbiome present in association with the healthiest and/or highest-yielding plants across crop or natural habitats (13, 18, 60, 111). At the same time, recent advances in ‘omics technologies (e.g., genomics, transcriptomics, proteomics) have generated a flood of data on the composition and functional activities of plant and soil microbiomes. Focusing on compositional data, microbiomes are commonly described using lists of microbial taxa and their relative (sequence) abundances, along with diversity metrics, dimensionality reduction analyses to capture compositional variation among samples, and multivariate statistical analyses to quantify the significance of variation among microbiomes or microbiome sample sets. Although they provide a superb overview of the "who's who" of plant microbiomes and are excellent for distinguishing compositional or diversity differences among microbiomes, such analyses are inadequate for capturing essential elements of microbiome organization, most notably patterns of coexistence among microbial populations across the microbiome. There is broad recognition that knowledge of the functional roles of plant and soil microbes in plant health, disease suppression, nutrient cycling, and carbon sequestration requires understanding the organization of the microbiome over time and space, not just what microbes are present. This reflects two important concepts: (*a*) The functional capacities of individual microbes are influenced profoundly by interactions with other organisms in the microbiome, and (*b*) management of microbiomes in agriculture or other habitats requires understanding not only which microbes are present but also how they are structured across plant or soil habitats.

Network analysis is a common approach for analyzing systems of interrelated components in diverse disciplines, including medicine, social science, and animal science (19, 39, 40, 71, 76, 79, 95). The essential approaches and mathematical foundations are the same across scientific fields; however, the use and interpretation of network metrics can vary. There have been many excellent reviews of network analyses over the past few years, offering rich insights into the applications of network analyses to different systems, including in plant and soil microbiomes (3, 12, 20, 45, 67, 85, 88). Although both coassociation and interaction networks have been used in the study of microbiomes (64, 75, 76), we focus here on coassociation networks. A coassociation network is constructed using data on the relative abundances of microbial taxa from across a collection of samples representative of a particular microbiome in time or space. Correlations in abundances among all pairwise taxon combinations across the collection of samples are determined, and these

CONCEIVING THE METAMICROBIOME AND ITS RELEVANCE TO AGRICULTURE

Plant and soil microbiomes exist as a metacommunity, i.e., a collection of communities across a landscape that are linked by the dispersal of diverse and potentially interacting species within the metamicrobiome. The metamicrobiome is the collection of microbes existing across a habitat and spanning a vast array of what could be conceived as individual microbiomes. Metacommunity theory focuses on the idea that species interact in complex ways within a spatially heterogeneous matrix of suitable and nonsuitable patches across the landscape, is well-established in ecology, and offers a rich body of models for studying the factors that structure natural communities. The concept of the metamicrobiome is especially relevant in agricultural systems because both the spatial scale of management and the management targets (plant or ecosystem health or productivity) are much larger than the scale of any individual microbiome. This means that understanding the organization of the metamicrobiome in space and time is key to devising effective management outcomes.

relationships are used to define the network. Thus, networks visually and quantitatively summarize patterns of coexistence among populations across a collection of microbiome samples, or a metamicrobiome (see sidebar titled Conceiving the Metamicrobiome and Its Relevance to Agriculture). By summarizing patterns of coexistence across individual microbiome samples, network analyses characterize biological organization that is missed when using traditional, single-sample-centered microbiome analytics and provide data and insights for addressing a completely distinct set of questions (**Figure 1**). Network analyses provide a platform for addressing fundamental questions about microbiome organization and can shed light on the ways in which the organization of microbiomes across a habitat may mediate functionality, ecology, and evolutionary dynamics within plant microbiomes.

In this review, we provide an introductory overview of the how and why of network analysis in microbiome research and highlight the distinct information types provided by network analyses in contrast to traditional microbiome metrics. In addition, we summarize recent work that provides novel insights into the roles of particular microbes, or collections of microbes, in the organization, functional capacities, and eco-evolutionary dynamics of plant and soil microbiomes.

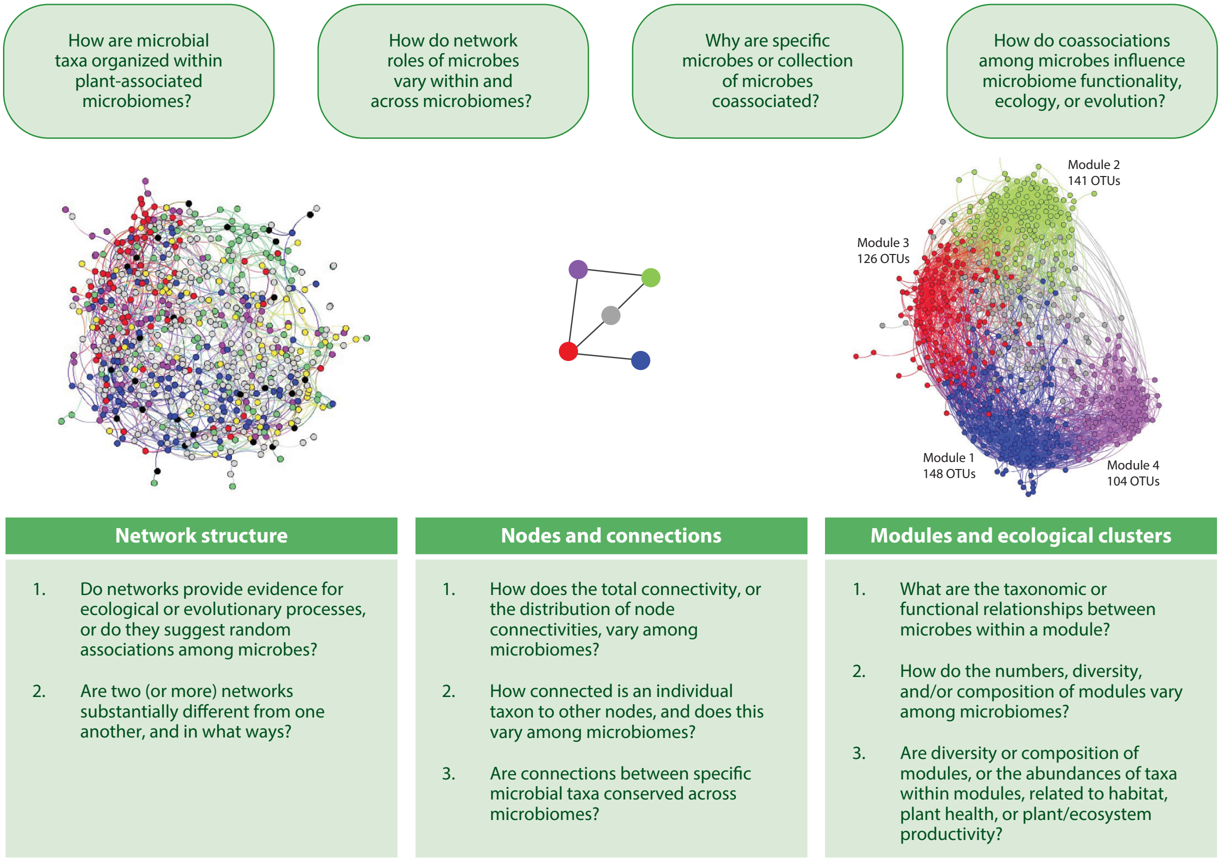
Nodes: the building blocks of the network that represent the objects used to evaluate the connections. In this review, nodes represent individual microbial taxa (ASVs, OTUs, or cultured microbes)

Edges: the connections between two nodes within the network. This can represent either a correlation of relative abundances or a direct interaction, such as nutrient competition, antagonism, or communication

NETWORK FOUNDATIONS

Network Basics: Foundational Structure and Characteristics

Fundamentally, networks are made up of two components: nodes and edges. Nodes generally represent the components of the system being studied [e.g., specific microbial taxa, ranging from amplicon sequence variants (ASVs) to operational taxonomic units (OTUs), genera, or even broader taxonomic categorizations], whereas edges represent relationships or connections between nodes. In this review, we focus on networks of microbial coassociation, where edges signify correlations in relative abundance between pairs of taxa across samples. This is in contrast to interaction networks, in which edges represent direct functional interactions between microbes (e.g., niche overlap in resource consumption, antibiotic inhibitory or cross-feeding capacities, etc.) (54, 77, 97, 112, 117, 118). Notably, coassociations among microbes do not provide explicit evidence for species interactions (17, 39, 55, 106; but see 16, 45, 64). To accommodate distinct types of microbial relationships captured in different kinds of networks, edges can have additional properties such as strength (the intensity of niche overlap between taxa), sign (a positive correlation in abundances versus a negative one), and/or direction of functional impact (e.g., taxon A has a negative impact on taxon B, but B has no impact on A).



How are microbial taxa organized within plant-associated microbiomes?

How do network roles of microbes vary within and across microbiomes?

Why are specific microbes or collection of microbes coassociated?

How do coassociations among microbes influence microbiome functionality, ecology, or evolution?

Network structure

1. Do networks provide evidence for ecological or evolutionary processes, or do they suggest random associations among microbes?
2. Are two (or more) networks substantially different from one another, and in what ways?

Nodes and connections

1. How does the total connectivity, or the distribution of node connectivities, vary among microbiomes?
2. How connected is an individual taxon to other nodes, and does this vary among microbiomes?
3. Are connections between specific microbial taxa conserved across microbiomes?

Modules and ecological clusters

1. What are the taxonomic or functional relationships between microbes within a module?
2. How do the numbers, diversity, and/or composition of modules vary among microbiomes?
3. Are diversity or composition of modules, or the abundances of taxa within modules, related to habitat, plant health, or plant/ecosystem productivity?

Figure 1

Biological and analytical questions addressed using microbiome network analyses. Figure adapted with permission from Reference 6. Abbreviation: OTUs, operational taxonomic units.

Network Analytics: Evaluation and Comparison Among and Between Networks

Module: a group of highly interconnected nodes with limited connections to nodes outside of the group

Complexity: the average links per node in the network

Modularity: a metric to summarize the number and isolation of modules within a network

Conceptualizing a microbiome as a network grants access to a wide range of metrics and analyses for characterizing the patterns of connection within the network, referred to collectively as a network's structure or topology. These metrics can be categorized at multiple scales (*a*) based on properties of individual nodes (such as the number of significant coassociations or interactions that one taxon has with other taxa in the network); (*b*) according to properties of a subset of nodes [such as a collection of coassociated nodes within a module or the frequency of various three-node subgraphs across a network (74, 79, 89)]; and (*c*) based on the structure of the entire network (measures of global network structure, such as complexity or modularity). Notably, some metrics do not fit neatly into this schema. For example, centrality metrics (e.g., betweenness centrality and closeness centrality) often provide a value for each node in the network yet depend on the global network structure to calculate each node's value. Because each metric in isolation illuminates only a facet of the network's structure, it is advisable to consider several metrics in exploring particular biological questions of interest.

What do researchers do with these metrics? They must first consider global network structure. Two key questions are (*a*) whether an individual network differs substantially from what

HIGHER-ORDER INTERACTIONS IN THE MICROBIOME

Higher-order interactions, i.e., cases in which the presence of one microbe affects an interaction between another two, are of particular interest in microbiomes. For instance, if species A inhibits species B but this inhibition is suppressed by the presence of species C, then we say that these species are involved in a higher-order interaction. Put another way, a higher-order interaction is when a node (species C) affects one or more edges between other nodes (the interaction between A and B). Although higher-order interactions have been well-established within synthetic microbial communities, their impact on the assembly and function of wild microbiomes remains unclear (4, 43, 48, 94, 119). Coassociation network construction relies primarily on observing pairwise coassociations (32) within large, naturally assembled communities, but configuration model randomizations can be used to identify higher-order interactions through network structures (especially three-node subgraphs, i.e., motifs) that appear more or less frequently in an empirical (experimental data-based) network than expected under randomizations that preserve the pairwise structure (77, 78).

might be expected in the absence of ecological and evolutionary forces (i.e., if it were constructed at random); and (*b*) whether two networks are different, and, if so, in what ways. Determining whether a network differs from random can be accomplished using randomizations of empirical network structure, with two approaches being particularly common. In the first, the number of microbial nodes and edges is preserved, whereas all other aspects of network structure are randomized (36, 46). In the second, degrees are preserved for each node, whereas edges are randomized (termed configuration models) (11). Network structures that differ from a configuration model randomization suggest the presence of higher-order interactions or structures (see sidebar titled Higher-Order Interactions in the Microbiome).

Comparing networks, e.g., between habitats or experimental treatments, is another common goal of network analysis (5, 41, 57, 68–70, 80, 90, 91, 122, 128). Differences in network structure or characteristics can be important in identifying factors that mediate microbiome assembly and how such factors vary among biological settings or in relation to plant health or productivity over time. It can be difficult to compare empirical networks statistically, as the data requirements to generate even one network can be immense, ranging from hundreds of thousands to millions of sequences among many replicate samples. When sufficient replicate networks are available, standard hypothesis-based statistics are appropriate on metrics that have been normalized for noninformative variation (e.g., network size). Absent these data, metrics of network structure can be compared qualitatively and, when substantially different from one another or from random expectation, differences can be seen as indicators of a role for biological processes in microbiome organization and provide a foundation for more precise hypotheses and further experimentation.

Betweenness centrality: indexes the degree to which an individual node connects to other nodes; the number of times a node lies on the shortest path between two other nodes in the network

Closeness centrality: the mean distance of a node to other nodes in the network

Degrees: the number of edges connected to a particular node

Connectivity: a metric or set of metrics that relates to how a node interacts with all other nodes within the network; often utilizes one or more of degree, betweenness, and/or closeness metrics

Modular hubs: nodes that have high connectivity to other nodes within the same module

Node Analysis: Characterizing Nodes by Connections

Although global network metrics are useful for comparing the overall structures of microbiomes, analyses of individual microbial nodes (ASVs or OTUs) within the network structure also play a significant role in microbiome network analyses. Nodes can be categorized in multiple ways, reflecting variations in the number, types, and strengths of interconnections among them (45, 64, 85). Hub nodes, or nodes with high connectivity within the overall network, are of particular interest and are identified using analysis based on one of three metrics (64): node connectivity (1, 110, 113), node influence (e.g., 63, 120), or link analysis (e.g., 21, 61). Highly interconnected nodes may be further differentiated as modular hubs or connectors when they link nodes within

Peripheral nodes: nodes that have few edges or low connectivity

Generalist edges: connections between two or more nodes that are conserved across distinct microbiome networks

Specialist edges: connections between two or more nodes that are unique to a particular microbiome network

Network connectivity: the mean connectivity among nodes in the network; the average number of edges per node in the network

or between modules, respectively, whereas peripheral nodes are distinguished by low connectivity with other nodes. These different node types have been suggested to reflect differences in the biological role of specific ASVs/OTUs within the microbiome (6, 62).

Among the distinct node types, hub nodes have received the most attention, specifically in the search for potential biological keystone species in the microbiome (1, 7, 16, 72, 73, 75, 82, 83). Microbial keystone species (or keystone taxa) are defined as taxa that exert considerable influence on microbiome structure independently of their abundance within the community, although empirical testing for biological keystoneness remains difficult (7, 16, 27, 66, 86). However, work with synthetic microbiomes has been useful in providing support for keystone roles for individual microbial taxa (for additional reading, see 22, 93, 121). However, in the absence of rigorous and difficult-to-collect data on species interactions, hub nodes within microbiome coassociation networks are hypothesized to represent candidate keystone taxa for further study (1, 128).

Network Substructures: Modules and Ecological Clusters

Coassociation networks are also frequently used to identify clusters of nodes, i.e., modules, whose pairwise relative abundances are significantly positively associated among samples (34, 42, 81), often with limited connectivity to nodes outside the module (34, 64). Negatively coassociated nodes can also be defined within modules, although these are less commonly considered in microbiome research. Modules can be identified using several approaches (65, 84, 89, 114) and represent an important substructure within the microbiome network in that they capture collections of nodes (microbial taxa) that are consistently coassociated across the microbiome landscape. Microbes that consistently coexist (positively coassociated nodes) have an increased likelihood of sustained species interactions and thus reciprocal selection or coevolution, suggesting that modules may represent foundational ecological units of both (coevolved) functionality and diversification within the microbiome. Negatively coassociated nodes may represent taxa that have mutually exclusive habitat preferences or that engage in antagonistic species interactions. The stability of module composition among distinct microbiome networks (e.g., over time or space and among distinct cropping systems or agricultural treatments) or the extent to which positive or negative coassociations between individual nodes or taxa are conserved among distinct microbiome networks (generalist edges versus specialist edges) is of particular interest in studies of microbiome assembly and dynamics (56, 73, 96, 110, 130, 133).

NETWORK ANALYSIS INSIGHTS INTO PLANT MICROBIOMES

Network analyses are becoming increasingly common in plant microbiome research. Here, we highlight key recent findings focusing on network connectivity, module composition and structure, and node analysis in plant and soil microbiomes and what they have shown us about their dynamics and assembly in relation to plant health and productivity.

Network Structure: Connectivity

Because network connectivity is a relatively simple metric of network structure and has been used as an index of ecological complexity (along with species richness), we focus first on it. The link of connectivity to ecological complexity is of importance, as it is used to infer potential for emergent processes and self-organization (62) within the microbiome. Simply put, greater network connectivity indicates consistent patterns of coexistence among taxa (or other units of biological organization, including, e.g., genes or biosynthetic gene clusters) across microbiome samples, which is suggestive of stable patterns of community self-organization. A high frequency of consistent coexistence among taxa (positive coassociations among nodes) across microbiome samples may also suggest selection for taxon assemblages (modules) exhibiting emergent processes in which the collective behavior of the assemblage is distinct from that of the individual components (62). In contrast, persistent negative coassociations among nodes may suggest antagonistic interactions between taxa or mutually exclusive habitat preferences. Collectively, such variation in network connectivity among microbiomes can provide insights into the roles of ecological and evolutionary forces in mediating microbiome assembly over space or time and provide a rich landscape for further hypothesis testing.

Shi et al. (99) provided one of the first assessments of network connectivity in plant-associated microbiomes, reporting on bacterial coassociation networks in the oat plant rhizosphere and bulk soil over time. They reported that microbiomes in the oat plant rhizosphere had significantly higher network connectivity (more links per node) than those in the surrounding bulk soil. The authors also found that although microbiome diversity decreased in the rhizosphere over time, network connectivity in rhizosphere (but not bulk) soil increased over the growing season. They suggest that the increased network connectivity within the rhizosphere reflects greater complexity within rhizosphere versus bulk soil microbiomes. Increases in network connectivity in the rhizosphere over time were argued to reflect increasing community organization, which is suggested to integrate both increasing roles for habitat selection reflective of shared niche preferences and increased importance of bacterial interactions in community assembly. Collectively, this study highlights how understanding the dynamics of network connectivity in distinct habitats can provide new insights into microbiome assembly.

Further work on the dynamics of network connectivity in plant-associated microbiomes reveals substantial variation among cropping and plant systems. For example, in contrast to the work described above, Liu et al. (70) found that bacterial networks associated with soybean were more connected in bulk soil than in the rhizosphere; they suggest that this partly reflects the greater bacterial diversity found within the bulk versus rhizosphere soil. Work by Almario et al. (2) on network connectivity in the phyllosphere bacterial microbiome of field-grown *Arabidopsis* from November to March confirmed the dynamic nature of network connectivity. However, in contrast to the results of Liu et al. (70), Almario et al. (2) found that networks achieved minimum connectivity and complexity in February, nearing the end of the *Arabidopsis* life cycle. They suggest that the reductions in network connectivity in the *Arabidopsis* phyllosphere microbiome over the winter months reflect a shedding of both nodes (ASVs) and node connections, coincident with an increase in the fraction of nodes and specific node–node connections retained from one month to the next. The result overall is lower microbiome network connectivity but an increasing fraction of connections (coassociations among taxa) that were maintained from month to month (generalist connections). This simplification of microbiome structure to a core group of coassociated ASVs perhaps reflects the strong selective filter of abiotic winter stressors, in which only the most stress-resilient taxa survive and dominate the phyllosphere habitat. Further work is needed to shed light on the roles of abiotic versus biotic factors in mediating organization of plant microbiomes and the consequences for microbiome functional capacities.

Researchers have also reported variation in network connectivity in hopes of identifying general trends in microbiome network structure in diseased versus healthy plants. For example, Wen et al. (125) report lower connectivity of rhizosphere bacterial microbiomes in *Ralstonia solanacearum*–infected tomato plants versus noninfected plants, whereas Hu et al. (56) found the opposite pattern among endophytic bacterial microbiomes in *Ralstonia*-infected tobacco. Similarly, Zeng et al. (132) also reported more-connected endophytic bacterial microbiomes (and greater positive:negative network connectivity ratios) in Verticillium wilt–infected versus noninfected cotton plants. Microbiome network connectivity has also been explored in the rhizosphere of tobacco plants grown in bacterial wilt suppressive and conducive fields (134). Considering bacterial and fungal networks separately, Zheng et al. (134) found that bacterial networks were more complex in the rhizosphere of tobacco plants grown in a *Ralstonia*-suppressive soil but that fungal networks had lower connectivity (reduced complexity) in the rhizosphere of plants grown in the suppressive versus conducive soil. The authors suggest that the high network connectivity of the disease-suppressive rhizosphere bacterial microbiome may be key to disease suppression. More complex and connected networks are hypothesized to be more robust to biotic and abiotic perturbations, including disease. The Zheng et al. (134) work showed further that the total abundances of highly connected keystone taxa within the suppressive soil microbiomes were negatively correlated with pathogen density. Further work is needed to explore the ways in which enhanced network connectivity and the abundances of highly connected nodes are related to pathogen densities and disease development in different pathosystems.

Crop management practices can also be considered sources of variation in microbiome network connectivity and complexity (26, 32, 52, 53, 100–102, 128, 133). For example, Banerjee et al. (8) compared fungal network structure between conventional and organic farming systems and showed that network connectivity and complexity were reduced in response to increased agricultural intensification (defined as high nutrient inputs, low crop diversity). Organic farming supported significantly greater microbiome network connectivity than either conventional or no-till farming systems. They suggested that the reduction in network complexity in more-intensive agricultural production systems may reflect the establishment of a more random microbiome dominated by fast-growing (r-strategist) microbes at the expense of putative keystone taxa. In contrast, the higher network connectivity and abundance of well-connected keystone taxa under organic farming may be indicative of a more complex and potentially interactive microbiome that exhibits greater resilience to biotic and abiotic perturbation and stress.

Among these and many other studies (1, 6, 38, 44, 60, 77, 97, 123, 128, 129), key take-home points include (*a*) the lack of a singular consistent response of microbiome network connectivity to disease or to disease suppression; (*b*) the likely critical role that differences in crop, pathogen, microbial target(s) (bacteria versus fungi versus intra-Kingdom networks), and microbiome habitat of focus (e.g., endophytic versus rhizosphere versus bulk soil) play in mediating variation in network connectivity; and (*c*) the temporal dynamism of network structures in the plant microbiome. Collectively, despite the lack of simple generalizations, these findings suggest that connectivity analyses, with their emphasis on microbiome organization versus microbial censuses, provide important new ways of thinking about the dynamics and assembly of plant microbiomes.

Network Structure: Modules

Analysis of modules, or specific collections of coassociated ASVs within a target microbiome, provides a useful statistical means to focus on specific assemblages that may represent distinct, coselected, and plausibly functional ecological units. Of particular interest to plant pathologists is the identification of modules that are positively or negatively associated with pathogen populations or diseased plants or modules associated with disease-suppressive soils. Identification of microbial modules associated with specific functionality can be an important step in determining novel targets for sustainable crop management. For example, network analyses were used to identify rhizosphere bacterial modules associated with the pathogen-suppressive capacity of the soil microbiome (96). They characterized pathogen-suppressive activity for every soil sample using three distinct metrics (the density of pathogen inhibitors, the frequency of pathogen inhibitors, and the mean inhibitory intensity of pathogen inhibition). Among four distinct bacterial modules identified in the microbiome network, one stood out as comprising OTUs whose relative

SYNTHETIC COMMUNITIES

Synthetic communities (SynComs) are simple microbiomes that are artificially created by coculturing collections of microbes under defined conditions (98, 107). In top-down approaches, a collection of microbes associated with a particular function are studied under controlled conditions (for example, a module within a microbiome network) (49, 92). With top-down approaches, researchers can use the SynCom to evaluate, for example, the resilience of collective functionality to incubation conditions, initial population densities, or community diversity and the presence or absence of different members using subtractive incubations. In contrast, bottom-up approaches rely on knowledge of the functional capacities of individual microbes and compose SynComs based on predictions of the role of species interactions in mediating the functional capacities of individual microbes (24, 49, 92). Both approaches can help identify emergent functions of microbial consortia and have become critical tools for understanding the assembly, dynamics, and especially functionality of microbiomes. One significant limitation in the use of SynComs to study plant-associated microbiomes is the current nonculturability of a substantial proportion of taxa detected using amplicon or metagenomic sequencing approaches.

abundances were significantly positively correlated with all three metrics of pathogen suppression; these OTUs are most abundant in soils in which pathogen suppression is greatest. In contrast, OTUs in the other three modules had relative abundances that were negatively correlated with one or more of the pathogen-suppression metrics. The authors suggest that the sole suppressive module represents an excellent potential source of pathogen-suppressive microbes. More importantly, because pathogen-suppressive phenotypes can be under positive selection due to microbial interactions in soil (58, 59), deconstructing the suppressive module to identify emergent properties of the collection of OTUs (or subsets of OTUs) may be important in devising management strategies to select for suppressiveness. This represents a novel dimension for further hypothesis testing, which may be explored systematically using synthetic community approaches (see the sidebar titled Synthetic Communities).

Identifying the factors that mediate positive or negative coassociations among populations in the microbiome is an important next step in understanding microbiome assembly. Specifically, why do ASVs or OTUs coexist within modules? The simplest explanation may be that modules represent organisms whose relative abundances are positively associated as a function of shared nutrient or habitat preferences. Work by Bakker et al. (5) characterized the relationships between relative abundance and soil nutrients (C, K, N, OM, pH) among OTUs comprising distinct modules in a rhizosphere microbiome network analysis. They found distinct differences in nutrient preferences among OTUs associated with each of the modules, including identifying some modules that were clearly associated with nutrient-rich rhizosphere habitats and others strongly associated with nutrient-poor rhizosphere habitats. Notably, although this work and related work by de Menezes et al. (31) suggest an important role for habitat in microbiome structure and modular assembly, this suggestion does not limit the potential that species interactions within the microbiome are also important to microbiome assembly and functional outcomes.

Illustrating the potential for habitat and species interactions to collectively mediate module composition and functional capacities, Bakker et al. (6) explored *Streptomyces* modules within the rhizosphere of four perennial prairie plant species growing in a long-term experimental prairie. They identified *Streptomyces* OTUs that clustered into five distinct modules in one prairie plant host (*Andropogon gerardii*) and tracked those same OTUs in the rhizosphere of three different prairie plant species. Two key findings were that (*a*) OTUs that clustered into a single module in *Andropogon* were only sporadically clustered with one another when in the rhizosphere of different plant hosts, and (*b*) the same collection of OTUs had different relationships with pathogen suppression by the soil microbiome when present in the rhizosphere of different plant hosts. That is, the abundances of the particular collection of OTUs are associated with distinct functional characteristics when in the rhizosphere of different plant hosts, and those OTUs coassociate with different OTUs in the rhizosphere of different plant hosts. This work highlights the significant role that biotic organization, as captured by modular assembly, can play in determining the functional capacities of the microbiome. Rather than a fixed association between a particular OTU and a function within the microbiome, understanding the compositional context—the module, or the assemblage of OTUs with which an organism coexists and potentially interacts over ecological and evolutionary time—may be critical to understanding or predicting the functional capacity of the microbiome. One important implication of this finding is the potential significance of microbiome history to functional capacities. That is, microbiome coassociation structure may impose a critical form of ecological memory on microbiome functional capacities, in which past states of a community (which taxa, having which functional capacities, have coexisted through time and space) influence present or future functional capacities of the community. In this way, understanding the structure of microbial coassociations over time and space within microbiomes is key to long-term management for beneficial outcomes.

Modules have also been investigated to determine whether there are disease-specific modules within the plant microbiome that can define a pathobiome. The pathobiome concept emphasizes the potential role of microbial assemblages and species interactions in mediating plant disease and disease symptoms, e.g., in both suppressing and enhancing disease development (9, 116). Network modules offer a powerful analytical tool for identifying such assemblages, and potential networks of positive or negative species coassociations, across the agricultural landscape. Qiu et al. (87) provide an excellent example of this approach. Specifically, they used network analyses to identify bacteria and fungi that are consistently positively or negatively correlated with pathogen populations in *Fusarium oxysporum* f. sp. *vasinfectum* (FO)–infected cotton plants in both field and greenhouse studies. They found 9 bacterial and 24 fungal OTUs whose relative abundances were significantly correlated (positively or negatively) with FO in the greenhouse, and 23 bacterial and 2 fungal OTUs strongly coassociated with FO in the field. Microbiome networks were more connected in FO-infected versus noninfected plants, and both positive and negative coassociations with FO abundances were observed among fungal and bacterial OTUs. OTUs whose relative abundances are positively correlated with pathogen abundances may facilitate infection, or they may simply be enriched by the presence of a stressed (and presumably leaky) plant. Similarly, the authors suggest that OTUs that are negatively associated with pathogen abundance or infection may serve as pathogen antagonists or may be specific to the habitat offered by a healthy plant. Further work focused on isolation of taxa positively or negatively associated with the pathogen to determine their impacts on infection and symptom development. By exploring taxa and consortia associated with healthy plants, or modules that are negatively associated with disease, the Qiu et al. (87) study highlights the potential for enhanced understanding of the complex interactions within the microbiome that contribute to suppressing or facilitating plant disease and a pathway to novel approaches for disease management. These modules may also provide important starting points for systematic exploration of emergent properties within soil microbiome modules, particularly in relation to disease development or suppression.

Creative work on network modules has also been performed across large geographic scales (23, 28, 73, 104, 127). For example, Toju et al. (108–110) compiled fungal microbiome data across eight distinct forest systems and identified metacommunity hubs, which were collections of highly positively coassociated arbuscular mycorrhizal fungi (AMF), saprotrophic fungi, and endophytic fungi that were suggested to collectively play key roles in mediating key ecosystem functions across diverse climatic regions. Such an approach is especially valuable for identifying specific microbes or microbial functional groups that may be useful inoculants to enhance crucial microbial functions across broad ranges of biotic and abiotic conditions. Although work within individual fields or cropping systems may identify locally coadapted microbial modules, larger-scale approaches spanning diverse ecosystems or geographic locations may be more useful for identifying generic associations of microbes that cross large scales of space and time. In this way, analysis of microbiome modules can provide very different types of insight and address different questions, depending upon the spatial and temporal scales of analysis.

Network hubs: nodes with high connectivity to the overall network

Modular analyses can also shed important light on the ways in which microbiome diversity is organized and how this may mediate microbiome function. Recent research by Fan et al. (37) used network analyses to define modules across a long-term fertilization experiment. They subsequently characterized the relationships between phylotype richness of bacteria, fungi, nematodes, and AMF in each module and (*a*) the abundance of key soil functional genes or (*b*) crop productivity. In one of the four network modules, which they designated a keystone module, phylotype richness of each of the microbial groups across the individual experimental plots was significantly positively correlated with crop productivity. Notably, although richness of fungal and AMF phylotypes was significantly negatively correlated with productivity overall, richness of fungal and AMF phylotypes within the keystone module was positively associated with crop yields. This work highlights the role that network analyses can play in both understanding the organization and biology of plant microbiomes and generating original hypotheses for further investigation.

Network Hubs

Identification of keystone microbes or network hubs has provided important insights into the potential roles of individual microbial taxa in microbiome organization and functionality. For example, Agler et al. (1) investigated the phyllosphere microbiome of *Arabidopsis thaliana* and determined that the effects of abiotic factors and plant genotype in mediating microbiome composition are due to the strong impact that they have on hub microbes. They used network analyses to identify hub microbes that were distinguished by their very high-degree and low-betweenness centrality. Through further experimentation, they found that two hub taxa were especially important in structuring the phyllosphere microbiome. Specifically, *Dioszegia*, a basidiomycete yeast fungus, and *Albugo*, an obligate biotrophic oomycete plant pathogen, were responsive to host genotype and the abiotic environment and transmitted these impacts to other microbes in the inter-Kingdom (bacterial, fungal, and oomycete) network via their impacts on leaf colonization and population dynamics. Similarly, Zheng et al. (134) identified keystone bacterial and fungal taxa in network analyses of *Rhizoctonia*-suppressive and -conducive soils and diseased and healthy root samples, defining keystone taxa based on high-degree, high-closeness centrality and low-betweenness centrality. They identified no keystone taxa in conducive soils or infected roots, although 9 and 13 bacterial keystone taxa were found in suppressive soil and healthy roots and 3 and 10 fungal keystone taxa were found in suppressive soil and healthy roots, respectively. Among these highly interconnected keystone taxa, *Pseudomonas* and *Streptomyces* were the most abundant in the suppressive soil and healthy roots, and the total abundance of all bacterial keystone taxa was negatively correlated with *Ralstonia* in the soil. This suggests the potentially significant role of these well-connected keystone taxa in microbiome function, in this case disease suppression. More broadly, these studies highlight further ways in which network analyses can play an important role in organizing the complexity of plant and soil microbiomes to highlight individual taxa or collections of taxa that are especially important to functional outcomes.

NETWORK INSIGHTS

Keystone nodes: highly connected nodes that represent microbial taxa hypothesized to have a disproportionate impact on the microbial community

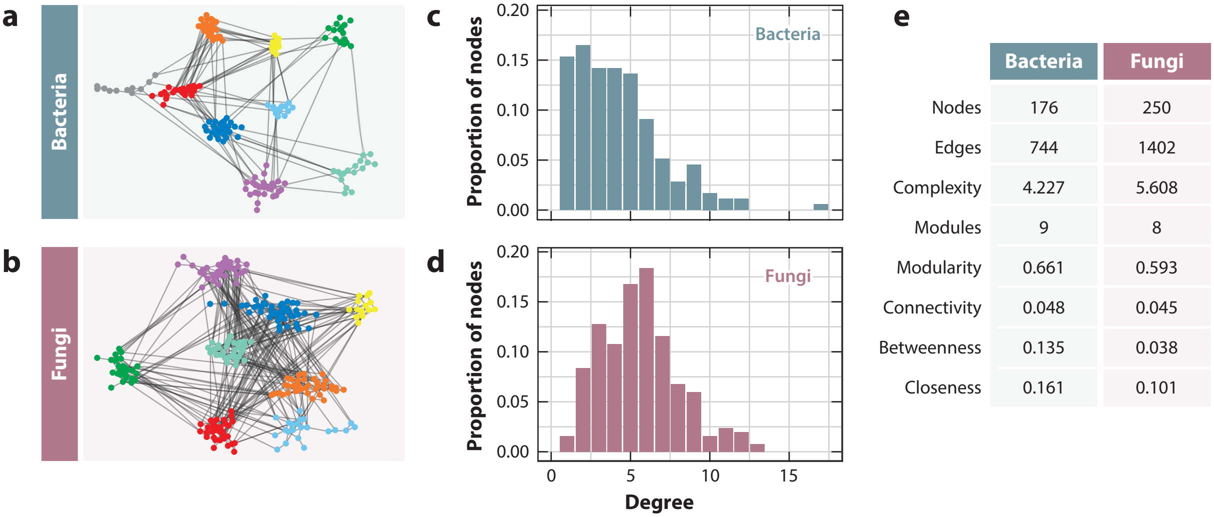
Beyond insights already revealed, network analyses have the potential for exploring and elucidating additional dimensions of the biology and organization of plant and soil microbiomes. Advances in understanding are facilitated by community-wide adoption of standardized data and metadata standards (15, 35, 115, 124) as well as timely provision of raw and processed data in open-access data platforms (103, 126) to enable integration of data across experimental systems and studies. With this in mind, we focus briefly on further questions that are suggested by network analyses and metrics, especially across experimental systems, and how they can (*a*) enhance our understanding of the assembly and biology of microbiomes and the microbes of which they are composed and (*b*) provide a platform for development of novel management strategies for suppressing plant diseases in agriculture.

Node Connectivity

Although many studies have sought to identify highly connected hub or keystone nodes (individual microbial taxa), understanding the consistency of connectivity for specific taxa among diverse microbiome sample sets may provide important additional insights into the biology and ecology of microbial taxa and their role in plant health. For example, are specific taxa consistently highly connected within diverse microbiome settings, or is the connectivity of the taxon idiosyncratic to the setting? That is, is the connectivity of specific microbial taxa related to microbiome setting, plant health or yield, or the disease-suppressive or -conducive nature of the microbiome? What does variation in connectivity for individual taxa in different microbiomes tell us about the biology or ecology of those taxa and how connectivity of individual taxa relates to agricultural outcomes? For example, nodes with little connectivity to others may suggest taxa that are inactive, incidental immigrants, or even relic DNA that is captured in the microbiome sample but not part of the functional microbiome. Alternatively, poorly connected taxa may be those whose activities are largely independent of other microbes. This question deserves further study, particularly for taxa whose connectivity may vary significantly among microbiomes. More broadly, recognizing the significance of coassociations among microbes to microbiome functional capacities, careful consideration of the patterns of connectivity of individual taxa across diverse microbiome settings may provide novel insights into the ecology and life history strategies of microbes within complex plant and soil microbiomes.

Node Distribution

Beyond the focus on individual nodes, considering the ways in which the distribution of node frequencies (the frequency of nodes observed at each node connectivity value) within a network might vary with treatment, plant health, habitat (rhizosphere, endosphere, bulk soil), and microbial target (bacteria, fungi, archaea, oomycetes) is also likely to provide new ways of thinking about microbiome organization. **Figure 2** highlights bacterial and fungal microbiome networks from the rhizosphere of prairie plants, including the networks (**Figure 2*a*,*b***), the distribution of node connectivities (**Figure 2*c*,*d***; frequencies of nodes observed at each degree) for bacterial and fungal microbiomes, and a summary of metrics for the bacterial and fungal networks (**Figure 2*e***). Comparing the fungal and bacterial networks, although average connectivity (**Figure 2*e***) is quite similar (0.048 and 0.045 for the bacteria and fungi, respectively), the distribution of connectivities among nodes is quite different. That is, a very high proportion of nodes for the bacteria have very low connectivities compared to the fungal network (proportion of nodes having 0–5 degrees = 74% and 50% for bacteria and fungi, respectively). In contrast, 44% of fungal taxa are connected with 6–10 other fungal taxa, whereas only 23% of bacterial taxa are connected to 6–10 other



	Bacteria	Fungi
Nodes	176	250
Edges	744	1402
Complexity	4.227	5.608
Modules	9	8
Modularity	0.661	0.593
Connectivity	0.048	0.045
Betweenness	0.135	0.038
Closeness	0.161	0.101

Figure 2

An example microbiome network analysis comparing bacterial and fungal coassociation networks sampled from the same rhizosphere communities. (*a*,*b*) Coassociation networks, (*c*,*d*) frequencies of nodes with varying degrees, and (*e*) network metrics of rhizosphere bacterial and fungal communities from experimental fields. Networks were constructed using amplicon sequence data grouped at the genus level; microbial abundance data were analyzed with SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) (53). Networks illustrated here incorporate only positive coassociations between (*a*) bacterial and (*b*) fungal genera. Genera were clustered into modules using the Louvain algorithm and are represented by nodes of similar color; unconnected nodes are not represented in the network images. Modules of bacterial and fungal communities were identified independently so that node colors in panels *a* and *b* are not related. Node degrees within (*c*) bacterial and (*d*) fungal networks are represented as the proportion of total nodes with a given degree.

bacterial taxa. The frequency distributions illustrate the ways in which the frequencies of low and moderately connected taxa differ between the bacteria and the fungi. Although theoretical work has shown that diverse systems become more stable and resilient to perturbation as the fraction of nodes that are connected to others increases (33), the question of how the distribution of connectivity among nodes may impact resilience has received little empirical study for plant microbiomes. That is, does having the same total number or frequency of connections within a community evenly distributed among nodes versus having a microbiome possessing a handful of highly connected nodes (perhaps keystone taxa?), leaving most nodes unconnected or with a very small number of connections, matter to plant health, microbiome function, or broader concepts in community biology (e.g., invasibility, resistance to perturbation, etc.)? Understanding whether or how the distribution of node connectivities varies with management, plant health or productivity, or habitat type may provide a novel path for exploring microbiome organization and understanding the factors that mediate functional outcomes in agriculture.

Modules as Fundamental Units of Ecology, Diversity, and Evolution

The extraordinary diversity and complexity of plant and especially soil microbiomes have posed a challenge to researchers seeking to understand microbiome dynamics and functionality. Although many studies have considered associations of taxon abundances within modules in relation to beneficial outcomes (e.g., disease suppression, crop yields, soil carbon sequestration), work by Fan et al. (37, 38) and others (3, 109) illustrates an intriguing role for microbiome network structure in organizing the complex diversity of the microbiome. Further systematic study of the ways in which module diversity metrics, ranging from simple consideration of numbers of modules within microbiomes and how numbers vary among microbiomes to more complex assessments of phylogenetic or functional diversity within or across modules (e.g., 37), may shed light on novel and important predictors of beneficial outcomes for plants or soil health. Moreover, because network modules define the potential for persistent species interactions and reciprocal coevolutionary dynamics among microbial populations across the landscape, it is worth exploring whether modules represent basic ecological or evolutionary/coevolutionary units within soil or plant microbiomes and, by extension, essential building blocks of microbiome diversity (6, 37, 38). Although coevolutionary theory is well-developed for metapopulations across the landscape (50, 51, 105), the application of metapopulation theory to network-defined modules offers a novel framework for hypotheses exploring species interactions and coevolutionary dynamics.

CONCLUSION

The significance of microbiomes to plant and ecosystem fitness is well-recognized, yet researchers struggle to identify the most important microbiome characteristics for predicting or managing soil or plant microbiomes for beneficial outcomes. This is likely a consequence of both the extraordinary complexity of microbiomes and the approaches that have been used most commonly in microbiome analysis. Network analysis offers an important tool for revealing elements of microbiome organization that are missed by traditional microbiome analyses that focus on diversity and composition, most notably in characterizing the patterns of coexistence among organisms across the microbiome landscape. Because the functional capacities of microbes are often significantly impacted by coexisting populations, understanding the dynamics of coexistence within the metamicrobiome is likely to be especially important in predicting functional outcomes. The systematic incorporation of network analyses into the vast troves of microbiome data being generated in agricultural and natural habitats will advance our capacities to manage and engineer microbiomes for beneficial outcomes and provide conceptual and mechanistic insights into the ecology and evolutionary biology of plant and soil microbiomes.

SUMMARY POINTS

1. Plant and soil microbiomes exist as a metacommunity, i.e., a collection of communities linked by the dispersal and movement of diverse and potentially interacting species within a habitat. Because interactions among coexisting microbes are critical to diverse microbial functions, understanding patterns of coexistence across the metamicrobiome is key to predicting functional outcomes.
2. Traditional analyses of microbiome composition and diversity fail to capture the organization and structure of microbiomes and, in particular, patterns of coexistence of microbial taxa across the microbiome.
3. Network analysis of microbiomes provides quantitative information on the organization of the microbiome as well as insights into the potential roles of individual populations and collections of microbes (modules) in microbiome functional outcomes.
4. Network analyses provide a significant foundation for hypothesis testing to advance basic understanding of the factors that mediate microbiome organization and the influences of microbiome organization on plant and ecosystem health and productivity.
5. Systematic applications of network analyses across environmental microbiome research systems are needed to provide broad understanding of the variations in microbiome organization and microbial network roles across habitats, space, and time.

6. Managing microbiomes in agriculture and the environment means managing the metamicrobiome; successful management requires enhanced understanding of the organization of microbial populations within microbiomes over time and space.

FUTURE ISSUES

1. Rapid advances in the applications of deep learning, machine learning, and artificial intelligence methods to microbiome data will significantly enhance capacities to detect microbiome organizational patterns associated with beneficial outcomes (more productive plants, enhanced carbon sequestration) and predictive modeling of microbiome dynamics in complex field settings.
2. Diverse data types, including multi-omics data sets capturing functional gene abundances as well as transcriptomic, proteomic, metabolomic, and other data, will expand our understanding of the functional organization of microbiomes.
3. Better integration of field and synthetic microbiome findings will propel successes in microbiome management using inoculation or other manipulation strategies.
4. Development of conceptual models to advance hypothesis testing on the ecology, evolutionary biology, and dynamics of plant and soil microbiomes, including through applications of metapopulation models, will enhance predictive and mechanistic understanding of environmental microbiomes.

DISCLOSURE STATEMENT

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Errata

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