

## Review

## Conceptual strategies for characterizing interactions in microbial communities

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## SUMMARY

Understanding the sets of inter- and intraspecies interactions in microbial communities is a fundamental goal of microbial ecology. However, the study and quantification of microbial interactions pose several challenges owing to their complexity, dynamic nature, and the sheer number of unique interactions within a typical community. To overcome such challenges, microbial ecologists must rely on various approaches to distill the system of study to a functional and conceptualizable level, allowing for a practical understanding of microbial interactions in both simplified and complex systems. This review broadly addresses the role of several conceptual approaches available for the microbial ecologist's arsenal, examines specific tools used to accomplish such approaches, and describes how the assumptions, expectations, and philosophies underlying these tools change across scales of complexity.

## INTRODUCTION

Aided by advances in next-generation sequencing methods and computational power, we are well on our way to successfully characterizing the vast and diverse microbial communities of our planet. Through collective sampling and sequencing efforts we have characterized microbial communities from a multitude of environments, ranging from soils, coral reefs, built environments, extreme environments, and various niches in the human body (e.g., Thompson et al., 2017; McDonald et al., 2018). These efforts have revealed the unequivocal importance of microorganisms in the maintenance of most ecosystems on the planet via processes such as nutrient cycling, biogeochemical reactions, and maintenance of metabolic homeostasis in animal hosts (Gilbert and Neufeld, 2014; Paerl and Pinckney, 1996; Hooper et al., 2012). As the tools that enable us to observe and characterize the vast microbial world continue to be refined, there is a need to better understand the mechanisms driving the formation, maintenance, and function of microbial communities. However, microbial members do not exist in isolation but instead interact to form a community; therefore, an improved understanding of the principles that explain our observations of the microbial world requires a firm grasp of their various interactions.

Understanding the interactions between members of a microbial community has been a fundamental goal in microbial ecology since the inception of the field (Brock, 1966; Tsuchiya et al., 1972). Microbial interactions are central to ecosystem function and are hence important to consider for understanding mechanisms of biodiversity maintenance (Bohannan and Lenski, 2000; Kerr et al., 2002), community structure (Faust and Raes, 2012), and community function (Fuhrman, 2009). By characterizing community interactions, we can identify keystone taxa (Banerjee et al., 2018). Of importance, analysis of organismal interactions has demonstrated that microbes may have an impact disproportionate to their abundance (Graham et al., 2016) and has led us to understand the importance of rare and/or conditionally rare taxa (which constitute the majority of microbial diversity) (Shade et al., 2014). Interactions can act as a force multiplier of the direct and indirect effects that a keystone or important taxon exerts on a microbial community and therefore can serve as a useful metric of true community contribution. At the more holistic level, elucidation of such interaction dynamics can provide ecologists with insight into how the combined effects of relatively simple microorganisms amplify to produce large-scale emergent functions (Bernabe et al., 2018). As an analogy, one can consider individual microbes as parts of a complex machinery; to reverse engineer its effect one must examine the parts individually as well as examine how each part corresponds to one another. Through the study of microbial individuals and interactions we can increase our understanding of the direct and indirect roles of specific members, predict how the community may respond to perturbations in the environment, and, perhaps, eventually engineer complex microbial communities for our benefit.

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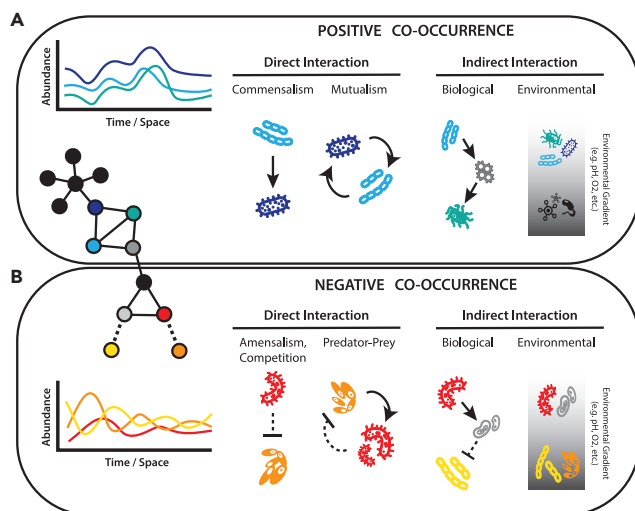


In theoretical ecology, a biological unit can be thought to interact with another biological unit in one of three ways: positively (+), negatively (–), and neutrally (0). When considering the bidirectionality of interactions between two microbes and the mechanism by which they interact, we can propose classically recognizable categories such as mutualism (++), commensalism (+0), amensalism (–0), predator prey/parasitism (+–), and competition (–) (Lidicker, 1979). Although such categorization suggests a finite nature of interactions that can be grasped for a given system, the study of real-world communities has revealed the complex, nuanced plasticity and dynamic nature of actual interactions. Interaction strengths (or even directionality) can change depending on a multitude of inter-specific, intra-specific, and environmental factors. For example, the mutualistic symbiosis between the dinoflagellate algae *Symbiodinium spp.* and its scleractinian coral host can shift to a parasitic relationship when ocean waters become excessively warm and/or eutrophic, causing *Symbiodinium spp.* to sequester more resources and reduce host fitness (Baker et al., 2018). Such issues can obfuscate investigations of ecological interaction dynamics even in the most manageable and well-characterized biological communities. Moreover, scaling from individual interactions to microbial systems introduces additional complexity owing to the massive abundance of diverse microbial taxa present in most natural environments, the majority of which remain uncharacterized (Zamkovaya et al., 2021). Fortunately, the microbial ecologist is now able to access an arsenal of tools to overcome such difficulties and test fundamental hypotheses of microbial interactions. Such tools can allow for the distillation of complex microbial datasets into manageable, conceptualizable systems. In this review we do not aim to extensively cover the technical aspects and methodology of the various analytical techniques or modeling methods in discussion. Rather, our goal is to (1) broadly describe the conceptual role of various tools in answering targeted questions about ecological interaction dynamics and (2) speculate on the future directions that microbial ecology may move toward from the use of these tools and anticipate how such tools may evolve as a response. We aim to contribute an assessment of how the study of interactions may contribute to a practical, useful understanding of global microbial dynamics.

## CHARACTERIZING THE MICROBIAL INTERACTIONS OF REAL-WORLD, COMPLEX COMMUNITIES

With the exception of highly sterilized or extreme environments, real-world microbial communities contain a large number of constituents that may be taxonomically and chemically diverse (Thompson et al., 2017; Nayfach et al., 2021; Shaffer et al., 2021). These complex communities are shaped not only by extrinsic factors but by constraints imposed and opportunities provided by the microbial constituents themselves (Anantharaman et al., 2016; Sung et al., 2017; Hug and Co, 2018; see review by Weiskopf et al., 2021). The study of such communities has been largely informed by traditional ecological approaches, and this holds true for network analyses, which have a long and rich history in the ecological literature (May, 1972, Cody et al., 1975). Co-occurrence networks provide an opportunity for microbial ecologists to leverage information about the presence (or absence) of members in these communities to explore the extent to which specific taxa, metabolites, and other microbe-associated traits interact antagonistically or synergistically. Network approaches can also be used to explore the importance of ecological unknowns, such as the role that components of “microbial dark matter” serve in community connectivity and stability (Zamkovaya et al., 2021). Pairing microbial data with extrinsic variables, such as nutrient availability, abiotic features, or host-associated physiological parameters allows for more nuanced comparisons of microbial communities (Bahram et al., 2018; Gibson et al., 2016; Kartzinel et al., 2019; Khan et al., 2020). More importantly, network analyses provide a framework for hypothesis-based exploration of mechanisms shaping the structure and function of complex microbial communities in real-world systems.

Approaches based on 16S rRNA amplicon or shotgun metagenomic sequencing provide a compositional snapshot of microbial communities (Gloor et al., 2017; Quinn et al., 2019). These compositional data can be harnessed to estimate the relative abundance of microbial taxa, and relative abundance values can in turn be used to identify taxa of biological interest or importance using correlation-, regression-, or dissimilarity-based methods. These approaches can incorporate phylogenetic information or remain phylogenetically agnostic (Lozupone and Knight, 2005), depending on the question of interest. Most tools developed for microbiome network analyses produce co-occurrence networks based on correlation metrics (Pearson, Spearman, etc.), and correlation values are typically interpreted as describing the “interaction” between two microbial taxa. A positive correlation may indicate a synergistic interaction in which the metabolites produced by one taxon are consumed by another or instead perhaps an interaction in which both taxa mutually benefit from the same secondary metabolites. Conversely, a negative correlation may indicate antagonistic interactions in which two microbes are competing for a limited resource or the products of one microbe inhibit the growth of another (Freilich et al., 2011; Berry and



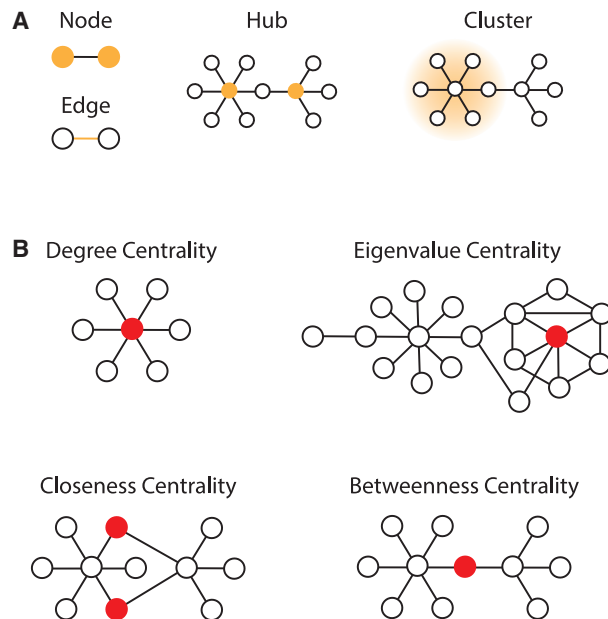
**Figure 1. Potential mechanisms of co-occurrence**

(A and B) (A) Positive or (B) negative interactions observed in co-occurrence networks can result from direct and/or indirect biological mechanisms including (but not limited to) commensalism, mutualism, and shared environmental preferences (cool colors) or amensalism, competition, predation, and disparate environmental preferences (warm colors).

Widder, 2014) (Figure 1). Correlations cannot provide information about the specific underlying mechanisms driving observed patterns of relative abundance, or even guarantee an interaction at all. Rather, the utility of co-occurrence networks lies in their ability to summarize a vast array of pairwise associations into network elements (edges and nodes) that can generate testable hypotheses. Furthermore, network features can be compared to identify biologically meaningful patterns and identify important hubs or keystone taxa in the community. A great deal of effort characterized by interdisciplinary collaboration is rapidly advancing this area of research. Here we provide an overview of commonly used methods for constructing co-occurrence networks from compositional data, examples of the application of co-occurrence networks for understanding the biology of real-world microbial communities, and the inherent limitations of network-based approaches.

Three key challenges faced during co-occurrence network analyses are (1) the compositionality of sequence-based data used to define microbial communities, (2) sparsity of such data, and (3) high dimensionality of microbial taxa paired with relatively low sample sizes. Fortunately, several approaches have been developed that consider these features. To address the issue of compositionality, raw data (e.g., 16S rRNA amplicon sequences) can be normalized (Gloor et al., 2017; Quinn et al., 2019). There are several ways to achieve this, the most common being a transformation from raw read counts to relative abundance (OTU<sub>i</sub> /  $\sum_{i=1}^N \text{OTU}_i - N$ ). This transformation, however, limits downstream statistical analyses since operational taxonomic units (OTUs) may no longer be considered independent and are limited to a non-Euclidean simplex. It is also particularly problematic when comparing heterogeneous samples. To circumvent these limitations, other transformation approaches have been proposed and successfully used in microbiome studies, such as the log-ratio transformation of raw OTU read counts (see Quinn et al., 2019). Centered log-ratios (clr) of the geometric mean are now widely used as an approach to examine the differential proportions of microbial taxa (Morton et al., 2019). As an alternative, the Phylogenetic ILR (PhILR) combines an isometric log-ratio (ILR) transformation with evolutionary tree information to transform microbial data to an unconstrained coordinate system (Silverman et al., 2017); the benefit of this approach lies in its flexibility for the user to apply conventional statistical analyses directly to transformed data.

Once transformed, correlation analyses can be performed on the data to infer pairwise interactions (positive or negative correlations) between taxa, which is the approach taken by many programs including (but not limited to) SparCC (Friedman and Alm, 2012) and CCLasso (Fang et al., 2015), many of which have been implemented in publicly available workflows, such as that of the R package NetCoMi (Peschel et al., 2021). However, such correlation-based methods are unable to consider indirect associations. A more robust approach capable of considering indirect associations is the use of probabilistic graphical modeling paired with undirected networks followed by stability-based model selection to infer putative pairwise



**Figure 2. Defining network components**

(A) Common network features include *nodes* (individual microbes), *edges* (correlation value between two nodes), *hubs* (individual nodes exhibiting a high number of edges and thus a disproportionate influence on a network), and *clusters* (a group of nodes that are tightly connected with strong edges; also called sub-networks).

(B) Common measures of network centrality include *degree centrality* (the number of edges connecting a single node to others), *eigenvalue centrality* (the influence of a node within the network), *closeness centrality* (a measure of the average shortest distance from one node to all other nodes), and *betweenness centrality* (a measure of the extent to which a node lies on the shortest path between other nodes).

interactions. This partial correlation-based method can be implemented by the program SPIEC-EASI (SParse InversE Covariance Estimation for Ecological ASsociation Inference) (Kurtz et al., 2015), which has been widely used to describe complex microbial interactions among soil communities (Toju et al., 2016), wildlife-associated microbial communities (Lutz et al., 2021), and interkingdom associations relevant to organismal health (Tipton et al., 2018). SPIEC-EASI is particularly useful in cases where data are sparse and highly dimensional, as is quite often the case in naturally occurring microbial communities. Another burgeoning area of microbial community analyses is the application of mutual information approaches and mediation models to reduce high-dimensionality metagenomic data and identify key features associated with variables of interest (Liu et al., 2017). Mediation models have been useful for studies in which a combination of microbial metagenomic data and host gene expression are of interest with respect to clinical or disease phenotypes. There are many approaches to reducing the dimensionality of features, and those applied to studies of microbial communities must also consider the previously mentioned challenges of sparsity and compositionality of these data. The package SparseMCMC developed by Wang et al. (2020), for example, combines Dirichlet (Hijazi and Jernigan, 2009) and linear log-contrast regressions to model (1) the relationship between microbial community composition and variable of interest (e.g., clinical phenotype) and (2) relationships between a treatment or intervention and the interactive effects of microbial community members. We note that the methods mentioned thus far rely on arbitrary thresholds of significance for network construction. Random matrix theory-based network approach (e.g., see the Molecular Ecological Analysis Pipeline [MENAP] developed by Deng et al. (2012)) automatically defines network thresholds based on the data structure itself rather than artificial selections (Zhou et al., 2010, 2011). This approach can be useful for users intending to conduct network comparisons (Shi et al., 2016; Yuan et al., 2021).

Regardless of the approach selected, a key goal of microbial community network analyses is to identify biologically meaningful features and interactions. It is therefore important to understand how network components (e.g., nodes, edges) and features (e.g., degree centrality) are interpreted (Figure 2). Nodes typically represent individual microbial OTUs or amplicon sequence variants, whereas edges indicate the

presence and magnitude of interactions between two or more individuals. With this understanding in mind, it becomes possible to identify the most interactive, and therefore likely to be most influential, members of the community. To achieve this, there are many network statistics that can be considered and helpful reviews of their technical application and/or biological interpretation are readily available (Röttgers and Faust, 2018, 2019; Faust and Raes, 2012; Banerjee et al., 2018). Within microbial ecology, centrality and network stability are measures that have been widely used owing to the ease with which these statistics can be biologically interpreted. Centrality can be defined by various metrics depending on the user's specific interests in network dynamics (e.g., betweenness centrality, closeness centrality, degree centrality, eigenvector centrality) (Figure 2). Measures of centrality are estimated using an adjacency matrix calculated from the same underlying data, and each represents a different process by which nodes are connected. However, some measures are more likely to be correlated than others (Valente et al., 2008). For example, a network containing many nodes with a high *closeness* centrality is also likely to contain more nodes with high *degree* centrality, contributing to a more highly connected network that is robust and resilient to random perturbations (although we note that targeted removal of highly connected nodes can also dramatically reduce network stability; see Albert et al. (2000)). Alternatively, networks containing many nodes with limited degree and closeness centrality are likely to contribute to less stable networks. Centrality metrics can also be used to identify keystone nodes within the network (we do note, however, that this is not always the case; see Banerjee et al. (2018) for further discussion on the identification of keystone nodes). The application of more advanced centrality algorithms such as MATria (Cickovski et al., 2019) can facilitate the identification of keystone and other important nodes by using iterative approaches to identify unified sets of central nodes that maximize levels of agreement between the various centrality metrics.

Ultimately, network analyses of complex microbial communities may be seen as a helpful tool but one that, when used in isolation, is insufficient for fully identifying mechanisms of microbial community assembly (see Hirano and Takemoto, 2019 for discussion on network limitations; see Faust, 2021 for open challenges in network construction and analysis). Regardless, when applied with acceptance of the limitations inherent to highly dimensional, sparse, and compositional datasets, networks can serve a useful role in identifying important members of complex communities, characterizing changes in network patterns within and between environments, and enabling hypothesis testing to gain deeper insights into the mechanisms underlying microbial associations.

## EXPLORING COMMUNITY INTERACTION DYNAMICS VIA TIME SERIES ANALYSIS

As with any biological community, a real-world microbial community is inherently dynamic (Gerber, 2014); its composition and the proportions of its membership are constantly shifting and evolving through time as it responds to its external environment. The same can be said for its various interactions, which are temporally associated phenomena by definition. The characterization of any biological interaction requires, at a minimum, the examination of the state of its members at two different points in time (or in space as a proxy). However, it is important to clarify here that microbial interactions are not necessarily fixed in time; they can display variations depending on sampled sub-intervals. Without consideration of such temporal nuances our analytical approaches may run the risk of homogenizing and oversimplifying such dynamics, or even failing to detect certain interactions at all.

Static network analyses incorporate multiple replicates of a community for network generation, often by collecting multiple samples across time points. The underlying assumption with such an approach is that interactions remain stable and constant for the system, and so the combination of information at several time points will improve the inferences of global interaction characteristics (Faust and Raes, 2012). Such an assumption can be justifiable and/or unavoidable in certain instances. For example, the gut microbiome is noted for its exceptional stability through time (Faith et al., 2013) and so longitudinal sampling schemes are often used successfully to increase the statistical power necessary for determining global interaction correlations. However, this assumption is more difficult to justify for communities in non-stable and dynamic environments. The microbial dynamics of communities within lakes and marine systems are strongly affected by distinct seasonal trends (Gilbert et al., 2012; Eiler et al., 2012; Kara et al., 2013), and interaction signals within such communities can therefore change significantly depending on the time of year. The sampling interval also plays an important role in determining which interaction trends become most obvious in these dynamic communities. The predominant interaction drivers of marine microbial communities can greatly shift depending on the examined timescale (hours, days, months, and years) (Fuhrman et al., 2015). For such instances, a well-executed time series analysis can serve as a useful tool for removing

the confounding effects of seasonality and time from the data, as well as for specifying interaction characteristics to specific timescales of interest.

Another important advantage of assessing the time-resolved changes in communities is the ability to identify interaction dynamics that are delayed in time. Time-delayed interactions can occur in microbial communities through a variety of ecological processes, such as succession (Marino et al., 2014; Fierer et al., 2010) and predator-prey cycles (Needham et al., 2013). In succession, colonizing taxa play a fundamental role in manipulating the environment to allow for the proliferation of subsequent taxa, leading to a set of predictable community states after colonization. In the predator-prey cycle, limitations in population growth rates cause lags in the association between the abundances of one population onto another. In both cases, certain initial microbial groups in a community play a fundamental role in the prevalence of microbial taxa in later stages, yet their individual population dynamics do not correlate in real time. Such interaction types are likely more prevalent than expected; in fact, a study of freshwater bacterioplankton interaction dynamics by Eiler et al. (2012) found a greater number of time-lagged co-associations within the community than contemporaneous relationships. Time-delayed interactions are an unmistakably key component to understanding how communities develop and function over time, yet they can be undetectable to us without the proper use of dedicated time series analysis methods.

Several robust methodologies exist that incorporate time series in the analysis of community interactions from microbial datasets. One popular approach, particularly when absolute abundance data are available, is the generalized Lotka-Volterra (gLV) method. Algorithms based on this approach primarily use sparse regression techniques to quantify interaction parameters of the classic Lotka-Volterra differential equations of population dynamics (Fisher and Mehta, 2014; Mounier et al., 2008; Stein et al., 2013; Marino et al., 2014; Bucci et al., 2016; Hosoda et al., 2021). As an example, a gLV based model combined with Tikhonov regularization enabled the quantification of species-species interactions in the intestinal microbiome to numerically predict ecological dynamics under time-dependent external perturbation and additionally characterize community stability (Stein et al., 2013). Another popular approach is the Local Similarity Analysis (LSA), a dynamic correlation-based network inference algorithm that allows for the quantification of temporal changes in the microbial composition between shifted time series to identify complex dependencies among taxa, as well as between taxa and environmental factors (Xia et al., 2013; Ruan et al., 2006; Gilbert et al., 2012; Eiler et al., 2012). For instance, LSA was applied by Chow et al. (2014) to examine and predict both protist-bacteria and virus-bacteria relationships of the San Pedro Ocean time series. In a similar vein, dynamic Bayesian networks (DBNs) are probabilistic graphical models that allow for the conditional dependence structure of the underlying data-generation process to change with time (Garcia and Kao-Kniffin, 2020; Lugo-Martinez et al., 2019; McGeachie et al., 2016). DBN models were applied to the infant gut microbial ecosystem to accurately capture known colonization shifts in dominance between three bacterial populations (*Bacilli* to *Gammaproteobacteria* to *Clostridia*) in the weeks immediately following birth (Lugo-Martinez et al., 2019). Finally, a promising approach yet to be fully utilized in the microbiome field is the equation-free modeling approach such as that of the S-map method (Cenci et al., 2019; Yu et al., 2020; Suzuki et al., 2017). The S-map method (and its various iterations) is based on the framework of Empirical Dynamic Modeling (Ye et al., 2015; Deyle et al., 2016; Ye and Sugihara, 2016), which generates a multivariate ecological time series without making assumptions about underlying microbial processes. The potential advantage of such an approach is that it is robust to non-equilibrium dynamics and non-linearity in multi-species ecological processes.

Although static “snapshot” approaches to community interaction inference certainly have their place in understanding the basic characteristics of community structure in real-world microbial communities, the incorporation of temporal information into analyses strengthens our ability to understand the fluidity of interactions shaped by shifting ecological and evolutionary processes. With the increasing availability of microbial time series with improved resolution and length, we expect that time series will become more widely used to facilitate the improved understanding of real-world microbial interactions through their dynamics, as well as for developing increased predictive capabilities of such communities.

## VALIDATING MICROBE-MICROBE INTERACTIONS USING CONTROLLED EXPERIMENTS WITH SIMPLIFIED, MOCK COMMUNITIES

Although empirically derived analyses such as co-occurrence networks and time series analyses provide a foundational framework for understanding the broad structure of the various interactions within microbial



communities, their interpretability can be limited when used as stand-alone tools. Observational inferences require validation, and in addition, empirical approaches provide limited information on the mechanistic basis of any given interaction in the community (Hirano and Takemoto, 2019). Experimental *in vitro* approaches, which involve the creation and observation of a synthetic microbial consortia under a controlled laboratory setting, provide an alternative approach that can compensate for some of these shortcomings. This reductionist methodology provides two key advantages: (1) the ability to physically simplify the system of study to include only targeted microbial members of interest and (2) the ability to constrain and manipulate the external environment for experimentation. When paired with network analyses, *in vitro* techniques can provide powerful insights into the specific physical and/or biochemical mechanisms underlying co-occurrence patterns between individual microbial species, as well as into the environmental context dependence of interaction dynamics (Das et al., 2018; Diner et al., 2016).

Synthetic microbial consortia can be defined as the composition of two or more genetically non-identical microbial members that are intentionally selected and combined to form an interacting community. Depending on the question at hand, such consortia can be designed with varying levels of phylogenetic complexity. A synthetic community may be composed of several distinct microbial species (Bell et al., 2005; Das et al., 2018) or, at the other end of the spectrum, may be composed of genetically engineered strains of a single species (Kerr et al., 2002; Mee et al., 2014). Although the former approach provides a more accurate representation of a particular set of inter-specific interactions occurring in a real-world microbial community of interest, its viability can be hampered by difficulties of culturing most microbial organisms (Staley and Konopka, 1985). Although technologies are being developed to make more microbial organisms culturable and isolatable for experimentation (Zengler et al., 2002; Stewart, 2012), they are yet to be widely adopted. Alternatively, the latter approach allows for the ability to remove phylogenetic variation in the system, which can be useful when the goal of a study is to examine the specific role of microbial functions and/or genes in driving interactions.

Once the synthetic microbial consortium is formed, it must be paired with an experimental setup that provides an appropriate external medium for the community and provides the ability to manipulate environmental characteristics of interest (e.g., temperature, nutrient concentration, pH, light, initial density of microbial members). Again, the experimental setup can be designed with varying levels of complexity depending on the question of interest and on the desired form of environmental control. For example, a marine microbial community environment may be reasonably replicated using liquid media co-cultures (Diner et al., 2016), whereas interactions within more specialized environments are best replicated using fabricated microbial ecosystems (EcoFABs; Zengler et al., 2019). Perhaps the experimental setup can also benefit from the highly precise manipulation of the environment at the micro-scales relevant to individual microbial interactions, which can be accomplished via promising new approaches involving microfluidic techniques (Leung et al., 2012; Park et al., 2011; Stubblefield et al., 2010; Lambert et al., 2017).

As a complement to the *in vitro* approach, computational models can be implemented to provide interpretations of experimental observations, which can then be used to predict outcomes. Several popular modeling methodologies exist for this level of scale. Dynamic flux-balance models, for example, can be employed to model the growth trajectory of each member in a consortium in response to a fluctuating parameter and to other members, such as to optimize the growth parameters of engineered strains in a microbial consortium for the production of ethanol from lignocellulosic biomass (Hanly, 2013). Constraint-based or genome-scale metabolic modeling (GSMM) uses flux-balance analysis to predict the growth and metabolite rate of a species under a specified, steady-state set of environmental conditions. This approach can be used to develop predictions of community-level fitness from the temporal dynamics of biomass concentration of the community and metabolite concentration of secreted products. Metabolite exchanges between species determine the spatiotemporal dynamics of the consortia, which can be applied to predict the community-level nature of biological interactions using GSMM from the multiple species (Zomorodi et al., 2014; Harcombe et al., 2014). Finally, agent-based modeling focuses on the individual modeling of microbial cells and the environment through time. Although not widely used (the rate kinetics that underpin them are not simply known for the majority of enzymatic activities), their ability to capture changes at the unit form of a level that leads to changes at a global community level can be used to infer the spatial organization of microbial colonies (Shashkova et al., 2016). Such integrated analyses pairing experimentation with computational modeling can allow us to observe how interactions occur in real time at the molecular level, learn the functional consequences associated with changes in the

community or environment, and extrapolate such knowledge toward understanding global microbial interaction principles.

It is important to note that, when using the *in vitro* approach, certain observed interaction characteristics may only pertain specifically to the synthetic microbial consortium in the experiment; the extrapolation of interaction signals can break down when the *in vitro* set up is placed outside of the defined system (Wolfe, 2018). Although several principles and mathematical algorithms have been proposed, it remains a challenge to break down and identify the unique signals achieved from the emergent properties of a consortium from its constituent parts to scale it at a higher level of microbial structure. The metabolic pathways that underpin microbial interactions are often still referred to as “dark matter,” with unannotated genes, proteins, and metabolites (Lloyd et al., 2018). Furthermore, we can expect that most real-world microbial interactions cannot truly be described by a single mechanism but instead are a result of a combination of several mechanisms with differing levels of contribution toward the overall signal. Instead, the primary value of *in vitro* studies may lie more in their ability to facilitate a more complete understanding of the categorical forms in which microbial interaction mechanisms often occur in a defined system, as well as in providing a sense of how the contributions of these mechanisms scale in relation to one another. We expect that, as this sub-field advances, *in vitro* studies will increasingly provide microbial ecologists with the ability to quickly identify the major molecular mechanisms underpinning a given microbial interaction of interest.

## CONCLUSIONS

Unlike in many other disciplines within the field of ecology, microbial ecologists are faced with the inability to make direct observations of the various interactions that occur in their systems of study. To overcome this fundamental obstacle, we must rely on various conceptual frameworks to aid us in our development of a working model of interaction dynamics, given our current level of technological capability and data. In conclusion, co-occurrence networks may be used to pare down complex and high-dimensional compositional data to a simplified, comprehensible description of co-occurrence patterns that represent possible interactions occurring within taxa. Time series analysis can characterize the level of fluidity that interaction structures can exhibit, as well as elucidate time-dependent interactions. Finally, *in vitro* experimental techniques using synthetic communities and modeling approaches can investigate the validity of predicted relationships as well as validating mechanisms of interaction. Although the result from one single approach may not be conclusive on its own, the combination of these approaches is a powerful method for identifying universal interaction principles that govern global microbial community dynamics. The rate of progress that has been achieved in this field is promising; we are already beginning to see the widespread use and refinement of these tools for application in answering diverse questions in microbial ecology. Moreover, the conceptual approaches discussed in this review are now being advanced further through the incorporation of multi-omic datasets, allowing for an even deeper understanding of microbial interactions via their metabolomes, transcriptomes, and full genomes (Ruiz-Perez et al., 2021; McDaniel et al., 2020; Aguiar-Pulido et al., 2016). We are optimistic that, similarly to how standardized sequencing protocols (Gilbert et al., 2014) and user-friendly bioinformatic platforms (Caporaso et al., 2010; Bolyen et al., 2019; Schloss et al., 2009; Schloss, 2020) have revolutionized the accessibility of basic microbiome analyses to a broader scientific community, many of the tools described here will follow a similar trajectory and become essential tools for any microbially inclined scientist to incorporate into their arsenal.

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## AUTHOR CONTRIBUTIONS

S.M.K. conceived of the overarching concepts of this review. All authors contributed to the writing of this manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.



**Glossary**

**Agent-based modeling**—A simulation framework involving the actions and interactions of autonomous agents to study the behavior of a system and identify what controls its outcomes.

**Centrality**—A method of measuring the degree to which a node is connected to the other nodes within a network, typically used in microbial ecology as a proxy for the influence of a microbial taxon onto the community.

**Constraint-based metabolic modeling**—A set of specific methods and tools used to perform genome-scale metabolic simulations on a matrix associating metabolites to reactions, which can be used to infer biochemical processes at the system level.

**Co-occurrence networks**—A visualization and analysis method that characterizes a microbial community through the depiction of associations found in the relative abundances of its constituent microbial taxa across a set of samples.

**Dynamic Bayesian networks (DBNs)**—A directed graph-based approach that can be used to estimate the dynamic dependencies between nodes (representing either microbial taxa or environmental variables), by modeling nodes as a function of variables in preceding time points.

**Dynamic flux-balance models**—A simulation modeling framework representing the cellular dynamics of a culture system and biochemical processes.

**Empirical dynamic modeling**—An equation-free modeling approach that aims to characterize and predict the behaviors and relationships found within dynamic systems via the reconstruction of attractor manifolds from time series data.

**Generalized Lotka-Volterra (gLV)**—A set of methods that estimate microbial interactions and growth rates via the Lotka-Volterra differential equations of population dynamics.

**Keystone taxa**—Highly connected microbial taxa with disproportionate influence on the maintenance of community structure and/or function.

**Local Similarity Analysis (LSA)**—An analytical method that employs dynamic programming to detect “local” associations that only occur in sub-intervals within time series, as well as to detect time-lagged associations.

**Microfluidic techniques**—A set of techniques and technologies that allow for systems with the capability for precise manipulations of fluids with volumes at the scale of microliters to nanoliters.

**Network stability**—The extent to which a network can lose individual nodes and still maintain its overall structure and function.

**Time-delayed interaction**—A type of biological interaction in which the effect of a microbial member on another member is only experienced by the receiving member after a time lag.

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