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Perspective

More than the sum of its parts: uncovering emerging effects of microbial interactions in complex communities

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Abstract

Microbial communities are not only shaped by the diversity of microorganisms and their individual metabolic potential, but also by the vast amount of intra- and interspecies interactions that can occur pairwise interactions among microorganisms, we suggest that more attention should be drawn towards the effects on the entire microbiome that emerge from individual interactions between community members. The production of certain metabolites that can be tied to a specific microbe-microbe interaction might subsequently influence the physicochemical parameters of the habitat, stimulate a change in the trophic network of the community or create new micro-habitats through the formation of biofilms, similar to the production of antimicrobial substances which might negatively affect only one microorganism but cause a ripple effect on the abundance of other community members. Here, we argue that combining established as well as innovative laboratory and computational methods is needed to predict novel interactions and assess their secondary effects. Such efforts will enable future microbiome studies to expand our knowledge on the dynamics of complex microbial communities.

Keywords: community dynamics; microbial interactions; microbiome research; secondary effects

Introduction

Microorganisms seldom live as isolated species. Rather they coexist in a community with other microorganisms, within small consortia or as part of complex cohorts that can span all domains of life (Faust and Raes 2012). Within these consortia, different types of intra- and interspecies interactions occur that can have beneficial, negative or neutral effects for one or multiple interacting participants. With that, interactions can range from being advantageous for all interacting organisms (mutualism) to having an overall negative impact on all microorganisms involved (competition) (Faust and Raes 2012). Ongoing discussions revolve around the prevalence of distinct microbial interactions. While some studies contend that negative interactions such as competition dominate (Foster and Bell 2012, Palmer and Foster 2022), others suggest this perspective might be biased. The challenge lies in distinguishing mutualistic and commensalistic interactions (Kehe et al. 2021, Kost et al. 2023). These studies propose that both positive and negative impact interactions are widespread within microbiomes, challenging the notion of negative interactions' overwhelming abundance. A multitude of conceptually different interaction mechanisms, such as the excretion of antimicrobial compounds or cross-feeding of metabolites and the formation of biofilms, are underlying each type of interaction. Traditionally, these interactions are defined at the molecular level, meaning that they include a direct exchange or conversion of molecules, such as primary or secondary metabolites, toxins, siderophores or signaling compounds between two or more organisms (Braga et al. 2016). The direct correlation between the exchange of molecules and a positive or negative impact on the exchanging organisms make these interactions relatively easy to quantify and classify. However, microbial interactions at the molecule level are solely centered around observing effects on the interacting players. The "emerging effects" of microbial interactions, which are less direct consequences of microbial interactions that can impact the entire community, are often overlooked, or not considered. While these emerging effects of microbial interactions are more challenging to qualify and quantify, their influence on complex communities and even their host environment can potentially be more impactful than the individual interaction itself.

Here, we suggest that future studies that aim to improve our understanding of the functioning of complex microbial communities should (i) combine innovative methods to uncover novel microbial interactions that are of relevance within an environment and at the same time also (ii) consider the emerging effects that these interactions have on other community members, and thereby the community itself. The gained information in positive and negative impacts of individual microbial interactions on the entire population will drastically increase our understanding of the dynamics in complex microbial communities and enable us to better protect and utilize the ecosystem functions and services that these communities provide.

Emerging effects of microbial interactions

The composition and activity of a microbiome is always a result of selection and adaptation to general changes in the ecosystem,

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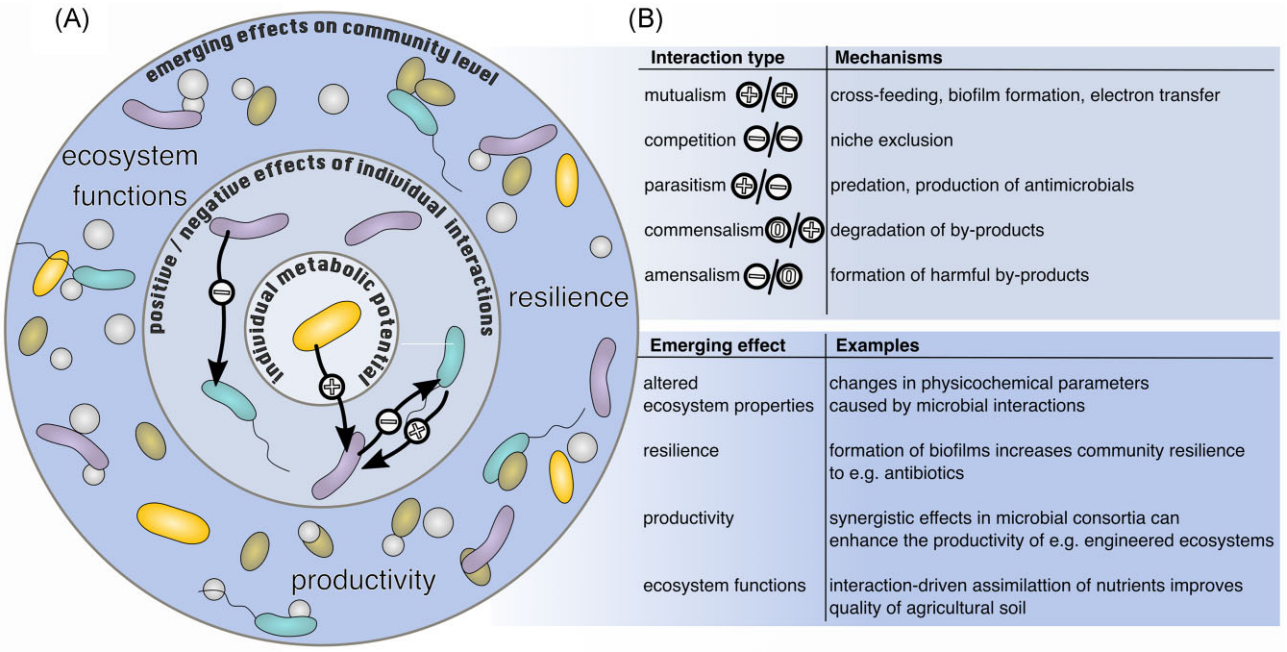

Interaction type	Mechanisms
mutualism ⊕/⊕	cross-feeding, biofilm formation, electron transfer
competition ⊖/⊖	niche exclusion
parasitism ⊕/⊖	predation, production of antimicrobials
commensalism 0/⊕	degradation of by-products
amensalism ⊖/0	formation of harmful by-products

Emerging effect	Examples
altered ecosystem properties	changes in physicochemical parameters caused by microbial interactions
resilience	formation of biofilms increases community resilience to e.g. antibiotics
productivity	synergistic effects in microbial consortia can enhance the productivity of e.g. engineered ecosystems
ecosystem functions	interaction-driven assimilattion of nutrients improves quality of agricultural soil

Figure 1. Microbial interactions at different scales. (A) Microbial communities are not only shaped by the metabolic potential of their members as well as individual microbial interactions but also the emerging effects that these interactions have on other community members and their environment. The manifold of positive and negative interactions taking place on a species-species level can cause further effects on the community level, such as a change in the net-productivity of communities, the ecosystem functions they provide as well as their resilience towards disturbances. (B) An overview of different interaction types and emerging effects of microbial interactions as well as examples for underlying mechanisms.

while these changes can be a direct result of e.g. manmade disturbances or climate change, they can also be an emerging effect of the microbial interactions within the ecosystem: While each type of interaction has a direct effect on one or multiple of the involved microorganism, it can also have direct or indirect impacts on the composition, functioning and productivity of the entire microbial community (Fig. 1). By impacting physicochemical properties of their habitat, microbial interactions indirectly affect other members of their consortium. This was underscored by a study that showed that numerous species thriving in a diverse, stable community struggle to coexist when paired in a simple two-species culture under identical circumstances (Chang et al. 2023). It concluded that the coexistence of multiple species is, in itself, an emerging effect of various interactions. Examples of other emerging effects are for instance, a syntrophic interaction like the interspecies electron transfer from *Geobacter metallireducens* to *Rhodopseudomonas palustris* that can enable carbon fixation by *R. palustris* (Liu et al. 2021). The subsequent decrease of CO_2 can lead to changes in the environments pH and thereby have implications for other members of the community. Furthermore, this pairwise interaction has a major impact on the pool of available organic carbon for the entire community. Likewise, the formation of biofilms as a direct result of microbial interactions is creating an entire micro-habitat with manifold of emerging effects on other microorganisms in the community including a more efficient cycling and recycling of nutrients, a higher level of resilience of species within the biofilm or an increase of horizontal gene transfer (Bamford et al. 2023). At the same time biofilms serve as grazing hotspots for predatory microorganisms and thus play an important role in trophic interactions within the microbial community (Costa et al. 2018).

These emerging effects of microbial interactions will subsequently impact the diversity, but also the resilience of entire microbial communities. Thereby, they have a direct impact on the ecosystem functions and services that each community provides. Most studies focusing on microbial interactions fail to account for these secondary effects, partially because we are lacking methods and tools to assess them. It is because of this, that future studies focusing on microbial interactions need to find novel ways to not only detect and describe direct interactions, but beyond that uncover the emerging effects of these interactions between microorganisms.

Pinpointing interactions in complex microbial communities

Interactions at the molecule level

To study direct interactions between microorganisms, traditional methods often rely on the ability to maintain the organism(s) of interest in culture under laboratory conditions. Thus, screening for most interaction mechanisms, e.g. the formation of antimicrobial compounds or harmful by-products as well as cross-feeding, is classically performed for isolated organisms (Lubbe et al. 2017). By observing a microorganism's impact on the growth or activity of other organisms, direct assumptions on interactions between both species can be made. However, to date, only a small fraction of the members in complex communities can be cultivated in the laboratory and, thus, the majority of microbial interactions on the molecular level can currently not be assessed. To overcome this shortcoming, cultivation approaches need to be improved and tailored more towards the isolation, and potentially co-isolation, of organisms that interact in their environment (Yan et al. 2023). Despite the fact that the way two species interact within a co-culture compared to within a complex community might differ, gaining more insight into (potential) interaction mechanisms is crucial to better our understanding of the dynamics within a microbiome. While classical cultivation and enrichment techniques are time-

consuming as well as labour and resource intensive, novel cultivation techniques should therefore be aimed to be higher in throughput or target specific species of interest. These techniques vary from cell sorting, membrane diffusion and microfluidic based cultivation techniques (Lewis et al. 2021). To be able to study interactions between microorganisms, more effort should be put on co-isolation and co-cultivation of interacting organisms. This is all the more relevant given that some microorganisms cannot live as pure isolates because they need to be in permanent contact with one or more partner(s). Two recently discovered prokaryotic clades, DPANN archaea and CPR bacteria, seem to consist of mostly obligatory symbionts as they have small genomes and miss genes regarded essential to survive alone (Hug et al. 2016, Dombrowski et al. 2019, Jaffe et al. 2020). Co-cultivation studies would help elucidating which species these microorganisms interact with and could make it possible to study the type of interaction, whether it has a positive or negative impact on the symbiont and at the same time allow the interaction to be ecologically relevant in the environment of origin.

At the same time, exploring ways to identify microbial interactions that do not rely on cultivation are key to obtain a broader view of the dynamics within complex communities. Labelling-based techniques which have been used to study microbial exchange of compounds for decades, initially with radioactive or stable isotope probing (SIP) of DNA, offer a way to monitor microbial interactions *in situ* (Dumont and Murrell 2005, Ziels et al. 2018) (Fig. 2A). By using isotopically labelled substrates (e.g. using ^{13}C) their uptake into cells can be detected. Different labelling techniques have been developed since then, such as RNA-SIP and protein-SIP (Jehmlich et al. 2008). While on the level of incorporation of nucleic acids, the flow of a labelled substrate across different trophic levels is challenging, protein-SIP allows to quantify the relative isotopic abundances in an organism's proteome and thus to draw conclusions about trophic interactions such as predation and cross-feeding between microorganisms. Recently, an ultra-sensitive, accurate and high-throughput protein-SIP method was described that allows for precise detection of translation, measuring activity in microbial communities (Kleiner et al. 2023). The more sensitive and accurate labeling-based methods become, the more informative they are for studying complex interaction networks in microbial communities.

Another recently developed labelling method is BioOrthogonal Non-Canonical Amino acid Tagging (BONCAT) (Fig. 2A) (Hatzenpichler et al. 2014). With BONCAT non-canonical amino acid homologues, either L-azidohomoalanine (AHA) or L-homopropargylglycine (HPG), are incorporated by microorganisms as replacement of nascent L-methionine during translation. Using a click-chemistry reaction, the incorporated amino acid homologue can be labelled fluorescently and subsequently visualized. BONCAT can be combined with FISH or FACS to identify or sort out the labelled microorganisms for further analysis. BONCAT incubations can be performed *in situ* and thus inform about the physiology and activity of microorganisms directly in their native environment and they can also be monitored over longer periods of time. With this, BONCAT can in principle give indications about not only the microorganisms that directly utilize a substrate, but also those who incorporate the amino acids later in time after being stimulated via predation, cross-feeding, or by-product degradation (Hatzenpichler et al. 2020). Even though this method comes with its limitations (see Hatzenpichler et al. 2020 for details), it in principle allows for pin-pointing primary metabolic interactions between microorganisms, and for determining emerging effects on other community members.

Prediction of physical interactions

Besides investigating interactions at the molecular level (i.e. observing and measuring the exchange of primary and secondary metabolites), microorganisms can also be physically attached and directly interact with one another. In this case, uncovering the exchange of metabolites might be more difficult, but identifying the interacting partners might be an easier first step to pinpoint the type of microbial interaction, e.g. guided by available genome data. To uncover such physical cell-cell interactions, cell sorting techniques can be used. During single-cell sorting two cells that are attached to each other will be treated as one sorting event and, depending on the technique used, can later be identified by sequencing, microscopy analysis or used for co-cultivation (Lewis et al. 2021). Making use of the latter, Cross et al. (2019) were able to co-sort episymbiotic CPR bacteria while targeting its actinobacterial host using reverse genomics, a technique using fluorescently labelled antibodies that target membrane proteins that are exposed to the outside of the host cell (Cross et al. 2019). The physically interacting microbes were then dispensed into microwell plates for cultivation and genomic analyses. Besides such targeted approaches, random cell-sorting of physically interacting microbe-microbe pairs combined with micro-well cultivation or single cell sequencing techniques can uncover microbial interactions. Similarly, cells in close proximity could be co-captured in microfluidic droplets (Fig. 2B), cultured within the droplets and after sorting the microdroplets, the small consortium of microorganisms in the droplet can be taxonomically identified and further studied (Ohan et al. 2019). As the generation of droplets is gentle on the sample, this method allows to not only capture cells that are tightly attached to each other, but also cells with weaker, more transient physical interactions.

Apart from directly investigating physical interactions through the co-cultivation of physically attached microorganisms, novel methods can be used to predict physical interactions of cells (Fig. 2B). Emulsion paired isolation-concatenation (epic)PCR is a method that is used to isolate cells from each other within a mixed sample using an emulsion mixed with acrylamide suspension. However, closely associated cells are captured together inside emulsion droplets and during polymerization, the droplets turn into polyacrylamide beads and the cell walls are broken up so that the DNA becomes freely available for fusion PCR and amplicon sequencing. With that, this technique can be used to identify endo- and episymbionts (Xie et al. 2022) or microorganisms that physically interact without the need of a cultivation step. Similarly, high-throughput chromosomal confirmation capture (Hi-C) crosslinks DNA molecules that are in close proximity. Hi-C could be used to identify microorganisms that exchange DNA or are subjected to horizontal gene transfer (Yaffe and Relman 2020). Potentially, also endo- and episymbionts could be identified via this method if there is a close association between the host's and symbiont's DNA (Fig. 2B). While these methods are potentially prone to also predict randomly attached cells, both epicPCR and Hi-C allow to make large-scale predictions on physically interacting microorganisms without the need of cultivation steps, rendering them promising sequencing-based approaches to generate hypotheses on physical microbial interactions in complex samples.

Assessing emerging effects of microbial interactions

While methods to experimentally explore microbial physiology and interactions are providing an increasing amount of information on complex communities, this information, in combination

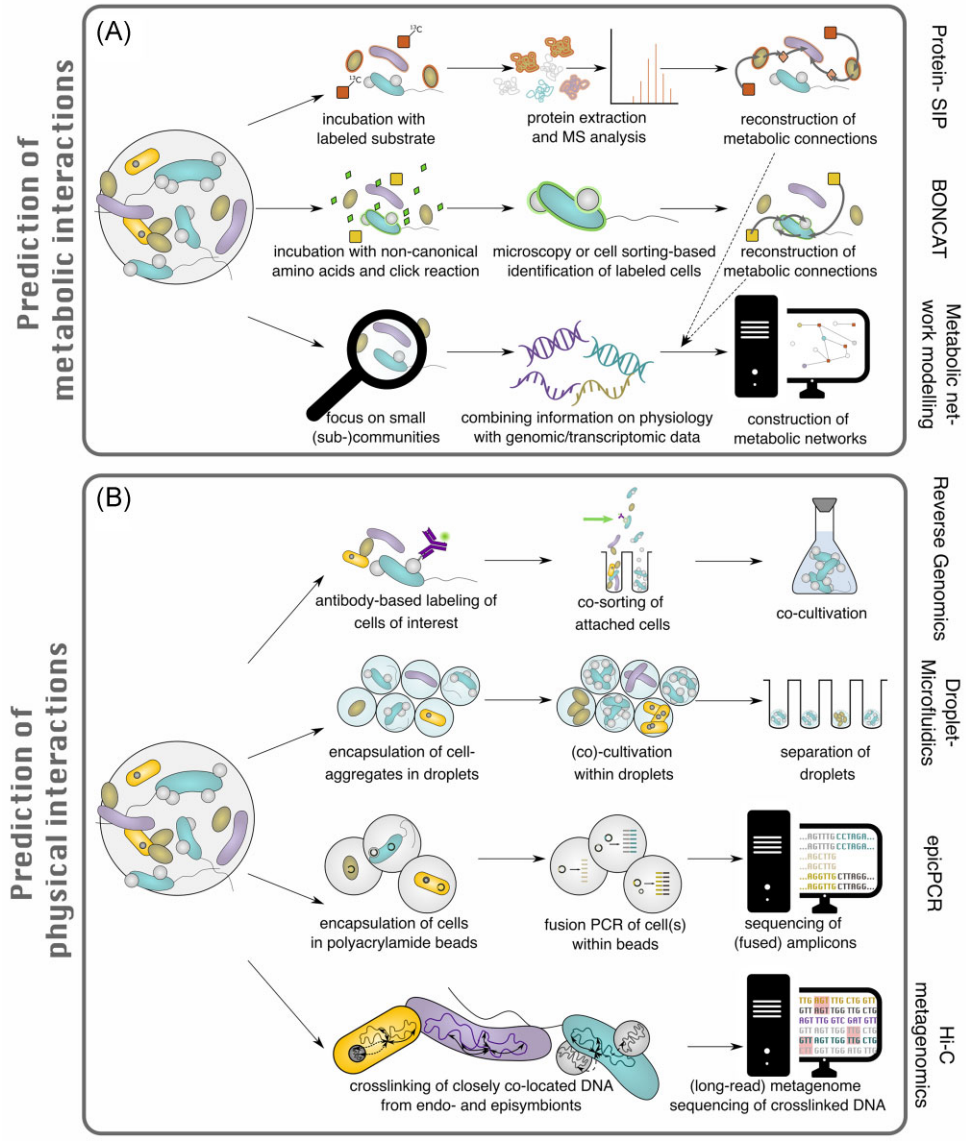

Figure 2. Examples of techniques that enable to study individual microbial interactions and their emerging effects. (A) Prediction of metabolic interactions. Protein-SIP: Communities are incubated with labelled substrates after which all proteins are extracted and analysed with mass spectrometry. Trophic interactions can be identified by following the labelled molecules. BONCAT: Communities are incubated (in situ or ex situ) with non-canonical amino acids homologues which will be built into active microorganisms. Via a bioortogonal click reaction the active cells are labelled fluorescently and identified via microscopy or cell-sorting. Active cells can be identified after different incubation time intervals to identify interactions such as predation, cross-feeding and by-product degradation. Metabolic network modelling: Metabolic network models can predict microbial interactions in communities when high quality meta-omic data combined with environmental data and data gathered from labelling experiments is used as input for metabolic network models. (B) Prediction of physical interaction via co-cultivation techniques or based on molecular signals. Reverse Genomics: Targeted microorganisms are labelled with specifically designed antibodies. Labelled cells and physically attached cells can be sorted from their community and co-cultivated together. Droplet-Microfluidics: Microorganisms in a community are encapsulated in droplets. Cells that physically interact are encapsulated conjointly. Microorganisms are (co)-cultivated within their droplet and the droplets can be separated from the other encapsulated community members for further (co)-cultivation. epicPCR: Microorganisms in a community are encapsulated in polyacrylamide beads. Cells that physically interact are encapsulated conjointly. Fusion PCR is performed on the cells in the beads and physically attached microorganisms can be identified. Hi-C metagenomics: Co-localized DNA from endo (and epi)-symbionts in a community is crosslinked, isolated and sequenced with long-read sequencing platforms. Both endo- and episymbionts can then be identified.

with genomic data, can further be used to fuel mathematical models that predict complex interactions and their emerging effects. Tools available to build genome-scale metabolic networks and community-based metabolic models are continuously being improved (Qian et al. 2021). Different mathematical models are described to predict interactions and dynamics in microbial ecosystems. Models can be either mechanistic or empirical but are more powerful when combined. This integration of multiple models is still in constant development (Qian et al. 2021), however, the main bottleneck of models and their integration is the complexity of microbial communities: The more microbial species are present in a community, the more data needs to be collected and the more dynamic and complex a model becomes (Qian et al. 2021). For this reason, synthetic communities are often designed to better control the quality and amount of information that can be fed to the models. This approach however, excludes by default all uncultured microorganisms and is thus mostly useful for well-known microbiomes such as the human gut and

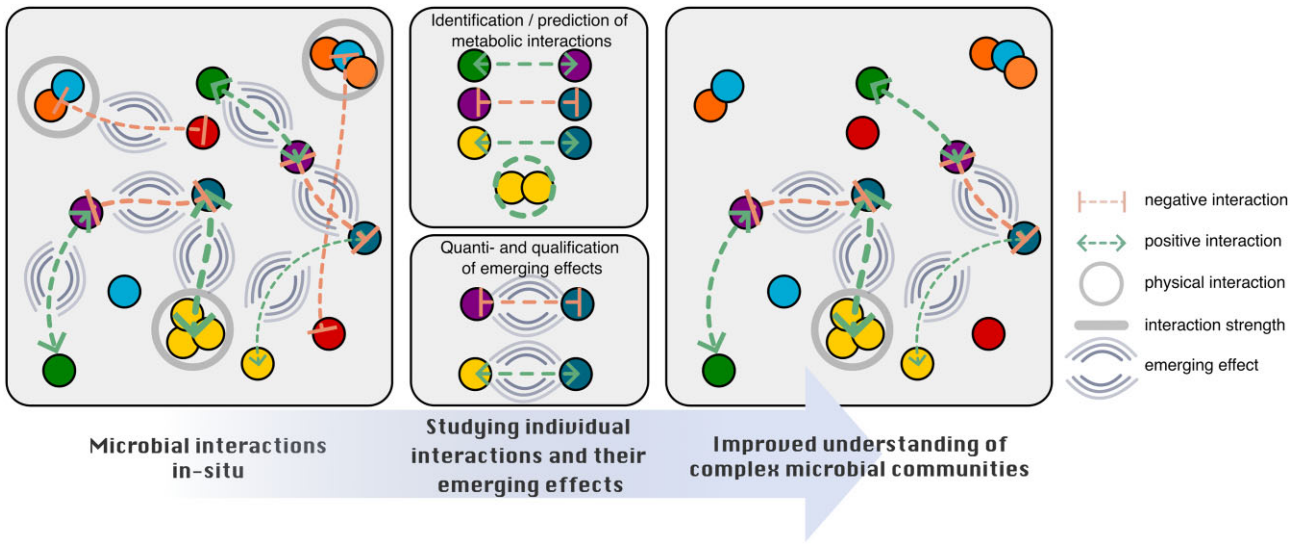

Figure 3. Suggested roadmap towards a better understanding of the impact of microbial interactions and their emerging effects on complex communities. To better understand the dynamics within complex microbial communities *in situ*, an improved understanding of individual microbial interactions using innovative approaches (see Fig. 2) as well as efforts to quantify and qualify the emerging effects that these interactions have on the entire community are needed.

plant-associated microbiomes (Hromada et al. 2021, Sun et al. 2023). Nonetheless, this controlled method offers a pathway to study general characteristics of microbial communities. By illuminating fundamental traits, it is possible to gain insights that transcend specific microbiomes, potentially shedding light on principles applicable to a wider range of ecosystems (Carlström et al. 2019). Despite its limitations in encompassing all microbial diversity, this method contributes significantly to understanding broader patterns governing these communities (Ratzke et al. 2020). For less defined microbiomes, many genomes, especially genomes of poorly characterized microbial lineages, have a large fraction of genes that cannot be properly annotated and both primary and secondary metabolic pathways remain incomplete and thus hard to integrate into metabolic models. With that, metagenomes and metagenome assembled genomes (MAGs) substantially increase the data input for new models, but the quality of such data using often incomplete and contaminated MAGs, is currently not yet optimal. Increasing the quality and level of annotation of MAGs will ensure more reliable models to predict microbial interactions. With evolving sensing technology, also parameters about the environment, such as temperature, pH and oxygen concentrations can be monitored more accurately and more frequently. High-quality (meta-) omics data, information from environmental monitoring studies, as well as data gathered from labelling experiments can later serve as input for newly developed models, that enable a better understanding of various environments. This can help to understand the dynamics of a certain microbial ecosystem and how they are impacted by the microbial interactions that are taking place (Beatty et al. 2021). Recently, a study concluded that the ecological community function often follows simple equations, enabling accurate prediction and optimization of ecological function (Diaz-Colunga et al. 2022). Coinciding, another study used community-function landscapes that could predict the community function based on species presence or absence, even without detailed knowledge of abundance dynamics or species interactions (Skwara et al. 2023). Furthermore, an additional study demonstrated that shared metabolic traits result in analogous community compositions, particular notable when substrates are metabolically alike. Their approach effectively predicts community composition in novel environments by leveraging insights from communities assembled in environments sharing similar metabolic characteristics (Vila et al. 2023). While the use of modeling to predict the effects of microbial interactions is to date still in its infancy, the rapid development of AI-based machine learning tools will certainly help to improve community-based metabolic models to predict complex interactions (Asnicar et al. 2023), and help to assess emerging effects of microbial interactions on larger scales and in different habitats (Jiang et al. 2022).

Besides modelling-based methods to predict microbial interactions and their effects, as well as methods to observe or measure species-species interactions, new methods to assess the emerging effects of these interactions are needed. For that, finding measurable indicators that reflect the state of a microbial community, are needed. Possible indicators, such as the functions provided by a microbial community could be estimated using metagenomic approaches. Instead of looking at the predicted metabolic capacities of its individuals, the "community potential", being the pool of (e.g. metabolic) capabilities present across a community, can give an indication of overall gained and lost functions in the presence or absence of specific microbe-microbe interactions. Here, the fact that generating and assessing meta-omic datasets and spatial information becomes much more feasible will help to assess the overall functioning of complex communities in nature as well as experimental settings.

Furthermore, microbial interactions do not only impact the functioning of their community but can also have an impact on the net-productivity of their community (Wu et al. 2023). This is of special relevance in communities that are used for biotechnological applications such as wastewater treatment plants or biogas facilities. In these facilities, syntrophic microbial interactions often play a key role and directly impact e.g. the yield of a certain product, the overall biomass production or the uptake of specific substrates (Mauerhofer et al. 2018). Similarly, negative interactions between competing microorganisms or the presence of predators could negatively impact individual microbes and

thereby decrease the productivity of the entire community. In engineered microbial communities, these parameters are usually easy to measure, and while the quantification of microbial growth or compound turnover rates is disproportionally harder to perform in a complex environmental setting, studies can certainly benefit from the well-established methods and knowledge of biotechnological processes to assess the productivity of natural communities as well.

The impact that microbial interactions between individual community members can have on the entire community, both positively and negatively, is incontrovertible. By affecting the environmental conditions of the microbial community, its species composition as well as the functional diversity and productivity of the community, microbial interactions can have a substantial impact. With the emerging effect of microbial interactions on various levels, it is likely that also the stability and resilience of the community, i.e. the ability of a community to bounce back to its original (functional) community composition, when facing disturbances will be affected. The impact appears most pronounced within intricate communities, as a noticeable trade-off was observed between a community's stability and its complexity (Yonatan et al. 2022). While measuring the resilience of a community used to be challenging (Shade et al. 2012) the integration of time series analyses with modeling-based approaches and metaomic studies enables scientists to assess and monitor microbial community resilience based on the their return time, degree or return rate of return and efficiency, as well as its response to changes in microbial interactions (Todman et al. 2016, Philippot et al. 2021).

Rethinking microbial interaction studies

While traditionally being tailored to investigate interactions between two already isolated microorganisms, microbial interaction studies have often neglected a large fraction of the interactions that occur in natural environments. The growing availability of multi-omic datasets, however, suggests that microbial interactions in nature are more complex than they can be depicted by classical species-species interaction studies. To understand the complex interaction networks in natural environments, as well as the emerging effects that these interactions can have on the entire community and even their environment (Fig. 3), future studies can refer to a broad set of new methods to predict, measure, test and visualize microbial interactions (Fig. 2). Clever combinations of available wet- and dry-lab methods, as well as innovative approaches to make use of already existing methods are needed to predict novel interactions. At the same time, utilizing methods to (co-)cultivate obligatory interacting microorganisms in high-throughput will not only expand the available collections of isolates for further experimental and imaging-based studies, but also allow the use of sequencing-based methods to further elucidate the genetic basis, and potentially the evolutionary history, of these microbial interactions.

While unravelling and understanding more and more interactions between microorganisms in an environment is crucial, we strongly believe that it is vital to also look beyond these individual interactions and focus on the secondary effects that they have on a community level. A concerted effort of traditional and novel methods to investigate microbial interactions, as well as an increased awareness for the presence and impact of their emerging effects on an environmental level, will greatly improve our understanding of the mechanisms underlying the dynamics in microbial communities (Fig. 3). The gained knowledge will enable us further to utilize and conserve the ecological functions and services that microbial communities in all sorts of environments provide.

Author contributions

Patricia Geesink (Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing), Jolanda ter Horst (Conceptualization, Investigation, Writing – original draft, Writing – review & editing), and Thijs J. G. Ettema (Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing).

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