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Review article

Higher-order interactions and emergent properties of microbial communities: The power of synthetic ecology

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A B S T R A C T

Humans have long relied on microbial communities to create products, produce energy, and treat waste. The microbiota residing within our bodies directly impacts our health, while the soil and rhizosphere microbiomes influence the productivity of our crops. However, the complexity and diversity of microbial communities make them challenging to study and difficult to develop into applications, as they often exhibit the emergence of unpredictable higher-order phenomena. Synthetic ecology aims at simplifying complexity by constituting synthetic or semi-natural microbial communities with reduced diversity that become easier to study and analyze. This strategy combines methodologies that simplify existing complex systems (top-down approach) or build the system from its constituent components (bottom-up approach). Simplified communities are studied to understand how interactions among populations shape the behavior of the community and to model and predict their response to external stimuli. By harnessing the potential of synthetic microbial communities through a multidisciplinary approach, we can advance knowledge of ecological concepts and address critical public health, agricultural, and environmental issues more effectively.

1. Introduction

Microbes are the earliest forms of life that emerged on Earth, and today, their descendants inhabit most habitats on the planet. During billions of years of activity, microbial presence has shaped the biosphere and is deeply intertwined with the evolution and maintenance of more complex organisms. For example, the activity of ancient photosynthetic bacterial communities capable of performing oxygenic photosynthesis ended up filling the atmosphere with molecular oxygen [*Great Oxygenation Event,* approximately. 2.4 billion years ago (BYA) [1] and paved the way for the emergence of eukaryotic cells 2-1 BYA [2–4], and later, of multicellular life 1.5–0.5 BYA [5]. In fact, eukaryotic cells appeared when two different microbes, belonging to the Bacteria and Archaea domains, developed an endosymbiotic relation [6]. In present days, microbial total biomass is the second largest in abundance after plants [7]. So, despite their small size as individuals, their impact as a whole is essential for life on Earth.

Microbial communities include diverse microbial populations that coexist and interact in a particular environment. Bacteria are major components in microbial communities, but also, Archaea and Eukarya are usually constituents of these communities. Viruses, whose reproduction depends on infecting a host, are the most numerous of the microbes and are considered important components of microbial communities. Even protozoa and micrometric animals, such as nematodes, rotifers, and tardigrades are part of such communities. Different communities drive many processes in their respective environment. For instance, trillions microbial cells inhabit different compartments of animal gut, forming the gut microbiome. These microbes influence a wide range of metabolic functions, thereby playing a crucial role in determining health and disease outcomes. In humans, the composition of the microbiome has been

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linked to obesity, Alzheimer's disease, Parkinson's disease, and colon cancer, among other illnesses [8]. Some herbivores rely on the cellulolytic activity of their gut microbiome to digest food. Microbes living in the soil form associations with plant's roots and affect their growth and health [9]. Aquatic environments are rich in microbial life that drives oxygen production and the biochemical cycling of elements, such as carbon, nitrogen, sulfur, and iron [10–12]. Therefore, the ecology of microbial communities is key to understanding processes that operate at a global scale, and how human activity impacts these processes.

The function of a microbial community results from intra- and inter-species interactions among the populations that constitute the community. Since the explosion of *-omics* technologies, the revelation of the vast diversity of microorganisms that can exist in a given community has significantly pushed forward the field of microbial ecology. However, this new knowledge opened the challenge of understanding the intricate interactions between members of the community and the continuous feedback from the environment, that is also being transformed by the community. One solution to this task is to simplify or build microbial communities in controlled environments in which their behavior could be studied in fine detail. The knowledge obtained from these experiments can help understand how microbial communities shape the environment and how they can be applied to improve biotechnological processes [13], agriculture [14,15], health [16], food safety [17,18], water treatment [19,20], and even to ameliorate the environmental changes derived from human activity [21,22].

Synthetic communities refer to simplified communities created by artificially mixing axenic populations of microorganisms [23]. Using synthetic microbial communities has become a trending and growing strategy to understand how complexity emerges from simplicity in microbial ecology and to apply microbial consortia's capabilities. The published research on synthetic communities grows considerably every year (Fig. 1). Although synthetic communities may sometimes be far from representing a natural microbial community, understanding which community traits depend on the ecological context and which are driven by a few important key species is a knowledge gap that can be tackled with these research tools.

The present review has two main goals: First, to present examples of model synthetic microbial communities, what has been learned from them, and underscore the importance of moving from pairwise interactions to higher-order interactions. Second, to serve as a guide to select methodological and modeling strategies to build and study synthetic communities (for further information, a few selected reviews on specific topics can be found on Table S1).

2. From natural to synthetic

The natural environment encompasses a wide range of gradients of physicochemical components such as temperature, oxygen, salinity, pH, nutrients, and antibiotics, to name a few. These gradients are dynamic, so the high degree of possible conditions at any given time and space gives rise to a wide array of niches. Different organisms have evolved to occupy these niches and can overlap, coexist, and interact with other species, forming diverse and complex communities in which redundancy provides stability and robustness towards perturbations. The biotic and abiotic interactions promote auto-organization in composition, time, and space; this process is known as community assembly. Different factors affect community assembly and can be grouped as dispersal (immigration and emigration of species between communities), selection (environmental factors that promote fitness advantages), speciation/diversification (accumulation of mutations that result in new species and ecotypes), and drift (stochastic changes in community structure) [24]. As these factors change, the composition of microbial communities also undergoes changes in a continuous cycle that may lead to different community states (Fig. 2) [25,26].

The diversity of physicochemical conditions and biotic interactions that drive community assembly result in various ecosystems (Fig. 3). Some of these unique microbial ecosystems can be useful to understand fundamental aspects of life. A remarkable example is that of microbial mats [27–29] and microbialites [30–32], which are some of the earliest complex ecosystems that evolved as far back as 4.2–3.4 BYA [33,34]. These communities develop a layered spatial structure that depends on physicochemical gradients generated by the community itself. In the case of microbialites, the layers can mineralize and grow into macroscopic structures that resemble corals. Nowadays, microbial mats are widespread in sediments of aquatic environments and active microbialites can still be found

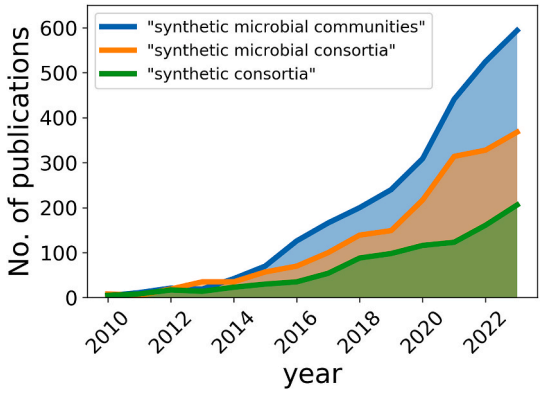


Fig. 1. Frequency of publications on microbial synthetic ecology in the last decade. The data was obtained by searching the terms in the legends in Google Scholar.

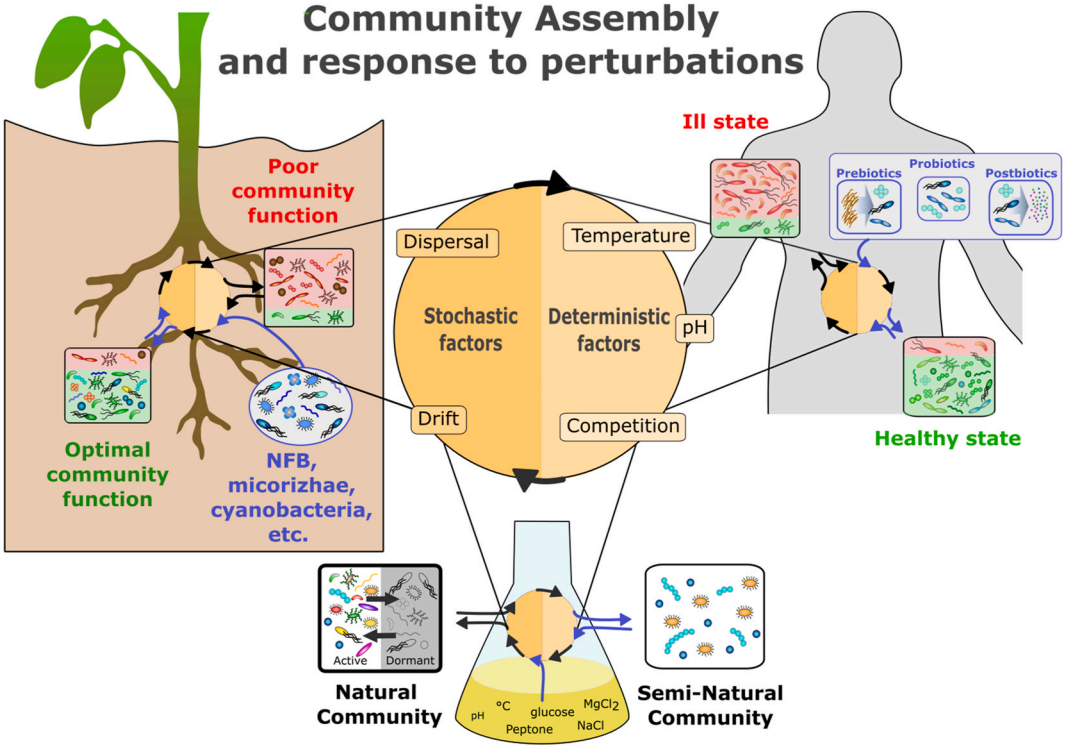


Fig. 2. The Dynamic Process of Community Assembly. Community assembly is a dynamical process influenced by deterministic and stochastic factors. Some factors can be abiotic, such as pH, temperature, salinity, and oxygen; or biotic, such as species interactions, selection, and speciation. As these factors change, microbial communities change their structure in response, leading to a continuous cycle of assembly. In the case of gut microbiota, an unhealthy community structure has been associated with various illnesses, such as diabetes, gut cancer, and inflammatory bowel disease. However, treatments with prebiotics, probiotics, and postbiotics can induce assembly into a healthy structure, promoting overall gut health. Similarly, plants establish symbiotic relationships with microbes, and environmental factors such as intense land usage, nutrient depletion, and pollution can lead to poor functional soil and rhizosphere microbiomes, resulting in suboptimal conditions for plant growth. However, the addition of beneficial microbes to the soil, plant roots, and seeds can result in better crop growth and yield by restoring microbial communities. Growing natural microbial communities in the laboratory is challenging due to the potential changes in community composition and function that can occur when culturing samples under imposed artificial media and conditions. These changes can cause the resulting microbial community to differ significantly from the original community in its natural environment, making it difficult to accurately represent the structure and function found in nature.

living in a few places [35]. Microbial communities can also thrive in extreme environments [36,37] such as underwater chimneys, thermal waters, or in the cold Antarctic deserts. The study of these communities offers insights into life's evolution, development, and adaptability, and make it possible to hypothesize on how life could also emerge in other planets [32]. Microbial communities also associate with plants [38–40], fungi [41], and animals [42–44], and are an essential component in soil [45,46]. These communities have been used as models to study the natural state of the system and many types of measurements can be performed directly *in situ*; however, the low accessibility and the impossibility of carrying out controlled experiments or test hypotheses under the unstable conditions of the natural environment is a significant limitation for generating a mechanistic understanding of community assembly and function.

Researchers strive to create suitable laboratory conditions that emulate the natural environment to enable the growth of previously unculturable strains [47,48]. While most microbial species are still considered non-culturable [49], there are many culturable strains spanning various branches of the microbial tree of life, which makes it possible to at least study a fraction of the microbial diversity under controlled conditions. A sample of a natural community that is inoculated in a well-defined growth environment will inevitably change; some taxa will be enriched while many others won't grow under the new imposed artificial conditions. After the assembly period, the resulting semi-natural community will differ from its natural counterpart [50–52], but it will also maintain some of its complexity (Fig. 2, bottom). For example, Reid et al., conducted a study to investigate the role of sulfate-reducing bacteria in arsenic methylation by cultivating them in the presence of arsenite, thus enriching microbial populations capable of metabolizing this compound and making it possible to identify which taxa participates in such function [53]. Finally, Winogradsky columns, are one of the oldest and most remarkable strategies for the cultivation of microbial mats in an artificial media inoculated from natural samples, enabling them to mimic the metabolic complexity and stratification observed in natural microbial mats [54].

Host-associated microbiomes offer an opportunity to retain some of the properties of the natural community. The host creates the required conditions to sustain its microbiome, so if it can be maintained *ex situ* in controlled conditions, it will be easier to access its

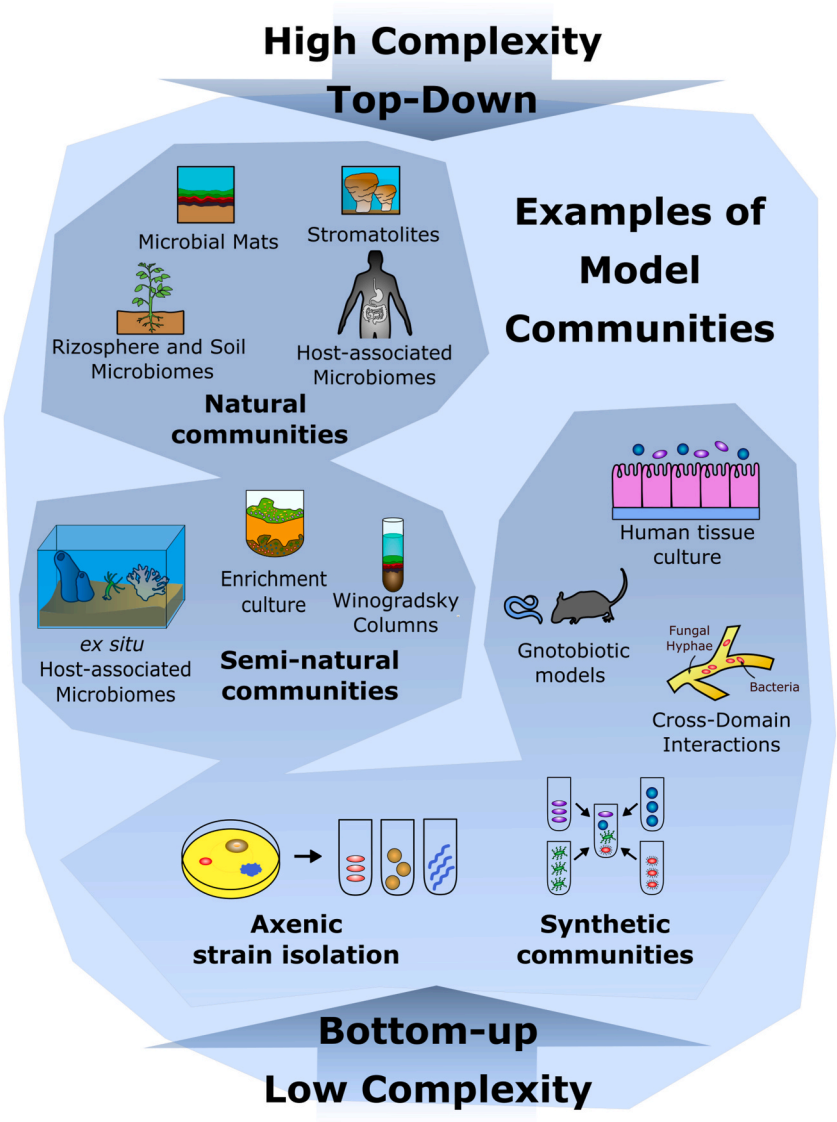


Fig. 3. The Spectrum of Microbial Ecosystems. Microbial communities can be categorized into natural, semi-natural, and synthetic, forming a continuum of states in-between. Examples of natural communities include microbial mats and stromatolites, which are among the oldest examples of complex ecosystems on Earth. The human gut microbiome is extensively studied for its relevance to human health, while soil and rhizosphere communities have a significant impact on agriculture and every other life form on Earth. Semi-natural communities, that develop in mesocosms, Winogradsky columns, and host-associated microbiomes offer practical options for studying complex communities with limited accessibility. The construction of synthetic communities from axenic cultures, offer a systematic approach to study the emergence of community behavior and interactions networks in highly controlled setups with varying levels of complexity.

microbiome. While some host species could be challenging to maintain *ex situ*, there are multiple examples of host-microbiome models that are simple in structure and that can be maintained artificially, such as sponges [55], hydras [56], and duckweed [57] (Fig. 3). Despite the increased representability of semi-natural communities, they can still be too diverse and complex for a mechanistic understanding of their emergent properties.

The logical step to simplify microbial communities further is by reverse-engineering natural microbial communities from their isolated components, in what is known as the bottom-up approach (Fig. 3). Although it's not yet possible to reach high levels of complexity, the use of synthetic communities has been widely applied to gain insights in many aspects of community behavior, such as responses to biotic/abiotic variables, to test and propose ecological theories and to increase community functionality for biotechnological applications. Some examples of synthetic communities are discussed throughout this review, but a larger comprehensive list of examples is shown in Table S2.

In what may have been the first example of a synthetic microbial community competition experiment, Gregory Gauss used two species of yeast and experimentally probed the competitive exclusion principle, which states that two species competing for the same resource cannot coexist [58–60]. In a more recent example, Ehsani et al., built a 10-member synthetic community to test the effect of initial evenness on community assembly. He found that the final community structure at the genus-level was similar regardless of initial evenness [61]. In a simpler set of 2- and 3-member synthetic microbial communities, Meroz et al., tested community stability at an evolutionary timescale (400 generations), concluding that what seemed to be a 'stable' community at short time windows, changed over longer timescale, but in a robust fashion rather than random divergence [62]. These studies suggest that communities follow some

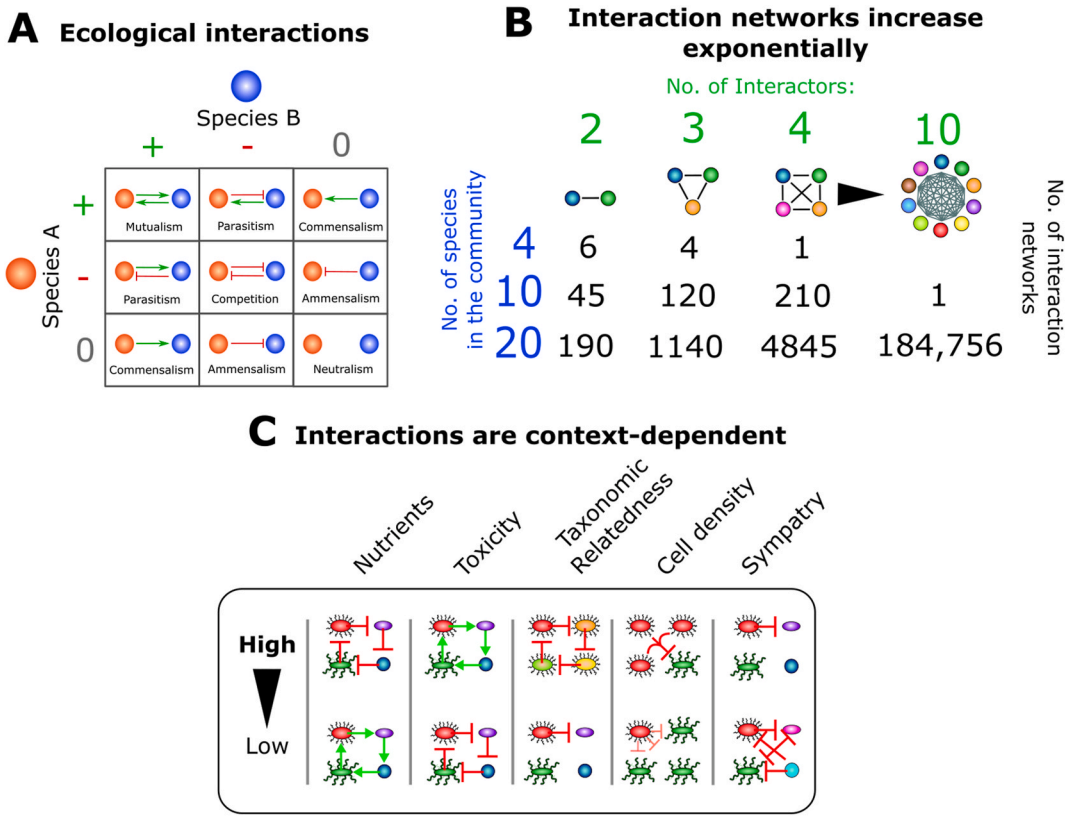


Fig. 4. Exploring the Complexity of Interactions: networks, properties, and patterns. A) Ecological interactions can be classified into six types: Mutualism, Competition, Commensalism, Ammensalism, Parasitism, and Neutralism. When taking directionality into account, nine possible interactions can occur between any two species. Positive effects are represented by green arrows while negative effects are represented by red arrows. B) As the number of interacting species in a community increases, the complexity of interaction networks also increases rapidly. This makes it challenging to design and characterize all possible interaction networks in synthetic communities. An example is shown for the possible interaction networks in communities with four, ten, and twenty species. C) Under stressful conditions, such as low nutrients or high toxicity, cooperation is promoted, while competition is observed under less stressful conditions. The prevalence of interference competition is thought to positively correlate with taxonomic relatedness. The strength of the antagonistic effect is cell density dependent. Pairwise antagonism assays between isolates from the same sampling site (referred to as "high sympatry") show less antagonistic interactions than those from different sampling sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

deterministic rules while assembling, and not just depend on the initial conditions of the system.

Synthetic communities also serve as tools to investigate the contribution of individual members to community-level functions. For instance, Sanchez-Gorostiaga et al., (2019) investigated amylase degradation in a seven-member community consisting of six *Bacillus* species and *Paenibacillus polymyxa*. Their results showed that the presence of *P. polymyxa* increased the amylolytic activity of the community through strong pairwise and higher-order interactions, unaccounted for in a null model that assumed additive contribution of the species [63].

Synthetic communities can be incorporated into host-microbiome systems by inoculation into germ-free hosts (also referred to as gnotobiotic), where the native microbiome is eliminated by a given treatment. This strategy adds a new complexity level bringing synthetic communities to a setup that is closer to their natural habitat. Synthetic communities assembled from duckweed (*Lemna minor*) associated isolates, have demonstrated structural stability and resemblance to the original duckweed microbiome. This shows that, under certain conditions, synthetic communities can successfully emulate their natural counterparts [57] in the case of hosts with a simplified microbiota.

Even though it is not yet possible to go from the isolated parts of a community to the complexity that exists in nature, general properties are being uncovered and new hypotheses are being proposed and experimentally tested by using synthetic microbial communities.

3. Types of microbial interactions

In microbial communities, a great variety of species cohabit and engage in diverse interactions. Historically, studying microbial interactions primarily focused on pairwise relationships, providing the foundation for understanding the dynamics of microbial communities. These pairwise interactions can result in positive (cooperation), negative (competition) or neutral outcomes for the involved species, with the latter occurring less frequently [64,65] (see Fig. 4A).

3.1. Cooperation

Various types of cooperative strategies exist among different organisms. Facilitation, which encompasses commensalism and mutualism, is the most prevalent form of positive interaction. Facilitation occurs when an organism creates a more favorable local environment, such as by alleviating nutrient stress through nutritional symbiosis (mutualism) or indirectly by reducing competition or predation pressure (commensalism) [66] (Fig. 4A). For instance, when organisms exchange metabolites that can be used for growth, it is referred to as cross-feeding [67]. In aquatic microbial communities, certain bacterial species produce metabolites essential for the growth of species that do not produce them (known as auxotrophs) [68,69]. This cooperation suggests that auxotrophy links community members through a complex web of metabolic interactions [69] (Fig. 4B). Additionally, there are examples of bacterial commensal interactions, such as *Pseudomonas putida,* which requires the presence of *Acinetobacter* spp. for biofilm production when growing on benzoate. A mutant of *P. putida* strain with an increased capability of attaching to *Acinetobacter* spp. exhibits an increased overall growth in the co-culture biofilm [70].

Cooperation that evolves between two species and that is preserved in nature because of their benefit to one another is expected only under very restrictive conditions. In fact, it has been suggested that negative interactions prevail in microbial communities [71,72]. The scarcity of positive interactions between species can be explained by competition for the same resources between species in communities [73]. Additionally, diversity results in numerous new and sporadic interactions that may not confer evolutionary advantages [74–76]. Nonetheless, there is now evidence that cooperation plays a more important role than previously believed [70,77–80].

Positive interaction can be used to assemble synthetic communities with higher yields of productivity. For example, in the yeast *Saccharomyces cerevisiae*, a co-culture of complementary auxotrophic strains rarely engages in syntropic interaction. By high-throughput screening of a large *S. cerevisiae* knock-out collection, some strains were identified that spontaneously formed syntropic interactions. Then, by splitting parts of the biosynthetic pathway of malonic semialdehyde (precursor of biodegradable polymers) among both strains, the production of malonic semialdehyde was higher compared to each strain holding the complete pathway [81]. These findings were later expanded to propose a biotechnological toolkit from the identified syntropic pairs [82].

3.2. Competition

The most studied interaction between bacteria is competition (Box 2). During competition, both organisms suffer a decrease in their fitness (ability to survive and reproduce) or die [83]. Competition can be classified into two types: exploitative competition (competition for resources) and interference competition (competition through active mechanisms i.e., antagonism) [84,85]. See Table S2 for examples.

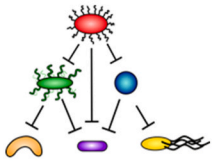
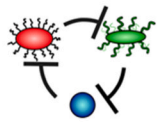
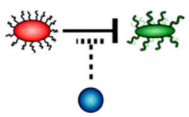
Resource or exploitative competition is indirect, since microbes that utilize nutrients more efficiently can limit their availability for other species. Bacteria capable of forming structures like biofilms fall into this category. Biofilms are structures that allow

microorganisms to anchor to surfaces and restrict nutrient availability to neighboring communities [86]. Another example of exploitative competition is the production of siderophores, molecules that sequester iron from the environment, thereby limiting its assimilation by other species [87].

In contrast, competition by interference is direct. In this case, a microbe produces a toxin, antibiotic, or compound that inhibits growth or kills the competitor. Extensive literature exists on competition by interference or antagonistic interactions [88–96]. Competition by interference is usually associated with microbes capable of producing antimicrobial compounds like antibiotics. Nonetheless, antagonism appears to be context-dependent (Fig. 4C). For example, a higher degree of antagonism is expected between closely related species or strains [92,94,97], possibly due to similar metabolic requirements between close kin, which may favor competition. The stress-gradient hypothesis states that stressful conditions will promote cooperation over competition [98], and this has been observed in synthetic microbial communities [99,100]. Interestingly, despite their role in bacterial warfare, antibiotics can also function as signaling molecules [101–103].

3.3. Higher-order interactions in microbial communities

In recent years, there has been a growing interest in studying complex microbial models, which has led to the discovery of new community properties that were not apparent when considering only pairwise interactions [104]. By expanding the number of strains involved in these interactions, researchers have gained insights into the complexity of microbial communities. Moving beyond pairwise interactions, the introduction of a third, fourth, or more interactors introduces a level of unpredictability to the dynamics [105] (Fig. 4B). In these more complex scenarios, higher-order interactions (HOIs) emerge (Box 2), influenced by the occurrence of indirect effects during the interactions (Fig. 5). This highlights the significance of considering interactions beyond pairwise relationships and sheds light on the complexity of microbial communities. Several microbial models involving three or more species have provided valuable insights into these complex interactions [96,99,106–111].

Competition	Dynamics	Examples	Network
Transitive: Transitive competition would occur when there is a direct and hierarchical interaction. For example, when species A is a better competitor than B (denoted here as A > B) and C (A > C), while B is better than C (A > B > C) (Gallien, 2017).	**Interaction chain:** Emerge when pairwise competitive interactions are embedded in a network (still pairwise) interactions. As in a trophic cascade, the indirect effects that result arise from changes in the density of a third (or further) species that interacts with both species of the focal pair (Levine, *et al.*, 2017).	Pérez-Gutiérrez, *et al.*, 2013; Wright & Vetsigian, 2016, Friedman *et al.*, 2017	
Intransitive: Intransitive competition occurs when the competitive superiority of species is not strictly hierarchical and not always direct. Arise for instance when the impact of one species on another requires or is modified by the presence of a third species (Wootton 1994).	**Cyclic interaction (rock - paper - scissors model):** Occur when species A > B and B > C, C is a better competitor than A, which generates an intransitive loop of competitive interactions A > B > C > A (Gallien, 2017). Although the interactions between the species remain fundamentally pairwise, the stabilized dynamics emerge from stringing these pairwise interactions together, so that changes in density propagate through the network to form a negative feedback loop that counteracts the initial perturbation (Levine, *et al.*, 2017).	Kerr, *et al.*, 2002; Kirkup & Riley, 2004	
	Higher-order interactions: Higher-order interactions emerge only in networks of three or more species. Such interactions do not necessarily stabilize, and can in fact destabilize, coexistence. The effect of one competitor on another depends on the population density of a third species. In a higher-order interaction, one competitor immediately modifies the competition between another two (for example, by changing individual traits) (Levine, *et al.*, 2017).	Lozano, *et al.*, 2019; Piccardi, *et al.*, 2019	

HOIs in microbial communities can lead to different outcomes, from coexistence to antagonism. For instance, one competitor can modify the dynamics between other interactors in a beneficial way, resulting in coexistence or growth promotion [99,108,112] (Fig. 5B). In this sense, the use of a four-species bacterial community that is able to coexist in media with a toxic concentration of

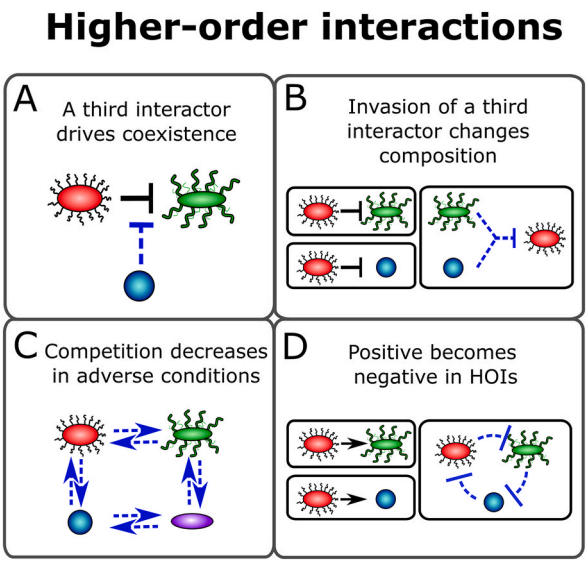


Fig. 5. Higher-Order Interactions Topologies Alter Species Dynamics. A) Indirect interactions between two species can be modified by the presence of a third interactor. B) The negative impact of an invasive species on two other organisms can be mitigated or avoided if those organisms are together. C) Adding more species to a community can increase the potential for coexistence in an environment where some species cannot survive alone. D) The addition of a third species can reverse a positive interaction, leading to a community with competition.

metal-working fluids through the degradation of this compounds, allowed the identification of the key member that performed toxin degradation, enabling the growth of the other species only when the four where together and modifying previous interactions (which also exemplifies facilitation) [99] (Fig. 5C).

Narisawa et al. (2008), Lozano et al. (2019), Gallardo-Navarro & Santillán (2019), and Aguilar-Salinas & Olmedo-Álvarez, (2023) reported examples where antagonist pairwise interactions change when a third species is involved (Fig. 5A). These studies explore three-species communities comprising an antagonist, a sensitive, and a resistant species. The presence of the resistant strain diminishes the antagonistic effect on the sensitive. Interestingly, Aguilar-Salinas & Olmedo-Álvarez (2023) focused on a three-species model involving species belonging to the phylum Bacillota (formerly Firmicutes), where the resistant strain was *Bacillus cereus*. Notably, Lozano et al. (2019) also showed that a *B. cereus* strain buffers an antagonist interaction [108]. Thus, the same HOI dynamics emerge across different experimental models and conditions, even when different mechanisms are involved (such as structural changes, exploitative competition, or resistance to antagonist compounds).

HOIs can also result in negative modification to community dynamics, as addition of a third interactor can lead to adverse effects. For instance, Abrudan et al. (2012) reported that different strains of *Streptomyces*, which exhibited coexistence in pairwise interactions, engaged in antagonism in the presence of a third strain [113] (Fig. 5D).

Recognizing HOIs as a vital component of microbial communities sheds light on how community dynamics stabilize and facilitate robust coexistence among species, considering their interactions with both other species and the environment [114]. Interactions within communities involve organisms at different taxonomic ranks and understanding HOIs helps explain these interactions. An attractive model of cross-domain HOIs is that of Mickalide & Kuehn (2019), which involves an *Escherichia coli* strain, the algae *Chlamydomonas reinhardtii*, and a ciliate *Tetrahymena thermophila*. *E. coli* can invade either the algae or the ciliate populations individually, but not when they co-occur. This model stands out because, unlike other HOIs examples, the influence or modification of the interaction occurs in two directions: the algae prevent *E. coli* from invading the ciliate population, and at the same time, the ciliate prevents the invasion of the algae population [112].

HOIs can manifest in very different dynamics that help or hinder the species engaged in them. These described HOIs contribute to the complex features observed in synthetic communities with a modest increase in diversity of just one or two species. These also shows how difficult it would be to identify HOIs involving more than four species and opens the challenge of uncovering to what extent HOIs can manifest in synthetic microbial communities.

3.4. Rock-paper scissors: transitive and intransitive interaction in the study of microbial interactions

The various competition dynamics described for HOIs include “transitive interactions” and “intransitive interactions” (Box 1). Transitive interactions involve direct competition, where species A outcompetes species B and C, and species B outcompetes species C (A > B > C) [115]. This dynamic resembles a trophic network with hierarchical relationships characterized by one-way interactions. In contrast, “intransitive interactions” are not strictly hierarchical and need at least three interactors (i.e., A > B > C > A). In this case, the impact of one species on another can be indirectly modified by a third species [116]. The Rock-Paper-Scissors model serves as a foundational example of intransitive interactions and is widely known [117].

Synthetic ecology - Synthetic systems are tools that help understand natural biological systems by constructing them, synthetic systems can be simplified to allow experiments that would be too difficult to interpret if done in their natural context [205]. Compared with individual organisms, synthetic systems can also resist losses in function as a result of environmental perturbation or invasion by other species [206].

Resistance/Resilience - Resistance can be considered the degree to which a system is able to cope with a disturbance or stress, is the inverse of sensitivity [207,208], meanwhile resilience has been defined as the rate at which a system return to its original state after a disturbance, also referee as *community recovery* [208].

Competition - One of the earliest definitions of competition was introduced by Elton and Miller (1954), which includes the negative effects of one interactor on another by reducing reproductive efficiency or increasing the mortality. But this definition only considers interference competition (by the effect of a metabolite) and does not consider the exploitative competition (for resources) [84,85,209].

Higher-order interactions - Higher-order interactions (HOIs) occur when the interactions between species are no longer fundamentally pairwise [105]. The presence of HOIs in ecological communities evidence that the dynamic behavior of a community cannot be predicted based on observations of interactions between pairs of species in isolation, implying that simple models are inadequate to predict the dynamic of complex communities [210].

Emergent properties - In bacterial communities, certain interactions drive the dynamics of these complex systems. In laboratory settings, when studying bacteria at a high level of organization, we can observe characteristics not identifiable by analyzing the component organisms in isolation. These are the so called “emergent properties” of a biological system [211].

Key concepts and definitions [205–211].

The Rock-Paper-Scissors model involves three *E. coli* strains: one strain produces colicin (C), which inhibits the growth of a second strain (S), while the third strain is resistant to colicin (R). This interaction can be represented as a network, where C displaces S through interference competition, S displaces R (due to growth advantage), and R displaces C (also due to growth advantage).

Kerr conducted confrontational tests and *in silico* simulations to demonstrate that the intransitive model leads to coexistence of the three strains in a local and structured environment [117]. This model has been utilized to study bacterial communities and is considered a significant example of bacterial dynamics within complex communities. Although the Rock-Paper-Scissors model primarily involves pairwise interactions, stability within the community is observed only in structured environments, with stability being lost in unstructured environments [117]. Despite its simplicity, this model laid the groundwork for subsequent models and studies [118].

4. Methods to study microbial communities and interactions

Commonly used methods for microbial ecology can be broadly categorized into culture-based, nucleic acid-based, and function-based approaches (see Fig. 6). Each category and its specific technical basis offer advantages and disadvantages. For instance, culture-based methods are limited to microbes that can be cultivated in laboratory media, excluding a significant portion of taxa. Nonetheless, pure cultures enable a profound understanding of microbial physiology and behavior. Nucleic acid-based methods give a complete picture of which microbes are present in a sample, but do not distinguish between genetic information derived from dormant, viable or even deceased microbes. Function-based methods alone measure the net productivity of the community but cannot provide insights into which specific taxa contribute to these functionalities or to what extent. In practice, these three approaches are often used in combination to overcome the trade-offs, allowing for a more comprehensive understanding of microbial ecology.

4.1. Culture-based methods

The ability to culture and isolate microbes in the laboratory has been the backbone of microbiological research, and these methods remain highly used in modern microbial ecology. In fact, different approaches have been developed to culture microorganisms through “culturomics” [119]. Microbes can be grown in both liquid (unstructured) and semi-solid (structured) media. Spatial structure is a key driver of community interactions and dynamics as it physically limits contact between microbial populations [120–122]. Competition for space is an important aspect of microbial behavior that can be studied through culture methods by analyzing the expansion of

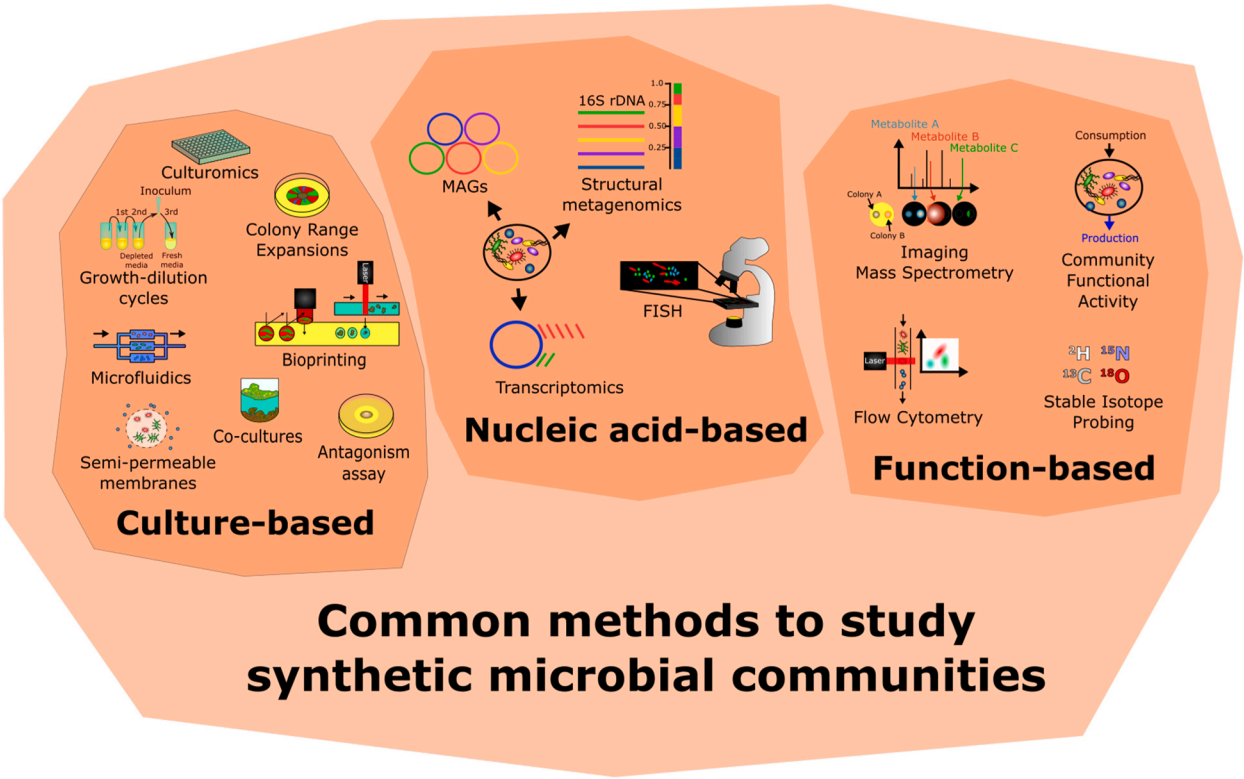


Fig. 6. From Cultivation to Sequencing: Methods to Study Microbial Interactions and Community Dynamics. Microbial interactions and community dynamics can be studied using various techniques, which can be broadly classified into three categories: culture-based, nucleic acid-based, and function-based methods. Culture-based methods involve cultivating microorganisms in laboratory conditions to study their behavior and interactions. Nucleic acid-based methods utilize DNA and RNA molecular technologies to identify and quantify microorganisms. Function-based methods assess the metabolic activity of microorganisms in a community, such as measuring enzyme activity or substrate utilization. Examples of commonly used techniques within each category are shown.

mixed colonies (range expansions) made of fluorescently labeled strains [123–127].

Growth-dilution cycles are used to re-supply nutrients and monitor the community for long periods of time. These cycles consist of growing a multi-species microbial culture, homogenizing, diluting, and seeding it for a new culture cycle. Between each cycle, a subsample of the culture can be analyzed by means of plating, DNA profiling, and mass spectrometry. It has been observed that after several cycles, microbial communities can reach ecological stability by either coexistence [128] or competitive exclusion [129]. A drawback for this method is that serial bottlenecks are being applied, and it can be argued that the community dynamics result from stochastic sampling between cycles, but this effect can be avoided using chemostats.

One of the current challenges is to develop synthetic communities with a higher number of species, interactions, and growth conditions to screen trough them fast and select adequate synthetic communities for a given purpose. High-throughput methods allow automatization and a high number of simultaneous tests [130].

The use of microfluidics has become widespread in the microbial ecology toolkit. It offers several advantages like miniaturization, finely controlled geometry, a wide array of possible configurations and the advantage of coupling with standard microscopes for observation [131–135]. For example, Gupta et al. (2020) explored contact-independent interactions or chemical communication in the absence of cell-cell contact between two strains of *E. coli* as a function of microscale distance. Said et al. (2020) took advantage of the design flexibility to investigate microbes unable to coexist through a modular approach, wherein microbes are cultivated in connected chambers and in which the imposed flow prevents undesired chemical interactions. This approach bears significance for potential biotechnological applications [135].

Bioprinting has gained significant traction as a collection of techniques enabling the precise deposition of microbes onto surfaces. One notable approach by Ceballos-González et al., involves controlled turbulent mixing of two “inks,” each containing a different microbial population. This method facilitates the control of the mixing patterns of both inks, thereby enabling to stamp multiple replicates of microbial communities. At low mixing conditions, two patches emerge, separated by one interaction front between both populations. At higher mixing conditions, multiple finer clonal patches emerge [136].

Alternative bioprinting strategies utilize laser-induced droplet formation for microbial isolation. Through this approach, a laser pulse induces the creation of small droplets from pre-treated samples, effectively encapsulating the microbes within [137]. These encapsulated microbes can subsequently be subjected to analysis or cultivation. Notably, this technique has proven effective for

sampling soil microparticles. A key advantage lies in the minute focal point of the laser, allowing the sampling of microhabitats without perturbation of the sample. Given that microbes predominantly inhabit micrometer-scale spaces [138–141], this would be a coherent sampling approach to avoid disruption of the native spatial structure. Bioprinting has demonstrated its efficacy in isolating a broader range of diversity compared to classical techniques, including rare or typically non-culturable taxa [142].

4.2. Nucleic acid-based methods

DNA and RNA-based sequencing methods provide valuable information on the presence and relative abundances of microbes and their functions. These methods circumvent the need to grow the microbes in the laboratory. For instance, Pacheco and Segre (2021), evaluated the intricate interplay of environmental compositions in a high-throughput manner to discern its impact on the growth yield and taxonomic configuration of synthetic microbial communities growing in up to 32 different carbon sources. By employing 16S amplicon sequencing, they successfully delineated the final community structure. Intriguingly, species thriving individually under specific nutrient conditions exhibited reduced growth when co-cultured with others under the same conditions, hinting at competitive interactions. On the other hand, species that coexisted in single carbon conditions may not coexist under multi-carbon conditions. Finally, nutrient complexity did not necessarily positively correlate with community diversity [143].

High-throughput sequencing data not only provides insights into microbial community and genetic structure but can also reveal potential community interactions. By comparing data from multiple sampling site, patterns of “avoidance” or “companionship” between taxa can be found. For example, the absence of a certain taxon when another is present could indicate antagonism or competition between the two. Borrowed from macroecology, co-occurrence patterns have proven useful in microbial ecology studies enhancing our understanding of community dynamics [144–147].

With the constant improvement and lower costs in sequencing technologies, it is now easy to sequence whole genomes from environmental samples, providing insights into the functional capabilities of microbial communities by analyzing its genetic repertoire [148]. For example, Thaumarchaeota is an abundant and ubiquitous phylum of Archaea that plays critical roles in the global nitrogen and carbon cycles. Through the utilization of Metagenomic-Assembled Genomes, Reji et al. (2020) were able to identify a marine Thaumarchaeota clade that lacked both ammonia-oxidizing and carbon-fixation pathways [149], highlighting the power of metagenomics for uncovering important features of natural communities.

Genes and genomes allow the identification of certain taxa, but they do not provide information of how these taxa contribute to the functional pool of the community. Metatranscriptomics reveals which genes are actively transcribed at a given moment, offering insights into the functional pulse of microbial communities. For instance, the study conducted by Arora-Williams et al. (2022) used metatranscriptomics for elucidating the functioning of microbial communities within aquatic dead zones (hypoxic regions in oceanic environments). Their findings revealed a positive correlation between hypoxia and various functions, including sulfur metabolism, denitrification, anoxygenic photosynthesis, and methanotrophy. They also employed metagenomics to deduce the probable taxa responsible for carrying out these functions. As oceanic dead zones become more prevalent nowadays, connecting shifts in community functions and composition will serve to understand how the biogeochemical cycles could be affected in the future [150]. A limiting factor of metatranscriptomics is that there are still many transcripts whose function is not yet characterized.

Apart from methods that rely on sequencing, Fluorescent *In Situ* Hybridization (FISH) offers an approach for identifying and localizing specific microbial species or groups within a sample. In FISH, a short, single-stranded nucleic acid probe is designed to be complementary to a specific target DNA or RNA sequence. The probe is labeled with a fluorescent dye and can be visualized and/or quantified under a microscope. By using probes labeled with different fluorophores and specific sequences, it becomes possible to detect multiple microbes simultaneously. FISH has been extensively used to study microbial distribution at the microscale in natural habitats [151–153] and controlled environments with synthetic communities [154].

4.3. Function-based methods

Community function is the result of the chemical reactions and genetic activation/repression within a microbial community. These interactions span cooperative or competitive dynamics, and higher-order/emergent properties shaping the overall behavior and stability of the community. Different parameters can be used to measure community function, including biomass generation, metabolic activity, enzyme production, biofilm formation, among others. By measuring these parameters, researchers can gain insights into the assembly and overall activity of microbial communities and how they respond to different environmental conditions.

Function-based methods allow us to assess the metabolic capabilities of microbial communities without requiring any prior knowledge of the constituent organisms. A prime illustration is the use of plates containing diverse carbon substrates, readily available commercially. This approach unveils the community’s active metabolic pathways. The study by Bittleston et al. (2020), is a good example of its application for the analysis of functional diversity among microbial communities isolated from pitcher plants. In combination with respiration measurements, their findings show that different communities have different functional patterns but similar respiration rates [155].

Not all function-oriented methods will uncover which species, taxa, or community is contributing to said function, and to overcome this, a combination of methods can be used. For example, Berga et al., (2017) used DNA-based methods to analyze changes in the microbial community structure in response to salinity perturbations, alongside measurements of respiration and carbon consumption. Despite alterations in community structure, respiration and carbon consumption remained unchanged [156], underscoring the role of redundancy in maintaining community function.

Stable Isotope Probing (SIP) enables researchers to track the flow of radioactively marked molecules through microbial

communities and the environment [157–159]. This technique, when used in combination with DNA-based methods, allows researchers to determine which specific taxa are actively performing certain functions [160,161]. A recent study by Kong et al., (2020) used SIP to investigate the utilization of ^{13}C-labeled rice residue by bacterial and fungal communities under two different fertilization regimes. By separating the heavy ^{13}C that bacteria incorporated into their DNA, the researchers were able to identify the main taxa involved in rice residue decomposition. Network analysis was further employed to identify co-occurrence patterns among bacteria and fungi, allowing them to reconstruct how these microbes interacted under different experimental conditions and fertilization regimes. This analysis identified shifts in keystone species under each fertilization regime [162].

Mass spectrometry (MS) is a crucial tool for profiling compounds produced by microbes [163,164]. These methods can help identify compounds involved in both intraspecific and interspecific interactions and characterize community composition even at the level of strains, where 16S rRNA sequence similarity can surpass 99.9 % [165,166]. Moreover, a variant of this technique, imaging mass spectrometry, enables the observation of the spatial distribution of compounds [167,168]. For example, compounds produces by *Pseudonocardia* were detected directly on the exoskeleton of leaf-cutter ants using imaging MS, providing insights into the chemical sources of interactions between ants, fungi and bacteria [169,170].

The methodologies outlined above heavily rely on bioinformatic analysis. In addition, ecological predictions, hypotheses, and theories are often intertwined with mathematical models based on differential equations and computational simulations, such as agent-based models. Network analysis, co-occurrence patterns and metabolic modeling have been widely used to analyze and understand microbial interactions [78,144,171–173]. These topics are beyond the scope of this review, but have been discussed elsewhere [174]. Models are useful tools that can help bridge the gap between what can be experimentally observed in simple communities and what is observed in more complex ones. Furthermore, models are often developed alongside synthetic community models (see Table S2).

5. Synthetic microbial communities and health

The study of synthetic microbial communities has gained significant attention due to its potential applications in health. Understanding the interactions within these communities can provide insights into the complex dynamics and emergent properties that impact human health and diseases [16]. Despite numerous studies on the human gut microbiome, translating these findings into improved health outcomes remains slow due to the microbiome's complexity, which is calculated to include from 300 to 1000 microorganisms, with a population density of 10^{11} cells/g and a total of $\approx 10^{13}$ cells in the colon [175]. Fecal samples are the source of strains used for the design of synthetic microbial communities. In addition to culturing bacteria to obtain collections of representative intestinal microbiota, an immense microbial data set has been obtained from both in vivo and *in vitro* systems through metagenomic studies of fecal samples.

Mice models are the most extensively studied, but many interesting models are used to build synthetic communities for the study of gut microbiota. Here we give some examples on the advances in generating microbial collections from the intestinal tract to experiment on synthetic communities, emphasizing the different experimental strategies used to understand the interplay between bacterial metabolisms.

5.1. Gut synthetic communities in human and animal models

The human gut microbiome is a complex and dynamic ecosystem consisting of hundreds of species that interact with each other and the human host. Bottom-up approaches to studying this ecosystem involve starting from a reduced microbial diversity to simplify experimental variables, facilitating the reconstruction of synthetic gut communities. Van Leeuwen et al. (2023) provide a systematic review of these synthetic communities, detailing current strategies to construct and test their functions [176].

Synthetic communities can be constructed to analyze different aspects of the dynamics and metabolic interactions. D'hoe et al. (2018) created a synthetic community from three abundant members of the human gut microbiome: *Blautia hydrogenotrophica*, *Faecalibacterium prausnitzii*, and *Roseburia intestinalis*. This study highlighted that community interactions can significantly alter individual bacterial behaviors and metabolic outputs [177]. Clark et al. (2021) developed a synthetic human gut microbiome comprising 25 prevalent bacterial species to study butyrate production, a key metabolite for gut health. Their findings underscored the importance of microbial interactions in metabolic functions [178]. Shetty et al. (2022) investigated microbe–microbe interactions within a 15-strain synthetic gut community, revealing significant temporal variations and niche overlaps among species, influenced by diet and other environmental factors [179].

Animal models are highly valuable to the study and validation of microbial communities when the experiments cannot be carried out in humans. A well-known model is the Oligo-Mouse Microbiota (OMM12) that provides insights into gut microbial dynamics by using a stable community of twelve bacterial species. These models are crucial for preclinical microbiome research, as demonstrated by **Weiss** et al. (2021), who utilized metabolic network reconstruction to understand strain-strain interactions [180]. Other simpler animal models offer a simplified and experimentally tractable model for studying gut microbiota. The worm *Caenorhabditis elegans*, which has a significantly simple and fully characterized nervous system, is used as a model to study the gut-brain axis. By feeding gnotobiotic *C. elegans* with synthetic bacterial communities, it was observed that interspecies interactions played a more important role in community assembly than host-microbe adaptation or gut environmental filtering [129]. Finally, the gut bacteria of honeybees. can be grown and genetically manipulated, providing valuable insights into microbial interactions relevant to both bees health and broader microbiome studies [181].

5.2. Applications of synthetic microbial communities to the development of probiotics and fecal microbiota transplants

Probiotics are defined as live microorganisms that are administered to produce a health benefit on the host. Probiotics are of considerable interest, particularly in the context of the human gut. However, their impact is difficult to assess since there is limited evidence supporting their benefits in the general population. One significant challenge is the anaerobic nature of many beneficial gut bacteria, making them difficult to cultivate and maintain during a useful shelf life. Vazquez-Castellanos et al. (2019) suggested possible *in silico* and *in vitro* approaches for the iterative design of anaerobic bacterial consortia to be used in modulating the gut microbiome [182]. Jansma et al. evaluated the impact that a probiotic strain had on a three-strain community representative of the upper small intestinal microbiota, regarding the metabolism of a tryptophan-derived metabolite, kynurenine. The strains *Pseudomonas fluorescens* and *E. coli* are capable of metabolizing kynurenine, but not *Streptococcus salivarius, Streptococcus thermophilus*, or *Lactobacillus casei*. Their results showed that probiotic supplementation directly affects kynurenine synthesis and in general the community's metabolism. Moreover, *L. casei* seemed to increase the resistance of the community to perturbations [183].

Fecal microbiota transplants (FMT) hold significant potential for therapeutic applications. However, challenges such as the anaerobic nature of gut bacteria and the complexity of microbial interactions make their impact difficult to predict. FMT has shown efficacy in treating conditions like recurrent *Clostridium difficile* infection, but its mechanisms remain poorly understood, necessitating further research [184,185].

5.3. Antibiotics and community dynamics

Antibiotic resistance poses a major public health threat, aggravated by the complex interactions within polymicrobial communities. Mounting evidence shows that inter-species interactions in microbial communities alter the susceptibility of its members to antibiotics, resulting in their survival upon treatment reviewed by Ref. [186]. An example of these is the work of Bottery et al. (2022) who used coculture assays and showed that β-lactamase production by a multi-drug resistant *Stenotrophomonas maltophilia* can provide imipenem protection to a *Pseudomonas aeruginosa* strain, sensitive to the imipenem antibiotic [187].

6. Synthetic microbial communities and agriculture

Microbial strains, and more recently consortia, are widely used to aid in plant health [188]. However, results are often inconsistent and unpredictable due to our poor understanding of the complex interactions that take place between the inoculated microbes, the native microbiota, and the host plant in nature [15]. Culture-independent approaches have allowed a comprehensive view of the great microbial diversity that inhabits the soil and interacts with plants [189]. Still, they do not allow for the generation of experimental systems to test the role of individual members of the community or their genes in community functions [190]. Synthetic ecology approaches and the development of model synthetic communities have the potential to aid in plant productivity by delivering inoculants with increased stability and success [14,191] and by increasing our knowledge of the relationship between members of the plant microbiome and plant traits reviewed in Ref. [190]. Notably, well-established, trackable model communities (i.e. where absolute measurements of each strain are possible) allow the identification of higher-order interactions and emergent community functions that impact plant health.

6.1. Synthetic communities as biofertilizers

Synthetic communities composed of plant-associated bacteria have been used as biofertilizers not only to increase the health and quality of plant products, but also to serve as models to understand the mechanisms that mediate symbiosis. Xin et al. (2024) explored the contribution of the bacteria associated to tea plants for theanine synthesis. Using amplicon sequencing, they first identified bacteria that potentially modulate nitrogen metabolism in high- and low-theanine varieties. Then, a synthetic community was generated mimicking the composition found in high-theanine varieties. This 21-strain community increased the theanine content and N metabolism in low-theanine tea varieties, and imparted tolerance to nitrogen deficiency in *Arabidopsis*, increasing root development in low nitrogen conditions via ammonium uptake [192].

Screening randomly assembled synthetic communities is a strategy to uncover patterns that mediate community properties as biofertilizers. Hu et al. (2021) used different consortia assembled from 8 plant growth promotion (PGP) model *Pseudomonas* spp. strains. This approach allowed the study of the effect of community richness (number of species) on the PGP capabilities. Additionally, by quantifying the viability of introduced strains, overall bacterial diversity, and correlations between consortia functions and PGP traits in plant assays, the authors established that the positive effect of consortia was mediated by shifts in the native bacterial communities [193].

6.2. Synthetic communities for biocontrol

Synthetic ecology approaches are also useful to generate communities that aid in the control of plant pathogens. Santhanam et al. (2015) used a synthetic a consortium to control sudden-wilt disease in *Nicotiana attenuata* caused by a co-infection of *Fusarium* sp. and *Alternaria* sp. Initial tests showed that a mix of 5 bacterial strains attenuated disease incidence in seeds and plant, outperforming fungicide treatments. A reductionist top-down approach was adopted where individual members were removed one at a time, showing that biocontrol could be recapitulated with a 3-strain community [194]. Similarly, Bedendsen et al. (2018) studied the plant protection

capabilities of microbes that were recruited in *Arabidopsis* plants infected with the biotrophic pathogen *Hyaloperonospora arabidopsidis* (Hpa). Culture-independent analyses identified strains from the genus *Xanthomonas*, *Microbacterium*, and *Stenotrophomonas* sp. as members that are recruited upon Hpa infection. Next, strains from these genera were isolated and used as a synthetic community to show that inoculation protects the plant from Hpa infection [195].

More recently, Li et al. (2021) explored high-and low-abundance bacteria found in plants of the legume *Astragalus mongholicus* with root rot symptoms caused by *Fusarium oxysporum*. Two synthetic communities were assembled consisting of strains enriched (SCI) or excluded (SCII) during infection. SCI, but not SCII, induced plant growth and protected the plants against *F. oxysporum* infection. The 13-strain SCI was then simplified through a top-down approach mediated by plant-selection; the resulting 4-strain synthetic community (SCIII) recovered from the plant displayed beneficial functions similar to the 13-strain community. Notably, the simpler synthetic community allowed the elucidation of specific roles of the individual members: while *Stenotrophominas* was able to inhibit fungal growth *in vitro*, *Advenella* sp. reduced disease incidence in the plant. Additionally, induction of systemic resistance was associated to the presence of low abundance strains via de JA signaling pathway [196].

Finally, using a screening approach, Emmenegger et al. (2023), used more than100 randomly assembled synthetic communities in an infection model with *A. thaliana* and a *Pseudomonas* sp. pathogen. Additionally, plant morphometric values and pathogen quantification data were analyzed using machine learning. This approach allowed to identify, from the initial pool of 36 strains, the individual members that mediate plant protection. Moreover, the results allowed the rational design of 2-member synthetic communities with synergistic plant protection, as well high higher-order (4-member) communities that displayed increased protection [197].

6.3. Model synthetic communities and emergent plant-beneficial properties

As exemplified by the work described above, synthetic ecology approaches and the use of synthetic communities can aid in the generation of effective biofertilizers and biocontrol inoculants. However, understanding the higher-order interactions and the resulting emergent properties in the plant microbiome requires the creation of model synthetic communities composed of trackable strains, which can be individually quantified even in experimental systems that involve the host plant. This implies a more robust development as an experimental system. Nui et al. (2017) assembled a 7-member synthetic community from maize roots. Members were obtained using a top-down approach through plant selection and subsequent isolation. The strains obtained resembled the natural root microbiota, as shown by 16S sequencing. Notably, additionally to the strains comprising the community, the authors developed seven selective media for absolute quantification of each member, contributing to a more robust and useful model system. Interestingly, by systematically removing individual strains and measuring community dynamics in the root allowed the identification of *Enterobacter cloacae* as a keystone species, since its absence causes community collapse in the plant root. Finally, the authors showed that the community displays biocontrol traits against *Fusarium*, a function that may depend on higher-order interactions, since it was found at lower levels in individual members [198].

Ren et al. (2015), assembled a 4-species community comprised of soil bacteria. The community was selected from testing biofilm formation of all 4-member combinations from 7 soil isolates. Interestingly, biofilm formation was induced in the community conformed by *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans* and *Paenibacillus amylolyticus* (SPMX synthetic community) as compared against individual members and all possible combinations of 2 and 3 members. This full combinatorial approach was key to defining that synergistic biofilm induction is a higher-order function that requires the presence of all strains. In vitro, all strains grew better in the multispecies biofilm, compared to the single-species biofilms, an effect not observed in the planktonic fraction [199]. Interestingly, the SPMX community was also able to colonize the roots of *Arabidopsis,* contributing to drought tolerance through the induction of the biosynthesis of the ABA phytohormone [200]. This function was also synergistic since it was not observed in the individual strains.

Well established synthetic communities composed of strains that are amenable to genetic dissection can serve as models to understand the genetic determinants for higher-order interactions that mediate plant beneficial functions. Lozano et al. developed the THOR community [108], composed of strains from the phyla Firmicutes, Proteobacteria and Bacteroidetes, which are dominant in the rhizosphere. To assemble the community, the *B. cereus* strain (Firmicutes) was first selected, and the other 2 members were selected from 20 candidate strains based on their high relative abundance in the rhizosphere and antagonistic interactions. One important criterion was the higher-order interaction displayed in the community, where the antagonistim between *Pseudomonas koreensis* CI12 and *Flavobacterium Johnsoniae* CI04 was inhibited in the presence of *Bacillus cereus* UW85. This HOI mediates the stability in the 3-member community. Additionally, the community displays other emergent properties such as motility and biofilm formation, which could impact community fitness in natural settings. While beneficial interactions with plants have not been reported for the THOR community, it has served as model to understand the molecular basis of community stability and interspecies interactions [201,202].

Based on these observations, higher-order interactions appear to be crucial for the stability and functions of plant-associated bacterial communities. For this reason, Gastelum et al. proposed that detecting emergent properties in mixed colony biofilms assembled through *in vitro* combinatorial approaches should result in the identification of communities presenting higher-order interactions. Using this rationale, a synthetic community from maize-associated bacteria was created consisting of 3 members: *Bacillus pumilus* NME155, *Bhurkolderia contaminans* XM7 and *Pseudomonas* sp. GW6. Notably, selective culture media were designed for absolute quantification of each strain, which allowed to follow community dynamics during biofilm development. Positive and negative pairwise interactions were detected between the different members, but the higher-order assembly resulted in a complex colony architecture – a proxy for biofilm formation – that was absent in individual and pairwise interacting colonies [203].

7. Perspectives

Microbes play a significant role in various domains including medicine, agriculture, and the food industry. They are crucial in addressing challenges related to climate change, antibiotic resistance, water treatment, crop productivity, biodiversity loss, and pollution. Engineered microbes, particularly synthetic microbial communities, serve as factories in many industrial and biotechnological applications. The deliberate and systematic development of these synthetic communities can advance medical and environmental sciences, opening doors to novel drugs, enzymes, bioproducts, and bioprocesses [204]. To achieve these advancements, it is essential to understand and disentangle the complexity of microbial communities.

The integration of current and emerging methodologies, theories, and models with a focus on synthetic communities will generate comprehensive data sets that will allow us to face significant technical and analytical hurdles. Additionally, emerging technologies such as artificial intelligence, machine learning, and single-cell approaches can provide new insights into microbial interactions.

These advancements will not only enhance our ability to harness the potential of synthetic microbial communities for practical applications but also deepen our understanding of ecological concepts and microbial dynamics.

CRediT authorship contribution statement

Oscar Gallardo-Navarro: Conceptualization, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Bernardo Aguilar-Salinas:** Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Jorge Rocha:** Writing – review & editing. **Gabriela Olmedo-Álvarez:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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