Trends in Microbiology

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Review

Emerging models to study competitive interactions within bacterial communities

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Within both abiotic and host environments, bacteria typically exist as diverse, multispecies communities and have crucial roles in human health, agriculture, and industry. In these communities, bacteria compete for resources, and these competitive interactions can shape the overall population structure and community function. Studying bacterial community dynamics requires experimental model systems that capture the different interaction networks between bacteria and their surroundings. We examine the recent literature advancing such systems, including (i) *in silico* models establishing the theoretical basis for how cell-to-cell interactions can influence population level dynamics, (ii) *in vitro* models characterizing specific interbacterial interactions, (iii) organ-on-a-chip models revealing the physiologically relevant parameters, such as spatial structure and mechanical forces, that bacteria encounter within a host, and (iv) *in vivo* plant and animal models connecting the host responses to interbacterial interactions. Each of these systems has greatly contributed to our understanding of bacterial community dynamics and can be used synergistically to understand how bacterial competition influences population architecture.

Highlights

Interbacterial interactions are a major determinant of microbial community composition, and consequently have a significant impact on ecological functions and host health.

Bacteria possess a diverse array of tools to compete with other bacteria, including both contact-dependent and contact-independent mechanisms.

Understanding the interactions and dynamics within bacterial populations may enable the manipulation of microbial communities for therapeutic or agricultural benefits.

Fully capturing antagonistic interactions within diverse communities and their environment in experimental model systems has been a challenge; however, new technologies and animal models are providing a more complete picture of bacterial population dynamics.

Introduction

Multispecies microbial communities are ubiquitous within nature, being found within diverse environments including soil [1], water [2], and plant [3] or animal hosts [4]. Within these communities, bacterial cell-to-cell interactions play a crucial role in shaping the overall population composition, organization, and interaction with the surrounding environment or host [5]. These interactions can be categorized as cooperative, where different bacteria provide mutual benefits to each other, or competitive, where one set of bacteria antagonizes or excludes another. Cooperative interactions include, but are not limited to, metabolite cross-feeding, intercellular signaling, and the secretion of shared adhesins or biofilm components, while competitive interactions include competition for nutrients, physical space, and other resources, as well as active antibacterial weapons that directly eliminate competitors [6,7]. Such weapons can be highly diverse in their mechanism of action ranging from contact-dependent toxin delivery systems that impact only the local area [e.g., the **type 6 secretion system** (**T6SS**) (see Glossary) and **contact-dependent inhibition** (**CDI**)] to the release of diffusible growth inhibitors (e.g., antibacterial compounds and **bacteriocins**) (Figure 1).

The development of systems to study bacterial cell–cell interactions is strongly motivated by the prospect of being able to modulate these interactions for the benefit of medicine [8–11] and agriculture [12–14]. However, fully capturing the complexity of polymicrobial communities is not trivial. In this review, we discuss recent advances in the development of artificial and animal model systems to study these communities and the interbacterial interactions driving their dynamics.

In silico models uncover theoretical principles underlying bacterial interactions

Computer simulations incorporating our understanding of metabolism, cellular behavior, and community composition have been used to generate predictions about the spatiotemporal

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dynamics of microbial communities [15]. The primary strength of these *in silico* models is that they allow numerous parameters to be systematically varied on a scale that is not experimentally feasible. For example, to understand the impact of cell density on interbacterial competition, instead of constructing multiple bacterial communities each with a different initial number of cells, researchers can simply re-run a single computational model varying the initial bacterial seeding density. Using this approach, it was found that a lower initial cell density results in greater separation of the competing bacteria and less contact-dependent bacterial antagonism [16].

One particularly useful form of *in silico* analysis commonly used to model bacterial communities is **agent-based modeling** (**ABM**). These models can incorporate the heterogeneity and structural organization of microbial communities [17,18]. ABMs allow computation of the collective behavior of a bacterial population based on underlying assumptions of the behavior of individual cells. For example, **exopolysaccharide** (**EPS**) was initially observed to act as a 'protective armor' for individual *Vibrio cholerae* cells against T6SS-mediated attacks [19]. An ABM was developed to simulate this behavior in the context of a multicellular population with mixed phenotypes to explore how this defense mechanism can provide collective protection to non-EPS-producing cells [20]. Two distinct mechanisms were explored: EPS sharing between cells and 'flank protection' where EPS producers physically separate the sensitive cells from attackers. The ABM demonstrated that both mechanisms were theoretically possible, and indeed both mechanisms could be directly observed in real communities of EPS-producing *Escherichia coli*. Moreover, ABMs can also incorporate the metabolic costs needed to exhibit different cellular behaviors. This type of integrative analysis has been used to demonstrate how the potential evolutionary benefit of EPS-mediated protection outweighs the metabolic costs to produce it [20], and are consistent with other studies demonstrating the benefits of protective barriers against T6SS and bacteriocins in *Pseudomonas* and *Klebsiella* [9,21].

In silico community modeling can also capture emergent population behavior, providing insights into how specific bacterial interactions shape the larger community organization. For example, mathematical modeling has demonstrated how T6SS-mediated attacks drive phase separation of constituent species within an initially well-mixed population, leading to clonal patches of cells [22]. This physical separation facilitates cooperation and increases species diversity within the larger bacterial community by limiting the extent of antagonistic contact-dependent interactions. Similar to phase separation, formation of dead cell boundaries or 'corpse barriers' that block contact-dependent antagonism also facilitate bacterial community diversity [23]. *In silico* bacterial community models varying the amount of time elapsed between a cell receiving a T6SS attack and that cell ultimately lysing have been used to understand the limits of corpse barrier formation. From this, it has been found that delivery of fast-lysing T6SS effectors enables greater dominance of T6SS attackers in the population [24] (Figure 2A, Key figure).

A theoretical model incorporating motility on a solid surface and contact-dependent weaponry has been used to find that bacterial cell motility can enhance toxin delivery through two separate, but related, processes [16]. First, motility increases the probability of an attacker coming into contact with a prey cell (genotypic mixing), and second, motility allows an attacker to move on to additional prey rather than repeatedly attacking the same one (target switching) [16]. While this model was specifically applied to twitching motility and CDI-mediated competition in *Pseudomonas aeruginosa*, similar models have been constructed for other organisms, antagonistic weapons, and cellular contexts. These analyses have found that antagonistic weapons can be more or less effective depending on the conditions [25]. Short-range weapons are useful when an attacking strain is outnumbered, facilitating invasion and establishment, while long-range weapons are highly effective when attackers are abundant. In this way, bacteria fight efficiently as groups or individuals

Glossary

Agent-based modeling (ABM): *in silico* modeling technique in which a microbial colony is represented as a group of discrete agents with predefined attributes and whose interactions are governed by a set of rules.
Auxin: a plant hormone that promotes the elongation of shoot cells in plants.
Bacteriocins: a diverse group of proteinaceous antibacterials that are active against specific bacteria.
Bone morphogenetic protein (BMP) pathway: a versatile cell signaling pathway impacting many developmental processes.
Colicins: bacteriocins, produced by *Escherichia coli*, which kill related bacterial species.
Contact-dependent inhibition (CDI): a type of two-partner secretion system used to deliver toxins that inhibit target-cell growth.
Exopolysaccharide (EPS): extracellular macromolecules excreted as a tightly bound capsule or loosely attached slime layer in microorganisms.
Horizontal gene transfer: movement of genetic information between organisms that is not directly from parent to offspring.
Ixotrophy: contact-dependent predatory strategy of filamentous bacteria in aquatic environments.
Microbe-associated molecular patterns (MAMPs): conserved microbe-specific molecules that direct a response of host cells.
Microbiota: a community of microorganisms that live in and on the human body.
Phytopathogens: microorganisms causing disease in plants.
Rhizosphere: the area of soil immediately surrounding plant roots where diverse microbial communities reside.
Stool-derived *in vitro* communities (SICs): *in vitro* bacterial communities cultured from stool samples which can be used in microbiota research.
Type IV pili (T4P): dynamic filamentous appendages involved in adherence, DNA uptake, motility, biofilm formation, and protein secretion.
Type VI secretion system (T6SS): a dynamic bacterial nanomachine that fires a toxin-loaded needle-like apparatus directly into target cells.

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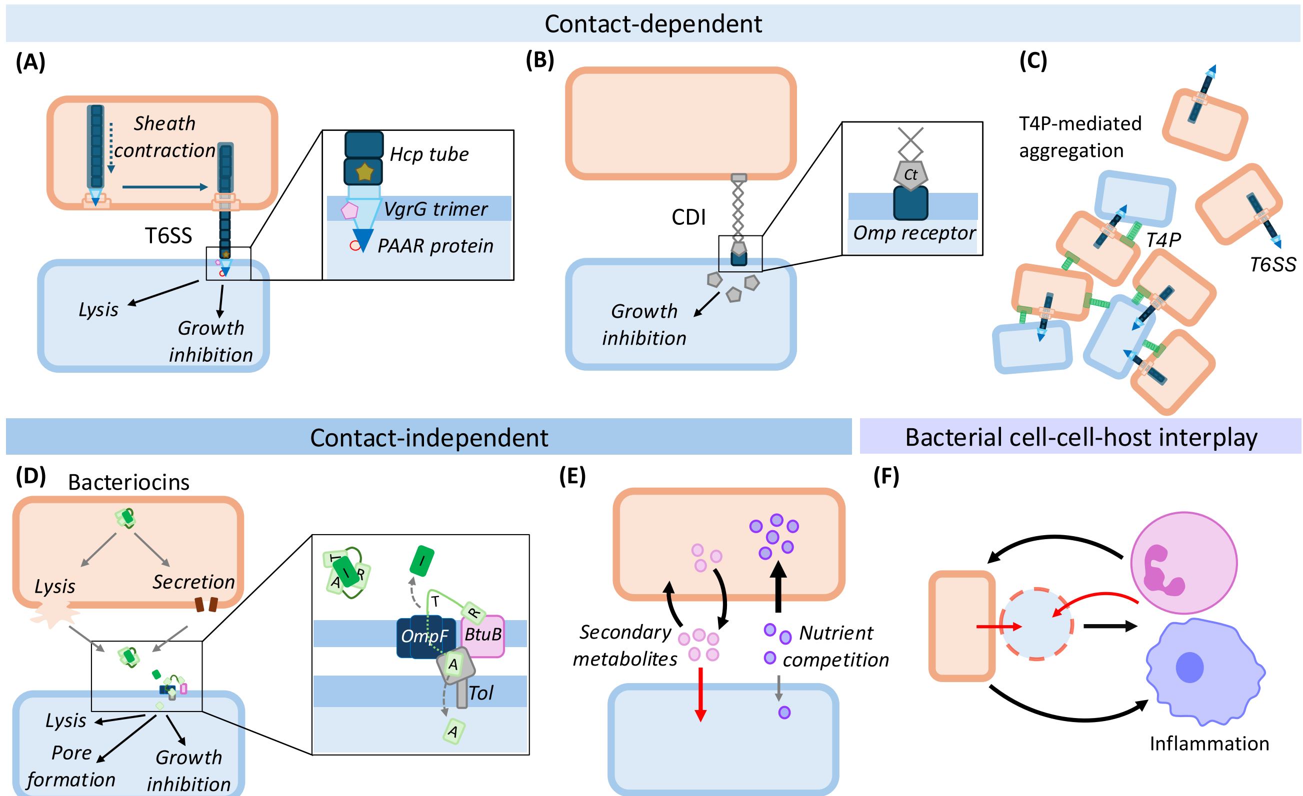

Figure 1. Types of bacterial competitive interactions. Bacteria possess a wide range of competitive interaction mechanisms. (A) The type VI secretion system (T6SS) is a membrane-anchored complex consisting of an inner tube (Hcp) polymerized from a spike (VgrG trimer), and Pro-Ala-Ala-Arg (PAAR) tip protein, surrounded by a sheath. Upon sheath contraction, the Hcp tube is propelled out of the cell and into adjacent cells, delivering a toxic payload of effectors associated with Hcp, VgrG, or PAAR. (B) Contact-dependent inhibition (CDI), a variant of the type V secretion system, is composed of an elongated spring-like β-helical structure carrying a C-terminal toxin domain (Ct), which binds to and translocates through one of several different Omp receptors in the outer membrane of sensitive cells. (C) Type IV pili (T4P) can mediate the aggregation of bacterial cells, facilitating the cell–cell contacts needed for contact-dependent processes like T6SS. T6SS^{+} bacteria that remain planktonic and free-floating deposit their effector payloads harmlessly into the extracellular space. (D) Bacteriocins are diffusible protein toxins released by producer cells either through a dedicated secretion system or cell lysis. There is a huge variety of different bacteriocins, but some of the best studied, Group A colicins, are depicted here. These colicins consist of a receptor-binding domain (R), which binds to an outer membrane receptor, and a translocation domain (T), which assists in delivery of an activity domain (A) into the target-cell cytosol. Along with the colicin, producer cells also make a cognate immunity protein (I), which offers protection from self-intoxication that is before target-cell entry. (E) Secondary metabolites play various roles in interbacterial communication, including autoinducers facilitating quorum sensing or short-chain fatty acids that restrict the growth competing bacteria. Similarly, asymmetric acquisition of environmental nutrients can give some bacteria a competitive advantage over others. (F) Bacterial growth and byproducts of bacterial killing (red arrow) can trigger environmental changes, such as host immune responses, which can feedback to modulate the bacterial community.

depending on the ecological context, hinting at why different bacteria may have evolved to use different antibacterial weapons.

Overall, *in silico* models help to generate predictions and identify potential emergent properties of bacterial communities. However, modeling will always be limited by our current understanding of the behaviors of individual cells. Discovery of new behaviors, as well as the validation of *in silico* predictions, ultimately require observing and experimenting with actual living bacteria.

In vitro models: a controlled environment to test hypotheses

Typically, to study interbacterial interactions it has been sufficient to simply mix interacting bacteria together, spot them onto a surface, and then either visualize the resulting community via

Key figure

Images of bacterial interaction outcomes using different models

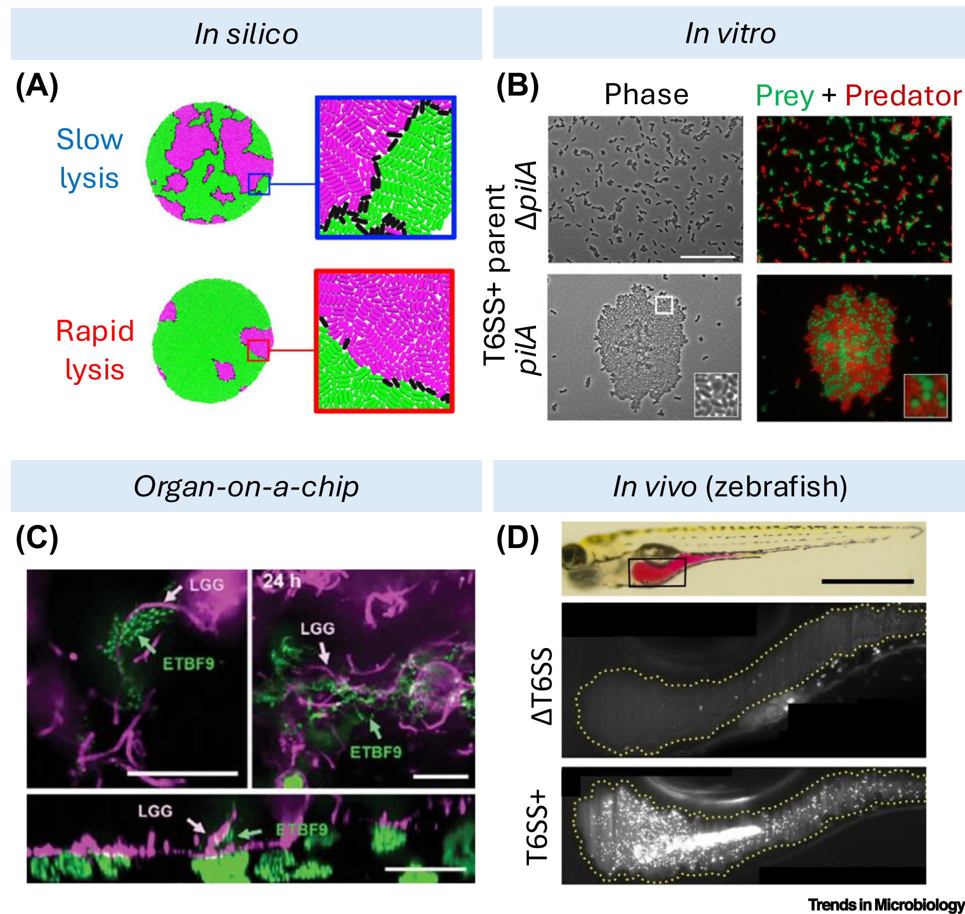

Figure 2. (A) Simulation snapshots comparing T6SS+ attacker (green) versus susceptible prey strain (pink) competition outcomes for slow and rapid prey lysis (black cells). Magnified sections highlight the reduced corpse barrier seen when prey strains undergo rapid lysis. Adapted from [24]. (B) Representative microscopy images of T6SS+ predator strains (red) either with their native major pilin gene (*pilA*, bottom panel) or lacking it (Δ*pilA,* top panel) alongside T6SS sensitive prey strains (green), highlighting T4Ps role in facilitating T6SS-mediated prey cell elimination. Adapted from [33]. (C) Top view (upper panel) and cross-section (lower panel) of *Lactobacillus rhamnosus* (LGG, purple) inhibiting attachment and proliferation of Enterotoxigenic *Bacteroides fragilis* (ETBF, green) through co-aggregation. In this way, LGG prevents colonization by pathogenic ETBF, preserving a healthy state. Adapted from [58]. (D) *Aeromonas* in the zebrafish larval gut 24 h after invasion by ΔT6SS mutant (lower panel) and T6SS+ (upper panel) *V. cholerae. V. cholerae* uses its T6SS to enhance zebrafish intestinal movements that lead to expulsion of resident *Aeromonas*. Adapted from [76].

microscopy or count the number of surviving viable cells of each species through selective plating and colony counting. This basic approach has been used to identify antagonistic systems [26], measure the delay between an interaction and its resulting effect [11], determine the species specificity of interactions [27], and characterize abiotic factors governing interactions [28]. Recently, droplet-based printing technology has enhanced this bacterial mixing approach to enable direct investigation of specific micron-scale patterns within bacterial communities and their impact on interbacterial interactions [29].

Bacterial mixing experiments can be coupled with genetic and genomic approaches to identify key genes mediating intercellular interactions. For example, a recent RNA-seq analysis of quorum-sensing mutants of *V. cholerae* identified a four-gene operon responsible for mediating bacterial antagonism independently of any known mechanism [30]. Similar approaches have also been used to understand the coordinated activation of different T6SS systems present in the same bacterium [31]. Agar spot overlay experiments and transposon mutagenesis experiments have been used to identify antimicrobial toxins produced by human gut Bacteroidales species [32].

Visualizing bacteria with distinct behaviors using time-lapse microscopy allows correlation of specific gene modifications with changes in the wider bacterial community organization. Observing bacterial populations in this way has revealed how the position of different strains and species in space can dictate interbacterial interactions and, consequently, the overall population structure [23,33–35]. In *V. cholerae,* the presence of functional **type IV pili** (**T4P**) induces auto-aggregation*,* enhancing the number of cell–cell contacts needed for T6SS-mediated antagonism [33] (Figure 2B), with similar observations being made in *P. aeruginosa* [34]. Removal of the T4P reduces aggregation and consequently the cell contact needed for T6SS-mediated killing. Interestingly, aggregation can also be used as a means of protection, as a genetic screen showed that *E. coli* mutants overexpressing fimbriae formed microcolonies that protected against T6SS attacks [35].

Fluorescence microscopy and quantitative image analysis can be used to visualize the macroscopic organization of bacterial populations [36], the micron-scale arrangements of bacterial communities [37], as well as the subcellular localization and assembly dynamics [38] of the systems mediating interactions between cells. Advances in high-resolution imaging has provided further mechanistic insight into how localization and assembly of these systems are controlled. 3D-structured illumination microscopy (SIM) of fluorescently labeled T6SS sheath in *Acinetobacter baylyi* identified nascent sheath assemblies forming at the cell–cell contact site of adjacent bacteria that were previously undetectable by standard widefield microscopy [39]. By systematically imaging knockout mutants of previously uncharacterized genes in the T6SS gene cluster and several known outer membrane proteins, it was found that periplasmic protein TslA and outer membrane porin OmpA were required for localization of these T6SS assemblies to cell–cell contact sites.

The nanometer-scale imaging capabilities of cryo-electron microscopy (cryo-EM) have been exploited to obtain structural images of secretion systems [40,41]. Such techniques were recently applied to study the complex multi-system interaction, **ixotrophy**, whose underlying molecular mechanisms had been poorly understood. The bacterium *Aureispira* CCB-QB1, was seen to deploy extracellular ‘grappling hooks’ to catch *Vibrio* prey by their flagella. This facilitated the close predator–prey contact required for T6SS-mediated prey killing [41]. Combining single-cell analysis with stable isotope-labeled prey revealed that prey components are taken up by the attacker and used as a nutrient source.

While the aforementioned studies have primarily focused on pair-wise interactions, it is also possible to study larger bacterial communities and their interaction networks [42,43]. Simplified synthetic microbial communities provide a useful tool to disentangle complex interactions. These communities, although reduced in their complexity, can be constructed to maintain the key features of natural systems (such as stability, metabolomic profile, fermentation pathways) and therefore preserve their core functionalities. For example, various **stool-derived *in vitro* communities** (**SICs**) recapitulate the *in vivo* response to infection and antibiotic treatment and,

in this way, provide a powerful, high-throughput tool for modeling *in vivo* **microbiota** responses to perturbation [10]. The Oligo-Mouse-Microbiota [OMM^{12}] is a widely used synthetic bacterial community, which can be exploited for *in vitro* studies [42,44] as well as in gnotobiotic mice where it recapitulates key phenotypes such as colonization resistance to pathogens [45] and immune development [46]. Synthetic microbial communities have also contributed towards research into environmental-associated microbial communities [47,48]. A defined five-species community of soil microbes showing long-term stability was used to explore how microbial communities evolve and how environmental stressors impact community diversity [47].

Despite the many advances made in studying bacterial interaction networks, capturing the native environment surrounding the various bacterial communities often remains elusive. Bacterial behavior and the extent of interbacterial interactions can be highly dependent on the community environment. These differences can be attributed to structural differences between 2D and 3D surfaces [49], diffusion of signals [50], physical forces [51], fluid flow rates [52], and host signaling [53]. Organ-on-a-chip approaches present a novel and powerful way to address some of these environmental factors by incorporating host elements into the system.

Organ-on-a-chip models enable investigation of spatial constraints underlying bacterial interactions

The recent advent of organoid and 'organ-on-a-chip' (OOC) models provides a bridge between *in vitro* and *in vivo* models, allowing recapitulation of *in vivo* 3D architectures in controlled, well-defined *in vitro* settings. Organoids are *in vitro*-grown 3D tissues that use differentiated, self-organizing cells. These models can capture host responses, making them valuable infection models able to provide insight into the bacterial–bacterial–host crosstalk in a physiologically relevant microenvironment. For instance, human respiratory epithelium organoids infected with *P. aeruginosa* virulence factor mutants have been used to provide mechanistic insight into how *P. aeruginosa* disseminates and invades mucosal surfaces. Using high-resolution microscopy in combination with functional measures of host responses, such as cilia beating frequency and epithelial barrier integrity, it was seen that, under physiological conditions, the T6SS promotes invasion of goblet cells. Following invasion, intracellular bacteria use their type 3 secretion system to induce goblet cell death and expulsion, resulting in epithelial disruption which promotes bacterial translocation and spread. Given that the lungs are the main entry portal for airborne pathogens, being able to visualize and track the events leading to pathogen colonization and breach of this barrier in a physiologically relevant setting may highlight new therapeutic opportunities [54].

Another organoid model, the 3D urine-tolerant human urothelial (3D-UHU) model, which recapitulates the critical human urothelial features alongside an innate epithelial immune response, has been used to model urinary-tract infections (UTIs) through inoculation of bacteria [55,56]. The 3D-UHU model has been used to determine bacterial factors involved in uropathogenic *Escherichia coli* (UPEC) invasion and intracellular bacterial community (IBC) formation, two key features of persistent and recurrent infections. Indeed, FimH was identified as being critical to IBC formation although dispensable for invasion [56]. Moreover, using this model to explore the host response to different uropathogens revealed strain and species specificities in cytokine and chemokine production as well as host cell morphologies. This variety in bacteria–host interactions highlights a need for more targeted therapeutics unlike the more general UTI treatment approach currently in use.

OOC models additionally incorporate microfluidic devices, allowing mechanical forces to be captured, such as cell and/or tissue contractions and intraluminal flow. OOCs can support co-culture with living gut microbes and are able to capture key host responses such as epithelial adhesion,

barrier function, mucus production, and cytokine release. OOCs can be pretreated with gut commensals before addition of pathogenic bacteria or some form of intestinal injury. Exploring host responses and visualizing bacterial community architecture can then provide insight into mechanisms of colonization resistance [57–59] (Figure 2C).

OOC technologies can be used to study bacterial interactions in the context of host tissue behaviors, such as host cell reorganization in response to infection by intracellular pathogens [60]. OOC technologies integrating patient-derived cell or microbiota samples might also be able to offer individualized insights into microbial composition and pathophysiology. However, OOC technologies will be intrinsically limited by the scope of the system they recapitulate. Capturing whole-body or multisystem effects of interbacterial and bacterial–host interactions will require the use of *in vivo* animal model systems.

In vivo models to explore bacterial cell–cell interplay within a host

The microenvironment in which a bacterial community are embedded has been shown to impact the bacterial community structure [61–63] as well as the specific bacterial cell–cell interactions that occur [64]. Moreover, in recent years, studies have begun to reveal how bacterial competition can modulate the host environment. As such, plant and animal models have been crucial to advance our understanding of bacterial interactions in the context of host factors. In Box 1, we discuss plant models more broadly, and consider three widely used animal models that have been exploited to study microbial communities: *Drosophila melanogaster* (fruit fly), *Danio rerio* (zebrafish), and *Mus musculus* (mouse). These different models have contributed significantly to our understanding of the importance of context-dependent spatial resolution, and the interplay between bacterial competition and the host.

There has been a growing interest in the importance of plant-associated microbiota to the field of ecology and evolution [65]. Plant-associated microbiota can benefit plant growth by increasing the nutrient supply and suppressing pathogens. In contrast, plant pathogens can cause significant damage to crops, resulting in major economic losses and threatening food security [66]. As such, understanding the interactions within plant-associated microbial communities is paramount to secure crop productivity (reviewed in [67]). In agreement, *in planta* competition assays have revealed the protective effects of *Pseudomonas putida,* a saprophytic soil bacterium that can colonize the root of crop plants including rice and corn. In this case, work has shown that *P. putida* uses its T6SS to drive killing of resilient **phytopathogens**, protecting the crop from necrosis [68].

Just as bacterial interactions can shape plant-associated bacterial community structure, the plant immune response also influences bacterial community composition [69]. In this example, colonization of *Arabidopsis* plants with the bacterium *Bacillus velezensis* triggers production of reactive oxygen species (ROS) that can restrict bacterial growth. ROS stimulates **auxin** production by *B. velezensis*, which mitigates ROS toxicity. As a result, *B. velezensis* has a competitive advantage over other soil microbiota. This host-mediated bacterial antagonism is also beneficial to the plant host as *B. velezensis* produces secondary metabolites that restrict fungal growth.

As with other *in vivo* models, use of plant infection models has highlighted the importance of environmental niche to bacterial interactions and community architecture. Different bacterial colonization structures have been observed, depending on the type of plant as well as the location on the plant (e.g., nodal or seminal roots) [70–72]. For example, coupling confocal microscopy and automated image analysis with spatial statistics revealed differences in the distribution of bacterial cells along the root, with bacterial community density, and by extension interbacterial interactions, increasing from the root tip to the plant-root cell border [71]. Additionally, antibacterial

Box 1. Modeling bacterial interactions within a host

Plant models

Plant models, which often include leaf infection models or studies of the rhizosphere, have enabled fundamental discoveries in the field of genetics (e.g., mendelian inheritance and transposable elements), immunology (e.g., NOD-like receptors), and bacterial secretion systems (e.g., the *Rhizobium leguminosarum* T6SS) [98,99]. The importance of studying plant-associated microbial communities is being increasingly recognized, having significant implications for agriculture (e.g., enhancing resistance to control diseases that impact crop production) [3,66]. *In planta* competition assays, where bacterial cells infiltrate and are co-incubated with leaves, allow direct interactions between bacteria to be visualized and quantified [68,73]. Plant models are amenable to *in situ* hybridization techniques (which can profile the spatial ecology of complex microbial communities along plant roots [94]), and gnotobiotic models can be set up with relative ease [71]. Although useful for studying plant-specific microbiota, plant infection models may have limited translatability to animal systems as plant microbial systems have fundamentally different cellular structures, distinct metabolic activities, and generally lower cellular turnover.

***Drosophila melanogaster* (fruit fly)**

Fruit flies have a simple microbiota comprising 5–20 species of stable colonizers, and their gut shows physiological similarity to that of mammals [100]. Moreover, they have fast generation times, and there are a wide variety of genetic manipulation tools, making them amenable to screening assays. Their innate immune system is also highly conserved with mammals [101]. Collectively, these features make fruit flies highly useful to model gut infection and study host–microbe interactions and bacterial interactions. Additionally, fruit flies can easily be reared germ-free and then associated with a defined consortium of bacteria, allowing interactions between specific bacterial species and the host to be measured [78,100]. New methodologies, such as the bellymount technique, which allows high-resolution imaging of the intact fly abdomen, can be used to investigate bacterial community dynamics in a living organism [62]. Despite these advantages, the physiology and anatomy of flies are different from humans (e.g., less complex immune system), which can limit direct translation of findings to humans.

***Danio rerio* (zebrafish)**

Zebrafish have been used in microbiota research for many years [102] and, more recently, have emerged as a useful model to study host–pathogen and bacteria–bacteria interactions [83,84,103]. Their rapid development, genetic tractability, and extensive genomic homology to humans make them a highly useful tool [103]. Zebrafish are amenable to live-cell imaging, meaning that bacterial community dynamics can be followed and the bacteria–host interactions visualized non-invasively. Germ-free models are available, and the larvae hindbrain ventricle (HBV) offers a naturally sterile injection site, allowing for investigation of defined microbial communities and their impact on the host in the context of a fully functional immune response. In addition to the HBV, other infection sites exist (including caudal vein, otic vesicle, tail muscle, tail fin, gut, yolk sac) offering an opportunity to model different types of infection (e.g., systemic or local). Lastly, until approximately 4 weeks post-fertilization, zebrafish have only an innate immune system, meaning that host–microbe interactions can be investigated independently of adaptive immune responses [103]. However, the unique physiology of the zebrafish model that enables such extensive manipulation and visualization is both a strength and weakness. A criticism of the model is that it is too different from mammalian systems to accurately represent disease from human-adapted pathogens.

***Mus musculus* (mouse)**

Mice are the most common mammalian model in biomedical research because of their close proximity to human physiology and clinical relevance. Mice are routinely used before advancing clinical trials testing pharmacologic treatments on humans. Genetic and molecular tools are widely available as well as germ-free and 'humanized' models, providing versatility. Murine models have greatly enhanced our understanding of bacterial pathogenesis and the protective role of the gut microbiota [104]. Bioluminescence imaging techniques can be used to track bacterial populations through specific metabolic labeling of the bacteria [105], and surgical procedures or stool analysis can provide a snapshot of the bacterial community at a specific location, allowing regional differences in bacterial community composition to be quantified [64,104]. However, use of mice is relatively low throughput (as compared to *Drosophila*, zebrafish), *in vivo* imaging techniques are invasive, and mice are naturally resistant to some human pathogens (e.g., *Shigella* sp., *Salmonella* Typhi, *Staphylococcus aureus*), requiring genetic manipulation (e.g., to 'humanize' a mouse) or antibiotic treatment (e.g., to clear microbiota) to generate a relevant model of human infection [106]. Moreover, there are significantly higher levels of ethical scrutiny that come into play when conducting research using mice and other vertebrate animals.

systems have also been demonstrated to be directly influenced by the plant environment, as the T6SS DNase effector produced by the soil bacterium *Agrobacterium tumefaciens* only confers a competitive advantage during *in planta* competition [73].

In animal models, a significant focus has been placed on developing techniques to facilitate non-invasive, *in vivo,* live-cell tracking of microbial community dynamics in space and time [62,74]. The

recently described bellymount technique for *Drosophila* enables longitudinal imaging of the *Drosophila* abdomen and has been used to explore the colonization patterns of the *Drosophila* gut commensal *Lactobacillus plantarum* [62]. Using this technique revealed different 3D patterns of *L. plantarum* depending on the location; in the proximal midgut lumen the bacteria coalesced into clumps, while in the distal midgut lumen *L. plantarum* sparsely dispersed as single cells, something which could not be captured without visualizing the bacterial community in this way [62]. The regional differences in *L. plantarum* community organization correlate with impaired bacterial viability after transit through the middle midgut.

Light sheet fluorescence microscopy (LSFM) offers gentle and fast superresolution, making it ideal for visualizing bacterial location and dynamics in a living model organism [75]. For example, using LSFM in combination with the zebrafish model has revealed how mechanobiological changes in the host environment drive variations in bacterial composition [76]. It was shown that T6SS-dependent interactions of *V. cholerae* with host epithelial cells induces peristaltic movements of the zebrafish gut, displacing microbiota and promoting its own colonization. Moreover, LSFM of the zebrafish gut revealed a surprising competitive strategy used by bacteria based on manipulation of bacterial spatial organization, namely, dissolution of aggregates [77]. Monocultures of *Enterobacter* ZOR0014 in the zebrafish gut are highly aggregated; however, in the presence of *Aeromonas*-MB4, a non-aggregating strain, *Enterobacter* clusters rapidly fragment into non-motile, slow-growing, individual cells which are less abundant (Figure 2D).

Use of *in vivo* models has illuminated how bacterial competition can modulate the host environment. In *Drosophila,* infection with *V. cholerae* encoding a T6SS led to reduced survival, enhanced intestinal damage, and higher diarrheal symptoms compared to infection with *V. cholerae* encoding a nonfunctional T6SS (or compared to infection of germ-free *Drosophila*) [78]. Further work in *Drosophila* revealed that T6SS-mediated antagonism towards commensals inhibits intestinal repair through activation of the **bone morphogenetic protein** (**BMP**) pathway, preventing regenerative stem cell proliferation [79,80]. Collectively, these works demonstrate how T6SS-mediated antagonism promotes pathogenesis. Similar observations have been made in zebrafish [81] and mice [82]. It is suggested that bacterial antagonism generates **microbe-associated molecular patterns** (**MAMPs**) which drive this response [81,82]. Infection of the zebrafish larvae hindbrain ventricle (HBV) has proven transformative for *in vivo* investigation of bacteria–bacteria interactions in the context of an innate immune response [83,84]. Recent work showed that host inflammation in response to T6SS-mediated bacterial antagonism is not specific to *V. cholerae,* with similar observations being made following *A. baylyi* T6SS-mediated antagonism. However, **colicin**-mediated antagonism, despite being more potent that T6SS (essentially clearing the HBV of colicin-sensitive bacteria), does not stimulate an inflammatory response, suggesting that different modes of interbacterial competition *in vivo* affect the host in distinct ways [81]. Beyond inflammation, work in adult zebrafish has found that *V. cholerae* T6SS-driven changes to the commensal microbial community cause changes to the intestinal metabolite composition [85]. This is important because metabolite changes can subsequently cause selection for or against certain bacterial species.

Just as bacterial interactions can drive changes in host response, the host response can also influence bacterial interactions. For example, *Salmonella* Typhimurium-induced inflammation alters gut microbial ecology, releasing growth-fueling metabolites that are used by *S.* Typhimurium and other members of the family Enterobacteriaceae, causing enterobacterial blooms. Mouse models of *S.* Typhimurium infection have found that the inflammatory environment influences contact-dependent [86] and contact-independent interactions [87,88]. Specifically, inflammation promotes **horizontal gene transfer** of a colicin-plasmid from *S.* Typhimurium to *E. coli* [86]. Inflammation also triggers production of colicin Ib (CoIIb) in *S.* Typhimurium and expression of

colicin receptors in *E. coli*, thereby driving colicin-dependent competition through simultaneously promoting CoIIb production and susceptibility [87]. Lastly, inflammation also induces microcin expression in the commensal *E. coli* 8178 which, when fused to salmochelin siderophores, inhibits *S.* Typhimurium growth [88].

Collectively, *in vivo* studies have allowed a third element of the bacterial cell–cell interaction network to be considered: the host. Because of the reciprocal nature of interactions between bacterial communities and their host environments, further development of tractable plant and animal models to study the host involvement of bacterial community dynamics will accelerate our ability to secure food crops and develop novel treatments for infection and dysbiosis.

Concluding remarks and future perspectives

Due to the complexity of cell–cell interactions, developing and predicting the fate of multispecies bacterial populations can be challenging. Here, we reviewed innovative theoretical and experimental approaches which have enabled fundamental discoveries into bacterial interactions and population dynamics.

The importance of the human gut microbiota, one of the densest, most diverse microbial ecosystems on earth, is being increasingly recognized and has crucial roles in host physiology [4]. Given the growing appreciation of the physiological context in shaping bacterial interactions, numerous studies have analyzed bacterial interactions and communities in humans, typically through metagenomic and metabolomic analysis of stool samples [89,90]. Such approaches have explored the evolutionary dynamics of bacterial interaction tools [89] and rates of DNA transfer among specific populations [90]. Beyond metagenomic and metabolomic analysis, other techniques have been developed which allow direct visualization of bacterial community architectures within their native host environment such as MiPACT-HCR [91,92], CLASI-FISH [93], and SEER-FISH [94]. Such techniques can be applied to host-associated communities, including those in the plant **rhizosphere** or in human sputum, to profile the spatial organization and interaction patterns of bacteria.

The current frontier of microbial community research lies in understanding how these community-scale structures feedback to and regulate the interaction systems in individual cells. That is, although significant progress has been made towards understanding how small-scale cell–cell interactions shape larger communities, it remains unclear what environmental or immune factors modulate these interaction systems and what portion of a given microbial community are affected. Indeed, many studies to date have relied (intentionally or not) upon strains with constitutive activation of cellular systems [95] to reduce experimental complexity, but behavioral heterogeneity likely plays a central role in many important biological processes, including bacterial persistence [96] and phase variation [97]. Combining *in situ* imaging techniques with reporters for individual cellular behavior would allow researchers to advance beyond the assumed homogeneity intrinsic to many model systems. Additionally, integrative approaches utilizing *in silico, in vitro,* OOC, and whole-animal models together has the potential to provide the holistic understanding of bacterial community dynamics at all size and complexity scales needed for precise modulation of microbial communities for agricultural or therapeutic benefit (see Outstanding questions).

Outstanding questions

Progress has been made towards disentangling bacterial interactions within the microbiome; however, our understanding of how viruses or fungi contribute to this is limited. How do within- and cross-kingdom interactions contribute towards the ecology of our microbiome?

How does the host immune response regulate bacterial cell–cell interactions and resulting community structure?

Interactions within a microbial population play crucial roles in community function. Could we engineer synthetic bacterial communities with defined interactions and functions to restore the microbiota to a healthy state following perturbations (such as antibiotic treatment, infection or inflammatory disease)?

Studies have begun to identify precise bacterial colonization patterns and interaction networks among specific bacteria and the immune system. How do these interactions scale up to generate stable, health-promoting communities?

Acknowledgments

We apologize to authors whose work could not be included due to space limitations. We thank Ho and Mostowy laboratory members for helpful discussions. M.V. is funded by a Bloomsbury Colleges PhD Studentship. Research in the B.H. laboratory is supported by MRC Grant (MR/T031131/1). Research in the S.M. laboratory is supported by a European Research Council Consolidator Grant (772853 – ENTRAPMENT) and Wellcome Discovery Award (226644/Z/22/Z).

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