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From microbiome composition to functional engineering, one step at a time

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SUMMARY Communities of microorganisms (microbiota) are present in all habitats on Earth and are relevant for agriculture, health, and climate. Deciphering the mechanisms that determine microbiota dynamics and functioning within the context of their respective environments or hosts (the microbiomes) is crucially important. However, the sheer taxonomic, metabolic, functional, and spatial complexity of most microbiomes poses substantial challenges to advancing our knowledge of these mechanisms. While nucleic acid sequencing technologies can chart microbiota composition with high precision, we mostly lack information about the functional roles and interactions of each strain present in a given microbiome. This limits our ability to predict microbiome function in natural habitats and, in the case of dysfunction or dysbiosis, to redirect microbiomes onto stable paths. Here, we will discuss a systematic approach (dubbed the N+1/N−1 concept) to enable step-by-step dissection of microbiome assembly and

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functioning, as well as intervention procedures to introduce or eliminate one particular microbial strain at a time. The N+1/N−1 concept is informed by natural invasion events and selects culturable, genetically accessible microbes with well-annotated genomes to chart their proliferation or decline within defined synthetic and/or complex natural microbiota. This approach enables harnessing classical microbiological and diversity approaches, as well as omics tools and mathematical modeling to decipher the mechanisms underlying N+1/N−1 microbiota outcomes. Application of this concept further provides stepping stones and benchmarks for microbiome structure and function analyses and more complex microbiome intervention strategies.

KEYWORDS microbiota, microbiome development, focal strains, inoculants, modeling, systems' analysis

INTRODUCTION: WHY MICROBIOME ENGINEERING?

Microbial communities, or microbiota, self-organize in a seemingly spontaneous way within the spatial, temporal, physical, chemical, and biological boundary conditions of their environments or hosts (i.e., their habitats). The microbiota within the context of these boundaries (i.e., "microbiomes," Fig. 1A, Box 1) (1) contribute to important ecological and biogeochemical processes (2), as well as to plant (3), human (4), and animal health (5–8). Alarmingly, an increasing body of knowledge has pointed to recent changes in microbiome functioning [for example, loss of diversity or functions (9, 10)] across all systems (e.g., soils, animals, and human guts) (11–13). From a human perspective, this may have potentially dire consequences for public health, agricultural production, and environmental quality. Underlying causes may include a variety of external factors and conditions, such as pollution, climate change, land and agricultural management, travel to foreign areas, extensive use of antimicrobial compounds, pharmaceuticals, and nutritional habits. These changes motivate a deeper understanding of microbiome structure and function across systems in order to potentially restore and maintain ecosystem function.

Advances in sequencing and bioinformatic tools have enabled unprecedented insights into the taxonomic and functional richness of microbiota (18, 19). However, their collective properties remain largely unresolved, leading to several important unanswered questions: how do external factors disrupt the tendency of natural communities to maintain compositional and functional homeostasis (10), and alter the state, the maintenance, the resilience, or temporal dynamics ("trajectories") of microbiomes? How can negative consequences on microbiome homeostasis be recognized, predicted, prevented, and, possibly, reverted? To rationally intervene in disrupted or dysfunctional microbiomes, we need a much better understanding of the processes that lead to their formation and maintenance, as well as experience using empirical tools that we might eventually deploy to invoke an intended recovery or stabilization. Currently, complex microbiome engineering (20) that would involve simultaneously modulating many taxa or factors is likely unfeasible. These challenges, therefore, pose the question of whether a step-by-step approach of learning from single focal strains—one that is rooted in established expertise with cultivable microbes in agricultural (21), environmental (22), nutritional (23, 24), and pathogen research (25) can provide the insights necessary to enable microbiome engineering.

Here, we discuss an iterative approach to rationally intervene in microbiomes and decipher general principles underlying microbiome assembly and functioning. We call this approach the N+1/N−1 concept, a framework to add or suppress individual microbiota members (*focal strains*) within the context of their habitat, and to probe outcomes and underlying functional processes (Fig. 1B). In this sense, N+1/N−1 can also be considered as an intended state to be achieved, as well as a starting point for developing more complex microbiome engineering strategies (Fig. 1C). Many of our ideas center around deploying single strains to, for example, enable specific system functionalities; or eliminate single strains (e.g., recurring pathogens; Fig. 1D). Through

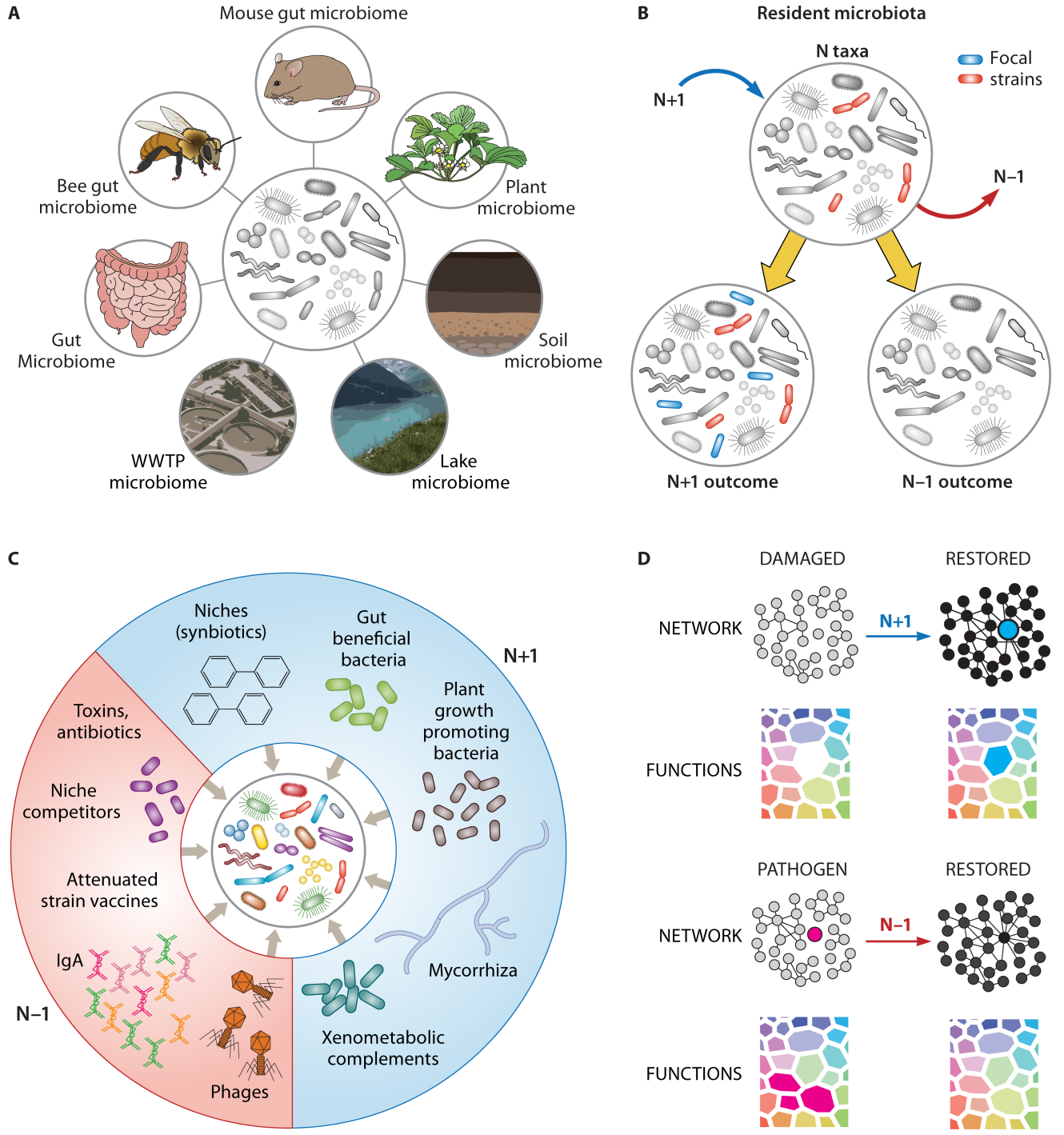


FIG 1 Concept terms in N+1/N–1 microbiome interventions. (A) All habitats (environments and hosts) are occupied by microbiota, (different) assemblages of microbial cells and taxa, collectively forming the microbiomes, as illustrated. WWTP, Wastewater treatment plant. (B) N+1/N–1 approaches intend to attain a state into which is introduced (+1) or eliminated (–1) a microbial taxon (focal strains) from the resident microbiota with its N (total) taxa, to study their outcomes, and understand the molecular and ecological mechanisms leading to the observed microbiome's properties. (C) Examples of specific applied N+1/N–1 intervention goals, to (D) e.g., complement microbiome functions in case of pollution damage, or restore functional networks by reducing pathogen loads. Part D modified after ref. (14).

successive cycles of designing, testing, and measuring (e.g., by removing or adding different strains, or altering nutrient profiles or habitat conditions), we can learn from

BOX1: GLOSSARY OF TERMS

Microbiota / microbial community: the collective (living) microorganisms in a habitat (including Bacteria, Archaea, and eukaryotic microbes). Generally not thought to include viruses, phages, or naked DNA (1).

Microbiome: the ensemble of all microbes integrated within the context of its habitat boundaries (environmental system or host); microbiota and their "theatre of activity: structural elements, metabolites/signal molecules, and the surrounding environmental conditions" (1).

Habitat: the array of biotic and abiotic factors, spatial constraints, physico-chemical conditions, and dynamic properties (flow, fluctuations) that characterize an environment or host.

Dispersal: flow of microorganisms into and out of a particular habitat (15, 16).

(Evolutionary) drift: random shifts in the relative abundance of taxa or genotypes.

Diversification: genetic change by horizontal gene transfer or mutation.

Niche: a set of biotic and abiotic conditions that determine the proliferation of a microbial cell or taxa in a given habitat. To differentiate: the potential and the realized niche (17).

Probiotic: live microorganism that, when administered in adequate amounts, may confer a health benefit on the host.

Prebiotic: substrate which is selective for the proliferation or maintenance of the probiotic within the target microbiome, either directly benefiting the probiotic or indirectly producing favorable niche conditions.

Synbiotic: combination of pro- and prebiotic(s).

Postbiotic: preparation of inanimate microorganisms and/or their components with a claimed health benefit on the host. Effective postbiotics must contain inactivated microbial cells or cell components with or without metabolites that contribute to observed health benefits.

Focal strain: a single microbial strain to be studied for its proliferation, activity within, or effects on a microbiota.

N+1 engineering: intervention in a microbiome of choice to add a focal strain, either for permanent engraftment, for temporary functional effects or for influencing the developmental trajectory of the microbiome.

N−1 engineering: intervention in a microbiome of choice to remove a focal strain, either to diminish its immediate effects (e.g., an infection) or to prevent its influence on the developmental trajectory of the microbiome.

ostensibly simple N+1/N−1 intervention tasks to improve our capability to rationally intervene in dysfunctional microbiomes.

This review begins with a detailed explanation of the N+1/N−1 concept; followed by discussions of how N+1/N−1 studies can be envisioned and designed, which tools are available to measure and quantify microbiome processes, and how computational models can be leveraged to understand the effects at the microbiome's system level. Since N+1/N−1 approaches are intimately related to natural processes of strain dispersal, we will then summarize what we might learn from natural N+1/N−1 occurrences to improve methods for microbiome interventions. Finally, we will present a number of case studies on N+1/N−1 intervention examples to summarize what has been learned, and how to go forward. Throughout, we emphasize the importance of systematic approaches across a broad spectrum of host- and environmental microbiomes to delineate general principles of microbiome intervention and differentiate them from system-specific traits.

THE N+1/N−1 CONCEPT

Precise design across a broad spectrum of host and environmental microbiomes

The N+1/N−1 concept is simple in its basic definition: to introduce ("+1") or eliminate ("−1") a focal strain of interest within a background targeted microbiome composed of N taxa (Fig. 1B). There may be various reasons (from an intervention point of view) to achieve N+1/N−1 states. For example, so-called suppressive soils have a lower incidence of agricultural plant diseases, which has been attributed to higher abundances of plant-beneficial bacteria (26). One might thus aim to complement disease-permissive soils with a specific plant-beneficial bacterial strain in order to ward off plant pathogens and enhance plant growth without intervention with phytochemicals (27) (Fig. 1C). As another example, one might strive to specifically remove a pathogen from the human gut after infection without the use of antibiotics (28), or to restore contaminated soils with detoxifying bacterial strains (29, 30).

Though simple, this N+1/N−1 concept opens the door to investigating the numerous experimental considerations and complex ecological processes that arise in microbiome engineering. For example, does introducing a focal strain into a microbiota mean that the strain should become permanently engrafted in that microbiota? Depending on the intervention goal, it might be sufficient to achieve only transient establishment(s), during which the focal strain can deploy its functionalities and then disappear. Depending on the dynamics or characteristics of the microbiome under scrutiny, adding or removing a focal strain may imply multiple successive interventions, in order to interfere with the microbiome development or its homeostatic processes. More generally, when aiming to introduce a strain that is beneficial to a host, it is critical to first understand how it disperses and proliferates in its habitat. This understanding in turn requires knowledge on the strain's niche, its competitors and predators, and when and where it best deploys its beneficial functionalities.

Indeed, strain dispersal, survival, proliferation, and decline are often multifactorial processes, which depend on the strain's genetic makeup, the microbiota composition, interspecific interactions, and characteristics of the habitat and niche. To generalize our understanding of these processes, N+1/N−1 studies present a tractable way to profile a wide range of different microbiomes and habitats (including those without and with hosts). As such, they can span both descriptive and mechanistic studies with native microbiomes as well as synthetic communities of reduced complexity that reproduce native microbiomes. Synthetic communities of reduced complexity (compared to natural microbiomes) have the advantage of better reproducibility, allowing more precise bottom-up *in vitro* systems control over the N+1/N−1 (knock-in and knock-out) composition and potential outcomes [see, for example, recent work on plant leaf synthetic microbiota (14)]. Ideally, such synthetic communities reproduce a native microbiome counterpart, and their composition can be guided by inference from multi-omics analysis of native microbiomes to have appropriate taxonomic and functional representation.

Importantly, N+1/N−1 approaches can bridge the gap between correlative and descriptive studies of microbiome composition (which are typically limited in their taxonomic and mechanistic resolution) and molecular studies focusing on causality in simplified systems (which improve reproducibility but may be biased by the composition of its microbial members). When considering the focal strains selected, an N+1/N−1 approach can more readily allow one to establish the molecular basis of their growth or demise within their respective microbiomes. The resulting mechanistic and ecological knowledge can then be extended by predicting the outcome of microbiome interventions. Failure to predict such outcomes would help to identify crucial remaining knowledge gaps and encourage the re-examining of established hypotheses. Conversely, successful predictions would drive the improvement of precision microbiome intervention tools.

N+1/N−1 experimental design

The first step in an N+1/N−1 study is the selection of focal strains. Ideal candidates are strains of relevance for a future application or therapy (e.g., plant-growth-promoting bacteria, probiotics, or xenobiotic metabolic complements; Fig. 1C) but could also consist of a presumed keystone taxon in a microbiota interaction network (31–33) or sentinel species (34). Interventions focused on pathogenic strains have the goal not to introduce the focal strain, but rather to remove it from a microbiome (N−1). Nonetheless, infective pathogens can also be studied through an N+1 framing, as they have evolved specifically to invade microbiomes. Understanding this invasive behavior may help to design strategies that lead to the pathogen's suppression or removal. For example, non-pathogenic strains that can fill the same niche may inhibit the pathogen from proliferating within the microbiome (35–38). We may also learn from pathogen invasion to guide strategies to improve the transient establishment of a (non-pathogenic) N+1 inoculant. Additional considerations in the selection of a focal strain may include genetic tractability (to enable comparison with microbiota properties in the presence of a mutated focal strain) and a good experience basis for physiological and functional screening (Fig. 2). Additionally, focal strains should minimize potential biological safety concerns, especially in the context of health or environmental intervention studies.

Any N+1/N−1 study needs a minimum of four components: the focal strain by itself, the resident microbiota by itself, the combination of the focal strain within the resident microbiota, and a defined habitat (Fig. 2). By comparing the growth of the focal strain in isolation with its growth in the presence of the resident microbiota, kinetic and physiological differences can be assessed, which are the basis for uncovering underlying regulatory mechanisms, metabolic changes, and ecological processes. Under the condition that one can achieve similar growth rates and phases of the focal strain in laboratory and native microbiome conditions, such comparisons can point to specific

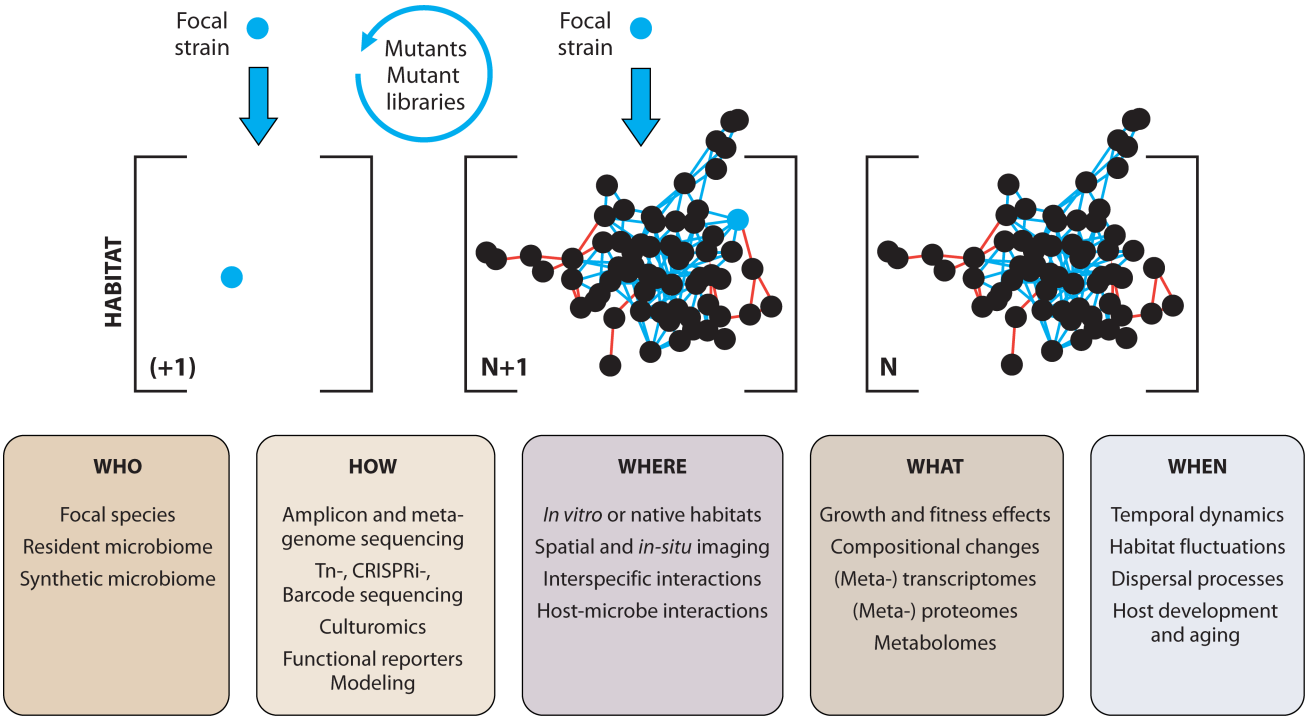


FIG 2 The who-and-how of N+1/N−1 microbiome interventions. Focal strains (symbolized by the blue circle) to be added into or removed from the target habitat (dark brackets) and its microbiota (N, represented here as a taxa co-occurrence network of positive: blue, and negative: red, connecting lines). Experimental designs need at least three conditions; the focal strain alone in the habitat (+1), the resident microbiota alone (N), and the inoculation condition (N+1). Examples of the details to define under "who," "how," etc. are explained in the main text. Iteration of the approach results from, e.g., follow-up experiments with mutant focal strains or altered habitat conditions and microbiota, in order to uncover molecular, ecological, or mechanistic processes.

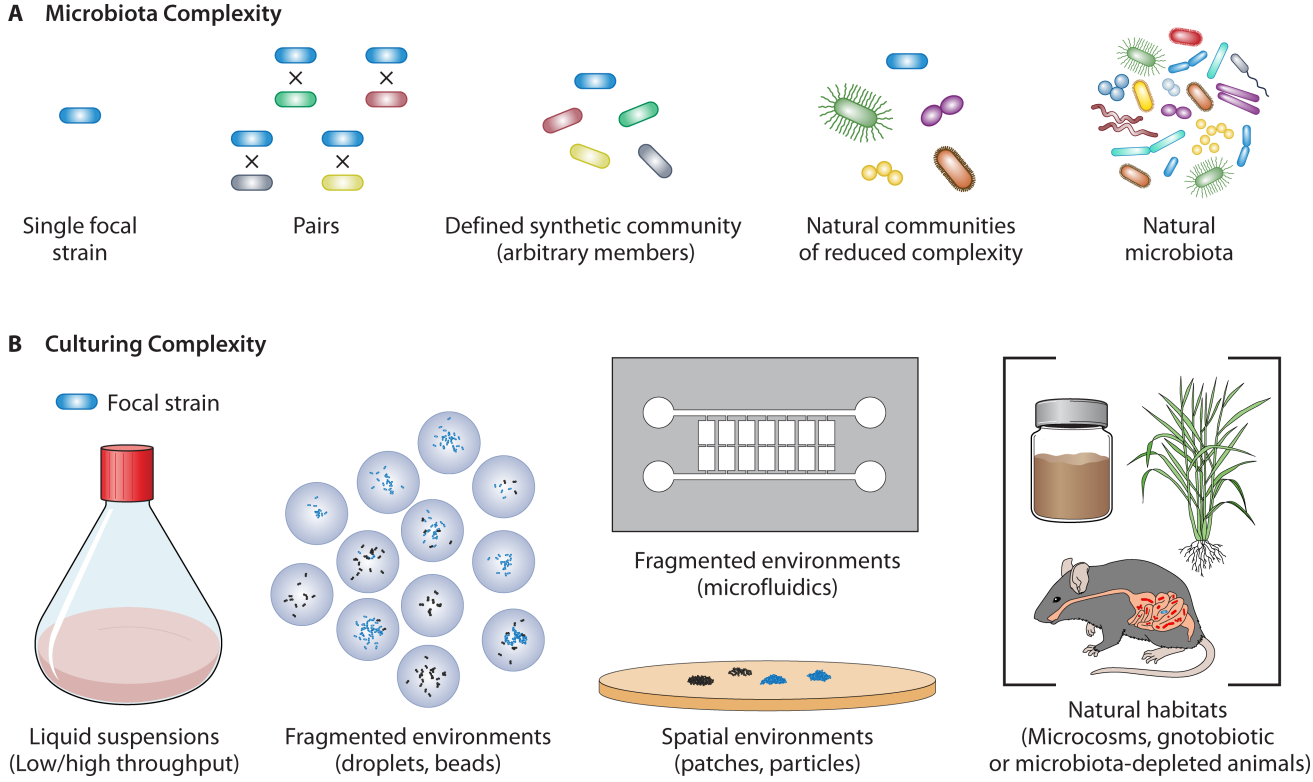


FIG 3 Potential lay-outs of N+1/N−1 studies. (A) Focal strains (here in blue) can be tested in conjunction with microbiota of increasing complexity to establish ecological and molecular mechanisms of their potential survival and proliferation, as well as their impact on the microbiome in the short or longer term. (B) Culturing systems can be tuned specifically to the main study objectives and/or methodological tools available.

adaptive mechanisms of the focal strain and to the prevailing host/habitat factors to which it reacts. These results can then be confirmed with focal strains mutated in identified characteristics or pathways.

The choice of resident microbiota into which the focal strain is introduced can range in complexity, different levels of which can be conducive to uncovering specific mechanisms and processes (e.g., interspecific interactions), and system-level properties (e.g., colonization resistance or niche availability) that control the focal strain's proliferation, survival, and impact (Fig. 3A). The simplest set-ups can consist of paired co-cultures between the focal strain and (native) culturable microbiota members (39), or even with any arbitrary culturable strains relevant to specific ecological processes (e.g., competition) (40). Combined information from co-cultures can to some extent predict higher-order interactions and the dominating community network (41, 42). As a next step in complexity, one could design simplified synthetic communities with members obtained either from a collection of culturable isolates (43) or from the native microbiome (38, 44–47). The advantage of choosing simplified communities is a more controllable, reproducible, and defined microbiome, which can help to produce N−1 states by composition and dissect effects of N+1 inoculations, treatments, or other system perturbations. However, such synthetic communities remain a simplification, of which it is important to understand the differences to the native microbiota. Multiple simplified microbiota have been compiled in recent years, representing a range of habitats. These include, for example, the Oligo-MM-12 (38), Oligo-MM-19 (48), and LCM communities (49) for the mouse gut microbiome, and a defined 104-strain hCom1 human gut microbiota mixture (50). Other model animals, such as the honeybee (46, 47), have also been used to study specific gut microbiota effects. Simplified representative synthetic communities have also been produced from cultured isolates for complex microbiomes that are inherently open in nature, like plants or topsoil. Examples here include the *At*-SPHERE [5 phyla, 430 plant

leaf and root isolates; reference (44)], or a 21-member (4 phyla) collection of soil isolates (45). Higher complexity can be achieved by cell "washes" from the natural habitat of the microbiota and their controlled reintroduction, but this comes at the loss of some reproducibility (45).

The final system choice to make with the selected focal strain(s) and resident communities is the habitat itself (Fig. 3B). In addition to the native habitat of the resident microbiota, one can test a range of different culturing conditions that allow for more precise control of the habitat and comparison across systems to identify consistent underlying mechanisms and processes. For example, liquid-suspended culturing is accessible and offers flexibility in terms of nutrients and growth conditions. It is also simple to parallelize in medium- to high throughput while maintaining flexibility to analyze microbiota (compositional) outcomes. At higher throughput, one could choose for fragmented and miniaturized growth in, e.g., encapsulated beads (51), microdroplets (41), or microchip formats (42). Spatial constraints are more easily implemented in microfluidic chambers (52–54), on surfaces (55), or "reprints" of native habitat structures (56).

In case the native habitat is preferred, one could reproduce this in a sterile form with the same overall conditions. When working with animal or plant hosts, one could deploy gnotobiotic or microbe-depleted individuals. This allows a controlled recolonization of the habitats, which is an extremely important element of N+1/N−1 studies. For example, gnotobiotic animal models permit growing microbiomes with known strain composition; in which case the natural habitat (e.g., animal gut) is colonized by a resident microbiota with reduced complexity. Cross-inoculation experiments are frequently possible, as demonstrated by human fecal microbiota grown in gnotobiotic mice as surrogate hosts, so-called "humanized microbiota" mice (57). Also, microbiota-depleted bees have been successfully recolonized by mixtures of their resident gut microbiota of defined composition and reduced complexity, to test specific host-microbiota and even behavioral effects (46, 47, 58). Similarly, gnotobiotic plants permit culturing of reduced complexity leaf or root microbiomes, which has been exploited to measure the effect of individual members of the *At*-LSPHERE microbiota collection by drop-out experiments (59). As an alternative approach to representative synthetic communities with cultured isolates one can also extract the mixture of resident microbiota from their original habitat and transplant this mixture in reproduced sterile or gnotobiotic habitats, as recently shown for soils (45). In certain cases, this results in reproducible high-complexity communities that can serve as resident background for testing N+1/N−1 scenarios.

Methods to monitor focal strains

The proliferation and physiology of an introduced focal strain within the microbiota and selected habitat can be measured with a variety of methods. For example, high-throughput sequencing and mass-spectrometry approaches can quantify changes in gene and protein expression as well as in metabolism between defined laboratory growth conditions and natural environments or the habitats intended for targeted application (60, 61). Instead of single focal strain genotypes, random insertion (36, 60) or CRISPRi mutant libraries (62) can be produced and inoculated, in order to gain functional evidence for the relevant mechanisms enabling the focal strain to proliferate. Kept in a library format of individual clones with each a single insertion mutant or CRISPRi-inhibited transcript, the complete library covers multiple times all coding content, such that positive and negative fitness effects and their modulation by habitat conditions and the microbiome can be detected. This is typically accomplished by growing the library under the conditions of the intended application, in comparison with defined laboratory conditions. Samples taken at different incubation time points are used for DNA extraction and sequencing of the marker insertion positions or CRISPRi-targets. Subsequently, the relative abundances of all marker insertion/target positions in the different conditions are quantified and compared among each other and to those in the

starting library. Depletion of marker insertion positions is interpreted as a sign of their negative fitness effect, whereas enrichment may point to fitness gain.

Fluorescently tagged focal strains may also be used to address specific questions on their *in situ* behavior or individual cell variability within a microbiome (63–65). Growth dilution markers [e.g., conditionally replicative plasmids (66), inducible fluorescent protein expression (67), or isotope labeling (68)], oscillation circuit-based molecular clockworks (69), and isogenic genomic barcoding tags can further inform on *in situ* growth rates and population bottlenecks (36, 70, 71). Fluorescent reporter systems can also reveal the actual spatial, temporal, and population-wide distribution of specific metabolic or physiological activities of the focal strain (54, 72–74), which can then be related to habitat substrate or niche availability and metabolic adaptation. Inducible off-on tags can be used to develop sentinel strains whose exposure to a specific stimulus in their habitat can be recorded and read out following their re-isolation (34, 75).

Microbiomes that can be observed over longer (e.g., evolutionary) timescales can be deployed to re-isolate clones of the focal strain during its colonization, survival, and/or population decline (76–78). The genomes of such isolates can be sequenced to identify the emergence of genetic or phenotypic traits that facilitated habitat adaptation (79). Ideally, this can be combined with *in vitro* growth experiments to validate assumed adaptive traits in niche colonization.

Methods to follow resident microbiota functioning and focal strain effects

Changes in the taxonomic composition of the microbiota can be quantified using amplicon sequencing of phylogenetic marker genes such as the 16S rRNA gene for Bacteria and Archaea, the 18S rRNA gene for microeukaryotes such as protists, or the internal transcribed spacer region for fungi. Depending on the compositional complexity of the resident microbiota, sequencing approaches may be replaced by, for instance, quantitative PCR. Although amplicon sequencing has the disadvantage of a lower taxonomic resolution, its convenience lies in its speed, cost efficiency per sample, and greater compositional depth. Interpretation of resident microbiota composition patterns is facilitated by numerous recent tools. By using a closed reference for mapping data (80), comparisons can be made across a large variety of studies. This can be explored in web interfaces such as the Microbe Atlas Database (https://microbeatlas.org/). The Microbe Atlas Database follows a single standardized approach to deduce taxonomic memberships, making it extremely powerful to compare taxa occurrences across habitats. Still, the current taxonomic resolution of Microbe Atlas and similar databases is limited because of their reliance on a single marker gene. In addition, only limited metabolic and functional microbiota information can be inferred from amplicon sequencing (81). Methods have been proposed to overcome this [e.g., reference (82)], but these depend on the availability of annotated reference genomes and ignore possible strain-level variations.

Alternative efforts aim to integrate taxonomic and functional information from metagenomic sequencing in the form of metagenome-assembled genomes [MAGs (83)]. As an example, mOTUs (metagenomic operational taxonomic units) is a community-benchmarked (84) metagenomic profiling tool that, in its latest version (85), leverages large-scale reconstruction of MAGs [e.g., reference (18)] and single-copy marker genes that can be identified in any metagenome (86). This makes mOTUs genome-independent, which is particularly useful for N+1/N−1 approaches involving resident microbiota that are not well represented by available reference genomes: a common issue across a wide range of environments and hosts (85). Ongoing efforts aim at mapping sequences of 16S rRNA genes and single-copy marker genes to their MAGs of origin, which would allow for quantifying gene functional compositions rather than inferring functions from taxonomic profiles, all while accounting for taxa without available reference genomes. These efforts will be facilitated by deploying long-read sequencing for metagenomics (87).

For simplified resident communities, amplicon sequencing can serve to follow compositional changes at high depth and resolution. It should be noted, however, that currently available sequencing strategies do not provide absolute but only relative strain abundances, except if combined with DNA spiking (88). Since the absolute population size is a crucial readout (e.g., for estimating growth), it can also be quantified using plate counts, flow cytometry, or quantitative PCR (89). Recent advances in flow cytometry have also demonstrated that community size quantification and population "typing" can be achieved simultaneously by applying machine learning classifiers, at least for low-complexity communities (90, 91). Such advances may greatly simplify the analysis of high-resolution time dynamic compositional changes emerging from N+1/N−1 experiments.

The analysis of diversity and compositional changes in the resident microbiota can be further coupled to pairwise random growth experiments with the inoculants, in order to understand how the focal strain may benefit or inhibit specific microbiota members. For small, defined microbial consortia, full interaction networks can be obtained by exhaustive (paired) co-culturing. Such networks are valuable tools to understand how a system is structured, contributing to hypotheses on, for example, the community-wide effects of centrally positioned microbes with high disruptive potential (31, 32, 59, 92, 93). While recent co-cultivation methods (42, 94) have enabled the screening of increasingly complex communities, obtaining networks for large, natural communities is still out of reach. Statistical methods based on co-occurrence (95–98) or time series data (99, 100) are useful instruments to start investigating such systems, allowing a first glimpse at the structure of the underlying ecological networks. More practically, they could guide the design of tractable synthetic microbial consortia—ensuring the predicted ecological network is well-covered—or help to strategically prioritize focal strains with a high predicted impact on the overall community. Schäfer et al., for example, systematically studied the impact of more than 200 individual strains on a defined synthetic community of 15 members to identify the major interacting species (39).

At a systems level, responses of the resident microbiome to the presence or absence of focal strains or to changes in biotic or abiotic factors can be followed by metatranscriptomics, metaproteomics, or meta-metabolomics. These methods are particularly applicable for observing changes in carbon or nutrient flows relevant to an N+1/N−1 engineering process (101).

Impact of spatial organization on the N+1/N−1 concept

Microbial habitats generally do not consist of homogenously mixed environments with ideally suspended individual cells of different strains but rather form highly spatially structured and dynamic assemblages of strains, biofilms, microcolonies, and with accompanying (dynamic) gradients in nutrients or electron acceptors. Unfortunately, studying spatial structures of complex microbiota is a challenging task that is currently almost exclusively accomplished by fluorescence *in situ* hybridization (FISH) techniques using probes to target different taxonomic groups (102–106), or extended to specific gene expression patterns in a spatial context (107). Despite its advantages, the application of FISH is limited in that the cells are inactivated through fixation and that probes are not species-specific. Together, these limitations complicate the interpretation of positive FISH-hybridization signals to corresponding functional activity. The use of genetically tagged fluorescently labeled focal strains is of an advantage here, as it can point to their live-specific spatial localization within the habitat and amidst resident strains. This approach can also be combined with suitable host models and live microscopy to obtain time-resolved information (108–110). Reduced complexity in the target microbiota and standardized habitats that enable non-invasive and live visualization would enormously simplify the characterization of the colonization process of a focal strain and could thus provide crucial dynamic information that is hard to obtain otherwise. This may be achieved, for example, by using microfluidic culturing systems, which enable control over spatial geometry, hydrodynamic and/or substrate

flow, material properties, nutrient sources, or gradients of electron acceptors. In addition, microfluidics approaches present opportunities to study bacterial cell adhesion, invasion, and virulence in habitats that mimic those of the host (111). Bead-encapsulation (51) or picoliter droplet culturing systems can be used to propagate microcolonies or micro-assemblages of different strains that reflect discontinuous growth environments (42). This might be coupled with sequencing strategies to detect specific compositional biases between inoculants and resident strains.

TOOLS AND STRATEGIES FOR N+1 AND N−1 ENGINEERING

Learning from nature

Despite its conceptual simplicity, successfully achieving N+1/N−1 states is a major challenge. The main reason is that the outcomes of focal strain inoculations are governed by complex ecosystem processes (which iterative N+1/N−1 cycles themselves aim to uncover). To better understand these processes, it is helpful to focus our attention briefly on what is generally known about the growth and development of microbiomes. More particularly, it is important to understand the characteristics of dispersal processes and how they can inform rational N+1/N−1 engineering efforts.

Microbiomes are inherently dynamic entities subject to processes that change their composition and functional gene repertoires. Here, we collectively refer to these as microbiome "developmental" processes. The development of microbiomes is, first of all, dependent on the microbial taxa that they encompass and their functionalities. Microbiomes are connected to the rest of the natural environment, and therefore, any microbiota is at any time subject to dispersal processes that lead to inflow of new strains (from "outside" the microbiome) and outflow (loss) of resident strains due to migration or cell death (Fig. 4A). For example, a growing plant will become colonized by microbes from the soil microbiome, an insect eating from a plant leaf will ingest microbes from the phyllosphere microbiome, and the microbes in its droppings (the insect gut microbiome) may find their way back into the soil. Existing resident and newly incoming strains undergo growth and decline as a function of selective pressures, available nutrients, or changes in the habitat, leading to fluctuations in taxon abundances, extinctions, and an eventual natural homeostatic state for the microbiome. Processes within the microbiome also contribute to its diversification, due, for example, to mutational drift, phage infection or predation pressure, and horizontal gene transfer processes, which can cause the sudden outgrowth or decline of resident strains (Fig. 4A) (15, 112–114).

Dispersal of strains can be considered as natural (albeit uncontrolled) N+1/N−1 events, most of which may go unnoticed or do not lead to strain establishment (though they may lead to the replacement of strains with others with very similar properties). For example, in the human gut, various strains of *Escherichia coli* succeed one another at relatively short time intervals (115). Progressive strain replacement has also been observed for plant-interacting pseudomonads, which evolve over time with their plant host, leading to the disappearance of ancestor strains (116, 117). Wastewater treatment systems, which act as open pools of inflowing material, collect a wide variety of microbial taxa with a specific set of recurrent core members (118). In some instances, however, incoming strains are able to expand their population at the expense of the stability of the resident microbiome. In these instances, one could speak of strain invasion, potentially leading to long-term deleterious consequences for the homeostatic processes of the natural microbiome (119). Pathogens are best known for their invasive behavior (Fig. 4B). For example, gut pathogens such as *Salmonella enterica* serovar Typhimurium (*S.* Tm) have evolved to exploit and release niches for their own establishment (36). Extreme distortion and collapse of microbiome homeostasis can also originate from within the existing microbiota by opportunistic strains. For example, several endogenous pathogens (such as *Clostridiodes difficile*) can occur at relatively low abundances in an undisturbed gut microbiome, but their outgrowth can arise when the composition, size, and function of the resident population are significantly impacted as a result of antibiotic treatment (120).

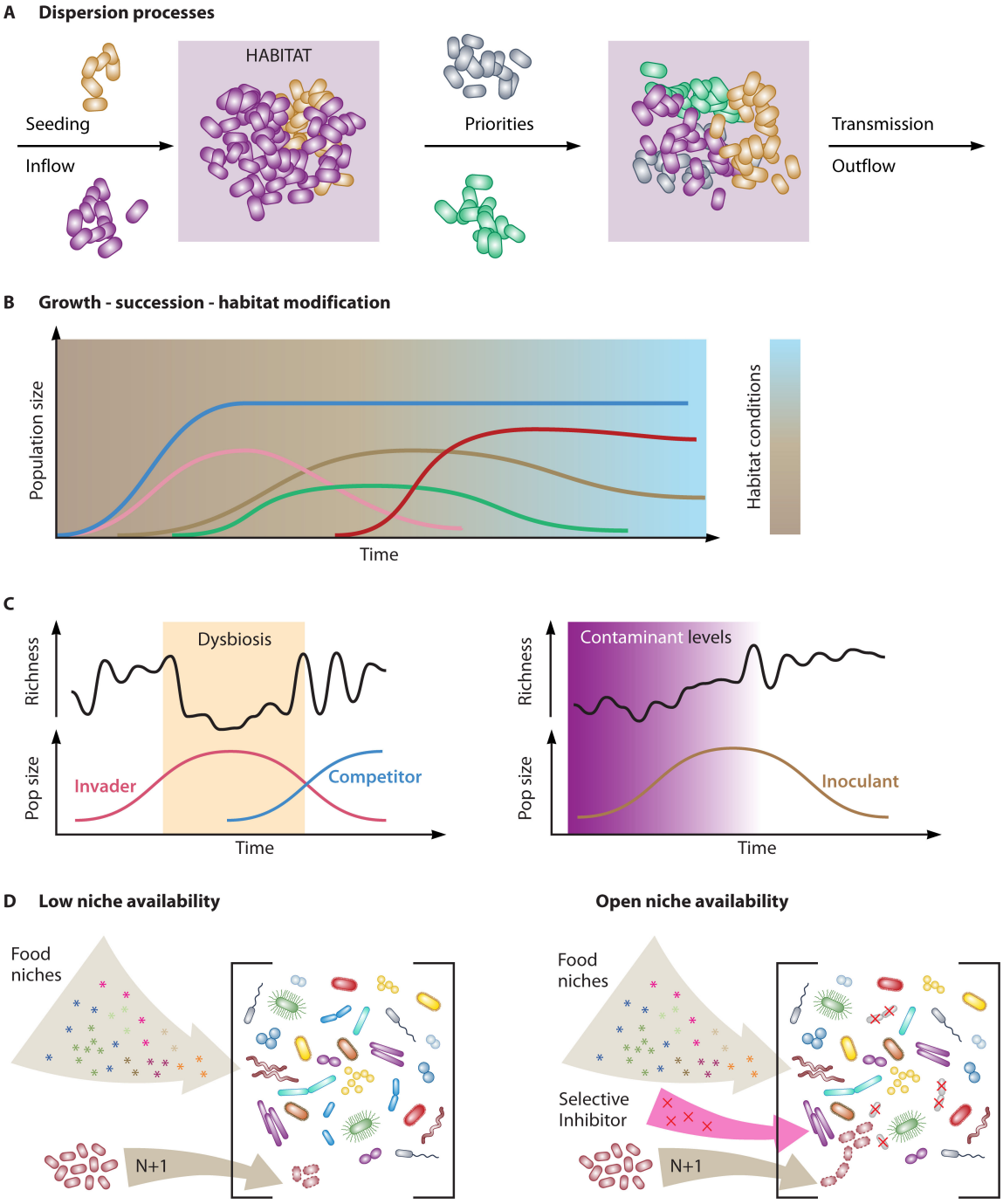


FIG 4 Concepts of microbiome development and N+1/N−1 engineering. (A) Dispersion processes leading to in- and outflow of strains into the habitats that form the microbiome boundaries. The initial establishment may be dependent on active seeding or transmission of specific microbial strains into the habitat, followed by the passive inflow of further strains, some of which may invade the established microbiota. Priority, selective, and random processes further determine the development of the microbiome. (B) Community development as a succession of different individual strain populations (colored lines), selected by the local environmental conditions, priorities, inflow, mutation, and available nutrients. Modification of habitat properties occurs as a consequence of growth, interspecific interactions, and emergent microbiome system-level properties. (C) Examples of N+1 interventions to restore microbiota-homeostasis: in case of a dysbiosed microbiome resulting from pathogen invasion or in case of a pollutant-stressed microbiome. Lines indicate N+1 population development and general microbiota richness. Gradients point to (reduced) pollutant levels resulting from inoculant activity. (D) Creating nutritional niches for N+1 proliferation, for example, by selective inhibition of a resident niche occupant (cells with red crosses).

Natural microbiomes strongly differ in their propensity, frequency, or range for dispersal, and individual strains likewise differ in their capability to establish in a new habitat, which has been associated to their growth competitiveness under available resources (121–123). Microbiomes existing in (more or less) open environments, such as soils, wastewater, or aquatic systems, as well as on skin, and plant leaves or roots, would intuitively be expected to be more strongly influenced by species dispersal (from other environments) than microbiomes in more closed environments, such as human, animal, or insect guts. Dispersal success and environmental filtering are further dependent on the occupation state of the habitat (124). Some habitats like guts or skin tissues of newborns and growing roots are rather pristine at the time of microbiota colonization and settlement (77, 113), whereas others may be fully occupied with microorganisms up to the system's carrying capacity. Intuitively, a pristine habitat may be easier to colonize than a system at carrying capacity, for which colonization resistance (i.e., the occupation of most available niches) needs to be overcome. Colonization processes of pristine habitats have been studied in detail and are typically characterized by waves of succession of different taxa and turnover until some form of steady-state or homeostasis is reached (45). For example, during human development, there is a pronounced rapid strain evolution and turnover of the microbiota in early childhood, when the human colonic microbiome follows a succession as the taxa gradually occupy the available niches (77). Colonic succession has been suggested to be strongly deterministic within the habitat conditions (45) but is further dependent on environmental cues. This has been attested by the permanent changes in gut microbiota composition in children with chronic undernutrition (125) or high-fat diets (126).

Modes of strain dispersal also play a role in successful establishment. Dispersal can take the form of active microbial motility, like gliding on surfaces, swarming, or swimming towards chemicals (110, 127) or light (128, 129), or can be a passive process. For example, bacteria can passively disperse via transport by fungal hyphae in soil (130, 131), by wind or water flow, association to particles, food uptake, or macroorganism activity (124, 132). Active motility typically allows for dispersal at smaller spatial scales, while passive transport allows for dispersal at larger spatial scales. Successful dispersal also entails overcoming ecological bottlenecks, which may determine the founder population size necessary for colonization. Finally, some dispersal mechanisms may have been selected naturally to ensure successful (or controlled) invasion and colonization of habitats, such as gut microbiota transmission through fecal pellets (133), maternal brood balls (134, 135), or breastfeeding (113).

In summary, N+1/N−1 intervention methods can thus take inspiration from these different processes to guide, for example, the design of specific requirements for windows of inoculation opportunity, niche availability, transmission, and temporal or long-term establishment.

Considerations for N+1/N−1 implementation

The processes underlying the outcomes of artificially introducing or eliminating strains from a microbiome will be similar to natural processes taking place during strain dispersal or microbiota transmission, except that by the engineering effort known limitations can be overcome (e.g., founder cell population size or niche availability). As a consequence, one can expect that the growth or demise of a focal strain in a target habitat will similarly depend on multiple factors, such as the state (composition, spatial structure, and functionalities) of the resident microbiota, the dynamic time window for inoculation opportunities (priority effects), physico-chemical parameters of the habitat, niche availability for the focal strain, selection for its metabolic capacities within the context of the habitat, its sensitivity to predation, and any specific competitive traits that a focal strain may deploy (136). To target a state in which N+1 focal strains can proliferate and survive within the target microbiome, it is necessary that appropriate nutrients are available to generate the metabolic energy necessary for their growth and that they can tolerate or adapt to any biological or physico-chemical stresses present in that habitat.

These considerations may lead to different approaches depending on the microbiome. Targeted colonization of the relatively pristine guts or skin tissues of newborns, or growing roots of fresh seedlings, will require different strategies than inoculation for restoration of disrupted microbiomes resulting from medical interventions or pathogen attacks or for reduction of pollutant loads in contaminated environments. Since N+1/N−1 strategies may be particularly envisioned in contexts of disrupted or dysbiotic microbiomes (Fig. 1C), nutrient availabilities and potentially unfavorable habitat conditions may play a crucial role in successful inoculant establishment and activity.

For example, one might aim to revert the consequences of an invading gut pathogen and the accompanying microbiome changes by the intake of a non-pathogenic niche competitor (37). This competitor would have to be able to proliferate in the dramatically altered gut nutrient conditions (137), in order to exclude the pathogen from its occupied niche (127) (Fig. 4C). As a different example, environmental changes imposed by diet shifts, fertilizers, antimicrobials, pesticides, or pollutants can disrupt microbiota composition and function, thereby altering the nutrient availability while imposing additional toxic stress. The goal of a focal strain here might be to reduce the toxic load, but to do so it must be able to take advantage of an excess of available nutrients to grow to a sufficiently large population to have an effect (Fig. 4C). Environmental toxicity can mask competition for resources and promotes facilitation between community members, which could help a focal strain establish (138).

In some cases, the intended outcome of an N+1 engineering strategy may be to achieve permanent establishment ("engraftment") of a focal strain within a microbiome, whereas in others the aim may rather be its transient and reversible presence. Examples of the latter may include the degradation of a toxic compound within a polluted system or the elimination of a plant pathogen during the vulnerable growth period of a crop (Fig. 1C). Alternatively, intended N+1 states may consist of engrafting specific genetic properties into the resident community members by means of the focal strain, but without necessarily maintaining it in the microbiota. This strategy has been followed, for example, to transfer pollutant degradation properties on mobile genetic elements into resident community members (139), but also to specifically inhibit functions in a targeted host within a microbiota (140, 141) or induce its killing (142). Temporal and controlled maintenance of focal strains has been a major goal of bioengineering efforts, which may involve genetically constructing or selecting strains with auxotrophies or inducible killing mechanisms that could restrict the survival of the focal strain outside the targeted habitat (142–145).

N+1/N−1 engineering tools

A number of tools are available to carry out an N+1/N−1 intervention process. In the easiest conception, N+1 engineering entails inoculating (i.e., mixing, injecting, swallowing, transplanting, etc.) a focal strain into a resident community at a defined dosage of live cells (Fig. 4D). Strain inoculation may be accompanied by a provision of unique carbon substrates or growth factors (synbiotics) exclusive for the focal strain that are added to the habitat or host, with the expectation that such substrates may at least temporarily favor its proliferation within the new habitat (146). Some studies have considered priming the focal strain before its inoculation, such that its adaptation period is minimal and does not hinder its proliferation within the target microbiome (147).

N−1 engineering, conversely, aims to deliberately eliminate, reduce, or inactivate a focal strain within a microbiome. Several means are conceivable for achieving this state, some of which rely on N+1 to achieve N−1 (in this context, introducing a focal strain to remove another, Fig. 4C). One approach, for example, has been used to eliminate a pathogenic strain by introducing a niche competitor at large numbers, which colonizes the microbiome and accelerates the clearance of the pathogen (143). Competitive niche exclusion is likely the reason underlying the frequent natural appearance of strain successions in the gut, which could thus be turned into a viable N−1 procedure (120). Similarly, the introduction of naturally occurring or engineered auxotrophic variants

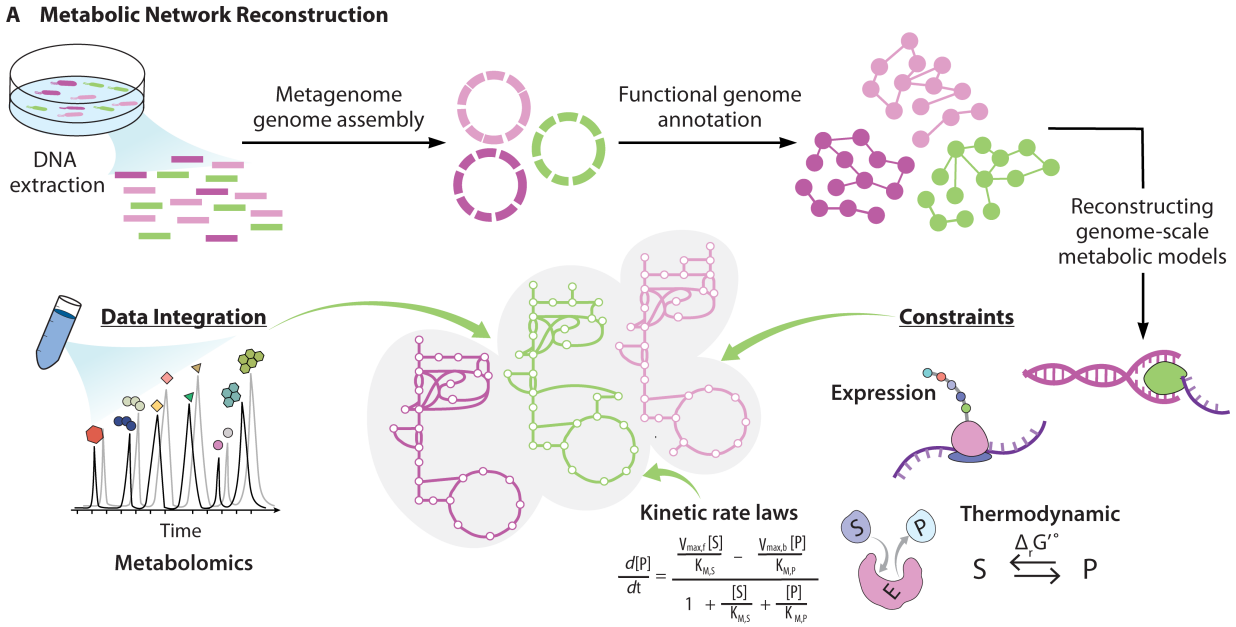
could drive the targeted extinction of a focal strain (148, 149). A recent success in the elimination of a recurring human gut pathogen, *C. difficile*, was obtained by re-inoculation not by a single focal strains but by complex microbiota retrieved from the distal gastrointestinal tract of healthy donors, a principle now known as fecal transplantation (150). Remarkably, this leads to the replacement of some 80% of strains present in the gastrointestinal tract before the transplantation and stable engraftment (more than 5 years) of almost two-thirds of the introduced donor taxa (151).

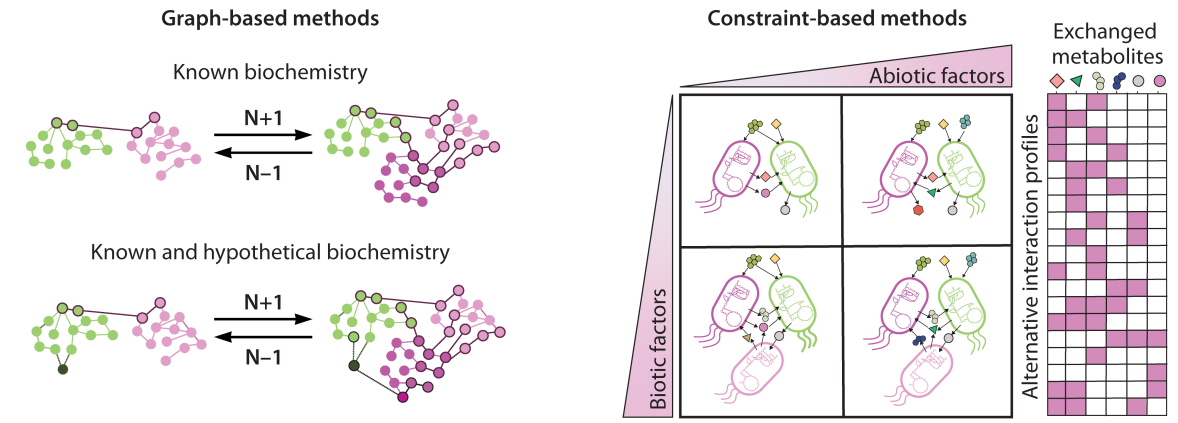
A second approach relies on eliciting an infection of the N−1 focal strain with a bacterial virus such as a lytic bacteriophage (phage), which can lead to its death. Phage infection is highly strain-specific and can be "self-propagating," in that infected and lysed target cells produce more phage particles that can then specifically infect neighboring cells (152). As a result of its selectivity, phage therapy could "spare" most of the members in a microbiome in contrast to broad-spectrum antibiotics (153). Phage killing of the focal strain could also be used to "open" the niche for colonization with non-pathogenic competitor strains of the same species, which are resistant to the applied phage. However, resistance to applied phages has been observed to rapidly arise and a combination therapy with antibiotics may, therefore, be necessary for effectively treating an infection (154). Phages are also considered as tools to restore dysbiosed microbiomes, in an approach termed phage rehabilitation (155, 156). As an alternative to infective phage particles, microbiomes may be dosed with purified phage proteins, such as endolysins, to target strain-specific lysis and removal (157, 158). Finally, purified phage tail-like particles known as tailocins are also being considered as tools to control or provoke subtle strain abundance changes within microbiomes (159).

Similarly, N−1 focal strains may be targeted by toxic proteins leading to their cell death. A proof of principle for this concept has been demonstrated, in which a donor strain transmitted a genetic construct into an (*E. coli* or *Vibrio cholerae*) focal strain by horizontal gene transfer, leading to the expression of a toxin that inhibited further cell division (142). In another example, *E. coli* engineered to produce a narrow-spectrum siderophore-bacteriocin Microcin Mccl47 was introduced into the mouse gut as a live biotherapeutic agent, which was able to selectively inhibit Enterobacteriaceae (160, 161). Lastly, N−1 approaches could also rely on purified bacteriocins with high host specificity (162, 163), or on drugs, especially if they display a narrow host spectrum (13, 164, 165). However, even narrow-spectrum agents may still be affecting commensal bacterial strains and, therefore, secondary effects on resident microbiota should be an integral part of N+1/N−1 studies.

Niche engineering

Apart from the biological tools needed for N+1/N−1 engineering, one should also consider the niches that are necessary for the focal strain to proliferate. Engineering the niches themselves can then be used as a tool to restore, implement, or prevent the growth of focal strains (or to combine both, Fig. 1C and 4D). For example, a wastewater treatment plant may in a sense be considered a (very rough) niche-engineered system to facilitate specific biogeochemical processes such as carbon removal, nitrogen transformation, or phosphate uptake, by controlling oxygen inflow and carbon dosage. Microbiome niche precision engineering has, until now, not been very well-developed, apart from a few examples including the synbiotic additions to inoculated strains mentioned above (146). For precise niche engineering, one would first have to map the available functional and spatial niches in a microbiome—a first approximation of which could be achieved by genome-scale metabolic models from individual isolates or metagenomes (Fig. 5A). In addition, one would also have to get an idea of the niche "size," i.e., the carrying capacity of the niche for functionally similar strains. As an example, most soils have a relative abundance of around 25% Proteobacteria, which may constitute hundreds of individual strains (166). At a typical bacterial cell density of 10^9 per gram of soil, it may thus be challenging to expect maintenance of an N+1 focal strain at much higher than 10^7 cells per gram. The true accessibility of a niche may





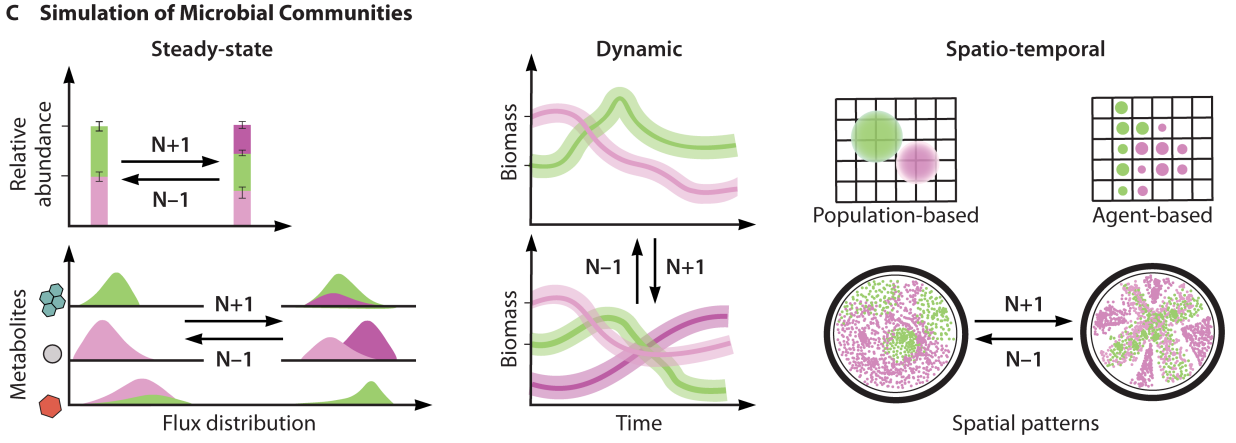


FIG 5 Computational tools for N+1/N−1 engineering. (A) Genome-scale metabolic networks incorporate all known biochemistry of an organism based on functional annotation from individual genomes or binned metagenome sequence data and can be contextualized by integrating additional constraints and data. (B) Metabolic interaction maps for the N+1/N−1 focal strains can be reconstructed by graph-based or constraint-based methods. (C) Microbial community function and development can be simulated at different scales to predict the effects of N+1/N−1 focal strain inoculations.

also be dependent on some window of inoculation opportunity. For example, an N+1 focal strain co-inoculated with resident community members simultaneously (so that all members need to grow) may find a larger niche than when inoculated into a steady-state community (essentially no net growth of any of the community members). Temporarily liberating niches to allow recolonization by others may, therefore, be a strategy to accompany N+1 /N−1 interventions (167) (Fig. 4D). This can be achieved by using abiotic compounds (antimicrobials, food additives, diet shifts, pesticides, or others) that target certain members of the community (37, 168, 169). When applied non-recurrently, these compounds can create a transient niche for the focal strain that can be occupied (37). In the case of mammalian-associated communities, the adaptive immune system can be harnessed via targeted vaccination approaches. While immunotherapies are intuitively an N−1 approach, recent evidence indicates some antibody responses can enhance microbial fitness, making it a feasible, targeted approach for N+ 1 interventions (108, 170–175).

N+1/N−1 computational tools

Computational models are powerful tools to better understand the inherent complexity of single species' metabolism and the emerging metabolic capabilities in the context of a microbiome (Fig. 5). These methods can be used as platforms to integrate and analyze data from the experimental designs outlined above, as well as to generate hypotheses and guide the design of experimental setups. Functional genome annotation based on sequence similarity has enabled the reconstruction of metabolic networks representative of all known biotransformation capabilities of an organism (Fig. 5A). These networks can be translated into mathematical models, which are known as genome-scale models (GEMs). GEMs can be coupled with constraint-based optimization methods to allow the estimation of feasible flux distribution profiles that support an observed phenotype (e.g., growth) and help gain mechanistic understanding (176). Additional layers of information can be added to GEMs, including but not limited to thermodynamic constraints (177), gene expression (178, 179), regulation (180, 181), kinetic rate law expressions (182), and various types of omics data, to make GEMs context- and application-specific. In the context of multispecies microbiota, reduced GEMs (183, 184) that preserve network properties can be considered to increase computational efficiency.

The information contained in metabolic networks and GEMs of single organisms can be leveraged to infer possible outcomes of N+1/N−1 interventions through the prediction of metabolic interactions between different species. Qualitative predictions of interspecific metabolic interactions can be made using metabolic networks and graph-based approaches (Fig. 5B). A metabolic network can be represented as a graph where each node represents a metabolite and each edge a reaction. Efficient graph search algorithms can then be employed to identify alternative pathways from a source metabolite in species A to a target metabolite in species B (185). The existence and multiplicity of such pathways can hint at possible cooperation among the species and can be informative of the resilience of a community to perturbations. Similarly, this approach can be employed to examine how these pathways are affected after the addition or removal of a focal strain. For example, one would expect that taxa with high connectivity act as metabolic hubs, whose elimination can radically affect community composition and function. Finally, community networks can be enriched with hypothetical biochemistry based on the enzymatic functions present in each genome (186, 187), allowing the discovery of yet uncharacterized community functions.

Constraint-based optimization methods can be used to infer metabolic interactions in a community (Fig. 5B). To do this, several tools have been developed under assumptions tailored to different types of interactions (e.g., positive or negative interactions, pairwise, or higher-order interactions) (188–190). These methods generally involve two steps. First, the external nutrients available to the community are specified. Such external nutrients are provided either by the metabolism of the host or by the environment. In the second step, the metabolic interactions are reconstructed given the external nutrients and

organisms' metabolic capabilities, i.e., their uptake and secretion reactions. This process yields valuable information on how abiotic (nutrient availability) or biotic perturbations (adding or removing a member) can alter the interaction networks. In addition, the generation of alternative interaction profiles helps to account for the uncertainty and variability of microbiome composition.

Simulation and design of microbial communities

Like a population of a single taxon in a closed environment, a microbial community in a habitat can be assumed to grow and converge to some stable state [arguably subject to the same dispersal and turnover mechanisms as described above (191, 192)]. Simulating the microbiome's composition and relative taxon abundances, and its global metabolic functioning, is a longstanding challenge. The least complex form of computational methods simulates microbial communities at a steady state without making any assumptions about the history or taxonomic successions in the community (Fig. 5C). Such methods allow for a variety of analyses, including capturing nutrient uptake and byproduct secretion (193), estimating internal metabolic fluxes (194, 195), predicting relative abundances (196), studying resource allocation to macromolecule synthesis (197), and designing synthetic communities with desired properties (198, 199). Similarly, such computational tools can be used to study how the addition or elimination of an existing member might cause the community to converge to a different steady state. Perturbations in community composition, i.e., biotic perturbations, can affect flux profiles, abundances, nutrient requirements, byproduct formation, or resource allocation.

The scope of computational simulations can then be extended by incorporating temporal species and habitat dynamics (Fig. 5C). Dynamic species models include two classes: ecological models and evolutionary models (200). Lotka-Volterra models are a classic example of the former. They include a phenomenological description of the system in the form of ordinary differential equations (201). Evolutionary models, on the other hand, study the evolution and variation of a genotype of species over long-time scales (200). Dynamic simulations based on GEMs can capture temporal variations in microbial abundances, extracellular metabolite concentrations, and macromolecule synthesis (179, 202–204). Such methods allow studying and predicting the effect of adding/removing a member as a function of time, where the simulations can be contextualized based on the initial conditions, growth properties, and species interactions (205).

To accelerate N+1/N−1 research efforts, another layer of complexity is needed, which accounts for the spatiotemporal dynamics of microbial communities (Fig. 5C). Both synthetic and natural microbiota have an inherent spatial component that significantly affects their evolution and function. Microbial cells take up diffusing nutrients from their environment to grow and divide, driving the community to expand into spatial structures such as biofilms or cellular aggregates. The niche architecture is, therefore, constantly changing due to the local variations in the concentrations of metabolites and the abundance of microbial cells. In addition, the close proximity of microbial cells creates a crowded environment and reduces diffusion, which affects nutrient availability and the metabolic capabilities of the organisms (206). Spatiotemporal modeling approaches can simulate such complex biophysical (crowded) cell environments and can help us understand the stochastic effects of the N+1/N−1 events that are not captured by other modeling frameworks. These approaches differ in the level of detail they capture, often involving a trade-off between spatial resolution and computational cost (201). These models can incorporate the various biophysical and mechanical properties of the systems, such as cell shoving (207, 208), production of extracellular polymeric substances (209, 210), crowding effects (206, 211), cell morphology effects (212), complex habitat geometries (213, 214), and (flow) hydrodynamics (209, 215). Spatially resolved modeling of the cell environment and cell-cell interactions can help to understand how perturbations in abiotic or biotic factors (N+1/N−1) affect the system's

emergent properties (201, 216). Finally, such models help to predict changes in microbial interactions and functions between well-mixed and spatially organized environments.

N+1/N−1 INTERVENTION APPLICATIONS

Plant-beneficial bacteria and/or arbuscular mycorrhizal fungi in the rhizosphere

Plant-beneficial bacterial inoculants in agriculture have long been studied as substitutes for chemical fertilizers and pesticides, being able to ward off phytopathogens or insect pests, or to provide further beneficial functions such as solubilization of nutrients or priming for plant defense and stress tolerance (26, 217, 218). In practice, however, plant-beneficial strains typically only transiently establish, e.g., within the rhizosphere microbiome (219). Hence, multiple inoculations are needed, leading to temporary population increases followed by progressive decline until the next inoculation (27). The reasons that underlie this transient rhizosphere colonization are multiple and intricate. The inoculant must persist and be active in order to be able to exert its plant-beneficial functions in the rhizosphere. This implies that the inoculant must adapt to the particular physico-chemical and biotic conditions in the rhizosphere environment, including varying soil pH (220), water availability (221), or micro- and macronutrient accessibility (222, 223), but also to plant immune responses that can involve the release of signaling compounds (224). Root exudates provide carbon-rich compounds at the root surface and soil interface, which can support bacterial growth at high numbers (225). Nonetheless, plant roots also secrete secondary metabolites such as coumarins, flavones, and benzoxazinoids that can promote or inhibit the growth of specific taxa (226–229). Bacterial inoculants, in turn, may release metabolites that allow them to shape the root microenvironment (230–233), as well as modify plant metabolism and defense (234–236). These processes then indirectly alter the root microbiota composition.

As discussed in general terms above, the success of a beneficial inoculant to invade the rhizosphere microbiome is dependent on niche competition for resources or space. This competition is often mediated by broad-spectrum diffusible antimicrobial compounds, such as phenazines or acylphloroglucinols (237), and by specialized weaponry, such as contact-dependent type VI secretion systems (238), bacteriocins (239), or tailocins (240, 241). In addition, the survival of the plant-beneficial inoculant can be affected by natural predators inhabiting the rhizosphere microbiome, such as bacteriophages (242, 243) or grazing phagotrophic protists (244). The establishment of a newcomer into an otherwise settled rhizosphere microbiome could further influence the microbiome composition by altering the structure of key microbial functional groups, such as the nitrogen-fixing bacterial community (245). These modifications can ultimately have repercussions on plant growth and development (245–247).

Agricultural practice could also benefit from the selective removal of undesired taxa, notably bacterial, fungal, or oomycete phytopathogens that hinder plant development, impair plant health, and reduce crop yields (217, 248, 249). This is the long-desired goal of biocontrol strategies aimed at removing plant pathogens. Most biocontrol techniques have focused on introducing specific plant-beneficial strains, which can directly compete for resources or space with the pathogen, causing niche exclusion (26, 237). However, there are also approaches that would potentially allow the selective removal of an undesired member of the rhizosphere or soil microbial community without the introduction of another individual. For example, bacteriophages that directly prey on specific bacterial plant pathogens such as *Ralstonia* or *Pectobacterium* species were introduced as seed, tuber, or soil treatments (250). Similarly, the direct application of taxon-specific bacteriocins or tailocins to leaves resulted in diminished populations of pathogenic *Pseudomonas* and *Xanthomonas* and reduced disease incidence (251–253).

Future strategies for successful introduction of plant-beneficial inoculants should implement interactions between the inoculant and the plant microbiota in genotype-specific plant breeding strategies and foster agricultural measures such as soil amendments or adapted cropping sequences aimed at sustaining inoculant establishment and

function (247, 254, 255). Many studies where bacteria are added to field soils have focused on the effects of inoculation (e.g., on plant growth or protection). However, few have followed how successful the added bacterial inoculum was in colonizing the soil or rhizosphere or have even examined the factors that determine whether or not it was successful.

Bioremediation and bioaugmentation in soil and wastewater systems

The concept of inoculating individual suspensions or mixtures of pure culture strains to induce or augment targeted metabolic processes within a microbiome (hence the term bioaugmentation) has been intensively and long practiced in the areas of soil and wastewater bioremediation (29, 256–258). Here, the underlying premise is that strains that have naturally adapted or evolved to use specific pollutants as carbon, nitrogen, or energy sources can be purified, isolated, and transplanted from one (contaminated) environment into another (259). Furthermore, specific cases of engineered bacteria with additional properties to their metabolic capacities, such as to better withstand toxicity, have been considered for bioaugmentation (22, 260–262). Strain inoculation has mostly been a trial-and-error approach (263–267), with emphasis on the outcomes of pollutant disappearance and bioavailability (263, 268–270), but little on understanding the underlying reasons or mechanisms for these outcomes. Fundamental questions as to whether strain characteristics predetermine success or whether there is a need for specific native ecological networks (given that strains do not come from the same site as where they are introduced) have traditionally not received the necessary attention (257). Only more recently have studies emerged that combine systematic efforts to determine inoculant properties or genetic factors that may support or disfavor their (temporary) establishment, to characterize the processes taking place during inoculation, survival, or strain demise, to unveil any commonalities among strains that may prime them for being effective colonizers, and to simultaneously uncover properties and reactions within the targeted microbiome [e.g., references (60, 61, 271, 272)].

Several studies using different inoculants intended for the degradation of aromatic compounds, for instance, have shown that physiological adaptations to a contaminated soil environment typically involve upregulation of stress defense systems, heavy metal resistance, nutrient scavenging, adhesion factors, and downregulation of motility (147, 273–277). Importantly, such adaptations are within the scope of the focal strains' inherent properties, leading to their growth and temporal establishment within the target microbiome (60, 272), which is to a large extent dependent on the bioavailability of the targeted compound (147, 277, 278). As it can be assumed that the presence of the pollutant for which the N+1 inoculant is intended also presents its specific colonizable nutrient niche, the proliferation of the inoculant is favored for as long as the compound is available, and no other factors limit its activity or lead to its decline. Though it is not straightforward to understand at which point inherent strain properties break down and result in the strain's decline, some studies have pointed to trivial yet difficult-to-overcome biological factors such as predation (256, 279, 280).

Unsurprisingly, studies using omics approaches have found evidence for the importance of a multitude of microbial genes for survival and proliferation in natural colonized habitats (60, 278). Their interpretation is complicated by the many unknown and hypothetical gene functions that appear to give selective benefit for growth and that point to multiple different mechanisms that enable N+1-inoculants to proliferate (273, 281). Obviously, contaminated soil is a very heterogeneous environment in which additional nutrients are limiting, which potentially hinders the population development of N+1 inoculants. Specific advantages for their proliferation may be found in spatially localized substrate provision from plant roots, leading to rhizo- (282, 283) and phytoremediation efforts (30, 281).

Food and nutrition

Probiotics are live microorganisms that, when administered in adequate amounts, may confer a health benefit on the host (284). The use of probiotics in the form of freeze-dried or liquid products, or cultured as part of food fermentation, is pervasive in modern nutrition and has a long tradition. This application has more recently been expanded with specific probiotic strains or strain mixtures, whose use has been claimed to result in potential benefits against obesity (285, 286), for stimulation of the immune system (287), or (re-)development of a homeostatic gut microbiota (113, 288–290). One could, therefore, consider the use of probiotics as an example of an N+1 intervention in the human gut microbiome. However, as in the two other application areas mentioned above, there is a strong focus concerned with clinical or health outcomes, whereas probiotic colonization itself remains complex and controversial (290). In addition, understanding the mechanisms of action, causalities, and impacts of probiotics on microbiome development is still limited and under debate.

Today, the most commonly used probiotics include *Bifidobacterium* and *Lactobacillus* strains (291), but also *Streptococcus salivarius*, *Enterococcus faecium*, *Akkermansia muciniphila* (286), or *Anaerobutyricum soehngenii* (292). Their application (in the sense of the N+1 concept) consists of high dosages (10^9–10^{10} live bacteria per intake), taken daily over months (287, 288). Available measurements of, e.g., stool content seem to indicate that the swallowed probiotics do not engraft within the (human) gut environment and need to sustain high constant titers to exert their action. For example, mothers in a mother-infant cohort ingested capsules with four probiotic strains (total 1.2×10^{10} colony forming units) daily for 6 months until birth, and then the newborns followed this pattern for 3 months. Significant probiotic strain survival (reaching up to 80% of relative abundances) was observed only when infants were breastfed, and mostly for *Bifidobacterium breve* (288). Pre- and probiotics, therefore, often accompany each other, with the purpose to provide specific carbon sources or other nutrients to the probiotic strain (146, 290, 293).

Despite demonstrated physiological effects on the host, there is also evidence that intake of probiotics may be harmful, particularly in sensitive groups or individuals (294). This risk highlights the need for a precise understanding of the mechanisms, causality, impact, and safety of probiotics (292). For example, the effects of *Bifidobacterium longum* administration may be dependent on the composition of the underlying gut microbiota and differences in intestinal cell wall adhesion (295). A more thorough and personalized understanding of the risks and benefits of these treatments may be achieved through targeted microbiota interventions in an N+1/N−1 framing.

Enteropathogenic infections in the animal gut

The composition and function of the microbiota in the animal gut are affected not only by available nutrients, interactions between its members, and physical constraints (size of the gut, gut transit times, body temperature, etc.) but also by the host immune defenses. The latter can specifically enhance the removal of unwanted microbes and the colonization efficiency of newcomers (296). Moreover, host genetic variation also contributes to microbial colonization efficiency (297).

The gut is a portal of infection by a significant number of food-borne enteropathogenic bacteria, which cause much morbidity and mortality worldwide (298). Of particular relevance are members of the Enterobacteriaceae, including pathogenic *E. coli*, *Shigella,* and *Salmonella* strains. These enteropathogens are ideal focal strains for N+1/N−1 studies, because of their high prevalence, their genetic accessibility, a vast body of knowledge on *E. coli* and *S. enterica* serovar Typhimurium growth physiology and metabolism, and a multitude of robust *ex vivo* and *in vivo* infection models [reviewed in references (168, 299, 300)]. Their study has greatly helped to understand the mechanisms promoting infection, which, as mentioned above, resembles a natural N+1 situation. In addition, studies on enteropathogenic infections have helped to understand the role of colonization resistance in preventing infectious diarrhea after pathogen ingestion.

Finally, many of the effects of the gut microbiota on host immune responses during the course of an infection and frequent spontaneous pathogen elimination when the host recovers from acute diarrhea have been detailed and summarized (168, 296).

The resident gut microbiota normally provides resistance against colonization of a food-ingested pathogen such as *S.* Tm, limiting its growth in the gut lumen and thereby preventing gut tissue invasion and enteric disease in many infected hosts. This has been inferred from epidemiological data (301) and mouse experiments. Many experimental infection models rely on microbiome perturbation prior to pathogen introduction to establish colonization and pathogenesis (169, 302, 303). Complex gut microbiota indeed efficiently protect mice from *Salmonella* gut colonization, and the effect of colonization resistance can be increased by introducing competitive *E. coli* strains (37, 38, 304, 305). Recent work suggests that strain-specific capacities to utilize poly-arabinose fibers, arabinose, or sugar alcohols like galactitol can enhance their gut-luminal growth (306, 307). This might provide a concept that can be used to create niches that would select for proliferation of added competitive Enterobacteriaceae strains and could prevent or reduce *S.* Tm diarrhea and its spread in food animal herds.

S. Tm gut colonization is further suppressed by the host's adaptive immune system, in particular, by secretory Immunoglobulin A, which crosslinks *S.* Tm daughter cells during division, leading to large aggregates. This prevents tissue invasion and favors more efficient clearing by the fecal stream (108). Moreover, recent work has further indicated that oral vaccination in combination with N+1 competitor *E. coli* strains can suppress *S.* Tm gut colonization (N−1) in otherwise susceptible mice (308). Finally, an intestinal colonization-competent auxotrophic *S.* Tm vaccine strain induced an immune response in mice and acted as a niche competitor (143). Such proof-of-concept studies in mice illustrate that combining orthogonal approaches can provide powerful tools or therapies to prevent infection via N+1/N−1 strategies.

OUTLOOK

The field of microbiome engineering is still in its infancy. Nonetheless, the wide variety of studies in a range of microbiome systems has already presented a wealth of information on potential strategies for microbiome interventions. What is clear from the overview presented here is that much can be learned from cross-microbiome efforts, coupled with general conceptual theory on natural microbiome development. While immediate success might be observed in specific applications related to potential health or environmental benefit, behavior and processes are often more complex than initially assumed. There is thus a strong need to start small: with single focal strains, including specific designs that aim to study strain behavior and survival in the context of the microbiome, and including the most appropriate approaches to understand strain and system function, as illustrated here. Such approaches could also embrace reintroduction attempts of strains that were depleted in the recent past (309), or that are geographically distinct (290).

Advancing general theory is difficult but will greatly benefit from the depth of data that can be achieved using omics techniques and analyses of clinical or environmental parameters. Crucial for understanding cross-system N+1/N−1 processes are computational approaches, consisting not only of the data analyses that underlie most of the experimental designs outlined above but also of specific computational efforts to simulate or compare N+1/N−1 inoculations with natural events. Ideally, one would be able to predict the growth and survival of focal strains within target microbiomes under the conditions of their native habitats. Considering that growth relies on energy generating and anabolic processes, much will be gained by the improvement and application of computational—and specifically genome-scale, models. The *in silico* behavior of an N+1 focal strain could thus be approached within its environmental context. Combining multiple individual genome-scale models into a coherent microbiome model is still a daunting task, but first efforts show how important metabolic features and fluxes between taxa can be approximated.

Finally, it is important to capture community assembly processes within the context of the habitat, as a function of physical processes, microbial activity, and taxonomic composition using computational frameworks. As discussed, a variety of approaches are available for this task, even for models that predict the compositional development of communities based on carbon, nutrient, and energy input or as a function of assumed (imposed) interspecific interactions. Models can further be discretized to allow the division of individual "agents" (virtual cells) of different taxa within defined space that add cell movement, and allow nutrient gradients, diffusion terms, or cell crowding effects. The agents can be subject to more general deterministic laws of growth physiology (e.g., Monod) or interspecific interactions (e.g., Lotka-Volterra), but can also be represented by reduced genome-scale models for increased computational efficiency while still maintaining a representative set of metabolic reactions. Harmonized strategies like the N+1/N– concept may thus eventually facilitate the comparison of ecological roles, organism characteristics, and effects of perturbations on a system scale across studies and microbiomes. Crucially, these advances will benefit greatly from appropriate data accessibility and stewardship practices in microbiome research (310–312).

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