Manipulating Bacterial Communities by *in situ* Microbiome Engineering

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4 **ABSTRACT (100-120 words)**
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8 Microbial communities inhabit our entire planet and play a crucial role in
9 biogeochemical processes, agriculture, biotechnology, and human health. Here, we
10 argue that “*in situ* microbiome engineering” represents a new paradigm of community-
11 scale genetic and microbial engineering. We discuss contemporary applications of this
12 approach to directly add, remove, or modify specific sets of functions and alter
13 community-level properties in terrestrial, aquatic, and host-associated microbial
14 communities. Specifically, we highlight emerging *in situ* genome engineering
15 approaches as tractable techniques to manipulate microbial communities with high
16 specificity and efficacy. Finally, we describe opportunities for technological innovation
17 and ways to bridge existing knowledge gaps to accelerate the development of *in situ*
18 approaches for microbiome manipulations. (Word count: 112)
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32 **KEY WORDS (2-8 words)**
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35 microbiome engineering, genome engineering, microbial communities, prebiotics,
36 probiotics, phage, xenobiotics, synthetic biology
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MAIN TEXT

Advances and roadblocks in microbiome research

Over the past decade, breakthroughs in high-throughput sequencing to read DNA from genomes have vastly outpaced our capabilities to edit genetic information. Developments in metagenomic and transcriptomic sequencing of mixed cell populations have enabled large-scale quantification of microbial community composition, function, and dynamics in a culture-independent manner [1]. However, we still lack a basic mechanistic understanding of the individual genetic factors that drive overall function and emergent ecological principles in these communities. To understand these complex communities and engineer them in useful ways, we must address critical technological and knowledge gaps in systems-level genetic manipulation [2].

Most microbial communities in nature exist in complex, dynamic consortia with highly interconnected networks of metabolic and ecological interactions that have yet to be unraveled. Consequently, the natural environments of most microbial communities are difficult, if not impossible, to recreate experimentally. In fact, a large majority of microbes have not been cultivated in the laboratory [3]. These microbes are not accessible for genetic studies, and their function and properties within their communities remain unknown. Even for culturable microbes, only a few genetic systems have been utilized in specific model strains [4]. Development of new genetic systems requires significant time and effort, and resulting tools are not necessarily transferrable between different microbes. Furthermore, studies of single model strains in laboratory conditions may not necessarily reflect behaviors relevant to natural environments. No general methods are available today to genetically engineer consortia of different organisms *in situ*. These challenges greatly limit the functional analysis and forward engineering of poly-microbial communities of any level of complexity.

Engineering microbial communities *in situ*

***In situ* microbiome engineering** (see glossary terms) methods allow for manipulation and study of microbial communities in their native context without the need for individual laboratory domestication. These approaches can be classified by several

characteristics: the magnitude of perturbation to community composition and function, specificity of perturbation to community members or processes, and degree of engineerability (Figure 1). While some approaches have low specificity and can lead to large-scale changes (e.g. **microbiota** transplants), others can be more easily designed to affect specific members while minimizing overall impact on the community (e.g. engineered probiotics). Here, we discuss contemporary *in situ* microbiome engineering approaches and offer *in situ* **genome engineering** as a new paradigm to directly manipulate communities with greater control of magnitude and specificity.

Contemporary methods for *in situ* microbiome engineering

Chemical, cellular and phage-based methods can be used to alter microbial communities *in situ*. Common examples of each approach are outlined in Table 1 and discussed in detail below.

Chemical modifiers of microbiomes

Biochemical availability can predictably affect microbiome composition and function [5]. **Prebiotics** are naturally occurring chemicals that selectively promote growth or activity in a community. Human-associated prebiotics are often non-digestible dietary polysaccharides that stimulate growth of commensal bacteria in the gut [6, 7]. Prebiotics can be used in other settings such as the food industry, where the polysaccharide β -glucan has been commercially sold to improve fish health and resistance to infection [8]. However, a major limitation of prebiotics is the inability to rationally predict or change the specificity of their manipulations. To address this shortcoming, approaches such as functional metagenomics, transcriptome and transposon sequencing have been used to identify gene-level fitness determinants of individual microbes in specific metabolic niches [9, 10]. These measurements have yielded more nuanced nutritional variation approaches that can be exploited to modulate the microbiome with higher precision [11]. For example, transposon sequencing of a model gut microbiome community revealed a specific dietary metabolite that could modulate the abundance of a single strain in the community [12]. Further, transcriptome sequencing of the gut bacterium *E. lenta* revealed specific genes

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4 that inactivate the cardiac drug digoxin and their transcriptional regulation, enabling
5 design of a dietary modification to reduce inactivation *in vivo* [13].
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8 Targeted biochemical modulation of microbiota can also utilize **xenobiotics**,
9 compounds foreign to an environment designed to modulate microbial function or
10 growth. For example, β -glucuronidase inhibitors have been used to reduce toxicity of a
11 chemotherapeutic by inactivating bacterial enzymes that reactivate the drug [14].
12 Asparaginase can be delivered *in vivo* to protect against infection by degrading
13 asparagine, a regulator of group A Streptococcus proliferation [15]. Finally, a small
14 molecule structural analog of choline can inhibit TMA production by microbes, reducing
15 levels of TMAO, a metabolite associated with cardiac disease [16].
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19 Antibiotics are a widely used class of xenobiotics that modulate microbial growth
20 by inhibiting essential cellular machinery. Most antibiotics target membrane integrity,
21 protein synthesis or replication processes [17]. However, due to the widely conserved
22 function of mechanisms targeted by antibiotics, they tend to affect a broad spectrum of
23 bacteria, causing large and potentially undesirable changes in the community [18].
24 Antibiotic use can lead to persistent alterations in microbiota composition over time, and
25 can further select for antibiotic resistance genes [19]. With the emergence of many
26 antibiotic-resistant pathogens, there is a critical need for new classes of antibiotics that
27 selectively target undesirable strains without promoting the spread of broad-spectrum
28 resistance. Antimicrobial peptides and secondary metabolites such as bacteriocins are
29 outstanding candidates for such novel antibiotics, as they display selective elimination
30 of particular strains and a diverse set of compounds are present in natural communities
31 [20, 21]. A better mechanistic understanding of specific biochemical processes and
32 strain-level genetic factors will improve novel strategies to modulate microbiome growth
33 and function with high specificity.
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52 **Cellular modifiers of microbiomes**

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54 Beyond biochemical approaches, live bacterial strains or communities can be
55 used to manipulate microbial ecosystems. In contrast to molecular modulators, these
56 cellular approaches can yield more nuanced interaction and function over space and
57 time. **Probiotics** are bacteria which can confer a benefit to a particular host
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environment [22]. For example, probiotic *Lactobacilli* have been used in livestock to decrease incidence of pathogenic infections [23]. In humans, probiotic bacteria alter the gut microbiota by competing for nutrients, producing antimicrobial compounds, or modulating host immunity [24, 25]. However, our fledgling understanding of probiotic mechanistic function has limited their value as a tool for predictive microbiome manipulation.

Genetically engineered probiotics have potential for more targeted community manipulations. For example, in the poultry industry, genetically modified *Salmonella* lacking virulence factors have been used to vaccinate chickens against infection [26]. In humans, synthetic biology tools can be applied to engineer probiotics with precise and novel functions. For example, bacteria have been modified to modulate microbiota or host physiology by secreting chemicals or proteins, including human interleukin-10 to reduce inflammation [27], NAFEs to reduce food intake and obesity [28], and bacterial quorum signals to modulate microbiota composition [29]. Wholly new functions can also be engineered; Danino et al. engineered an *E. coli* probiotic as an orally administered diagnostic of liver metastasis in mice through production of a detectable signal in urine [30]. More complex synthetic biology circuits such as combinatorial logic [31] or memory [32] circuits can be layered upon these simple designs to improve the precision, specificity and controllability of desired perturbations.

Mixtures of bacteria can also be utilized to manipulate microbiomes. Microbiota transplantation is the beneficial transfer of live bacteria from one environment into another. This approach has been recently popularized through successful clinical trials of fecal microbiota transplants to treat recurrent *C. difficile* infections [33]. It is hypothesized that transplantation may replace the existing microbial community with a more infection-resistant community from a healthy donor, but the mechanisms of this process remain largely unclear [34]. A more refined approach to transplantation is the transfer of synthetic communities, which could replicate the functions of complex consortia, but contain defined members that are amenable to detailed genetic and biochemical studies [35-37]. We envision that novel experimental tools to delineate interspecies and host-microbe interactions and improved metabolic and ecological

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4 modeling [38], combined with functional studies in gnotobiotic animals [39], will enable
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6 better design of microbial consortia to precisely manipulate microbial communities.
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9 10 ***Phage-based modifiers of microbiomes***

11 **Bacteriophages** (phages) are the most abundant, diverse, and rapidly
12 replicating life forms on earth [40]. Phages infect a host microbe, hijack its replication
13 machinery to reproduce, and then replicate via stable genomic integration (lysogenic
14 cycle) or lysis of the host and dissemination (lytic cycle). This life cycle makes them
15 ideal genetic engineering candidates to selectively eliminate strains or transfer specific
16 genes in microbial populations. Indeed, natural phages have been used to limit growth
17 of undesirable or pathogenic bacteria in humans [41], agriculture [42], food processing
18 [43], and aquaculture [44]. Phages have been further genetically engineered to deliver
19 specific DNA payloads or to alter host specificity. For example, phages have been
20 designed to deliver biofilm dispersal enzymes [45] or genes that increase antibiotic
21 sensitivity [46]. By delivering the CRISPR-Cas RNA-guided nuclease system with
22 phages, designated strains can be selectively eliminated based on their genetic content
23 [47]. Furthermore, phage host ranges can be modularly engineered by swapping phage
24 tail components [48]. Altering phage populations represents another avenue for
25 microbiome modulation; exposing the gut microbiome to antibiotics alters its associated
26 virome and ecological networks [49]. Quantitative characterization of phage ecology,
27 combined with advances in forward genetic engineering of phage function, will allow for
28 more complex manipulations of microbial communities *in situ*.
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45 46 ***In situ* genome engineering: an emerging frontier in microbiome modulation**

47 Despite much progress, contemporary methods for modifying microbiomes have
48 not seen widespread success in achieving desired manipulations. We attribute these
49 shortcomings to two major knowledge and technical barriers. First, we lack a fine-scale
50 understanding of how individual microbial species function in the context of their natural
51 environments, and a subsequent large-scale understanding of emergent ecosystem
52 function. Second, we lack techniques to efficiently manipulate microbial communities
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4 over a large range of magnitudes and specificities. These barriers have limited the
5 effective design and physical implementation of manipulations.
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8 Current methods face a variety of outstanding engineering challenges. For
9 example, chemical manipulations, such as transformation, are specific to particular
10 microbial strains and biochemical processes and cannot be broadly applied. Cellular
11 approaches require colonization of a foreign strain into an ecologically competitive
12 environment, which may be difficult to engineer or lead to unwanted consequences.
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17 Rather than targeting specific strains or functions, one could instead directly
18 modify the metagenomic content of a community to achieve a desired manipulation.
19 While genomes vary greatly between microbes, the **metagenome** of a community is
20 more constant [50], and governs its biochemical and cellular function. Compared to
21 currently available techniques, direct genomic manipulation could enable perturbations
22 with magnitude and specificity tunable over a much greater range. For example, a
23 metabolic pathway could be added directly to the genome of a native microbe, rather
24 than introducing a foreign strain containing the pathway, thus reducing off-target effects
25 and achieving a high-specificity manipulation. Alternatively, the same pathway could be
26 targeted to a broad range of native organisms to achieve a large-magnitude
27 manipulation. Advances in materials science to manipulate and polymerize chemical
28 building blocks enabled the proliferative use of plastics in the 20th century; we
29 analogously envision that direct, tunable manipulations of the genetic building blocks of
30 microbial communities will enable novel bioengineering applications. We advocate for
31 the development of *in situ* genome engineering approaches, or techniques to directly
32 manipulate genetic information and engineer new functions in complex microbial
33 communities (Figure 2).
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48 Because complex communities are difficult to recapitulate in the laboratory, new
49 approaches are needed for genome engineering *in situ*. In nature, microbial genomes
50 are in constant flux as a result of abundant horizontal gene transfer events mediated by
51 **mobile genetic elements** [51]. These horizontal gene transfer events have been
52 increasingly recognized for the important roles they play in the evolution of individual
53 genomes [52] and of entire microbial communities [49]. Furthermore, these events
54 occur rapidly, on timescales less than a week between bacterial species in the gut
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4 microbiome [53]. Thus, natural horizontal gene transfer processes, including
5 **conjugation**, natural transformation, and phage delivery are viable entry points for *in*
6 *situ* genome engineering.
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10 Conjugation via plasmids or transposable elements enables transfer of genetic
11 material through direct cell-to-cell contact. An *in situ* conjugative system could utilize a
12 transiently introduced donor cell carrying an engineered mobile plasmid or transposable
13 element that would be transferred to native microbiota. Secondary conjugations could
14 be engineered to occur following the first transfer to promote further propagation.
15 Bacteria can also uptake extracellular DNA through natural transformation machinery
16 [54] or chemical and physical transformation processes, such as abrasion with mineral
17 particles [55], which creates small membrane pores that allow DNA into the cytoplasm
18 [56]. Phages can also be utilized to transfer and insert genes into bacterial genomes.
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27 **Building an expansive *in situ* genome engineering toolbox**

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29 We envision a suite of genetic tools that will significantly expand our ability to
30 activate stable and controllable synthetic gene circuits in complex natural microbiomes.
31 First, natural horizontal gene transfer vectors could be engineered with tunable host
32 ranges and dynamics. These vectors could then be augmented with existing and
33 emerging synthetic biology tools, such as transcriptional and translational regulatory
34 parts, logic gates, and genome editing tools to add, remove, or modify particular
35 functions and ecologies. Finally, maintenance of these engineered vectors could be
36 precisely controlled, resulting in propagation across specific members of a community
37 or destruction via controlled kill switches. Recent studies suggest that such mobile
38 genetic element-mediated transfer may be a tractable approach to manipulate diverse
39 communities. Plasmids can be broadly mobilized into naturally occurring soil bacteria,
40 transferring to bacteria from 11 different phyla [57] with efficiencies of up to 1 in 10,000
41 cells within a few days [58, 59]. Furthermore, viral tagging experiments have revealed
42 that in nature, a single bacterial host can harbor dozens of different phage populations,
43 suggesting that viruses can be isolated and engineered for a broad range of hosts [60].
44 Here, we highlight key components that need to be developed to form the foundation for
45 this new *in situ* genome engineering toolbox.
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New replicative or integrative plasmids are needed for stable propagation of exogenous DNA in the microbiome. Recent sequencing efforts have demonstrated that plasmids are prevalent in microbial communities [61]. However, the host ranges of most plasmids remain unknown, and their associated proteins and modes of regulation are poorly characterized [62]. Few, if any, new plasmids are being developed, as almost all current vectors are based on plasmids isolated during the pre-genomic era. Characterizing more natural plasmids with various host ranges will elucidate the principles underlying their host specificity, transfer dynamics, and stability, and will aid in the construction of new synthetic vectors [63].

Tunable gene regulation systems with varying strengths and host-specificities are needed to better control the activity of synthetic circuits across microbial communities. A repository of characterized regulatory parts such as promoters and ribosome binding sites for diverse microbes does not yet exist and needs to be developed. Furthermore, basic measurement techniques to assess genetic circuit function across many species in parallel have not been developed. New strategies will be needed to engineer genetic circuits with broad and defined host ranges [64]. Existing and novel genetic parts, combined with community-level measurement strategies, will yield design principles for building genetic circuits with predictable performance in different hosts.

Recent advances in genome editing have enabled programmable modification of microbial genomes. RNA-guided CRISPR-Cas9 systems have been used to site-specifically edit bacterial genomes [65], while LI.LtrB group II introns, retrotransposons that undergo RNA intermediary steps, have been successfully repurposed for targeted gene editing of multiple bacterial species [66]. While these techniques enable powerful interrogation of the genome, new tools to enable strain- and site-specific genomic integration of large synthetic constructs are still required, as current approaches are often inefficient or difficult to target to desired genomic sites. Additionally, increased knowledge and functional annotation of microbial genomes, coupled with advances in modeling techniques to predict the effects of particular genomic modifications, will be necessary to maximize the utility of existing genome editing tools.

More sophisticated genetic devices are also needed to perform higher function processes. Programmable transcriptional and post-transcriptional regulators [67, 68],

combined with new chemical sensing pathways, will enable sophisticated genetic circuitry such as memory devices and kill switches to control engineered functions in complex communities [69]. Other strategies, such as a gene drive to propagate engineered function in higher order organisms [70], conceptually similar to bacterial *in situ* genome engineering, may be further developed for microbial populations.

A better understanding of the function and dynamics of natural mobile genetic elements will enable strategies for long-term persistence of mobile genetic elements. A major challenge in utilizing these natural elements is ensuring efficient delivery, transfer, and stability of the system over time. Delivery of mobile genetic elements and subsequent transfer between endogenous microbiota *in situ* could minimize perturbations to the overall structure of a given community. Mobile elements found in the wild employ elegant strategies to ensure their long-term presence; for example, an *E. faecalis* conjugative plasmid expressing a bacteriocin enhances niche colonization of its host, facilitating transfer to other *E. faecalis* strains in the mammalian gut [53]. Similar strategies which couple a niche or metabolic advantage to a mobile element could be employed to engineer selection and stability over time.

Finally, many bacterial immune systems such as CRISPRs and restriction endonucleases prevent foreign DNA from infiltrating the cell. Active immune evasion is required to enable efficient transfer and propagation of engineered DNA, which may require sequence recoding to avoid restriction enzyme digestion or modification of DNA methylation patterns to match that of the recipient cells. In fact, methylation-matching has been shown to increase gene transfer rates by several orders of magnitude [71]. Experimental and design tools for bacterial immune evasion are needed to predictably manipulate gene transfer efficiencies *in situ*.

Design principles, knowledge gaps and applications of *in situ* microbiome engineering

Successful *in situ* microbiome engineering will require an expanded understanding of basic ecological principles, new systems measurement methods and genetic parts, and the application of existing and new quantitative modeling frameworks (Figure 3, Key Figure). These three major knowledge areas directly inform and influence

each other; for example, underlying ecology provides a starting point for determining new genetic parts for systems engineering. The performance of these parts can then be used to parameterize quantitative models of complex microbial systems, which could ultimately reveal hidden or underlying ecological interactions. However, as outlined in Figure 3, key knowledge gaps still remain to be addressed.

The application of *in situ* microbiome engineering to basic science questions will enable a new class of experiments to elucidate the determinants of individual and community function. In individual strains, these new tools will allow for genetic studies of unculturable microbes and the mechanistic basis of microbial fitness and function. In communities, these tools will enable large-scale perturbations of community composition and interactions at unprecedented resolution and scale. Further, *in situ* microbiome engineering has numerous applications to the addition, deletion or modification of community-level functions and properties (Figure 4). In human-associated communities, microbiome manipulations could improve health and nutrition via removal of detrimental host interactions (e.g., chronic inflammation) and addition of beneficial processes (e.g., producing essential nutrients). Altering community-level properties such as resilience to infection could yield novel ecology-based treatments for infectious diseases. In an agricultural setting, targeted manipulations could increase crop yield by accelerating nutritional absorption or enhance bioremediation by removing toxins. In human-made environments such as buildings, pipelines, and ship hulls, engineered communities could enable exclusion of strains with undesirable properties (e.g. pathogenic or biofouling) and augmentation of materials with desirable “smart” properties such as self-healing, chemical production, and recording exposure to biochemical compounds. Finally, synthetic communities with defined functions could replace natural communities in certain settings to enable predictability and control over particular biochemical processes. Such communities could potentially be used to colonize environments lacking endogenous microbiota, such as other planets, to improve habitability for humans.

Safety and regulation

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4 The manipulation and engineering of microbial ecosystems in natural
5 environments will require significant advances in our ability to reliably predict
6 engineering outcomes and safeguard against undesirable events. Current
7 manipulations of microbial ecosystems, intended or not, are widespread and subject to
8 a complex litany of regulatory policies with varying stringency. New policies and
9 regulatory frameworks will be required to appropriately evaluate the safety and
10 implementation of emerging approaches such as genetically engineered probiotics and
11 mobile vectors. Analogous to conversations around the use of gene drives to cause
12 forced inheritance of engineered traits in a population [72], or CRISPR-Cas9 for gene
13 editing purposes [73], the societal implications of microbiome engineering technologies
14 and their regulation must be carefully considered, evaluated, and communicated to the
15 public in order for these open-environment engineering approaches to be rationally
16 evaluated and safely adopted.
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30 **Concluding remarks**

31 *In situ* microbiome and genome engineering offer exciting opportunities at the
32 frontier of population and ecological engineering with applications in basic science,
33 human health, agriculture, and beyond. As we move from understanding and
34 engineering individual organisms to entire ecosystems, we envision that these emerging
35 techniques will reveal a vast diversity and elegance underlying natural microbial
36 ecosystems, and will correspondingly suggest wholly new strategies to manipulate
37 microbial communities (See Outstanding Questions box).
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GLOSSARY

Bacteriophage/phage: a virus that infects and hijacks a bacteria's machinery to reproduce; can integrate stably into the bacterial genome.

Conjugation: a mechanism of genetic material transfer via direct cell-cell contact.

Genome engineering: technologies or approaches to alter genetically inheritable information in a targeted or specific manner.

***In situ* microbiome engineering:** manipulation of microbial communities in their native environment.

Metagenome: the collection of genes, genomes and inheritable information present in a given environment.

Microbiome: encompassing term referring to the microbiota, metagenome, and surrounding environment of a microbial community.

Microbiota: the set of microorganisms present in a given environment.

Mobile genetic elements: genetic information that can be transferred between cells; includes conjugative plasmids, transposons, bacteriophages.

Prebiotics: naturally-occurring chemicals that can promote growth or activity in a community in a selective manner.

Probiotics: bacteria that can confer a benefit to a particular host environment.

Xenobiotics: biochemical compounds unnatural to an environment that can promote or limit the growth or function of specific microbial community members.

Table 1. Commonly utilized *in situ* microbiome engineering methods

Method	Class (common formulations)	Predominant Targets	Mechanism of Action
Prebiotics (chemical-based)	Dietary fibers (Inulin), Polysaccharides (oligosaccharides)	Firmicutes (Lactobacillus) Actinobacteria (Bifidobacteria)	Promote bacterial growth; mechanism generally unknown
Antibiotics (chemical-based)	β -lactams (Cephalosporins, Carbapenems)	Clostridium, Staphylococcus, Streptococcus	Block cell wall synthesis
	Aminoglycosides (Kanamycin, Streptomycin)	Klebsiella, Pseudomonas	Block protein synthesis
	Macrolides (Erythromycin, Azithromycin)	Chlamydia, Legionella, Mycoplasma	Block protein synthesis
	Glycopeptides (Vancomycin)	Enterococcus, Clostridium, Staphylococcus	Block peptidoglycan synthesis
	Quinolones (Ciprofloxacin, Levofloxacin)	Neisseria, Pseudomonas, Streptococcus	Block DNA replication
	Metronidazole	Bacteroides, Clostridium	Block DNA/RNA synthesis
Probiotics (cellular-based)	Firmicutes (Lactobacillus), Actinobacteria (Bifidobacteria), Proteobacteria	Variable, broadly targeting	Compete for nutrients, produce antimicrobials, modulate environment
Microbiota transplants (cellular-based)	Fecal microbiota transplants	Variable, broadly targeting	Replace native community; mechanism generally unknown
Bacteriophages (phage-based)	Specific phage strains or cocktails of phages	Variable, but strain-specific	Cell lysis (lytic); genomic integration (lysogenic)

Figure 1: *In situ* microbiome perturbations vary in magnitude, specificity and degree of rational design required.

A variety of approaches, based on chemical (green), cellular (blue) and DNA (orange) methods can be applied to manipulate microbial communities in their native context. Each method can vary in its magnitude of perturbation to the native microbiome, shown increasing on the horizontal axis; its specificity of targeting to particular community members, shown increasing on the vertical axis; and its degree of required rational design, shown with increasing shading density. Particular combinations of magnitudes and specificities may be desirable for given target applications. Chemical-based approaches such as *xenobiotics*, *prebiotics*, and *nutritional variation* yield relatively broad spectrum changes, with varying magnitudes. *Antibiotics*, a class of xenobiotics, can yield larger magnitude changes with higher specificity. Cellular-based

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4 techniques such as *probiotics* and *engineered probiotics* can yield low magnitude, specific
5 perturbations, while large-scale microbiota transplants or synthetic communities can yield to
6 larger, but less specific changes. Finally, DNA-based methods such as *phages* can yield highly
7 specific, albeit low magnitude perturbations, while *engineered mobile DNA* can yield
8 perturbations over a large range of magnitudes and specificity. This flexible control of magnitude
9 and specificity implies that engineered mobile DNA may be a desirable and tractable method for
10 manipulating microbial communities in comparison to other methods.
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21 **Figure 2. The *in situ* genome engineering toolbox.**

23 The genomic content of native microbial communities can be directly engineered via *in situ*
24 genome engineering. As we depict in the top panel, mobile genetic elements can be delivered
25 and transferred to an endogenous microbiome, where they elicit desired function via a
26 combination of regulation and actuation strategies; these elements can then maintain
27 themselves over time to achieve long term desired function. A toolbox of existing and novel
28 genetic tools will enable engineering of mobile genetic elements for *in situ* genome engineering
29 methods. *Transfer* methods such as phages, plasmids and transposons can be used to deliver
30 and circulate engineered DNA sequences to microbial communities, via processes such as
31 transduction, transformation and conjugation. *Regulatory* parts, including transcription and
32 translation parts and sensors of endogenous and exogenous chemical ligands will enable the
33 construction of more complex genetic devices to tune host-range and endow higher order
34 functions such as logic and memory. *Actuation* of genomes, including addition and removal of
35 genes, modulation of expression, targeted mutations, and episomal modifications will allow for
36 changes to underlying community metabolic function, or introduction of wholly new functions
37 such as reporting on the state of an environment. These functional manipulations can further
38 alter communities at the ecological level by altering the abundances of specific strains or
39 introducing competitive or cooperative interactions. Maintenance of these mobile elements
40 allows for dynamic and long-term control of engineered genetic content; with replication,
41 integration and optimized immune evasion, vectors can be stably propagated over time.
42 Alternatively, lysis can quickly disseminate phages across a community, or engineered circuits
43 such as kill-switches could be used to eliminate circuits as a safe-guard.
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4 **Figure 3 – Key Figure. Principles and knowledge gaps in *in situ* genome engineering.**
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6 Key principles for *in situ* microbiome engineering include *community ecology* (blue), *systems*
7 *engineering* (red), and *quantitative modeling* (orange). On the left, we illustrate the direct
8 interplay between each of the three principles; these directional interactions and knowledge that
9 inform one-another are denoted via color coded arrows and text. On the right, we detail specific
10 *biological and ecological* and *engineering and modeling* knowledge gaps.
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4 **Figure 4. Applications of *in situ* genome engineering.**
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6 *In situ* genome engineering approaches could be utilized to address fundamental basic science
7 questions and key applications in medicine, health, food, farming, water and energy.
8 Engineered vectors introduced into native environments could allow for manipulation of
9 metagenomic content, and would propagate within the community over time. These engineered
10 vectors could then endow these communities with desirable alterations to community-level
11 function, stability, and dynamics, detailed in the upper right panel.
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OUTSTANDING QUESTIONS

How do specific biological factors such as genetic content or biochemical interactions lead to emergent ecological properties, including spatial variation, dynamics and stability?

What is the extent of horizontal gene transfer in microbiomes, and how does this impact intraspecies heterogeneity, genomic stability, evolutionary selection and community function?

How can standardized synthetic biology genetic parts for genetic transfer, regulation, and genome editing be characterized and designed across many different bacteria?

What systems engineering principles can be leveraged to increase transferability and composability of these parts into more complex genetic devices across many different hosts?

What are appropriate quantitative modeling frameworks to predict specific microbiome manipulations?

Figure 1

Perturbation to the Native Microbiome

Figure 2

Principles for *in situ* microbiome engineering

Biological & ecological knowledge gaps

- community transcriptome & metabolome
- community spatiotemporal distribution
- quantitative inter/intraspecies interactions
- natural environment for growth
- evolutionary selection pressures
- degree of horizontal gene flow
- stability of genome/metagenome
- determinants of community stability
- impact of intraspecies heterogeneity

Engineering & modeling knowledge gaps

- repertoire of synthetic biology parts
- interoperability/composability of parts
- transferability/scalability of designs
- community-scale design rules
- predictive modeling frameworks
- relevant systems-level timescales

Figure 4

Applications of *in situ* genome engineering

Table

Method	Class (common formulations)	Predominant Targets	Mechanism of Action
Prebiotics (chemical-based)	Dietary fibers (Inulin), Polysaccharides (oligosaccharides)	Firmicutes (Lactobacillus) Actinobacteria (Bifidobacteria)	Promote bacterial growth; mechanism generally unknown
Antibiotics (chemical-based)	β-lactams (Cephalosporins, Carbapenems)	Clostridium, Staphylococcus, Streptococcus	Block cell wall synthesis
	Aminoglycosides (Kanamycin, Streptomycin)	Klebsiella, Pseudomonas	Block protein synthesis
	Macrolides (Erythromycin, Azithromycin)	Chlamydia, Legionella, Mycoplasma	Block protein synthesis
	Glycopeptides (Vancomycin)	Enterococcus, Clostridium, Staphylococcus	Block peptidoglycan synthesis
	Quinolones (Ciprofloxacin, Levofloxacin)	Neisseria, Pseudomonas, Streptococcus	Block DNA replication
	Metronidazole	Bacteroides, Clostridium	Block DNA/RNA synthesis
Probiotics (cellular-based)	Firmicutes (Lactobacillus), Actinobacteria (Bifidobacteria), Proteobacteria	Variable, broadly targeting	Compete for nutrients, produce antimicrobials, modulate environment
Microbiota transplants (cellular-based)	Fecal microbiota transplants	Variable, broadly targeting	Replace native community; mechanism generally unknown
Bacteriophages (phage-based)	Specific phage strains or cocktails of phages	Variable, but strain-specific	Cell lysis (lytic); genomic integration (lysogenic)