Probiotic and microbiota engineering for practical applications

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Abstract

The last decades have witnessed amazing advances in engineering individual microbes for practical applications. However, engineering microbial consortia is a recent development, opening a new path for diverse applications, including curing gut diseases, solving food shortages, enabling sustainable bioproduction, mitigating pollution, and potentially addressing the climate crisis. In this perspective article, I will summarize recent technological innovations in synthetic biology that enable probiotic and microbiome engineering. Additionally, I will cover associated issues such as biosafety and biocontainment when the probiotics and microbiota are engineered. Given the complexity of global problems that are difficult to solve using a single microbe, this perspective will guide the research community to a better future with exciting potential applications.

Keywords: antibiotic resistance spread; biocontainment; biosensor; computational model; horizontal gene transfer; microbiome; engineering tools

Introduction

Microbiome is an emerging topic of research. As a variety of systems and synthetic biology tools become available for researchers, we start to better understand the dynamics of complex microbiota, build a synthetic microbial community, and engineer microbiota in situ. For example, targeted, specific strain 'knockout' in a community of diverse microbes has been demonstrated by combining machine-learning-based predictive CRISPR tools with liposome-based DNA delivery methods [1]. As genomics has advanced by utilizing simple gene knockout tools to understand the function of the deleted gene [2], such targeted strain elimination tools will help us understand the role and function of individual microbial members in an environment, potentially enabling in situ microbiota engineering. In addition, computational models allow us to understand and engineer the complex interactions among microbes, phages, metabolites, and environmental factors [3]. Furthermore, systems biology analysis tools such as omics tools and microcalorimetry have facilitated the elucidation of microbial interactions, refined the computational models, and discovered the mechanisms of microbial thermogenesis [4]. Soon, it may be possible to build a synthetic consortium of hundreds of microbes that can be used for diverse applications to solve global problems, including climate crisis, pollution, food shortage, diminishing natural resources, sustainable manufacturing, and health issues [5].

Similarly, engineering probiotics has been facilitated by an increasing number of synthetic biology tools, including genome engineering methods, microbial sensors, sophisticated genetic circuits, and predictable computational models and tools for gene expression control. Despite these available engineering tools, the gut environment is heterogeneous and complex due to the constantly changing microbiota and chemicals, making practical applications of probiotics challenging. In essence, engineered probiotics should be tested in the dynamic gut environment by considering the gut microbiota and the real-world environment. Additionally, biocontainment and

biosafety of genetically engineered microbes (GEMs) should be addressed for the approval of GEMs for biomedical applications [6].

In this perspective article, I will discuss the challenges and opportunities of probiotic engineering in the context of the gut microbiota. I will briefly summarize state-of-the-art technologies that enable the engineering of probiotics and gut microbiota, including the development of biosensors, microbiome engineering tools, and biocontainment approaches. In addition, I will explain the relatively new concept of the gut microbiota–brain axis and the unexplored issue of antibiotic resistance spread via horizontal gene transfer of antibiotic resistance genes (ARGs) originated from research labs [7, 8]. I aim to write a perspective article to encourage the research community to think about new promising directions and relevant problems, as opposed to a comprehensive review of the current probiotic and microbiota engineering, which has been discussed by many other colleagues.

Developing microbial sensors and genetic circuits for probiotic engineering

Since 2000, diverse microbial sensors have been developed and used in synthetic biology research. However, most biosensors have been engineered to build 'toy' systems where non-practical sensors such as the IPTG-inducible *lac* promoter are used, and a limited number of real-world, application-relevant biosensors have been developed [9, 10]. Although the number of practically useful microbial sensors is still very small for biomedical applications, exciting novel biosensors have been developed by mining and engineering natural biosensors [11-14] or designing them de novo [15] although de novo-designed biosensors have been mostly tested in cell-free systems [16]. For probiotic-based diagnostics, the recent advances show great promise. For example, Inda-Webb et al. have developed GEMs that detect inflammation-associated molecules and produce luminescence [17]. Notably, the emitted light is converted to a wireless signal in a sub-1.4 cm^3 device that contains the GEM and all non-biological components, making it compatible with ingestion and enabling wireless communication between the animal gastrointestinal tract and the signal receiver outside the animal body. This device will allow for non-invasive detection of diseases, including inflammatory bowel disease, and remote personalized care by supporting communication between patients and doctors.

Recording detected signals permanently is another interesting topic of recent research. Sheth et al. developed a biological recorder by using the CRISPR adaptation system, demonstrating stable recording of temporal signals such as metabolites in the environment [18]. Similarly, time-ordered recordings of transcriptional events have been demonstrated by using engineered RNA barcodes based on prokaryotic retrons [19]. In this system named Retro-Cascorder, retrons are reverse transcribed into DNA, and the integration of barcodes using the CRISPR system allows for reconstructing transcriptional event timing. Additionally, Schmidt et al. used barcoded CRISPR arrays to enable multiplexed recording and provided a non-invasive method for reconstructing transcriptional histories of isogenic bacteria in vivo [20].

Significant technological progress in developing therapeutic probiotics has recently been made [21-23]. For example, Isabella et al. has engineered a probiotic strain *E. coli* Nissle (EcN) by expressing an enzyme that converts phenylalanine into benign metabolite to treat the metabolic disease phenylketonuria [24]. Importantly, the authors showed the GEM's efficacy in both mice and primates, and the company Synlogic has successfully finished the first and second clinical trials, allowing it to perform its phase 3 clinical trial. Another notable example is the development

of engineered probiotics limiting *C. difficile* infection in mice by restoring bile salt metabolism in response to microbiome dysbiosis induced by antibiotics [25]. The diseases that can be treated by GEMs are not limited to metabolic diseases or infection. Harimoto et al. developed a dynamic, programmable encapsulation system for therapeutic microbes that showed an improved anti-tumor efficacy in animal models [26]. Ho et al. used a cruciferous vegetable diet and myrosinase-expressing GEMs to convert glucosinolates of cruciferous vegetables into sulphoraphane with known anticancer activity, showing proliferation inhibition of colorectal carcinoma in murine models [27]. In addition to engineered probiotics, native or engineered gut commensal microbes can be used to prevent pathogen colonization [28] and growth impairments of malnourished children [29].

Notably, biosensors and genetic circuits for practical applications in the gut should meet the following requirements. First, their response time must be within the proper range for relevant applications. For example, to quickly sense and treat life-threatening pathogen infection, the sensing and responding time should be at the range of several minutes, rather than several hours. Second, engineered probiotics should stably maintain the added function in the complex and competitive gut environment. If a longer residence time of administered probiotics is desired, genetic stability against mutations should be ensured by implementing redundant genetic circuits [30]. Importantly, we should consider the tradeoff between genetic stability increased by back-up circuits and added metabolic burdens imposed by redundancy. Third, biocontainment of engineered probiotics should be ensured, as further discussed below and in our recent review [31]. Importantly, permanent colonization of GEMs would not necessarily beneficial, given their evolvability and biocontainment issues.

Developing microbiota engineering tools

While omics approaches have advanced our understanding of complex microbiota, including the diversity of species, metabolites, and potential functions such as metabolic pathways, the dynamics of their complicated interactions and the role of individual players in the microbial community have yet to be elucidated [5]. Recently, microbiome or microbiota engineering tools have been developed to address this important issue. For example, Ronda et al. engineered horizontal gene transfer events to modify the mouse gut microbiome in situ [32]. Rottinghaus et al. used machine-learning-based, predictable CRISPR tools to specifically kill or isolate a strain of interest in the microbiota with the strain level accuracy. To deliver the CRISPR machinery, the authors applied liposome that can be fused to most bacteria, promising broad applications [1]. Similarly, Rubin et al. developed a method for engineering the genome of specific organisms in microbiota [33]. Notably, the authors used environmental transformation sequencing and DNA-editing CRISPR-Cas transposase systems to enable sequence-specific genome editing in a community.

Given engineering microbiota mostly involves developing methods for manipulating non-model organisms, the recent tools for engineering non-model microbes are worth noting [34]. For example, Jin et al. developed a method that can identify the gene transfer methodology for diverse non-model microbes [35]. Importantly, the authors applied their technology to delete a gene for bile acid synthesis in a microbiota, discovering the gene's role in regulating gut inflammation. Patel et al. demonstrated the common synthetic genetic elements can work in both eukaryotes and prokaryotes [36]. Although this study used model hosts, including *Pseudomonas putida*, their tool may facilitate engineering non-model organisms, broadening its utility for microbiome

engineering. Building and characterizing a defined synthetic consortium of 104 bacteria, Cheng et al. also provided insights into microbiome-associated phenotypes [37]. Notably, despite the exciting recent advancements in developing microbiota engineering tools, future research will benefit from more in situ engineering tools with strain specificity and high spatiotemporal resolution as well as an improved in-depth knowledge of the interaction dynamics among gut microbiota, phages, gut metabolites, and the animal host.

Gut microbiota–brain axis

It has become evident that gut microbiota can affect gut-brain communication (i.e., the existence of gut microbiota-brain axis) [38], and the concept of probiotics as delivery vehicles for neurochemicals has been proposed [39]. Despite the rise of research interest in the gut-brain communication and use of probiotics for practical applications, discovery and development of microbial sensors and metabolic pathways for neurotransmitters are major technical hurdles that need to be resolved. Recently, microbial neurochemical sensors with high specificity have been developed in EcN by using protein structure prediction algorithms, such as Rossetta, and screening biosensor libraries [12], addressing those issues. Such microbial neurotransmitter sensors can be used as a non-invasive diagnostic tool. Alternatively, as discussed below, dynamic genetic controllers can be developed in native gut microbes or probiotics to sense and respond to fluctuating gut neurochemical levels, potentially modulating brain activity and function.

Neurotransmitters play a major role in controlling brain function and behavior, including stress responses, pain perception, mood changes, and cognition. These neurochemicals had long been believed to be only associated with the host, but two papers were published to demonstrate that these neurotransmitters can be detected [40] and metabolized [41] by gut bacteria. More recently, Yano et al. discovered that gut microbiota plays an important role in controlling serotonin, a neurochemical affecting mood and happiness [42]. Notably, more than 90% of the body serotonin is produced by enterochromaffin cells in the colon [43], not by brain cells. If specific biosensors for serotonin can be developed in probiotic microbes, happiness or good mood might be maintained for a relatively long time by using probiotic GEMs containing dynamic controllers that keep serotonin concentrations within desirable levels (Figure 1) [12]. To achieve such an ambitious goal, researchers should develop more microbial sensors with high specificity, dynamic controllers with rapid response time, and actuators (e.g., enzymes) with sufficiently high activity in the complex, constantly changing gut environment. Obviously, practical applications of the gut microbiota-brain axis concept will require a better understanding of complex interactions among the brain, gut, and gut microbiota as well as further development of genetic sensors, circuits, and microbiota engineering tools that allow for investigation of neurochemicals in the gut and their impact on the animal host [12]. Notably, the field of gut microbiota-brain axis research is at its infancy, but the future scientific advances may change the paradigm of medical research.

Biocontainment and biosafety of GEMs

Microbial biocontainment is essential for engineering safe living diagnostics and therapeutics. To this end, probiotic microbes could be engineered to rely on a nonstandard or nonnatural amino acid that would not be present in natural environments [44]. However, this approach would be impractical for many applications because these relatively expensive compounds, which might also be harmful to the animal host or the environment, should also be supplied until the microbes' designed mission for practical applications is accomplished. In

addition, the required extensive genome engineering, such as eliminating a specific stop codon from the entire genome, has yet to be done for application-relevant, non-model organisms, as opposed to only *E. coli*.

As alternative approaches, kill switches have been developed in microbes, but these genetic circuits are highly susceptible to mutational inactivation due to the selective pressure, leading to enriching escape mutant populations and incomplete killing [45]. Importantly, kill switch systems must satisfy the NIH guideline for the escape rate: 10^{-8} [46]. To address these issues, Rottinghaus et al. leveraged several strategies to maximize kill switch efficacy and maintain genetic stability, including use of multiple induction modalities (i.e., both chemical and environmental signals), functional redundancies within the kill switch circuit, an antibiotic-independent plasmid maintenance system, genomic knockouts of SOS response genes to mitigate mutagenesis, and provision of intra-niche competition by a non-kill switch EcN strain to better mimic the gut environment [30]. Importantly, with these combined approaches, the authors demonstrated complete elimination of kill switch strains within the gut of mice upon chemical induction, biocontainment after excretion with temperature induction, and genetic stability of the system both *in vitro* (i.e., more than 224 generations or 28 days of cultures) and *in vivo* (more than 8 days under selection).

Despite these advances in biocontainment technologies, studies investigating the efficacy and stability of biocontainment genetic circuits in the real-world environment such as soil and surface water microcosms are extremely limited, let alone the field tests. For example, although Rottinghaus et al. performed the kill switches' efficacy test in mice [30], their killing efficiency and long-term stability remain poorly characterized under stressful and nutrient-poor environmental conditions that may promote the selection of nonfunctional mutants or the suboptimal circuit performance. The potential unintended escape and propagation of GEMs in the built and natural environment might pose risks to human and environmental health, which will depend on the engineered biosynthetic pathways and functions of GEMs. Although the consequence of GEM releases is difficult to assess, we must simulate the environmental conditions to test the invasiveness, potential disruption of ecosystem functions, and unintended effects of the products (e.g., chemicals and proteins) produced by the GEM. In addition to the discussed technological progress, educating the public about the advantages and risks of GEMs in an openly manner will be critical for regulatory approval and public acceptance of GEM-associated technologies. With further technological advances and ongoing open discussions among governments, researchers, and the public, I expect more engineered probiotics and microbiotas to be approved for diverse applications by regulatory agencies.

Conclusion and future perspectives

The Human Microbiome Project and related research efforts have generated an enormous amount of data about the composition of gut microbial communities and demonstrated that changes in the composition correlate with many human diseases. Although many correlational studies have enhanced our understanding of complex dynamics of the gut microbiota and the host, functional studies are still lagging, but necessary to translate these findings into new therapies. To address this issue, we can construct chemical synthesis and degradation pathways in probiotic strains, and test whether they can affect the gut environment and host responses. I envision a future medical research field where researchers can find designer probiotics in a probiotic catalyst handbook to

manipulate gut (and systemic) chemical levels. In a complex gut chemical network, each probiotic serves as a live source or sink for a given chemical, which is equivalent to gene overexpression or knockdown, respectively, in a gene regulatory network study of an organism. Importantly, this novel concept allows us to mimic the situation where gut microbes perturb gut chemical levels, even prior to identifying the responsible, native microbes. Additionally, this is a generalizable approach even if the identified gut microbes are unculturable and difficult to genetically modify. This designer probiotic-based approach will also enable researchers to investigate the microbiota-host interaction. For medical applications, those designer probiotics can be engineered to have a controller that enables them to keep concentrations of a target chemical at the human-defined level, providing medicines without spikes and valleys resulting from pharmacotherapy (Figure 1).

The human gut houses up to 100 trillion microbes, with 10^{11} - 10^{12} cells/mL, the highest density known for any habitat [47]. The intestinal epithelial cells face the continually changing environment: not only ingested and digested compounds, but also replicating microbes as well as their metabolites. The gut is truly a complex environment where countless interactions, which are challenging to elucidate, occur among microbes, chemicals, and host cells. Easily and broadly applicable approaches are needed to understand the complex interplay among the gut microbiota, gut chemicals, and the host, as gene knockout/overexpression methods have advanced our understanding of a single organism for several decades. As I discussed above, I propose to engineer the gut microbiota by introducing designer probiotics that synthesize and degrade the target molecule. My rationale behind this choice is as follows. First, the native gut strain-targeting engineering requires prior identification of producers or consumers of a given chemical in the complex microbiota, which is a challenging task. Second, even if the microbial suspects are identified, their targeted engineering tools must be available for targeted killing or killing system delivery [1]. Third, using designer probiotics, we can easily ‘knock-in’ metabolic genes in various combinations without direct manipulation of the native gut microbes, allowing us to study any gut microbiota regardless of variability in the native microbial composition. Given that the gut microbiota is different from one individual to another, the probiotic-based tool can be applied more easily and broadly than other methods that require direct manipulation of the native gut microbes.

This short perspective article summarizes the advances in tools for engineering probiotics and microbiota mainly for biomedical applications as well as the relevant biosafety and biocontainment issues (Table 1). However, as I have previously discussed [5], those tools can be applied to engineer soil or water microbiota. For example, ssCRISPR gRNAs, along with the liposome-mediated cargo delivery method, can be used in diverse applications, including improving the health of livestock, plants, and humans, identifying and isolating microbes with unique characteristics, investigating the roles of individual microbial community members, and tailoring microbiota for improved functions [1]. Specifically, this tool can shorten and simplify microbial isolation processes, which currently involve complex tailored media and serial culture systems, and it can facilitate the discovery of microbes with novel characteristics. In addition, this new technique has vast implications in designing strain-specific antimicrobials and combating the growing concern of antibiotic- and bacteriocide-resistant microbes in not only clinics but also crop fields by delivering the CRISPR payload to microbes in diverse ecosystems. In another recent study, sentinel microbes could monitor specific, environmental DNA sequences for diverse applications in forensics, epidemiology, and ecology [48]. Leveraging such tools, I envision that the entire planet will be viewed as a huge bioreactor [5] where probiotics, supporting the planet

health, can fix carbon dioxide and nitrogen to mitigate greenhouse gas problems and nitrogen fertilizer-related issues, degrade plastic wastes in the environment [49], sustainably produce high-value compounds from wastes [50], and kill plant pathogens without affecting other beneficial microbes [1].

Plasmids have long been used in biological studies as a simple way of genetically engineering microbes. Researchers typically rely on antibiotic resistance for continued maintenance of plasmids, but the use of antibiotic resistance cassettes, released with dead experimental microbes by research laboratories into the environment for many decades, can contribute to the potential spread of antibiotic resistance via horizontal gene transfer in the environment [5-8]. Additionally, antibiotics are most likely to be absent in the GEM's application site (e.g., contaminated soil for bioremediation and patient gut for disease treatment), making the site selection-free and leading to the loss of plasmids needed for GEM's designed functions [7]. I suggest that we should develop and adopt a new pipeline for antibiotic resistance gene-free plasmid (ARGFP)-based cloning and maintenance [5-8]. For example, such a platform can consist of an auxotrophic gene marker for plasmid selection and an essential gene for plasmid addiction or maintenance in the background of a double-knockout cloning strain, as demonstrated in a recent report [7]. Given the unknown, significant percentage of biological researchers' contributions to antibiotic resistance spread through the unrestricted release of ARG-containing plasmids for many decades, the ARGFP-based method can revolutionize the field's practice, potentially contributing to addressing the critical problem of antibiotic resistance spread and multi-drug resistant pathogens [5-8]. Such responsible research activities will also help scientists and engineers gain the public trust again [6].

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Declaration of interest

The author is a co-founder of Moonshot Bio, Inc. The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Table 1. Genetic tools for engineering microbes and microbiota. HGT, horizontal gene transfer. ARGFP, antibiotic-resistance-gene-free plasmid.

Tools	Notes	References
Microbial biosensor	Useful for non-invasive detection of disease markers and dynamic control of chemical levels	[17, 48]
Genetic recorder	Used to record detected signals; genetic memory device	[18-20]
Therapeutic function	Typically, therapeutic proteins or metabolic enzymes	[24-27]
Strain- or species-specific microbiota engineering tool	Enabling in situ microbiota engineering with target specificity at a strain or species level; facilitating targeted microbe killing, genome engineering, and isolation	[1, 33]
HGT-based microbiota engineering tool	Modification of a broader range of microbes in the environment, typically via less-specific conjugation	[32, 34]
Tool for detecting or controlling neurochemicals	Neurochemicals are found in the gut and metabolized by gut microbes, necessitating development of microbial sensors and enzymes for gut microbiota-brain axis study	[12, 42]
Biocontainment tool	Genetic stability with low escape rates should be ensured	[30, 44, 45]
ARGFP-based cloning	Useful to minimize antibiotic resistance spread via HGT caused by plasmids released from research laboratories	[7]

Figure legend

Figure 1. Engineered microbes to keep gut chemical concentrations at the human-defined levels. **A.** An engineered microbe contains a dynamic controller that maintains the target chemical within the desirable level in mice. **B-C.** Circuit responses at low (B) and high (C) levels of the target chemical are shown. Black, active; gray, inactive. Blunt arrow, repressing; pointed arrow, activating; deg, degradation gene; syn, synthesis gene; rep, repressor gene; chem, target chemical to control.

** outstanding interest

[1] The authors developed microbiota engineering tools for targeted strain knockout or selected strain isolation from a community of microbes by leveraging machine-learning-based CRISPR tools and liposome-based DNA delivery techniques.

[17] The authors developed a device for non-invasive diagnostics, which is compatible with ingestion and enables wireless communication between the gastrointestinal tract and the signal receiver outside the animal body.

[19] The authors demonstrated time-ordered recordings of transcriptional events using prokaryotic retrons, which are reverse transcribed into DNA, followed by the integration of barcodes by the CRISPR-Cas system.

[25] The authors developed engineered probiotics that reduced *C. difficile* infection in mouse models by restoring bile salt metabolism in response to dysbiosis.

[30] The authors developed kill switches with high mutational stability by implementing multiple strategies, demonstrating genetic stability of the circuits in vitro for at least 28 days of continuous cultures and in vivo for at least 8 days under selection.

[33] The authors combined environmental transformation sequencing with DNA-editing CRISPR-Cas transposase systems to demonstrate sequence-specific genome editing in a community context.

[35] The authors developed a tool that can identify the gene transfer methodology for diverse non-model microbes, applying it to delete a gene for bile acid synthesis in a microbiota and discovering the gene's role in controlling gut inflammation.

[36] The authors showed that common synthetic genetic elements could work in eukaryotes and prokaryotes for microbiome engineering.

[37] The authors provided insights into microbiome-associated phenotypes by building and analyzing a defined synthetic consortium of 104 bacteria.

*** special interest**

[7] The authors developed a streamlined cloning method without using an antibiotic resistance gene, showed a comparable cloning efficiency to that of antibiotic-resistance-gene-based cloning, and demonstrated long-term plasmid maintenance in vitro and in vivo, potentially enabling broad applications while reducing the risk of antibiotic resistance spread due to the antibiotic-resistance-gene-containing plasmids discarded into the environment from research laboratories.

[12] The authors developed specific microbial sensors for neurochemicals in a probiotic strain by leveraging protein structure simulation programs and screening mutant libraries although the protein's crystal structure was unavailable.

[15] The authors provided a computational design strategy that enables the creation of sense-and-respond systems by designing and building protein binding sites de novo.

[16] The authors created protein-based, modular biosensors de novo by using the thermodynamic coupling of ligand binding to sensor activation.

[20] The authors utilized barcoded CRISPR arrays to record transcriptional histories of bacteria in vivo.

[26] The authors developed a programmable encapsulation system for engineered bacteria with an improved anti-tumor efficacy in animal models.

[32] The authors engineered horizontal gene transfer events, enabling microbiome engineering in situ.

[48] The authors developed sentinel microbes to monitor environmental DNA sequences for potential applications such as forensics, epidemiology, and ecology.

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