Synthetic biology in probiotic lactic acid bacteria: At the frontier of living therapeutics

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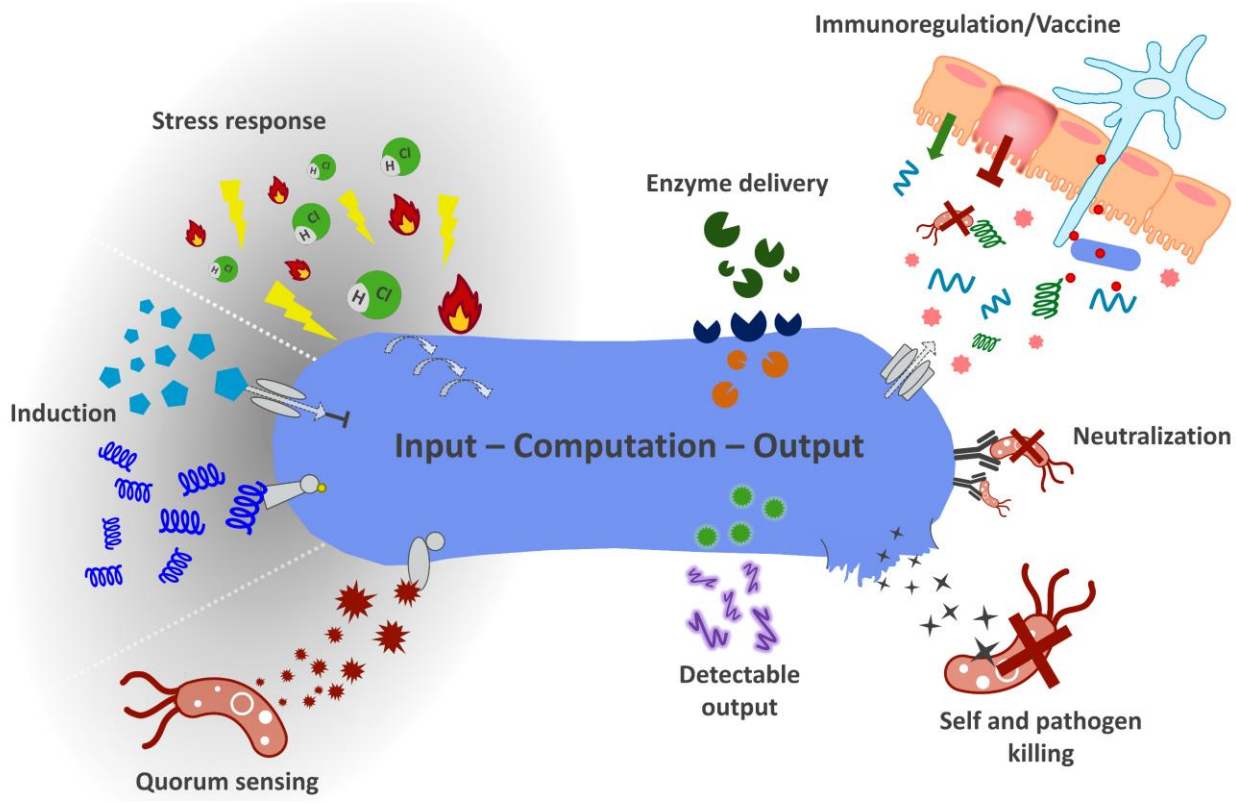
Abstract

The trillions of microbes hosted by humans can dictate health or illness depending on a multitude of genetic, environmental, and lifestyle factors that help define the human ecosystem. As the human microbiota is characterized, so can the interconnectivity of microbe-host-disease be realized and manipulated. Designing microbes as therapeutic agents can not only enable targeted drug delivery but also restore homeostasis within a perturbed microbial community. Used for centuries in fermentation and preservation of food, lactic acid bacteria (LAB) have a long history of safe, and occasionally health promoting, interactions with the human gut, making them ideal candidates for engineered functionality. This review outlines available genetic tools, recent developments in biomedical applications, as well as potential future applications of synthetic biology to program LAB-based therapeutic systems.

Highlights

- Genetic tools in LAB can be highly strain- and species-specific
- Genome-engineering tools have enabled engineering of more complex functions in LAB
- Therapeutics in development are largely focused on GI disorders and vaccines
- Advances in *E. coli* engineering provide a framework for future LAB engineering

Graphical Abstract



Figures

Figure 1. Summary of current genetic tools and modes of action for probiotic therapeutics

The various synthetic biological components corresponding to input (left), computation (center), output (right) are outlined. Environmental signals like heat, low pH, and UV-irradiation can initiate a stress cascade. Concentration gradients of salts, sugars, and antimicrobial peptides can induce or repress gene expression. Quorum-sensing machinery can be co-opted to control population-wide responses. These gene circuits can be used to actuate several therapeutic outputs. Enzymes can be secreted, displayed, and cytoplasmically expressed; production of cytokines, antimicrobial peptides, hormones, antigens, and enzymes can manipulate immunoregulatory signals; pathogens can be neutralized or killed by the production of antibodies and toxins; and detectable outputs can be produced to diagnose subclinical disease states.

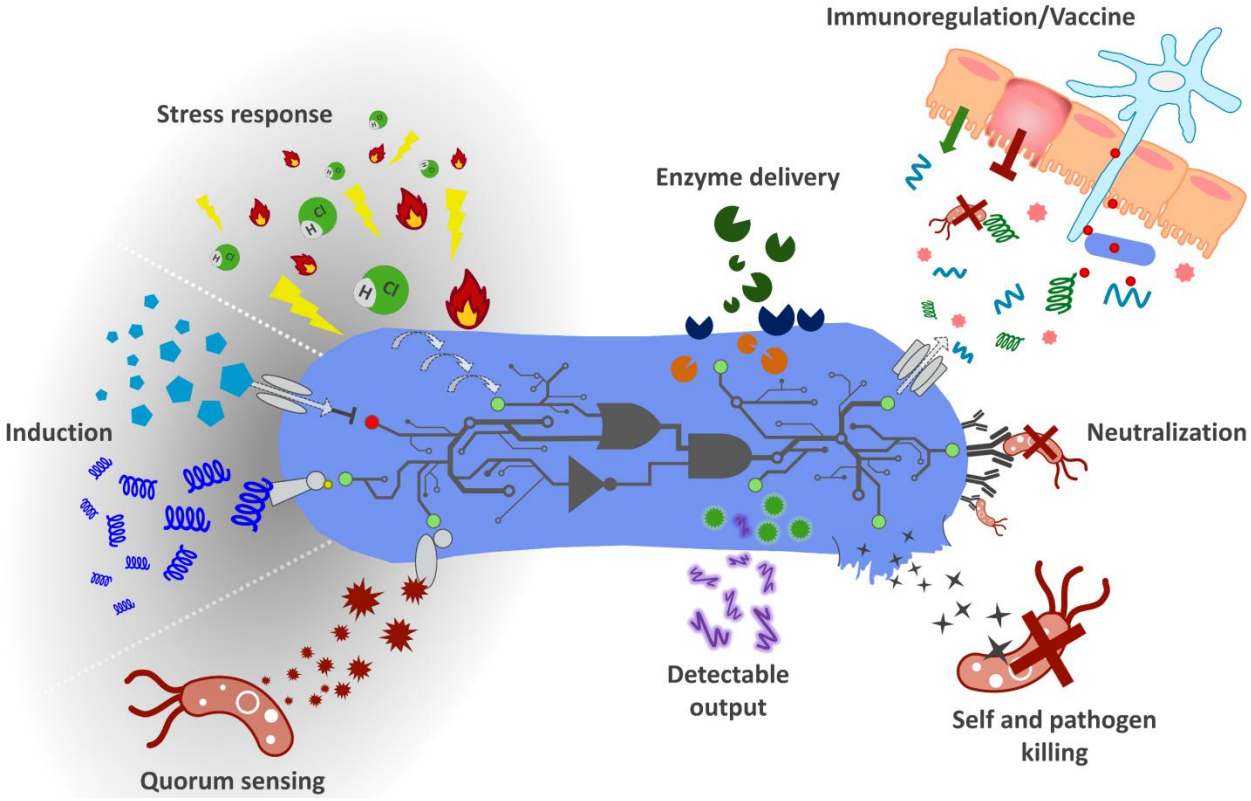


Figure 2. Target applications of probiotic therapeutics

Microbes can confer benefits depending on the route of administration and their relationship with the human microbiota (top left). These benefits are outlined for LAB therapeutics targeting the intranasal/aerodigestive (top right), oral (center right), and urogenital/rectal (bottom left) mucosal membranes.

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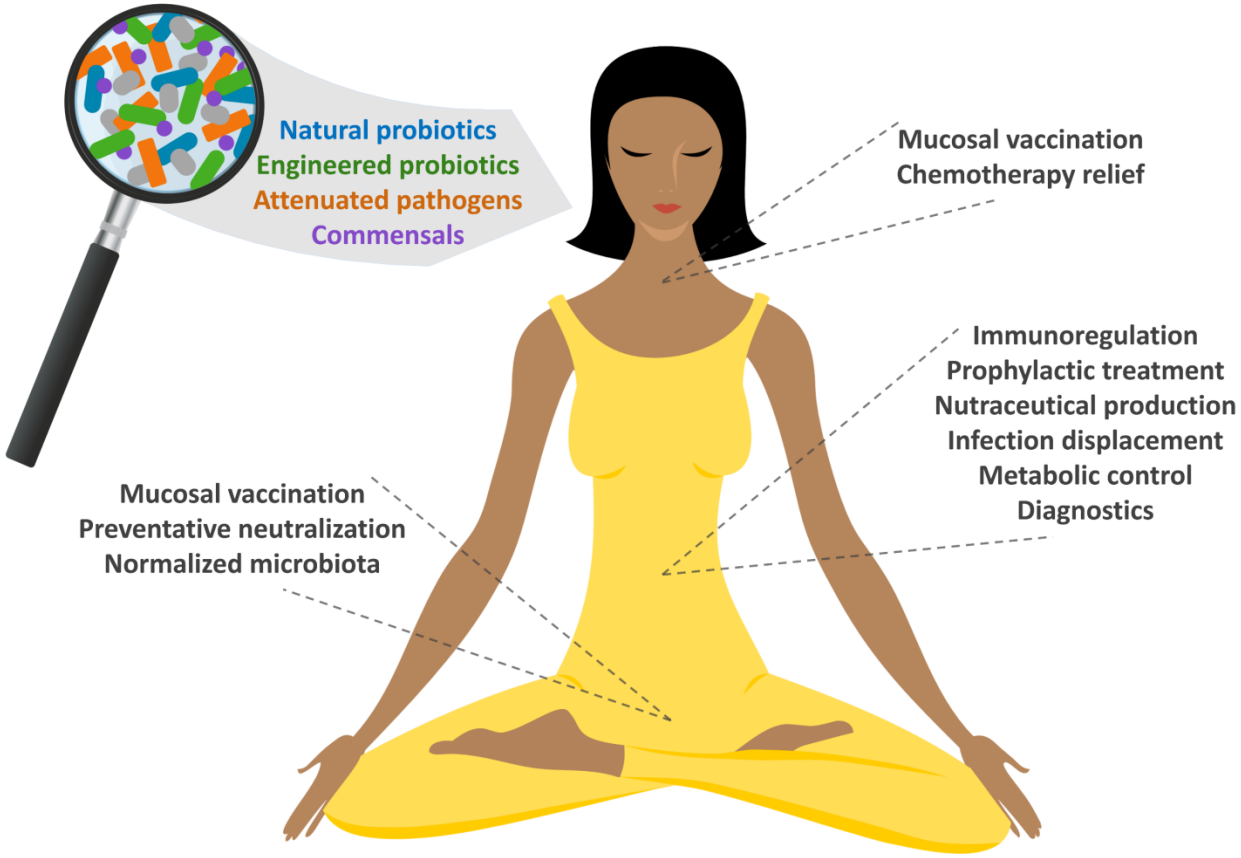

Introduction

The advent of next-generation sequencing has provided a new lens into the vast and diverse microbial taxa present in and on the human body, with their collective genomes termed the microbiome. Consequently, the continued resolution of the microbiome in the post-genomic era has modified the ontological definition of an individual, evoking a re-formation of human beings as holobionts, pluralistic ecosystems in which humans are not only hosts *to* but members *of* a community functioning as a whole being.

With this framework, microbiota health is interminably linked to human development and wellness, and in turn, microbial dysbiosis can stem from, or spur, disease. With the largest microbial community present in the gastrointestinal (GI) tract, a perturbed gut microbiota can invite opportunistic overgrowth, nosocomial infection, immunodysregulation, and metabolic imbalances, each of which have been associated with a multitude of diseases [1]. The gut microbiota has been further associated with aging, asthma and atopy, cardiovascular disease, neuropsychiatric disorders, cancer, and childhood diseases. Although historically contextualized within traditional medicine and home remedies, recent explorations into treatments of these conditions have sought to leverage the microbe-host connection by restoring homeostasis through the supplementation of *good bugs*, colloquially termed probiotics.

Lactic acid bacteria (LAB), largely from the order Lactobacillales and long used in food and agricultural processing, have become the centerpiece of probiotics research [2]. Extending beyond LAB to *Bifidobacterium*, non-pathogenic *Escherichia coli* Nissle 1917 (EcN), and some yeasts, conventional probiotics have been employed in several successful clinical trials to combat foodborne enteric pathogens and impart general immunoregulatory benefits [3]. While probiotics are often sold as over-the-counter health supplements, they have an opaque connection to wellness, resulting in symptom alleviation or general health promotion without a designated mode-of-action. An adjuvant approach, conventional drug therapy (i.e. small molecules) along with prebiotic and probiotic supplements, can sometimes provide synergistic benefit. For example, prebiotic and/or probiotic supplementation was more effective at eradicating *Helicobacter pylori* or *Clostridium difficile* infections when antibiotics were often not sufficiently efficacious [4,5]. This adjuvant strategy is now in a second iteration as LAB probiotics are being directly engineered to express these conventional drugs, acting as living therapeutics. This review highlights recent publications and outlines the toolsets being implemented in probiotic microorganisms, with particular emphasis on LAB, to localize, tune, control, and remember functional outcomes with therapeutic, preventative, and diagnostic potential. While this review focuses on the most recent advances in LAB engineering, a comprehensive and historical perspective of LAB, their relationship to human health, and the fundamentals of their expression systems can be found in *Lactic Acid Bacteria: Microbiological and Functional Aspects* [6].

Developing synthetic biological parts libraries for LAB probiotics

Expression systems

A cornerstone of synthetic biology is the forward engineering of cellular behavior using well-defined parts libraries. Supported by several decades of basic and applied research due to its importance in the fermented foods industries, *Lactococcus lactis* has emerged as a model and platform LAB for synthetic biology. Early gene cloning vectors constructed using plasmids isolated from *L. lactis* strains – pWV01 and pSH71 (as well as pAMβ1 from *Enterococcus faecalis*) – are still the backbone for more advanced genetic tools for gene expression, inducible systems, chromosomal integration, and recombineering.

A considerable number of constitutive promoters and terminators of varying strength have already been isolated from *L. lactis* and have provided sufficient gene expression for initial characterization studies. Using promoter mutagenesis, synthetic constitutive promoter libraries were generated in *L lactis* and *Lactobacillus plantarum* with wide dynamic ranges [7,8]. Alternatively, inducible promoters offer conditional expression and are often more useful for synthetic biological applications. The nisin-controlled expression (NICE) system is the most widely used and reviewed, constructed based on the autoregulatory production of the antimicrobial peptide nisin. One drawback to the NICE system is leaky basal expression, which can limit its utility to applications that require tight control or for expression of toxic proteins. The P_{Zn}-*zit*R expression system [9] and the stronger zinc-regulated expression (Zirex) system [10] can each be implemented simultaneously with the NICE system to provide tight co-expression under non-toxic Zn^{2+} levels. The agmatine-controlled expression (ACE) system, adapted from the eponymous locus in *L. lactis* [11], also offers a tightly-controlled and dose-responsive alternative to the NICE system. Another widely used induction system is based on the *Lactobacillus sakei* sakacin-P regulatory machinery and is activated by the inducing pheromone/quorum sensing molecule IP-673/SppIP. This system has been shown to be compatible with many LAB (but not *L. lactis*) for protein expression, secretion, and surface display. Some variations of classical *E. coli* expression systems like the *lac* operon with the $P_{lacSynth}$ promoter have been evaluated in lactobacilli as well [12].

Since the synthetic biological applications of LAB currently being explored are focused on gastrointestinal applications, promoters that can be induced by gut-associated physiological signals are being explored as means to controlling gene expression *in vivo*. Some examples implemented in *L. lactis* for therapeutic expression studies include pH-dependent promoters P_1 and P_{170} [13], sugar-regulated xylose-induced expression system (XIES) [14], heat shock-responsive *dnaJ* promoter [15], and reactive oxygen species (ROS)-responsive superoxide dismutase A promoter (P_{SodA}) [16]. Most recently, the stress-induced controlled expression (SICE) system uses the *L. lactis groESL* promoter [17], which has been demonstrated to be induced *in vitro* under conditions that simulate those encountered in the intestines [18]. These studies show potential of metabolite or conditional induction systems for *in vivo* gene activation, circumventing the need for expensive and labile peptide-based induction molecules.

Genome editing tools

Chromosomal integration has long been achieved in *L. lactis* for both knock-out and knock-in functionalities [19]. For example, integration of the *nisRK* genes has greatly improved protein expression levels at sub-lethal nisin concentrations and has proven to be critical for implementing of NICE to other LAB. Additionally, targeted knock-in of therapeutic genes to replace thymidylate synthase (*thyA*) facilitate constitutive protein expression under P_{ThyA} while concurrently creating a thymidine auxotrophy [20]. However, these tools are typically implemented for single gene manipulation. Genome-scale mutations, such as those required for pathway- and gene circuit-engineering require additional tools [21]. Recently, genome-wide modifications were made in *L. lactis* and *L. reuteri* using single-stranded DNA (ssDNA) recombineering [22]. While effective, the efficiency of this system is impeded by the innate mismatch repair mechanisms that function to correct mutations. Echoing work performed in *E. coli*, ssDNA recombineering was later combined with CRISPR-Cas9 technology in *L. reuteri*, vastly improving mutational efficiency. Here, an *L. reuteri* prophage-derived ssDNA-binding protein (RecT) facilitates mutagenesis by hybridizing oligonucleotides to the lagging strand of the DNA replication fork. The Cas9 nuclease is then targeted to the wild-type gene sequence for elimination, allowing enrichment of mutant alleles [23]. An alternative proposal for chromosomal manipulation in LAB is the pathway engineering vehicle for lactic acid bacteria (PEVLAB) system [24]. By leveraging species-specific plasmid copy control, the PEVLAB system confers high-copy numbers in *E coli* for efficient cloning and single-copy availability in *L. lactis* for smaller edits (< 10 base pairs) using ssDNA recombineering. These copy numbers also accommodate large DNA manipulation (> 100 base pairs) through λred/RecET recombination and chromosomal integration through homologous recombination.

LAB therapeutics currently in development

Synthetic biological circuits developed in lab strains of *E. coli* [25] have shown to be easily ported to probiotic EcN for therapeutic applications [26]. While a few other non-probiotic Gram-negative strains are also being explored as therapeutic vehicles (*Salmonella enterica* serovar Typhimurium [27], *Bacteroides fragilis* [28], and *Bacteroides thetaiotaomicron* [29]), food-grade probiotic Gram-positive LAB may be more desirable as oral or mucosal therapeutic delivery vectors since they are inherently non-pathogenic and can survive traversal through the harsh gastric environment. Thus, much application focus has been placed on using LAB to treat idiopathic GI disorders that have few current treatment options or as mucosal vaccines, which are promising alternatives to parenteral injections. In fact, mucosal administration may be ideal because both chronic low-grade inflammation and acute infection response begin at the mucosal layer, where localized immune response contributes about 80% of all immunocytes to tissues [30].

Mucosal vaccines present antigens to evoke protective immune responses at the aerodigestive, intestinal, and urogenital mucus membranes. Using the expression tools described in the *Expression systems* section, LAB chassis have been successfully used to deliver antigens and offer protection against subsequent challenge with pathogens. Cytoplasmic [31],

surface-anchored [32], and secreted [17] forms of the human papilloma virus (HPV)-16 E7 antigen have been administered intranasally in mice with recombinant *L. lactis*. Similarly, though delivered by oral administration, recombinant *Lactobacillus gasseri* expressing the C-terminus region of streptococcal M6 protein (CRR6) induced a protective antigenic-like response in mice under *Streptococcus pyogenes* challenge [33]. Heat-shock protein (HSP) is an important self-antigen in diabetes, and the onset of diabetes mellitus (DM) type I was reduced in non-obese mice using *L. lactis* expressing HSP65 with tandem repeats of P277 (HSP65-6P277) [34]. Though not quite a vaccination, antibodies can offer protection by neutralizing viral particles. To this end, a *Lactobacillus jensenii* strain shown to colonize the cervicovaginal mucosa of rhesus macaques was engineered to display short-chain variable fragments and single-domain antibodies against conserved CD4i epitopes to neutralize human immunodeficiency virus type 1 (HIV-1) variants [35]. Healthy vaginal flora are dominantly lactobacilli, so an added benefit of urogenital administration is the restoration of these populations, preventing the onset or recurrence of bacterial vaginosis. Opportunistic gut infections are notoriously difficult to treat, especially when they have acquired antibiotic resistance. Probiotics can innately displace or prevent such overgrowth, and engineering target specificity can synergistically enhance their functions. For instance, *L. lactis* engineered to express enterocins in response to the cCF10 pheromone, conveyed strong and specific antimicrobial activity against *E. faecalis*, including vancomycin-resistant strains [36].

Efforts to prophylactically control obesity, diabetes, and most prominently, inflammatory bowel disease (IBD) has dominated LAB therapeutics that target the intestinal mucosa. Oral administration of recombinant *L. lactis* constitutively expressing immunosuppressive cytokines like IL-10, IL-27, and TGF-β1 has shown early promise for attenuating IBD in mice at levels 1/10,000th of that administered systemically. Similarly, to restore balance to intracellular communication in model IBD mice, alpha-melanocyte-stimulating hormone (α-MSH) hormone has been secreted from *Bifidobacterium longum* [37] and *L. casei* [38], as well as porcine insulin-like growth factor I (IGF-I) from *L. lactis* [39]. Other proteins such as mouse heme oxygenase-1 (HO-1) [40] have been secreted by *L. lactis* to promote anti-inflammatory cytokines or suppress pro-inflammatory cytokines in murine IBD models. Additionally, in an effort neutralize pro-inflammatory cytokines, *L. lactis*-secreted recombinant IL-6 single chain variable fragment (rIL6scFv) was able to bind commercially available mouse IL-6 [41]. Alternatively, serine protease inhibitors have received attention for IBD treatment, as elevated proteolytic activity is observed in inflamed tissue models. Using *L. lactis*, secreted elastase-specific protease inhibitor (Elafin) and secretory leukocyte protease inhibitor (SLPI) provided a dose-dependent and stronger anti-inflammatory response than cytokines with the same mouse IBD model [42]. There are also a number of investigations in which recombinant LAB expressing antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (Kat), have neutralized ROS implicated in IBD pathogenesis [2]. Interestingly, these enzymes showed combinatorial anti-inflammatory activity when cytoplasmically expressed in *Streptococcus thermophilus* and orally administered to mice in a fermented milk mixture [43]. Mucositis is a side-effect caused by chemotherapeutic drugs and radiation therapy and an

additional application for engineered probiotics that target inflammation. Recently, progression of intestinal mucositis was halted in mice by engineered *L. lactis* that secreted human pancreatitis-associated protein I (PAP) [44]. Oral mucositis was also effectively reduced with *L. lactis* engineered to secrete human Trefoil Factor 1 (hTFF1), delivered in a mouthwash [45] that is beginning Phase II clinical trials [46].

Obesity is a chronic metabolic disorder highly correlated with inflammation and often triggers autoimmune diseases like IBD. The aforementioned HSP65-P277 [34] also prevented hyperglycemia, and with impaired insulin regulation being associated with reduced HSP expression, *L lactis* expressing HSP may offer treatment for obesity-related DM type II. To more directly treat diabetes and obesity, human commensal *L. gasseri* were engineered to constitutively secrete glucagon-like peptide 1 (1-37) (GLP-1(1-37)), known to reprogram intestinal epithelial cells into glucose-responsive insulin-secreting cells. Daily oral administration reduced hyperglycemia in a rat model of diabetes [47]. Other metabolic therapeutics aim to capitalize on the microbial populations in the small intestine, as they offer early intervention in digestion. L-Arabinose isomerase (L-AI) expressing *L. lactis* has been tested as treatment for hyperglycemia since this enzyme can convert ingested galactose to D-tagatose, an anti-hyperglycemic agent [48]. Similarly, phenylalanine ammonia lyase (PAL) is in the early stages of being developed as an enzyme-replacement therapy for phenylketonuria (PKU), and it was recently expressed in *L. reuteri* fed to model PKU mice [49]. Because the microbial density and diversity in the small intestine is low, due to a thin loose mucosal layer, high shear-luminal flow, and continual exposure to low pH gastric contents, LAB may be uniquely suited to deliver therapeutics to this region of the gut [50].

Gene circuits for living therapeutics

Synthetic gene circuits may offer tunable autonomous decision making, not only for successful delivery and production of biotherapeutics, but also to ensure patient safety. Although auxotrophy is the favored strategy for biocontainment of synthetic organisms, gene circuits can provide added protection through alternate containment mechanisms. For instance, presence of quorum-sensing molecule N-acyl homoserine lactone (AHL) from *Pseudomonas aeruginosa* was used to induce autolysis in EcN [51]. This suicidal circuit design could be adapted for regional containment of synthetic LAB as well. Alternatively, spatial patterning has been demonstrated with *L. lactis* expressing fluorescent proteins in mixed producer-responder populations where protein expression was controlled using the dual inducing and antimicrobial activities of nisin [52]. Analogous to band-pass filter in electronic circuits, only cells that fall within a defined nisin concentration range express the reporter gene, either because nisin concentration is too low for induction or it is too high and cells lyse. Notably, since observed fluorescence was dependent on a concentration gradient of an external factor, it is potentially adaptable for biocontainment purposes. Various other “kill-switches” for *E. coli* may be adaptable to LAB as well [53].

While there is significant interest in using live microbes as therapeutics, there are several technical hurdles that must be overcome before they can be widely and safely used in humans.

Introduction of recombinant genes often results in metabolic burden in engineered microbes that adversely affects their ability to compete for a niche in the crowded gut environment. Chromosomal integration can overcome the need to continually select for plasmid maintenance, but integration tools are not available in all species and strains [21]. Genetic circuit design implementations demonstrated in *E. coli* can be used as guides to illuminate the path forward for future studies with LAB. For instance, a two-part tetracycline-inducible trigger element and transcriptional memory switch derived from phage *λ* allowed engineered *E. coli* NGF-1 to avoid mutations and sustain detectable inducible populations without antibiotic selection for 200 days in mice [54]. As an alternative to chromosomal integration, the hok/sok toxin-antitoxin system has been used to retain plasmids for 72 hours *in vivo* in engineered EcN [55].

Currently, the only FDA-approved and commercially available microbiota-based therapy is fecal microbial transplant to treat *C. difficile* infections [56]. However, there are over 170 microbiota-based therapeutics in development [57], which is very encouraging for the future of engineered living therapeutics. The gene expression systems and genome editing tools outlined above have provided a strong foundation upon which we can build the next-generation of therapeutics that not only leverage the recent advances in synthetic biology in model microbes like *E. coli*, but also leverage the unique physiology of LAB. The ability of synthetic LAB to deliver prophylactics, target regional infections, produce small molecules and peptides, and remember diagnostic information may open the door to a new era of autonomous and precise medicine. As the public opinion of genetically modified organisms improves, living therapeutics will likely gain greater traction in a wide suite of biomedical applications.

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Annotations

[24]

•• Expanding beyond single gene and promoter manipulation, the PEVLAB system was systematically developed for full gene network manipulation in lactic acid bacteria. A shuttle plasmid with inducible copy control leverages engineering functions in *E. coli* and can then be integrated into *L. lactis*. The platform suggests the adoption of multiplex automated genome engineering and ssDNA-based engineering for small parts editing and Red/ET recombineering combined with selection/counter-selection for larger parts.

[35]

- Recently identified, broadly neutralizing antibodies against epitopes of the HIV-1 viral envelope were made into active antibody fragments and optimally expressed in *L. jensenii*. While all fragments bound the CD4i sites, the m36.4 antibody also inhibited HIV-1 in a neutralization assay.

[47]

- GLP-1(1-37) has been previously shown to stimulate the reprogramming of rat and human intestinal cells into β-cells that can produce insulin in response to glucose levels. When chromosomally integrated into *L. gasseri* ATCC 33323 and subsequently fed to diabetic rats, constitutive secretion of GLP-1(1-37) significantly reduced hyperglycemia. The co-expression of β-cell and enteroendocrine markers were observed to measure the extent of reprogramming.

[52]

•• The dual signaling and antimicrobial features of nisin were leveraged to create a circuit for spatial band-pass protein expression. Green fluorescent protein expression was induced using the NICE system in which an external nisin concentration gradient determined circuit outcome. Nisin-producing cells and nisin-sensitive responder cells were mixed to produce various spatial patterns by observing green or red fluorescence.

[54]

- The mouse commensal strain *E. coli* NGF-1 was conferred with a memory switch and an environmental response trigger. Here, Cro protein expression was driven by tetrathionate, a product of ROS during inflammation. A phage-lambda-based CI/Cro memory switch was turned on along with *lacZ* expression. This was able to measure subclinical inflammation in a TRUC mouse model and was retained in the off state for 200 days.