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# Living Therapeutics: The Next Frontier of Precision Medicine

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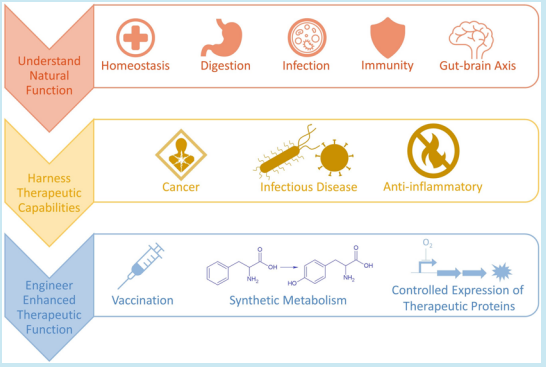
**ABSTRACT:** Modern medicine has long studied the mechanism and impact of pathogenic microbes on human hosts, but has only recently shifted attention toward the complex and vital roles that commensal and probiotic microbes play in both health and dysbiosis. Fueled by an enhanced appreciation of the human−microbe holobiont, the past decade has yielded countless insights and established many new avenues of investigation in this area. In this review, we discuss advances, limitations, and emerging frontiers for microbes as agents of health maintenance, disease prevention, and cure. We highlight the flexibility of microbial therapeutics across disease states, with special consideration for the rational engineering of microbes toward precision medicine outcomes. As the field advances, we anticipate that tools of synthetic biology will be increasingly employed to engineer functional living therapeutics with the potential to address longstanding limitations of traditional drugs.




**KEYWORDS:** *microbiota, microbial engineering, living therapeutics, synthetic biology*



Throughout history, humans have relied on natural and manufactured compounds to maintain health and to treat and cure disease. The majority of these drugs are small molecules, chemically extracted or synthesized and screened for their impact on biological processes. More recently, biopharmaceuticals, or "biologics", have emerged as a powerful class of disease-fighting agents that includes molecules such as enzymes, peptides, cytokines, and antibodies, and are generally characterized by increased size, specificity, and functionality compared to small molecules. Whereas many small molecule drugs can be chemically synthesized, most biologics must be manufactured in living cells in facilities following strict guidelines for purification and formulation to ensure no potentially harmful cellular contaminants remain in the final product. These procedures increase production costs which is passed on to consumers, limiting accessibility to these powerful drugs. Technologies that reduce costs, improve sustainability, and promote expanded development of biological therapeutics are required to broaden the availability of these drugs. In this review, we discuss microbes as living therapeutics that can circumvent limitations of both small molecule drugs and purified biologics through their intrinsic features as well as with engineered functionality. We explore advances and limitations and discuss key considerations for the continued development and engineering of microbial prophylactics and therapeutics.

## MICROBIOTA COMPOSITION AND FUNCTION

**A Brief History.** Pathogens have been used for therapeutic purposes dating back to ancient Egypt,[1] and gained widespread attention due in part to the development of Coley's toxins:[2] a cocktail of *Streptococcus pyogenes* and *Serratia marcescans* used to treat sarcoma. More recently, toxin-deficient *Clostridium* spp.[3] and modified *Salmonella enterica* ser. Typhimurium[4] have been pursued as cancer treatments. Despite some success, the therapeutic use of pathogens comes with the risks naturally associated with these infectious agents. For therapeutic development, nonpathogenic microbes are attractive alternatives. The human body is home to diverse, complex communities of microbes that are beneficial, and often essential, to many host processes (Figure 1). These commensal microbes, collectively termed the human microbiota, maintain homeostasis, protect against infection, and ensure proper physiological development of the host. While recent global research efforts including the Human Microbiome Project in the United States[5] and the METAgenomics of the Human Intestinal Tract (MetaHIT) program in Europe[6] have aimed to uncover the composition and function of these microbial communities in detail, scientists have been isolating and studying gastrointestinal microbes since the development of anaerobic culturing systems in the 1940s.[7] More recently, rapid development in DNA sequencing technology has made possible the fast and

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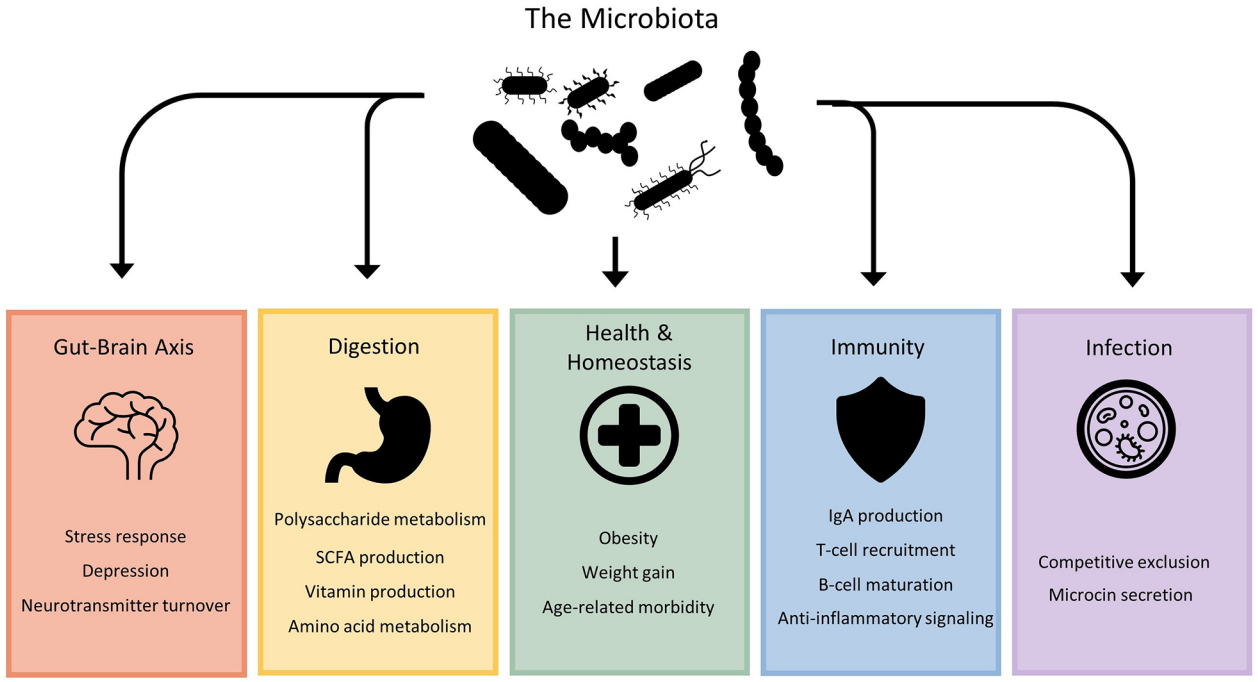


**Figure 1.** Natural functions of the human commensal microbiota.

accurate identification of members of nearly any microbial community, even unculturable species,[8] *via* amplification and sequencing of the 16S rRNA gene. This technique has been used to characterize microbiota of the gastrointestinal tract,[9,10] vagina,[11] and skin.[12] Methods to improve throughput using DNA microarrays[13] and to gain insight into the function and physiology of a community using culturomics[14] or fluorescence *in situ* hybridization[15] have also been developed.

**The Human Gut Microbiota.** The best studied and most diverse human microbial community is the gut microbiota: the collection of microbes that inhabit the gastrointestinal tract. The gut microbiota consists of hundreds of species of microbes from many different phyla, although members of the Firmicutes and Bacteroidetes account for ∼80% of individual organisms.[16] While there is significant conservation of a set of core commensal species between individuals and the composition of an individual's gut microbiota is fairly consistent over time,[17,18] several factors including age,[19] ethnicity,[20] environment,[21] and diet[22,23] can influence the diversity and composition of a given microbial community. Understanding how these commensal species interact with each other and with the host, as well as understanding what happens when the natural human−microbe homeostasis is disrupted, is an important first step in utilizing these microbes as drugs.

*Digestion.* One of the best-known contributions of gut microbes to human health is that of digestion. In the anaerobic environment of the distal gut, bacteria form biofilms on food particles and help metabolize oligosaccharides and polysaccharides.[24] They are also responsible for the metabolism of amino acids[25] and glycated proteins,[26] and digest starches otherwise resistant to hydrolytic enzymes in the gut.[27] These bacteria-mediated digestive processes produce short-chain fatty acids (SCFA), vitamin K, and other metabolites that are crucial in salvaging energy and nutrients from ingested foods.[28] SCFAs in particular are key regulators of energy metabolism, intestinal pH, and water and sodium absorption.[29−31] Altered SCFA levels and the corresponding disruption of SCFA-mediated fluid absorption and G-protein coupled receptor signaling cascades can contribute to the worsening of numerous diseases including diarrhea, colitis, and colorectal cancer.[32−34]

*Immunoregulation.* A healthy gut microbiota is also an important regulator of gastric immune system development and maintenance. A comparison of the gut microbial communities of germ-free and conventional mice revealed that peptidoglycan containing *meso*-diaminopimelic acid, found more commonly in Gram-negative bacterial cell envelopes, is recognized by the host epithelial cell receptor NOD1, and induces maturation of intestinal lymphoid follicles that produce IgA-expressing B cells in healthy, but not germ-free, mice.[35] Additionally, the role of the gut microbiota in immune development appears to be dependent on host-specific microbial colonization, as transplantation of a human fecal sample in germ-free mice resulted in decreased CD4+ and CD8+ T cells, fewer intestinal dendritic cells, and decreased antimicrobial peptide production as compared to conventional mice.[36] In addition to directly impacting the maturation of the intestinal immune system, gut microbes play an important role in regulating inflammatory responses. Certain commensal bacteria in the gut, including Gram-negative members of the *Bacteroides* and *Acinetobacter* and the Gram-positive *Faecalibacterium prausnitzii*, promote secretion of the anti-inflammatory cytokine IL-10 and reduce production of IL-12 and IFN-$\gamma$.[37−40] Decreased diversity in the microbial community, caused by neonatal antibiotic treatment, for example, has been associated with increased susceptibility to inflammatory diseases such as allergic asthma,[41,42] atopic eczema,[43] and Crohn's disease.[38] Microbial diversity in the gut can also be used as a predictor for immune status and disease progression in patients infected by HIV.[44]

*Gut−Brain Axis.* Beyond the gut, increasing evidence points to a gut−brain axis and a strong link between gut microbiota composition and neurological activity and development,[45,46] controlled by stimulation of the vagus nerve.[47] Germ-free mice exhibit altered stress response and motor function compared to standard mice, and display correspondingly altered levels of


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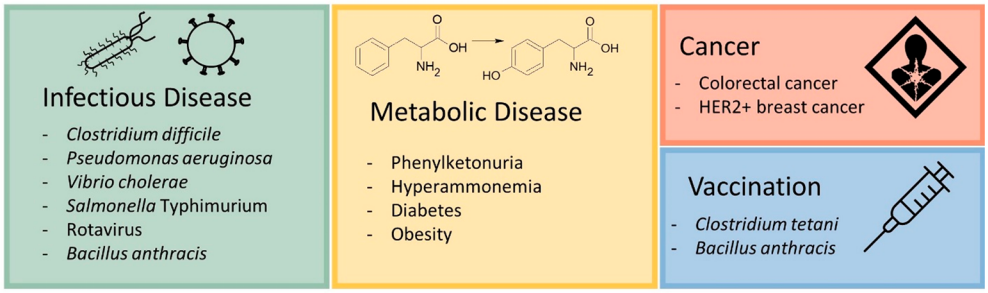


**Figure 2.** Medical applications of engineered commensal bacteria. The center panel depicts the conversion of phenylalanine to tyrosine. Deficiency in the enzyme catalyzing this reaction is a cause of phenylketonuria, which is has been addressed using engineered commensal bacteria.

numerous neurochemicals.[48,49] These abnormal phenotypes can be recapitulated by treating healthy mice with antimicrobials to disrupt the natural gut microbiota.[50] All of these effects can be fully or partially corrected by full fecal transplant of a healthy microbiota[49,50] or by colonization with a single species such as *Bifidobacterium infantis*[48] at an early age, but not in adulthood, highlighting the importance of early establishment of a healthy gut microbiota.

In line with the findings that gut microbiota composition has significant impact on brain function, several neurological conditions have been linked to gut dysbiosis. Interestingly, fecal transplantation from mice showing depressive symptoms into germ-free hosts recapitulated the depressive behaviors of the donor mice, supporting a strong, potentially causative, link between microbiota composition and depressive disorders.[51] Autism spectrum disorders (ASD), for which gastrointestinal dysfunction is a frequent comorbidity, have been similarly linked to dysbiosis of the gut microbiota.[52,53] Comparisons of the microbiota compositions of ASD patients with neurotypical patients reveal significant changes in the relative abundance of several genera, though results of these studies have not always been consistent.[54,55] As in major depressive disorder, fecal transplantation from human ASD patients into neurotypical germ-free mice recapitulates symptoms,[56] and fecal microbiota transfer therapy of healthy donors to ASD patients showed short-term and long-term efficacy on gastrointestinal and ASD symptoms in a small clinical trial,[57,58] indicating both a potential causative link between gut microbiota composition and disease, and potential for microbe-based therapeutic development. Correlations have also been established between the composition of the gut microbiota and other neurological conditions, including Alzheimer's disease[59] and schizophrenia,[60] though these are still developing areas of research.

## GUT MICROBES AS THERAPEUTICS

As metagenomic, transcriptomic, and culturomic techniques have enhanced and broadened our understanding of how the microbiota functions under homeostatic conditions, there has been a corresponding increase in the capacity to utilize members of the microbiota as therapeutics or prophylactics (Figure 2). Such uses range from restoration of microbial homeostasis *via* wholesale fecal transplantation to administration of single, rationally engineered strains to carry out specific novel functions to combat disease. A wide variety of human health conditions can be treated in this manner, from bacterial and viral infection to metabolic disorders and cancer supporting the broad therapeutic potential of commensal microbes.

**Microbes as Vaccines.** Traditional vaccines are comprised of a killed or live attenuated pathogen, a carrier, and adjuvant or immunomodulator which helps the antigen stimulate the adaptive immune system to raise a response against the pathogen and confer immunity to the patient. Because attenuated or killed vaccines often still result in adverse effects, approaches have been developed utilizing recombinant antigenic proteins. These proteins represent isolated, non-pathogenic fragments of the virulent microbe and are therefore inherently safer but, partly due to their presentation outside of the context of the full organism, may not be immunogenic enough to stimulate a sufficient protective response. To address this limitation, strategies combining the immunogenic properties of attenuated pathogens with the antigenic properties of heterologously expressed proteins have been pursued,[61−66] though concerns about the safety of these attenuated pathogens persist. Certain human commensal microbes are attractive alternative delivery vehicles for vaccine antigens, as they typically do not display any inherent pathogenicity within their native microenvironment. Importantly, it has been observed that antibodies are raised against the natural gut flora in mammals,[67] suggesting that these microbes, though not pathogenic, may promote a sufficient immune response to confer lasting immunity against the delivered antigen. Strategies using adherent *Lactobacilli*, transient *Lactococci*, and orally colonizing *Streptococcus gordonii* have been discussed in detail elsewhere.[68,69] Here we discuss a few key examples of the intersection of microbial engineering and vaccine development in the past decade (Figure 3).

*Bacterial-Mediated Antigen Delivery.* Tetanus, or lockjaw, is a potentially fatal infection of *Clostridium tetani* secreting tetanus neurotoxin, which binds to inhibitory neurons and prevents neurotransmitter release, causing paralysis.[70] A nontoxic cleavage product of tetanus toxin, tetanus toxin fragment C (TTFC), has been used as an antigen in attenuated pathogen delivery systems,[63] but for reasons discussed above, a system using a nonpathogenic commensal microbe is desirable. In pursuit of this alternative, *Lactobacillus casei* and *Lactobacillus plantarum* have been engineered to express and deliver TTFC[71] (Figure 3A). A three-dose intranasal priming regimen of $5 \times 10^9$ cells/dose expressing TTFC intracellularly from a plasmid followed by an identical three day boosting regimen 4 weeks later raises toxin-specific serum IgG and mucosal IgA in mice, and results in significant spleen and cervical lymph node (CLN) activation upon antigenic challenge up to 3 weeks after the booster regimen is completed. Microbes expressing surface-bound TTFC required an additional three-day booster to raise the same response. The authors speculate that this is likely due to increased susceptibility of the displayed antigen to proteolytic degradation compared to intracellular TTFC. Additionally, the authors suggest that the large amount of accumulated TTFC


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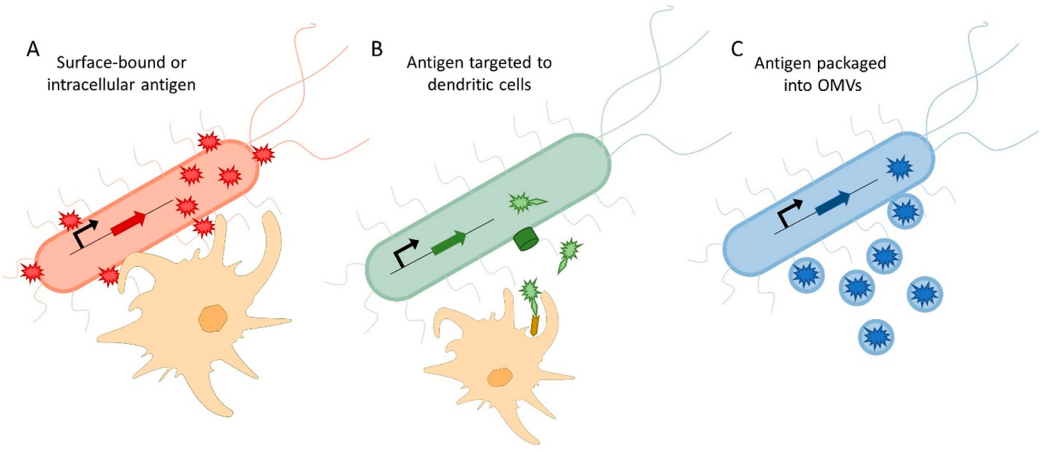


**Figure 3.** Vaccination strategies using engineered commensal microbes as antigen delivery or presentation vehicles. (A) Intracellular or surface-bound antigen expression uses the bacterial chassis as an adjuvant to promote immune cell recognition and uptake. (B) Antigens engineered with dendritic cell-targeting peptides are secreted and targeted to immune cells for uptake. (C) Antigens are packaged into outer membrane vesicles to improve immune cell recognition and uptake of recombinant antigens.

resulting from lysis of bacteria producing the antigen intracellularly may be beneficial to induce a protective immune response more rapidly. Oral dosing generated a toxin-specific immune response in only 9 of 16 mice, compared to 16 of 16 dosed intranasally. This study demonstrates the potential of engineered commensals as delivery vehicles for exogenous antigens, though further optimization to improve immunogenicity and duration of immunity are required. More recent studies have used similar surface-tethered antigen strategies to generate potential vaccines against influenza[72] and *Leptospira* infections.[73]

*Enhanced Antigen Presentation.* Engineered commensal microbes have also been combined with clever antigen design to improve their efficacy as vaccine delivery vehicles (Figure 3B). Standard anthrax vaccination is known to cause adverse side effects due to the use of aluminum as an adjuvant,[74,75] and requires a burdensome dosing regimen, which includes five intramuscular doses over 18 months and yearly booster doses thereafter.[76] To address these issues, the human commensal species *Lactobacillus acidophilus* has been modified to express *B. anthracis* protective antigen (PA) and present it to mucosal dendritic cells as an orally dosed vaccine.[77] While *L. acidophilus* secreting PA alone produced a mild immune response, a strain producing PA fused to a 12 amino acid dendritic cell targeting peptide elicited a strong specific immune response in mice comparable to that observed in mice administered the standard vaccine. More recently, a similar strategy using a modified *Lactobacillus* to target the *Mycobacterium tuberculosis* antigens to dendritic cells showed promise as a booster to the existing *M. tuberculosis* vaccine.[78] While improvements to both the strain and fusion protein are required to increase efficacy and reduce the dosing requirement, this treatment highlights the potential of synergistic microbial and protein engineering for vaccine development.

Another alternative engineered microbial vaccine system centers on packaging of vaccine antigens into outer membrane vesicles (OMVs) (Figure 3C). The Gram-negative commensal *Bacteroides thetaiotaomicron* (*Bt*) has been modified to package *Salmonella* Typhimurium and Influenza A virus (IAV) antigens into OMVs for use as a vaccine.[79] *S.* Typhimurium OmpA and SseB and IAV spike protein H5 were fused to the *Bt* outer membrane protein OmpA to facilitate OMV packaging and expressed in *Bt* from a plasmid. Intranasal administration of 70 $\mu$g modified OMVs once per month for three months raised increased specific IgG and IgA immune responses in C57BL/6 mice compared to empty OMVs. Importantly, these OMV-based vaccines did not cause any adverse systemic or injection site inflammatory responses. In the same study, oral administration *Bt* OMVs carrying human keratinocyte growth factor-2 was shown to significantly attenuate colitis in a mouse model, further demonstrating the flexibility of this technology.

**Gut Microbes as Anti-infectives.** *Fecal Microbiota Transplantation.* When gut microbial homeostasis is disrupted, often by antibiotic treatment, the potential for opportunistic infection by pathogenic microbes increases. Infection with the Gram-positive obligate anaerobe *Clostridioides difficile* after antibiotic treatment is the most common nosocomial infection in the United States, with more than 500 000 cases annually.[80] Standard treatment for opportunistic *C. difficile* infection consists of cessation of the inciting antibiotic followed by administration of vancomycin or fidaxomicin.[81] Despite the initial effectiveness of this treatment, more than 25% of patients relapse after antibiotic discontinuation.[82] Alternative or additional treatments include intravenous IgG,[83] vaccination,[84] and administration of single microbial strains like *Lactobacillus plantarum*[85] or *Saccharomyces boulardii*.[86] These treatments have limited ability to prevent recurrence, require multiple repeat dosing, or both. The most recent clinical guidelines for diagnosis and treatment of *C. difficile* infection recommend fecal microbiota transplantation for treatment of recurrent infection.[81] Microbiota transplantation results in long-term remodeling of the recipient's gut microbiota to resemble that of the healthy donor.[87] Recipients generally respond rapidly to this single-administration treatment, with 74% experiencing disease resolution in 3 days.[88] Additionally, this therapy has a higher rate of response than many alternative treatments, with a primary cure rate of 91% that increases to near 100% in patients not infected by the hyper-virulent 027 strain of *C. difficile*.[89] Elimination of *C. difficile* by restoration of a healthy gut microbiota is believed to be due to several factors, including blocking of *C. difficile* outgrowth and direct interference with the *C. difficile* life cycle by metabolites like secondary bile acids and SCFAs.[90]


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*Single-Strain Therapy.* While administration of individual microbial strains is not the most effective treatment for challenging infections such as *C. difficile*, the nonpathogenic Gram-negative *Escherichia coli* Nissle has shown promise as a therapeutic to treat several gastrointestinal conditions. Nissle has been used clinically to treat inflammatory bowel diseases such as Crohn's disease and ulcerative colitis,[91] and also to restore the natural impermeability of intestinal epithelial cells after disruption by irritable bowel syndrome.[92] This strain can also interfere with the ability of many pathogens to infect the gut, including *Salmonella* Typhimurium,[93] *Candida albicans*,[94] *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila*, *Listeria monocytogenes*,[95] and pathogenic *E. coli*.[96] Secretion of microcins, a class of antibiotics characteristic of nonsporulating bacteria,[97] and NF-κB-induced expression of the antimicrobial peptide human beta-defensin-2[98] are believed to be responsible for this potent anti-infection activity.

*Engineered Systems: Quorum Sensing.* *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen that is a common source of nosocomial infection in the respiratory and gastrointestinal tract,[99] and is difficult to treat due to antibiotic resistance genes and efflux pumps.[100] Using synthetic biology tools developed over decades of exploration and engineering of the workhorse lab bacterial strain *E. coli*, the probiotic *E. coli* strain Nissle was engineered to detect, target, and kill pathogenic *P. aeruginosa in vivo*. *P. aeruginosa* secretes *N*-acyl homoserine lactone (AHL) for quorum sensing.[101] A gene circuit was constructed in Nissle to sense this effector and induce expression of four gene products: CheZ, which promotes motility toward the AHL-secreting colonies,[102] pyocin S5, which disrupts the cellular integrity of *P. aeruginosa*,[103] dispersin B (DspB), which destabilizes biofilms, and Lysis E7, which induces self-lysis of the Nissle and release of accumulated pyocin and DspB. This engineered strain significantly improved survival after *P. aeruginosa* challenge in a *C. elegans* model, and significantly reduced *P. aeruginosa* burden both therapeutically and prophylactically in a mouse model.[104]

*Vibrio cholerae*, the Gram-negative bacteria that causes cholera, also utilizes quorum sensing mechanisms to control expression of its virulence factors: cholera toxin and toxin coregulated pilus.[105] Two secreted signaling molecules, autoinducer-2 (AI-2) and cholera autoinducer-1 (CAI-1), act as the messengers controlling gene expression. At low cell density, virulence factor expression is upregulated, but at high density, resulting in high AI-2 and CAI-1, virulence factor expression is downregulated, and *V. cholerae* escapes the host *via* the excretory system. *E. coli* Nissle, which naturally expresses AI-2, was engineered to also constitutively express the *cqsA* gene, which encodes the final enzyme in the CAI-1 synthesis pathway, to synthetically mimic conditions of high *V. cholerae* density and downregulate virulence factor expression.[106] In a mouse model, oral delivery of this engineered Nissle strain dramatically improved infection outcome when dosed prophylactically 8 h before infection (92% increased survival, 80% reduction in cholera toxin), with reduced efficacy when probiotic and pathogen were administered concurrently (27% increased survival).

The gastroenteritis-causing *Salmonella* Typhimurium induces intestinal inflammation by secreting effector molecules using its Type III secretion systems.[107] This inflammation induces release of reactive oxygen species, which oxidize thiosulfate to tetrathionate, conferring growth advantage.[108] The gene cluster responsible for *Salmonella*'s tetrathionate sensing and utilizing mechanism, *ttRSBCA*, was transferred to the chromosome of *E. coli* Nissle, resulting in a persistent, inflammation-sensing probiotic in a mouse model.[109] Further work added a tetrathionate-inducible microcin H47 expression system, expanding the utility of *E. coli* Nissle as an *S.* Typhimurium killer, significantly reducing *S.* Typhimurium fitness in culture.[110] These studies demonstrate the powerful therapeutic potential of both natural and engineered probiotic gut bacteria.

*Engineered Systems: Neutralizing Antibody Fragments.* Engineered commensal microbes expressing neutralizing antibody fragments have also been pursued as therapeutic or prophylactic agents against bacterial and viral infections. Microbial therapies are attractive low-cost alternatives to traditional treatments to combat gastrointestinal infections, which are more common in resource-poor areas.[111] One such infectious disease is rotavirus induced diarrhea, which kills more than one million children in the developing world each year, and is traditionally treated by vaccination[112,113] or antibody therapy.[114,115] Antibodies targeting rotavirus, including a llama variable heavy chain antibody fragment (VHH) reduced morbidity in rotavirus-induced diarrhea disease models.[116] Notably, VHH antibody fragments are well-suited to treatment of gastrointestinal conditions due to their inherent temperature- and acid-resistant properties, and can be easily expressed in bacteria due to their small size. *Lactobacillus casei*, a transient human commensal bacterium, was engineered to express a surface-tethered antirotavirus VHH as an orally dosed prophylactic.[117] Mice receiving 4 days of $1 \times 10^8$ CFU engineered *L. casei* per day displayed significantly reduced diarrhea frequency compared to mice treated with wild-type *L. casei* when challenged with 20× diarrhea dose 50 (DD50) of rotavirus. Treated mice receiving a low dose more akin to natural exposure (4× DD50) showed an 88% reduction in diarrheal frequency and 99.9% decreased virus after 3 days compared to mice treated with wild-type bacteria. Interestingly, a strain of *L. casei* engineered to secrete, rather than display, antirotavirus VHH did not significantly alter the frequency of diarrhea symptoms or reduce the viral load in treated mice. This contrasts with previously discussed studies, in which surface display was a less successful protein delivery technique.[71] In this case, the advantage conferred by surface display over secretion may be due to increased avidity of bacteria decorated with antibodies compared to secreted monomers. Importantly for application in challenging locations, freeze-dried doses of engineered *L. casei* proved equally effective as fresh preparations at preventing rotavirus infection.

*Bacillus anthracis* spores pose a significant threat as agents of bioterrorism and, though typically associated with aerosolized dispersal, can be spread through the gastrointestinal tract. This route of exposure is most common in developing nations,[118] once again motivating the creation of cost-effective countermeasures and treatments. A high-affinity single-chain variable fragment (scFv) antibody against the *B. anthracis* PA toxin protected against infection in a mouse model[119] and *Lactobacillus casei* was modified to express and attach this scFv to its surface noncovalently.[120] This provides both surface-tethered and free secreted scFv due to the relatively weak interaction of the scFv fusion with the bacterial cell membrane. Two oral doses of $2.5 \times 10^9$ CFU of engineered *L. casei* was sufficient to significantly reduce the effects of *B. anthracis* edema toxin (ET) after oral challenge. Interestingly, strains of *L. casei* secreting either free or covalently surface-tethered toxin-neutralizing scFv had no significant impact on symptoms of


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ET challenge. The authors note that improving scFv stability and altering surface tether length could resolve this issue. While further development is needed to improve efficacy, this treatment, along with the rotavirus therapy discussed previously, underscore the promise of engineered commensal microbes as *in situ* therapeutic antibody delivery vehicles.

**Gut Microbes as Anti-inflammatory Therapeutics.** In addition to engineering microbes to interfere with pathogenic infection, microbial therapeutics have also been developed to mitigate symptoms of inflammatory gastrointestinal diseases. As with anti-infective microbial drugs, increased understanding of how the natural gut microbiota impacts and influences host processes has led to advances in design and engineering of members of the microbiota as drugs.[121] Building from the notion that gut immune development depends on microbiota composition,[36] it has been shown that seeding of germ-free mice with a selected fraction of healthy human fecal samples results in a significant increase of regulatory T cells (FoxP3 + CD4 + $T_{reg}$) compared to unseeded mice.[122] The 17 members of this fraction were identified as members of the class *Clostridia* by 16S rRNA sequencing. These strains produce SCFAs that induce TGF-$\beta$1 expression and expansion of microbiota-specific $T_{reg}$ cells. Oral administration of these *Clostridia* strains decreased disease burden in mouse models of diarrhea and colitis and reduced levels of pro-inflammatory cytokine transcripts, providing evidence that rational manipulation of the gut microbial community could be an effective strategy to mitigate inflammatory symptoms in the gastrointestinal tract.

Other members of the gut microbiota can be leveraged to fight inflammation not by their inherent anti-inflammatory properties, but rather by their utility as protein producers. Interleukin-10 (IL-10) is an anti-inflammatory cytokine normally expressed by $T_H2$ and $T_{reg}$ cells to control inflammatory responses. Systemic administration of recombinant IL-10 has been shown to be a safe and potentially effective treatment of Crohn's disease in humans.[123] Based on this, *L. lactis* strains that secrete biologically active interleukins, including IL-10, were developed.[124−126] Following induced colitis, a daily dosing regimen of $2 \times 10^7$ recombinant *L. lactis* reduced inflammation in the colon, yielding therapeutic effect with 1 U of IL-10 compared to the $1.25 \times 10^4$ U required with systemic administration of recombinant protein. A thymidine-dependent version of this strain with the IL-10 production machinery inserted in the chromosome was deemed safe and biocontainable, and showed an ability to decrease symptoms associated with Crohn's disease in a clinical trial.[127] *L. lactis* has since become a popular and effective vehicle for *in situ* protein production to treat inflammatory bowel disease, with strains developed to produce anti-inflammatory proteins IL-27,[128] heme oxygenase-1,[129] and the *Yersinia* effector LcrV,[130] all for the treatment of colitis. Further, *in situ* production of protease inhibitors,[131,132] superoxide dismutase to reduce levels of inflammatory reactive oxygen species in the intestine,[133] and even TNF-neutralizing single domain antibody fragments[134] have all shown promise as live microbial therapeutics for inflammatory bowel diseases.

**Gut Microbes to Treat Metabolic Disorders and Cancer.** *Phenylketonuria.* Since the late 1990s, the expanded functional potential of commensal bacteria has been leveraged to combat metabolic disorders in the gastrointestinal tract (Figure 4). Phenylketonuria (PKU) is a genetic disorder causing a deficiency in phenylalanine hydroxylase (PAH) and subsequent accumulation of phenylalanine in the blood, which can have severe neurological consequences.[135] Traditional therapy for this condition requires strict diet control and severely limited protein intake from birth to maintain manageable serum phenylalanine levels. Alternative treatment is highly desirable, as the required dietary restrictions are difficult to maintain for the duration of life. *E. coli* has been used to produce large amounts of a replacement enzyme, phenylalanine ammonia lyase (PAL). When dosed orally, PAL mitigates phenylalanine accumulation and associated cognitive defects,[136] but requires daily injection, which limits its utility. In pursuit of a better solution, the probiotic bacterium *Lactobacillus reuteri* was functionalized to produce PAL *in situ* in a mouse model of PKU.[137] The *Anabaena variabilis* gene encoding PAL was codon optimized and transferred into an *L. reuteri* expression plasmid under control of a *Lactobacillus* constitutive promoter. When dosed daily for one or 2 weeks as a lyophilized powder mixed with feed, PAL-expressing *L. reuteri* significantly reduced serum phenylalanine levels compared to untreated control mice. Though no evidence of the engineered probiotic could be found in the stool of treated animals eight months post-treatment, the utilization of erythromycin resistance and constitutive expression of therapeutic protein limited the potential of this therapeutic to progress into the clinic. With these issues in mind, a clinically applicable strain of *E. coli* Nissle, SYNB1618, was generated to treat PKU[138] (Figure 4B). Genes encoding PAL, the high-affinity phenylalanine transporter PheP, and L-amino acid deaminase (LAAD), which metabolizes phenylalanine to phenylpyruvate, were integrated into the Nissle chromosome under control of variably inducible promoters: PAL and PheP under anaerobically inducible control to facilitate expression in the distal gut, and LAAD, which requires oxygen to function, under arabinose control to be expressed in culture and supply phenylalanine-degrading effect immediately after dosing. Key synthetic biology considerations, including therapeutic gene redundancy and placement near essential genes to avoid homologous recombination-mediated gene loss, ensure retention of therapeutic genes during manufacturing and through the duration of treatment. As an additional layer of biocontainment, SYNB1618 contains a deletion of the gene encoding 4-hydroxy-tetrahydropicolinate synthase, which causes the strain to require exogenous diaminopimelate for cell-wall maintenance and biosynthesis, preventing growth of dosed Nissle in the patient and greatly reducing the chances of environmental escape. Cynomolgus monkeys receiving an oral dose of $7 \times 10^{11}$ CFU SYNB1618 and challenged with an oral dose of phenylalanine comparable to the contents of a standard meal showed a 58% reduction in serum phenylalanine compared to mock treated controls. Seven days post-treatment, no evidence of SYNB1618 remained in the stool, indicating successful biocontainment. In a Phase 1 clinical trial, healthy and PKU patients dosed with a solid oral formulation of $7 \times 10^{10}$ CFU SYNB1618 showed increased levels of phenylalanine degradation products TCA and HA, indicating functional SYNB1618. These patients also displayed no serious adverse events, and all patients cleared the engineered Nissle within the expected clearance window. This treatment has moved into Phase 2 clinical trials to assess safety, tolerability, and ability to lower serum PKU levels in PKU patients (synlogictx.com).

*Hyperammonemia.* A similar strategy has been employed to combat hyperammonemia, or increased serum ammonia. This condition is caused by defects in ammonia-metabolizing enzymes or by damage to the liver, and the associated inability to metabolize gut-derived ammonia can result in life-threatening


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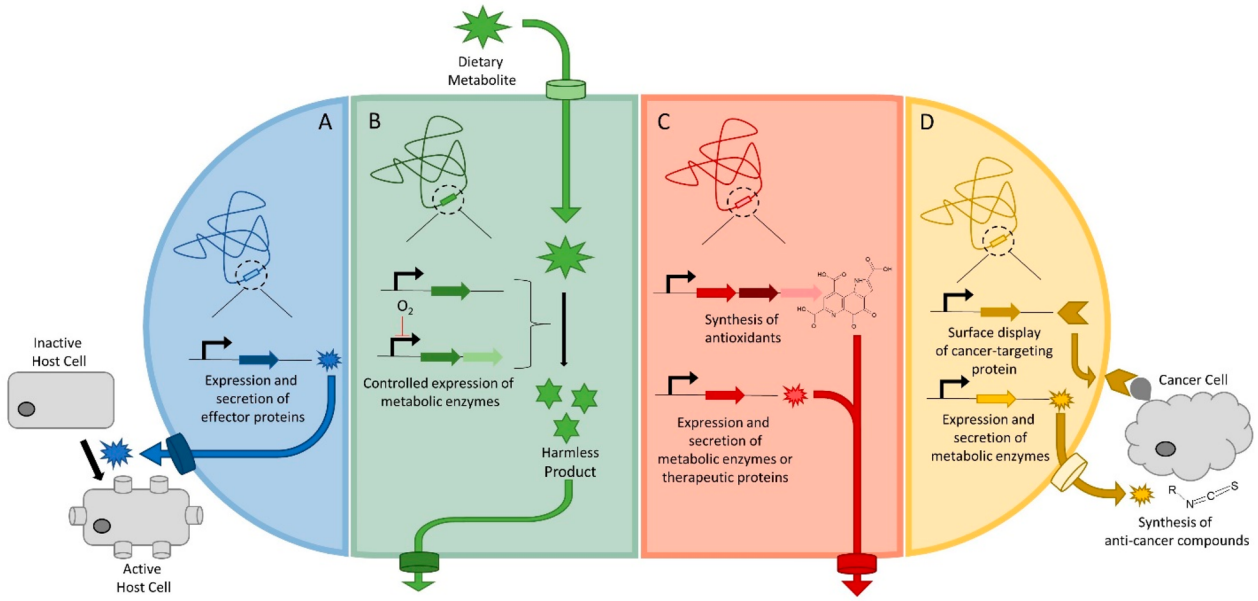


**Figure 4.** Strategies to combat metabolic disease and cancer using engineered commensal microbes. (A) Synthetic expression and secretion of effector proteins can facilitate activation of host cells into functional cells to alleviate disease conditions (*e.g.*, insulin secretion from activated epithelial cells to combat diabetes). (B) Engineered metabolite transporters and enzymes can alleviate disease conditions in which natural metabolism is disrupted by converting the pathogenic metabolite into a harmless derivative (*e.g.*, phenylalanine metabolism in phenylketonuria). (C) Genetic components encoding therapeutic proteins or metabolic pathways to generate small molecules can be used to combat numerous disease conditions including inflammatory bowel disease, obesity, and cancer. (D) Multiple genetic components can work synergistically to target the engineered microbe to a disease site to deliver therapeutic compounds.

complications, including hepatic encephalopathy.[139,140] To combat this condition, a modified strain of *E. coli* Nissle was constructed that can metabolize ammonia from the gut into L-arginine.[141] To maximize ammonia utilization, the ArgR repressor was deleted from the Nissle genome, and a feedback resistant *N*-acetylglutamate synthase enzyme, ArgAY19C, was inserted into the chromosome. This gene was placed under the control of the $P_{fnrS}$ promoter: a native *E. coli* promoter that is active only under anaerobic conditions. To control the biosafety of the modified organism, the thymidylate synthase gene ThyA was deleted, requiring external thymidine supplementation for survival of the strain. Daily $1 \times 10^9$ CFU doses of the strain, SYNB1020, significantly reduced serum ammonia levels and increased survival in both chemically induced hepatic encephalopathy and OTC $spf^{ash}$ mice deficient in liver ammonia processing capacity. In a Phase I clinical trial, daily doses of up to $5 \times 10^{11}$ CFU were well-tolerated, and the administered SYNB1020 cleared within 14 days of the final dose. These advances demonstrate both the scientific and translational promise of engineered commensal bacteria for the treatment of metabolic disorders.

*Diabetes and Obesity.* Diabetes is another gut-associated metabolic disorder with a traditional treatment involving significant dietary restriction. Type I and Type II diabetes are characterized by elevated blood glucose, either due to autoimmune destruction of insulin-producing beta cells, or by cellular resistance to insulin signaling and reduced beta cell function, respectively.[142] Mitigation of symptoms involves close monitoring of blood glucose and supplementation of insulin, in addition to strict dietary and physical exercise regimens. As in the case of PKU, alternative treatments are desirable to ease the disease burden on patients. Glucagon-like peptide 1 (GLP-1) has been shown to convert adult intestinal epithelial cells into insulin-secreting cells *in vitro* and in a mouse model,[143] and bacterially produced GLP-1 has proven effective at inducing insulin secretion in the human epithelial cell line Caco-2.[144] To improve the suitability of this technology for stable, safe therapeutic use, a gene cassette encoding GLP-1 fused to a Lactococcal signal peptide under control of the *Lactobacillus* S-layer protein promoter (SlpA) was integrated into the genome of the probiotic *Lactobacillus gasseri*[145] (Figure 4A). When fed to rats twice daily for 50 days in an induced diabetes rat model, this engineered strain successfully restored insulin and glucose sensing and uptake to levels not significantly different from healthy control rats. This work demonstrates the capacity of engineered microbes to treat metabolic disease by modulating host gut physiology.

Microbes have also been developed to supplement metabolic function in the prevention of obesity. *N*-acyl-phosphatidylethanolamines (NAPEs) are synthesized in response to consumption of food and are metabolized into *N*-acylethanolamides (NAEs), which act as anorexigenic lipids.[146] NAPE synthesis is inhibited under high-fat dietary conditions, potentially contributing to the adverse health effects associated with these diets. Intraperitoneal administration of NAEs has been shown to reduce food consumption and signs of obesity in rats fed high-fat diets,[147] but this is an unlikely route for a human therapeutic, so an alternate way to deliver these therapeutic compounds is desirable. A modified strain of *E. coli* Nissle was therefore constructed, expressing the *Arabidopsis thaliana* *N*-acyltransferase enzyme At1g78690, which catalyzes the final step in NAPE synthesis[148] (Figure 4C). This strain, when administered to mice daily as a $10^{11}$ CFU oral bolus for 7 days, reduced food intake by 15% compared to mock treated mice. Treated mice also exhibited reduced weight gain and fat mass, which was dependent on NAPE synthesis, not reduced food intake. The effects of this treatment persisted over at least 12 weeks, though the engineered Nissle was absent from the feces after 8 weeks,


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and no long-term effect on the gut microbiota composition was observed.

Another approach to managing obesity focuses on the metabolic dysbiosis that results from poor diets. Overconsumption of dietary fructose from high fructose corn syrup, for example, can induce metabolic dysbiosis, including elevated serum glucose and lipid levels, increased oxidative stress, and obesity.[149,150] A modified strain of *E. coli* Nissle, engineered to produce the antioxidant pyrroloquinoline quinone (PQQ) and to express the fructose-metabolizing enzyme fructose dehydrogenase (Fdh), demonstrated efficacy in reducing the adverse effects of a high-fructose diet in a rat model[151] (Figure 4C). Administration of the Nissle strain, bearing a plasmid containing the *Gluconobacter frauteuri* Fdh gene and the *G. suboxydans* PQQ operon under constitutive *tac* promoter expression, resulted in significantly reduced blood glucose and lipids, improved oxidative stress response, reduced markers of liver injury, and maintenance of healthy weight compared to untreated rats when dosed orally once per week at $10^9$ CFU/dose for two months in combination with high dietary fructose. Together, these treatments demonstrate the therapeutic potential of engineered microbes for the treatment of metabolic disorders, with the possibility of long-term efficacy and decreased dosing frequency.

*Cancer.* Infections have long been known to increase cancer risk in affected individuals.[152] More recent studies have found that dysbiosis of the gut microbiota also plays a role in the development of certain cancers.[153−155] Monitoring gut microbiota composition can be a tool in early detection of colorectal cancer,[156] and the presence of certain species increases cancer risk.[157] As with other microbiota-linked conditions, fecal transplants from mice with colorectal cancer increase disease risk in healthy mice while healthy microbiota transplants decrease disease in high-risk mice.[158] The microbiota impacts cancer risk through many pathways, including through IL-6 inflammasome signaling,[159] induction of DNA damage,[160,161] and short-chain fatty acid (SCFA) signaling pathways.[33,162−165] The gut microbiota also influences host response to chemotherapeutics and immunotherapy, either improving efficacy by raising a more potent immune response,[166−168] or adversely affecting metabolism of the drug.[169] Understanding how the microbiota impacts cancer development and treatment is key to the development of microbe-based cancer therapeutics.

Beyond their role in digestive energy salvage, gut commensal microbes play a crucial part in processing a wide range of dietary compounds into health-promoting agents. Most notable among these compounds are plant secondary metabolites that are often ingested as inert precursors and require enzymatic activation and transformation to exert their anti-inflammatory, anticancer, or other beneficial effects. Understanding the metabolic pathways driving this activity is key to harnessing and enhancing the therapeutic potential of gut microbial species. One class of dietary compound that has been studied in great detail is glucosinolates, chemicals found in cruciferous vegetables that require activation by the plant enzyme myrosinase to transform into highly reactive isothiocyanates (ITCs). In plants, ITCs serve a defensive purpose while in humans, evidence suggests that they help prevent gastrointestinal and other cancers.[170] There is no human ortholog to myrosinase, and studies have confirmed that glucosinolate metabolism in humans is mediated by a number of different gut bacteria, but until recently, no bacterial myrosinase had been identified. *E. coli* Nissle was engineered into a tumor-targeting producer of plant myrosinase as a treatment for colorectal carcinoma[171] (Figure 4D). This strain works in two ways to achieve specific antitumor activity. First, constitutive expression and YebF-mediated secretion of the *Armoracia rusticana* myrosinase I1 converts dietary glucosinolate into chemopreventive sulforaphane in the mildly acidic condition of the colon. Second, surface display of the *Streptococcus gallolyticus* Histone-like protein A (HlpA) binds to heparan sulfate proteoglycan on the surface of the tumor cells, facilitating localization of the engineered Nissle at the tumor site. In a mouse model of colitis and colorectal carcinoma, weekly oral administration of this bifunctional engineered microbe reduced the number of colorectal tumors by 75% compared to untreated mice. Importantly, in the absence of dietary glucosinolate, the number of colorectal tumors found in treated mice was not significantly different than in untreated control mice, highlighting the cooperative effect of dietary glucosinolates and engineered microbes designed to enhance their metabolism. Genome-wide transposon insertion studies have since revealed a gene cluster in *Bacteroides thetaiotaomicron, BT2160-BT2156*, that is responsible for the metabolism of glucosinolates into chemopreventive ITCs,[172] opening the door to new opportunities to develop microbe-based therapeutics and diagnostic tools.

In addition to treating colorectal cancer with oral administration of engineered gut microbes, it is also possible to treat systemic cancers using similar therapies administered intravenously. The preferential localization of obligate anaerobes in tumor tissue[173−177] enables systemic administration of nonpathogenic bacteria without inducing bacteremia or sepsis.[178] Specifically, the human gut commensal *Bifidobacterium longum* localizes to tumors following intravenous administration and has been engineered to metabolize orally dosed 5-fluorocytosine (5-FC), a nontoxic antifungal medication, into the chemotherapeutic drug 5-fluorouracil (5-FU) as a therapy for the treatment of solid tumors.[179] 5-FU is commonly used in the treatment of various cancers, but toxicity and poor pharmacokinetics of the drug limits its usefulness.[180] This approach capitalizes on the tumor-localizing ability of *B. longum* to convert 5-FC into 5-FU only at the tumor site, thus minimizing systemic toxicity.[181] When dosed intravenously for 4 days, in combination with daily oral dosing of 5-FC, tumor volume was significantly reduced in mouse models of induced and transplanted tumors. Preclinical efficacy was achieved in this model after 4 days of $5 \times 10^8$ CFU/day of engineered *B. longum*, followed by 72 days of oral 5-FC. This therapy, named APS001F, has since moved into a Phase I clinical trial to assess safety in human patients (clinicaltrials.gov, 2012). More recent work utilized the proven tumor localization and safety of systemically administered *B. longum* to facilitate *in situ* delivery of trastuzumab-derived HER2-targeting single chain variable fragment (scFv) antibodies for the treatment of breast cancer.[182] In an effort to mitigate both the significant cost of trastuzumab therapy and the potentially serious side effects of this drug, *B. longum* was engineered to secrete active trastuzumab-derived[183] scFv at high levels under the control of the *B. longum* rpsP promoter directly within HER2+ tumors. Intravenous administration of $6 \times 10^8$ CFU/dose engineered *B. longum* twice per week for 3 weeks significantly suppressed tumor growth in a mouse model of HER2+ cancer with no noted adverse effects. These studies lend credibility to the prospect of using engineered human commensal microbes as systemic therapies, in addition to their utility as drugs administered to the gastrointestinal tract.


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## MICROBIAL COMMUNITIES AND THERAPEUTICS OUTSIDE THE GUT

While the gut microbiota is the most thoroughly studied human commensal microbial population, and its members have been most widely modified for potential therapeutic use, other microbial communities play an important role in human health and have potential therapeutic applications. Here, we briefly discuss microbial communities outside of the gut, highlighting their function and therapeutic applications, with reference to other reviews that have covered these communities in greater detail.

**The Vaginal Microbiota.** As with most other mucus membranes on the human body, the vagina is home to a diverse community of microbes. 16S rRNA sequencing of healthy women across age and ethnicity revealed that lactic acid-producing bacteria, primarily *Lactobacillus spp.*, are the dominant members of this community.[184,185] Stability of vaginal microbiota composition is influenced by many factors, including time in the menstrual cycle, sexual activity, and the initial community composition.[186,187] Composition stability is enhanced in pregnant women compared to nonpregnant women, though the dominant *Lactobacillus* species may shift.[188] As with other microbiota, dysbiosis of the vaginal microbiota is associated with many health risks.[189] This dysbiosis, termed bacterial vaginosis (BV), is characterized by increased diversity with reduced abundance of the dominant *Lactobacillus* and a corresponding increase in vaginal pH.[190,191] BV is associated with increased risk of sexually transmitted diseases,[192] human papilloma virus,[193] HIV,[194,195] and preterm birth in pregnant women.[196] Vaginal administration of probiotic *Lactobacillus* can disrupt BV and return the microbial community to a healthy state by interfering with biofilm formation.[197,198] Studies using a porcine vaginal mucosa model revealed that lactic acid produced by the vaginal commensal *Lactobacillus crispatus* inhibits the colonization and growth of *Gardnerella vaginalis* and *Neisseria gonorrheae*, species characteristic of BV.[199] *L. crispatus* strain CTV-05 has progressed into Phase 2 clinical trials for use as a probiotic treatment for BV.[200]

The resident *Lactobacilli*, in addition to their inherent ability to revert dysbiosis of the vaginal microbiota, have great potential as engineerable live biopharmaceuticals for delivery of therapeutic proteins. A strain of *Lactobacillus jensenii*, another common vaginal commensal, modified to secrete the HIV entry inhibitor cyanovirin-N (CV-N), decreased the transmission rate of simian immunodeficiency virus (SIV) in macaques.[201] Daily vaginal application of a cream containing this engineered strain for 5 days reduced the rate of SIV infection by 63% over untreated control macaques. *L. jensenii* was further modified to express a single-domain broadly neutralizing anti-HIV antibody.[202] Integrated into the minor capsid gene of the *L. jensenii* genome, the secreted antibody blocks CD4 epitopes exposed upon HIV binding, inhibiting progression of HIV attachment and neutralizing the virus in *in vitro* assays. Development of a live, self-replenishing microbial HIV treatment could increase prevention rates and decrease adverse side effects associated with long-term use of HIV medication. These advances, combined with the progression of vaginal probiotics through clinical trials, are evidence of the promise of live microbial therapeutics of the vaginal microbiota.

**The Oral Microbiota.** The oral microbiota is composed of more than 700 species of bacteria that colonize the teeth and soft tissue of the mouth.[203,204] Significant efforts to understand the "core" or "healthy" oral microbiome have been made to establish a baseline for understanding the function, and the potential therapeutic utility, of the bacteria of the oral cavity.[205] These species survive by formation of protective biofilms, which have been implicated in many oral diseases including dental caries and periodontal disease. Systemic infections can also occur when normally nonpathogenic microbes enter the bloodstream through disrupted tissue.[206] Ordinarily, these oral bacteria prevent colonization by opportunistic pathogens, either passively by obstruction of binding sites or actively *via* effector secretion. This is evident in the case of *Streptococcus salivarius*,[207] a commensal species that secretes salivaricin compounds with antimicrobial activity. When dosed orally in combination with an antimicrobial mouthwash, oral colonization of halitosis-inducing bacterial strains was significantly reduced in human patients.

The commensal species of the oral microbiota also have potential as delivery vehicles for therapeutic proteins. Lactic acid secretion by the pathogenic bacterium *Streptococcus mutans* is involved in the formation of dental caries, but requires colonization by *S. mutans* for pathogenesis.[208] Previous work has proven the efficacy of immunoglobulins targeting adhesins on *S. mutans*, preventing colonization and associated dental caries in humans.[209] The human oral commensal microbe *Lactobacillus zeae* was modified to produce surface-tethered scFv versions of these adhesin-targeting antibodies for prevention of *S. mutans* colonization.[210] The engineered *L. zeae* strain caused rapid agglutination of *S. mutans in vitro* and, when administered to rats by oral swab every other day for 2 weeks, persisted in the oral cavity for the duration of the study (7 days after the final dose), and significantly reduced both the *S. mutans* burden and the formation of dental caries compared to rats treated with wild-type *L. zeae* or *L. zeae* harboring an empty plasmid.

**The Nasal and Nasopharyngeal Microbiota.** The nasal cavity and nasopharynx are also home to diverse communities of microbes, termed the nasal and nasopharyngeal microbiota, respectively. These communities, while physiologically close to the oral cavity, are clearly distinct from the microbes colonizing the mouth. *Corynebacteriaceae* and *Staphylococcaceae* are abundant in the nasal cavity, while *Streptococcaceae* become more common through the nasopharynx and into the oral cavity.[211,212] There is significant variability between the nasal and nasopharyngeal microbiota of individuals, so much so that a "core" set of species has not been identified.[212] A metagenomic study of twin pairs has revealed that the composition of these communities is more closely linked to environmental factors than to genetics.[213] As in the gut, disruption of the nasal and nasopharyngeal microbiota is associated with many diseases, including asthma,[214] chronic rhinosinusitis,[215] and bronchitis.[216,217] These microbes also play an important role in protecting their hosts from respiratory infections, including from the potential pathogen *Staphylococcus aureus*.[218] The common nasal commensal species of the *Corynebacterium* genus, used in small studies as nasal probiotics, interferes and competes with *S. aureus* colonization, evicting established *S. aureus* colonies in up to 70% of treated patients.[219,220] Additionally, *Staphylococcus epidermidis* combats *S. aureus* colonization, both indirectly through bacterial resistance and directly by secretion of the serine protease Esp.[221] This protease acts both prophylactically, preventing formation of new *S. aureus* biofilms, and therapeutically, breaking down existing biofilms. The discoveries of multiple nasal and nasopharyngeal commensal species with inherent antipathogen activity supports the


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potential of these microbes to treat and prevent disease at these body sites.

**The Skin Microbiota.** The skin is the largest and most environmentally exposed organ in the human body, and is colonized by a diverse assortment of microbes.[222,223] Due to the large size and varied environmental conditions of the skin (*i.e.*, moist/dry, warm/cold, *etc.*), the composition of the skin microbiota varies greatly based on location on body site and individual. These microbes can act as an additional barrier to infection and have anti-inflammatory effects,[224] but can also delay wound healing *via* formation of biofilms.[225] Additionally, altered skin microbiota is associated with acne, atopic dermatitis, and psoriasis.[226−229] Cutaneous infection with *Leishmania* also results in dysbiosis of the skin microbiota, which is transmissible from the site of infection.[230] This transmitted dysbiosis contributes to worsened inflammation upon subsequent *Leishmania* infection. Efforts to develop probiotic treatments for body odor, psoriasis, and wound healing, among others, are under way.[225,231] Notably, skin microbiota transplantation improved atopic dermatitis outcomes in a mouse model,[232] reminiscent of the gut microbiota transplant therapy for *C. difficile* infection. The resident commensal microbes of the skin microbiota may be promising candidates for engineering of microbe-based topical or cutaneous therapeutics. As study of the skin microbiota continues, and efforts to understand and utilize the resident commensal strains as probiotics advance, opportunities for engineering of commensal-based therapeutics will grow in parallel.

## REFINEMENT AND CONTROL OF LIVING THERAPEUTICS

**Engineering and Regulation.** *Genetic Engineering.* The recent and historical examples discussed in this review highlight the utility of engineered microbes to serve as therapeutic or prophylactic treatments for a broad range of human diseases. In order to continue building on the achievements in this area, development of species-specific genetic components is needed to more efficiently engineer diverse microbial species for applications as live therapeutics. In this section, we will broadly discuss key design considerations and recent developed synthetic biology tools for the engineering of commensal microbes as drugs. Comprehensive reviews of available genetic components[233] and principles for the design of synthetic gene circuits[234] are available elsewhere. Advances in DNA sequencing and bioinformatics have enabled the identification of candidate genetic components from native microbial genomes[235,236] and DNA assembly techniques such as Gibson Assembly[237] and Golden Gate Assembly[238] have allowed for the rapid construction and screening of libraries of genetic parts. Historically, microbes have been engineered in the context of a controlled laboratory setting using plasmids and antibiotic resistance genes to screen and select variants, or for use in large industrial processes where antibiotic use is common to prevent contamination from the environment.[239] While the use of plasmid-based selection systems remains indispensable for the processes of screening and optimizing engineered microbes, bacteria generated for therapeutic applications require alternative approaches for two main reasons. First, plasmid stability in bacterial culture relies on antibiotic resistance, which is a selection strategy that cannot be efficiently replicated in a therapeutic setting, *e.g.*, within the human gastrointestinal tract. Second, the use of antibiotic resistance cassettes increases the risk for horizontal gene transfer into native microbial communities, which could contribute to the growing antibiotic resistance crisis. These considerations require strategies enabling the integration of genetic cassettes into the genome of the target microbe. A number of studies have identified species-specific integration machinery in several strains of bacteria, including *Lactobacilli spp.*[240,241] and *Bacteroides thetaiotaomicron*.[242] Others have opted for more general approaches, developing phage-based[243] or CRISPR/Cas9[244,245] systems for genomic integration to improve the efficiency and specificity of integration. Advances and limitations of integration of synthetic genes into microbial genomes have recently been discussed elsewhere.[246]

*Metabolic Engineering.* Many engineered microbes designed to serve therapeutic or prophylactic purposes rely on combinations of natural or synthetic genetic components to alter their metabolism to produce a therapeutic protein or encode new metabolic pathways. Microbes engineered for industrial production of commodity chemicals, biofuels, and recombinant drugs are typically designed for maximal production of the desired product or maximum flux through a certain metabolic pathway.[247,248] This may not be ideal in therapeutic settings, as overexpression of non-native genes may be detrimental to normal microbial function or toxic to the host. These workflows also traditionally reach an end point of cell lysis for extraction and purification of biological products.[249,250] There is, therefore, a requirement for a therapeutic-specific set of genetic parts and design criteria that support fine-tuned control of metabolism, gene expression, and therapeutic secretion.

In the case of engineered microbes designed for replacement therapy, *e.g.*, of a missing host enzyme, it may be sufficient to merely set the expression of the gene(s) to a level that is therapeutic to the host, but not detrimental to either the host or the engineered microbe. In cases involving more sophisticated gene circuits, it may be desirable to control the expression of genes in response to a stimulus: a disease marker, dietary metabolite, or coadministered compound. Foundational concepts and techniques for static and dynamic control of synthetic gene expression, independent of or dependent on cellular environment, respectively, have been discussed in detail elsewhere.[251] More recent advances in static gene control of non-lab-strain organisms include the design of synthetic promoter and ribosome binding site (RBS) libraries for the Bacteroides genus that allow for 1 000 000-fold range of gene expression.[252] Recent contributions to the toolset of the Bacteroidetes are motivated by the natural abundance of this phylum[16] which has contributed to its frequent use as a target for engineering. Tunable sets of promoters and RBS have also been developed to control gene expression in *Lactobacillus plantarum*,[253] though these tools are significantly impacted by plasmid copy number. To address the potential issue of decreased gene expression from a single-copy integrated gene compared to a high copy number plasmid, promoters have been engineered with feed-forward loops to stabilize gene expression independent of copy number.[254]

There has also been rapid development of dynamic gene expression control tools in the past decade for both lab strain and non-lab-strain organisms. Strategies to enable dynamic control of synthetic genes in *E. coli* using quorum sensing mechanisms,[255] co-opting stress response to accumulation of toxic intermediates,[256] and using high-sensitivity, low-cross-talk small molecules[257] have been developed. Further, recombinase-based DNA memory switches that record exposure to environmental stimuli and CRISPR interference (CRISPRi)-


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based inducible promoters that offer gene expression control over orders of magnitude have been developed for *Bacteroides thetaiotaomicron*.[258] Noncoding RNA and CRISPRi-based expression control strategies are broadly useful, as they can control expression at the transcriptional, translational, and protein level, and are effective in a diverse set of species.[259] Finally, temperature-inducible switches have been designed to enable targeted control of gene expression by ultrasound or in response to fever.[260] With recent advances in identification and design of genetic parts, computational design and prediction algorithms to automate the gene circuit design process have been developed for *E. coli*[261] and *Bacteroides thetaiotaomicron*,[262] further improving the efficiency and accessibility of microbial engineering.

In many cases, an engineered microbial drug must secrete a therapeutic protein, enzyme, or metabolite to achieve efficacy, which differs from the end point cell lysis that is common to industrial production with microbes.[249,250] This difference necessitates the development of species-specific genetic secretory elements to facilitate extracellular delivery of microbially produced drugs. Motivated by a desire to better understand the function of bacterial pathogens, the identity and mechanism of bacterial secretion systems have been well described.[263,264] With this foundational knowledge of bacterial secretion, computational prediction[265−268] and public databases[269−271] have been developed, broadening the set of available secretory elements for use in microbial engineering. In addition to co-opting native bacterial secretion signals, it is also possible to improve secretion through addition of synthetic secretion signals, enabling high efficiency secretion of proteins in non-native species.[272]

**Biocontainment.** In addition to the identification and development of genetic components to enable precision engineering of microbes for therapeutic purposes, the need to establish biocontainment strategies for these drugs is an ever-present challenge. Preventing the escape of non-natural components of engineered strains, both within the host and in the broader environment, will be a critical step in implementation and approval of this quickly growing class of biotherapeutics. In both cases, systems must be in place to ensure that the engineered microbe remains in the intended niche and does not transmit its synthetic genes to neighboring microbes through horizontal gene transfer (HGT). The rise of antibiotic resistance is one of the most serious concerns regarding HGT,[273] though this concern is mitigated by the use of genomic integration to eliminate plasmid-based antibiotic resistance cassettes as discussed above. Core concepts and considerations for biocontainment of engineered organisms have recently been discussed in detail elsewhere.[274] Here, we highlight some key examples and considerations relevant to the use of engineered microbes as therapeutics.

For engineered microbes that have reached clinical trials, auxotrophy is a commonly used biocontainment strategy. Deletion or replacement of an essential gene, such as thymidylate synthase[121,135] or 4-hydroxy-tetrahydropicolinate synthase,[132] results in the engineered strain losing viability in the absence of an external supplement. Even in these cases, however, it is possible that environmental levels of the auxotrophic marker may be high enough to permit growth of engineered strains. In order to reduce the chance of environmental supplementation, strategies in which the engineered strain requires nonstandard amino acids to function[275] and layered approaches requiring multiple orthogonal supplements[276] have been developed. Similarly, modular "kill switches" have been designed, encoding logic functions that require the presence of a selected set of environmental signals in order to repress the expression of a toxic gene.[277] These strategies significantly reduce the risk of environmental escape but are limited by their requirement for an external supplement, which may be disadvantageous in the context of an engineered microbe designed as a one- or two-dose treatment. Designing inducible kill switches that are natively in the "off" state and turn on in response to a stimulus may circumvent the requirement for continual supplementation while retaining the reduced risk of environmental escape. Temperature-responsive kill switches using the CcdB/CcdA toxin-antitoxin system have been designed to trigger at ambient temperature after fecal elimination,[260,278] which may be useful additions to transient engineered microbe therapies that are expected to quickly pass through the gastrointestinal tract.

Using the end point of cell death as the standard of biocontainment may not always be sufficient, however, as bacterial DNA may persist in the host or the environment even after death of the microbe. To address this potential concern, a programmable CRISPR system has been designed to degrade user-specified DNA sequences in response to a defined transcriptional program.[279] This system, designed and optimized in *E. coli*, can be integrated into the microbial genome, and can be targeted to degrade plasmid or genomic DNA and induce cell death, degrading DNA and reducing cell numbers by over 100-fold. This system has the potential to be flexible, as it could be programmed to respond to a change in disease state, conditionally ending delivery of therapeutic products, or could be linked to environmental cues to facilitate killing in the event of escape into the environment or outside of the target niche.

## CONCLUSIONS

In this review, we have discussed the history, state-of-the-art, and future prospects for the use of native and engineered microbes as mediators of human health, highlighting the roles of community composition, microbial metabolism, and the host. Finally, we have discussed the state of the field of microbial engineering, detailing the advances in community manipulation, synthetic biology tools, and design considerations to ensure the efficacy, control, and safety of microbe-based therapeutics. With multiple such drugs advancing into and along the clinical pipeline, the future of these living therapeutics promises to be impactful and expansive. The increasing ease of genetic manipulation of nondomesticated microbes and an expanding suite of synthetic biology tools will increase the flexibility of living therapeutics and support the rapid application of these technologies to new and emerging disease threats. The renewability, ease of production, stability, and ease of administration make microbe-based drugs attractive alternatives to traditional therapies, while decreased costs for both the producer and consumer should expand the availability of these drugs to a wider population.

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## Notes

The authors declare no competing financial interest.

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