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Review

Discovery and delivery strategies for engineered live biotherapeutic products

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Genetically engineered microbes that secrete therapeutics, sense and respond to external environments, and/or target specific sites in the gut fall under an emergent class of therapeutics, called live biotherapeutic products (LBPs). As live organisms that require symbiotic host interactions, LBPs offer unique therapeutic opportunities, but also face distinct challenges in the gut microenvironment. In this review, we describe recent approaches (often demonstrated using traditional probiotic microorganisms) to discover LBP chassis and genetic parts utilizing omics-based methods and highlight LBP delivery strategies, with a focus on addressing physiological challenges that LBPs encounter after oral administration. Finally, we share our perspective on the opportunity to apply an integrated approach, wherein discovery and delivery strategies are utilized synergistically, towards tailoring and optimizing LBP efficacy.

Highlights

The physiological microenvironment of the gut influences the efficacy of live biotherapeutic products (LBPs) and both discovery and delivery strategies can be used to overcome physiological challenges in the gut.

Multi-omics illuminates colonization mechanisms of nonengineered LBPs to inspire engineering strategies.

Functional genomics generates and tests engineered LBPs in a high-throughput manner to provide improved strains.

Pharmaceutical formulations can be used to control the interactions between LBPs and their physiological microenvironment, creating modular technologies and approaches that can be applied to all LBPs.

Genetic engineering approaches can improve LBP delivery through overcoming physiological challenges, enabling molecular interactions with host surfaces, controlling therapeutic functions in response to local physiological cues.

Tailoring the development of LBPs for human application

LBPs are an emerging therapeutic modality that encompass living microbes (e.g., bacteria, yeast), are not vaccines, and are used to prevent, treat, or cure human diseases [1]. LBPs may include a single microbial strain or a consortium of multiple microbial strains. The functions of LBPs may be conferred through processes innate to the microbe(s) or enabled through genetic engineering. In all cases, LBPs perform specific therapeutic functions, thus distinguishing them from probiotic supplements [2]. One of the first examples of a genetically engineered LBP was a *Lactococcus lactis* engineered to secrete interleukin-10 to locally treat intestinal inflammation through *in vivo* optimization in small animals, introduction of **biocontainment** (see Glossary) strategies, and evaluation in human clinical trials [3–5]. While there are currently no FDA-approved LBPs, many current clinical trials utilize either consortia- or single strain-based LBPs for the treatment of recurrent *Clostridium difficile* infection [6,7] amongst a variety of other indications, including cancer, inflammation, metabolic disorders, and rare diseases. Over 50% of current clinical trials using LBPs for cancer treatment are in combination with immunomodulatory antibodies, exemplifying their importance in immune regulation [8]. This validates the potential for LBPs to target a wide variety of indications and substantiates the need to optimize the discovery and delivery process to rapidly expand LBP translation to the clinic [9].

In this review, we focus on genetically engineered LBPs that are administered via the oral route, due to their rapid emergence in clinical trials and because of their unique potential to be tailored for specific therapeutic functions using omics-based methodologies and formulation strategies. We primarily focus on studies performed in several traditional probiotic **chassis** organisms while highlighting rising LBP chassis candidates in the field. We close by providing a perspective on the challenges and opportunities in the clinical translation of novel LBPs.

LBPs provide a wide range of beneficial functions, such as regulation of the mucosal immune system, *in situ* production of therapeutics, nutrient/toxin metabolism, or even act as living

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diagnostics [10,11]. Unlike other therapeutic modalities (e.g., small molecules, biologics, gene therapies) [12], LBPs are living, growing, and dynamically interacting with their hosts. As such, design of LBPs presents unique challenges such as their required survival within the host, their potential interactions with the host immune system, and their close competition with existing members of the gut microbiome [13]; these challenges stem from the unique physiology of the gut and the need for LBPs to navigate and synergistically interact with the host (Figure 1, Key figure).

A major limitation in the translation of LBPs is the lack of quantitative and high-throughput approaches to enable discovery of LBP chassis or components (e.g., **promoters**, **colonization factors**) that can navigate host physiology and efficiently perform therapeutic functions. Another distinct limitation, is the lack of strategies that enable controlled LBP delivery (e.g., target site localization, controlled transit times) to the gut to provide therapeutically optimal LBP concentrations. In this review, we consider gut physiology (Box 1) to identify major challenges (Figure 1) that limit LBP efficacy. We then highlight how these physiological challenges can be circumvented, overcome, or even exploited via: (i) discovery of LBP chassis or genetic components using quantitative omics methodologies, and (ii) novel delivery strategies, including pharmaceutical formulations or genetic engineering approaches. Finally, we provide our perspective as to how discovery and delivery can be integrated to enable the rational design, development, and implementation of next-generation LBPs.

Overcoming physiological challenges in LBP discovery

In this section, we discuss omics-based strategies (Table 1) for evaluating and engineering the interactions between LBPs and both the indigenous microbiota (e.g., competition for nutrients or **niches**, nutritional **cross-feeding**) and the human host (e.g., binding to gut surfaces, immune recognition, **metabolic crosstalk**) (Figure 2).

Genomics

Genomics is the study of the structure, function, and evolution of genomes within a given organism or community of organisms [14]. Genomics analyses can be used to identify LBP chassis that contain **colonization**-promoting gene functions [15,16] and identify interactions between the LBP and gut **symbionts** [17] (Figure 2B). For instance, *in silico* analysis of the *Bifidobacterium bifidum* genome revealed genes encoding for glycosyl hydrolases and ABC-type transporters that are inherent features of mucus-colonizing bacteria [18]. These genes enable degradation and transportation of mucin-derived host glycans, thus ensuring nutrient availability in the gut for this LBP chassis [19]. To translate these predictions to function (e.g., colonization) in competitive gut environments, *in vivo* studies can validate these *in silico* hypotheses; approaches such as shotgun metagenomics can describe compositional shifts in the microbiota *in vivo* and therefore identify microbe–microbe interactions. For example, oral administration of *Lactobacillus plantarum* shifted the composition of the microbiota towards an increase in *Bacteroides* and a decrease in Firmicutes in a mouse model of inflammatory bowel disease [20]. Further mechanistic understanding of how microbe–microbe interactions challenge LBPs can be revealed through genomics-assisted computational modeling of the microbial community [21] as well as experimental approaches. Pairwise combinations of a 12-member synthetic human gut microbiota were cultured to parameterize the generalized **Lotka-Volterra equations**, revealing how specific species drive community stability [22]. Looking forward, similar mathematical models can be constructed for LBPs, enabling discovery of interspecies interactions and prediction of LBP colonization. Separately, in studies involving human fecal microbiota transplants, metagenomic analyses have enabled the identification of colonization factors that govern the occupation of specific niches in the human gut microbiome [23–25], providing clinically translatable data that can be considered in future engineered LBP design.

Glossary

Adhesin: components or appendages of bacteria that facilitate adhesion or adherence to other cells or to surfaces.
Auxotrophy: the inability of an organism to synthesize a particular compound required for its own growth, requiring an external supply of that compound for survival.
Biocontainment: the prevention of engineered microbes from entering, being metabolically active in, or growing in environments outside of the host.
Biofilm: a protective film primarily composed of polysaccharides, proteins, nucleic acids, and lipids that is secreted by a microbe and enables microbial adherence to surfaces.
Chassis: an organism that contains and supports the genetic components encoding for a desired engineered function.
Colonization: when microbes continuously grow and maintain metabolic activity in/on a host.
Colonization factor: a gene, or set of genes, that enable a microbe to colonize a host.
Cross-feeding: intra- and interspecies exchange of nutrients.
Genetic tractability: the amenability of a microbe for genetic manipulation.
Hydrogel: a water-containing gel composed of a network of crosslinked polymer chains.
Lotka-Volterra equations: a pair of first-order nonlinear differential equations, frequently used to describe the dynamics of biological systems in which multiple species interact.
Metabolic crosstalk: interaction between the host and the microbiota that can determine fate and function of the microbe and the disease state of the host.
Microparticle: a particle between 1 and 1000 μm in size, often composed of biocompatible lipids and/or polymers, which can encapsulate drug molecules or microbes.
Niche: the position of a species within an ecosystem encompassing both the physical and environmental factors required for survival and the interactions with other species.
Pharmacokinetics: the time course of a drug moving through the distinct compartments of the body.
Promoter: a sequence of DNA that controls the expression level of downstream coding regions.

Transcriptomics

Transcriptomics is the study of the structure, function, and evolution of the transcriptome (i.e., the entirety of RNA transcripts produced by the genome) of a given organism or community of organisms under a variety of conditions [26]. Here, we focus on transcriptomics analyses performed on microbes placed under conditions relevant to human gut physiology to uncover gene regulation pathways relevant to overcoming physiological challenges (Figure 2C). Transcriptomic analysis of *Akkermansia muciniphila* demonstrated that several systems involved in bile acid resistance (e.g., ABC transporters, RND transporters, hopanoid synthesis, exopolysaccharide synthesis) were differentially regulated while under a bile acid challenge [27]. Further, a transcriptomic study of *A. muciniphila* cultured in the presence of mucin showed upregulation of fructosidase, β-galactosidase, and hexosaminidase, which convert mucin to various oligosaccharides, which are then converted into monosaccharides to be used in glycolysis [28]. A similar transcriptomic study has also been conducted on a smaller community consisting of *Faecalibacterium prausnitzii*, *Blautia hydrogenotrophica*, and *Roseburia intestinalis*, revealing that each species exhibited different metabolic activities when cultured individually or together and showing that these strains competed for fructose and cross-fed formate [29]. Other transcriptomic analyses of mucin utilization have also been conducted on *Bacteroides thetaiotaomicron*, *Ruminococcus gnavus*, and *Bacteroides fragilis* [30–32] to understand how different chassis adapt and utilize mucin structures in the gut. Collectively, presence and/or activation of certain cellular processes can provide competitive nutritional advantage to LBP chassis over other microbes and these patterns can be identified via transcriptomics.

Transcriptomic analysis can be performed for other physiological stresses such as acid [33], oxygen, and other microbes [34,35]. *Lactobacillus acidophilus* profiling in germ-free mice revealed that carbohydrate, nucleotide, and amino acid metabolism genes, as well as genes encoding for mucus-binding proteins, surface **adhesins**, and surface-layer (S-layer) proteins, are differentially regulated in the gut [36]. These genes exhibited differential spatial expression patterns, indicating that *L. acidophilus* alters gene expression, depending on niche association. Once identified by transcriptomics, these **regulatory networks** can be synthetically optimized to further increase chassis fitness or can be recombinantly introduced in other less-fit LBPs to resist physiological challenges [37,38].

Proteomics

Proteomics is the study of the structure, function, and evolution of the proteome (i.e., the entire set of proteins expressed) by an organism or community of organisms at a given time and under a variety of conditions [39]. Here, we primarily focus on proteomic studies that evaluate the protein expression profiles of single microbial strains in response to physiological challenges relevant to the human gut (Figure 2D). Previously, proteome responses of several LBPs in the presence of bile [40], nutrient availability [41], and oxygen gradients [42] have been captured. A proteomics study conducted on the surface proteins of different *Lactobacillus* strains revealed strain-specific adaptation to physiological challenges such as bile, immune responses, and other microbes [43]. Identifying and characterizing surface proteins with immunomodulatory activities can provide insight into circumventing immune challenges in the gut. Recent proteomic studies conducted on *L. acidophilus* surface proteins identified several S-layer proteins and S-layer-associated proteins with immunomodulatory characteristics [44]. LBP surface protein content (both quantity and type) may drive LBP chassis' long-term adaptation in the gut, allowing colonization to reach 'steady-state'. Proteomics enables high-throughput analysis of the proteins involved in overcoming physiological challenges specific to the gut environment, which can assist in selecting a chassis for LBP design or incorporating engineered elements utilizing these proteins.

Quorum-sensing molecule: a molecule that signals the presence of related microbes nearby; often induces the expression of virulence-related genes.
Regulatory networks: sets of macromolecules (e.g., proteins, RNA) that interact to control the level of expression of various genes in an organism.
Regulatory part: a DNA sequence that influences the rate of transcription of nearby genes.
Symbiont: microbe associated with the host without implication of benefit or harm.
Transformability: capacity for a microbe to be transformed with foreign genetic material.

Key figure

Physiological challenges in the human gut

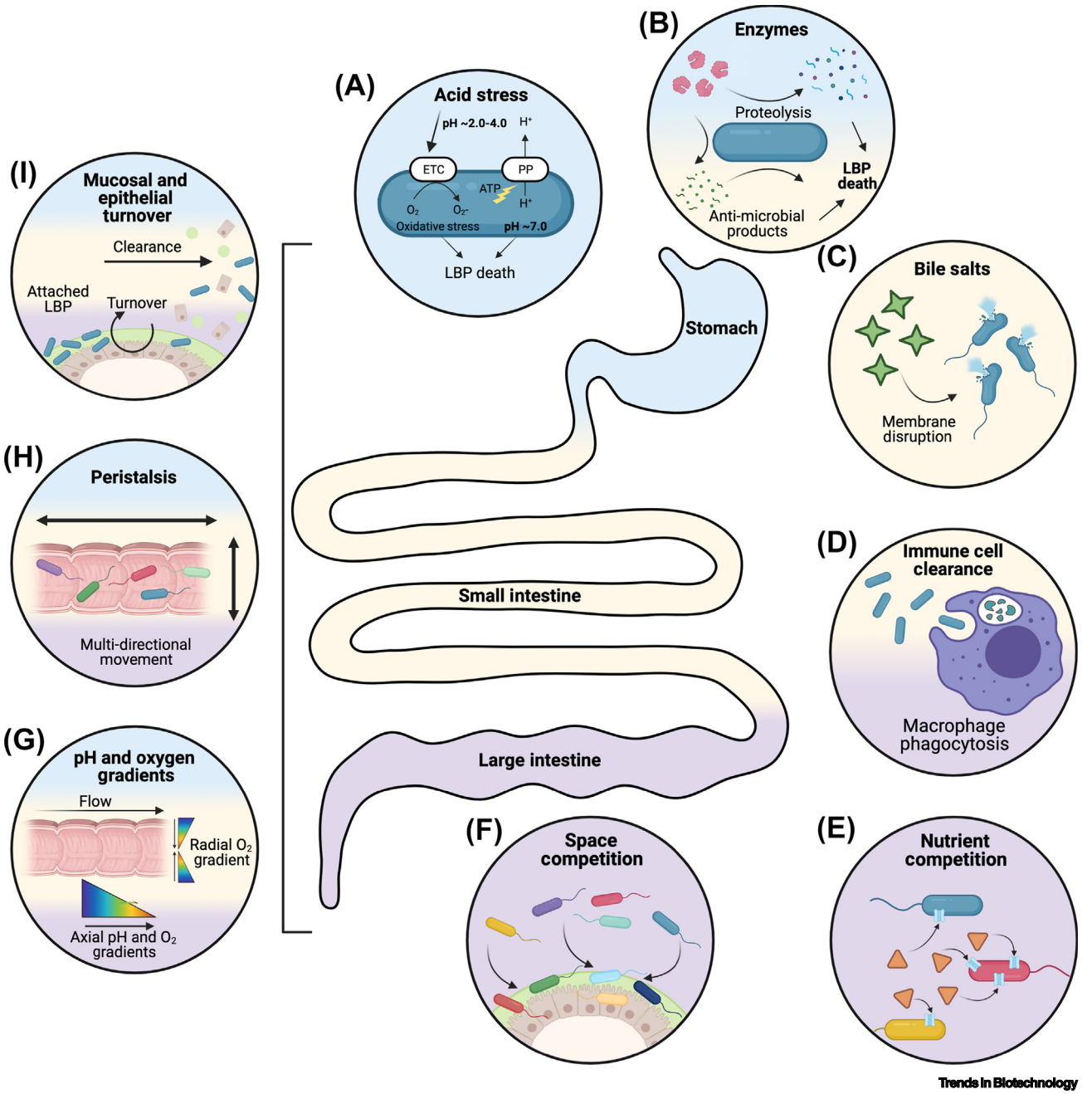

Figure 1. Upon oral administration, live biotherapeutic products (LBPs) will encounter various physiological challenges during passage from the stomach (blue), through the small intestine (yellow), and to the colon/large intestine (purple). There are a variety of chemical challenges secreted in the gut, such as (A) acid (stomach), (B) digestive enzymes (stomach and small intestine), and (C) bile salts (small intestine), which either disrupt essential LBP components (e.g., cell wall) or cause internal stresses that lead

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Box 1. Host–LBP interactions: physiological challenges

The dynamic interactions between LBPs and the host microenvironment govern LBP fate and function. Following oral administration, LBPs encounter various physiological challenges (see Figure 1 in main text) that decrease survival, prevent colonization, and alter therapeutic function of LBPs. In the stomach, acids induce protein denaturation [94] and enzymes cause proteolysis of the LBP [95]. In the small intestine, enzymes persist and bile salts, which solubilize cell wall lipids and proteins, are secreted [96]. As LBPs enter the colon (large intestine), LBP clearance via immune cells (macrophages) occurs [97]. Since the colon is home to the largest number of microbes (~10^{14} CFU) in the body, it is a highly competitive (for both space and nutrients) environment for LBPs [98]. Furthermore, since many LBPs are fermented in optimal *in vitro* conditions prior to administration, processed during formulation, and stored at various temperatures, LBPs must rapidly adapt to new nutritional sources and chemical environments (oxygen or pH differences) while competing with microbiota members that have evolved to thrive in that specific environment. A separate set of challenges such as peristalsis, mucus/epithelium turnover, and both pH and oxygen gradients are dynamic and persist through the stomach to the colon [99]. The physical contractions associated with peristalsis breakdown and transport food through the gut, which leads lead to rapid transit times (2 h in the stomach, 2 h in the small intestine, and 6 h in the colon [99]); this limits LBP interactions with target tissues or sites. Mucus and epithelium turnover are active processes that occur dynamically and at different rates in the stomach, small intestine, and colon; mucus turnover contributes to gut transit of LBPs and influences residence time [100]. Oxygen gradients in the gut (~21% in the stomach with gradual reduction to almost 0% in the colon), can affect LBP performance since LBP metabolism and thus therapeutic function can be influenced by oxygen content [101]. Similarly, gut pH (generally 1.0–2.5 in stomach, 7–7.4 in small intestine, and 6–6.7 in colon [102]), can affect LBP growth and metabolism. Collectively, chemical challenges (e.g., stomach acids, enzymes, bile salts) can lead to the rapid reduction of viable LBPs, physical challenges (e.g., peristalsis, mucus turnover) can limit the ability of viable LBPs to colonize or persist in specific parts of the gut, and competitive challenges (e.g., nutrient competition, space competition) can affect LBP metabolism and their therapeutic functions.

Metabolomics

Metabolomics analyses may be categorized into two distinct approaches: (i) untargeted metabolomics wherein all molecules (e.g., sugars, lipids, fatty acids, phenolic compounds) other than DNA, RNA, or proteins in an organism or community are evaluated; and (ii) targeted metabolomics wherein specific and known molecules are evaluated in a given organism or community under a variety of experimental conditions [45]. Here, we primarily discuss targeted metabolomic approaches because they can be used to distinguish which mechanisms microbes use to overcome specific physiological challenges in the gut (Figure 2E). Metabolomics can be used to reveal the ability of the LBP to tune its metabolic functions to transform host-, microbiota-, and diet-derived and xenobiotic compounds to facilitate survival in the gut [46]. For example, a metabolomic study of 22 *L. plantarum* strains revealed the extent of bile acid deconjugation in a strain- and substrate-specific manner. In addition to mitigating bile stress, deconjugated bile acids restrict proliferation of certain microbial species, including opportunistic pathogens, therefore reducing LBP competition for nutrients and space [47]. Similarly, a metabolomics study on a mucin-degrading *R. gnavus* strain and the resistant-starch-degrader *Ruminococcus bromii* investigated their cross-feeding dynamics in the presence of host-derived (mucin) and diet-derived (resistant starch) sugars, revealing that these strains compete for malto-oligosaccharides [48]. Additionally, the metabolic capacity of the LBP can simultaneously benefit the host and enable LBP energy acquisition in the gut. Metabolomic profiling of *L. acidophilus* and *Lactobacillus gasseri* characterized the metabolic ability and extent of degradation of dietary oxalate, which is a toxic compound involved in kidney disorders, including primary hyperoxaluria [49]. Similarly, metabolomic studies on *B. thetaiotaomicron* revealed that sphingolipid biosynthesis and outer membrane vesicles were essential for maintenance and development of immune system and gut symbiosis [50,51]. Collectively, metabolomic analysis of LBPs reveals mechanisms to overcome physiological challenges at the molecular scale.

to LBP death. (D) Immune cells in the gut (small intestine and colon) can actively sense, interact with, and clear exogenous LBPs. Competition, arising from the existing microbiota (large intestine), can limit LBP ability to access sufficient (E) nutrients, for growth and metabolism, or (F) space, for adherence, growth, and colonization. Physiological challenges can also be ubiquitously encountered through the gut, such as chemical gradients (G) (pH and oxygen) or physical phenomena, including (H) peristalsis and (I) epithelial/mucosal turnover. These physiological aspects, and how they serve as challenges to LBPs upon oral administration, are discussed in more detail in Box 1.

Table 1. Omics strategies for discovering novel LBP design elements

Omics science	Characterizes/ quantifies	Organisms investigated	Application to LBPs	Refs
Genomics	DNA	*Bifidobacterium bifidum*, 36 *Bifidobacterium* spp., *Bacillus coagulans*, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, *Bacteroides uniformis*, *Bacteroides vulgatus*, *Blautia hydrogenotrophica*, *Collinsella aerofaciens*, *Clostridium hiranonis*, *Desulfovibrio piger*, *Eggerthella lenta*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Prevotella copri*	Colonization factors and *in vivo* adaptation mechanisms via comparative genomics	[15,18]
			Intermicrobe interactions via profiling compositional changes in native and synthetic communities	[21,22]
Transcriptomics	RNA	*Saccharomyces cerevisiae*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus rhamnosus GG*, *Lactobacillus crispatus*, *Bacteroides fragilis*, *Akkermansia muciniphila*, *Ruminococcus gnavus*, *B. thetaiotaomicron*	Intermicrobe interactions via differential expression analysis in synthetic communities	[34,35]
			In vivo adaptation mechanisms by differential expression analysis in gut-like conditions	[30,33,103]
			Genetic parts for engineered LBP design via differential expression analysis in response to defined gut cues	[31,37,38]
			Colonization factors via spatially resolved differential expression analysis	[104]
Proteomics	Proteins	*Lactobacillus acidophilus*, *Lactobacillus salivarius*	*In vivo* adaptation mechanisms via differential proteome analysis in gut-like conditions	[40–43,105]
			Genetic parts for engineered LBP design via proteomics of engineered strains	[106,107]
Metabolomics	Metabolites (Excluding DNA, RNA, or protein)	*L. acidophilus*, *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. vulgatus*, *B. hydrogenotrophica*, *C. aerofaciens*, *C. hiranonis*, *D. piger*, *E. lenta*, *E. rectale*, *F. prausnitzii*, *P. copri*, *R. gnavus*, *Ruminococcus bromii*	*In vivo* adaptation mechanisms via metabolomics of gut-adapted strains	[48,49,51]
			Intermicrobe interactions via metabolic profiling of synthetic communities	[22,29]

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Table 1. (continued)

Omics science	Characterizes/ quantifies	Organisms investigated	Application to LBPs	Refs
Functional genomics	Engineered gene function	*Escherichia coli* Nissle, *B. thetaiotaomicron*, *S. cerevisiae*, *Saccharomyces boulardii*, *B. fragilis*	Promoter design	[34,55,59]
			Pathway optimization	[56,57]
			In situ genetic tractability	[53,54]
			In vivo adaptation mechanisms	[62,63]

Functional genomics

Functional genomics uses genetic engineering for high-throughput investigation of gene functions, **regulatory parts**, and **transformability** [52] (Figure 2F). Engineering functions into gut-adapted bacteria can minimize the burden of physiological challenges [53]. For example, a technique called metagenomic alteration of gut microbiome by *in situ* conjugation (MAGIC) demonstrated genetic engineering of gut bacteria through horizontal gene transfer; afterwards, identities of modifiable gut bacteria were elucidated by metagenomic sequencing [54]. Although this approach may enable high-throughput identification of genetically modifiable bacteria in the gut environment, it may not be feasible for therapeutic purposes without additional safeguards in place due to biocontainment concerns. Another approach for ensuring activity of therapeutic functions in the presence of physiological challenges is to characterize genetic regulatory parts under gut-mimicking conditions. For instance, using transcript barcoding, activity levels of 30 promoters in *Escherichia coli* Nissle 1917 (EcN) were characterized *in vitro* and *in vivo* [55]; this approach can enable better predictions of engineered function *in vivo* to aid in regulatory part selection [56]. A similar approach can be applied to LBPs engineered to express complex metabolic pathways. For example, nine promoters were combinatorically assembled *in vivo* in *Saccharomyces boulardii* to achieve rapid optimization of β-carotene and violacein productivities [57]. Broadly, high-throughput characterizations of regulatory parts can identify functions that 'turn on' in response to defined gut microenvironments, which can enable control over LBP function in hosts. Functional genomic screens, either loss-of-function (i.e., transposon insertion sequencing) or gain-of-function (i.e., genomic fragment library), enable identification of colonization factors in LBPs [58]. Considerable efforts have also recently focused on the engineering of *Bacteroides*; broadly, these efforts have focused on developing a genetic toolkit, including ribosome binding sites, promoters, CRIPSR-Cas systems, and recombinases to achieve tunable expression of innate and heterologous genes and demonstrated a range of gene expression, up to 10 000-fold with constitutive promoters and up to 100-fold with inducible promoters [59–61]. High-throughput screening of 2100 *Bacteroides vulgatus* clones containing fragments of the genome of a natural colonizer, *B. fragilis,* revealed a unique class of polysaccharide utilization loci that enabled colonization of *B. vulgatus* by allowing it to better utilize gut mucins [62]. In another study, a metagenomic library generated from healthy infants and their mothers was expressed in EcN. Upon competition of these strains *in vivo*, it was revealed that expression of genes involved in polysaccharide utilization, acid tolerance, and mucin utilization enabled increased colonization of EcN in germ-free and gnotobiotic mice [63]. Altogether, multi-omic analyses provide a powerful platform for identifying synthetic 'parts' that enable an engineered LBP to change its behavior in response to the host's immune system, disease state, microbiota composition, and metabolic signaling pathways.

Overcoming physiological challenges in LBP delivery

Here, we discuss pharmaceutical formulations and genetic engineering approaches (Figure 3) that can modulate LBP interactions with physiological surfaces, overcome physiological challenges, and address biocontainment issues as they relate to delivery of LBPs.

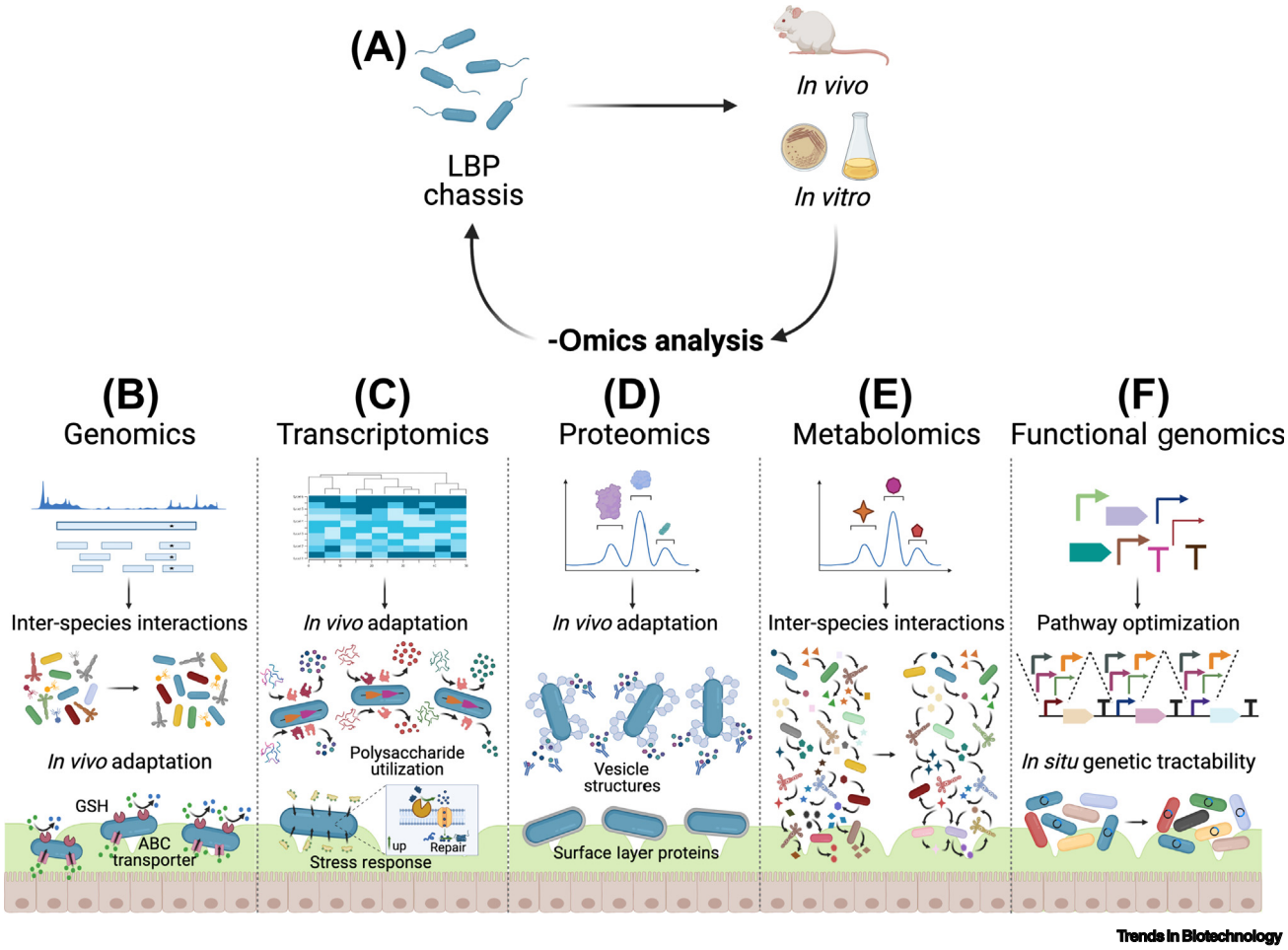

Figure 2. Discovery approaches to overcome physiological challenges and control microenvironment interactions. (A) Discovery of live biotherapeutic products (LBPs) can be guided by investigating chassis in *in vitro* and *in vivo* settings, using a variety of 'omics' pipelines. (B) Genomics measures DNA composition at both the organism and community levels and can be used to determine the presence and abundance of LBPs in various locations in the gut and under various microenvironment conditions. These insights identify factors that drive LBP adaptation to the gut and control interactions with other microbiota members. (C) Transcriptomics can identify genes that are differentially regulated in response to physiological challenges in the gut. These differentially regulated genes illuminate *in vivo* adaptation mechanisms of LBPs. (D) Proteomics can identify proteins in LBPs (as well as their subcellular localization) that facilitate *in vivo* adaptation. Since proteins on LBP surfaces mediate extracellular interactions, proteomics can provide insight into how LBPs interact and communicate with the dynamic gut microenvironment. (E) Metabolomics can identify the metabolic activity of LBPs in a multispecies community through identification of metabolites and their fluxes, which in turn illuminates how an LBP chassis adapts to gut microenvironments. (F) Functional genomics can be used to achieve pathway optimization and high-throughput strain screening for engineered LBP applications. Pathway optimization enables high activity of the engineered function even under the burden of physiological challenges. High-throughput strain screening can identify candidate chassis that are already gut colonizers and that can receive genetic payloads *in situ* through horizontal gene transfer.

Formulation strategies

Pharmaceutical formulations are used to control interactions between a therapeutic and its physiological environment [12]. For LBPs, pharmaceutical formulations can be used to mitigate chemical challenges, interface with physiological tissues, and target specific areas that provide competitive or therapeutic advantages. In recent work, coating of probiotics via self-assembly of biocompatible lipids in a 15-minute vortex step (Figure 3A) enhanced probiotic protection against enzymes, low/high pH, antibiotics, and ethanol. The lipid coating increased LBP residence time in mice and improved therapeutic efficacy in two models of murine colitis [64]. This coating approach has also been used with biocompatible mucoadhesive polymers to protect and improve *in vivo* survival of coated microbes [65]. Some LBP formulations are inspired by

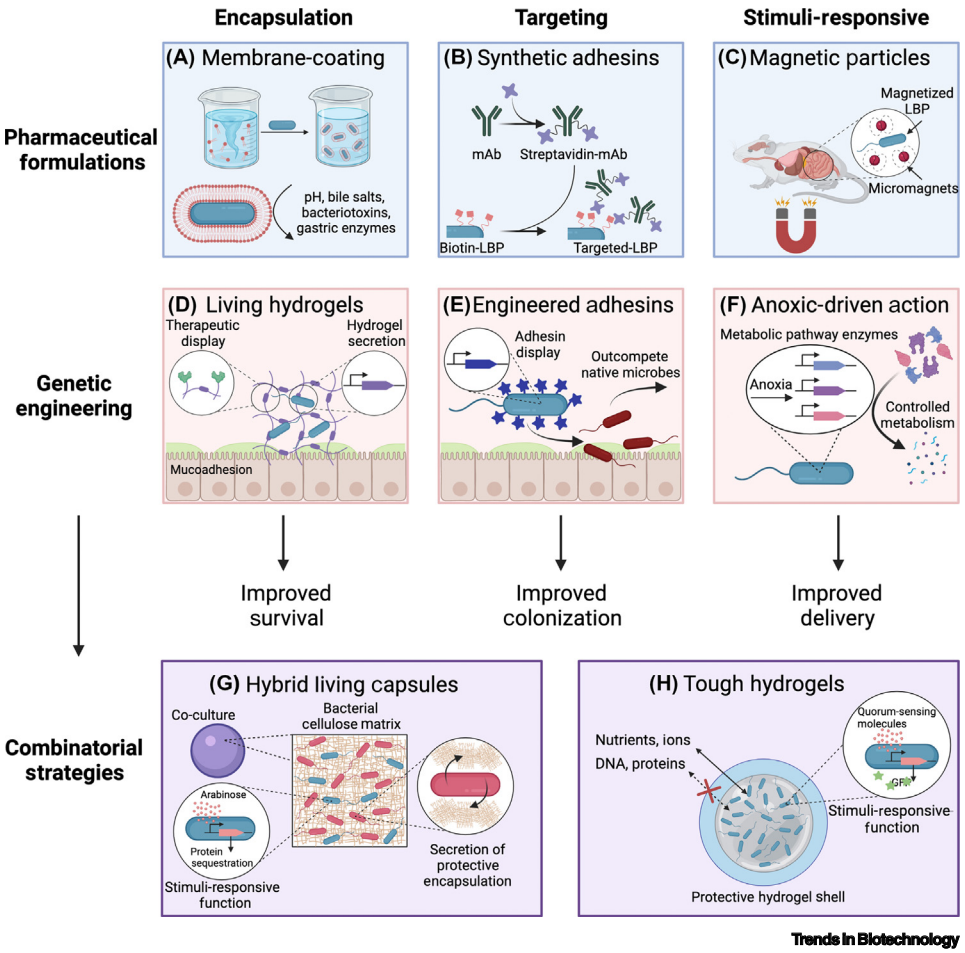

Figure 3. Formulation and genetic engineering strategies to improve live biotherapeutic product (LBP) delivery. Pharmaceutical formulations and genetic engineering strategies can be leveraged to both overcome physiological challenges and utilize physiological microenvironments to improve LBP delivery and LBP function. Examples of pharmaceutical formulation approaches include (A) encapsulation via phospholipid bi-layer membrane LBP coatings, (B) targeting via synthetic adhesin-surface modification of the LBP, and (C) endowing stimuli-responsive functions by using external magnets to direct the movement of magnetized LBPs. Examples of genetic engineering approaches include (D) engineered LBPs that secrete an encapsulating protective and mucoadhesive hydrogel with conjugated therapeutic modalities, (E) LBPs engineered to express adhesins with high binding affinities on the microbial surface for targeting, and (F) the expression of several enzymes for controlled metabolism in response to anoxic conditions for stimuli-responsive therapeutic function. Recently, pharmaceutical formulations and genetic engineering strategies have been combined to (G) encapsulate genetically engineered LBPs in a hydrogel bead, which allows diffusion of nutrients into the bead to maintain the engineered function while achieving biocontainment, and (H) enable coculture of a bacterial cellulose-secreting probiotic organism with engineered LBPs to enable protective encapsulation of the LBP while maintaining its engineered function.

the innate abilities and functions of gut symbionts. Natural **biofilm** formation was induced in *Bacillus subtilis* as a self-encapsulation approach. The biofilm-coated LBP had improved resistance against low pH and gut enzymes *in vitro* and enhanced LBP mucoadhesion *in vivo*, leading to 17-fold greater intestinal colonization [66]. Other biofilm-inspired formulations have also been described; alginate-based **microparticles** were used to simultaneously encapsulate probiotic microbes, enable secretion of small molecule therapeutics and probiotic by-products, and limit diffusion of chemical challenges into the microparticle [67]. Inspired by adhesins, a surface modification approach was used to decorate the exterior of EcN with anti-MUC2 antibodies (Figure 3B). This involved the chemical conjugation of biotin to primary amines on the microbe

surface and subsequent introduction of streptavidin conjugated antibodies. This approach enabled molecular targeting to mucus and accelerated EcN colonization [68]. Stimuli-responsive materials can also be used to direct LBP function. Recently, externally applied magnetic fields were used to manipulate the gut transit of EcN that was orally coadministered with micromagnets (Figure 3C) [69]. Importantly, this approach facilitated targeted microbe localization and enabled stable colonization in mice, without the use of antibiotics. The encapsulation approaches, bio-inspired strategies, and stimuli-responsive functionalities described here, and in other recent work [70,71], could improve delivery of LBPs by increasing survival following oral administration, prolonging residence time, accelerating colonization speed, or specifying the location of colonization.

Genetic engineering strategies

Genetic engineering strategies can be applied towards improving delivery by overcoming physiological challenges, facilitating site-specific targeting, enabling increased survival and persistence in the gut, and controlling drug release. EcN was genetically engineered to form a living **hydrogel** through secretion of curli fibers that encapsulate and protect the LBP *in situ*, promote mucoadhesion, and mimic biofilm formation, thereby mitigating LBP clearance due to mucosal turnover, peristalsis, space competition, and acids/enzymes [72]. This platform was also developed to display therapeutic trefoil factors conjugated to the curli fibers to promote mucosal healing (Figure 3D) [73]. Similar to the surface-modified adhesins described earlier, LBPs can be genetically engineered to display ligands on their surface to enable specific molecular interactions within the host; recent work described the genetic engineering of *L. lactis* and EcN to express a variety of mucus-binding proteins on the microbial surface [74]. Being naturally resistant to stomach acids and gastric enzymes, spore-forming bacteria (e.g., Clostridia, Firmicutes) are also being studied as potential chassis [75] or for identifying proteins involved in the process of colonizing the late gastrointestinal tract [76]. Further, lactic acid bacteria were engineered to surface-express a fragment of the *Clostridioides difficile* adhesin, SlpA (Figure 3E); this enabled the LBP to outcompete and prevent colonization of *C. difficile* in hamster and piglet *in vivo* models of infection [77].

LBPs can also be genetically engineered to sense and respond to the dynamic environment of the gut. For example, recent work described a genetically engineered lactic acid bacteria that releases both antimicrobial and antibiofilm proteins in response to the *Pseudomonas aeruginosa*-specific **quorum-sensing molecule**, 3-oxo-C12-homoserine lactone [78]. Recently, the dynamic oxygen gradients in the gut were leveraged to initiate therapeutic function by engineering EcN to express phenylalanine-degrading enzymes in response to anoxic environments for the management of phenylketonuria (Figure 3F) [9]. Additionally, the LBP was genetically engineered to be auxotrophic to enable biocontainment. **Auxotrophy**, or the inability to produce an essential metabolite, is a key element often utilized in genetically engineered microbes for human application to enable biocontainment without the need for plasmid-based, antibiotic resistance markers. In other work, two genetically engineered functions, molecular targeting, and stimuli-responsive drug secretion, were combined in a single LBP. Here, EcN was genetically engineered to: (i) selectively bind to the colorectal surface antigen, heparan sulfate proteoglycan; and (ii) secrete the enzyme myrosinase, which converted dietary glucosinolate into the chemotherapeutic sulforaphane. This approach resulted in a sevenfold reduction in tumor occurrence in a murine model of colorectal cancer [79]. Collectively, these and other recent examples [59,80] highlight the potential of using genetic engineering approaches to control LBP interactions with the host on a molecular scale, or secrete therapeutics in response to external cues.

Combination approaches: pharmaceutical formulations to deliver genetically engineered LBPs

Synergistic strategies that combine both pharmaceutical formulations and genetic engineering strategies to improve LBP delivery have begun to emerge. Recent work utilized the material

properties of bacterial cellulose (e.g., biodegradability, barrier properties), secreted by *Gluconacetobacter hansenii*, and the **genetic tractability** of EcN to generate hybrid living capsules that combined both strains in a single delivery system. EcN, genetically engineered to perform a variety of sense-and-respond functions (e.g., biomolecule sequestration, enzymatic catalysis) was cocultured with *G. hansenii*, which naturally produced a cellulose-based capsule that enabled the encapsulation and protection of the genetically engineered EcN (Figure 3G). Importantly, EcN maintained its genetically engineered functions while encapsulated within the *G. hansenii* bacterial cellulose-based capsule [81]. Further, genetically engineered *E. coli* was encapsulated into crosslinked sodium-alginate beads containing polyacrylamide on the terminal exterior; this created a 'tough' hydrogel-like shell on the exterior of the bead (Figure 3H). This formulation approach protected the encapsulated microbes from external challenges (e.g., antibiotic, low pH) while ensuring: (i) biocontainment of the genetically modified LBP via prevention of release/escape of the encapsulated LBP, and (ii) maintenance of genetically engineered functions (e.g., response to external chemical stimuli, secretion of signaling molecules, sensing of heavy-metal contaminants) [82]. This example, which utilizes biomaterial encapsulation of genetically engineered microbes, points towards possible next-generation LBP delivery systems wherein biomaterial-mediated biocontainment can be achieved and genetically engineered functions can be maintained. Biocontainment is a critical component in designing genetically engineered LBPs with several other approaches, including nutritional auxotrophy, kill switches, transcriptional regulation pathways, and devices to remove genetically engineered constructs, which have been extensively reviewed elsewhere [10,83]. Further, although stable gut colonization may be desirable in some cases, deriving a therapeutic effect from an engineered LBP is not necessarily dependent on colonization and several genetically engineered LBPs studied in clinical trials were intentionally designed not to colonize the gut for the purpose of improving LBP biocontainment [3,84]. We envision that both genetically engineered approaches and formulation approaches will continue to be developed and potentially combined in single LBP systems, ideally leading to the establishment of a biocontainment toolbox that can be used for a variety of specific strains, applications, and end-functions.

Concluding remarks and future perspectives

The challenges encountered by LBPs upon oral administration and during gut transit are multifactorial (Figure 1) and can affect LBP functions (e.g., survival, site-specific targeting, *in situ* drug production, transit time, therapeutic action). These complexities present challenges to both LBP design and delivery, as no single set of criteria is sufficient to design or deliver LBPs that are appropriate for all use cases. However, the myriad of distinct biomolecular environments in the gut are advantageous, as they enable LBPs to be tailored to reside or perform functions at specific sites.

Multi-omics analyses play a major role in discovery of novel microbial chassis, colonization factors, and regulatory parts that enable LBPs to overcome gut physiological challenges (Figure 2 and Table 1). Multi-omics-based LBP discovery has primarily been accomplished in two ways. First, analyses are performed on wild type, nonengineered symbionts, or probiotic strains. This process illuminates the natural mechanisms microbes use to overcome physiological challenges. Second, and more recently, candidate gene edits are delivered at high throughput to an LBP chassis and gene edits with beneficial effects are recovered by applying omics-based analyses and innovative selection conditions. It is expected that the recent application of machine learning techniques towards analysis of microbiome data [85] will extend to LBP design. Furthermore, advances in single-cell [86] and spatially resolved [87] omics analyses can provide greater insights into the heterogeneity of microbial behaviors *in vivo*. Additionally, while most LBP discovery efforts to date have focused on bacteria, other kingdoms of life (e.g., fungi, viruses, and archaea) may provide other therapeutic opportunities.

Outstanding questions

How can discovery approaches and delivery strategies be synergistically combined to facilitate rational development of LBPs?

How can genome-scale, ecological, and physiologically based pharmacokinetic modeling be integrated to achieve predictive design of LBPs?

What are the optimum *in vitro* conditions that can predict and represent *in vivo* activity of engineered functions using 'omics' analysis?

How can predictive models and 'omics' data be combined to minimize the metabolic burden created by the engineered function?

How can functional genomics selections be applied to select for LBPs that improve host health?

How can multi-omics approaches be used for optimization of LBP formulations?

How can host and microbiota response to delivery formulations be quantified?

For LBPs that integrate discovery approaches and delivery strategies, how can the most important and efficacy-driving LBP features be identified?

How can pharmaceutical formulations be integrated into existing manufacturing processes for LBPs?

As LBPs have been discovered, developed, and evaluated in humans, two distinct strategies to improve their delivery have emerged. The use of pharmaceutical formulations and genetic engineering strategies (Figure 3) are actively being developed and employed to control LBP location (target site), duration (residence time), and concentration (dose) to improve safety and efficacy. Pharmaceutical formulation strategies (e.g., encapsulation, target-functionalization, stimuli-responsive control) can potentially be applied to all LBPs since they rely on using physical and chemical modifications to the chassis. Moreover, formulation approaches are modular and can be modified for specific LBPs (e.g., material–microbe compatibility), delivery functions (e.g., release), or host tissues (e.g., disease sites). However, genetic engineering approaches can be uniquely applied to LBPs since, unlike other therapeutics, LBPs are living and actively sense, respond, and perform therapeutic functions dynamically within the host. Together, the use of pharmaceutical formulations or genetic engineering strategies offer advantages that can

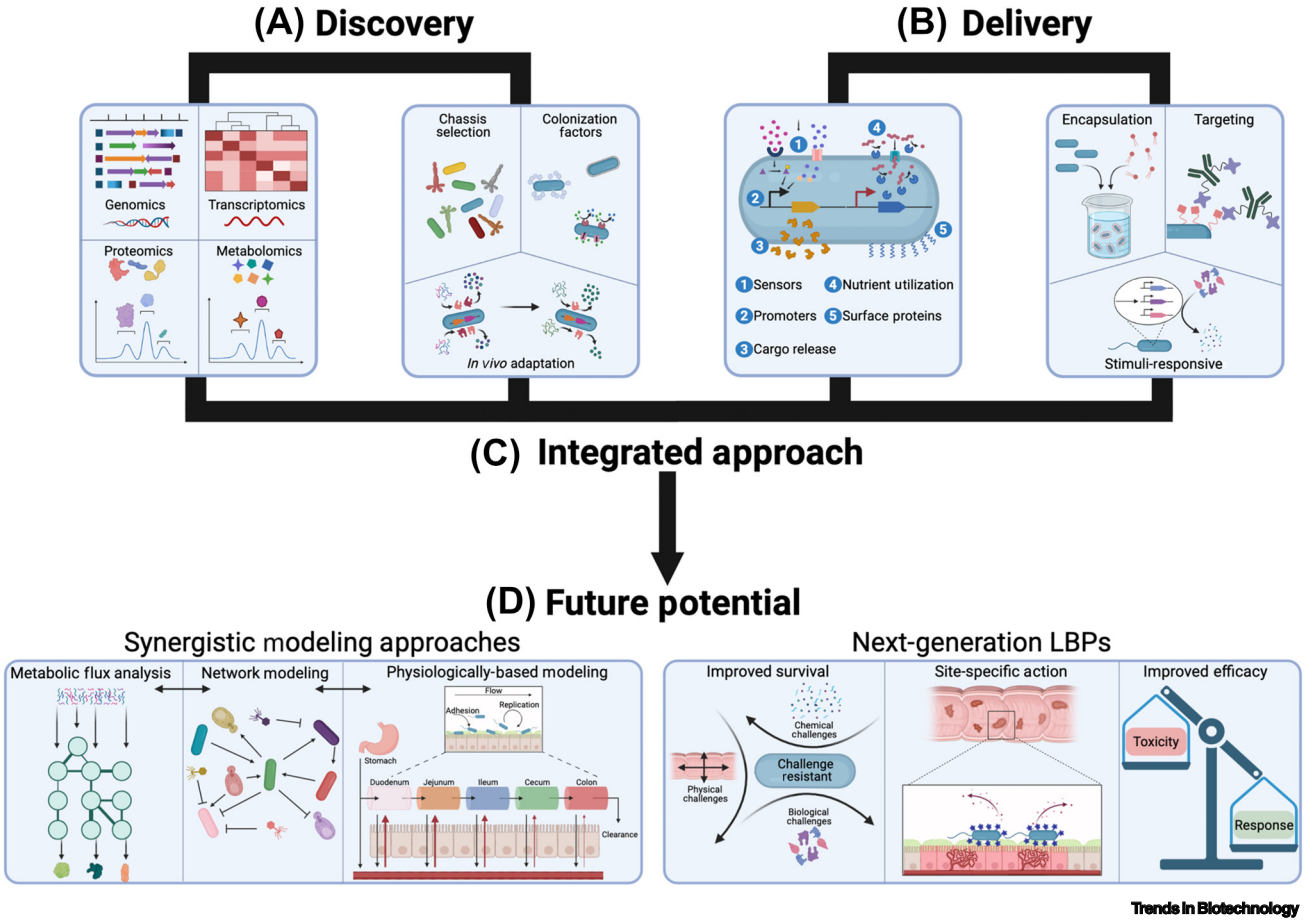

Figure 4. Integrating discovery and delivery for future live biotherapeutic product (LBP) development. (A) Recent trends in the discovery of LBPs utilize multi-omics (e.g., genomics, transcriptomics, proteomics, metabolomics) strategies to uncover potential new microbial chassis, colonization factors, and adaptation mechanisms to overcome the physiological challenges of the gut. (B) Recent trends in the delivery of LBPs utilize both pharmaceutical formulation and genetic engineering strategies to encapsulate, target, and/or generate stimuli-responsive functions to overcome physiological challenges. (C) The current strategies can be integrated wherein discovery and delivery occur simultaneously for synergistic LBP design (e.g., chassis selection, genetic engineering, formulation approaches). (D) Synergistic LBP discovery and delivery has the potential to inform more sophisticated modeling approaches wherein micro-scale approaches (e.g., metabolic flux analysis, network modeling) are combined with macro-scale approaches (e.g., physiologically based pharmacokinetic modeling) to enable better prediction for LBP efficacy. Synergistic LBP discovery and delivery also has the potential to pave the way for next-generation LBPs that have both improved survival and site-specific action through genetic engineering and pharmaceutical formulations approaches, ultimately leading to more efficacious LBPs, with increased therapeutic response and decreased off-target toxicities.

synergistically cooperate to provide unmatched control over LBP delivery and function; indeed, recent examples (Figure 3C) highlight the potential of combining these approaches to address unmet needs in LBP delivery.

Distinct challenges in the clinical translation of LBPs from academic laboratories to generally available therapies include the translation from small animal models to humans and the ability for model systems to predict *in vivo* utility. Each type of model system (e.g., *in vitro*, *in vivo*, *in silico*) has both unique benefits and limitations as it relates to generating multi-omics data. *In vitro* culture and adhesion models allow for identification of specific parameters that may influence colonization and/or survivability in the gut, such as metabolite consumption or microbial surface-binding proteins [74,88–90]. By contrast, *in vivo* models enable the generation of physiologically relevant multi-omics data; however, the identification of specific parameters influencing LBP performance can be more challenging. *In silico* models allow for the rapid generation of diverse sets of data, however, can be limited by assumptions in the computational model [91,92]. Further, the colonization profiles observed in mice or other animal models do not always reflect colonization profiles observed in humans, necessitating translational studies and predictive modeling to scale to humans.

Traditionally, LBP discovery (Figure 4A) and delivery (Figure 4B) have occurred sequentially, with delivery considerations arising after the discovery process. However, we posit that these operations can synergize with each other if performed simultaneously (Figure 4C) (see Outstanding questions). For example, multi-omics analyses described earlier can be applied to LBPs residing in the delivery vehicle to describe their effects on microbial physiology and design LBPs that are highly compatible with delivery formulations. Also, the impact of LBP formulations on the gut microbiota and host can be queried, enabling researchers to select LBPs that minimize detrimental effects. Moving forward, more sophisticated quantitative approaches are needed to accelerate the process of designing and formulating engineered LBPs for therapeutic application; the utilization of predictive modeling may enable this (Figure 4D). Specifically, modeling approaches that integrate gut physiology (commonly used in **pharmacokinetic** modeling) with microbial metabolism and growth (commonly used by microbial ecologists) will enable rapid evaluation of different engineering and delivery strategies *in silico* before experimental testing [93]. By performing LBP discovery with delivery strategies in mind, and vice versa, we expect that engineered LBPs will be able to treat diseases with much tighter therapeutic windows and with more strict requirements for site specificity.

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Declaration of interests

No interests are declared.

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