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Drugging the microbiome and bacterial live biotherapeutic consortium production

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Research leading to characterization, quantification, and functional attribution of the microbes throughout the human body has led to many drug-development programs. These programs aim to manipulate a patient's microbiome through the addition of new strains or functions, the subtraction of deleterious microbes, or the rebalancing of the existing population through various drug modalities. Here, we present a general overview of those modalities with a specific focus on bacterial live biotherapeutic products (LBPs). The bacterial LBP modality has unique concerns to ensure product quality, thus, topics related to manufacturing, quality control, and regulation are addressed.

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Introduction

Use of human-commensal bacteria as a drug precedes the modern discovery of bacteria, dating to medicinal concepts more than 1000 years ago. Early in the 20th century, immunologist Ilya Metchnikoff posited that colonic florae influence mental capacity, establishing the gut–brain-axis hypothesis. In the 1950s, Eiseman utilized fecal microbial transplant (FMT) administered by retention enema to treat antibiotic-associated diarrhea [1]. Intense anaerobic microbiology development in the 1960s through 1980s yielded discovery of new gastrointestinal (GI) microbes, development of GI simulators, and understanding of colonization resistance to pathogens. More recently, the Human Microbiome Project [2] and the European MetaHit Project helped shape an understanding of human health conditions driven by imbalances in microbial diversity or relative abundances (collectively 'dysbiosis'). Parameterization of GI ecosystems launched current investments into 'drugging the microbiome' via myriad modalities targeting indications ranging from bacterial gut infections (e.g. *Clostridioides difficile*) to more distant interactions between the gut and brain, skin, lung, heart, and metabolism [3•]. This article reviews microbiome therapeutic modalities with a particular focus on bacterial live biotherapeutic products (LBPs) and their manufacture.

Modalities for microbiome modulation

Classic pharmaceutical development typically values disruptive technologies (e.g. stem cells) first as tools and targets [4], and later as therapies only when sufficiently derisked. This pattern is emerging for the microbiome. Drugs can affect the GI microbiome and vice versa [5–8]. Anti-infectives have been used to treat microbial disease for generations, however, current knowledge recognizes that broad-spectrum antibiotics can lead to alteration of the microbiome that increases susceptibility to pathogens [4,9], including colonization by drug-resistant (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp) 'ESKAPE' pathogens [10] or by promoting inflammatory diseases [11]. Narrow-spectrum antibiotics may spare the microbiome, targeting specific pathogens [12], but feasibility is challenged in development and clinical deployment.

Antibiotics may modulate microbiome constituents [13], but are inherently subtractive and therefore cannot directly provide absent microbes and their associated unique functions. To provide multiple novel beneficial activities, and to displace deleterious microbes and their activities, modification of the microbiome via introduction of new bacteria is a rational drug- development approach. Probiotics contain dietary microbes that are typically low abundance and transient in the GI tract versus true commensal organisms [14]. In clinical studies, probiotics hinder post-antibiotic microbiome restoration [15•] and can have adverse consequences in sick patients [16]. Regulated as over-the-counter nutritional supplements and not as pharmaceuticals, probiotics can have inadequate safety and quality oversight [17,18]. Additional modalities beyond anti-infectives and

probiotics are likely required to produce meaningful patient benefit.

The LBP portfolio contains multiple approaches ranging from FMT to fermented organisms. Several FMT products are in development. Despite perceptions that these crude preparations are effective [19], they contain and transmit many uncharacterized elements beyond bacteria [20–27] as recognized by U.S. Food and Drug Administration (FDA) safety alerts on the observed transmission of toxigenic *E. coli* [28]. Many FMT products have been explored in uncontrolled production and clinical trial settings with attendant limitations. Where rigorous controlled trials have been performed, efficacy with FMT is ambiguous [29••].

Fermented commensal and recombinant organisms are under development as LBPs [30], with compositions ranging from single to multiple organisms. For specific, single-activity targets such as host-enzyme deficiencies, single-organism LBPs may be effective [31]. For complex diseases such as inflammatory bowel disease, the heterogeneity of GI species found in patients opposes the single 'silver bullet' microbe hypothesis: in large datasets, no single species was found common across all subjects [2]. Certain commensal or recombinant microbes could provide specific activities for rare diseases [32], however, one challenge to these products is sustained delivery of the target activity in amounts required to achieve clinical effect. Failure modes may include insufficient specific activity, or lack of compatibility with the patients' existing microbiomes. Consortia of several microbes may be designed as a drug to provide multiple functions to address complex diseases, to ensure broad compatibility, to provide diverse redundancy, or to serve as an ecological scaffold sufficient to disrupt a dysbiotic microbiome. Microbial consortia products adopting these various strategies are in mid- to late-stage clinical development, with significant proof-of-concept for treating infectious diseases [33•,34].

Microbes as regulated LBPs have a decades-long precedent as live attenuated bacterial vaccines protecting against diseases caused by *Salmonella typhi*, *Vibrio cholerae*, and *Mycobacterium tuberculosis* (Bacille Calmette-Guérin). Accordingly, development of single-organism LBPs is well-established, except for the microbial phenotypes in the human GI microbiome. The remainder of this review will focus on these organisms and production of associated consortia products.

Donor-derived versus designed consortia live biotherapeutic products

Donor-derived LBPs rely upon donor materials (e.g. stool samples) as the source for the formulated microbes. Designed LBPs rely upon cultivated microbes as the active ingredient. The current candidates in late-phase clinical development rely upon the donor-derived model. This method of drug manufacture provides for a ready-made mixture of human-commensal microbes that require no upstream processing. As with blood- and tissue-derived products, a multitier control strategy is minimally necessary to ensure patient safety. Donor health screening, donation management, and final product testing must be components of the control strategy, but may be insufficient, as demonstrated by U.S. Food and Drug Administration (FDA) alerts related to the transmission of infectious diseases associated with minimally purified FMT [28]. A separate class of donor-derived products incorporates further purification methods targeting latent pathogen inactivation as an additional control for product safety [35].

In one strategy, designed LBP consortia provide a reductionist set of bacteria intending to shift a microbiome composition [36]. Multiple design principles are utilized to define component bacteria during discovery and development of these products. Understanding keystone species of health or disease as a structuring concept is one method [37]. Other methods include incorporation of specific functions for the target clinical indication [38,39•], or catalyzing a community shift that enables colonization resistance [40]. Consortia complexity is limited by many technical aspects that are the reality of Current Good Manufacturing Practices (cGMP) manufacturing, including production, quality control, drug formulation, and product stability.

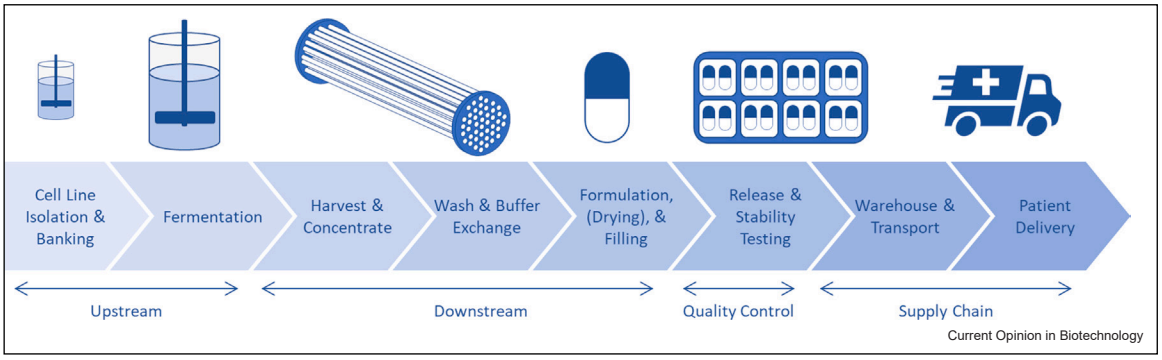
Manufacturing of designed consortia live biotherapeutic products

The manufacture of LBP consortia requires unique consideration across disciplines, including upstream and downstream bioprocessing, formulation development, quality control and analytical development, facility organization and operation, and the emerging regulatory landscape. A generalized block-flow diagram for LBPs is presented in Figure 1.

Upstream bioprocessing

Unlike typical *Lactobacillus* and *Bifidobacter* probiotics or common biotechnology industry microbes, the diversity of microbes, which may be considered for LBPs, is expansive [2], and many of interest are strict anaerobes. These species often lack aerotolerance, dying rapidly upon exposure to oxygen. When preparing cell banks, the unique physiology of human-commensal bacteria provides specific challenges for cryopreservation [41]. Many commensal taxa have fastidious nutritional requirements and have been considered difficult to cultivate. Historical laboratory cultivation relies upon medium components that are unrecognizable to Current Good Manufacturing Practices (cGMP) expectations, for

Figure 1



Generalized block-flow diagram for LBP manufacture and distribution. Upstream operations, including cell-line isolation and banking and fermentation to expand the bacterial culture(s). Downstream operations include harvest and concentration of the cultivated cells, buffer exchange to remove spent fermentation broth, and formulation to preserve cell viability. Drying and blending operations may be necessary before filling, depending on the target product. Release testing ensures final adherence to product-quality attribute specifications. Warehousing, transport, and storage before patient delivery is informed by product stability and may require significant control of environmental conditions to preserve viability.

example, sheep blood, bovine brain/heart infusion, or rumen fluid. Development of enhanced cultivation techniques and media formulations to manufacture LBP strains requires a combination of new microbiological methods and machine-learning techniques [42–46]. In some cases, cocultivation of two or more bacteria may enable symbiotic cultivation of a desirable fastidious microbe. However, depending on the precision required for drug manufacture, the dynamics of mixed fermentations may be difficult to control. For example, simply the branching and degree of polymerization of polysaccharides influence the cultivation dynamics of consortia [47].

Bioprocessing will also encounter a familiar strategic decision: durable versus single-use equipment. Among the typical considerations, the arithmetic for bacterial LBPs is driven by the short cycle time of the bacterial processes. Table 1 presents additional unique considerations.

Downstream bioprocessing

The downstream purification operations for LBPs generally focus on concentration of harvested fermentation broth, and washing into a stability-promoting buffer. A major challenge for LBPs is preservation of viability through processing, storage, and drug delivery. While buffer-exchange operations such as centrifugation and tangential flow filtration are well-known and generally robust for bacteria, application of these methods within a pharmaceutical manufacturing environment for a variety of anaerobic bacteria requires consideration of the oxygen exposures during processing to preserve viability and decontamination between strains. Refrigerated storage and dehydration is often required, which is usually accomplished via lyophilization or spray-drying, both established technologies [48].

Formulation

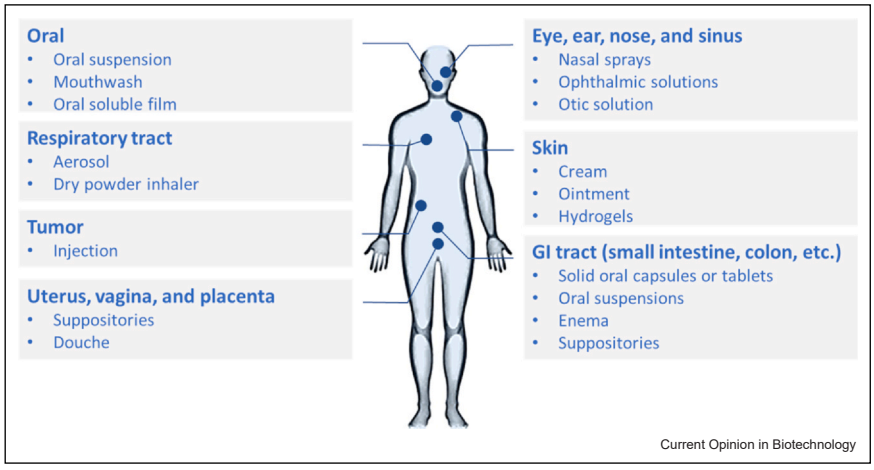
Formulations of bacterial LBPs must achieve the following goals: preserve the viability of the composition

Table 1

Unique considerations for the evaluation of durable versus single-use technology (SUT) for bacterial LBPs.

Consideration	Durable equipment	SUT
Fast turnover of bacterial processing	Increased time spent per year on cleaning and sterilization cycles	Increased consumable costs per year, and increased supply-chain risks due to high utilization rate
Multiplicity of strains for consortia products	Increased cleaning and sterilization validation, including with spore-forming microorganisms.	Increased assurance against contamination. Increased flexibility for process permutations
Heat and mass transfer of bacterial fermentations	Easily designed for sufficient transfer	Larger fermentation scales may suffer from slow heat transfer, especially during temperature induction Anaerobic bacteria usually have far lower heat and mass transfer requirements.
Oxygen sensitivity	Easily designed to prevent oxygen ingress	Oxygen-barrier properties and degassing strategies key to maintain viability

Figure 2



Target microbiomes, routes of dose administration, and types of drug formulations that may be considered for bacterial LBPs.

throughout the intended shelf life, enable potent delivery of the drug to target, and limit risks associated with biological outgrowth of either the product or bioburden.

Oral formulations consisting of encapsulated dried bacteria blends have been investigated, including for strict anaerobic bacteria [49•,50]. Oral dosing for the upper alimentary canal must achieve fast disintegration and dissolution to promote distribution. Conversely, lower intestinal delivery via oral administration requires protection against the acidic environment of the stomach and consideration of the oxygen gradient along the gastrointestinal tract. In the case of live oral bacterial vaccines, this is achieved using sodium bicarbonate to temporarily neutralize stomach acid [51]. Other approaches may include the use of capsules that contain intrinsic delayed release properties, tablets or capsules that are coated with polymers providing enteric protection, or microencapsulated particles [52]. The oral dosing of bacterial spores is facilitated by the natural resistance of spores to gastric stress [35]. Pediatric formulations may present different challenges owing to physiological differences in child digestive tracts, and in the strong preference for oral solutions/suspensions [53].

Nonoral formulation and administration methods continue to emerge and are described for GI, dermatological [54–56], vaginal [57,58], or nasal [59,60] delivery. Figure 2 shows additional routes of administration and drug formulations that have been considered for bacterial LBP development.

Formulations, in combination with drug packaging, must stabilize the live bacterial components to preserve viability throughout storage and dosing. Preservatives and physical conditions (e.g. low water activity) typically used in drug formulation for the purposes of microbial control may be counterproductive for the successful preservation of bacterial LBPs. Bacterial spores have emerged as a product form that facilitates both dosing and stability [35] owing to their resistance to various environmental stresses encountered during storage, for example, thermal, osmotic, and oxidative stress. Controlled freezing and drying by lyophilization is a method of bacterial preservation known for over one hundred years, and is supplemented with new technologies, for example, using polymeric film entrapment [61].

Quality control

Assays to define the safety, identity, strength, purity, and pharmaceutical-quality elements ('SISPQ') of LBP preparations require new interpretations and implementation.

Safety and identity are interlinked. Each strain's full genome and plasmids should be assessed for suitability, including presence of (pro)phage, antibiotic-resistance genes, especially those on mobile elements, and toxin genes [62–64]. Phenotypic assessments for toxin production may be warranted. For human-commensal strains, preclinical toxicology studies in animals have limited applicability due to poor competition of human commensals against an established host-adapted microbiome [65].

Dose strength can be measured via viability assays on solid or in liquid media, or by flow cytometry with a properly demonstrated viability stain [66]. Multiple forms of a strain (e.g. both spore and vegetative) may each require quantification to ensure reliable dosing. Mixtures of bacteria likely require enumeration of each strain to further ensure consistent dose strength and enable tracking of per-strain stability. Assessments of potency based on mechanisms of action may be appropriate and especially straightforward for LBPs containing organisms providing a specific activity.

Microbiological purity is a significant concern for LBPs. Established modalities have guidance for acceptable levels of general bioburden and specific organisms of concern [67,68], but existing guidance is incomplete owing to the live-product microbes that complicate detection. The various formulations and delivery methods outlined in Figure 2 have inherently different risk profiles ranging from low-risk nonaqueous oral dosages to higher-risk ophthalmic solutions and injectables. Consortia LBPs made from axenic fermentations of fastidious organisms have the potential for (cross-)contamination, and therefore mitigation strategies are essential for shared equipment, parallel workstreams, and multiproduct facilities. Classical media prescribed for bioburden testing [69–71] may not be useful for product testing due to nonselectivity and obscuring of nonproduct bioburden content. Viability-based bioburden measurements will need to be carefully developed for selectivity toward potential contaminants leveraging attributes that do not overlap with the intended product strains. Additional methods may be necessary, which specifically detect high-risk strains or high-risk functionalities via, for example, nucleic acid amplification. These methods may be applied to other strains manufactured within a multiproduct facility or microbes observed during environmental monitoring. Species presenting a risk to the intended patient population may require additional consideration. In one case, a suppression technique involving phage lysin specifically active toward a product strain was demonstrated to enable counter-selectivity and therefore detection of low-level nonproduct contaminants [72].

Application of emerging technologies can suffer from a lack of well-considered controls to ensure consistent performance. A review of various microbiome-profiling methods [73•] highlighted the effects of technical sources of variability on the quantification of microbial profiles and therefore on the importance of controls. The 2019 National Institute for Standards and Technology (NIST) Workshop on Standards for Microbiome Measurements focused on measurement-assurance tools for next-generation sequencing and viability methods and has led to ongoing collaborative work in the field. Controls for flow-cytometry technology have also been reported to address technical variation [74•].

Regulatory landscape

The regulatory landscape for bacterial LBPs is emerging. In the United States, the U.S. Food and Drug Administration (FDA) has issued guidance regarding the manufacture of LBPs for early clinical trials [63], and U.S. Food and Drug Administration (FDA) scientists have authored papers outlining manufacturing expectations throughout the clinical development process, including the use of INDs even for commercial probiotic supplements now intended for clinical studies and the use of FMT [75•,76]. Firmicutes make up the largest part of the gut microbiome and many are spore-forming organisms. It is likely that many LBPs will contain spore-formers as a component, perhaps with spores as the intended dosage form. The U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) have issued guidance on the use of spore-forming microorganisms for drug manufacture [77,78].

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