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Live Biotherapeutic Products: where are we?

Edited by
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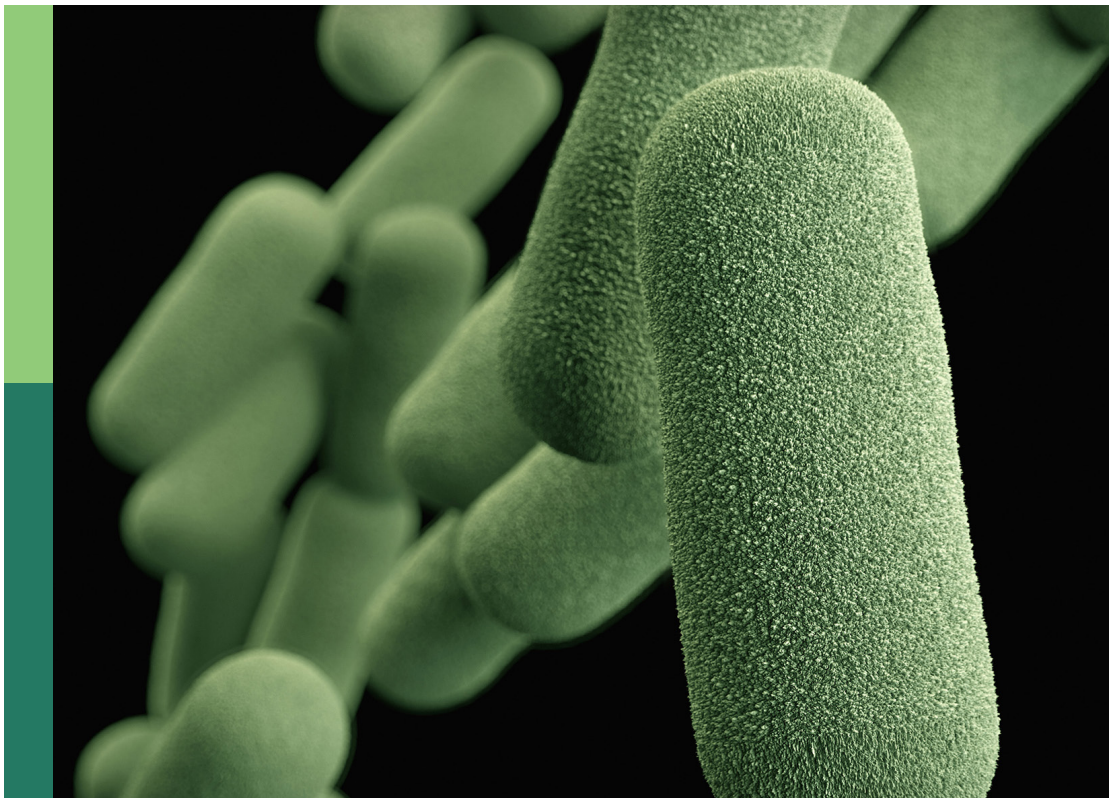

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Live Biotherapeutic Products: where are we?

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Editorial: Live Biotherapeutic Products: where are we?

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Live Biotherapeutic Products, fecal transplantation, safety, *Clostridium difficile*, regulatory procedure

Editorial on the Research Topic
Live Biotherapeutic Products: where are we?

In the fourth century BCE, Ge Hong recognized the utility of "yellow soup", a fecally originated product in managing diarrheal disease (Zhang et al., 2012). In Europe, the first recorded use of Fecal Microbiome Transplantation (FMT) took place in veterinary medicine. The procedure was carried out by Fabricius Acquapendente (1537–1619), an Italian anatomist and surgeon, who transferred gastrointestinal contents from a healthy animal to a sick one. This treatment, known as "transfaunation", became a widespread treatment for animals and was used for restoring normal rumination in cattle (Borody et al., 2004). Interest in the use of the restoration of the microbiome as a means of treating *C. difficile* infection (CDI) recurrences has re-ignited in the past decade. In this Research Topic, various aspects of this management process are discussed from a variety of perspectives.

The FDA's approval of Rebyota and Vowst in 2022 and 2023, respectively, was considered a landmark in terms of the development of novel therapies for treating recurrent *Clostridiodes difficile* infections. Navakelke and Chopra discussed the potential of Live Biotherapeutic Products (LBP) for human conditions, acknowledging that we have reached a turning point in recognizing the truly amazing breadth of conditions that can be managed.

Prior to the approval of these two LBPs there were significant challenges posed by regulators in terms of guidance and analytical frameworks. These were elegantly highlighted by the Microbiome Therapeutics Innovation Group (MITG) and Barberio. It was clear that existing regulatory frameworks for governing LBPs had significant gaps. These included microbial identification, potency and bioburden. The MITG led collaborative efforts to engage experts in the field to hold discussions with the FDA. These meetings discussed catalyzing improvements in LBP analytics and refreshing the regulatory landscape. It was clear that a multi-faceted approach to the continued development of LBPs is essential.

Slijkerman et al. discussed the challenges faced when expanding LBP development while ensuring product safety and effectiveness. Previous recent experiences with non-regulated fecal materials for transplantation were associated with infections leading to some deaths notably among immunocompromised patients. Thus the focus shifted from traditional methods to developing safe products for use in fecal transplantation, namely live microbial products (LMPs). LMPs are subdivided into specific subcategories and their product characteristics led to the development of appropriate guidelines. LMPs are subject

to GMP-compliant manufacturing guidance. However, there are no guidelines at present for injectable LMPs. Safety-related quality analysis is often inappropriate. Current FDA guidelines require screening for 29 pathogens to be absent from currently approved LBPs. This list is updated as necessary. Slijkerman et al. highlighted LMP guidelines and their different subcategories.

Thomas et al. presented the value of microbial consortia used in laboratory studies. Keratinocyte dysfunction is intrinsically involved in skin barrier repair and wound healing. Their study suggested that probiotic supplementation with two strains each of *Lactobacillus* and *Bifidobacterium* spp. may improve wound management. Interestingly the metabolome profile of these strains exerts a positive effect on key cell lines. These observations are encouraging for managing skin functionality.

Allergic conditions are a major global health problem. The role of the microbiome on immune function and many subsequent conditions in early life is becoming clearer. Tarrant and Finlay examined the potential of LBPs for the prevention and clinical management of childhood allergies. However, the present findings are inconsistent and somewhat limited. The authors discussed the current research and highlighted future possibilities. It has been shown that LBPs can mediate allergy susceptibility. These include several mechanisms such as Th1/Th2 balance, SCFA-induced inactivation of GPR41/43, and HDAC inhibition. It is these outcomes that suggest that LBPs may have value in managing allergic conditions.

Chronic conditions carry a significant burden due to elevated mortality and economic impact. Postbiotics are bioactive compounds that could be used as a possible therapeutic approach for chronic diseases. Asefa et al. discussed the potential of postbiotics in managing non-communicable diseases such as diabetes, cancer, obesity and certain cardiovascular conditions. The authors discussed the various mechanisms that could exert beneficial effects. These include immune response, immune modulation, and reduction of inflammation, leading to improved gut barrier function to improve permeability. A deeper understanding of these effects could enhance health outcomes in various at-risk populations.

LBPs have been extensively studied in *Clostridiodes difficile* infection (CDI). Sehgal and Feuerstadt discussed the effectiveness of LBPs in the treatment of recurrent CDI. Over the past decade our understanding of rCDI has shown that the relationship of dysbiosis (a disturbance of gastrointestinal microbial flora) provides an ideal setting for *C difficile* infection and for recurrent infections. Antibiotics have generally been effective in treating the causative infection, but can do nothing toward rebalancing the dysbiosis. The value of replacing lacking key species by using LBPs has shown the importance of reversing the microbial imbalances and developing eubiosis. The use of fecal microbiome therapy (FMT) has had variable success over the past decade. The introduction of healthy, human-derived feces into patients suffering from rCDI, as with the aforementioned products Rebyota and Vowst, has shown comparable effectiveness and safety. These are safe and effective when administered after standard antimicrobial therapy. These LBPs offer a new and safe approach to rCDI.

Lactobacillus is the dominant species in a healthy vagina and provides the first line of defense against pathogens. Vaginal dysbiosis is characterized by the loss of lactobacillus species, and is associated with genital diseases such as bacterial vaginosis, aerobic vaginosis, vulvo-candidiasis, sexually transmitted infections and pregnancy complications. Conventional treatments do not restore the protective flora of the vagina. Valeriano et al. examined the potential of LBPs in remedying dysbiosis. Lactobacillus species display a range of genotypic and phenotypic features, which include lactic acid production, inhibition of pathogens to epithelial cells, and other essential characteristics.

Consortia of specific organisms are being studied to develop Vaginal Microbiome Therapy (VMT). Valeriano et al. showed that these products are undergoing the standard range of FDA regulatory steps including the assessment of efficacy and tolerability. An appreciation of the many vaginal conditions and their causative organisms and disturbed environment is likely to improve the management of a range of conditions, which carry a significant morbidity and have significant financial implications with the use of VMT.

Finally, Navalkele and Chopra highlighted the application of LBPs in the management of different infectious diseases. As previously discussed, the microbiome consists of many microorganisms, including bacteria, fungi, viruses, and parasites. These organisms are found throughout the body. The most abundant location for these organisms is the gastrointestinal tract. The microbiome supports many bodily functions, as demonstrated by the well-established gut-brain axis. Dysbiosis is associated with immune-mediated conditions such as obesity, cancer and many others. The replacement of antibiotic resistant species found in the gastrointestinal tract by LBPs holds significant promise.

Perhaps the most relevant application of specific microbiome changes is in the management of COVID-19, a novel approach in which LBP LL-37 can prevent the binding of the virus to the host cell receptor ACE2 thus reducing the risk of infection. Other infections that are under investigation include those of the respiratory and urinary tracts, HIV, bacterial vaginosis and candidiasis. The status of the various clinical trials was summarized by these authors.

Overall, Live Biotherapeutic Products hold significant promise as therapeutic or preventative options for various human diseases and conditions. The development of these products is subject to evolving regulations, including those related to manufacturing processes and safety. As these become more defined, LBPs will build on the work of Ge Hong and his “yellow soup”.

Author contributions

GT: Writing – original draft, Writing – review & editing.

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Lactobacillus johnsonii and host communication: insight into modulatory mechanisms during health and disease

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Lactobacillus johnsonii is a commensal bacterium that has been isolated from vaginal and gastrointestinal (GI) tracts of vertebrate hosts, including humans, rodents, swine, and poultry. *Lactobacillus*-based probiotic supplements are popular because of the health advantages they offer. Species such as *L. johnsonii* are particularly interesting due to their potential health-promoting properties. Here, we reviewed the research on specific strains of *L. johnsonii* that have been studied in the context of health and disease and delved into the underlying mechanisms that aid in preserving host homeostasis. The utilization of *L. johnsonii* strains has been widely linked to numerous health benefits in the host. These include pathogen antagonism, control of mucosal and systemic immune responses, reduction of chronic inflammation, modulation of metabolic disorders, and enhanced epithelial barrier. These findings suggest that *L. johnsonii* plays a critical role in maintaining host homeostasis, highlighting its potential as a probiotic.

KEYWORDS

***Lactobacillus johnsonii*, gut microbiota, gut-lung axis, probiotics, microbiota metabolites**

Abbreviations: AD, atopic dermatitis; BV, bacterial vaginosis; BSH-L, bile-salt-hydrolase; BBDP, BioBreeding diabetes-prone rats; BBDR, BioBreeding diabetes-resistant rats; BMDC, bone marrow-derived dendritic cells; COPD, chronic obstructive pulmonary disease; DHA, docosahexaenoic acid; DSS, dextran Sulfate Sodium; EHEC, enterohemorrhagic *Escherichia coli*; FMT, fecal microbiota transplantation; HFD, high-fat diet; LAB, lactic acid bacteria; LPS, lipopolysaccharides; Msp1/p75, major secreted protein 1/p75; MCP1, monocyte chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; OUT, operational taxonomic units; RHE, reconstructed human epidermis; RSV, respiratory syncytial virus; TD1, Type 1 diabetes; TJ, tight junction; Treg, regulatory T cells.

1 Introduction

Lactobacillus johnsonii is a Gram-positive, homofermentative, non-spore-forming rod-shaped host-adapted bacterium (Zheng et al., 2020) with lactic acid being its predominant end product from sugar metabolism (Lebeer et al., 2008). Several strains of this species have been isolated from vaginal and gastrointestinal (GI) tracts of vertebrate hosts, including humans, rodents, swine, and poultry (Ravi et al., Pridmore et al., 2004; Leonard et al., 2014; Wu et al., 2016; Guerrero-Preston et al., 2017; Dec et al., 2018; Zhang et al., 2019; Ahire et al., 2021; Reed et al., 2022). The abundance of this bacterium in various niches is often influenced by external factors such as diet, antibiotic treatment, and invading microbes (Mason et al., 2012a; Mason et al., 2012b; Antonissen et al., 2016; Thompson et al., 2023). *L. johnsonii*, like other well-known *Lactobacillus* species, is of particular interest due to its potential health-promoting properties, which mark this specie as a probiotic candidate, defined by the FAO/WHO as ""live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014).

As a commensal bacterium, *L johnsonii* needs to survive, colonize, multiply and exert its function in the acidic and high bile concentrated conditions in the gut (Stavropoulou and Bezirtzoglou, 2020). For these purposes it has developed resistance and tolerance mechanisms against stressors, while also competing with other indigenous microbes in this niche (O'Flaherty et al., 2018; Zhang et al., 2019; Stavropoulou and Bezirtzoglou, 2020; Bagon et al., 2021). *L. johnsonii* is surrounded by an outer packaged protein shell called S layer. In addition, extracellular peptidoglycan, teichoic acids, and capsular and exo-polysaccharides help to protect and keep cellular integrity and adherence to the host, while the mechanism for stress sensing and export systems complemented the stress resistance machinery (Lebeer et al., 2008). Furthermore, *L. johnsonii* can adapt to the host's nutritional environment because its genome encodes a high number of the phosphotransferase system (PTS) and ATP-binding cassette (ABC) transporters as well as amino acid protease and peptidases that enable the uptake and utilization of a variety of sugars and amino acids available in the host GI tract microenvironment (Fujisawa et al., 1992; Lebeer et al., 2008; Zhang et al., 2019; Boucard et al., 2022). *In vitro* studies have shown that *L. johnsonii* L531 can produce higher levels of short-chain fatty acid (SCFA) (butyric acid, acetic acid) and lactic acid, having an impact on the metabolic profile and the gut resident microbiota (He et al., 2019). These metabolites are known to promote the maturation of the host immune system and regulate the onset and progression of inflammatory responses (Rooks and Garrett, 2016; Richards et al., 2016).

The inter-strain variations in carbohydrate utilization profile, as well as cell wall composition, determine *L. johnsonii*'s health-promoting and immunomodulatory properties (Fujisawa et al., 1992; Zhang et al., 2019; Schar-Zammaretti and Ubbink, 2003; Guinane et al., 2011). As a result, while *L. johnsonii* is a good probiotic candidate, the different strains of this specie must be independently investigated per the Food and Agriculture Organization of the United Nations (FAO), which guidelines demand to include the source of isolation, characterization, and a credible case presented for their health effects, to be called 'probiotic' (Hill et al., 2014).

L. johnsonii strains such as NCC 533 (also known as La1) is a commercially available probiotic. Several studies, including *in vitro*, animal models, and clinical trials have shown NCC 533 binding properties to host mucosal cells, as well as its ability to inhibit gut pathogens, stimulate the immune system and metabolic functions, enhance the mucosal barrier and improve human intestinal microbiota (Neeser et al., 2000; Granato et al., 2004; Pridmore et al., 2004; Bergonzelli et al., 2006; Yamano et al., 2006; Inoue et al., 2007; Denou et al., 2008). Several of these health-promoting activities are also observed in other *L. johnsonii* strains when administered to different animal models (La Ragione et al., 2004; Kingma et al., 2011; Fonseca et al., 2017; He et al., 2019; Charlet et al., 2020; Zou et al., 2020). Notably, the survival capacity and safety of *L. johnsonii* strains N6.2 and 456 supplementation have been studied in healthy human volunteers (Marcial et al., 2017; Davoren et al., 2019). *L. johnsonii* strain N6.2 is under clinical trials for its probiotic effect on Type I diabetes (T1D) onset in children, adolescents, and adults (Clinical Trial: NCT03961854, 2019-2023; Clinical Trial: NCT03961347, 2020-2026), while *L. johnsonii* strain MH-68 have shown promising results in the glycemic control and immunomodulation (Wang et al., 2022).

This review summarizes the existing scientific literature on the mechanisms by which *L. johnsonii* affects health and disease progression. Our goal is to comprehend the effects of *L. johnsonii* on various health outcomes.

2 *Lactobacillus johnsonii*: impact on health and disease

2.1 *L. johnsonii* and the modulation of Gastrointestinal health

Different regions of the GI tract, such as the mouth, stomach, small intestine, and colon, have unique environmental conditions, including variations in pH, nutrients availability, and oxygen levels. These variations created distinct niches for different microorganisms to thrive (Thursby and Juge, 2017). Scientists are studying how gut bacteria affect health and its potential role in treating gastrointestinal disorders (Bidell et al., 2022). *L. johnsonii* strains as probiotics have been shown to enhance gut health in humans and animals (Marcial et al., 2017; Yang et al., 2022b; Yang et al., 2022c). Microbes can colonize various regions of the GI tract and impact other microbial communities throughout the entire digestive system.

2.1.1 *L. johnsonii* and the Gastrointestinal epithelial barrier

The intestinal epithelial barrier regulates immunity, nutrient absorption, digestion, and hormone production as well as metabolic processes (Lee et al., 2018). The tight junction (TJ) complex between epithelial cells maintains the intestinal barrier, regulates

selective paracellular transit of ions, water, and solutes, and limits the transit of microorganisms, food allergens, and macromolecules (Lynch and Pedersen, 2016; Lee et al., 2018). Several studies have demonstrated the capacity of different *L. johnsonii* strains such as MG, L531, BS15, and 135-1-CHN to enhance the barrier function by upregulating TJ related genes (ZO-1, Occludin, and Claudin-1) (Xin et al., 2014; Liu et al., 2015; Mu et al., 2017; Chen et al., 2021; Lyu et al., 2023), as well as direct interaction with the Junctional Adhesion Molecule-2 (JAM-2) (Bai et al., 2022). Postnatal administration of *L. johnsonii* N6.2 to T1D-prone rats showed no morphological differences between groups in the structure of the villus however, an upregulated expression of claudin-1 and decreased expression of occludin was observed in the *L. johnsonii*-supplemented group, as well as decreased intestinal pro-inflammatory response, showing the ability of *L. johnsonii* N6.2 to ameliorate the intestinal barrier dysfunction (Valladares et al., 2010). The oral administration of *L. johnsonii* promoted the activation of the TLR1/2-STAT3 pathway and increased the number of anti-inflammatory macrophages, leading to IL-10 release and improvement of DSS-induced colitis in mice (Jia et al., 2022). In contrast, clinical studies evaluating the effect *L. johnsonii* NCC 533 supplementation in patients after intestinal resection for Chron's disease reported that *L. johnsonii* NCC 533 failed to prevent endoscopic recurrence after six months (Marteau et al., 2006; Van Gossum et al., 2007). These studies demonstrated the potential benefits and limitations of *L. johnsonii* in improving intestinal barrier function and reducing epithelial inflammation (Figure 1).

2.1.2 *L. johnsonii*: Control of pathogens and regulation of the immune response in the GI

Oral microbiota equilibrium can be affected by inflammatory conditions, such as periodontitis (Manos, 2022). It has been reported that oral pathobiont *Porphyromonas gingivalis* is highly expanded during chronic periodontitis and is associated with several inflammatory disorders, from atherosclerosis to colitis. It plays an important role in establishing and expanding gut pathobionts, highlighting the importance of the oral-gut axis in the development of GI tract pathologies (Kitamoto et al., 2020). *Lactobacillus* bacteria and specifically *L. johnsonii* strains, have been used as an alternative approach to the control of pathobionts associated with periodontitis and dental cavities because of their anti-biofilm activity, which alters the ability of pathobionts to colonize (Jaffar et al., 2016; Giordani et al., 2021). Controlling oral pathogens and oral inflammatory diseases could also impact individuals' gut microbiota composition and overall health (Imai et al., 2021).

Extensive research on various *L. johnsonii* strains demonstrates the pathogen-inhibiting property of this bacterium in the GI tract, often via secretion of antimicrobial molecules, lowering the pH of the environment and competing for similar niches. The supplementation of *L. johnsonii* has modulated several intestinal pathogens, such as *Helicobacter pylori*, *Salmonella* spp., pathogenic *Escherichia coli*, and *Clostridium perfringes* (Figure 2).

The ability of *L. johnsonii* to inhibit *Helicobacter pylori* infection has been widely studied. Supplementation of *L. johnsonii* in animal models infected with *H. pylori*, resulted in reduced pathogen load,

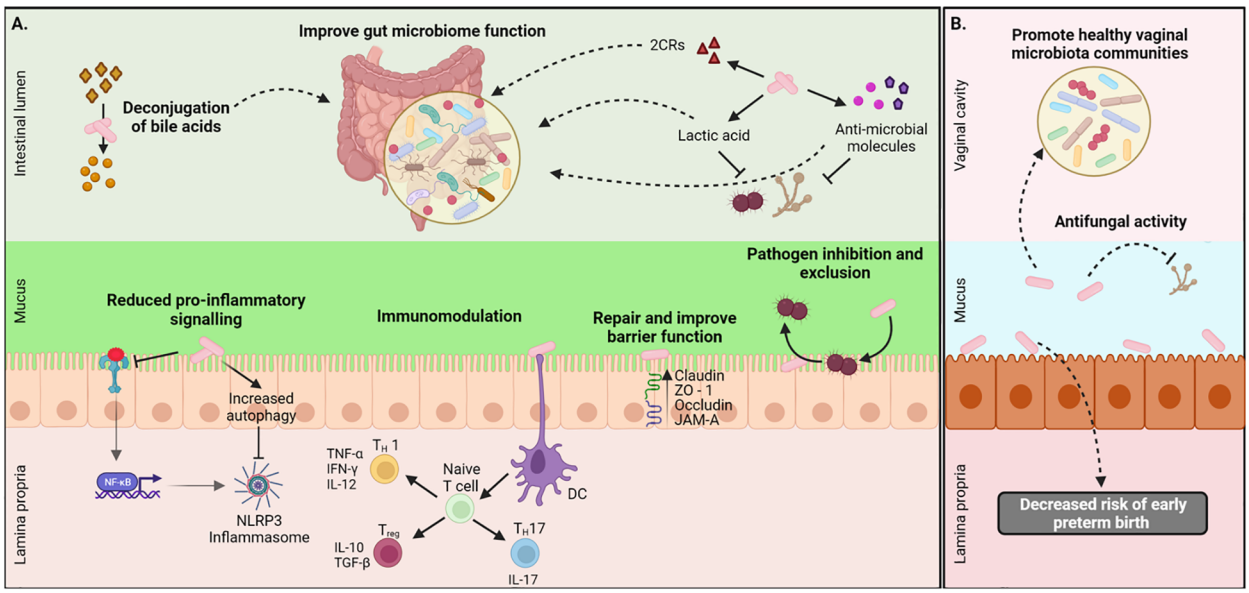

FIGURE 1
Local health benefits conferred by *L. johnsonii* administration. **(A)** *L. johnsonii* secretes metabolites like lactic acid, hydrogen peroxide, antimicrobial peptides, and bile salt hydrolases (BSH) that facilitate pathogen inhibition and improved gut microbiome function. *L. johnsonii* also inhibits pathogen-induced activation NLRP3 inflammasome via inhibition of TLR4-mediated signaling and promotion of autophagy. It interacts with epithelial cells and repairs barrier function by increasing the expression of tight junction proteins like claudin and occludin. *L. johnsonii* also has immunomodulatory functions. For example, it stimulates dendritic cells (DC), resulting in downstream modulation of both pro-and anti-inflammatory cytokine secretion and thus mediating a Th1/Th2/Treg immune balance response. **(B)** *L. jonhsonii* colonize the vagina of healthy women were display its antifungal properties to promote a healthy vaginal microbiota. Created with BioRender.com.

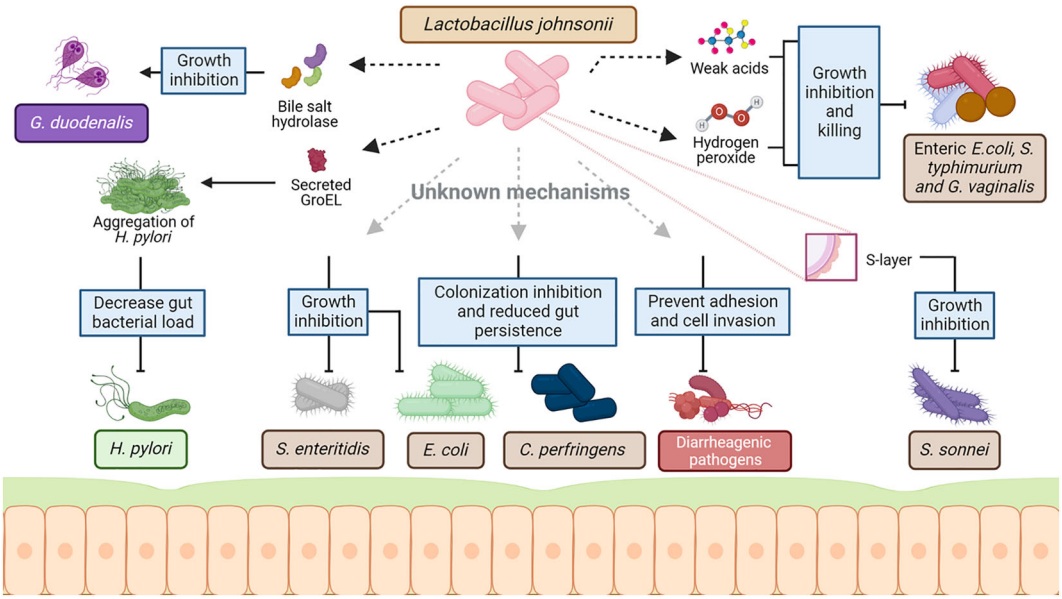

FIGURE 2
Mechanism of interaction between *L. johnsonii* and commensal and pathogenic bacteria. As a host-associated species, *L. johnsonii* interacts with the resident microbiota as well as invading pathogens to establish stable colonization in the niche. This interaction is multifaceted and involves both secreted and cell surface molecules of *L. johnsonii*. *L. johnsonii* produces bacteriocins and bacteriolysis that target *Lactobacillus* and *Enterococcal* species in a strain-specific manner. Weak acids like lactic acid produced by *L. johnsonii* act together with hydrogen peroxide, inhibiting and killing enteric, vaginosis-associated, and uropathogenic pathogens like pathogenic *E. coli, S. typhimurium* and *Gardnerella vaginalis*. Several cell surface structures of *L. johnsonii* are also involved in antimicrobial activities. GroEL is a surface-associated protein that triggers aggregation of *H. pylori* upon secretion by *L. johnsonii*. This aggregation is hypothesized to cause the rapid exclusion of *H. pylori* from the GI tract upon *L. johnsonii* administration. Similarly, S- layer protein on *L. johnsonii* cell surface can inhibit *S. sonnei* growth. Other cell surface-associated structures like EPS, LTA, EF-t,u, and specific carbohydrate-binding receptors – all involved the in adhesion of *L. johnsonii* to epithelial cells and mucin – are hypothesized to play a role in competing with commensal microbes and pathogens for mucosal binding sites. *L. johnsonii* also inhibits the growth of other pathogens like *S. enteritidis* and reduces gut persistence of *E. coli* and *C. perfringens* through a yet unknown mechanism. Created with BioRender.com.

mobility, and aggregation in the gastric mucosa (Sgouras et al., 2005; Isobe et al., 2012; Aiba et al., 2015; Aiba et al., 2019). *L. johnsonii* encode for and secretes a cell surface structure protein named GroEL, which triggers *H. pylori* aggregation under *in vitro* conditions, (Bergonzelli et al., 2006). This interaction could lead to the rapid exclusion of *H. pylori* observed in different *in vivo* studies (Sgouras et al., 2005; Isobe et al., 2012; Aiba et al., 2015; Aiba et al., 2019). A clinical trial using *L. johnsonii* Lj1 fermented milk in *H. pylori*-positive volunteers showed reduced antral gastritis, inflammatory score in the gastric mucosa, and decreased density of *H. pylori* (*Pantoflickova et al., 2003*). In contrast, oral supplementation with *L. johnsonii* NCC 533 supernatants did not control *H. pylori* persistence in humans (Michetti et al., 1999). However, heat killed/lyophilized as well as viable *L. johnsonii* No.1088. were shown to reduce gastrin-mediated acid production, by decreasing the number of gastrin-positive cells in mice stomach (Aiba et al., 2015) and its combination with anti-*H. pylori* urease immunoglobulin Y (IgY) significantly reduced *H. pylori* infection (Aiba et al., 2019). More studies are needed to determine the necessity of viable bacteria to report a positive effect of *L johnsonii* in *H. pylori* treatment.

In addition to pathogen exclusion, *L. johnsonii* supplemented mice resulted in reduced *H. pylori*-related inflammation by diminished gastric mucosa inflammatory leukocyte (neutrophils, lymphocytes, macrophages) infiltration and proinflammatory chemokine and cytokine expression (macrophage inflammatory protein 2, keratinocyte-derived cytokine) (Sgouras et al., 2005). Additional *in vitro* studies showed that the incubation of *H. pylori*-infected human adenocarcinoma *AGS* cell lines with *L. johnsonii* NCC 533 cultures supernatants reduced the expression of *H. pylori*-induced IL-8, without affecting the bacterial viability (Sgouras et al., 2005). These studies showed the immunomodulatory effect of *L. johnsonii* in the control of *H. pylori*-related inflammation.

Current therapies for *H. pylori* infection include antimicrobial agents and inhibitors of gastric acid secretion, such as proton pump inhibitors (PPI) and vonoprazan. In a mouse model, these drugs decreased the population ratio of *L. johnsonii* (*Nadatani et al., 2019*). Interestingly, *L. johnsonii* supplementation in a model of indomethacin-induced small intestinal damage in combination with PPI or vonopazan, protects mice from intestinal injury (Nadatani et al., 2019). These data illustrate the distinct characteristics of *L. johnsonii* and its potential used as part of therapeutic protocols to alleviate the adverse effects of medications and synergistically reduce detrimental bacterial growth and tissue inflammation.

Different studies suggest that *L. johnsonii* L531 has the potential to control other intestinal pathogens, such as *Salmonella* sp. (He et al., 2019; Xia et al., 2020; Yang et al., 2020; Chen et al., 2021; Yang et al., 2022b). Oral supplementation with *L. johnsonii* L531 to newly weaned piglets, one week before challenged with *Salmonella enteric*

serovar Infantis, reduced diarrhea severity, intestinal inflammation, and tissue damage. The modulation of the inflammatory response led to epithelial protection and reduced abundance of *Salmonella* in the ileum mucosa (He et al., 2019; Yang et al., 2022b). The protective effects of *L. johnsonii* on *Salmonella* sp. immunopathogenesis, have been associated with the inhibition of the NOD pathway, the modulation of endoplasmic reticulum stress, and the promotion of autophagy degradation (Yang et al., 2020; Yang et al., 2022b); as well as the regulation of NLRC4 and NLRP3 inflammasome, proinflammatory cytokines expression via NFκB signaling and inhibition of mitochondrial damage (Xia et al., 2020; Chen et al., 2021).

In silico studies identified three potential gene products in *L. johnsonii* NCC 533 genome that may catalyze the known antimicrobial factor hydrogen peroxide (H_2O_2) synthesis. *L. johnsonii* NCC 533 and other *L. johnsonii* strains produced H_2O_2, which is hypothesized to play a role in the elimination of *Salmonella enterica* serovar Typhimurium SL1344 *in vitro* (*Pridmore et al., 2008*). Additionally, it has been suggested that H_2O_2 and lactic acid produced by *L. johnsonii* act co-operatively to kill enteric, vaginosis-associated, and uropathogenic pathogens, such as enteric pathogenic *E. coli, S. typhimurium* and *Gardnerella vaginalis* (Atassi and Servin, 2010). Acidification of the microenvironment is an anti-microbial mechanism employed by several lactic acid bacteria (LAB). Lactic acid and other weak acids produced by lactobacilli have been known to exhibit pathogen-inhibitory function by reducing the pH in the surrounding environment (Peter, 1993; Servin, 2004). Interestingly, *L. johnsonii* NCC 533 inhibits *Salmonella enterica* serovar Typhimurium SL1344 growth only at a low pH of 4.5, but not at pH 6.5 (Fayol-Messaoudi et al., 2005) (Figure 3).

L. johnsonii NCC 533 has been shown to control pathogens by producing bile-salt-hydrolase (BSH) (Travers et al., 2016). This enzyme hydrolyzes the amino bonds of conjugated bile salts to generate deconjugated bile salts (cholic, deoxycholic, and chenodeoxycholic acids) (Begley et al., 2006; Travers et al., 2016). It has been shown that BSH might play a role in antiparasitic activity against *Giardia* sp., a protozoan intestinal parasite that causes giardiasis, by inhibiting the proliferation of *Giardia* sp. trophozoites (Travers et al., 2016; Allain et al., 2017). The BSH present in the supernatants of *L. johnsonii* NCC 533 prevent *Giardia* sp. growth *in vitro* by converting bile's non-toxic components into highly toxic components to *Giardia* sp. (Travers et al., 2016). Furthermore, mice treated with recombinant BSH during *Giardia duodenalis* infection presented decreased numbers of trophozoites in the small intestine, showing the antiparasitic effect of the BSH-L enzyme and suggesting that the mechanism by which *L. johnsonii* controls intestinal parasite infection is through the production of specific metabolic enzymes (Allain et al., 2017).

L. johnsonii can prevent the adhesion and cell invasion of several diarrheagenic bacteria, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), *Yersinia pseudotuberculosis* and *Salmonella typhimurium*, to intestinal epithelial cells (Bernet et al., 1994; Liu et al., 2015). This broad inhibitory effect of *L. johnsonii* strains was initially attributed to the non-specific steric interference of receptors needed for pathogen colonization.

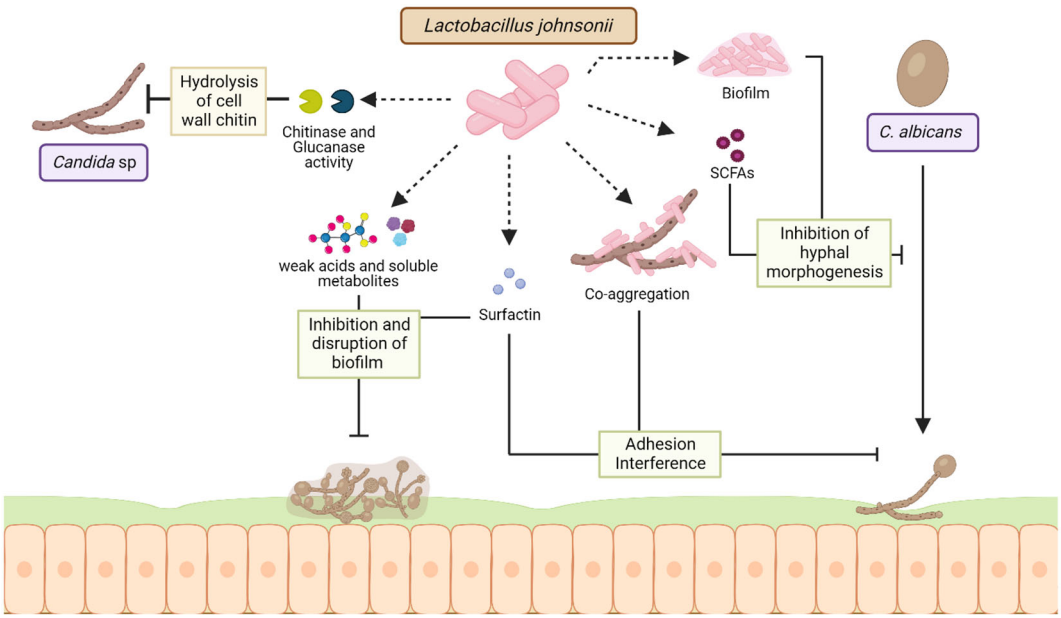

FIGURE 3
Mechanisms of interaction between *L. johnsonii* and *Candida* sp. *L. johnsonii* antagonizes the growth of *C. albicans* both *in vitro* and in the GI tract via secreted and cell surface molecules. Chitinase and glucanase-like hydrolytic enzymes secreted by *L. johnsonii* can degrade the fungal cell wall, causing rapid decreases in *Candida* viability during co-culture. Acidification of the niche due to weak acids and other soluble metabolites produced by *L. johnsonii* can inhibit the formation of *Candida* biofilms and disrupt established *C. albicans* biofilm structure. Strains of *L. johnsonii* also encode the surfactin gene, a biosurfactant that prevents biofilm formation and inhibits *C. albicans* adhesion. Additionally, *L. johnsonii* biofilm structure and production of SCFAs like butyric acid can inhibit *C. albicans* hyphal morphogenesis, thereby affecting its pathogenicity. Finally, *L. johnsonii* and *C. albicans* co-aggregate *in vitro*, a characteristic hypothesized to interfere with adherence and trigger rapid exclusion. Created with BioRender.com.

However, there is evidence suggesting the involvement of a more direct inhibitory mechanism by a recent work of Zang et al., which showed that the S-layer protein of *L. johnsonii* F0421 inhibited *Shigella sonnei* adhesion to HT-29 cells (Zhang et al., 2012). Thus, *L. johnsonii* strains can regulate the colonization of intestinal pathogens by controlling their adherence to the mucosal epithelium.

L. johnsonii has also been shown to provide protection against *Citrobacter rodentium*-induced colitis in an animal model by modulating the innate immune signaling pathways, as well as inflammatory responses and ER stress (Zhang et al., 2021). *L. johnsonii* administration in abiotic mice did not abrogate *Campylobacter* sp. jejune growth but reduced the expression of pro-inflammatory cytokines (such as IL-6, MCP1, and TNF) in the intestinal tract (Bereswill et al., 2017). *L. johnsonii* NJ3 supplementation of mice infected with enterohemorrhagic *E. coli* increased the diversity of the intestinal microbiota and improve the diarrhea index, body weight, and liver index (Hu et al., 2021).*In vitro* studies have shown that *L. johnsonii* L531 inhibit NLRP3 activity by promoting autophagy leading to reduced *Escherichia coli*-induced cell damage (Zou et al., 2020).

In the last decade, the number of antibiotic-resistant pathogenic bacteria and the search for alternative therapies to help control bacterial infections have increased. Probiotics, as well as fecal transplantation from healthy individuals, is an alternative therapy for the treatment of antibiotic-resistant bacteria and for re-establishing healthy gut microbiota in individuals with chronic diseases (Reyman et al., 2022). Studies by Ekmekciu et al. compared the efficacy of fecal microbiota transplantation (FMT) from healthy mice to oral supplementation with *L. johnsonii* in mice subjected to broad-spectrum antibiotic treatment for eight weeks. The antibiotic treatment diminished immune cell populations in the intestine, mesenteric lymph nodes, and spleen. In contrast, after antibiotic treatment, FMT and *L. johnsonii* supplementation increased CD4+, CD8+, and regulatory T cells (Tregs) cells in the small intestine and the spleen. Treatment *with L. johnsonii* also maintains colonic IL-10 production (Ekmekciu et al., 2017). This study showed the potential of *L. johnsonii* supplementation in individuals with dysbiosis caused by antibiotic treatment and its use as a therapeutic intervention for bacterial infection with an antibiotic-resistant phenotype.

Gut microbiota dysbiosis can exacerbate intestinal fungal infections, and *Candida* sp. is the most frequent cause of yeast infection (Charlet et al., 2020; Jawhara, 2022). *L. johnsonii* and *Bacteroides thetaiotaomicron* interact with *Candida* sp. and promote fungal cell wall degradation via chitinase-like and mannosidase-like activity, inhibiting fungal growth (Charlet et al., 2020). It has been shown that the administration of these two bacterial during DSS-induced colitis controlled the growth of pathogenic *E. coli*, *Enterococcus faecalis*, and *Candida glabrata* in the intestine, intestinal inflammation by downregulating intestinal IL-1β, TLR9, and NF-κB activation and upregulating IL-10 (Charlet et al., 2020). In a different approach, Bertolini et al. observed that changes in the microbial composition and function induced by dietary sucrose generated an increased abundance of *Lactobacillus* sp. and decreased *Candida albicans* burden in a murine model of oropharyngeal candidiasis during immunosuppression (Bertolini et al., 2021). The same authors showed that *L. johnsonii* MT-LB4 has an inhibitory effect on *Enterococcus faecalis* and planktonic *Candida albicans* growth *in vitro* (*Bertolini et al., 2021*). Furthermore, the production of oleic acid and palmitic acid by *L. johnsonii* during interaction with colonic epithelial cells has been associated with anti-inflammatory and antifungal properties in a DSS- induced colitis mice model (Charlet et al., 2022). Studies have shown that *L. johnsonii* MT4 exhibited pH-dependent and pH-independent antagonistic interactions with *C. albicans*, by inhibiting its growth and biofilm formation via nutrient competition and the production of metabolites with anticandidal activity with a similar sequence to antifungal compounds, such as Bacillomycin D, Surfactin, glucanase, and Msp1/p75 (Vazquez-Munoz et al., 2022). *L. johnsonii* JCM1022 inhibits *C. albicans* hyphal morphogenesis *in vitro*, via butyric acid production (Tang et al., 2010). Therefore, *L. johnsonii* in the GI tract can control the growth of fungal pathogens and hinder biofilm formation through *L. johnsonii*-derived metabolites, as well as anti-inflammatory and anti-fungal properties (Figure 3).

2.2 *L. johnsonii* and autoimmune diseases

Dysbiosis of the gut microbiota has been hypothesized to promote autoimmune disorders, such as type 1 diabetes (TD1) (Chagwedera et al., 2019). T1D results from the destruction of insulin-producing β cells via autoreactive T cells, which affects the self-regulation of blood sugar in the body. Notably, T and B-cell-deficient rodents fail to develop T1D, even when carrying predisposing genetic mutations (Christianson et al., 1993). Evidence shows that the resident gut microbiota is involved in the progression of T1D and that altering the gut microbiota using probiotics can be a therapeutic tool to help manage T1D (Vaarala et al., 2008; Dovi et al., 2022).

It has been observed that rats which spontaneously develop TD1 due to genetic predisposition (BioBreeding diabetes-prone rats -BBDP), have increased susceptibility to infections (Roesch et al., 2009). One of the differences between BBDP rats and BioBreeding diabetes-resistant rats (BBDR) is their gut microbiota, specifically, *Lactobacillus* and *Bifidobacterium* abundance, which are dominant bacterial communities that negatively correlated with the onset of T1D (Lai et al., 2009; Roesch et al., 2009; Valladares et al., 2010). Interestingly, oral administration of *L. johnsonii* N6.2 to BBDP rats, decreased the incidence of diabetes by altering intestinal microbiota, decreasing the host intestinal oxidative stress response, and modifying the intestinal pro-inflammatory response, while *Lactobacillus reuteri* fails to mediate the resistance to T1D (Valladares et al., 2010). This was further accompanied with changes in dendritic cell phenotype that contributed to the Th17 lymphocyte's immune polarization in mesenteric lymph nodes and spleen (Lau et al., 2011).In addition, it was described that *L. johnsonii* N6.2 derived lipids promoted a tolerogenic-migratory DC-like phenotype that could enhance regulatory T cells responses and prevent the initiation of the autoimmune process (Cuaycal et al., 2023). TLR9 activation seems to be implicated in this

polarizing-tolerogenic mechanism (Kingma et al., 2011). In addition, to reshape the Treg/Th17 commitment, *L. johnsonii* N6.2 can modulate the assembly of the inflammasome, evidenced by lower levels of mature caspase-1 in BBDP rats (Teixeira et al., 2018). Immunoregulatory properties of *L. johnsonii* N6.2 derived H_2O_2 abolished the activity of the rate-limiting enzyme for tryptophan catabolism, indoleamine 2,3-dioxygenase (IDO) known by its capacity to induce the proinflammatory cytokine IFNγ (Valladares et al., 2013). A pilot clinical study with this strain supports the safety and tolerance of *L. johnsonii* N6.2 administration in healthy humans' patients (Marcial et al., 2017). However, few clinical studies have supported the benefits of probiotic supplementation in patients with T1D (Dovi et al., 2022). In a clinical study, patients diagnosed with T1D (onset age 6 to 18 years old) were supplemented daily for 60 days with placebo or a capsule containing active probiotics including *L. johnsonii* MH-68. The probiotics mix had a positive impact on glycemic and glycated hemoglobin levels in the blood, increased the presence of beneficial bacteria species, such as *Bifidobacterium animalis*, *Akkermansia muciniphila* and *Lactobacillus salivarius* and reduced inflammatory cytokines in the serum of patients with TD1. Glycemic control and immunomodulation persisted 3 months after stopped probiotics intake (Wang et al., 2022). Although probiotics cannot cure T1D, they can help manage symptoms and be used as a supportive treatment for T1D and other autoimmune diseases.

Furthermore, it has been suggested that *L. johnsonii* can release bioactive molecules with immunomodulatory effects (Harrison et al., 2021). Microbial extracellular vesicles have been reported in feces, blood, and urine and show different patterns depending on the individual's health status. There is an increasing interest in studying these microbial extracellular vesicles as possible biomarkers for disease assessment and as immunomodulators of disease over the use of live organism (Park et al., 2021; Diez-Sainz et al., 2022; Yang et al., 2022a). *L. johnsonii* N6.2-derived nanovesicles (NV10) are rich in glycerophosphoglycerols and contain several unique and differentially expressed proteins compared to the bacteria cellular membrane (Harrison et al., 2021). *L. johnsonii* N6.2 extracellular vesicles could upregulate IL-10 expression in macrophages, promoting the M2 tolerogenic phenotype through STAT3 activation, while in the human pancreatic cell line Blox5, it reduced cytokine-induced apoptosis (Teixeira et al., 2022). *L. johnsonii* N6.2 derived phospholipids modified bone marrow-derived dendritic cells (BMDCs) transcriptional signature, triggering the expression of anti-inflammatory cytokine Il10 (Cuaycal et al., 2023), suggesting that *L. johnsonii* N6.2 nanovesicles' phospholipids components might have an immunomodulatory function. Interestingly, human pancreatic islets treated *in vitro* with *L. johnsonii* N6.2 extracellular vesicles showed significant upregulation of the expression of glucose transporter Solute Carrier Family 2, Member 6 (*SLC2A6)*, also known as glucose transporter 6 (GLUT6), suggesting that *L. johnsonii* can induce glucose uptake by pancreatic islets under high glucose conditions, and increase insulin secretion (Teixeira et al., 2022). These studies showed possible mechanisms by which *L. johnsonii* generated changes at a location distant to the gut via extracellular vesicle and possible mechanisms of *L. johnsonii* N6.2 to attenuate the onset of T1D by immunoregulation (Figure 4).

It is important to investigate the effects of *L. johnsonii*-derived extracellular vesicles and live *L. johnsonii* to understand the different outcomes generated among them and the specific role of the extracellular vesicles in the modulation of inflammatory responses and autoimmune diseases.

2.3 *L. johnsonii* and metabolic diseases

Metabolic diseases due to poor diets and obesity also cause significant disease manifestations. Diet can affect gut microbiota composition and function, as well as metabolic processes that can lead to the development of metabolic syndrome, cardiovascular disease, and type 2 diabetes (Ginsberg and MacCallum, 2009; O'Toole and Shiels, 2020; Wicinski et al., 2020). Recent studies have evaluated the effect of *L. johnsonii* N6.2 supplementation in a high-fat diet (HFD) rat model to induce metabolic syndrome. The authors observed that *L. johnsonii* N6.2 in combination with phytophenols reduced mTORC1-activating phosphorylation of AKT and other genes expression downstream mTORC1 signaling pathway in HFD-fed females (Kling et al., 2018). mTOR and AKT functions are associated with glucose and lipid metabolism, which are involved in metabolic syndrome (Saxton and Sabatini, 2017), suggesting that *L. johnsonii* N6.2 supplementation may help to diminish fat deposition and could modulate the development of the metabolic syndrome.

The close relationship between the gut microbiome and obesity has been extensively studied (Wicinski et al., 2020). An elevated prevalence of obesity worldwide is associated with increased non-alcoholic fatty liver disease (NAFLD) (Wong and Ahmed, 2014). A study by Jinge et al. investigated the effect of *L. johnsonii* BS15 administration on the development of NAFLD in obese male mice. The authors observed that *L. johnsonii* BS15 supplementation protected mice from hepatic steatosis and hepatocyte apoptosis when exposed to a HFD. The protective effect was attributed to enhanced liver antioxidative defense, as well as inhibition of insulin resistance and decreased expression of acetyl-CoA carboxylase 1, fatty acid synthase, and peroxisome proliferator-activated receptor γ. Long-term alterations were observed in the gut microbiota of obese mice, with an increased abundance of *Lactobacillus* sp. and specifically *L. johnsonii* after 63 days of supplementation. After 119 days of probiotic supplementation with *L. johnsonii*, obese mice showed decreased serum LPS levels, and reduced intestinal permeability and pro-inflammatory response by downregulating TNFα expression (Xin et al., 2014). These studies confirm the crucial role of the microbiome in maintaining metabolic homeostasis in the host and reducing associated illnesses, as well as the long-term effect of *L. johnsonii* supplementation in the gut microbiota composition and functionof host metabolism and inflammatory status (Figure 4).

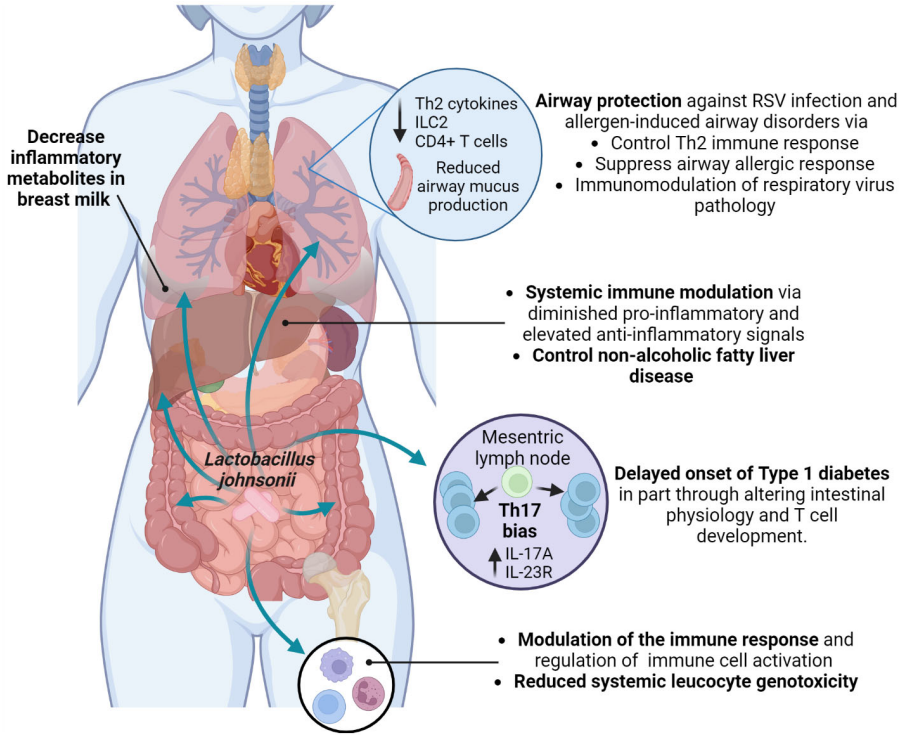

FIGURE 4
Systemic health benefits conferred by *L. johnsonii* administration. *L. johnsonii* immunomodulatory properties are related to its capacity to alter microbiota composition and function, changing the bacterial communities' metabolites profile. In addition, *L. johnsonii* express and produce substances and release vesicles with local and systemic anti-inflammatory and metabolic effects that modulate susceptibility to Th2 (allergic) responses and RSV infection in the airways and impacted onset and clinical manifestations of autoimmune and metabolic diseases. Created with BioRender.com.

2.4 *L. johnsonii* and the Reproductive system

The vaginal microbiome is a dynamic ecosystem influenced by external or environmental stressors (sexual activity and personal hygiene) and intrinsic physiological conditions, such as hormonal changes, sexual development, pregnancy, and disease states. *Lactobacillus* sp. is the most abundant microorganism in the vaginal bacterial community and is a well-known pH acidifier (Greenbaum et al., 2019). Although recent data suggest that bacterial vaginosis (BV) results from polymicrobial disruption of the vaginal microbiota, the alkaline pH in BV patients has been related to decreased lactic acid production by *Lactobacillus* sp. (Greenbaum et al., 2019). One of the *Lactobacillus* specie that colonized the vagina and intestine of healthy women is *L. Johnsonii (Dobrut et al., 2018). L. johnsonii* UBLJ01, isolated from the vagina of healthy women, was found to inhibit the growth of *Gardnerella vaginalis*, *Proteus mirabilis*, and *Candida albicans (Ahire et al., 2021)*. The therapeutic effects of *L. johnsonii* B-2178 and *Lactobacillus acidophilus* were tested in a rat model of vulvovaginal candidiasis and observed that both lactobacilli reduced *C. albicans* vaginal load and hyphae formation and significantly reduced proinflammatory cytokines IL-17 and IFNγ. Interestingly, only *L. johnsonii* B-2178 protected the vaginal mucosa epithelium from histopathological changes (Elfeky et al., 2023), suggesting that the presence of *L. johnsonii* in the reproductive tract may help to control the growth of pathogens and maintain a healthy environment (Figure 1).

2.5 *L. johnsonii* and the perinatal and infant health

Clinical studies during the prenatal, perinatal, and infant periods are relevant since infancy is a critical period when the human microbiome starts to establish, and alterations in the microbiome composition during early life can impact overall host homeostasis and promote the development of disease risk factors. In the last decade, there has been increased interest in studying the short- and long-term effects of pre- and post-natal microbiome alterations in mothers and newborns (Fujimura et al., 2016; Fonseca et al., 2017; Fonseca et al., 2021). The study of maternal and infant microbiomes is an opportunity to explore the critical role of *L. johnsonii* in physiological outcomes during pregnancy and the infant's health.

Supplementing with probiotics during pregnancy can alter the composition of the gut and vaginal microbiota, breastmilk microbes, impact mother and infant immunity, and types of molecules that can be passed to the newborn (Rautava et al., 2012; Kuang and Jiang, 2020; Fonseca et al., 2021; Lehtoranta et al., 2022). A study evaluating the effect of prenatal supplementation with *L. johnsonii* MR1 in mice observed changes

in the gut microbiota and the systemic metabolic profile of supplemented mothers and their offspring. Offspring from *L. johnsonii*-supplemented mothers showed an expansion of bacteria belonging to *Lachnospiraceae* and *Muribaculaceae* families, similar to *L. johnsonii*-supplemented mothers. In addition, the systemic metabolic profile of mothers and offspring, as well as the mother's breastmilk metabolic profile, displayed similarity in the decreased presence of inflammatory metabolites (9,10-dihydroxyoctadecenoic acid (DiHOME), linoleic acid metabolite, and guanosine) (Fonseca et al., 2021). Similar metabolite changes were found in clinical studies with birth cohorts and showed that increases in systemic metabolites, such as DiHOME were associated with severe allergic disease in children (Fujimura et al., 2016). Likewise, clinical studies have shown that prenatal probiotic supplementation prevents infection, preterm delivery during pregnancy, and the manifestation of GI disorders and allergic responses in newborns (Baldassarre et al., 2018; Navarro-Tapia et al., 2020) (Figure 4).

To our knowledge, there are no clinical studies evaluating the effects of *L. johnsonii* administration during pregnancy. However, the presence of *Lactobacillus gasseri/Lactobacillus johnsonii* in the vagina of pregnant women has been associated with a decreased risk of early preterm birth (Tabatabaei et al., 2019). These data from animal models and clinical studies emphasize the potential role of *L. johnsonii* in women's reproductive health, including controlling pathogens and promoting healthy pregnancies. Additionally, early-life *L. johnsonii* exposure may be critical in establishing a healthy microbiome. Thus, the study of prenatal *L. johnsonii* supplementation represents an opportunity to assess the potential benefits in mothers and infants. Testing its use prenatally in mothers with vaginal dysbiosis and postnatally in infants born via C-section could be especially interesting.

2.6 *L. johnsonii* and the Respiratory system: gut-lung axis

The gut-lung axis concept postulates that alterations in the gut microbiota affect lung homeostasis. A correlation between the composition of the gut and lung microbiota from birth to adulthood suggests an interconnection. Altering the gut microbiome affects lung immunity and microbiota composition (Markey et al., 2018; Yagi et al., 2022). This could also be an effect generated by systemic microbiome-derived metabolites or even the previously described extracellular vesicles.

Exposure to environmental factors impacts the gut microbiome composition and has been associated with increased risk of asthma development (Fujimura et al., 2016; Yagi et al., 2022). Early-life exposure to livestock or pets significantly diversifies the gut microbiome and reduces allergy and asthma risk, highlighting the link between environment and microbiome composition and function (Ownby et al., 2002; von Mutius and Vercelli, 2010). House dust from dog owners was found to confer protection against ovalbumin and cockroach allergen-induced airway diseases when orally administered to mice (Fujimura et al., 2014). Notably, this protection in mice models was associated with an increased abundance of *L. johnsonii* MR1 in the gut (Fujimura et al., 2014; Ravi et al., 2023). Mice supplemented with *L. johnsonii* MR1 before an airway-allergen or respiratory syncytial virus (RSV) challenge presented reduced Th2-airway-related immune response and reduced mucus deposition in the airways (Fujimura et al., 2014; Fonseca et al., 2017). This effect was related to an attenuated proinflammatory phenotype in dendritic cells and increased pulmonary Treg cells due to altered systemic metabolic profile (Fujimura et al., 2014; Fonseca et al., 2017). Furthermore, maternal *L. johnsonii* MR1 supplementation protected the neonates from severe RSV immunopathology, presenting a significant decrease in airway mucus deposition, Th2 cytokines production, as well as reduced numbers of innate lymphocyte cells 2 (ILC2) and CD4+ T cells in the lung. Furthermore, offspring born from *L. johnsonii*-supplemented mothers maintain the immunomodulatory effect until adulthood. Adult offspring were infected with RSV and showed reduced RSV immunopathology, suggesting that prenatal *L. johnsonii* supplementation impacts mother and offspring gut microbiome composition and function and metabolic profiles that might alter long-term the mucosal and systemic immune response (Fonseca et al., 2021). This study emphasizes the importance of the mother's microbiome and the transfer of gut microbiota and immune-modulatory metabolites from mother to offspring to control allergic disease and respiratory pathogens during infancy (Fonseca et al., 2021). Pre- and post-natal probiotics have been recommended for patients with a high risk of developing allergic diseases (Fiocchi et al., 2015). Overall, these studies emphasize the importance of the gut microbiota (gut-lung axis) in maintaining respiratory health by delivering metabolites, regulating metabolism, improving immune system maturation, and possibly lung development. *L. johnsonii* may improve lung health and modulate the immune response to pathogens. Clinical studies are needed to assess its potential use in controlling inflammation in the respiratory tract (Figure 4).

2.7 *L. johnsonii* and skin barrier

Similar to the gut, skin microorganisms play an essential role in educating the cutaneous innate and adaptive immune response, and skin microbiota dysbiosis has been associated with skin diseases (Byrd et al., 2018), suggesting that manipulation of skin microbiota could help control skin pathologies, such as atopic dermatitis (AD) and eczema. Interestingly, reshaping of the gut microbiota, metabolic functions, and immune responses by oral probiotic interventions has been proposed to positively impact the clinical manifestations of inflammatory skin disorders such as AD (Fang et al., 2021). However, AD patients have skin dysbiosis characterized by a high prevalence of *Staphylococcus aureus (Brussow, 2016)* and a lower presence of *Lactobacillus* species in the skin, as well as increased abundance of *Clostridium difficile* and bifidobacterial species in the gut (Melli et al., 2020). A connection between the gut microbiome and the skin microorganism community has been suggested, which could potentially impact the immune response of patients who have inflammatory skin conditions.

The benefits of altering skin microbiota by directly applying pre-and probiotics have been reviewed previously (Al-Ghazzewi and Tester, 2014). The microbe-microbe interactions and immunological action of a topical lotion containing heat-treated *L. johnsonii* NCC 533 were assessed in an *in vitro* reconstructed human epidermis (RHE) model. Non-replicative *L. johnsonii* NCC 533 reduced *Staphylococcus aureus* colonization and boosted cutaneous innate immunity by inducing the expression of antimicrobial peptides, such as cathelicidin and β-defensin (Rosignoli et al., 2018). In addition, the topical use of heat-killed *L. johnsonii* NCC 533 in 21 patients with AD and swab positive for *Staphylococcus aureus*, reduced *S. aureus* load and the AD overall score in an open-label, multicenter clinical study (Blanchet-Rethore et al., 2017). These studies showed an alternative use of *L. johnsonii* to control skin pathogens and boost the skin innate immune response. The authors pointed out the importance of non-replicating bacteria in this interaction with the host and argued that heat-killed *L. johnsonii* NCC 533 maintains its ability to stimulate cytokine production and induce the expression of antimicrobial peptides. It is possible that heat-killed *L. johnsonii* activates innate immune receptors by interacting directly with the skin epithelial cells in a TLR2-dependent but TLR4/MD-2-independent manner (Elson et al., 2007), helping to control *Staphylococcus aureus* growth. It is important to note that this intervention is not considered probiotic-mediated, as it does not contain live *L. johnsonii*. The interconnected nature of skin and gut microbiome interactions has not been thoroughly examined; however, they likely interact through their influence on local and systemic immune responses. Additional research is needed to better understand the potential skin health benefits of *L. johnsonii*, offering a valuable research opportunity.

2.8 *L. johnsonii* anticarcinogenic activity

Recent findings have highlighted the importance of probiotics for cancer treatment (Slizewska et al., 2021). The gut microbiome's composition and function are linked to clinical response to immunotherapy for antitumor treatment (Weersma et al., 2020). Furthermore, a reproducible shift in bacterial richness and metabolic pathways has been consistently identified across different cohorts of individuals with colorectal cancer, which opens the possibility of using microbial signatures as biomarkers for intestinal cancer (Thomas et al., 2019). Microbiome-derived metabolites, such as short-chain fatty acids (SCFA), decreased inflammation and cancer cell proliferation (Ocadiz-Ruiz et al., 2017), and regulate the onset and progression of inflammatory responses (Richards et al., 2016). *L. johnsonii* is essential for influencing intestinal microbiota composition and metabolic activity, producing compounds with anticarcinogenic activity, stimulating the immune system, and modulating cell proliferation and apoptosis (Slizewska et al., 2021). Interestingly, *in vitro* and *in vivo* studies have shown that *L. johnsonii* L531 can produce high levels of SCFA, such as butyric, acetic, and lactic acids, affecting the metabolic profile and gut resident microbiota (He et al., 2019). Additionally, a comprehensive analysis of operational taxonomic units (OTU) in a mouse model of ataxia-telangiectasia, a genetic disorder associated with B cell lymphoma, showed that the less cancer-prone mouse colony had higher *L. johnsonii* colonization. Short-term restorative oral treatment with *L. johnsonii* RS-1 decreased systemic genotoxicity and inflammatory state in mice prone to developing cancer by diminishing hepatic T and NK cells, pro-inflammatory cytokines IL-1β and IFN-β levels, and elevated anti-inflammatory cytokines TGF-β and IL-10 (Yamamoto et al., 2013). This study shows the capacity of *L. johnsonii* strains to regulate the inflammatory response in cancer, like other beneficial bacteria that decrease inflammation and cancer cell proliferation and possibly modulate the efficacy of anticancer therapy (Lee et al., 2021). However, the mechanism by which each probiotic intervention exerts its anticarcinogenic activity must be clarified.

3 Concluding remarks

L. johnsonii is a commensal bacterium that has been isolated from vaginal and gastrointestinal (GI) tracts of vertebrate hosts, including humans, rodents, swine, and poultry. *Lactobacillus*-based probiotic supplements are popular because of the health advantages they offer and species such as *L. johnsonii* are of particular interest due to their potential health-promoting properties. *L. johnsonii* possesses exceptional properties that help it to maintain homeostasis in the host by controlling the expansion of pathogens, modulating metabolic pathways, and regulating the immune response systemically and locally. The modulation and restoration of healthy microbiota by *L. johnsonii* offer positive outcomes and represent an important tool to aid treatments and control specific pathologies' development by directly modulating microbiota composition and function and, consequently, local and systemic immune responses. While several of these health-beneficial properties have been investigated *in vitro* settings and animal models (Table 1), there is still insufficient scientific evidence in humans to support these claims (Table 2). Studying the

TABLE 1 *Lactobacillus johnsonii* studies in animal models and *in vitro* experiments.

Ref.	Model	Strain(s)	Principal Outcome(s)
(Ahire et al., 2021)	*In vitro*	*L. johnsonii* UBLJ01	*L. johnsonii* formed biofilms *in vitro* and had a standard antibiotics susceptibility. Secreted exopolysaccharided and inhibited pathogens growth (*E. coli, Gardnerella vaginalis, Proteus mirabilis*, and *C. albicans*).
(Sgouras et al., 2005; Bergonzelli et al., 2006)	*In vitro* and *H. pylori* infected C57BL/6 mice model	*L. johnsonii* NCC 533	GroE protein facilitated *L. johnsonii* NCC 533 binding to epithelial cells and mucus proteins in a pH-dependent manner and aided *H. pylori* aggregation. *H. pylori* induces pH-dependent IL-8 secretion. *In vivo* studies

(Continued)

TABLE 1 Continued

Ref.	Model	Strain(s)	Principal Outcome(s)
			showed that *L. johnsonii* NCC 533 administration attenuated *H. pylori*-associated gastritis by reducing proinflammatory chemokine, cytokine expression, and immune cell infiltration. *H. pylori*-induced IL-8 secretion is reduced *in vitro* in the presence of neutralized *L. johnsonii* NCC 533 culture supernatants, without loss of *H. pylori* viability.
(Aiba et al., 2015) (Aiba et al., 2019)	Human gut microbiota-associated mice model and germ free mice model	*L. johnsonii* No.1088	*L. johnsonii* No.1088 suppressed gastric acid production and inhibited the growth of *Helicobacter pylori*.
(Yang et al., 2022c)	Chronic diarrhea in rhesus macaques (RMs. *Macaca mulatta*)	*L. johnsonii*	RMs with chronic diarrhea showed a microbiome depleted in *L. johnsonii*, *L. reuteri* and *L. amylovorus*. *L. johnsonii* isolated from asymptomatic RMs possessed probiotic genes encoding lactate dehydrogenases, mucus-binding proteins, bile salt hydrolase and bile salt transporter.
(Mu et al., 2017)	MRL/lpr mice (lupus nephritis model).	Mix of 5 *Lactobacillus* strains including *L. johnsonii* 135-1-CHN	Lactobacillales supplementation had a sex-dependent anti-inflammatory effect. It restored the gut mucosal epithelial barrier, diminished IL-6, upregulated IL-10 and IgG2 levels, and skewed the Treg-Th17 balance in the kidney towards Treg, leading to immunosuppression
(Xin et al., 2014)	High fat diet (HFD) mice model	*L. johnsonii* BS15	*L. johnsonii* BS15 protected mice from hepatic steatosis and hepatocyte apoptosis, enhanced the liver antioxidant defense system, and increased the expression of the fasting-induced adipose factor. *L. johnsonii* BS15 administration modulated gut barrier function and gut microbiota, as well as downregulated TNFα expression in the liver.
(Isobe et al., 2012)	*Helicobacter pylori* infection model of Mongolian gerbil	*L. johnsonii* NCC 533	*L. johnsonii* NCC 533 impaired *Helicobacter pylori* colonization and ameliorated gastritis.

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TABLE 1 Continued

Ref.	Model	Strain(s)	Principal Outcome(s)
(Nadatani et al., 2019)	Mice Indomethacin (IND)-induced intestinal damage.	*L. johnsonii*	*L. johnsonii* administration protected from IND-induced intestinal damage and reduced IL-1β expression.
(He et al., 2019) (Yang et al., 2020) (Yang et al., 2022b) (Xia et al., 2020) (Chen et al., 2021)	Piglets model of *Salmonella* sp. infection and *in vitro* studies	*L. johnsonii* L531	Supplemented piglets had reduced diarrhea severity, restored tight junctions (ZO-1, Occludin, and Claudin-1), exhibited *Salmonella* sp. clearance, and restored SCFA. Attenuated tissue damage and inflammation and contributed to the maintenance of intestinal homeostasis by reducing expression of pro-inflammatory innate cytokines (IL-6, IL-1β, IL-8, and TNFα) and NOD-related proteins (NOD1/2, RIP2), regulating NLRC4 and NLRP3 inflammasomes assembly and NF-κB signaling pathway (TLR4, MyD88, p-IκBα, and p-p65), reduced ER stress and cellular damage, as well as inhibition of mitochondrial damage and mitophagy, and modulating autophagy degradation.
(Liu et al., 2015)	*In vitro* studies with IPEC-J2 cells	*L. johnsonii* P47-HY	*L. johnsonii* P47-HY supplementation improves the integrity of the gut barrier by stimulating the production of cytoprotective heat shock proteins and fortified cellular defense against enterotoxigenic *Escherichia coli* by regulating tight junction proteins and direct interactions with pathogens.
(Zhang et al., 2012)	*In vitro* studies with HT-29 cells	*L. johnsonii* F0421	*L. johnsonii* F0421 inhibits adherence of *Shigella sonnei* in a dose dependent manner. S-layer proteins on *L. johnsonii* F0421 have a role in this exclusion adhesion process.
(Bereswill et al., 2017; Zhang et al., 2021)	Mice model *of Campylobacter jejuni* infection	*L. johnsonii*	Prophylactic supplementation of *L. johnsonii* did not alter *Campylobacter jejuni* growth, but diminished colonic apoptosis and attenuates colonic hyperplasia, as well as reduced systemic proinflammatory mediators (IL-6, MCP1, TNFα and

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TABLE 1 Continued

Ref.	Model	Strain(s)	Principal Outcome(s)
			nitric oxide) and immune cell infiltration in the colonic tissue *L. johnsonii* restored abnormal expression of antimicrobial peptides (lysozyme) and abrogated ER stress–related cell apoptosis.
(Hu et al., 2021)	Mice model *of E. coli* infection	*L. johnsonii* NJ13	Ameliorate the diarrhea index and increased body weight. Improved microbiota structure (reduction of *Helicobacter pylori* and *Shigella*) and increasing in butyric acid-producing bacteria and *Lactobacillus*.
(Ekmekciu et al., 2017)	Secondary abiotic mice	*L. johnsonii*	*L. johnsonii* recolonization increased CD4+ and CD8+ T cells populations in the small intestine and spleen, and sustained IL-10 production in the colon. A minor increased of the frequency of intestinal regulatory and memory/effector T cells and activated dendritic cells was observed.
(Travers et al., 2016; Allain et al., 2017)	Mice model of *Giardia duodenalis* infection *in vitro* studies	*L. johnsonii* NCC 533	*L. johnsonii* La1 genome possessed probiotic genes encoding *bile-salt-hydrolase* (*bsh*) enzymes. BHS enzymes identified in the supernatants of *L. johnsonii* La1 prevent *Giardia duodenalis* growth *in vitro*.
(Zhang et al., 2021) (Jia et al., 2022) (Charlet et al., 2020) (Charlet et al., 2022)	Mice model of colitis	*L. johnsonii*	*L. johnsonii* supplementation alleviated induced colitis in different mice models. <u>*Citrobacter rodentium*-induced colitis model</u>: *L. johnsonii* pretreatment regulated inflammation by diminishing systemic proinflammatory cytokines (TNFα, IL1β, IL6, IL17a, IFNγ and MCP1) and immune cells infiltration (T cells and macrophages) in the gut. Restored concentrations of antimicrobial peptides such as lysozyme and attenuates ER stress-related cell death. <u>DSS-induced colitis model:</u> *L. johnsonii* supplementation alleviated the severity of diarrhea, altered gut microbiota composition by increasing the presence of SCFA-producing bacteria, as well

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TABLE 1 Continued

Ref.	Model	Strain(s)	Principal Outcome(s)
			as bacteria with anti-inflammatory, immunomodulatory and antifungal properties.
(Bertolini et al., 2021)	Mice model of fungal infection *in vitro* coculture model	*L. johnsonii* MT-LB4	*C. albicans* infection in immunocompromised mice was associated with enterococci relative abundance. *Lactobacillus* sp. depletion with antibiotics showed a negative correlation between these bacteria genera and the opportunistic bacteria *Enterococcus* in *Candida*-infected mice. *L. johnsonii* has an inhibitory effect on *Enterococcus faecalis* and planktonic *Candida albicans* growth.
(Vazquez-Munoz et al., 2022)	*In vitro*: Coculture of *L. johnsonii* and *C. albicans*	*L. johnsonii* MT4	*L. johnsonii* MT4 has genes encoding products with anticandidal properties (bacteriocin, hydrolases, biosurfactant). *L. johnsonii* MT4 reduced the metabolic activity of *C. albicans* biofilms in a dose–response pattern and impacted its *Candida* dimorphic transition.
(Roesch et al., 2009) (Lai et al., 2009) (Lau et al., 2011; Teixeira et al., 2018) (Valladares et al., 2010) (Valladares et al., 2013) (Kingma et al., 2011)	Bio-Breeding diabetes-prone (BBDP) Rats and non-obese diabetic (NOD) mice	*L. johnsonii* N6.2	*L. johnsonii* bacteria abundance in stools samples differs between diabetes-prone and diabetes-resistant rats. Two cinnamoyl esterases enzymes isolated from *L. johnsonii* N6.2 have potential to mitigates diabetes symptoms. Supplementation with *L. johnsonii* N6.2 isolated from Bio-Breeding diabetes-resistant (BBDR) rats, delays the onset of TD1 in BBDP rats. *L. johnsonii* N6.2 supplementation in BBDP rats pulsed dendritic cells to mediate Th17 bias and modulates the assembly of the inflammasome. H_2O_2 produced by *L. johnsonii* N6.2 abolished the rate-limiting enzyme of tryptophan catabolism, indoleamine 2,3-dioxygenase (IDO).
(Harrison et al., 2021) (Teixeira et al., 2022)	*In vitro*	*L. johnsonii* N6.2	*L. johnsonii* N6.2-derived nanovesicles are rich in glycerophosphoglycerols and contains several unique and differentially expressed proteins compared to the

(Continued)

TABLE 1 Continued

Ref.	Model	Strain(s)	Principal Outcome(s)
			bacteria cellular membrane. IgA and IgG antibodies against protein domains from nanovesicles were generated in the plasma of individuals supplemented with *L. johnsonii* N6.2. Nanovesicle-derived bioactive molecules suppressed cytokine-induced apoptosis and promoted a tolerogenic immune environment by skewing macrophages to a M2 tolerogenic phenotype associated to STAT3 activation, expression of AHR-dependent genes, and IL-10 secretion.
(Cuaycal et al., 2023)	*In vitro*	*L. johnsonii* N6.2	Bone marrow-derived dendritic cells (BMDCs) showed an upregulation of maturation-migratory and immunoregulatory related genes when incubated with *L. johnsonii* N6.2 purified phospholipids. These BMDCs presented a tolerogenic-migratory DC-like phenotype, suggesting its capacity to induce a regulatory T cell response.
(Kling et al., 2018)	Rat model of obesity (HFD)	*L. johnsonii* N6.2	*L. johnsonii* N6.2 reduced AKT phosphorylation and downregulated various genes that are part of the downstream signaling pathway of mTORC1 in female rats.
(Elfeky et al., 2023)	Vulvovaginal candidiasis rat model	*L. johnsonii* B-2178	*L. johnsonii* B-2178 reduced *C. albicans* vaginal load and hyphae formation, as well as pro-inflammatory cytokines IL-17 and IFN-γ and NF-κB, while minimized the epithelium damage and restored normal vaginal architecture.
(Fonseca et al., 2017) (Fonseca et al., 2021) (Fujimura et al., 2014)	Mice model of asthma and Respiratory Syncytial Virus (RSV) infection. Neonatal mice model of RSV infection. *In vitro* studies.	*L. johnsonii* MR1	Intestinal *L. johnsonii* MR1 presence was linked with allergic, and RSV reduce immunopathology in mice exposed to house-dust from homes with pets. *L. johnsonii* MR1 oral supplementation to adult mice altered gut microbiome communities and systemic metabolic profile that reduced RSV immunopathology, airway Th2 inflammatory response,

(Continued)

TABLE 1 Continued

Ref.	Model	Strain(s)	Principal Outcome(s)
			and dendritic cell function, as well as increased pulmonary Treg cells. Prenatal supplementation with *L. johnsonii* MR1changed the gut microbiota and the systemic metabolic profile of supplemented mothers and their offspring. *L. johnsonii*-supplemented Mothers and their Offspring showed expansion of Lachnospiraceae families as well as, changes in the systemic and breastmilk's metabolic profile, that presented reduced levels of inflammatory metabolites. The neonates born from supplemented mother showed reduced RSV immunopathology and dampened Th2 immune response.
(Rosignoli et al., 2018)	*In vitro* human epidermis (RHE) model	Heat-treated *L. johnsonii* NCC 533	Heat-treated *L. johnsonii* suspensions reduced the binding of *Staphylococcus aureus*. Heat-treated *L. johnsonii* induced the presence of antimicrobial peptides.
(Yamamoto et al., 2013)	Mice model of Ataxia-telangiectasia	*L. johnsonii* RS-1	*L. johnsonii* restoration diminished genotoxicity by reducing hepatic NK and T cells, pro-inflammatory cytokines IL-1β and IFN-β and increasing expression of anti-inflammatory cytokines TGF-β and IL-10.
(Hsieh et al., 2012)	*In vitro* and rat model	*L. johnsonii* MH-68	*L. johnsonii* MH-68 suppressed *H. pylori* urease activity, dampened its adhesion capacity to epithelial cells and inhibits bacteria growth *in vitro*. *L. johnsonii* MH-68 supplementation effectively decreased *H. pylori* load in the gastric mucosa and lowered the expression of IL-8 and lymphocyte infiltration.

APCs ,antigen presenting cells; AHR, aryl hydrocarbon receptor; BSH, bile-salt-hydrolase ; BBDP, Bio-Breeding diabetes-prone; BBDR, Bio-Breeding diabetes-resistant; CFU, colony formation units; DSS, dextran Sulfate Sodium; DHA, docosahexanoic acid; ER , endoplasmic reticulum ; IND, indomethacin; KC, keratinocyte-derived cytokine; HFD, high fat diet; MIP-2, macrophage inflammatory protein 2; mTORC1, mTOR complex 1; MOI, multiplicity of infection ; NK, natural killer cells; NLRC4, NLR family apoptosis inhibitory protein CARD domain-containing protein 4; NSAID, NLRP3, non-steroidal anti-inflammatory drugs; nucleotide-Binding Domain, Leucine-Rich–Containing Family, Pyrin Domain–Containing-3; OA, oleic acid; PA, palmitic acid; PUFAs, polyunsaturated fatty acids; RSV, respiratory syncytial virus; RM, rhesus macaques; SCFA, short chain fatty acids; SLE, systemic lupus erythematosus; TCR, T cells receptor; TD1, type 1 diabetes.

TABLE 2 *Lactobacillus johnsonii* studies in human cohorts.

Ref.	Study design	Strain(s)	Principal Outcome(s)
(Davoren et al., 2019)	Healthy humans (11) with normal diet	Daily 100mL of yogurt containing 10^{10} CFU of *L. johnsonii* 456 for 7 days	Daily consumption as part of yogurt for 7 days impacted the microbiota composition, elevating the presence of lactic acid bacteria and *L. johnsonii* 456 DNA unique sequences were still detected in human fecal samples weeks after intake was stopped.
(Marcial et al., 2017)	Randomized, double-blind, placebo-controlled trial	*L. johnsonii* N6.2 (5×10^8 CFU per capsule) during 8 weeks with 4 weeks washout period Placebo: skim milk	*L. johnsonii* N6.2 impacted the innate and adaptative immune systems and effects on the tryptophan metabolism are dependent on the baseline microbiota composition, specifically the lactic acid bacteria population.
(Wang et al., 2022)	Randomized, double-blind placebo-controlled trial	Probiotic mix including *L. johnsonii* MH-68 Placebo: insulin therapy without probiotic mix	Probiotics mix changed the microbiota composition of TD1 patients, increasing *Bifidobacterium animalis, Akkermansia muciniphila*, and *Lactobacillus salivarius*, reduced fasting blood glucose levels and serum proinflammatory cytokines.
(Marteau et al., 2006; Van Gossum et al., 2007)	Multicenter, randomized, controlled trial/Randomized, double blind, placebo-controlled trial	*L. johnsonii* NCC 533 (10^{9-10} CFU)	Supplementation failed to prevent early endoscopic recurrence after post-ileocecal resection of macroscopic lesions in patients with CD.
(Michetti et al., 1999; Pantoflickova et al., 2003)	Randomized, double-blind study/Randomized, double-blind, placebo-controlled trial	*L. johnsonii* LJ1/ *L. johnsonii* NCC 533 supernatant	*L. johnsonii* LJ1 reduced *H. pylori-associated* gastritis, *H. pylori* load, and increased mucus production. *L. johnsonii* NCC 533 supernatant inhibited *H. pylori* growth *in vitro*, but not *in vivo*
(Tabatabaei et al., 2019)	Nested case-control study (94 women with spontaneous preterm birth cases)	*Lactobacillus gasseri/ Lactobacillus jonhsonii*	*Lactobacillus gasseri/Lactobacillus jonhsonii* oligotype was associated with a decreased risk of early spontaneous preterm birth.
(Blanchet-Rethore et al., 2017)	Open-label, multicenter clinical study	Heat-treated *L. johnsonii* NCC 533, non-replicating probiotic. Lotion	Application of *L. johnsonii* NCC 533 lotion in patients with atopic dermatitis, reduced Staphylococcus aureus colonization as well as atopic dermatitis lesions.

CFU, colony forming units; CD, Crohn disease; TD1, Type 1 diabetes.

microbiomes of pregnant women and their infants presents an opportunity to investigate the significant role of *L. johnsonii* in impacting physiological outcomes and infant health. Hence, to validate the efficiency of *L. johnsonii* as a therapeutic probiotic, it is necessary to conduct more randomized clinical trials that encompass diverse populations, including individuals of different sexes, ages, and dietary habits. Other important parameters to consider include health status, underlying diseases or conditions, dosage, route and frequency of administration, the location of the study, and ensuring an adequate sample size for accuracy (Dronkers et al., 2020).

These trials should also adhere to intent-to-treat principles, conduct prospective evaluation, and use an adequate control group (Evans, 2010; Lim and In, 2019) to generate scientific evidence of the mechanism of action of *L. johnsonii* and validate its benefit during health and disease.

Author contributions

LA: Writing – original draft, Writing – review & editing. KR: Writing – original draft, Writing – review & editing. GH: Funding acquisition, Writing – original draft, Writing – review & editing. NL: Funding acquisition, Writing – original draft, Writing – review & editing. WF: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Live biotherapeutic products: a capstone for prevention of recurrent *Clostridiodes difficile* infection

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Clostridiodes difficile infection (CDI) continues to be one of the leading causes of healthcare-acquired diarrhea and infections, and recurrence is the biggest challenge in its management. As technology and research have led to a better understanding of the pathophysiology of *C. difficile*, we have come to appreciate the role that the gastrointestinal microbiota plays in infection onset and the prevention of recurrence. The gut microbiota is disrupted in those with CDI, which allows further propagation of the infection leading to recurrence, if the microbiota deficiency is unable to regrow itself. While antimicrobial therapy is necessary for treatment of any CDI, these therapeutics do not address the underlying disturbance of microbiota. Microbial remodulation therapies have been developed supplementing the microbiota deficiency that exists after the standard of care antimicrobial resulting in a reduction of recurrence. Fecal microbiota transplantation (FMT) was the initial attempt for this type of therapeutic and proved to be safe and effective, however never achieved FDA approval. In light of this, live biotherapeutic products (LBPs) were developed by pharmaceutical companies through a more standardized and regulated process. These products are safe and efficacious in reducing CDI recurrence when given after a standard of care antimicrobial, eventually leading to FDA approval of two products that can now be used widely in clinical practice.

KEYWORDS

***Clostridioides (Clostridium) difficile* infection, *difficile*, fecal micriobiota transplantation, FMT, live biotherapeutic product**

1 Introduction

Clostridioides difficile infection (CDI) continues to be a leading cause of antibiotic associated and healthcare related diarrhea in the United States (Lessa et al., 2015; Magill et al., 2018). With an estimated half million annual infections, approximately 30,000 deaths in the United States and a crude overall incidence rate of 121.2 cases per 100,000 persons,

the burden of CDI is tremendous (Lessa et al., 2015; Centers for Disease Control and Prevention (CDC), 2019; Guh et al., 2020). One of the biggest challenges in managing CDI is recurrence. After an initial episode, recurrence rates have been estimated to be as high as 35%, and this rate increases with each successive episode, including up to 45% after the first recurrence and ~60% after the second recurrence (McFarland et al., 2002; Pepin et al., 2005; McDonald et al., 2018).

The treatment of CDI has historically included antimicrobials as a singular therapy. The American College of Gastroenterology guidelines published in 2021 recommend the use of vancomycin, fidaxomicin or metronidazole for non-severe initial infection (Kelly et al., 2021a). For severe initial infection (defined as a white count of >15,000 cells/mm^3 and/or serum creatinine >1.5 mg/dL) treatment with either vancomycin or fidaxomicin is recommended. For an initial recurrence, a taper-pulse regimen of vancomycin or a course of fidaxomicin should be considered. As technology and research have led to a better understanding of the pathophysiology of CDI, we have come to appreciate the role of the microbiota in preventing recurrence and with this, additional therapeutics have been developed supplementing the standard of care (SOC) antimicrobial resulting in a reduction of rates of recurrence (Seekatz and Young, 2014).

The vegetative phase of *C. difficile* releases toxins causing symptoms including abdominal pains, fevers and diarrhea. Standard of care antimicrobials, such as vancomycin and fidaxomicin, control the vegetative phase of the infection. The spore phase is more resilient transferring from patient to patient, and also remaining within a patient's system after SOC antimicrobials for extended periods. Antimicrobials have little to no impact on the spore phase, however, a healthy diverse microbiota can eradicate this phase decreasing the likelihood that it reconverts, or germinates, back into the vegetative phase, causing a recurrence (Seekatz and Young, 2014).

With this in mind, the treatment landscape for recurrent CDI (rCDI) has evolved dramatically in the last ten to fifteen years. A disruption of the gut microbiome composition, known as dysbiosis, allows spore germination, reduces the inherent resistance against colonization *with C. difficile* allowing vegetative growth with toxin production (Seekatz et al., 2016). Prior studies have demonstrated that low microbial diversity, particularly a decrease of the bacterial phyla *Bacteroidetes* and *Firmicutes*, plays a vital role in the pathogenesis of CDI (Chang et al., 2008). Thus, while antimicrobial therapy is necessary for treatment of CDI, these treatment modalities cannot directly address or correct the underlying dysbiosis. With SOC antimicrobials alone, we rely on natural regrowth of the deficient *Bacteroidetes* and *Firmicutes*, which frequently does not occur appropriately resulting in the high rates of recurrence of CDI. In fact, vancomycin itself can further deplete the diversity of the microbiota leaving patients prone to getting CDI recurrence (Louie et al., 2009; Ajami et al., 2018). In the light of this, therapies targeting intestinal microbial restoration following antimicrobials, thereby treating both phases of the infection, have rapidly evolved and are now widely available.

Therapies replenishing the gut microbial diversity are designed to engraft desirable microorganisms that are deficient, regaining the so called "colonization resistance." This was originally achieved via fecal microbiota transplantation (FMT) but in recent times, the more sophisticated approach of live biotherapeutic products (LBPs) have become available. Broadly, FMT involves the transfer of stool from a healthy screened donor into the intestinal tract of the recipient, whereas the United States Food and Drug Administration (FDA) approved LBPs widely screen donors, sample the stool for consistency of microbes to be transferred and have standardized approaches for administration. This pivotal change in management underlies the progress achieved within the therapeutics for rCDI. This manuscript will outline the differences between thes two FDA approved LBPs, and clinical trial evidence related to the efficacy of LBPs.

2 Fecal microbiota transplantation

The principle of FMT, as it relates to CDI, is to replenish the entire microbial diversity supplementing the deficiencies of desirable bacteria within the intestinal tract, particularly *Bacteroidetes* and *Firmicutes*. This introduction of a broad array of microbes aims to reverse the dysbiosis created by CDI establishing metabolic equilibrium that minimizes the likelihood of future recurrent episodes. Guidelines from the Infectious Disease Society of America and Society of Healthcare Epidemiology of America in 2021, along with the American College of Gastroenterology 2021, recommend the use of FMT after two or more recurrences of CDI that have been treated with appropriate antibiotics (Johnson et al., 2021; Kelly et al., 2021a). The American Gastroenterological Association guideline discussing fecal microbiota-based therapies published in 2024 advised consideration for fecal-microbiota based therapies, such as FMT and LBPs, in adults with "recurrent *C. difficile*" (Peery et al., 2024). This effectively includes patients with first recurrence, expanding the indications for these treatments.

Although the clinical trials considering FMT are heterogenous in reporting of methodology and design there are now many studies considering FMT for the prevention of recurrence of CDI (Feuerstadt et al., 2022a). The efficacy for FMT to prevent rCDI has been seen to vary, being more than 85% in many early observational studies but closer to 72% in controlled clinical trials (McGovern et al., 2021). An observational study of the largest stool bank in the United States that utilized centralized screening and processing, assessed 5,344 patients between 2014 and 2018 reporting a clinical resolution rate of 78% (Osman et al., 2022). Another study reported prospectively collected data from the AGA FMT National Registry, reporting that across 259 patients, they had 1 month follow up data on 222 with clinical resolution rates of 90% at that time. Of the 112 patients that had data available for 6-month follow up, 96.4% remained without recurrence (Kelly et al., 2021b).

A recent systematic review and meta-analysis evaluated the efficacy of FMT and LBPs for the prevention of rCDI in prospective clinical trials and compared this to the data from randomized clinical trials (Tariq et al., 2023). Across 19 clinical trials, clinical resolution with FMT or LBP was seen in 78% of patients, and among these, a resolution rate of 72% was observed in the 10 trials

that included a control arm. While data from most studies have been encouraging, there has been significant heterogeneity in study design, screening of donors, composition of microbiota that was administered, assessment of outcomes and mode of administration among these clinical trials. Given this lack of standardization across studies with FMT, efforts focusing on microbiota therapeutics that are standardized with consistent microbial consortia have created the sub-category of LBPs (Table 1). This advancement of technology has resulted in better studies and resulted in the FDA approval of two products that will be discussed below.

3 Fecal microbiota transplantation versus live biotherapeutic products

3.1 Donor screening

Screening of potential donors for FMT has evolved from local screening and rudimentary methods of stool processing in an endoscopy suite prior to a colonoscopic application of the blended material to more rigorous screening and packaging of the samples with the development of stool banks. For FMT, the screening process usually includes a rigorous history to exclude chronic usage of medications that can impact the microbiota, chronic diseases thought to be associated with dysbiosis of the microbiota, such as diabetes mellitus, metabolic syndrome, and a variety of blood tests including testing for human immunodeficiency virus, hepatitis A, hepatitis B and hepatitis C and stool testing for CDI, ova and parasites (Bakken et al., 2011). For FMT, there are no "standard" screening criteria for the donor and it is recommended that screening be comprehensive (Peery et al., 2024). Donor screening criteria are not uniform across individual institutions or stool banks and is site dependent (Tariq et al., 2018). Stool banks require FDA oversight under an investigational new drug (IND) application, therefore, meeting a standardized minimum requirement that is not necessarily met by local sites. Donor screening for LBPs has undergone rigorous assessment by the FDA having comprehensive safety checks to ensure consistent expansive safety within each sample distributed.

TABLE 1 Key features of difference between fecal microbiota transplantation and live biotherapeutic products.

		Fecal Microbiota Transplantation	Live Biotherapeutic Products
I.	*Donor Screening*	++	+++
II.	*Sample Screening*	?	+++
III.	*Product Manufacturing Procedure*	?	+++
IV.	*Clinical Trial Data*	+	+++
V.	*Safety Data*	+	+++
VI.	*Ease of Access*	?	+

+ Good.
++ Better.
+++ Best.
? Unknown/Not applicable.

3.2 Sample screening

FMT procedures typically require comprehensive donor screening, but once the sample is donated, there is no further assessment of the microbial contents of what will be administered to the recipient. It is assumed that since this is a broad spectrum of microorganisms that sufficient levels of *Bacteroidetes* and *Firmicutes* are being administered, in the case of CDI, but the volume and proportion of these bacterial phyla, or any other microorganisms is not regularly assessed. In contrast, once the sample has been obtained from donors for LBPs, there is a quality assurance that what is administered is consistent with the proprietary formulation that was used in the FDA overseen clinical trials. Thus, LBPs have more consistent end-products, resulting in more predictable safety and efficacy.

3.3 Product manufacturing procedure

The manufacturing process is very heterogenous for individual institutions that are not engaging with a stool bank for the samples they will administer. There was no codified format for production of these samples and each institution mixed the samples differently dependent upon resources available to the clinical care team. Moreover, the process of administration, be it through colonoscopy or oral administration, added an extra layer of heterogeneity in its use. Stool banks are more regulated, and follow good clinical practice for their production under their IND application with the FDA (FDA, 2013). The LBPs are approved by the FDA, and as a result, always are produced under Good Manufacturing Practice, having regulation and uniform manufacturing procedures that are approved by FDA.

3.4 Clinical trial data

Most trial data for FMT varies in terms of the tools used for diagnoses, the time measured to recurrence, FMT preparation techniques, modes of administration, criteria for an adverse event and outcomes assessed - all of which have led to significant heterogeneity of this data. Trial data pertaining to LBPs is more uniform by virtue of its FDA trial oversight, standardized inclusion criteria, processing techniques and formulation as well as administration, making the data more codified, reliable and reproducible.

3.5 Safety data

Adverse events following FMT are common, but are mostly mild and include abdominal discomfort, diarrhea, nausea, vomiting and constipation (Saha et al., 2021; Kelly et al., 2021b). Serious

adverse events have been seen in <10% patients and the majority are thought to be unrelated to FMT (Saha et al., 2021; Kelly et al., 2021b). A small number of bloodstream infections caused by organisms in FMT have been documented as well (Solari et al., 2014; DeFilipp et al., 2019), however most of such cases have occurred in patients who are immunocompromised and therefore at a high risk of bacterial translocation. In contrast, safety data extracted from LBP trials have revealed a similar frequency of events with the majority being mild to moderate adverse events of similar gastrointestinal nature and thus far there are no reports of infectious diseases being transmitted by LBPs (Lee et al., 2023). Further, controlled clinical trials require more stringent safety reporting, including solicited adverse events and adjudication by independent Drug and Safety Monitoring Boards.

3.6 Ease of access

The last, but perhaps most clinically relevant factor is ease of access of both of these therapies. As stated above, FMT cannot be undertaken by a physician or a practice without an IND through the FDA or via a stool bank with an approved IND with the FDA. While LBPs, being approved by the FDA, can be administered by any licensed and practicing physician in the appropriate clinical setting. The question of cost and insurance coverage is one that is still being defined.

With this foundational knowledge in mind, let's discuss these two ground-breaking new LBP therapies in more detail.

4 Fecal microbiota live-JSLM (Rebyota™, RBL)

RBL was the first FDA approved LBP consisting of a broad consortium of spore and non-spore forming bacteria, including a minimum threshold of *Bacteroides*. It is a single, rectally administered dose containing 150mL of therapeutic material and 10^7 microbes per mL or 15 x 10^8 microbes per treatment.

4.1 Clinical trial data

A phase III prospective, randomized, double-blind, placebo-controlled trial (PUNCH CD3) assessed the efficacy of RBL in patients with the one or more recurrences (Khanna et al., 2022a). Diagnosis for entry into the study was at the discretion of the local investigator including PCR and EIA/glutamate dehydrogenase (GDH). Patients were treated with SOC antibiotics for a minimum of ten days, followed by a washout period of one to two days, after which they were randomized to either receive a single dose of RBL or a single dose of placebo via rectal instillation. Following 8-weeks, the overall model adjusted efficacy of the SOC+RBL was 70.6% compared with 57.5% for the SOC+placebo. A Bayesian statistical analysis leveraging data from the phase II randomized controlled trial was utilized, demonstrating a posterior probability of superiority of 0.991 and statistical significance. The PUNCH CD3 open label study, was reported as an interim analysis assessing 300 patients with first recurrence and beyond who had medical co-morbidities that were not included in the original phase 3 trial including irritable bowel syndrome and inflammatory bowel disease. Of these patients, 74.6% had a clinical resolution at 8 weeks after treatment with RBL; of those 84.0% remained responsive 6 months after treatment (Khanna et al., 2022b). There were no concerning adverse or serious adverse events observed in either trial.

4.2 Constitution of fecal microbial structure and resultant metabolome

To further prove the durability of these results with the clinical success demonstrated by this trial, the fecal microbial constitution of patients was investigated. Patients submitted stool samples prior to treatment, and then at 1, 4 and 8 weeks, 3 and 6 months after receiving study treatment (Blount et al., 2021). The microbiome of patients who received RBL showed a rapid shift with an increase in favorable *Bacteroides* and *Clostridia* and decrease in the proinflammatory *Gammaproteobacteria* and *Bacilli*. Furthermore, this change was durable during the 6 month follow up.

Bile acids are known to play a critical role in the life cycle of *Clostridioides difficile*. Primary bile acids trigger the spore germination and permit growth of *C. difficile* while secondary bile acids inhibit this process and interfere with the propagation of the infection (Winston and Theriot, 2016). Further testing the credibility of RBL, the stool samples collected from participants were also evaluated for changes in bile acid compositions (Papazyan et al., 2021). Here, they found that primary bile acids predominated before treatment, while secondary bile acids were more prevalent after treatment, and these changes were more significant in the patient population treated with RBL, further validating its downstream biochemical effects.

4.3 Administration

The product is administered 24-72 hours after completion of the SOC antimicrobial course for CDI. The application kit consists of a broad microbial consortium in 150mL of material contained within a small plastic bag, tubing and a clamp on the tube. Prior to administration, the patient is appropriately positioned in a left lateral decubitus or knee to chest position (Feuerstadt et al., 2023). One end of the tube is inserted into the bag containing the microbial consortium while the other end is lubricated and placed into the patient's rectum. The bag is slowly raised by the provider administering the treatment and the clamp attached to the tube is opened to allow free flow of the material through gravity. The material flows in over 2-3 minutes and the patient is then maintained on their side for 10 minutes for observation.

RBL is very different from traditional FMT for several reasons, most of which were outlined as part of the FMT versus LBP section. It is probably most important to understand that, although RBL is a broad consortium, there is sampling of the post-donation

consortium to ensure consistency of safety and efficacy with what has been tested previously in clinical trials, including ensuring a minimum threshold of *Bacteroides*. A marked difference between this type of product and the heterogenous FMT, with no sampling prior to administration. The better structured trials and consistency of administered product should result in wider acceptance and application by clinicians.

5 Fecal microbiota spores, LIVE BRPR (Vowst™)

Vowst (VOS) is distinct from RBL as this is a microbiota-based LBP that is derived from donor stool and subjected to an ethanol purification process that results in the isolation of *Firmicutes* spores. This is a consortium of microorganisms that only produce spores. VOS is administered orally as four capsules a day for three consecutive days after the patient has completed a course of SOC antimicrobials and a bowel lavage.

5.1 Clinical trial data

A phase III, prospective, double blind, randomized, placebo-controlled trial (ECOSPOR III) included patients with two or more recurrent episodes of CDI diagnosed by enzyme immunoassay or cell cytotoxicity neutralization assay (Feuerstadt et al., 2022b). Patients enrolled received ten to twenty-one days of SOC, followed by a washout period, after which magnesium citrate was administered the day of, or the night before intervention. Patients were randomized to receive either VOS or placebo and were monitored for recurrence for 8 weeks. By the end of this 8-week time period, 88% patients who received SOC+VOS demonstrated sustained clinical response, as compared with 60% of those who received SOC+placebo ($p<0.001$). There were no concerning safety signals within this trial.

A subsequent phase III, prospective, single arm, open-label trial (ECOSPOR IV) investigated the safety of VOS through weeks 24 by expanding their inclusion criteria and formulating two separate cohorts of patients (Sims et al., 2023). They included patients from the ESCOPOR III trial who recurred as part of the "first" cohort, and the "second" cohort included those rCDI patients diagnosed via PCR assay and/or those with first recurrence of the infection. They found an overall efficacy of 91.3% at 8 weeks and 86.3% at 24 weeks. Those with first recurrence had a 93.5% efficacy at 8 weeks.

5.2 Constitution of fecal microbial structure and resultant metabolome

The fecal microbiota composition of enrolled patients was also investigated to detect the microbial changes resulting from the administration of VOS. Stool specimens were obtained at baseline, which was within three days of completion of SOC, and then at 1, 2 and 8 weeks. The composition of microbiota in patients who received SOC+VOS revealed a higher prevalence of *Firmicutes* including *Ruminococciae and Lachnospiriciae* and a decrease in the proinflammatory *Enterobacteriaceae*. This was evident by week 1 and persisted through week 8. This supported the evidence of engraftment of the product with a desirable microbial response. Further, the investigated metabolomic profile revealed that secondary bile acids were significantly more prevalent in the SOC+VOS arm compared with SOC+placebo at 1 week and 8 weeks, exhibiting an overall favorable downstream effect and providing a good metabolic explanation for the success of those treated with VOS.

5.3 Administration

VOS should be administered two to four days after completion of SOC antibiotics to ensure wash out of the SOC antimicrobial. The day prior to administration, or at least 8 hours before the first dose, patients should drink 10oz of magnesium citrate or 250 mL of polyethylene glycol (if with renal insufficiency). VOS is consumed orally, with a total of 4 capsules taken daily for 3 days on an empty stomach prior to the first meal of the day.

6 Important considerations for phase III trials of LBPs

With an understanding of the clinical trials for RBL and VOS, it is also important to consider some key factors that might impact these trials, and how these factors might affect outcomes measured. It is pertinent to note that in each trial, both the intervention arm and the placebo arm received SOC antibiotics prior to randomization. The LBP was an "add-on" to the standard of care to improve its efficacy. The "placebo" arm frequently implies that patients did not receive further active intervention following SOC antibiotics. The duration of antimicrobials did differ among the trials ranging from ten to twenty-one days in the VOS trial and a minimum of 10 days in the RBL. It is unclear how this might impact outcomes, but longer courses of antimicrobials, such as vancomycin, might not be better, given the impact of vancomycin on the microbiota (Vrieze et al., 2014).

When considering treatment of a disease, if the disease is not properly diagnosed, one can't expect a treatment targeting that disease to work. Therefore, diagnostic testing plays an essential role. Unfortunately, the diagnostic tools we have for CDI are sub-optimal. The polymerase chain reaction (PCR) assay, detecting the genes that code for the toxin, is the most commonly used assay for CDI in the United States but has a tendency to over-diagnose the infection, being positive when the *C. difficile* is not necessarily releasing toxin (Magill et al., 2018). Alternatively the enzyme immunoassay (EIA), a test that detects the toxin itself, has an unacceptably low sensitivity (Khanna et al., 2017). Therefore, a negative test does not necessarily rule out CDI. Finally, a cell culture cytotoxin neutralization assay is an accurate tool for diagnosis, but not widely available in clinical practice. Testing in the clinical trials

need to balance accuracy while also mimicking what is available for the majority of practitioners. Ultimately, the diagnostics used in the trial have an impact on accurate patient selection and therefore the ability of interventions to show they work in the correct patients.

The number of recurrent episodes used for initial enrolment was not uniform among the LBP trials. The pivotal trial for RBL included patients with one or more recurrent episodes while the VOS trial included patients with two or more rCDI episodes. It is believed that with each recurrence, the dysbiotic state of the patient becomes more severe. Therefore, if we are trying to use a therapy that restores the deficiencies, one might argue that with more recurrences, it might require more therapy to achieve the same effect in getting the microbiota above the threshold where it can effectively resist germination of *C. difficile* and a recurrence.

A washout period is the time between completion of antimicrobials and administration of the LBP. The purpose of the washout period is to ensure that the SOC antibiotics have been eliminated from the patient so they do not significantly alter the newly administered organisms given within the LBPs. This washout period ranges between 24 and 72 hours, which could further be confounded by the specific antimicrobial therapy that was received prior to LBP.

Further, only the VOS trial used a bowel purge to further eliminate the SOC antimicrobial from the patient. This was performed with magnesium citrate. Dosing of each LBP also varies significantly. While RBL is a 150mL enema given once and is a broad consortium of microorganisms including a minimum threshold of *Bacteroidetes*, VOS is administered in the form of capsules over 3 days and is a narrow consortium of *Firmicutes* microorganisms.

7 Discussion

For years, treatment avenues for CDI have been limited to SOC antimicrobial therapies, including vancomycin and fidaxomicin. While these antibiotics are effective and are supported by current clinical guidelines (Johnson et al., 2021; Kelly et al., 2021a), they only treat a portion of the infection and can further contribute to the disruption of the gut microbial environment. They also cannot act effectively on the *C. difficile* spores, which can remain in a patient's system and if the microbiota is unable to properly regrow and rediversify, these spores can germinate causing a recurrence.

With the discovery of the pivotal role that the intestinal microbiota plays in the pathogenesis of CDI, recent therapeutics sought to modulate the microbiome and its resultant metabolome hindering recurrence of CDI. Fecal microbiota transplantation was the first microbial remodulation therapy to be developed. While evidence for FMT was reassuring, the lack of standardization across studies raised concerns with its safety along with risks of transmission of other pathogenic organisms (DeFilipp et al., 2019). With the need for more uniform therapies, that a wider array of providers would feel comfortable using, the LBPs were developed, which were created in a more regulated fashion having much more rigidly structured clinical trials testing their safety and efficacy. Clinical trial data for both RBL and VOS was encouraging, proving not only their clinical success, but also their sustained efficacy at a microbial and metabolomic level ultimately resulting in FDA approval of both for the prevention of recurrence of CDI. With this approval, there should be much wider access for these effective therapies for both providers and patients. As the frequency of their administration increases in clinical practice, both products should result in a significant reduction in the rates of rCDI and hopefully the burden of CDI on our patients and healthcare system.

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The potential of live biotherapeutic products in allergic disease: current findings and future directions

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With the global prevalence of allergic disease continuing to rise at an alarming rate, the need for effective and safe therapeutics is paramount. Given the critical role of the early-life microbiota on immune development, emerging research suggests the potential use of live biotherapeutic products (LBP) for the prevention and treatment of childhood allergy. However, findings are limited and inconsistent. Therefore, the present review critically evaluates the current animal and human data on the therapeutic value of LBPs in allergy, the underlying immunological mechanisms by which LBPs may mediate allergy susceptibility, limitations of the current research that need to be addressed, and future research directions. Accordingly, LBPs may protect against allergic disease through several immunological and physiological mechanisms during early-life, including regulation of Th1/Th2 balance, SCFA-induced activation of GPR41/43 and HDAC inhibition, and maturation of epithelial barrier integrity. Taken together, current findings indicate powerful immunomodulatory properties of LBPs on allergic immune response, with LBPs offering exciting potential as a novel therapeutic tool for childhood allergy. However, the efficacy of LBPs in allergy is complex and influenced by many population and methodological factors, resulting in varied therapeutic benefits. While research thus far has focused on traditional probiotic strains, greater investigation into microbial consortiums selected from the microbiota of non-allergic infants may provide greater promise as a therapeutic tool for allergic disease. Further investigation, particularly into long-term efficacy, strain-specific effects, optimal supplementation regimes, and use of multi-strain consortiums, is necessary before findings can be translated into clinical applications to tackle childhood allergic disease.

KEYWORDS

allergic disease, asthma, live biotherapeutic products, microbiome, probiotics, early life, childhood allergy

Introduction

Atopy, an exaggerated IgE response to an allergen, affects 1 in 5 individuals worldwide (Bantz et al., 2014), with prevalence rapidly rising across the globe (Gutowska-Ślesik et al., 2023). Particularly in developing countries, rates of childhood allergy, including asthma, allergic rhinitis, atopic dermatitis and food allergy, collectively known as the atopic march, have seen drastic increases (Wong et al., 2013; Eghtesad, 2020; Edwards-Salmon et al., 2022), with 30% of individuals now estimated to be affected by atopic disease (Sánchez-Borges et al., 2018). Asthma, for instance, is the most common chronic childhood disease, with prevalence expected to affect 400 million people globally by 2025 (Masoli et al., 2004). Accordingly, the economic burden of allergic disease is considerable, with asthma alone costing the US $50 billion per year (Hester et al., 2016), and pediatric food allergies costing $25 billion (Gupta et al., 2013). Allergic disease has significant impacts on quality of life, with asthma accounting for 13.8 million missed school days in the US, and ~250,000 premature deaths globally per year (Pawankar, 2014). Unfortunately current treatment options are costly, often ineffective long-term, and are associated with many adverse effects, such as corticosteroid dependency (Lefebvre et al., 2015; Volmer et al., 2018). Moreover, little attention has been paid to the prevention of allergic disease, with current interventions focused predominately on treatment. Thus, atopic disease represents a major global health and economic burden, and the need for safe and effective therapeutics, particularly prophylactics, is paramount.

Live biotherapeutic products (LBPs), defined as the use of live microorganisms including bacteria, viruses and fungi for the prevention or treatment of disease (Cordaillat-Simmons et al., 2020), may represent one such potential therapeutic tool for childhood allergy. In recent years, emerging research has demonstrated a link between the early-life intestinal microbiota and risk of allergic disease. Significant differences in microbiome composition are repeatedly reported between infants with allergic disease compared to healthy counterparts (Kourosh et al., 2018; Boutin et al., 2020; Lee-Sarwar et al., 2023). Moreover, perturbations to microbiome colonization in early-life, i.e., via antibiotic use, are associated with increased allergy risk in childhood (Ni et al., 2019; Patrick et al., 2020). Accordingly, germ-free mice show enhanced susceptibility to allergic disease, compared to those with an established microbiota (Rodriguez et al., 2011), highlighting the important role of the intestinal microbiome in allergy development. The clear link between early-life microbiome development and allergy susceptibility has led to the exciting potential of microbiota-targeted interventions, including LBPs, for the prevention and treatment of atopic disease. However, research is still in its infancy, with many issues, including the identification of optimal bacterial strains, yet to be addressed before clinical recommendations on the therapeutic use of LBPs for childhood allergy can be made.

Consequently, this review will critically evaluate the role of the early-life intestinal microbiome in atopic disease and the current available research regarding the therapeutic potential of LBPs in allergy. Moreover, the underlying mechanisms by which LBPs may modulate allergic immune response, current challenges of LBP application in allergy and future research directions, will be assessed.

At this point, it is important to note the distinction between probiotics and LBPs, with probiotics defined as live microorganisms that confer benefit to host health, and LBPs as live microorganisms used for the prevention or treatment of disease (Cordaillat-Simmons et al., 2020). Accordingly, this distinction is not based on the content of the product, but it's regulatory status as a therapeutic for disease. Therefore, although some probiotic bacteria may fall under the category of LBPs due to their therapeutic effects on disease, not all probiotics can be classified as LBPs.

In addition, it should be noted that aside from the intestinal microbiota, the development of both the oral and respiratory microbiomes during early-life also influences immune function and allergy susceptibility (Teo et al., 2018; Arweiler et al., 2021; Cui et al., 2021; Ho et al., 2021). However, given the emerging data on the use of intestinal-targeted LBPs in allergy, this review will focus predominately on the role of the gut microbiome in allergy, and specifically, the potential of LBPs for the prevention and treatment of allergic disease.

Early-life intestinal microbiome shapes immune development and allergy susceptibility

The early-life gut microbiota profoundly impacts immune development, having long-lasting effects on disease outcomes later in life (Gensollen et al., 2016; Iyer and Blumberg, 2018). It is well established that the first ~1000 days of life are a critical window of opportunity in which the maturing gut microbiota drastically shapes immune programming, and that perturbations to the microbiota during this critical window can have lasting impacts on immune function and subsequent disease risk, including allergy susceptibility (Cukrowska, 2018; Romano-Keeler and Sun, 2022). Accordingly, disruptions to microbiome maturation during the early-life critical window, through factors such as caesarean-delivery (Huang et al., 2015), method of feeding (Hu et al., 2021), prenatal (Cait et al., 2022) and infant antibiotic use (Ni et al., 2019) and pet exposure (Hesselmar et al., 2018), influence immune development and subsequent risk of atopy. For instance, both early-life antibiotic exposure (Lu et al., 2023) and caesarean-delivery (Liang et al., 2023) are associated with increased risk of asthma in childhood, reflecting the integral relationship between the gut microbiota and immune education.

Immune development begins in-utero, and despite the longstanding belief that the womb is sterile, recent findings suggest microbiota colonization commences in-utero (Stinson et al., 2019; Mishra et al., 2021) and influences fetal immune programming (Mishra et al., 2021). Neonatal immune regulation is initiated prenatally, in part driven by transplacental transfer of microbiota-derived metabolic signatures, such as short-chain fatty acids (SCFAs), which are able to cross the placenta and influence fetal T cell development (Kimura et al., 2020; Husso et al., 2023). For instance,

low serum acetate levels during pregnancy are associated with impaired Treg production in neonates (Hu et al., 2019). In accordance, prebiotic supplementation increased fecal acetate concentration of pregnant dams, which passed through the placenta and was detected in amniotic fluid, and elevated Treg cell production in the fetus, thus having pro-tolerogenic effects (Brosseau et al., 2021). Moreover, several bacterial strains, including *Staphylococcus* and *Lactobacillus* isolated from second-trimester fetal tissues were found to induce *in-vitro* activation of memory T cells in the fetal mesenteric lymph node, supporting the role of prenatal microbial exposure on fetal immune programming, particularly T cell development (Mishra et al., 2021). Notably, Th1/Th2 balance is a critical component of allergic inflammation; Type 2 allergies involve over-activation of Th2 cells in response to an antigen, triggering the production of type 2 cytokines, namely IL-4, IL-5 and IL-13, signaling an inflammatory cascade resulting in antigen specific-IgE production, mast cell activation and eosinophil degranulation, ultimately leading to the manifestation of allergy symptoms (Caminati et al., 2018; Akdis et al., 2020).During pregnancy, the fetal immune system presents a Th2-dominant phenotype, and the Th1 response is suppressed, resulting in Th2 polarization (Lee C-L. et al., 2011). However, microbial exposures during the pre- and post-natal period, promote a gradual shift from Th2 dominance to Th1/Th2 homeostasis (Qian et al., 2017), fostering immunotolerance to antigens. As such, maternal abundance of *Prevotella*, a SCFA-producing genus, during pregnancy is associated with reduced risk of food allergy in infants (Vuillermin et al., 2020), emphasizing the impact of the maternal prenatal microbiome on infant immune programming. Dysregulation of this Th2-Th1 shift in early-life, due to a variety of environmental and genetic factors, can result in excessive Th2 activation and improper maturation of Th1/Th2 balance (Martino et al., 2018; Krusche et al., 2020; Zhang et al., 2021) thus leading to increased allergy susceptibility. For instance, maternal antibiotic exposure during pregnancy is associated with an increased risk of both asthma and atopic dermatitis (AD) in offspring (Zhong et al., 2021), highlighting the influential role of the maternal microbiota on fetal immune development.

Microbiota-initiated immune programming continues into the post-natal period, shaped by many early-life microbial exposures such as mode of delivery, antibiotic use, pet exposure, urban vs. rural habitation, and method of feeding, as seen in Figure 1 (Kumbhare et al., 2019; Donald and Finlay, 2023). Breast milk, for instance, contains a multitude of bioactive components, including commensal bacteria, antibodies, human milk oligosaccharides (HMOs) and immune cells, which promote maturation of both the infant immune system and microbiota, and consequently help protect against immune-mediated disorders including allergy (Lokossou et al., 2022). Breast milk-transmitted maternal IgA is found to bind to intestinal bacteria (Sterlin et al., 2019) which not only helps prevent infection (Guo et al., 2021), but also dampens antigen-stimulated immune response by suppressing T helper cell activation (Koch et al., 2016), thus contributing to early-life immune regulation via microbiota-dependent mechanisms. Accordingly, sIgA treatment ameliorates allergic inflammation and promotes oral tolerance in a mouse model of food allergy (Kizu et al., 2015). Similarly, HMOs found in breast milk are shown to prevent the development of asthma in a murine model (Bozorgmehr et al., 2023), which is thought to reflect their prebiotic effect on the infant microbiota, promoting increased SCFA production, as well as supporting mucus production and epithelial barrier integrity (Tarrant and Finlay, 2023). In addition, a study comparing breastfed and formula fed infants demonstrated that breast milk promotes immunotolerance to antigens by increasing the production of Treg cells and suppressing T helper cell differentiation and cytokine production (Wood et al., 2021). Notably, here breastfed infants demonstrated significantly increased fecal abundance of SCFA-producing bacteria, compared to formula-fed, indicating the microbiota-dependent mechanism of breastfeeding in allergy protection.

Moreover, environmental exposures during both the pre- and post-natal period have significant impacts on infant microbiota development and thus subsequent allergy risk (Sbihi et al., 2019). For instance, growing up on a farm is found to be protective against asthma, atopic dermatitis and allergic rhinitis (Feng et al., 2016; Botha et al., 2019; Depner et al., 2020) due to increased exposure to farm-associated microbes, such as *Lachnospiraceae* and *Ruminococcaceae* (Kirjavainen et al., 2019; Depner et al., 2020), in early life resulting in enhanced microbial diversity and maturation, thus influencing immune-programming and allergy outcomes. Conversely, urban habitation is repeatedly associated with increased risk of allergic disease (Botha et al., 2019; Lehtimäki et al., 2021) with urbanized infants showing significant differences in intestinal microbiota composition to that of rural infants (Lehtimäki et al., 2021). Notably, this urbanized-microbiota signature is correlated with elevated inflammatory marker concentrations by 1 month of age, including CXCL8, CCL2 and CCL17, decreased levels of anti-inflammatory cytokine IL-10, and increased risk of asthma, atopic dermatitis and allergic sensitization throughout childhood (Lehtimäki et al., 2021). Specifically, infants exposed to pets or farm environments in early life show elevated fecal abundance of SCFA-producing bacteria (Gio-Batta et al., 2020; Yang et al., 2022). Notably, SCFAs have powerful anti-inflammatory and allergy-protective effects, through their ability to regulate T helper cell balance and support epithelial barrier maturation (Ratajczak et al., 2019), as discussed later in this review.

Consequently, maturation of the infant intestinal microbiota, influenced by a variety of environmental exposures commencing in the womb and continuing throughout the early-life critical window, drives immune programming, thus influencing subsequent immunotolerance and risk of allergic disease.

Given our growing understanding of the profound impact of the infant microbiota on immune programming and allergy susceptibility, determining the therapeutic potential of microbiota-targeted interventions, such as LBPs, for the prevention and treatment of allergic disease is of significant value.

Mechanisms of LBPs in allergy

Regulation of Th1/Th2 balance

One mechanism by which LBPs may influence childhood allergy susceptibility, is by regulation of Th1/Th2 cell

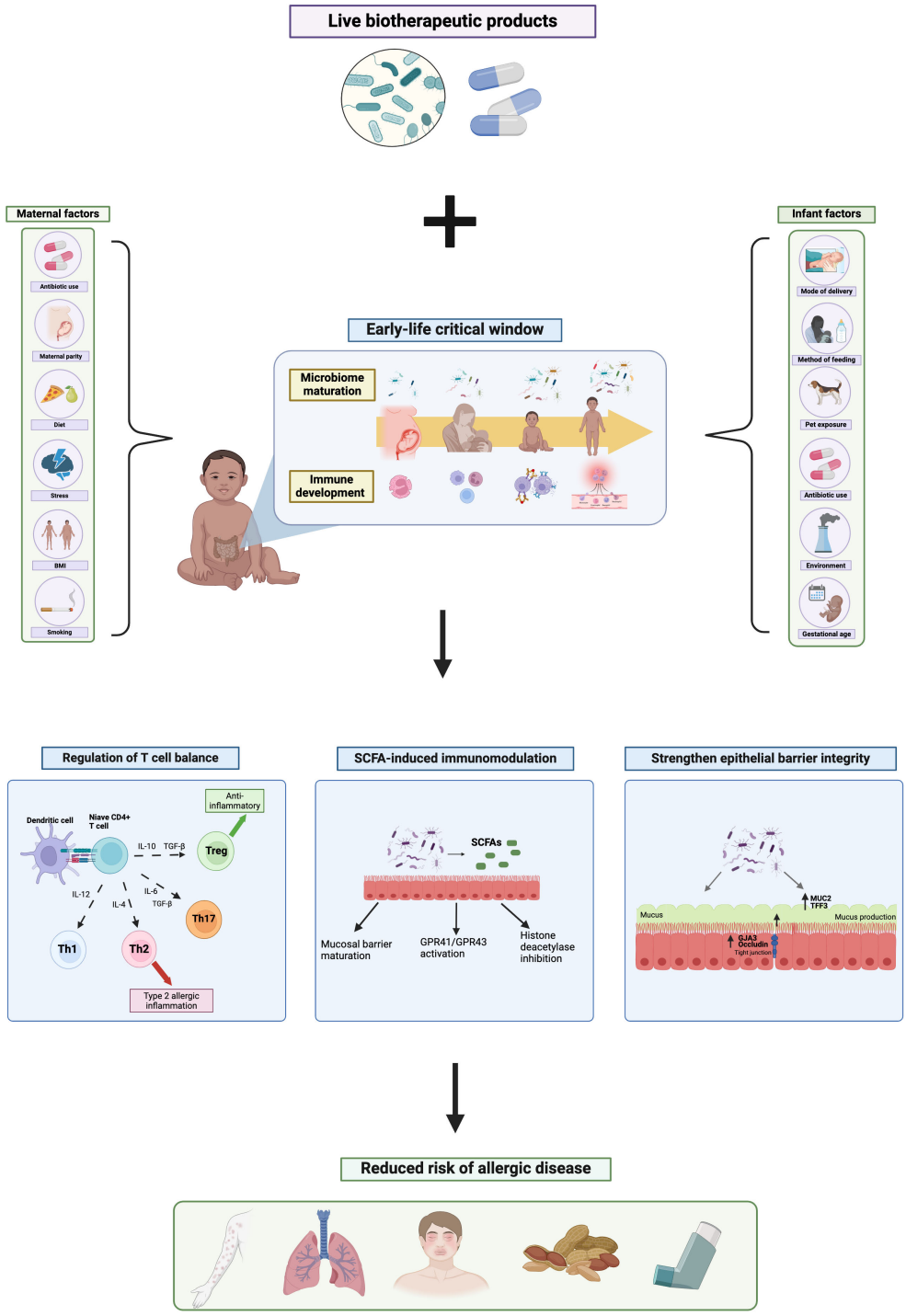

FIGURE 1
The mechanistic role of live biotherapeutic products on the early-life gut microbiome and risk of allergic disease. Created with BioRender.com.

homeostasis. As mentioned above, T helper cell imbalance is a critical component of allergic immune response, with allergic inflammation ascribed to enhanced activation of Th2 cells in response to an allergen, triggering the production of type 2 cytokines and IgE, eosinophil and mast cell degranulation, and ultimately allergy symptoms. Regulatory T cells on the other hand, exhibit anti-inflammatory effects by suppressing over-activation of Th2 cells, maintaining Th1/Th2 homeostasis, and promoting immunotolerance to allergens. Probiotic bacteria are able to bind to toll-like-receptors (TLRs), including TLR2, TLR4 and TLR6, on dendritic cells (Ren 2016; Bermudez-Brito) and stimulate dendritic cell maturation towards IL-10 production, resulting in Treg differentiation and thus immune regulation (Hisbergues et al., 2007). For instance, *Lactobacillus plantarum* treatment induced the production of anti-inflammatory IL-10 and IL-12 from allergen-stimulated bone marrow-derived dendritic cells (BMDCs) isolated

from asthmatic mice, via activation of TLR4 and TLR2 signalling pathways (Adam et al., 2010). Similarly, in another study, not only did *Escherichia coli* Nissle (EcN) supplementation prevent allergic inflammation in a mouse model of asthma, when treated to murine BMDCs, EcN was able to activate dendritic cell maturation towards IL-10 production, up-regulation expression of DC maturation factors CD40, CD80 and CD86, and suppress type 2 cytokine production in a TLR4-dependent manner (Adam et al., 2010). These findings translate *in-vivo*; *Bifidobacterium*, *Clostridia*, and *Lactobacillus* supplementation suppresses Th2 skewing and stimulates intestinal Treg production in mouse models of peanut and food allergy, demonstrating anti-inflammatory effects (Atarashi et al., 2013; Barletta et al., 2013; Li et al., 2020; Shi et al., 2020). Furthermore, *Lactobacillus* supplementation is found to reduce airway inflammation in murine model of both asthma (Atarashi et al., 2013; Barletta et al., 2013), and food allergy [45,46), inducing the suppression of type 2 cytokines and enhancing Treg production. This immunomodulatory effect is also seen in human studies; treatment of lactic acid bacteria including *Lactobacillus lactis, Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus GG* to dendritic cells isolated from allergic patients, significantly induced IL-10 and IL-12 production, indicating enhanced Treg differentiation, and suppressed type 2 cytokines following allergen challenge (Mohamadzadeh et al., 2005). Moreover, when co-cultured with naive CD4+ T cells, Lactobacillus casei treatment significantly increased the production of IFN-y in dendritic cells isolated from asthmatic patients (Ratajczak et al., 2007), a Th1 cytokine which has powerful inhibitory effects on Th2 cell activation, thus regulating T helper cell balance. Taken together, probiotic bacteria are able to regulate T cell differentiation away from Th2 polarization and towards Treg production via activation of TLR signalling, consequently promoting immunotolerance to antigens.

Despite this, it is important to note that the longstanding the Th2-centered paradigm of allergy is somewhat simplistic and reductionist. It is now known that varying endotypes of allergic disease result from complex interactions between many heterogeneous T cell subsets, including Th1, Th17, Th22, Th9, Tfh and Tregs as well as supporting immune cells such as ILC2s and neutrophils. For instance, intrinsic atopic dermatitis, a sub-type accounting for approximately 20% of AD cases, is characterized by over-activation of Th1 cells, stimulating TNF-a and IFN-y production, resulting in epithelial cell apoptosis and the manifestation of eczematous skin lesions (Kabashima-Kubo et al., 2012; Tokura et al., 2018). Moreover, while the majority of asthmatic patients present the type 2 asthma phenotype, a smaller percentage are characterized by neutrophilic asthma involving enhanced activation of Th17 and Th1 cells and neutrophilic inflammation (Pelaia et al., 2015; Boonpiyathad et al., 2019). Thus, although Th2 polarization accounts for a significant proportion of allergic inflammation, one should consider allergic immune response beyond the simple Th1/Th2 dichotomy. Therefore, future research should determine the mechanistic role of LBPs on the regulation of many heterogeneous T cell subsets involved in differing allergy endotypes. Nevertheless, LBPs can have powerful impacts on regulating Th1/Th2 balance, ultimately promoting immune tolerance and contributing to allergy protection.

SCFA-induced immunomodulation

A further mechanism by which LBPs may influence immune response and reduce risk of allergic disease is via the metabolites they produce, namely short-chain fatty acids. *Bifidobacterium*, *Lactobacillus, Bacteroides* and *Clostridia* in particular, ferment undigestible oligosaccharides and proteins in the colon and cecum to produce SCFAs. Accordingly, probiotic supplementation is repeatedly found to increase fecal SCFA concentration in both adults (Gaisawat et al., 2019; Kusumo et al., 2019) and children (Kim et al., 2015; Berni Canani et al., 2016). SCFAs, including acetate, butyrate and propionate, are known to have a variety of beneficial and anti-inflammatory properties including neuroprotection, supporting glucose homeostasis, cardiovascular protection and immunoregulation. SCFAs have been repeatedly linked to allergy protection, with high fecal butyrate concentration associated with reduced risk of eczema at 1 year of age (Kim et al., 2015), and food allergy and asthma at 6 years (Roduit et al., 2019). In addition, butyrate supplementation is found to significantly reduce airway inflammation in a murine model of asthma (Theiler et al., 2019; Vieira et al., 2019) and food allergy (Vonk et al., 2019). Furthermore, allergic infants demonstrate reduced genetic potential for intestinal butyrate production, having a lack of genes encoding for enzymes that ferment undigestible carbohydrates into butyrate, compared to non-allergic counterparts (Cait et al., 2019). Interestingly, formula milk supplemented with *Lactobacillus rhamnosus* is found to significantly increase fecal abundance of SCFA-producing bacteria, as well as fecal butyrate concentration in infants with CMA (Berni Canani et al., 2016). Of note, in this study, the most allergen-tolerant infants showed the largest increase in fecal SCFA concentrations, supporting the mechanistic role of microbial-derived SCFAs in allergy protection.

SCFAs are thought to influence allergy risk via several pathways, including via activation of G-protein coupled receptors 41 and 43 (GPR41/43), found on several immune cells, IECs and lung epithelial cells (Li et al., 2018). SCFA-induced activation of GPR41/43 has anti-inflammatory effects via p38 MAPK signaling pathways (Kobayashi et al., 2017). For instance, propionate treatment was found to ameliorate airway inflammation in a murine model of asthma via activation of GPR41 pathways (Trompette et al., 2014). Similarly, in a mouse model of CMA, Lactobacillus acidophilus supplementation not only alleviated allergic inflammation, but also increased fecal SCFA concentration and stimulated activation of GPR41 and GRP43 receptors (Wang et al., 2019), emphasizing the pro-tolerogenic effects of probiotic bacteria via SCFA-induced GPR41/43 activation.

In addition, SCFAs may also provide allergy-protective effects via inhibition of histone deacetylase (HDAC). HDACs are enzymes involved in the deacylation of histone proteins, and are key regulators of T cell differentiation (Haery et al., 2015), thus play a key role in allergic immune response. In allergic individuals, allergen exposure up-regulates HDAC expression, stimulating activation of the

mTOR-S6K signaling pathway, resulting in elevated IL-4 and IL-6 production, and consequently Th2 and Th17 cell differentiation. In addition, expression of HDACs suppresses FoxP3 transcription in CD4+ T cells, resulting in decreased Treg production (Deng et al., 2020; Lee et al., 2022), contributing to the T cell imbalance seen in allergy. Accordingly, patients with allergic rhinitis (Wang et al., 2015) and asthma (Butler et al., 2012) show increased HDAC1 expression compared to healthy counterparts. Notably, microbial-derived SCFAs, particularly propionate and butyrate, are powerful HDAC inhibitors (Lin et al., 2015). SCFAs are able to cross the intestinal epithelial barrier and inhibit HDAC expression in intestinal epithelial and immune cells, thus up-regulating FoxP3 transcription, suppressing IL-4 and IL-6 production, and ultimately promoting the proliferation of anti-inflammatory Treg cells (Arpaia et al., 2013; Furusawa et al., 2013). In support of this, butyrate supplementation reduces airway inflammation in a murine model of ILC2-driven asthma, inducing IL-13, IL-5 and ILC2 suppression via HDAC inhibition, and independently of GPR41/43 activation (Thio et al., 2018). In addition, the authors reported murine supplementation of butyrate-producing *Clostridia* significantly increased pulmonary propionate and butyrate levels and alleviated asthma. Moreover, another group demonstrated that acetate supplementation promoted the generation of Treg cells and alleviated asthma in a murine model via HDAC inhibition and FoxP3 acetylation (Thorburn et al., 2015). Hence, LBPs may exhibit allergy-protection through SCFA-induced HDAC inhibition, thus regulating T cell balance.

Taken together, LBPs may exhibit allergy protection via the production of microbial-derived metabolites SCFAs, which promote Treg production and immunotolerance through both GPR41/43 signaling pathways and HDAC inhibition. Of note, if the allergy-protective effects of LBPs derive from enhanced production of SCFAs, then future research may also investigate the potential for direct SCFA supplementation as a 'post biotic' metabolite for the prevention and treatment of allergic disease. The direct use of SCFA metabolites may bypass certain practical limitations of LBPs, such as cultivation and storage of live organisms.

Strengthening epithelial barrier integrity

Alongside their immunomodulatory actions, LBPs may also influence allergy susceptibility through physiological mechanisms, such as strengthening epithelial barrier integrity. During allergic sensitization, an otherwise harmless antigen, crosses the mucosal barrier and is presented by antigen-presenting cells, triggering Th2 cell activation and the type 2 inflammatory cascade, ultimately resulting in allergy development (Caminati et al., 2018). Thus, epithelial barriers act as protection preventing certain antigens to cross, enter systematic circulation and interact with immune cells. Accordingly, epithelial barrier dysfunction is commonly associated with allergic disease (Gon and Hashimoto, 2018; Kortekaas Krohn et al., 2020), and increased intestinal barrier permeability is a hallmark feature of food allergy (Parrish et al., 2022). Notably, many probiotic species are able to support the integrity of the intestinal epithelial barrier by stimulating mucus production and stabilizing epithelial tight junctions. For instance, several *Lactobacilli strains* are found to up-regulate MUC3 expression in intestinal epithelial cells, resulting in increased mucus production (Mattar et al., 2002; Das et al., 2016). Similarly, *Streptococcus thermophilus* and *Lactobacillus acidophilus* treatment in HT29 intestinal epithelial cells (IECs) was found to improve epithelial barrier properties by increasing the expression of tight junction proteins actinin and occludin and improving transepithelial resistance (TER), which subsequently protected IECs from *E.coli*-induced damage (Resta-Lenert and Barrett, 2003), highlighting the powerful role of LBPs in strengthening epithelial barrier integrity.

Moreover, alongside their direct effects on epithelial barrier function, LBPs may also act indirectly on the intestinal epithelial barrier via the metabolites their produce, specifically SCFAs. Microbial-derived SCFAs, are known to play a protective role in maintaining epithelial barrier integrity, by up-regulating the expression of MUC2 in IECs, thus stimulating mucus secretion (Burger-van Paassen et al., 2009; Giromini et al., 2022). In addition, SCFAs are found to maintain tight junctions through enhancing the expression of tight junction protein-related genes, GJA3 and occludin (Gao et al., 2021). Therefore, not only may LBPs directly promote intestinal barrier maturation, but also indirectly via their resulting metabolites.

Collectively, these findings indicate that LBPs may confer allergy protection by promoting intestinal barrier maturation and mucosal homeostasis, which may prevent leakage of antigens through the mucosa, reduce allergenic exposure to APCs, and consequently protect against sensitization in the gut. Despite this, further research is needed to confirm the importance of epithelial barrier integrity in allergy development in order to validate this mechanism.

Although the discussed pathways represent the principal mechanisms by which LBPs may influence allergic immune response, this is not an exhaustive list. Other potential mechanisms to consider include IgA-induced regulation of mucosal homeostasis and allergen neutralization (Scheurer et al., 2023), and direct inhibition of mast cell degranulation (Oksaharju et al., 2011; Forsythe et al., 2012; Forsythe, 2016).

Current findings on the role of LBPs in allergy

Animal data

A multitude of animal studies demonstrate beneficial immunomodulatory effects of LBPs on allergic inflammation, as summarized in Table 1. For instance, *Lactobacillus rhamnosus GG* supplementation was found to alleviate airway hyper-responsiveness, suppress Th2 cytokine release, and decrease immune cell infiltration in the lung, thus dampening Th2 cell response in a murine model of asthma (Wu et al., 2019). In a food allergy model, mono-colonization of Clostridia in germ-free mice not only inhibited sensitization to food allergens, but also increased CD4+Foxp3+ Treg cell numbers, IgA and IL-22 production in the lamina propria, ultimately restoring intestinal epithelial integrity (Stefka et al., 2014), highlighting the

TABLE 1 Summary of mouse studies assessing the therapeutic impact of LBPs in allergic disease.

Reference	Model	Bacterial strain	Treatment duration	Treatment outcome
(Barletta et al., 2013)	Peanut allergy model, peanut extract sensitization	VSL#3 mixture containing *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Bifidobacterium breve, Bifidobacterium longum*, and *Bifidobacterium infantis*, and *Streptococcus salivarius* subspecies *thermophilus*	Daily treatment for 20 days in adult mice.	- Ameliorated anaphylaxis symptoms - Supressed Th2 inflammation - Induced TGF-B activation and production of FOXP3 expressing Treg cells
(Shi et al., 2023)	Food allergy model, ovalbumin sensitization	*Lactobacillus rhamnosus* Probio-M9	n/a	- Reduced OVA-specific IgE, histamine and mMCP-1 - Dampened Th2 inflammation - Increased faecal abundance of *Firmicutes Bacteroidota* - Increased faecal SCFA levels
(Shin et al., 2016)	Atopic dermatitis model, 1-chloro-2,4-dinitrobenzene sensitization	*Multi-strain consortium containing: Lactobacillus acidophilus* CBT LA1, *Lactobacillus rhamnosus* CBT LR5, *Lactobacillus plantarum* CBT LP3, *Bifidobacterium bifidum* CBT BF3, *Bifidobacterium breve* CBT BR3, *Lactococcus lactis* CBT SL6, and *Streptococcus thermophilus* CBT ST3	Daily treatment for 8 weeks in adult mice	- Alleviated atopic dermatitis skin lesions - Reduced serum IgE levels - Reduced type 2 cytokines - Elevated proportion of $CD4^{+}Foxp3^{+}$ Tregs
(Wu et al., 2019)	Asthma model, ovalbumin sensitization	Lactobacillus *rhammosus GG* ATCC53103	Mice were either treated with *L. rhamnosus* a) prior to allergic sensitization daily from day 1-14, and 45-60, or b) post allergic sensitization daily from days 14-28 and 60-75	- Alleviated airway hyper-responsiveness - Supressed Th2 cytokines in BALF, as well as IL-17, TNF-α and HMGB1 - Reduced levels of infiltrating immune cells in BALF including eosinophils, lymphocytes, neutrophils and monocytes.
(Stefka et al., 2014)	Food allergy model, peanut extract sensitization, germ-free mice	Clostridia isolated from murine faecal samples	Administered twice, once before weaning and once two weeks after	- Inhibited sensitization to allergen - Increased CD4+Foxp3+ Treg cell numbers - Elevated IgA and IL-22 production in the lamina propria
(Arrieta et al., 2015)	Asthma model, ovalbumin sensitization	Multi-strain consortium consisting of *Feacalibacterium prausnitzii* ATCC 27766, *Veillonella parvula* ATCC 10790, *Rothia mucilaginosa* ATCC 49040 and *Lachnospira multipara* DSM-3073	Adult mice treated with microbial mixture on days 1, 7 and 14, then paired for breeding. Asthma model ran in offspring.	- Alleviated airway inflammation as measured by histological scoring of lung tissue - Reduced proinflammatory cytokines in lung tissue including IL-6, IL-17A, INF-y and TNF-a - Supressed OVA-specific IgE levels in serum - Decreased immune cell infiltration in BALF, including neutrophils and lymphocytes - Elevated faecal SCFA concentration
(Neau et al., 2016)	Cow's milk allergy model, β-lactoglobulin sensitization	*Bifidobacterium animalis* subsp. *lactis* P17, *Bifidobacterium animalis* subsp. *lactis LA*306, *Bifidobacterium bifidum* P122, *Lactobacillus rhamnosus* LA305, *Bifidobacterium longum* subsp. *infantis* LA308, *Lactobacillus salivarius* LA307	Daily supplementation of 1 of the selected bacterial strains alone for 41 days in 4-week-old mice.	- Out of the 6 strains assessed, *L. rhamnosus* LA305, *L. salivarius* LA307, and *B. longum* subsp. *infantis* LA308 significantly reduced serum IgE levels, protected against allergic sensitization and reduced mast cell degranulation - *Lactobacillus rhamnosus* LA305 induced Th1 and Treg responses - *Bifidobacterium longum* subsp. *infantis* LA308 induced Th1 responses - The remaining 3 strains had no impact on allergic inflammation
(Wang et al., 2019)	Cow's milk allergy model, β-lactoglobulin sensitization	*Lactobacillus acidophilus* KLDS 1.0738	Administered 3 times per week for 4 weeks	- Supressed hypersensitivity allergic response - Reduced IL-4, IL-6, IL-17, and TNF-β production, and increased anti-inflammatory cytokines, IFN-γ and TGF-β - Increased faecal SCFA concentration - Activated SCFA receptors, GPR41 and GPR43, in spleen and colon

(Continued)

TABLE 1 Continued

Reference	Model	Bacterial strain	Treatment duration	Treatment outcome
(Feehley et al., 2019)	Cow's milk allergy model, β-lactoglobulin sensitization	Fecal microbiota transplant (FMT) from either a) infants with cow's milk allergy or b) healthy controls, into germ-free mice	One-time FMT	- Mice colonized with FMT from healthy donors were protected from anaphylactic responses, whereas colonization with CMA-infant derived FMT induced drops in core body temperature and clinical manifestations of anaphylaxis in mice. - Significant differences were found between both microbiota composition of cow's milk allergic vs healthy control humans, and in mice. -Unique transcriptome signatures in the ileal epithelium were found between healthy and CMA colonized mice - *Anaerostipes caccae* specifically was correlated with protection against an allergic response to cow's milk allergen
(Mauras et al., 2019)	Cow's milk allergy model, β-lactoglobulin sensitization	FMT from either a) infants with cow's milk allergy or b) healthy controls, into germ-free mice	One-time FMT in 3-week-old mice	- Mice treated with FMTs from CMA-infants showed increased clinical scoring of CMA including diarrhoea, skin puffiness/scratching and anal inflammation, compared to mice colonized with healthy-donor FMT. - Mice colonized with FMT from CMA-infants showed elevated serum IgE and colonic gata3 mRNA (marker of Th2 cell activation), compared to mice treated with FMT from healthy infants. - Treatment with healthy-donor FMT significantly increased *Bifidobacteria/Lachnospiraceae* ratio in mice. - Specifically, an enrichment of *Bifidobacterium* and *Anaerostipes* (butyrate-producing) was found in mice colonized with healthy-donor FMTs and protected from allergic response.

n/a, unknown or unavailable.

pro-tolerogenic properties of Clostridia and the potential mechanistic role on epithelial barrier integrity. Furthermore, four bacterial genera (*Faecalibacterium, Lachnopsichia, Rothia and Vieonella*) found to be significantly decreased in abundance in asthmatic children compared to non-allergic counterparts, were reported to reduce airway inflammation in a murine model of asthma, indicating powerful asthma-protective properties (Arrieta et al., 2015). Similarly, a microbial consortium of seven strains of *Bifidobacteria* and lactic acid bacteria significantly reduced serum IgE, Th2 cell cytokines, and AD-like skin lesions in a mouse model of atopic dermatitis (Shin et al., 2016). Notably, the immunological alleviation of AD was thought to reflect LBP-induced production of CD4+Foxp3+ Treg cells, providing an immunoregulatory effect on Th2 cell polarization. Moreover, Shi et al., 2023 (Shi et al., 2023), demonstrated that *Lactobacillus rhamnosus* administration significantly reduced allergic inflammation in an OVA model of food allergy, including reductions in OVA-specific IgE, histamine and murine mast cell protease 1 (mMCP1), and regulation of Th1/Th2 balance. In addition, fecal SCFA concentration increased following *Lactobacillus* supplementation, supporting the mechanistic role of microbial-derived SCFAs in allergy protection (Shi et al., 2023).

Furthermore, a handful of animal studies have assessed the efficacy of fecal microbiota transplantation (FMT) for the treatment of allergic disease, demonstrating promising results.

Feehley et al., 2019 (Feehley et al., 2019) reported that treatment of germ-free mice with FMTs derived from healthy infants protected against anaphylactic responses to cow's milk allergen, whereas FMT from infants with CMA resulted in reductions in core body temperature, indicating anaphylactic allergic response. Accordingly, significant differences in fecal microbiota composition were observed between both infants with CMA compared to healthy controls, and the respectively colonized mice. Similarly, Mauraus et al., 2019 (Mauras et al., 2019) demonstrated that FMT derived from CMA-infants significantly increased clinical manifestations of food allergy in sensitized germ-free mice, including diarrhea, skin puffiness/scratching and anal inflammation, as well as serum IgE levels and colonic gata3 mRNA (a marker of Th2 cell activation), compared to mice treated with FMT from healthy infants. Notably, the observed allergy-protection following healthy-donor FMT was associated with increased *Bifidobacteria/Lachnospiraceae* ratio including enhanced abundance of *Anaerostipes* (Mauras et al., 2019), indicating an allergy-protective role of this butyrate-producing genus and hinting to underlying SCFA-dependent mechanisms. Collectively, these findings suggest encouraging potential for harnessing FMT as a therapeutic tool for allergic disease. However, it is important to note that data of FMTs in allergy is limited and preliminary, with only a handful of small-scale animal studies conducted to date. Therefore, substantially more research is needed, with several challenges yet to

be overcome before the therapeutic efficacy of FMT in allergy can be assessed in human trails.

Despite some promising results, it should be noted that contrasting animal findings demonstrating no beneficial effects of LBPs on allergy are also reported, although more sparse. For instance, Neau et al., 2016 found that *Bifidobacterium animalis* subsp. *lactis* had no impact on Th2 inflammation in a mouse model of CMA, yet *Bifidobacterium longum* subsp. *infantis* did (Neau et al., 2016), indicating strain-specific effects. Nevertheless, taken together, animal data suggests a powerful immunomodulatory role of microbial intervention in the prevention and management of atopic disease, indicating promising potential of LBPs. However, despite this, data from human trials is much more inconsistent; The more positive findings from animal studies likely reflects the greater number of cofounding variables, population heterogeneity, and complexity found in human trials, which are limited in animal research.

Human data

Given our ever-evolving understanding of the tightly intertwined relationship between the gut microbiota and immune maturation, an increasing number of randomized controlled trails (RCTs) have assessed the role of various LBPs in the prevention and treatment of allergic disease, as shown in Table 2. While findings are inconsistent, several studies indicate promising potential for the therapeutic value of LBPs in childhood allergy. In terms of allergy prevention, maternal supplementation of either a) *Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999 or b) *Lactobacillus paracasei* ST11 and B longum BL999 during the last two months of pregnancy and first two months of breastfeeding was found to significantly reduce the incidence of atopic dermatitis in infants at 2 years of age, indicating powerful allergy-preventive effects (Rautava et al., 2012). However notably, these bacteria had no effect on risk of atopic sensitization to a panel common food and plant allergens, suggesting the benefit is disease-specific. Similarly, in a RCT, maternal pre- and post-natal supplementation of *Lactobacillus rhamnosos GG, L. acidophilus* and *Bifidobacterium animalis subsp. lactis* lead to significant reductions in the incidence of atopic dermatitis in offspring (followed to 6 years of age), compared to control (Simpson et al., 2015). However, no effect was found on childhood incidence of atopic sensitization, asthma and allergic rhinoconjunctivitis, again indicating the protective efficacy is disease-specific. Accordingly, the World Allergy Organization (WAO), recommends probiotic supplements for pregnant and breastfeeding women, and infants, at high-risk of allergy, for the prevention of atopic dermatitis (Fiocchi et al., 2015). This clinical recommendation however, does not currently extend to the prevention of other atopic diseases, due to insufficient clinical data.

In terms of treatment of existing allergy, *Bifidobacterium bifidum* supplementation significantly reduced food allergy symptoms and serum IgE levels, and elevated serum IgG2, anti-inflammatory responses and restore gut microbiota composition in a RCT of infants aged 1–12 months with cow's milk allergy (CMA) (Jing et al., 2020). Moreover, *Lactobacillus rhamnosus* GG-supplemented formula milk was found to improve tolerance acquisition in infants with CMA (Canani et al., 2012). Notably, enhanced cow's milk tolerance was associated with shifts in intestinal microbiota composition, including increased abundance of *Blautia, Roseburia, Coprococcus* and *Oscillospira* following *Lactobacillus rhamnosus GG* supplementation, all of which are known SCFA producers, as well as significant increases in fecal butyrate production (Berni Canani et al., 2016). Thus, these findings suggest that LBP supplementation may improve antigen tolerance through shifting microbiome composition towards increased abundance of SCFA-producing bacteria, supporting the mechanistic role of SCFAs in allergy protection. Similarly, supplementation with *Bifidobacterium Longum* BB536, *Bifidobacterium Infantis* M-63 and *Bifidobacterium breve* promoted oral tolerance to cow's milk, as well as reduced circulating CD4+ cells, Th2 cell activation and basophil degranulation in infants aged 6–12 months with CMA (Strisciuglio et al., 2023), highlighting the powerful immunomodulatory role of *Bifidobacteria* in suppressing Th2 cell polarization. Interestingly in this study, immunotolerance to cow's milk persisted beyond both the LBP treatment period and 45-day wash-out period, suggesting that LBP-induced immunomodulation during the early-life critical window of immune development has lasting effects on immunotolerance to antigens beyond treatment period. In terms of asthma, *Lactobacillus gasseri* treatment was found to significantly improve peak excitatory flow rates (PEFR) and asthma severity scores, and decrease TNF-a, IFN-g, IL-12, and IL-13 release from HDM-stimulated PBMCs in asthmatic children, compared to control (Chen et al., 2010). Accordingly, LBP's may demonstrate powerful pro-tolerogenic effects in existing allergy, providing significant therapeutic value for allergy management.

However, despite some promising findings on the role of LBPs in allergy prevention and management, data is inconsistent, and many contrasting studies demonstrate no protective effect on allergy outcomes. For instance, maternal and infant supplementation of *Lactobacillus reuteri* throughout the last month of gestation and first year of life, had no impact on the incidence of atopic sensitization, IgE-associated eczema or asthma in children at high risk of allergy, followed till 6 years of age (Abrahamsson et al., 2013). Moreover, maternal supplementation of *Lactobacillus GG* during pregnancy till 6 months postpartum had no effect on the development of sensitization or allergic disease in offspring at 3 years of age (Ou et al., 2012). However, interestingly in this study, although maternal *Lactobacillus GG* supplementation did not reduce risk of allergy in offspring, a reduction in the severity of maternal allergic disease was found, indicating LBP's potential for allergy-protective immunomodulation in adults, beyond the early-life period. Furthermore, *Lactobacillus acidophilus* treatment for the first 6 months of life had no impact on susceptibility to atopic sensitization or allergic disease at 2.5 years of age in infants at high risk of allergy (Prescott et al., 2008). Inconsistency in findings likely reflects a multitude of factors including heterogeneity in bacterial strains assessed, timing, dosage and duration of supplementation, follow-up periods evaluated and population variables such as genetics, geographical location and individual

TABLE 2 Summary of human randomized controlled trials assessing the therapeutic impact of LBPs in allergic disease.

References	Allergy assessed	Population	Bacterial strain	Treatment duration	Treatment outcome
(Rautava et al., 2012)	Eczema Atopic sensitization	205 mother-infant dyads	Either: a) *Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999 Or b) *L paracasei* ST11 and *B longum* BL999	Maternal daily supplementation from last 2 months of gestation through till 2 months postpartum	- Supplementation of both probiotic treatments significantly reduced the risk of eczema development during the first year of life - No effect on risk of atopic sensitization in infants
(Simpson et al., 2015)	Atopic dermatitis Asthma Allergic rhinoconjunctivitis, Atopic sensitization	163 mother-infant dyads	Milk containing *Lactobacillus rhamnosos GG, L. acidophilus La-5* and *Bifidobacterium animalis* subsp. *lactis*	Maternal daily supplementation from 36 weeks of gestation till 3 months postpartum	- Reduced the incidence of atopic dermatitis in children at 6 years of age, compared to control - No impact on the risk of asthma, allergic rhinoconjunctivitis, or atopic dermatitis at 6 years old
(Canani et al., 2012)	Cow's milk allergy (CMA)	55 infants aged 1-12 months with CMA	*Lactobacillus rhamnosus GG*- supplemented formula milk	Daily consumption of *Lactobacillus rhamnosus GG*-supplemented formula milk, containing 1.46 × 10^7 CFU/100 mL, for 12 months	- Significantly improved tolerance to cow's milk challenge in infants with CMA, at 6 and 12 months follow up - Enhanced cow's milk tolerance was associated in shifts in microbiota composition to increased abundance of *Blautia, Roseburia, Coprococcus* and *Oscillospira*
(Strisciuglio et al., 2023)	Cow's milk allergy	8 infants aged 6-12 months with CMA	Consortium of *Bifidobacterium Longum* BB536, *Bifidobacterium infantis* M-63 *Bifidobacterium breve* M-16	Daily treatment for 45 days	- Bifidobacterium treatment significantly decreased the proportion of naïve T cells, activated CD4+ T, Th2 activation and basophil degranulation in blood, compared to control, indicating immunotolerance. - Probiotic-induced immunomodulation persisted after 45-day wash-out period
(Chen et al., 2010)	Asthma Allergic rhinitis (AR)	105 children aged 6-12 years old	*Lactobacillus gasseri* A5	Daily supplementation for 8 weeks	- Significantly decreased clinical symptom scoring of asthma and atopic rhinitis - Treatment improved pulmonary function and peak expiratory flow rates (PEFR), compared to control - Significant reductions in TNF-α, IFN-γ, IL-12, and IL-13 production from HDM-stimulated PBMCs isolated from allergic children
(Abrahamsson et al., 2013)	Asthma Allergic rhinoconjunctivitis Eczema Atopic sensitization	184 mother-infant dyads, with a family history of allergy	*Lactobacillus reuteri* 57730	Maternal daily supplementation from 36 weeks gestation, followed by infant supplementation from birth-12 months of age	- *L. reuteri* supplementation had no impact on risk of asthma, allergic rhinoconjunctivitis, eczema or atopic sensitization during 7 year follow up - Decreased Th2 and Th1 cytokines at 6 months of age, however this effect did not persist any later
(Ou et al., 2012)	Atopic sensitization Asthma Atopic dermatitis Allergic rhinoconjunctivitis	191 mother-infant dyads	*Lactobacillus rhamnosus GG* 53103	Maternal treatment of 1x10^{10} CFU daily from 24 weeks of gestation till 6 months postpartum	- No impact on risk of any allergic disease assessed in children infants followed till 36 months of age - Significantly reduced maternal allergic scoring, particularly in mothers with IgE >100 kU/L

(Continued)

TABLE 2 Continued

References	Allergy assessed	Population	Bacterial strain	Treatment duration	Treatment outcome
					- Improvement in maternal allergy symptoms was accompanied by an increase in plasma IL-12
(Prescott et al., 2008)	Atopic dermatitis IgE-mediated food allergy Asthma Atopic sensitization	153 infants followed from birth- 2.5 years of age, born from mothers with allergic disease	*Lactobacillus acidophilus* L10/LAVRI-A1	$3x10^9$ CFU daily from birth- 6 months of age	- No impact on risk of atopic dermatitis, IgE-mediated food allergy, asthma, or atopic sensitization at 2.5 years of age
(Marlow et al., 2015)	Atopic dermatitis	331 mother-infant dyads	Either a) *Lactobacillus rhamnosus HN001* or b) *Bifidobacterium animalis subsp. lactis HN019*	Maternal supplementation of either a) or b) from 35 weeks gestation till 6 months postpartum in breastfeeding mothers. All infants received daily supplementation corresponding to maternal treatment, from birth- 2 years of age.	- Toll-like receptor (TLR) genotypes of the infant influenced both pre-disposition to eczema and efficacy of probiotic treatment in reducing eczema development - 26 TLR single nucleotide polymorphisms (SNPs) interacted with *Lactobacillus rhamnosus* reduced risk of eczema - 2 TLR SNPs interacted with *Bifidobacterium animalis subsp. lactis* resulting in reduced risk of eczema, eczema severity or atopy
(Wang and Wang, 2015)	Atopic dermatitis	210 children aged 1-18 years old with atopic dermatitis	Either: a) *Lactobacillus paracasei* GMNL-133, b) *Lactobacillus fermentum* GM090, or c) *Lactobacillus paracasei* GMNL-133 and *Lactobacillus fermentum* GM090 combined	Daily supplementation of the following for 3 months: either *a) Lactobacillus paracasei* GMNL-133, b) *Lactobacillus fermentum* GM090 *or c) Lactobacillus paracasei* GMNL-133 and Lactobacillus *fermentum* GM090 combined	- Supplementation of *L.fermentum* and *L.paracasei* combined significantly reduced atopic dermatitis severity, as measured by SCORAD scoring. This benefit remained at 4 months post probiotic cessation - All treatment groups showed reductions in serum IgE, IL-4, TNF-α, urine eosinophilic protein X, and 8-OHdG levels, and increases in I FN-γ and TGF-β
(Yan et al., 2019)	Atopic dermatitis	Infants aged 4-30 months with atopic dermatitis	Heat-treated *Lactobacillus paracasei GM-080*	Daily supplementation for 16 weeks	- *L.paracasei* supplementation had no impact on atopic dermatitis severity, including SCORAD, itching, and IDQOL, compared to control
(Gore et al., 2012)	Atopic dermatitis	137 infants aged 3-6 months with eczema	Either:*a) Lactobacillus paracasei* CNCM I-2116 or b) *Bifidobacterium lactis* CNCM I- 3446	Daily supplementation of either *Lactobacillus paracasei* or *Bifidobacterium lactis* for 3 months	- No significant differences were found in eczema severity between probiotic and control treated infants
(Niers et al., 2009)	Eczema	123 mother-infant dyads with a family history of allergy	Consortium of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, and *Lactococcus lactis* W58	Maternal daily supplementation for last 6 weeks of pregnancy, and infant supplementation daily from birth- 1 year of age.	- Significantly reduced the risk of eczema development within the first 3 months of life - After 3 months of age, eczema incidence was similar in probiotic and control groups - Probiotic-induced eczema prevention during first 3 months of life was sustained at 2 years of age - Probiotic treatment significantly decreased IL-5 levels in blood, compared to control

(Continued)

TABLE 2 Continued

References	Allergy assessed	Population	Bacterial strain	Treatment duration	Treatment outcome
(Gorissen et al., 2014)	Asthma Allergic rhinitis	83 children from the above study (Abrahamsson et al., 2013) followed up at 6 years of age	Consortium of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, and *Lactococcus lactis* W58	Maternal daily supplementation for last 6 weeks of pregnancy, and infant supplementation daily from birth- 1 year of age.	- Follow up at 6 years of age found probiotic supplementation had no impact on the development of asthma or allergic rhinitis
(Huang et al., 2018)	Asthma	160 children aged 6-18 years old with asthma	Either: a) *Lactobacillus paracasei* GMNL-133, b) *Lactobacillus fermentum* GM090, or c) *Lactobacillus paracasei* GMNL-133 and *Lactobacillus fermentum* GM090 combined	Daily supplementation of the following for 3 months: either *a) Lactobacillus paracasei* GMNL-133, b) *Lactobacillus fermentum* GM090 *or c) Lactobacillus paracasei* GMNL-133 and Lactobacillus *fermentum* GM090 combined	- All probiotic-treated groups showed significant reductions in asthma severity - Supplementation of *L.paracasei* and *L.fermentum* combined significantly improved PEFR scores and decreased serum IgE levels
(Rose et al., 2010)	Asthma Atopic sensitization	131 infants aged 6-24 months old with a family history of allergy and two episodes of physician-diagnosed wheezing	*Lactobacillus rhamnosus* GG	Twice daily supplementation of 10^{10} CFU of *L.rhamnosus* GG for 6 months	- No difference in the incidence of atopic dermatitis or asthma-related events in children between probiotic-treated and control group - Probiotic treatment showed mild immunomodulatory effects on atopic sensitization: IgE specific for aeroallergens was reduced in probiotic-treated children compared to control
(Giovannini et al., 2007)	Asthma Allergic rhinitis	187 children aged 2-5 years old with asthma and/or allergic rhinitis	*Lactobacillus casei*-supplemented milk	Daily supplementation of 10^{8} CFU for 12 months	- No effect on asthma outcomes in children, including number or asthma episodes, severity questionnaires and serum IgE - In allergic rhinitis, probiotic treatment significantly decreased the number of AR episodes reported, and duration of diarrhoea episodes - No benefit on serum IgE levels in children with AR

allergy risk. Such complex interplay between many influencing factors makes it difficult to compare across human trials and draw conclusions on the efficacy of LBPs in the prevention of allergic disease.

Critical evaluation of current research

Given the plethora of factors which influence infant microbiota colonization, these likely also impact the therapeutic efficacy of LBPs in childhood allergy, reflecting such inconsistent findings from human trials. Therefore, establishing the overall allergy-protective value of LBPs for the general population is highly complex and nuanced. Several issues and methodological limitations in the current research likely contribute to contrasting results and should be considered in future studies.

Firstly, the allergy-protective efficacy of LBPs may vary depending on geographical location and population genetics; For instance, a meta-analysis of RCTs reported that Asian children aged 1–18 years old showed reduced AD severity (as measured by SCORAD) following *Lactobacillus* supplementation, whereas this LBP had no impact on allergy outcomes in European populations (Huang et al., 2017). The fact that the same treatment can have differential effects on allergy outcomes between varying populations may be explained by a plethora of factors that influence microbiota and immune development, such variation in diet, microbial exposures, genetics, and lifestyle. In a similar vein, Marlow et al., 2015 (Marlow et al., 2015) demonstrated that the impact of *Lactobacillus rhamnosus* and *Bifidobacterium animalis subsp. lactis* on eczema and atopy outcomes was dependent on variations in TLR genetic polymorphisms of the infants. Here, specific TLR genotypes influenced both the infants pre-disposition to atopy and the efficacy of the LBP supplementation, suggesting the allergy-protective effects of LBPs may vary depending on individual's genetics. Considering this, future RCTs should assess the therapeutic value of LBPs across varying geographical locations and genotypes to determine their applicability amongst a range of heterogenous populations.

Moreover, heterogeneity between bacterial strains assessed, makes it difficult to compare across studies and draw conclusions. The efficacy of LBPs is highly species and strain-specific, thus adding to inconsistency in findings. For instance, *Lactobacillus*

paracasei GMNL-133 supplementation was found to significantly reduce severity of atopic dermatitis, as measured by SCORAD, in children aged 1–18 years, providing a therapeutic benefit which continued beyond the treatment period (Wang and Wang, 2015). However in contrast, supplementation of differing strains of the same species, *Lactobacillus paracasei* GM-080 (Yan et al., 2019) and *Lactobacillus paracasei* CNCM I-2116 (Gore et al., 2012), had no impact on atopic dermatitis severity, thus demonstrating strain-specific variation in the allergy protective efficacy of this species. Although methodological and population differences between studies may contribute to these contrasting findings, strain-specific variation should be considered when evaluating the therapeutic value of LBPs in allergy. Therefore, future research should compare the therapeutic benefits of varying standardized bacterial strains across a range of allergic diseases, to determine optimal strains for allergy protection.

Furthermore, it is important to consider the timing of LBP supplementation, as therapeutic benefits may vary depending on when the supplementation is administered during the perinatal period. For instance, a 2016 meta-analysis reported that the preventive efficacy of LBPs on allergy development was highest when supplemented to both the mother-infant dyad pre- and post-natally, whereas infant supplementation alone had no overall impact on allergy outcomes (Wang et al., 2016), suggesting that microbial exposure during the prenatal period is important for fetal immune development and subsequent immunotolerance. However in contrast, another meta-analysis examining the role of LBPs on asthma risk revealed that only neonatal probiotic supplementation reduced asthma risk, whereas maternal combined pre- and post-natal supplementation did not (Du et al., 2019). As immune development begins in-utero, and maternal microbial exposure during pregnancy is known to influence fetal immune programming via transplacental transmission (Mishra et al., 2021; Husso et al., 2023), it is likely that initiation of probiotic intervention during pregnancy (and continued throughout early infancy), may confer the most immunotolerance to offspring. Nevertheless, greater research is necessary to compare timing and duration of LBP supplementation and determine the optimal supplementation regime to yield the greatest allergy protection in infants.

Moreover, the therapeutic value of LBPs in allergy prevention may depend on the child's risk status and pre-disposition to atopy development. Several trials report that LBP supplementation is most effective at reducing risk of allergy development in populations that are already pre-disposed to increased risk of atopic disease e.g., aforementioned TLR polymorphisms (Marlow et al., 2015) and family history of atopy. For instance, a meta-analysis demonstrated that pre- and post-natal probiotic supplementation had greater efficacy in reducing allergy and food hypersensitivity susceptibility in families at high risk of allergy, compared to those with no predisposition (Zhang et al., 2016). These findings are applicable for informing future clinical recommendations of LBP interventions for mother/infants with a known risk of allergy. In addition, future research should examine the impact of LBPs on allergy outcomes of children with a low-risk status, to determine their value to the general population.

In addition, when evaluating LBP efficacy, it is important to recognize the method of LBP administration and possible synergistic effects with other nutrients. For instance, breast milk contains human milk oligosaccharides (HMOs) which have powerful prebiotic properties and are known to support the growth of many intestinal commensal bacteria (Musilova et al., 2014; Salli et al., 2020; Walsh et al., 2020). To this regard, breast milk can be considered a natural 'synbiotic' containing both commensal bacteria and prebiotic HMOs to support microbial function. Therefore, the allergy-protective efficacy of LBPs may be influenced by whether infants are formula- or breast- fed. Moreover, aside from breast milk, the type of formula in which infants receive may have a cofounding influence on the colonization stability and efficacy of LBPs; As lactose is known to have a bifidogenic effect (Yılmaz-Ersan et al., 2016; Gallier et al., 2020), the varying concentration of lactose between formula milks, particularly amino-acid based/hydrolyzed vs. regular formula, may influence the stability and function of LBPs in the colon. Accordingly, a meta-analysis found that beneficial effects of LBPs on allergy outcomes were reported more by studies in which infants received amino acid-based or hydrolyzed formula, compared to those receiving standard formula or probiotics alone (Sestito et al., 2020), indicating a possible synergistic effect of amino-acid based/hydrolyzed formulas with LBPs. Consequently, method of feeding, including breastfeeding or formula type, should be taken into consideration when drawing conclusions on the protective role of LBPs in allergic disease.

Furthermore, the lasting protective effects of LBPs on long-term allergy outcomes are unclear. While several studies suggest that LBP supplementation protects against atopic disease throughout childhood, others suggest this protection is short-lived. For instance, Niers et al., 2009 (Niers et al., 2009) demonstrated that pre-and post-natal supplementation of a microbial consortium, consisting of *Bifidobacterium bifidum, Bifidobacterium lactis and Lactococcus lactis*, till 1 year of age significantly reduced incidence of eczema, including reduced IL-5 production, at 2 years old. However, this protective effect was no longer present by 6 years of age (Gorissen et al., 2014), demonstrating only temporary benefits within infancy. Notably, as the first 1000 days of life are considered as the early-life critical window in which microbiota colonization shapes immune system programming (Gensollen et al., 2016; Iyer and Blumberg, 2018; Romano-Keeler and Sun, 2022), microbiota and immune development are still ongoing at 1 year of age (the time of probiotic cessation in this study), thus it is possible that longer-term probiotic intervention throughout the entirety of the critical window is necessary to provide long-lasting allergy protection. In contrast however, other studies have demonstrated long-lasting allergy protection following early-life probiotics; Pre-natal and infant supplementation of *Lactobacillus rhamnosus* from pregnancy till 2 years of age (notably a much longer treatment duration than Neirs et al.,2009 (Niers et al., 2009), was found to significantly reduce both atopic dermatitis and hay fever susceptibility at 11 years of age, indicating long-term protective effects of this LBP (Wickens et al., 2018). Along with this inconsistency, many probiotic trials lack long-term follow-ups, making it difficult to draw conclusions on the long-term

protective efficacy of probiotic supplementation beyond infancy. This likely reflects logistical issues in maintaining participant follow up and high dropout rates. Nevertheless, future research should emphasize long-term follow up in order to determine the lasting therapeutic role of early-life LBP supplementation on allergy outcomes throughout the lifespan.

Lastly, various methodological limitations limit current findings and should be addressed in future research; Studies tend to recruit mothers and infants at high risk of allergy, which although has a valid rationale, creates selection bias and makes it difficult to determine the protective efficacy of LBPs against allergic disease in low-moderate risk individuals. In addition, many trials are limited by short follow-up periods, meaning the long-term impact of LBPs on allergy outcomes beyond infancy and throughout life are unclear. Moreover, several studies fail to account for cofounding variables including infant diet, breastfeeding vs. formula feeding, lifestyle factors and external microbial exposures (e.g., cesarean-delivery & antibiotic use), which can impact infant microbiota composition and thus LBP colonization and function. Such limitations should be addressed in robust and well-designed long-term RCTs.

Future research directions

Despite some promising data indicating an allergy-preventive role of LBPs, findings are limited and inconsistent, thus the WAO conclude that aside from eczema, there is currently not enough supporting evidence to enable clinical recommendations on the use of LBPs for the prevention of childhood allergic disease (Fiocchi et al., 2015). Therefore, greater research in the form of robust RCTs in a variety of heterogeneous populations, is necessary to greater determine the therapeutic applicability of LBPs in a variety of allergic diseases, in order for clinical recommendations to be made.

Regarding future research directions, the optimal bacterial strains, dosage, duration and timing of intervention to best prevent allergic disease are currently unknown, thus further research is necessary to specifically determine the aforementioned criteria. Well-controlled studies should directly compare multiple standardized bacterial strains, dosages and supplementation durations to determine the optimal intervention regime for both allergy prevention and management.

In addition, future RCTs should evaluate standardized atopy assessments, include mother-infant dyads of both high and low allergy risk, involve long-term follow-ups, account for potential synergistic co-founding variables, and assess populations across varying geographical locations and genotypes.

In terms of determining the optimal bacterial strains for allergy protection, the majority of strains assessed thus far are known probiotic species (e.g., lactic acid bacteria), yet further investigation into the compositional differences of the gut microbiota of allergic compared to healthy infants, may provide greater insight into specific 'allergy protective' microbes. As it is well established that intestinal microbiota composition during the early-life critical window varies significantly between healthy vs. allergic infants (Kourosh et al., 2018; Boutin et al., 2020; Lee-Sarwar et al., 2023), examination of the specific microbial communities that differ will enable greater understanding of the bacterial strains involved in allergy susceptibility and hint to their use as a LBP. As such, selection of a consortium of bacterial species present in healthy infants, but not allergic, may have greater relevance and efficacy as a therapeutic tool for allergic disease, compared to traditional generalized probiotic strains.

Furthermore, future research should continue to determine the potential therapeutic role of FMT in allergic disease. Currently, FMT is FDA-approved only for the treatment of recurrent *Clostridium difficile* (*C.diff*) infection (US Food and Drug Administration, Fecal FA, 2023), showing well-tolerated and successful results (Hui et al., 2019; Hvas et al., 2019). Given both the efficacy of FMT in *C.diff* infection and preliminary findings from animal models, FMT holds promising potential for an array of other microbiota-associated diseases, including allergy. Accordingly, further investigation to validate the efficacy and safety of FMT in allergy models, as well as enable a greater understanding of what characterizes an 'allergy-protective' microbiota composition and the underlying immunological mechanisms, is needed to prior to conduction of clinical trials. In addition, future research should address challenges of donor selection and standardization procedures, along with determining the long-term implications of FMT. With growing acceptance and understanding, FMT offers exciting potential as an alternative therapeutic tool not only for allergy, but also other difficult-to-treat microbiota-mediated and immunological diseases such as autoimmunity, as a means to restore the 'normal' microbiota and regulate immune response, thus warrants further exploration.

Moreover, given the established allergy-protective capability of microbial-derived SCFAs (Roduit et al., 2019; Theiler et al., 2019; Vieira et al., 2019; Vonk et al., 2019), combining LBPs with SCFAs, alongside microbial-supporting prebiotics such as HMOs, may aid the immunomodulatory function of LBPs and thus increase their efficacy in allergy prevention. Accordingly, further research should investigate the potential of synbiotic mixtures combining well-researched probiotic strains with prebiotics such as HMOs, GOS or FOS, to aid colonization stability and function. Moreover, future research should assess whether microbial-derived metabolites, SCFAs, which represent a key mechanism by which LBPs modulate immune response, may be used in combination with LBPs as a holistic 'post-biotic' to support microbial function and optimize allergy protection.

If SCFAs represent an effective functional metabolite by which LBPs exert anti-allergy effects, then directly harnessing them as a therapeutic tool may also be an exciting avenue for childhood allergy prevention.

Likewise, in addition to SCFAs, future research should also investigate the potential allergy-protective effects of other microbial-derived metabolites. While SCFAs are a predominant and well-established group of anti-inflammatory metabolites, preliminary findings suggest that other metabolites produced by colonic bacteria, such as poly-γ-glutamic acid (γPGA) and tryptophan metabolites, can yield immunoregulatory effects that may offer value as a therapeutic for allergy (Kim et al., 2009; Lee K. et al., 2011; Losol et al., 2024). For instance, γPGA, a metabolite

produced predominantly by *Bacillus subtilis* and *Staphylococcus epidermidis* (Jose Anju et al., 2018) is found to have an anti-inflammatory role in atopic dermatitis (AD) through its anti-microbial effects against *Staphylococcus aureus* (Inbaraj et al., 2011; Park et al., 2018), a key species implicated in the development of AD (Kim et al., 2019). Supportingly, murine supplementation of γPGA induced Treg production, regulated Th1/Th2 cell balance, enhanced natural killer cell activity via TLR4 signalling, and suppressed secretion of type 2 cytokines from epithelial cells via GPR-activation, resulting in the prevention of AD-like symptoms in mice (Lee K. et al., 2011; Lee SW. et al., 2013). Similarly, tryptophan metabolites such as indole derivatives and kynurenine produced from microbial metabolism of L-tryptophan, particularly by *Escherichia coli, Clostridium*, and *Lactobacilli* (Williams et al., 2014; Li et al., 2021), are also found to have immunomodulatory effects, including inhibition of mast cell degranulation, as well as regulation of T cell differentiation and dendritic cell immunogenicity (Mezrich et al., 2010; Zelante et al., 2013; Kawasaki et al., 2014; Losol et al., 2024), all of which may promote immunotolerance to antigens. Therefore, emerging data suggests that alongside traditional SCFAs, other microbial-derived metabolites may have notable immunoregulatory effects and hold therapeutic value in allergic disease. However, these findings are preliminary, and significantly more research is needed to determine the long-term efficacy of such metabolites on allergy outcomes, their impact on microbiota composition, and exact immunological mechanisms before translation to human trials. Accordingly, the potential synergistic effects of combining both LBPs and microbial metabolites as a holistic 'pre- and post-biotic' to optimize allergy-protection warrants further exploration and holds exciting promise. Moreover, a comparison of the immunomodulatory functions of various microbial metabolites, such as SCFAs, γPGA, indole derivatives, kynurenine, and others, to determine the most effective metabolite for allergy protection, would yield significant value.

In a similar regard, the synergistic effects provided by a consortium of bacterial species combined may increase efficacy against allergy. A meta-analysis of human trials reported that the preventive effects of probiotic supplementation on atopic dermatitis development were most pronounced when a combination of multiple species were administered together, rather than a single species alone (Zuccotti et al., 2015). Accordingly, commensal intestinal microbes interact through complex community dynamics to collaboratively promote microbiota euboisis (Ha et al., 2014; Coyte and Rakoff-Nahoum, 2019). For instance, *Lactobacilli* aids mucus binding of *Bifidobacteria* (Ouwehand et al., 2000), enabling greater functionality than when alone. Moreover, a major means of microbial interaction is via metabolic cross-feeding, in which certain intestinal bacteria feed off the metabolic products, such as SCFAs (Hirmas et al., 2022; Culp and Goodman, 2023), of other microbes, resulting in a complex network of producers and consumers cooperating to support microbiota functionality. As such, the use of multi-strain microbial cocktails supports the natural microbial interplay of the human microbiome, thus likely enhancing LBP efficacy. Therefore, future research should examine the use of multi-strain bacterial consortiums in allergy therapeutics, compared to traditional single-strain interventions, and determine which strains function best synergistically to provide optimal pro-tolerogenic effects.

Furthermore, although it is well established that the intestinal microbiota plays a crucial role in immune development and allergy susceptibility, what constitutes a microbiota composition that promotes and maintains immunotolerance to allergens is still unclear. Greater research to further establish this and the underlying immunological mechanisms, i.e. IgA-dependent pathways, is of significant value to inform microbiota-targeted interventions for reducing allergy risk.

Lastly, the prophylactic vs. therapeutic potential of LBPs in allergy remains unclear. While the majority of research thus far assesses the role of LBPs in the prevention of allergy development, greater research should examine whether LBPs can be used therapeutically to promote immunotolerance to pre-sensitized allergens in existing allergic disease. Although sparse, a small number of studies thus far have assessed the therapeutic role of LBPs on existing allergy; Supplementation of *Lactobacillus paracasei* and *Lactobacillus fermentum* both combined and alone were found to decrease asthma severity scores and serum IgE levels, and improve peak excitatory flow rates (PEFR) in asthmatic children aged 6–18 years old (Huang et al., 2018). Similarly, 8 week supplementation of *Lactobacillus gasseri* in children aged 6–12 years old, significantly reduced clinical symptoms of asthma, improved pulmonary function and PEFR and reduced allergen-stimulated production of inflammatory cytokines including IL-12, IL-13 and TNF-α (Chen et al., 2010). However, it is important to note the limited follow-up periods of these studies. In fact, RCTs with longer follow-up periods have reported negative results, including *Lactobacillus salivarius* at 4 month follow-up (Lee S-C. et al., 2013), *Lactobacillus rhamnosus GG* at 6 month follow-up (Rose et al., 2010), and *Lactobacillus bulgaricus* and *Streptococcus thermophilus* at 12-month follow-up (Giovannini et al., 2007), all demonstrating no benefits on allergy severity. This suggests the therapeutic effects of LBP supplementation in managing existing allergic disease may be temporary, and not persist beyond the supplementation period. Long-term daily supplementation may provide longer lasting benefits for the management of allergy symptoms. Consequently, greater research is necessary to decipher the therapeutic, compared to prophylactic, role of LBP interventions in promoting immunotolerance and managing existing allergic disease.

Conclusion

Overall, LBPs demonstrate powerful immunomodulatory effects during the early-life critical window and have exciting potential to be harnessed as a therapeutic tool for childhood allergic disease. Both animal and human data suggest that LBPs may act through several immunological and physiological mechanisms to influence allergic immune response, including but not limited to, TLR-induced regulation of Th1/Th2 balance, the production of microbial-derived SCFAs and resulting immunomodulation via GPR41/GPR43 activation and HDAC inhibition, and strengthening of epithelial barrier integrity. However, despite highly promising animal data, findings from

human intervention trials are mixed and inconsistent. The efficacy of LBPs in allergy protection is not straightforward and reflects a complex interplay of multiple factors including microbial strain, duration/timing, population genetics, infant risk status, maternal and lifestyle factors, and allergy phenotype. Despite this, LBPs hold exciting potential for both the prevention and treatment of allergic disease across the globe. Future research, particularly regarding optimal maternal/infant supplement regimes, and the efficacy of multi-strain consortiums and synbiotics, is necessary to translate findings into clinical applications, representing a fruitful and promising field of investigation.

Author contributions

IT: Conceptualization, Writing – original draft, Writing – review & editing. BF: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Clinical application of live biotherapeutic products in infectious diseases

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Live biotherapeutics products (LBP) are a novel range of therapeutic options in medicine. In this review, authors discuss basic composition and mechanism of action of LBP, provide a comprehensive focused overview of published *in vitro* and *in vivo* studies on efficacy of LBP for prevention and treatment of infectious diseases such as viral (HIV, COVID-19), bacterial (*C.difficile* infection, bacterial vaginosis, multi-drug resistant organisms) and fungal (Candida) organisms. This review should be of interest to clinicians to understand the broad application of LBP in infectious diseases world beyond recurrent *C.difficile* infection and to researchers on unexplored prospects of LBP and the need for further investigation in this emerging field to improve its clinical application.

KEYWORDS

live biotherapeutic product, live biotherapeutic product in infectious diseases, live biotherapeutic product for *Clostridioides difficile*, live biotherapeutic product for multidrug resistant organisms, live biotherapeutic product for viral infection, live biotherapeutic product for bacterial infection

Introduction

The human microbiome refers to a variety of microorganisms ranging from bacteria, yeasts, viruses, protozoa, archaea, and eukaryotic microbes colonizing the body (Aroniadis and Grinspan, 2024). These microbial communities inhabit the oral, nasopharyngeal, gut, genitourinary and skin lining. The interaction between the host and microbial communities is known to play an important role in human health and diseases. Gut microbiota, the most abundant of all microbiomes, has been associated with a wide variety of metabolic and immune mediated diseases like inflammatory bowel diseases, obesity, cancer, neurological disorders. Since birth, multiple factors such as diet, environment, genetics, antibiotics influence the composition of human gut microbiome (Aroniadis and Grinspan, 2024). Understanding this microbial community and complex host-microbiome interaction has been challenging. Recently, metagenomic studies have led to better understanding of composition of the gut microbiome and impact of alteration in gut microbiome aka dysbiosis on subsequent development of diseases and disorders. As a result, microbiome

engineering is now an emerging novel technique designed to alter microbial composition or its activity for therapeutic purposes to help restore normal health function. Live biotherapeutic products (LBPs) are such an example of genetically engineered microbes used for diagnostic and therapeutic purposes.

The US Food and Drug Administration (Food and Drug Administration, 2016) defines LBPs as "a biological product that 1) contains live organisms such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is not a vaccine". Recombinant LBP is defined as "a live biotherapeutic product composed of microorganisms that have been genetically modified through the purposeful addition, deletion, or modification of genetic material". In contrast to LBPs, prebiotics, probiotics and synbiotics are commonly used as functional foods and dietary supplements, without need for regulatory approval process and lack robust clinical trials data on efficacy against any specific disease prevention and treatment (Table 1).

Host microbiome and association with infection

The human microbiome can resist pathogens from developing infection via direct and indirect pathways. Microbiome can result in direct killing of pathogens, without host involvement, by producing metabolites, toxins and depriving pathogens of essential nutrients (Zhang et al., 2021). For example, vaginal microbiome predominantly consists of Lactobacillus species which produces lactic acid (Kwon and Lee, 2022). Acidic vaginal environment causes inhibition of growth of harmful bacteria like *E.coli*, *N.gonorrhea* and *C.trachomatis*. Lactic acid can also prevent viral infection such as inactivation of HIV surface charge preventing its attachment to host cell. Indirectly, host microbiome can activate innate and adaptive immune responses and stimulate anti-pathogen metabolite production to resist pathogenic infection. Common skin colonizer like *S.epidermidis* can induce CD8 T cells providing antimicrobial protection and helping with tissue repair (Zheng et al., 2020). In a similar fashion to host microbiome, LBPs can be a single microbial strain, or a community of microbial strains designed to interact with host ecosystem using innate microbial properties or through genetic modification (Heavey et al., 2022). These engineered LBPs can function by inhibiting growth of pathogenic organisms or toxins, stimulating production of beneficial molecules to prevent cancer, to modifying metabolism and mucosal immune system to decrease inflammation (Tan et al., 2020). A study performed on human colonic explants pretreated with a single-strain live biotherapeutic product (SS-LBP) of *Bacillus velezensis* strain ADS024 followed by *C.difficile* toxin exposure demonstrated protease secretion by ADS024 resulting in lower toxin B levels and toxin-mediated cell apoptosis (O'Donnell et al., 2022). Thus, SS-LBP has potential for protecting against colonic mucosal damage and preventing recurrent CDI post standard antimicrobial treatment.

Use of live biotherapeutic products in infectious diseases

Clostridioides difficile infection

Clostridioides difficile is one of the most common healthcare-associated infections with high recurrence rates and accounting for 15%-20% all-cause mortality. Exposure to antimicrobial therapy results in gut microbiota dysbiosis and higher primary bile acid concentration increasing the risk for *Clostridioides difficile* infection (CDI) and its recurrence. Treatment guidelines for CDI recommend oral antimicrobial therapy options such vancomycin, fidaxomicin and fecal microbiota transplant (FMT) for recurrent

TABLE 1 Comparison between prebiotics, probiotics, synbiotics and live biotherapeutic products.

	Prebiotics (Davani-Davari et al., 2019)	Probiotics (O'Toole and Cooney, 2008)	Synbiotics (Swanson et al., 2020)	Live biotherapeutic products (Rutter et al., 2022)
Definition	"A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health"	"Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"	"A mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host"	"Biological product that 1) contains live organisms such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is not a vaccine"
Use	Dietary supplement	Dietary supplement	Drug, food or supplement	Medicinal use for range of diseases such as *C.difficile* infection, colitis, inflammatory bowel diseases, cancer, phenylketonuria
Examples	Fructo-oligosaccharides (FOS), Galacto-oligosaccharides (GOS), Lactose, Inulin-enriched FOS	*Lactobacillus spp, Bifidobacterium* spp., *Saccharomyces, Bacillus* spp., *Escherichia coli, Enterococci, Weissella* spp.	*Streptococcus thermophilus* in combination with FOS/GOS/ Inulin-enriched FOS	*E.coli* Nissle 1917, *Lactococcus lactis*, *Salmonella typhi* VXM01
Regulatory approval requirement	No	No	No	Yes

CDI (rCDI). However, antimicrobial CDI treatment has failed to show sustained response in preventing rCDI and the potential spread of pathogenic organisms to recipients via FMT remains a concern.

Live biotherapeutic products have been extensively studied in treatment and prevention of recurrent CDI (Pettit et al., 2024). Microbiota based suspension has been demonstrated to enhance normal gut microbiota (commensal *Bacterioidia* and *Clostridia*) and reduce harmful microbes (*Gammaproteobacteria*, *Bacilli* and *Erysipelotrichia*), thus restoring normal gut flora as well as beneficial secondary bile acid concentrations (Orenstein et al., 2023).

RBX2660 containing a consortium of spore and non-spore forming fecal microbiota, live-jslm (Rebyota), has been studied in a phase 3 clinical trial (PUNCH CD3) administered as a single-dose rectal administration to adults 24-72hrs after completion of standard antibiotic therapy for rCDI (Khanna et al., 2022). Compared to placebo, RBX2660 demonstrated safety and 70.6% efficacy in preventing rCDI at week 8 with sustained effects for up to 6 months post treatment (Lee et al., 2023). Similar findings were observed in prevention of rCDIs in high-risk elderly patients treated with RBX2660 (Tillotson et al., 2022). Fecal microbiota spores, live-brpk or VOS (Vowst Oral Spores) containing suspension of Firmicutes spore forming colonies has been studied in phase 3 ECOSPOR III clinical trial to assess CDI recurrence after completion of anti-CDI therapy (Sims et al., 2023). Treatment of adult patients with oral dose of four VOS capsules for 3 days showed lower rCDI rates compared to placebo and sustained response for up to 24 weeks post treatment. Similar efficacy was seen in older adults through week 8.

A non-frozen oral encapsulated LBP (RBX7455) containing processed fecal microbiota-based suspension of RBX2660 from a single donor successfully completed phase 1 clinical trial demonstrating its safety and efficacy in preventing recurrent CDI (rCDI) in adults for up to 6 months post treatment (Fischer and Ray, 2024).

The phase 2 clinical trial of encapsulated high-dose oral formulation (10 capsules daily for 14 days) containing 8 well-characterized nontoxigenic nonpathogenic commensal *Clostridia* strains (VE303) has demonstrated efficacy in preventing rCDI in adults and primary CDI in high-risk elderly patients (Louie et al., 2023).

Treatment with nontoxigenic strain *C. difficile*-M3 (NTCD-M3) spores possess the ability to colonize gastrointestinal tract inhibiting proliferation of pathogenic *C.difficile*. A phase 2b clinical trial performed with daily oral formulation of NTCD-M3 spores for 7 days showed 95% efficacy in preventing rCDI for 6-weeks post treatment (Gerding et al., 2015).

Gastrointestinal infections other than *C. difficile*

Studies performed in *in vitro* and *in vivo* models have demonstrated antibacterial activity of recombinant human lactoferrin produced by *Lactobacillus casei* (Tan et al., 2020). Similarly, *Lactococcus lactis* is known to secrete anti-enterococcal peptide capable of inhibiting enterococcal growth exerting antimicrobial activity against *E.faecalis* and multidrug resistant *E.faecium* strains.

Traveler's diarrhea is caused by enterotoxin produced by Enterotoxigenic *E.coli* strain. Paton et al. constructed nonpathogenic *E.coli* CWG308 strain expressing glycosyltransferase genes from *Neisseria meningitidis* or *Campylobacter jejuni* resulting in production of modified lipopolysaccharide (mLPS) lacto-N-neotetraose (Paton et al., 2005). Structural similarity of mLPS to Shiga toxin's natural receptor resulted in binding and neutralization of enterotoxin in both human and porcine *in vitro* models as well as *in vivo* rabbit ligated ileal loops.

To combat cholera disease, *E.coli* Nissle 1917 constructed to express cholera autoinducer 1 resulted in reducing cholera toxin binding and intestinal colonization of infant mouse model along with significant growth inhibition of *V.cholerae* (Kelly et al., 2009; Duan and March, 2010). Similarly, *E.coli* Nissle 1917 strain engineered to produce antimicrobial peptides Microcin H47 in presence of tetrathionate, an indicator of intestinal inflammation, has demonstrated ability to inhibit growth of Salmonella species.

Multidrug-resistant organisms

Antibiotic exposure, infections with multidrug-resistant organisms (MDROs) can result in alteration of gut microbiota (Panzer et al., 2024). Patients harboring MDROs are known to develop long-term gut colonization with these organisms increasing risk for reinfection and transmission to others. In view of lack of robust data on decolonization strategies for MDROs, use of LPB to decolonize gut without risk for dysbiosis is a promising alternative (Ducarmon et al., 2021).

Engineered *E.coli* Nissle 1917 modified to overproduce microcin I47 has demonstrated antimicrobial activity against multiple MDRO, with highest efficacy against Enterobacteriaceae (Sonnenborn, 2016; Charbonneau et al., 2020). Preclinical animal model study demonstrated significant decline in intestinal concentration of carbapenem-resistant *K.pneumonaie* without change in native microbiome after seven days of daily oral LBP treatment (Mortzfeld et al., 2022). Genetically engineered *E.coli* Nissle 1917 strains with ability to produce dispersin B, an antibiofilm enzyme, has demonstrated decline in *Pseudomonas aeruginosa* concentrations in animal models and reduction in Enterococcal and Salmonella species in murine models through secretion of antimicrobial peptides (Hwang et al., 2017).

Coronavirus disease 2019 infection

Coronavirus disease 2019 (COVID-19) caused by the virus SARS-CoV-2 has accounted for high morbidity and mortality. Currently, omicron variants continue to circulate in communities worldwide causing mild to severe life-threatening infections including breakthrough infections. Outside of antivirals, LBP has been studied as potential treatment options for COVID-19.

Oral LL-37 is an LBP generated by combination of *Lactococcus lactis* (drug delivery agent) and Human antimicrobial peptide LL-37 (Zhao et al., 2023). In general, Human LL-37 is known to exert antiviral activity by disrupting lipid envelope, causing cell death.

Studies have demonstrated LL-37 prevents binding of SARS-CoV-2 spike protein to host cell receptor ACE2 reducing risk for COVID-19 infection. In an investigator-initiated prospective open-labeled randomized case-control single-center clinical trial, authors compared the effect of oral LL-37 vs placebo (*L.lactis*) on negative conversion time (NCT) of SARS CoV-2 nucleic acid test. Adult patients with mild Omicron BA.5 infection received oral LL-37 (n=129) or placebo (n=109) for seven days. Treatment with oral LL-37 resulted in significantly reduced NCT when initiated within 6 days of infection without any safety concerns. Additional studies are needed to demonstrate if early initiation of oral LL-37, reducing SARS CoV-2 viral load, could potentially have other beneficial roles such as shorter duration of clinical symptoms and lower risk for long COVID.

Respiratory tract infections

Viral respiratory tract infections are known to cause many diseases from common cold infections to severe pneumonia. Other than influenza and COVID-19, there is lack of robust data on antiviral treatment recommendations for other viruses. Respiratory microbiome is known to resist viral infections with antipathogenic properties, immune activation and maintenance of epithelial barrier integrity. *In vitro* topical testing has demonstrated *Lactobacillaceae* strains to directly inhibit respiratory viruses and stimulate interferon regulatory pathways. Further *in vitro* analysis of three strains *Lacticaseibacillus casei* AMBR2, *Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1 has shown to have combined anticytopathogenic effects against respiratory syncytial virus, influenza viruses and human coronaviruses-229E. The combination of these three strains suspended in oil delivered in form of a throat spray was investigated in healthy volunteers (Spacova et al., 2023). Study confirmed microbiome throat spray resulted in immune activation and colonization of throat with all 3 *Lactobacillacea* strains for 30 minutes post application. Further clinical trials are needed to assess the safety and efficacy of these products for practical use.

Urinary tract infections

Urinary tract infections (UTIs) can range from uncomplicated cystitis to pyelonephritis. It is the one of the most common bacterial infections in the world accounting for high-cost burden to the healthcare industry. Frequent use of antimicrobials for treatment of UTIs has resulted in a rise in antimicrobial resistance and limitation in available effective treatment options. Live biotherapeutic products have been studied as potential options for treatment and prevention of UTIs.

Normal urogenital flora is known to have protective factors which prevent ascending infections. Less UTIs were observed after vaginal application of *Lactobacillus crispatus* causing restoration of native microbial flora. Asymptomatic bacteriuria (ASB) *E.coli* strain has been shown to possess bacterial interference properties by inhibition of uropathogens via unknown mechanism to prevent UTIs (Rudick et al., 2014). Additionally, intravesicular or intravaginal ASB *E.coli* instillation has shown to possess analgesic activity similar to intravesicular lidocaine. Clinical studies performed in spinal cord injury patients, neurogenic bladder patients with chronic catheterizations, and recurrent UTIs showed significantly lower UTIs after inoculation of ASB *E.coli* strain 83972. Furthermore, ASB *E.coli* strain 83972 has shown comparable results to ciprofloxacin in reducing NU14 bacteriuria in mice models. ASB E.coli strain also demonstrated lower UTI allodynia in infections with pathogenic *E.coli* as well as other common gram negative and gram-positive uropathogens. Overall, LBPs have therapeutic benefit for UTIs as well as symptomatic relief in interstitial cystitis patients, reducing the risk for unnecessary antibiotic use in these patient population.

Human immunodeficiency virus

Every year more than 30,000 new human immunodeficiency virus (HIV) infections are diagnosed in the United States. Currently, prevention of HIV relies mostly on safe sex and needle use practices. Pre-exposure HIV prophylaxis with oral tenofovir-emtricitabine based antiretrovirals and long-acting cabotegravir injectable agents are available. Adherence to medications, risk of viral resistance and breakthrough infections remains a concern.

Normal vaginal flora has been known to possess beneficial properties resisting urogenital infections such as sexually transmitted diseases and HIV. Lactobacilli strains, commonly found in 17%-41% of vaginal flora, produce hydrogen peroxide and lactic acid reducing the risk of acquiring sexually transmitted infections and HIV-1.

Recombinant LBP MucoCept is a genetically engineered *Lactobacillus* strain, *L. jensenii* 1153-1666 designed to prevent HIV infection in women by secreting modified HIV-1 inhibitor protein, cyanovirin-N preventing mucosal viral entry (Lagenaur et al., 2015). Study in rhesus macaque model inoculated with vaginal MucoCept showed 63% reduction in acquisition of chimeric simian-HIV and six-fold reduction in animal viral loads with breakthrough infections. Potent, stable, easy-to-use, rapidly disintegrating vaginal tablets of MucoCept successfully colonized 83% of macaques after 21 days. Clinical trials are underway to test novel once-monthly MucoCept vaginal rings as a non-antiretroviral option for prevention of HIV-1 infection in women.

Bacterial vaginosis

Bacterial vaginosis (BV) is one of the most common vaginal infections in reproductive age women associated with high recurrence rate and increased risk for HIV infection due to genital inflammation and epithelial barrier disruption. The condition is known to occur due to imbalance in bacterial flora causing overgrowth of anaerobic bacteria and reduction in hydrogen-peroxide-producing Lactobacilli species, which normally predominate vaginal flora. Among Lactobacilli species, *L.crispatus* is known to be predominant with anti-inflammatory properties and suppression of proinflammatory bacterial species reducing the risk for HIV acquisition. Standard antibiotic treatment of BV has been shown to reduce proinflammatory cytokines, however it increases Interferon-gamma-induced protein (IP)-10 associated with HIV acquisition risk and does not restore beneficial Lactobacillus species. Studies with Lactobacillus species-based probiotics have been unsuccessful in lowering genital inflammation and BV recurrence.

Live biotherapeutic containing *L.crispatus* strain CTV-05 (LACTIN-V) has been investigated in phase 2 clinical trial post standard topical metronidazole treatment of BV (Armstrong et al., 2022). Compared to placebo, vaginal application of LACTIN-V resulted in lower rates of recurrent BV sustained up to 3-months post last treatment dose. Study findings showed LACTIN-V lowered concentrations of genital pro-inflammatory cytokines and chemokines, suppressed inflammatory bacterial vaginosis-associated bacteria and diminished epithelial barrier disruption. LACTIN-V is a promising treatment option for preventing recurrent BV and an appealing non-antiretroviral option to prevent HIV acquisition in women.

Candidiasis

Candidiasis is a fungal infection caused by the Candida species, predominantly *C.albicans*, a common part of normal human microbial flora. Opportunistic infections can range from mild thrush to life-threatening invasive diseases. Treatment options are limited to 3 antifungal classes and emerging resistance remains a concern. *Lactobacillus casei rhamnosus* Lcr35 (Lcr35) is known to possess anti-pathogenic properties against *Candida albicans* by preventing its adhesion to epithelial cells (Poupet et al., 2019). *In vitro* testing on Caco-2 monolayer, an enterocyte-like cell line, failed to show any effect of Lcr35 on *C.albicans* growth or biofilm formation. However, *in vivo* study on *C.albicans* infected Caenorhabditis elegans worm showed protective efficacy of Lcr35. Mechanistic study demonstrated Lcr35 mediated activation of host's immune response (DAF-16/Forkhead Box O transcription factor, upregulation of p38 MAP Kinase signaling pathway) and antifungal response as likely mechanism of action against *C.albicans*. Study findings provide insight into exploring potential therapeutic options in future for treatment of Candidiasis.

Discussion

Infectious diseases are caused by pathogenic bacteria, fungi, viruses or parasites. Management of infectious diseases ranges from symptomatic treatment to antimicrobial therapy for days to weeks. Currently, widespread use of antimicrobial therapy has resulted in a rise in antimicrobial resistance and its spread. Moreover, challenges encountered with lack of effective treatment options have become a rising concern. Live biotherapeutic products containing modified normal microbiota demonstrating ability to restore microbial flora and kill pathogenic organisms are ideal non-antibiotic treatment strategy to resolve ongoing challenges.

Preclinical and clinical studies with LBP have been successful in prevention and treatment of many infectious diseases (Table 2).

TABLE 2 Clinical application of live biotherapeutic products in Infectious Diseases.

Indication	Live Biotherapeutic Product	Mode of delivery	Clinical evidence
Prevention of recurrent *C.difficile* infection	Fecal microbiota spores, live-brpk or VOS (Vowst Oral Spores)	Oral capsules	Phase 3 ECOSPOR III clinical trial, FDA approved
	RBX2660, consortium of spore and non-spore forming fecal microbiota, live-jslm (Rebyota)	Rectal administration	Phase 3 clinical trial (PUNCH CD3), FDA approved
	RBX7455, fecal microbiota-based suspension from single donor	Oral capsules	Phase 1 clinical trial
	VE303, nontoxigenic nonpathogenic commensal Clostridia strains	Oral capsules	Phase 2 clinical trial
	Nontoxigenic *C.difficile*-M3 spores (NTCD-M3)	Oral capsules	Phase 2b clinical trial
Prevention and treatment of bacterial gastrointestinal infections (other than *C.difficile*)	Lactobacillus casei, Lactobacillus lactis, nonpathogenic *E.coli* CWG308, *E.coli* Nissle 1917	Unknown	Preclinical models
Gastrointestinal decolonization of Enterobacteriaceae including multidrug resistant organisms	*E.coli* Nissle 1917	Oral capsules	Preclinical models
Treatment of COVID-19 infection	Human LL-37 (*Lactococcus lactis* and human antimicrobial peptide LL-37)	Oral capsules	Prospective open-labeled randomized case-control single center clinical trial
Prevention and treatment of respiratory tract viral infections	*Lacticaseibacillus casei* AMBR2, *Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1	Throat spray	Phase 1 clinical trial
Prevention and treatment of urinary tract infections	*Lactobacillus crispatus*, Asymptomatic bacteruria *E.coli* strain 83972	Intravaginal or intravesicular application	Preclinical models
Prevention of HIV	*L. jensenii* 1153-1666 (MucoCept)	Vaginal tablets	Preclinical models
Prevention and treatment of bacterial vaginosis	*Lactobacillus crispatus* strain CTV-05 (LACTIN-V)	Vaginal application	Phase 2 clinical trial
Treatment of Candidiasis	*Lactobacillus casei rhamnosus* Lcr35 (Lcr35)	Unknown	Preclinical models

Most robust data is available in prevention of rCDIs. Faecal microbiota transplantation has been successful in preventing recurrent CDIs. However, reports of extended-spectrum beta-lactamase-producing *E.coli* infection and Shiga-toxin producing *E.coli* infection have been reported post FMT (Park and Seo, 2021). Incomplete screening of donor stool for pathogenic organisms has raised safety concerns related to FMT. Additionally, obesity and immune-mediated disorders have been linked with donor microbiome engraftment. In contrast, the FDA Center for Biologic Evaluation and Research (CBER) regulates the development of LBP including clinical trial studies to ensure its safety and efficacy (Cordaillat-Simmons et al., 2020). Facilities conducting manufacturing, processing and packaging of LBP operate under regulations of current good manufacturing processes to provide safe and reliable products. Recently, recombinant LBPs Rebyota and Vowst Oral Spores have been FDA approved for use in prevention of recurrent CDI.

In general, clinical development of LBP is a rigorous process of conducting genome sequencing of preferred clinical bacterial strain, detecting for any transferrable resistant genes, restricting replication of the microorganism in the human body and determining its biodistribution outside the site of action (Charbonneau et al., 2020). The major challenge in LBP development remains the lack of full understanding on interaction between host, microbiota, and environmental factors (Rouanet et al., 2020) Oral administration of LBP has been the most common mode of delivery thus far and considered safe and convenient for patient use. However, numerous physiological challenges encountered in human gastrointestinal tract including but not limited to stomach acids, bile acids, various enzymes, immune cells, native colonic flora, chemical environment, peristalsis, mucosal and epithelial regeneration can alter LBP functions (Heavey et al., 2022). Thus, determining appropriate delivery methods and dosage forms of LBP for treatment and prevention of diseases remains a mystery (Pot and Vandenplas, 2021).

Regardless, LBPs have tremendous potential for clinical application in both infectious and non-infectious diseases fields. The availability of safe and effective alternative non-antibiotic treatment options for management of infectious diseases will be a game changer. Use of LBPs for treatment and prevention of commonly encountered infections such as UTIs, respiratory viral infections, rCDI, and other GI tract infections can have significant healthcare and economic impact. With the current challenges of antimicrobial resistance, LBP are essentially the holy grail for management of infectious diseases.

Further studies are necessary to overcome ongoing challenges. To improve the development of LBP, application of multi-omics (e.g. genomics, transcriptomics, proteomics, metabolomics) studies are essential for better understanding of host-LBP-microbiome interactions, to identify effective delivery methods and dosage forms of LBP for preventative and therapeutic purposes (Heavey et al., 2022). Many of the current studies have focused on the use of recombinant *E.coli* and *Lactobacillus* strains. Additional exploratory work will be crucial to discover other beneficial microbes in the vast human microbiota and their potential clinical applications.

In conclusion, with rising antimicrobial resistance and limited antimicrobial treatment armamentarium, LBPs are essential and promising non-antimicrobial treatment options for management of infectious diseases. Advances in omics strategies and genetic engineering technology are necessary to perfect these products and use them in the real-world setting.

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Navigating regulatory and analytical challenges in live biotherapeutic product development and manufacturing

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The recent FDA approvals of Rebyota™ and Vowst™ represent landmark milestones within the burgeoning field of live microbiota-based products. Future microbiota-based treatment approaches also hold significant promise for treating patients with a myriad of diseases and disorders, yet substantial hurdles hinder their development and utilization. Foremost, existing regulatory frameworks governing live biotherapeutic product (LBP) manufacturing development have notable gaps, requiring comprehensive expansion and refinement. Along with regulatory challenges, hurdles remain in the optimization and validation of analytical methodologies essential for characterizing LBPs, including for microbial identification, potency, and bioburden. To address these challenges, Microbiome Therapeutics Innovation Group (MTIG) spearheaded collaborative efforts, engaging industry leaders and the FDA in discussions aimed at catalyzing improvements in LBP analytics and refining the current regulatory landscape. Extrapolating on feedback from these discussions, this review highlights challenges and identifies critical gaps. Specific recommendations for future regulatory guidance are proposed, as are recommendations for interactions that developers can take now with regulatory agencies to support the development of maturing guidance. Key analytical factors to consider in LBP development are reviewed, highlighting strengths and weaknesses of various methodologies. Collaboration among regulatory and government agencies, industry, and academia, facilitated by coalitions like MTIG, will be instrumental in ushering the microbiota-based therapeutics field into the next phase of approvals and advancements, ultimately benefiting patients.

KEYWORDS

live biotherapeutic product, microbiome therapeutics, regulatory, analytical testing, manufacturing, drug development, potency, bioburden

1 Introduction

As scientists unravel the causal relationships between commensal microbial communities and human health, opportunities for innovative therapies continue to expand. Early interventions were focused on fecal microbiota transplants (FMT), but the modalities investigated have expanded to include prebiotics, probiotics, postbiotics, donor-derived microbiota-based biotherapeutic products, and defined single/consortium live biotherapeutic products (LBPs) (Table 1). These modalities vary widely in their regulatory frameworks. For example, probiotics are considered foods/supplements, lack a commonly accepted definition among regulatory agencies (Table 1), have not been approved as drugs by the FDA, and require an Investigational New Drug (IND) application filed if used in a clinical setting to treat, prevent, or cure disease (Food & Drug Administration, 2006, 2016, 2018, 2023a; World Health Organization, 2006). Probiotics used "off label" and without an IND have posed safety hazards in some cases, with the FDA issuing warning statements (Food & Drug Administration, 2023a). In 2013 the FDA ruled that FMT would be regulated via the IND pathway (Merrick et al., 2020). In broad strokes, the 2013 ruling provided a pathway to therapeutic approval for live microbiota therapeutics, and two have now been approved: Ferring's Rebyota™ and Seres Therapeutics' Vowst™, representing landmark milestones for the field (Food & Drug Administration, 2023b). Both are donor-derived microbiota-based biotherapeutic products designed to address dysbiosis within the gut microbiome for the prevention of recurrent *Clostridioides difficile* (rCDI) infections, and both are a significant advancement beyond FMT in that they have controlled manufacturing processes, defined analytical testing methods, and established clinical performance (Lavoie et al., 2023).

Despite the promise of these approvals, substantial challenges persist for development of additional microbiota-based modalities, particularly with respect to understanding regulatory guidance and expectations. For instance, in 2016 all live microbiota-containing therapeutic modalities, including those now approved, appeared to fall within the FDA's LBP classification, whereas there are now indications that this classification may be limited to modalities whose manufacturing starts from laboratory-defined microbial strains (Food & Drug Administration, 2016). Since no such products are yet approved, this review will focus on unresolved questions associated with the analysis and regulation of these laboratory-defined single or consortia LBPs. To date, FDA's key guidance for LBPs is limited to only one document, issued in 2016 (Food & Drug Administration, 2016). While the lack of subsequent guidelines or further standardization has enabled continued innovation, it can also lead to unclear expectations and differing baseline assumptions which may delay clinical availability of new drugs. These gaps motivate diligent communication between LBP developers and regulators and demand further expansion and refinement of guidance as the field matures.

Throughout development and manufacturing of defined single or consortia LBPs, characterization of purity, potency, and identity is critical for patient outcomes and safety, as well as the quality documentation required for regulator assessment. Unfortunately, the selection and validation of analytical assays remains challenging. Likewise, health authority expectations and the applicability of existing Good Manufacturing Practice (GMP) guidance for manufacturing of these products is incomplete and often not harmonized across regions. To address these and other issues, Microbiome Therapeutics Innovation Group (MTIG) hosted a workshop at the 2023 Microbiome Connect conference to engage industry leaders and the FDA. Here we will expound upon these discussions to provide industry views on opportunities to improve analytics of LBPs, motivate foundational research across academia and industry, highlight current regulatory gaps, and propose specific interactions between regulators and developers to collaborate on maturing guidance in the field.

TABLE 1 Nomenclature.

Term	Definition
Live biotherapeutic product (LBP)	A biological product that 1. contains live microorganisms, such as bacteria, viruses or yeast; 2. is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3. is not a vaccine (Food & Drug Administration, 2016)
Donor-derived microbiota-based biotherapeutic product	Microbiota-based product that is derived during the manufacturing process from donor materials (e.g. stool samples) as the source for the formulated microorganisms
Defined consortia live biotherapeutic product	Fermented live biotherapeutic product that is derived from multiple cultivated naturally occurring microorganisms of defined, standardized composition (Ducarmon et al., 2021). Alternatively termed "designed" consortia live biotherapeutic product (McChalicher and Aunins, 2022)
Defined single live biotherapeutic product	Fermented live biotherapeutic product that is derived from a single cultivated naturally occurring microorganism as the source for the formulated product. Alternatively termed "designed" single live biotherapeutic product (McChalicher and Aunins, 2022)
Probiotics [1]	Whole, live microorganisms that are ingested with the intention of providing a health benefit (such as supporting digestion and nutrient adsorption in the intestine) (Food & Drug Administration, 2006) OR Live microorganisms, which when administered in adequate amounts, confer a health benefit to the host (World Health Organization, 2006) OR Whole, live bacterial strains or other microorganisms intended for non-therapeutic health benefits, commonly used in the US as dietary supplements, and as such not currently subject to FDA approval (Microbiome Therapeutics Innovation Group, 2024)

[1]Probiotics lack a commonly accepted definition, both generally and among regulatory agencies.

2 General needs and gaps in analytical testing

Analytical testing for the comprehensive characterization and release testing of any LBP encompasses key parameters such as identity, potency, purity (including microbial bioburden and contamination control), and stability (Food & Drug Administration, 2016). Definitions for these parameters have been recommended by the FDA, but specific analytical methods are not standardized. When developing such methods, it is imperative to ensure precision, accuracy, selectivity, specificity, and operational robustness in quality control processes to demonstrate lot-to-lot product consistency.

Assays traditionally used for biologics, including compendial methods, may perform poorly with respect to the above attributes when applied to LBPs, stemming from the complexity of the starting raw materials and various additional factors, such as biological diversity, number of strains, strain-to-strain interference, etc. Because of this, assay optimization and acceptance criteria for qualification and validation must be well-justified for each specific LBP, route of administration, and target population. The complexity and novelty of these assays may result in high costs for development and implementation, especially during early-phase development. Consistent execution will rely upon well-trained QC technicians and an emphasis on retaining staff with specific expertise. A balance of innovation, cost considerations, and regulatory standardization will be necessary, understanding that all methodologies will require a rigorous level of validation to support licensure and marketing authorization. Therefore, as the field continues to mature, successful end-to-end development requires mechanisms for active collaboration with regulators and within the industry in excess of the typical phased interactions to enable meaningful interpretation of clinical results and validation performance for both test methods and manufacturing processes. An overview of the challenges and potential solutions at each step of the product development cycle, from LBP identification/characterization through the marketing approval stage is presented in Figure 1 and discussed in detail below.

3 Evolving microbial identification methods

Traditional methods of microbial identification (ID) testing are based on cell morphology, colony morphology, and metabolic phenotypes, and are often insufficient to characterize LBPs, especially some multi-strain products displaying overlapping phenotypes or diverse growth requirements. Alternative ID methodologies include 16S rRNA gene sequencing, taxon-specific quantitative PCR [qPCR], MALDI-TOF mass spectrometry, and other biochemical or functional assays (Vandeputte et al., 2017;

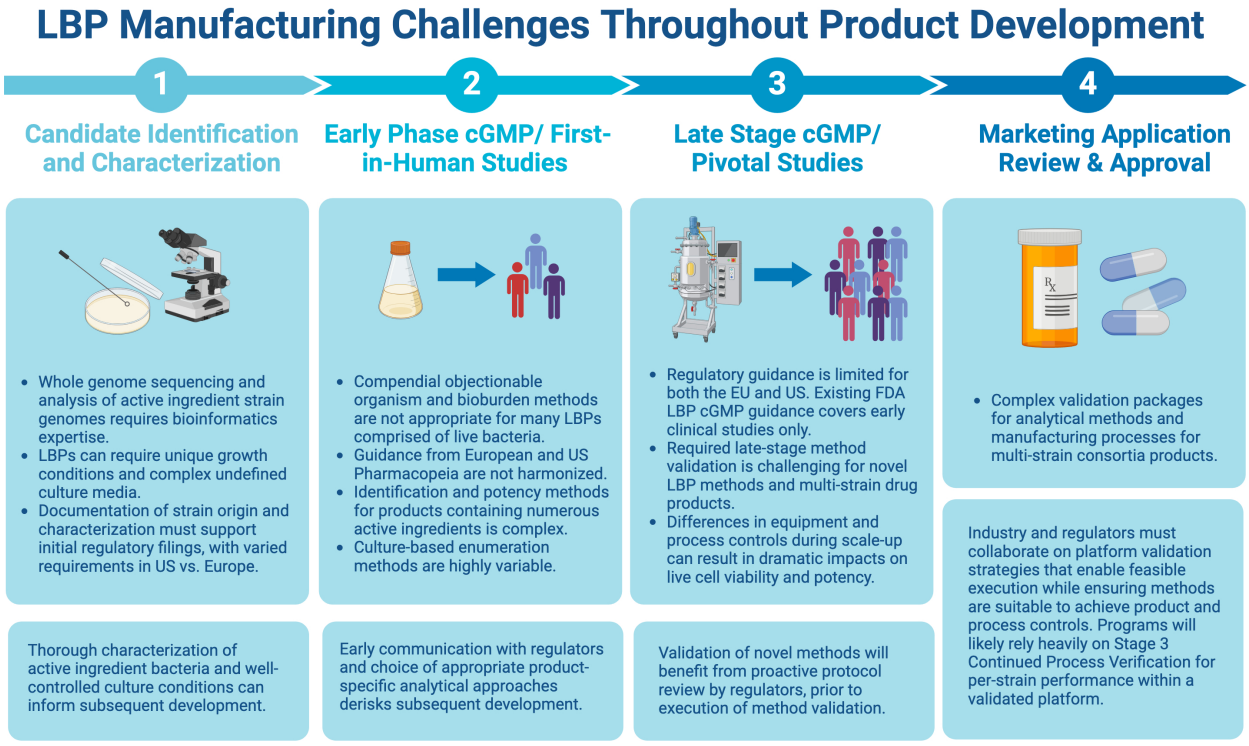

FIGURE 1
Challenges and potential solutions at each step of the product development cycle. Figure created with BioRender.com.

Galazzo et al., 2020; Jian et al., 2020; Paquet et al., 2021; Coryell et al., 2023). Some of these analytical assays are more easily validated than others based on previous use for established modalities, thus drug developers must assess overall suitability for the proposed use, including performance criteria (e.g., sensitivity and selectivity), implementability, and operational robustness. For multi-strain LBP release testing, ID methods may overlap with expectations for per-strain quantification to support potency.

There are a variety of factors to consider while selecting the optimal ID testing method, including accuracy, sensitivity, potential biases, and cost-effectiveness (Vandeputte et al., 2017; Galazzo et al., 2020; Jian et al., 2020; Coryell et al., 2023). The requirement for additional expertise and the higher cost of alternative methods such as MALDI-TOF can be a barrier for sponsor companies. Developers may consider combining methods to provide a more comprehensive characterization. For example, to overcome ID and enumeration challenges associated with LBPs, MALDI-TOF has been combined with colony forming unit (CFU) enumeration to simultaneously identify and enumerate viable active ingredient bacteria (Coryell et al., 2023).

With LBPs, product-specific ID is required by the FDA and European Medicines Agency (EMA), and the FDA recommends using at least two complementary methods for both ID and active ingredient assessments (Paquet et al., 2021). For example, a qPCR assay may be used to delineate strains with identical 16S rRNA gene sequences. Overall, using an ID method that can ensure a qualitative yes/no on batches produced over time is desirable.

To ensure assays have adequate sensitivity and specificity, benchmarking standards/controls are important throughout the development and manufacturing process. Standards are useful for any molecular-based assay used: 16S rRNA sequencing, MALDI-TOF, or the more exploratory option for LBPs, metagenomics sequencing. The use of appropriate sequencing controls is critical, including internal "spike-in" standards to address any variability in assay methods performance (Tourlousse et al., 2017). Nevertheless, the route remains unclear for validating sequencing as an approval-enabling product specification and further evaluation is needed to assess the suitability of any method for routine product release testing in the context of a commercial LBP.

4 Challenges in potency testing

Throughout the manufacturing process, including upstream fermentation, final release testing, and long-term storage, monitoring of LBPs for viability is imperative. Stabilization may be a challenge with certain formulation techniques and compositions, and viability of actives may be impacted when not under ideal conditions. Many LBPs are developed using a viable cell specification for potency release testing of drug substance and drug product, including monitoring of potency during stability studies. As more LBPs advance through late clinical and commercial development, an increase in understanding of their mechanism of action may facilitate identification of other functions or characteristics that are critical to clinical efficacy and that can be deemed gold-standard measures of potency (Paquet et al., 2021)

All methods of viable cell enumeration via CFU testing of active ingredient strains generally need to be tailored to the LBP. The different strains present in multi-strain LBPs may have unique growth requirements, diverse colony morphologies, or strain-to-strain interferences which can affect method performance. Further, common culturing methods and media may not even be feasible or amenable to validation for some strains. To address some of the challenges and shortcomings of traditional enumeration methods, alternative methodologies for LBP potency testing can be considered, including propidium monoazide (PMA) viability qPCR, flow cytometry quantification and sorting, impedance-based methods, or other biochemical or functional assays (Kobayashi et al., 2009; Reyneke et al., 2017; Galazzo et al., 2020; Chen et al., 2024). All of these alternative methodologies also have technical challenges/shortcomings in their application for potency testing but are valuable to explore.

Numerous factors must be carefully evaluated in establishing potency testing. In addition to release criterion, potency is a key metric of long-term stability and consistency between drug substance and drug product. Further, performance of potency test methods and product critical quality attributes should be considered when establishing specifications and testing strategies for dosage unit uniformity (U.S. Pharmacopeial Convention, 2011).

5 Bioburden and contamination control strategies

Monitoring microbiological impurities in LBPs is especially important as LBP manufacturing methods often include growth-promotion operations for fastidious organisms and frequently exclude operations for inactivation or clearance of non-product organisms. Guidance for acceptable levels of bioburden and specific microorganisms of concern is available for drugs in general but is incomplete for LBPs (Food & Drug Administration, 2016; U.S. Pharmacopeial Convention 2016b; Paquet et al., 2021). Compendial test methods, while recommended in FDA guidance for early clinical trials with LBPs, have not been developed for LBPs. Consequently, their potential lack of specificity may lead to challenges in accurately enumerating bioburden, especially when product-strain breakthrough occurs and confounds the results (Food & Drug Administration, 2016; U.S. Pharmacopeial Convention, 2016a). The European Pharmacopoeia does include LBP-specific chapters related to bioburden and specified microorganism analysis, however, these are not harmonized (European Pharmacopoeia, 2011; Franciosa et al., 2023). Compendial methods for anaerobic bioburden testing are not yet established, and not currently recommended in FDA guidance, though sponsors may need to consider incorporating such testing based on risk assessment of manufacturing operations, facility performance, product characteristics, route of administration, and the proposed patient population (McChalicher and Aunins, 2022).

A risk-based approach should be taken to application of USP <1111>, which provides criteria for existing bioburden limits with specified microorganisms based on sample type and route of administration (U.S. Pharmacopeial Convention, 2016b). Some LBPs warrant tighter limits than those traditionally applied based on

route of administration and dosage form. In assessment of risk, challenges inherent to LBP production processes, analytical limitations, and the characteristics of the target population should be considered. Supplemental methodologies for bioburden measurements, such as nucleic acid amplification techniques, may enhance detectability of strains for functionalities which present as a high risk for the intended target product profile (McChalicher and Aunins, 2022).

In addition to bioburden levels, regulatory agencies seek data from developers on the risk of transferability of antibiotic resistance to other bacteria and the risk of causing infection, both of which may be impacted by the levels of contamination, and thus would be part of any safety documentation (Paquet et al., 2021).

6 Additional gaps and heterogeneity in the evolving global regulatory framework

Regulatory oversight from the EMA in the EU and the FDA in the US, along with the pertinent ICH guidelines, govern the manufacturing of LBPs (Cordaillat-Simmons et al., 2020). In the US, production of investigational new drugs and biological products are subject to current GMP required under section 501(a)(2F)(B) of the FD&C Act and the IND regulations at 21 CFR Part 312, but the only LBP-specific guidance is a 2016 treatise on Chemistry, Manufacturing and Control (CMC) in early clinical trials (Food & Drug Administration, 2016). No updated guidance is available that considers the field evolution since then, and there remains no guidance for later stage or commercial CMC.

As in the US, there remains a guidance gap in the EU. The EMA coordinates with member states of the European Economic Area and the European Commission to provide regulatory guidelines governing medicinal products (Pharmabiotic Research Institute, 2022). Volume 4 of EudraLex contains guidance on cGMP for medicinal products for human use, and provides principles and guidelines for ensuring the quality, safety, and efficacy of medicinal products during their manufacturing process (European Commission, 2024). Since it does not include a specific definition for LBPs, the applicability of EudraLex Volume 4, e.g., Annex 1 and 2, and Part II remains unclear. Indeed, the only European regulations specific for LBPs are provided by European Pharmacopoeia (Ph.Eur.), via the general monograph 3053 on LBPs for human use, providing rather high-level dispositions on manufacturing and testing, and the two analytical monographs 2.6.36 and 2.6.38 on microbiological examination of LBPs regarding enumeration of contaminants and tests for specified microorganisms, respectively (Franciosa et al., 2023).

These gaps in US and EU guidance leave critical topics unaddressed, such as bioburden and cross contamination control (as discussed), clean room classification, biocontainment, and suitable controls regarding batch-to-batch and product-to-product facility changeover. One critical unaddressed challenge centers on defining the limits for bioburden detection, as this could impact either patient safety or efficacy. More data in that area will drive the type of standards and classifications the industry needs for bioburden controls. Additional shortcomings include guidance on controls pertinent to multi-product/multi-strain facilities and validation methods for platform methods or processes, as well as products containing spore-forming microorganisms (Food & Drug Administration, 2016; European Commission, 2024). A final gap centers on process control and qualification of material suppliers, including medium components often new to cGMP, and the validation status of computerized systems that haven't been used previously for GMP manufacturing.

The FDA is currently taking an open approach, not providing general guidance for LBPs beyond that for early phase clinical studies, in this newly evolving field. A strength of this approach is that it allows for continued innovation, yet until further guidance is released some ambiguity will remain. To that end, agencies in general need validated assays, relevant data and scientific rationale from LBP developers before solidifying regulation.

7 Avenues that developers can pursue now to navigate regulatory requirements

In the short term, developers will need to closely communicate with regulators and propose modifications to established drug development guidance as needed. Bilateral discussions with regulators on all critical steps of their product development should start early in the drug development lifecycle (Charbonneau et al., 2020; Cordaillat-Simmons et al., 2020). This will reduce the risk of a hold for an IND or other delays in advancing promising drug candidates through all phases of development.

It is likely LBP regulations will follow the patterns observed recently for other advanced therapies. For example, gene therapy and cell therapy faced gaps in applicable regulatory guidance and received special and specific regulation by the FDA in part due to their novelty and complexity, and potential safety risks (Eisenman, 2019; Beetler et al., 2023; U.S. Pharmacopeia, 2024a, 2024b). The FDA collaborated with experts, industry, and academia to develop guidelines specific for these emerging fields after clinical success and demonstration of market acceptance (Eisenman, 2019; Beetler et al., 2023).

Likewise, the novelty and complexity of LBPs will ultimately warrant special and specific regulation. By fostering dialogue and knowledge-sharing among industrial, academic, and regulatory stakeholders now, evidence-based LBP regulations may be developed. It will be up to the industry to push this consensus-building, just as they did with gene therapy.

8 Recommendations for longer-term development of regulatory guidelines

To move toward more specific regulatory guidance, FDA's guidance on "Early Clinical Trials with LBPs: CMC Information" should be expanded to cover missing topics particular to LBPs, including bioburden and objectionable organisms, multi-product/multi-strain facilities, LBP-specific technical handling steps, and other topics as discussed here (Food & Drug Administration, 2016).

In addition to addressing these gaps, regulations for later stages, including validation strategies for platform test methods and production processes, may be developed from existing drug development regulations, such as 21CFR Part 600 for biologics (Code of Federal Regulations, 2014), 21 CFR Part 210 for cGMP for manufacturing drugs, and 21 CFR Part 211 for cGMP for finished pharmaceuticals (Code of Federal Regulations, 2024a; b) Regulators can draw on the lessons learned from approval of Rebyota™ and Vowst™. Programs such as FDA's Breakthrough Therapy designation and Priority Review are intended to provide accelerated pathways to development and approval of therapies meeting the associated criteria, as was the case for both Rebyota™ and Vowst™. Clear, consistent, and timely updates by regulators as expectations for LBPs evolve ensure sponsors can achieve the intended benefits of these programs.

9 Discussion

Improving analytical technology for LBPs and continued collaborations for strategic implementation are critical for advancing this field. Facilitating these collaborations are organizations such as MTIG, an acknowledged liaison to the FDA, and Pharmabiotic Research Institute (PRI), Europe's Microbiome Regulatory Science Expertise Center, both of which support the regulatory development of microbiome therapeutics by fostering communication among regulatory agencies, industry experts, and other stakeholders. Contributions by USP, National Institute of Standards and Technology (NIST), and National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) can also move the field forward. Workshops, conferences, publications, and white papers are highly useful for facilitating and capturing the value of such collaborative projects.

Highlighting the benefit of these collaborations, the roundtable workshop led by MTIG inspired some solutions, presented here, in the ongoing effort to address challenges and future directions. Here we proposed specific interactions that developers and regulators can consider to collaborate on maturing guidance. For example, developers should communicate with regulators on all critical steps of their product development, early and often in the development life cycle, even proposing modifications to established drug development guidance as needed. In general, regulatory agencies need LBP developers to provide relevant data, scientific rationale, and qualified or validated assays (depending on the phase of development) before solidifying regulation (Parenteral Drug Association, 2012). Developers can thus support guideline development with these actions. By addressing the challenges and shortcomings of analytical methods used in LBP ID, potency, and bioburden testing, we proposed technical strategies and corresponding regulatory factors for developers to consider.

There is also a proactive role available to the FDA, EMA, and other regulatory bodies, as well as organizations such as MTIG, PRI, and the European Microbiome Innovation for Health (EMIH), to promote understanding of regulatory requirements via workshops and/or educational training programs. For instance, a working group to explore and develop regulatory guidance for the end-to-end process from cell banking to formulation and then finished product management would be beneficial. Another key to advancing these regulatory concepts will be established method standards, as are being championed by NIST (Olson et al., 2015; Forry et al., 2024).

To move the field forward, with LBPs in the pipeline, there is a clear need for more specific regulations, not only for commercial products but across the various stages of clinical development, from early clinical to Phase 3 process validation and commercialization. Most likely there will not be a one-size-fits-all solution since each LBP has unique properties. Nevertheless, there are likely enough commonalities in different LBP modalities to work toward consensus for some aspects. In order to achieve this goal of more defined LBP regulations, collaboration and active engagement between the FDA, EMA, industry, academia, and other relevant governmental agencies is essential. Data sharing and collaboration among developers and researchers as they relate to establishing some consensus in regulatory guidelines are strongly encouraged, as this would accelerate knowledge accumulation and facilitate evidence-based decision-making. Analytical improvements and regulatory solutions are the keys to success in this dynamic field. Novel solutions and a spirit of collaboration will carry this field forward into the next phase of LBP approvals.

Author contributions

MTIG: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing. DB: Conceptualization, Project administration, Writing – original draft, Writing – review & editing.

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Conflict of interest

The Microbiome Therapeutics Innovation Group MTIG is an independent 501(c)(6) coalition of companies leading the research and development of FDA-approved microbiome therapeutics and microbiome-based products to address unmet medical needs, improve clinical outcomes, and reduce health care costs. Author DB is the owner of Edge Bioscience Communications, a freelance, contract scientific/medical writing and consulting company. Corresponding author NK is Founder and Chief Executive Officer of Siolta Therapeutics.

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Vaginal dysbiosis and the potential of vaginal microbiome-directed therapeutics

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A healthy vaginal microbiome (VMB) is dominated by *Lactobacillus* spp. and provides the first line of defense against invading pathogens. Vaginal dysbiosis, characterized by the loss of *Lactobacillus* dominance and increase of microbial diversity, has been linked to an increased risk of adverse genital tract diseases, including bacterial vaginosis, aerobic vaginitis, vulvovaginal candidiasis, sexually transmitted infections, and pregnancy complications such as preterm birth. Currently, antibiotics and antifungals are recommended first-line treatments with high cure rates, but they also can lead to high recurrence and resistance development. As an alternative, lactobacilli have been utilized to restore the vaginal microbiota. In this review article, we discuss vaginal dysbiosis in various gynecological infections and potential interventions based on Live Biotherapeutic Products (LBPs) with a focus on those that use intravaginal treatment modalities to modulate the VMB. Based on these, we provide insights on key factors to consider in designing phenotypic and genotypic screens for selecting bacterial strains for use as vaginally administered microbiome-directed therapeutics. Lastly, to highlight current progress within this field, we provide an overview of LBPs currently being developed with published clinical trial completion for recurrent BV, VVC, and UTI. We also discuss regulatory challenges in the drug development process to harmonize future research efforts in VMB therapy.

KEYWORDS

dysbiosis, vaginal microbiome, vaginal health, live biopharmaceutical products, lactobacilli

Introduction

The vaginal microbiome (VMB) is composed of all the microorganisms inhabiting the vagina. The composition of the vaginal microbiome is dynamic, changing throughout a woman's lifespan, with notable shifts occurring during hormonal changes, such as menstruation, pregnancy, and menopause. Ravel et al. (2011) performed one of the earliest in-depth analyses of the VMB in reproductive-aged women of varying ethnic

groups using next-generation sequencing. They proposed a classification based on Community State Types (CSTs), where the CSTs and their subtypes describe the temporal changes in the composition of the VMB (Ravel et al., 2011; Holm et al., 2023). CSTs I, II, III, and V are characterized by the dominance of different *Lactobacillus* species, such as *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, respectively. Healthy reproductive-aged females are generally CST-I (*L.crispatus*-dominated) and CST III (*L. iners*-dominated). In contrast, CST IV and its subtypes characterize VMBs dominated by facultative anaerobes, such as *Gardnerella vaginalis* and *Fannyhessea vaginae* (formerly *Atopobium vaginae)*, resulting in a high-diversity vaginal microbiome with a reduced abundance of lactobacilli. Current prospective studies on reproductive outcomes of young, healthy women confirm the CSTs classification (Haahr et al., 2016; Kroon et al., 2018). However, due to the dynamic nature of the human VMB and the complexity of the various state types, not all women neatly fit into each CST. A modular approach was proposed in a Belgian cohort describing the co-occurrence of bacterial taxa based on network correlation analysis (Lebeer et al., 2023), where common species associated with bacterial vaginosis (BV) and a healthy VMB can be present in asymptomatic women. In addition, some women, especially those of Black or Hispanic background, may have a natural VMB dominated by non-*Lactobacillus* species. As more research is performed on defining a healthy VMB, more detailed sub-types based on the VAginaL community state typE Nearest CentroId clAssifier (VALENCIA) have been proposed. VALENCIA separates CST III and CST IV groups based on the relative abundance of different bacterial species (France et al., 2020). In addition, metagenomic community state types (mgCSTs) enable the separation of the same species based on the assembled metagenomes and functional diversity (Holm et al., 2023). Nevertheless, in all these findings, it is agreeable that a healthy VMB contributing to positive reproductive outcomes harbors a low-diversity microbial community dominated by different members of the *Lactobacillus* genus.

Furthermore, as our understanding of the VMB increases, it has been determined that vaginal dysbiosis that progresses from CST IV and its subtypes render women more susceptible to other infection-related issues, including BV (France et al., 2020), aerobic vaginitis (Donders et al., 2017), and sexually transmitted diseases (Edwards et al., 2019; Cheng et al., 2020; Wang et al., 2023). Vaginal dysbiosis also potentially increases the risk of other gynecological conditions associated with adverse pregnancy outcomes, such as miscarriage and preterm birth (Grewal et al., 2022; Gudnadottir et al., 2022).

A deep understanding of the composition and function of the VMB and its interaction with pathogens and the host immune system is necessary for the development of novel treatment options for vaginal dysbiosis. Recently, live biotherapeutic products (LBPs) have emerged as a promising and innovative class of therapeutic agents with broad applications, including addressing issues related to the VMB. The United States Food and Drug Administration (FDA) defines LBPs as biological products containing living organisms, such as bacteria, designed to prevent, treat, and cure human diseases or conditions, excluding vaccines. We aim to highlight key factors that can be important to delineate between probiotics with limited or no therapeutic claims as compared to these LBPs designed as drugs by revisiting the currently known consequences of vaginal dysbiosis that need attention and discuss the recent advancements in the application of LBPs for common gynecological conditions. Further, we outline the desirable characteristics of LBPs in treating vaginal dysbiosis and describe the challenges linked to LBP development for vaginal health.

Vaginal dysbiosis and infectious causes of gynecological inflammation

In a healthy vagina, the vaginal microenvironment harbors immune cells, such as natural killer cells, macrophages, and dendritic cells, abundance of which increases during inflammation (Monin et al., 2020; Anahtar et al., 2015). Lactobacilli are crucial for maintaining the vaginal mucosal layer's integrity as a physical defense against pathogens. However, vaginal dysbiosis occurs depending on both biological and behavioral factors. Sexual activity may be a primary cause of some of these infections. However, even sexually inactive individuals can experience dysbiosis in their lifetime due to the use of antibiotics, immunosuppression, low estrogen levels, or unsuitable hygiene habits (Figure 1). Vaginal dysbiosis is generally characterized by a long-term high-diversity state, where non-lactobacilli members of the vaginal community flourish. In a dysbiotic vaginal microbiome, increased microbial diversity, along with a decrease in beneficial lactobacilli, is accompanied by immunomodulatory changes that affect the natural barrier and contribute to further alterations in the microbiome, vaginal homeostasis, and host immunity.

Bacterial vaginosis

The most common dysbiotic state in the vagina occurs with BV, especially in reproductive-aged women worldwide. Its risk for susceptibility to other obstetric and gynecological diseases, such as preterm birth and sexually transmitted infections (STIs), renders a high global economic burden fueling the impetus for screening and therapeutic studies (Peebles et al., 2019). Nugent scoring based on microscopic evaluation of Gram-stained vaginal smear samples provided standardized measures for assessing vaginal health (Nugent et al., 1991; Coleman and Gaydos, 2018). Although the Nugent scoring is proposed as a gold standard and allows comparability between ethnic groups, it requires time and expertise, for which the Amsel criteria is more preferred especially in resource-limited settings (Bhujel et al., 2021). Clinically, the Amsel criteria are more commonly used, where 3 out of the 4 conditions are met for a diagnosis: 1) thin, homogenous discharge; 2) pH of vaginal fluid above 4.5; 3) clue cells on microscopy (more than 20% of epithelial cells); 4) positive KOH test (Amsel et al., 1983; Donders, 2010). However, given the lower specificity and sensitivity of the Amsel criteria (37 to 70%) as compared to Nugent scoring (94-97%) (CDC, 2021), variations in clinical diagnosis can affect the understanding of the contribution of

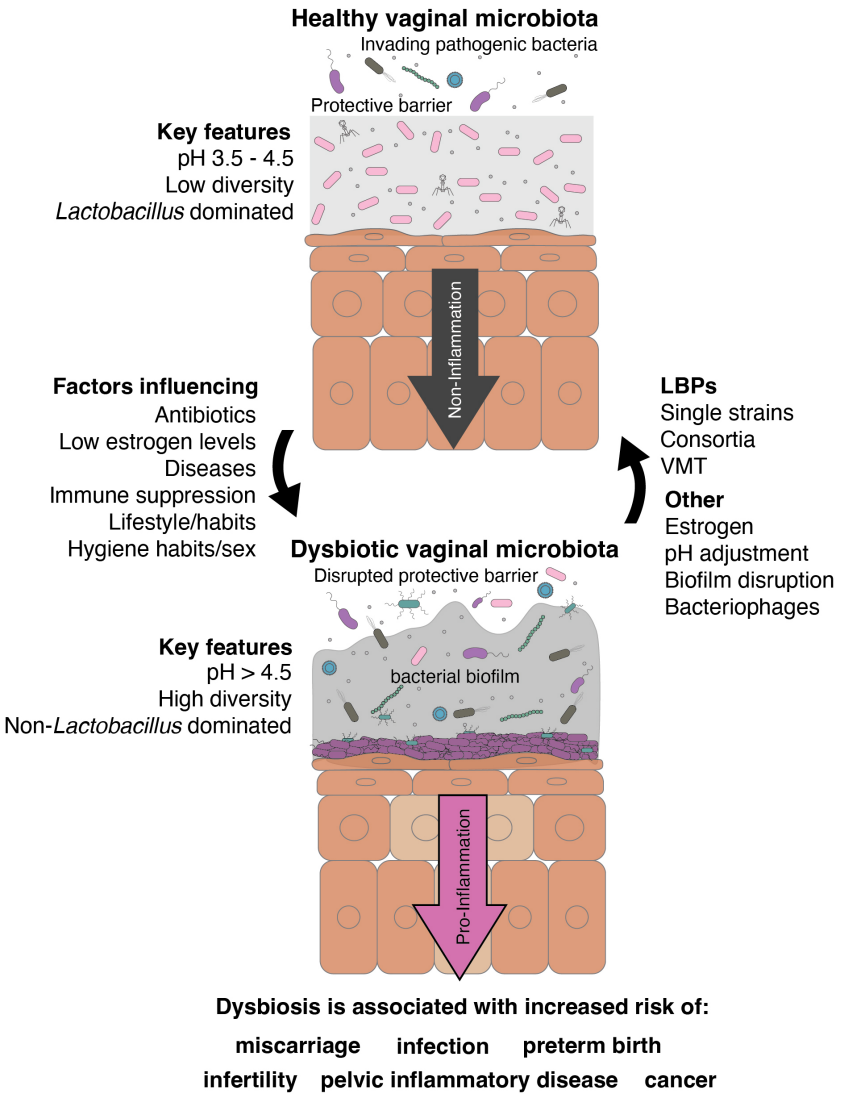

FIGURE 1
Graphical summary of the role of native lactobacilli in the vaginal tract and their potential role as LBPs in vaginal dysbiosis.

ethnic differences to the prevalence of the condition and the etiology of the disease relevant to the microbiome.

First-line treatment for BV is mainly clindamycin or metronidazole, with cure rates decreasing from >90% (Joesoef et al., 1999) to 50-80% (Eschenbach, 2007) in recent reports of known BV cases, accompanied by high recurrence rates (Bradshaw et al., 2006; Coudray and Madhivanan, 2020; Verwijs et al., 2020). The variability in response to antibiotic treatment may be due to differences in how efficient the inconsistency the BV-associated biofilm is disrupted (Bradshaw and Sobel, 2016; Wu et al., 2022; Sousa et al., 2023). Further, within biofilms, BV-associated bacteria may possess inherent antibiotic resistance mechanisms, such as pumping, chemical neutralization, and matrix barriers (Hardy et al., 2017; Nourbakhsh et al., 2022). This poor clearance can possibly lead to a high recurrence rate, where more than half of the women experience BV symptoms again within one year of the antibiotic treatment and likely enrichment of antibiotic-resistance genes in the vaginal environment (Beigi et al., 2004a; Bradshaw et al., 2006).

During BV, the vagina fosters a pro-inflammatory environment, which can be partly attributed to the immunomodulatory effects of the anaerobic bacteria and their metabolites. Notably, short-chain fatty acids (SCFAs) are elevated during BV and their abundance is associated with increased inflammation (Delgado-Diaz et al., 2020; Schwecht et al., 2023). The presence of SCFAs such as acetate, propionate, butyrate, and succinate combined with biogenic amine production in the absence of lactobacilli increases vaginal pH >4.5 (O'Hanlon et al., 2011), making it favorable for growing anaerobic bacteria that produce virulence factors that can degrade mucin, compromise the vaginal epithelial barrier integrity, and stimulate pro-inflammatory responses (Aldunate et al., 2015). Further, the recognition of bacterial cells by the toll-like receptors (TLRs) on the vaginal epithelial cells launches an inflammatory response (Aldunate et al., 2015; Delgado-Diaz et al., 2020). Aside from the production of amines, recent research found that specific amino acid metabolites secreted by BV-associated bacteria, such as imidazole propionate, can interfere with vaginal epithelial cell function and

further contribute to inflammation via the mTOR signaling pathway (Berard et al., 2023).

Within the context of the vaginal microbiome, the molecular and pathophysiological mechanisms of BV development remain largely unknown. What is known so far is that women suffering from BV are characterized by a dysbiotic vaginal microbiome dominated by a diverse group of anaerobic bacteria, such as *Gardnerella vaginalis*, *Fannyhessea vaginae*, *Prevotella bivia, and Sneathia amnii* (Fredricks et al., 2005; Muzny et al., 2019; Carter et al., 2023). The VMB is also depleted of *Lactobacillus* spp., except for *L. iners*, often found adapting and coexisting with BV-associated bacteria (Zheng et al., 2021). *G. vaginalis* and *Prevotella* have been touted as primary instigators (Machado et al., 2013; Muzny et al., 2018, 2019; van Teijlingen et al., 2024) of BV and biofilms are formed with other important anaerobic bacteria during pathogenesis (Bradshaw and Sobel, 2016; Hardy et al., 2017). Due to the nature of the polymicrobial infection, metabolic environmental shifts lead to continued host inflammatory responses and disrupted epithelial cell barrier, which become further aggravated, leading to secondary infections and adverse gynecological disease sequelae.

Aerobic vaginitis

Aerobic vaginitis, due to its similarity to BV, can be misdiagnosed and incorrectly treated. It has been associated with complications such as vaginal inflammation, bacteriuria, lower urinary tract infections, and acute pyelonephritis (Kaambo et al., 2018). AV is characterized by an increase in enteric aerobic commensals not generally found in BV, such as group B *Streptococcus agalactiae* (GBS), *Staphylococcus aureus*, *Escherichia coli*, and enterococci, in the vaginal microbiome (Donders et al., 2017). Compared to the normal vaginal flora, these aerobic bacteria increase by 3- to 5-fold and are associated with vaginal mucosal inflammation due to the disruption of *Lactobacillus* dominance (Fan et al., 2013). The main symptoms are similar to those of BV but present as negative on the KOH test (Donders et al., 2015). Severe inflammation is apparent in vaginal smears, where an increase in intermediate and basal cells occurs. It is accompanied by increased turnover of the superficial epithelial cell layer and possibly epidermal desquamation, vaginal epithelial atrophy, small erosions, and ulceration (Petrova et al., 2015). Immune system dysregulation is the main factor contributing to AV pathogenesis (Oerlemans et al., 2020a). Similarly, reduced estrogen levels and localized immunity make AV prevalent in postmenopausal women (Stika, 2010). Pathogenic members of AV, such as GBS, are an essential focus, as they can occasionally cause morbidity in older adults, pregnant women, and patients with underlying medical conditions (Kwatra et al., 2014; Brigtsen et al., 2015). GBS colonization of pregnant women is considered a significant cause of severe neonatal infections, including neonatal sepsis, meningitis, and pneumonia (Krauss-Silva et al., 2014). In addition to GBS, *Enterococcus faecalis* has also been associated with preterm birth, low birth weight, and puerperal sepsis, resulting in significant maternal and neonatal morbidity and mortality (Cools et al., 2016; Kim et al., 2014).

As such, preferred treatments for AV are kanamycin and quinolones, which have intrinsic activity against disease-causing pathogenic bacteria while potentially minimizing interference with the vaginal microbiota (Tempera and Furneri, 2010). So far, the use of meclocycline and kanamycin for the treatment of AV allows a focus on Gram-negative bacilli rather than Gram-positive cocci, as these drugs are not absorbed and may leave extra vaginal lactobacilli even in patients with severe AV (Tempera and Furneri, 2010). Likewise, clindamycin for treating BV is also used for AV treatment due to its broad spectrum of activity against several aerobic Gram-positive coccal species; however, infection control with clindamycin is short-lived and may not cover all species associated with AV (Sousa et al., 2023). On the other hand, non-absorbable, broad-spectrum antibiotics such as carbapenems and clavulanic acid-beta-lactam combination (amoxiclav) are very effective for rapid, short-term improvement of severe symptoms from aerobic infections, especially deep cutaneous vulvovaginitis caused by GBS or methicillin-resistant *S. aureus* (MRSA) (Donders et al., 2015). Some beta-lactam antibiotics, however, are associated with less efficacy in eradicating *E. coli* vaginal colonization, leading to a high frequency of recurrent urinary tract infections (Stapleton, 2016). Nevertheless, due to significant inflammation in AV patients, including leukocyte and parabasal cell infiltration, frequent and prolonged use of antibiotics may result in adverse side effects. It may need to be accompanied by local administration of estrogens or a combination of probiotics and a very low dose of local estriol for postmenopausal or immunocompromised patients (Donders et al., 2015).

Vulvovaginal candidiasis

Like BV and AV, VVC is another significant public health concern. In the US alone, more than 70% of women experience fungal infection at least once in their life, with inflammation of the vagina and vulva as the primary concern (Nyirjesy et al., 2022). They are commonly colonized in the vagina by *Candida albicans* and non-albicans species such as *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis*. Other risk factors for VVC include antibiotic usage, lifestyle choices such as sexual activity, serum glucose levels in diabetic and pre-diabetic females, estrogen balance, immunoregulatory deficiencies, allergies, and gene polymorphisms (Satora et al., 2023). Thus, VVC classifications help to form treatment modalities based on uncomplicated and complicated VVC. Those with uncomplicated VVC are infrequent, with mild to moderate symptoms. *Candida albicans* is usually the main culprit in uncomplicated VVC. On the other hand, non-albicans candidiasis mainly occurs in those who are immunocompromised, leading to more severe and recurrent VVC (Nyirjesy et al., 2022).

Immunity against candidiasis is complex and involves multiple processes, including pathogen recognition through receptors such as Toll-like receptors (TLR) and C-type lectin receptors (CLR), active innate and adaptive immune cells such as macrophages, dendritic cells (DC), and T cells, as well as the production of cytokines and chemokines such as IL-4, IL-6, IL-10, IL-12, IFN-γ, and TNF-α (Netea et al., 2015; Pathakumari et al., 2020; Zhou et al.,

2021a). A prospective study found higher serum levels of cytokines, including IL-4, IL-6, and IL-10, and lower levels of IFN-γ, TNF-α, and IL-17F in patients with recurrent VVC compared to those with VVC. This indicates that Th1/Th2 immunity may play an essential role in VVC (Ge et al., 2022). The host immune response against candidiasis has been comprehensively summarized in other reviews and will not be discussed in detail in this review (Netea et al., 2015; Pathakumari et al., 2020; Zhou et al., 2021a).

However, *Candida* spp. has evolved mechanisms for immune evasion and dissemination by which it can hijack macrophages and instigate hyphal escape via pyroptosis (Uwamahoro et al., 2014) and macrophage extracellular trap formation (ETosis) (Netea et al., 2015; Olivier et al., 2022). To escape immune detection, *Candida* spp. executes morphological switching from non-infectious yeast to hyphae inside the macrophages. It instigates hyphal escape by producing pore-forming proteins such as candidalysin (Olivier et al., 2022), leading to inflammation and macrophage rupture, consequently releasing the pathogen. Further, *Candida* spp. can neutralize acidic growth environments by releasing ammonia derived from the catabolism of amino acids that may promote evasion and survival, although regulated by mitochondrial respiration (Westman et al., 2018; Silao et al., 2024). Other non-canonical strategies promoting colonization and evasion include β-glucan masking, which is triggered by stressors, nutrients, antifungal drugs, and other factors such as nitrogen availability and quorum sensing molecules (Pradhan et al., 2019). Such adaptations may contribute to the possibility of recurrence aside from other factors that can promote or induce VVC.

Even in healthy females, *Candida albicans* can co-exist as commensal members of the *Lactobacillus*-dominated VMB. Vaginal lactobacilli are known to produce anti-*Candida* factors, specifically with growth inhibition and targeting of hyphal components. Nevertheless, it has been reported that candidiasis occurs more frequently with an *L. iners*-dominated microbiome (CST III) as compared to an *L. crispatus*-dominated flora (CST II) (Tortelli et al., 2020). *L. crispatus* can inhibit the growth of *C. albicans* through the production of lactic acid and other anti-*Candida* molecules (Jang et al., 2019) and has the potential to reduce hyphal morphogenesis (Wang et al., 2017). Lactobacilli can further inhibit *C. albicans* virulence by preventing fungal adhesion to vaginal epithelial cells and hindering biofilm formation (Takano et al., 2023). *Candida* has been found to co-occur with high abundance of lactobacilli and BV-associated bacteria (Liu et al., 2013). *Candida* colonization is more commonly asymptomatic and may not always progress to VVC (Tortelli et al., 2020). However, some studies have implicated lactobacilli-rich VMBs associated with symptomatic VVC (Beigi et al., 2004b; McClelland et al., 2009). Nevertheless, with focus on the species level of lactobacilli, it has been more linked to the presence of an *L. iners*-rich VMB than an *L. crispatus*-rich VMB (Tortelli et al., 2020). In this context, the exact mechanism leading to the switch between commensal and pathogenic forms of *C. albicans* is not fully understood, especially in the context of *Lactobacillus-Candida* interactions in the vaginal environment (Takano et al., 2023; Silao et al., 2024).

Azole antifungal agents are used as first-line treatment, leading to adverse consequences such as the rise in multidrug-resistant *Candida* spp. Thus, despite standard treatment, the high prevalence of VVC and yeast colonization still puts this high on the World Health Organization's (WHO) "critical priority" group. A review of evidence conducted by Nyirjesy et al. (2022) identifies the need for novel treatment approaches for recurrent VVC where no alternative treatments, even with oral probiotics, have solid evidence for support yet. Nevertheless, oteseconazole, another antifungal drug that finished a phase 3 study, presents a new option for recurrent VVC with fewer adverse effects that was approved in the USA for women who are not of reproductive potential (Martens et al., 2022; Siddiqui et al., 2023).

Vaginal dysbiosis and the susceptibility to sexually transmitted diseases

A healthy, vaginal epithelium is usually highly protective against STIs caused by viruses such as HPV and HIV, parasites such as *Trichomonas vaginalis*, and bacteria such as *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and *Chlamydia trachomatis*. In the study of HIV infection, the vagina has been thought to be a markedly more effective barrier than the rectum. Many layers of stratum corneum cells are shed each day, thereby reducing the ability of pathogens to reach target cells deeper in the epithelium. Further, along with the inhibitory properties of the resident vaginal lactobacilli, epithelial and immune cells constitutively produce low levels of antimicrobial peptides (e.g., SLPI) and cytokines such as the anti-inflammatory IL-1RA that contribute to homeostasis (Cone, 2014; Muzny et al., 2020).

However, BV and microbial diversity can modify the risk of STIs via their interaction with mucosal immunity within the female genital tract and modification of its protective epithelial barrier (Velloza and Heffron, 2017). Dysbiosis-associated bacteria like *Gardnerella* spp. and *Prevotella* spp. can break down the mucosal barrier through sialidase production and host cell lysis, making women more susceptible to gynecological infections (France et al., 2022). *In vivo*, this microbial shift triggers a local immune response and inflammation, reflected in increased pro-inflammatory cytokine production of interleukin (IL)-1α, IL-1β, IL-6 and IL-8 in the vagina with BV-associated bacteria in CST IV such as *P. amnii*, *Sneathia* sp. and *Mobiluncus mulieris* as compared to *Lactobacillus*-dominated communities (Marconi et al., 2014; Anahtar et al., 2015). *In vitro*, cervical epithelial cells produce higher concentrations of IL-6 and IL-8 when co-cultured with *G. vaginalis*, *P. bivia*, and *P. amnii* compared to *L. crispatus*, whereas *F. vaginae* also elevates IL-6, IL-8, and TNF-α cytokines (Doerflinger et al., 2014).

Human immunodeficiency virus

BV contributes to the proinflammatory cytokines in the vaginal tract of HIV-infected women (Mitchell et al., 2008). However, with these inflammatory states and disrupted barriers, infectious particles such as HIV can traverse through the genital epithelium via tears in the squamous epithelium or transcytosis across the

single cell layer of the endocervix, ultimately infecting underlying CD4+ target cells in the submucosa (Vyshenska et al., 2017). The net outcome of these interactions favors HIV infection and replication by attracting target cells, which will subsequently become infected and further propagate the infection.

Recent publications have demonstrated that women with non-*Lactobacillus*-dominant VMB were at a greater risk of HIV infection, where increased mucosal inflammation enhanced the rate of sexual transmission of HIV in the female genital tract (Vitali et al., 2017; Wessels et al., 2018; Wang et al., 2023). On the other hand, *L. crispatus* is associated with reduced inflammation due to an immunoregulatory environment, exclusion of BV-associated bacteria, and reduced HIV risk. However, *L. iners* has been associated with fewer immune benefits and lower HIV protection or exclusion of BV-associated bacteria, possibly providing a more intermediate transitional stage to dysbiosis (Armstrong and Kaul, 2021). It is possible that women can develop a persistent long-term state where BV is frequent and that this state is associated with more frequent HIV infection. BV is more prevalent and persistent among HIV-infected women, particularly among those who are immunocompromised (Jamieson et al., 2001; Schellenberg et al., 2012).

Human papillomavirus

A dysbiotic VMB poses similar risks for the increase in HPV infection and persistence (Mitra et al., 2016; Vyshenska et al., 2017; Muzny et al., 2020). CST-III (*L. iners*-dominated) and CST-IV (dominated by facultative anaerobes) are suggested to be risk factors for persistent HPV infection (Mei et al., 2022) due to low protective immunity with the loss of healthy vaginal lactobacilli. In addition, HPV proteins E6 and E7 enhance IL-10 expression and macrophage type 2 production (Kremleva and Sgibnev, 2016). Cytokines such as IL-6, IL-8 and TGFβ-1 could also potentially influence the growth of different vaginal microbes (Kremleva and Sgibnev, 2016).

During this time, strict anaerobic bacteria start to colonize the vagina and destroy the protective barrier of the cervical epithelium. This event promotes HPV entry of host cells, paving the way for subsequent integration of the HPV virus into the host DNA and accompanying cell division for virus replication. Nevertheless, a *Lactobacillus*-dominated VMB, especially with *L. crispatus* (Cheng et al., 2020), protects against HPV and prevents HPV persistence and subsequent disease development (Zhang et al., 2018; Muzny et al., 2020). In another study, an *L. gasseri*-dominated microflora (CST II) is also associated with faster HPV clearance (Mei et al., 2022). Without these protective mechanisms in place, the immune system cannot cope with the strong immunological pressure brought about by dysbiosis, allowing tumor evasion strategies that lead to cervical intraepithelial neoplasia or cervical cancer development in unresolved HPV infections (Piersma, 2011; Zhou et al., 2021b).

Trichomonas vaginalis infection

Trichomonas vaginalis infection is associated with an increased risk of HIV infection and cervical and prostate cancer (Dessì et al., 2019; Margarita et al., 2020). The vaginal microbiota also plays a role in mediating susceptibility to *T. vaginalis* parasite infection, increasing the risk of trichomoniasis among women with BV or a Nugent score > 3 (Hinderfeld et al., 2019; Balkus et al., 2014; Margarita et al., 2020). Here, the loss of lactobacilli leads to an increased pH, creating an environment more favorable for *T. vaginalis* growth and pathogenicity (Margarita et al., 2020). Studies have also demonstrated an association between prevalent trichomoniasis and detection of *Prevotella* and *Sneathia* (non-*amnii*) species. These bacterial species may mediate susceptibility by producing nicotinamide metabolites that promote *T. vaginalis* infection (Jarrett et al., 2019). In contrast, suppression of *L. iners* in the initial interaction with *T. vaginalis* occurs but shows the possibility of adapting and surviving after longer exposure to *T. vaginalis* (Chiu et al., 2021). According to this relationship, interventions that decrease BV incidence and promote eubiosis could potentially contribute to reductions in trichomoniasis incidence (Balkus et al., 2014).

Vaginal dysbiosis and its effect on adverse pregnancy outcomes, infertility, and IVF failure

Dysbiosis-induced cervicovaginal inflammation has been strongly linked to adverse pregnancy outcomes such as preterm birth (Fettweis et al., 2019). The most common infectious diseases correlated with pregnancy complications are BV, VVC, and AV (Gao et al., 2021). The mechanism for which VVC during pregnancy is still speculative (Bagga and Arora, 2020), but pregnancy-related factors such as elevated levels of estrogen and progesterone, glycogen deposition, low vaginal pH, and decreased immunity that affect the vaginal milieu remain risk factors for VVC (Tellapragada et al., 2017; Gao et al., 2021). This is observed in a prevalence study of 1119 pregnant women in different regions of the world, of which 30% is colonized by vaginal *Candida* based on culture (Disha and Haque, 2022). Despite the lack of precise mechanisms, the weakened immunity state of the female cervicovaginal tract during pregnancy remains conducive to *Candida* infection that can instigate prostaglandin release, leading to uterine contractions and premature birth. However, several large cohort studies and systematic reviews did not show any association between VVC and preterm birth (Schuster et al., 2020; Blomberg et al., 2023; Gigi et al., 2023).

AV, on the other hand, is characterized primarily by a high abundance of GBS and *E. coli*, which in severe cases may be accompanied by desquamative inflammatory vaginitis, is linked to pregnancy complications such as ascending chorioamnionitis, early membrane rupture, and preterm delivery (Donders et al., 2002, 2011; Ma et al., 2022). A significant reduction in the incidence of preterm birth has been shown in women treated with clindamycin for BV and AV (Lamont et al., 2003; Larsson et al., 2006). However, multiple large, randomized trials did not find reduced incidence of preterm birth after clindamycin treatment (Bellad et al., 2018; Subtil et al., 2018), but preterm birth rates were much higher in the participants who failed antibiotic treatment (McGregor et al., 1994;

Kekki et al., 2001; Sabol et al., 2006). In these cases, antibiotic resistance is likely to occur, and the normal vaginal flora is not maintained. Therefore, the current consensus in the field is that there is no significant reduction in the risk for preterm birth with clindamycin therapy (McClure and Goldenberg, 2019; Smaill and Vazquez, 2019). However, the mixed results may still warrant further investigation. Disruption of the vaginal microbiota has been proven to affect the incidence of preterm birth (Jacobsson et al., 2002). More recent studies of the vaginal microbiome of pregnant women with whole genome shotgun sequencing also reveal the similarities of *G. vaginalis* to the *Bifidobacterium* genera. In contrast, an *L. crispatus*-dominated VMB was significantly correlated to full-term birth, along with *L. gasseri* and *Bifidobacterium breve* (Feehily et al., 2020). However, current evidence shows that the overgrowth of BV-associated bacteria such as *G. vaginalis* generates proteolytic enzymes such as sialic acidase and proline aminopeptidase to break down the protective components of the vaginal tract such as mucin and the vaginal secretions and the fetal membranes (Cauci et al., 2008). The loss of protection allows pathogenic adhesion to the vaginal mucosa and invasion of the uterus, whereas the affected elasticity of the fetal membranes potentially leads to early rupture (Gao et al., 2021). Further, the protective low pH in the vaginal microenvironment is disrupted due to the lack of lactobacilli metabolic activity and concurrent biogenic amine production by BV-associated bacteria, possibly allowing more favorable conditions for opportunistic bacteria and pathogens to grow (Nelson et al., 2015; Borgogna et al., 2021). The parasitic *T. vaginalis* infection occurs in these conditions in symbiotic interactions with *Mycoplasma hominis*, leading to severe complications such as preterm delivery and low birth weight (Dessì et al., 2019; Margarita et al., 2020).

Continued vaginal dysbiosis can also affect the local immune response, leading to preterm birth. Within the mucosal layer, secretory immunoglobulin A (sIgA) antibodies and immune cells such as macrophages, B and T lymphocytes are present (Gomez-Lopez et al., 2017). However, with the process of infection during upstream movement or within the disrupted amniotic cavity, bacterial endo- and exotoxins generated further aggravate the condition by the stimulation of increased levels of proinflammatory cytokines such as IL-1α, IL-1β and TNF-α (Bogavac et al., 2010; Gao et al., 2021). Such conditions could potentially dysregulate hormonal balance which trigger the body's synthesis of prostaglandin and matrix metalloenzymes leading to early contractions and dilation of the cervix, and destruction of collagen matrices that maintain the fetal membranes (Bogavac et al., 2010; Triggs et al., 2020; Gao et al., 2021). Incidentally, one bacterium gaining notoriety in association with adverse pregnancy outcomes involves the opportunistic pathogen *Fusobacterium nucleatum*, as it has been detected in placental and fetal tissues in pregnancy complications involving both ruptured and intact fetal membranes (Han et al., 2010; Bohrer et al., 2012; Wang et al., 2013). Even though it is initially an oral and vaginal commensal, it has been shown to have invasive properties in murine models that bind and infect epithelial and endothelial cells of the fetal-placental units, with TLR4 inflammatory cascades that lead to poor outcomes such as preterm birth, chorioamnionitis, neonatal sepsis, stillbirth, and preeclampsia (Han et al., 2004; Vander Haar et al., 2018). Studies in mice also show potential glycan cross-feeding mechanisms between *F. nucleatum* and BV-associated bacteria, potentiating sialidase activities in the dysbiotic vaginal microbiome (Agarwal et al., 2020).

Likewise, BV-associated bacteria have been linked to increased susceptibility in acquiring more severe inflammatory infections from sexually transmitted infections affecting the upper female genital tract, including the cervix, uterus, fallopian tubes, and ovaries (Wiesenfeld et al., 2002; Edwards et al., 2019). BV-induced pelvic inflammatory disease is a risk factor for ectopic pregnancies, and chronic infections can severely affect the reproductive organs, resulting in infertility and low success rates with IVF treatment (Haahr et al., 2016). Thus, the endometrial microbiome became another center of focus. Although lower in biomass as compared to the vaginal microbiome, the commensal endometrial microbiome possibly supports the intricate balance that regulates embryo implantation, a natural inflammatory event, and pregnancy development (Odendaal et al., 2024). Despite the technical and sampling limitations, it is necessary to further understand the influence of the cervicovaginal microbiota and utilize restorative LBP therapies such as lactobacilli, to resolve such inflammatory infectious or even non-infectious gynecological conditions, and to improve maternal health by treating vaginal dysbiosis in reproductive-aged women (Haahr et al., 2022).

LBP mechanisms of action and screening for desired characteristics

Beneficial and antipathogenic effects of lactobacilli for LBP use are based on the traditional probiotic traits of lactobacilli such as in the production of lactic acid to maintain a low vaginal pH, production of antimicrobial compounds (i.e., bacteriocins, hydrogen peroxide) and stimulation of the immune system to help to maintain bacterial balance in the vaginal tract (López-Moreno and Aguilera, 2021). In addition, by adhering to the vaginal epithelia, vaginal lactobacilli may inhibit the attachment of pathogenic bacteria and utilize the same nutrients as pathogens, thereby restricting their growth (Lehtoranta et al., 2022). Given so, an understanding of the local vaginal *Lactobacillus* species and their niche provides insight into the selective criteria for LBP development (Figure 2).

Ecological fitness, competence and growth

Lactobacilli display distinct strain differentiation in both genotypic and phenotypic characteristics within the same species. Within vaginal species, each genome encodes species-specific protein families. Among the most common vaginal lactobacilli—*L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*—*L. iners* exhibited the smallest average genome size at 1.3 Mbp, lacking integral membrane proteins, transcriptional regulators, acetyltransferase GNAT family members, and certain ABC transport proteins.

However, they possessed unique ABC transporter permeases, thiol-activated cytolysin (inerolysin), and other protein families absent in other *Lactobacillus* species (Rampersaud et al., 2011; Macklaim et al., 2013). On the other hand, *L. crispatus*, known for its extensive genome size among vaginal lactobacilli, exhibited unique proteins such as DNA polymerase, bacteriocins, and toxin-antitoxin systems, absent in other vaginal strains. Generally, *L. crispatus* harbored all common protein families with the other *Lactobacillus* species, but no protein families were significantly more abundant in *L. crispatus* (Damelin et al., 2011). For *L. gasseri*, proteins related to copper resistance/homeostasis, toxin-antitoxin systems, and pediocin immunity were exclusive across all tested strains. In the case of *L. jensenii*, protein families involving families associated with ABC transport, transcriptional regulation, and a citrate transporter were unique. However, certain protein families such as the glucitol/sorbitol phosphotransferase system were present in other vaginal lactobacilli but were missing in *L. jensenii* (Merhej et al., 2009; Mendes-Soares et al., 2014).

Pan et al. (2020) also compared phenotypic assays of *L. crispatus* and *L. gasseri* in healthy vaginal microbiomes and human gastrointestinal tracts. Variations in the growth patterns and fermentative capacities are observed within the same species, where vaginal lactobacilli performed well in a simulated vaginal fluid, demonstrating higher lactic acid resistance at a later growth phase was necessary for the maintenance of low vaginal pH. Their findings underscored that the isolation source of a strain could influence its potential probiotic functionality, emphasizing its consideration in probiotic formulation (Pan et al., 2020). Similarly, clinical evidence shows that specific oral probiotic strains or their combinations may elevate vaginal lactobacilli counts in healthy women or women with BV and/or VVC and support natural VMB during/after recovery from antibiotics/antifungal treatment (De Alberti et al., 2015; Heczko et al., 2015; Mezzasalma et al., 2017). Nevertheless, current clinical research also shows that this has not always been the case in other BV, GBS infection and VVC cohorts, where there was no consistent improvement and vaginal colonization of orally administered probiotic strains (Hanson et al., 2014; Husain et al., 2020; Farr et al., 2020). As far, it seems that given the longer length, higher dosing, and necessity for lactobacilli to withstand gastric and bile acids for treatment via the oral route, the direct intravaginal route of antibiotic and LBP therapy is deemed more effective, relevant to the source of the strain.

Analysis of *L. crispatus* strains from various environments revealed genetic adaptations of strains to their ecological niches. Certain well-adapted metabolic and growth capacities to the vaginal tract can provide an advantageous basis for colonization and provide means for restoration of the natural vaginal lactobacilli. Notably, a vaginal isolate, *L. crispatus* PRL2021, demonstrates superior ecological competence in a simulated vaginal fluid compared to other vaginal *Lactobacillus* species, indicating its potential in higher growth performance in the vaginal microbiome and competitive mechanisms (Mancabelli et al., 2021). *L. crispatus* strains from

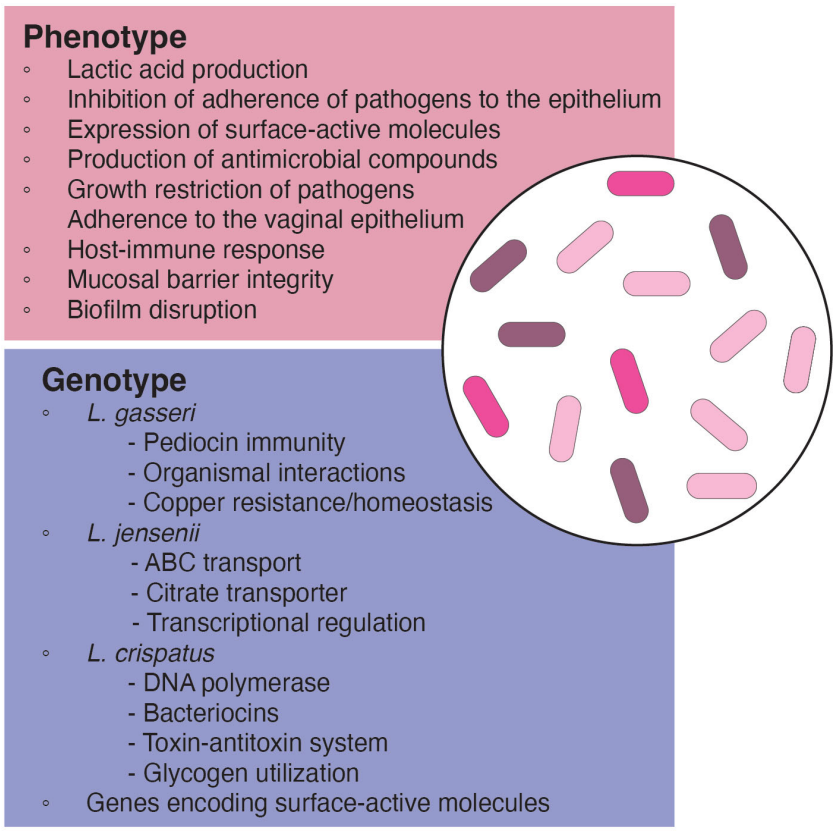

FIGURE 2
Phenotypic and genotypic characteristics of relevant species and strains for use in LBP development.

Lactobacillus-dominated and dysbiotic vaginal microbiomes were also studied, but with no phenotypic distinction observed between strains, specifically with organic acid production (Abdelmaksoud et al., 2016; van der Veer et al., 2019). However, they observed a disproportionally higher abundance of gene fragments encoding for glycosyltransferases among strains isolated from dysbiotic microbiomes, suggesting a role for cell surface glycoconjugates in shaping vaginal microbiota-host interactions. Additionally, they noted the ability of *L. crispatus* to grow on glycogen, correlating with the presence of a full-length pullulanase type I gene (van der Veer et al., 2019). In all studies, glycogen utilization deemed favorable for selection as it is the most abundant carbohydrate source in the vaginal milieu, supporting both growth and lactic acid production.

Antimicrobial production

Lactic acid has both antimicrobial and immune modulatory functions. It does not only acidify the vaginal environment but is also associated with lower levels of pro-inflammatory cytokines (e.g., IL-1α, IL-6, IL-8, and TNFα), which help to generate a homeostatic environment (Aldunate et al., 2015). Several *in vitro* studies have demonstrated that lactic acid is a potential antiviral and bactericidal compound, inhibiting replication/growth of genital STI pathogens and pathobionts, including *C. trachomatis*, *N. gonorrhoeae*, GBS, HPV and HIV (Muzny et al., 2020). In this context, acidity and protonated lactic acid are responsible for the anti-trichomonadal effects of *L. gasseri* (Pradines et al., 2022) alongside other lactobacillar bacteriostatic and bactericidal compounds in the vagina (Brotman et al., 2012). Other antimicrobial compounds such as hydrogen peroxide-producing enzymes are common among vaginal *L. crispatus* strains (van der Veer et al., 2019). The rationality of hydrogen peroxide production as a significant factor in vaginal health has been debated due to the lack of oxygen in the healthy vaginal tract (Tachedjian et al., 2018), and that physiological concentrations of hydrogen peroxide (<100 uM) do not affect BV bacteria (O'Hanlon et al., 2011). Nevertheless, given certain physiological factors causing vaginal dysbiosis such as in AV and VVC, it may still be relevant as the natural vaginal strains still maintain the core functional genes. In this context, vaginal *L. crispatus* supernatants are strongly inhibiting of *Candida albicans* growth, virulence gene expression and hyphal formation (Wang et al., 2017). Furthermore, bacteriocins have also been found in certain vaginal lactobacilli. Lactocin 160 from a vaginal *L. rhamnosus*, induces transient pore formation via disruption of the chemiosmotic potential on the cytoplasmic membrane of *G. vaginalis* (Turovskiy et al., 2009), whereas Gassericin E from *L. gasseri* EV1461 affects both related and non-related strains, inhibiting the growth of BV pathogens (Maldonado-Barragán et al., 2016).

Modulation of host innate and adaptive immune responses

Further, LBP host colonization and the inhibition of pathogen adhesion are important selective traits for maintaining mucosal barrier integrity (Boris et al., 1998; Osset et al., 2001; Delgado-Diaz et al., 2022). Lactobacilli can induce host defense against pathogens through the formation of microcolonies that attach to epithelial cell receptors and form a physical barrier to pathogen attachment (Lebeer et al., 2010; Petrova et al., 2013). For instance, *L. gasseri* inhibits *T. vaginalis* colonization of vaginal epithelial cells through the action of its cells and a cell-surface aggregation-promoting factor (Pradines et al., 2022). Similarly, genomic studies of *L. crispatus* also found genes encoding fibronectin-binding *L. crispatus* protein (LACT01268) and *L. crispatus* adhesins (LACT01712 and LACT02327) that have been found to interfere with fibronectin-binding and pilus components of *G. vaginalis* (Koumans et al., 2007; Yeoman et al., 2010). One such fibronectin-binding protein encoded by *L. crispatus* the LEA protein, which can potentially exert inhibitory effects by competing with the same attachment sites as the pili of *G. vaginalis* (Edelman et al., 2012; Ojala et al., 2014). Core proteins of *L. crispatus* may play a crucial role in protecting the vagina from pathogens and bacterial vaginosis and highlight intricate mechanisms through which *L. crispatus* maintains vaginal health.

At the same time, potential LBPs demonstrate the ability to improve host innate immune responses. TLRs on vaginal epithelial cells regulate the production of cytokines by responding to molecular patterns (MAMPs) present on the bacterial surface. For instance, *Gardnerella* can induce an epithelial cell response through nuclear factor kappa B (NF-κB) activation via a TLR2-dependent signaling pathway (Anton et al., 2022). In AV, inflammatory responses can also be induced through TLR4 activation of NF-κB (Mirmonsef et al., 2011; Rose et al., 2012). Given this, TLR2/TLR6 modulation has been suggested to play an anti-inflammatory role in certain situations (Li et al., 2013). To ameliorate the low activation of TLR2/TLR6 typically observed in an inflammatory state, *Lactobacillus*-dominated commensal communities may be an important part of maintaining homeostasis and inducing an immunomodulatory role to reduce vaginal inflammation (Nasu and Narahara, 2010; Hearps et al., 2017). Surface active molecules such as bacterial exopolysaccharides (EPS) and peptidoglycan on the cell surfaces are also present in *L. crispatus* (Ojala et al., 2014; Chee et al., 2020). EPS can enhance the ability of vaginal VK2 cells in producing anti-candidal human defensin-2 protein, and likewise improving colonization potential (Donnarumma et al., 2014). *In vitro* evidence also shows the ability of the vaginal *L. crispatus* peptidoglycan to stimulate CD207 expression in Langerhans cells, the antigen presenting dendritic cells in vagina, to reduce HIV receptor entry (Song et al., 2018).

Lastly, it has been demonstrated that certain *Lactobacillus* species such as *L. delbrueckii* and *L. rhamnosus* taken as oral probiotics may dampen lipopolysaccharide (LPS)-induced expression of HLA-DR, CD86, CD80, CD83, and IL-12 in human dendritic cells (Esmaeili et al., 2018; Odendaal et al., 2024). Although it is not yet well understood, it may be a process through which the microbiota modulates immunotolerance in early pregnancy (Inversetti et al., 2023; Odendaal et al., 2024). Nevertheless, further investigation is needed to explore the mechanisms associated with local immunomodulation within the cervicovaginal microbiome, and their relevance to the implantation regulation and pregnancy development.

Currently available LBPs and ongoing developments

Traditional probiotics vs. LBPs

In the US, alternative treatment options are classified only as vaginal probiotics, due to the more straightforward track for getting them out to the market. Currently, there are seven different vaginal probiotics available for BV support. The AZO Complete Feminine Balance™ and Jarro-Dophilus® Women contain similar strains of *L. crispatus* LbV 88, *L. jensenii* LbV 116, *L. gasseri* LbV 150N, and *L. rhamnosus* LbV 96, which are a mix of the most common *Lactobacillus* spp. found in the vagina and has been documented to have increased recovery rates from BV (Laue et al., 2018). On the other hand, Bio-K+® Women's Health, Jarrow Formulas® Fem-Dophilus® *1 Billion*, Jarrow Formulas® Fem-Dophilus® *5 Billion*, RepHresh™ Pro-B™ Probiotic, and UltraFlora® Women's utilize strains isolated from the urogenital tract, such as *L. reuteri* RC-14 and *L. rhamnosus* GR-1, which did not improve BV cure rates as a dietary supplement, but can contribute to improved vaginal flora composition (Hummelen et al., 2010).

Moving forward, it is crucial to consistently differentiate between using *Lactobacillus* strains as a traditional probiotic and its use as an LBP. More relevant regulatory functions and expectations are necessary for the drug development track, considering that the target populations are diseased individuals who may be immune-sensitive (Cordaillat-Simmons et al., 2020). As far, *Lactobacillus* bacteremia is rare and mainly correlated to intestinal translocation in severely immunocompromised patients (i.e. uncontrolled diabetes mellitus, malignancy or have undergone organ transplantation), urogenital pathologies, congenital and valvular heart diseases or those who have undergone invasive procedures, that may be more susceptible to probiotic use (Salminen et al., 2004; Rossi et al., 2022; Salminen et al., 2006; Bapna et al., 2023; Kullar et al., 2023). There are currently no clinical reports of *Lactobacillus* bacteremia with intravaginally administered LBP strains, but vaginal lactobacilli, specifically *L. jensenii*, has been reported in polymicrobial bacteremia with *Veillonella montpellierensis* during pregnancy in an immunocompetent but anemic patient at 33-weeks of gestation (Toprak et al., 2022). Another recent case involves *L. jensenii* and *P. bivia* polymicrobial bacteremia leading to renal and perinephric abscesses with an otherwise immunocompetent patient but has undergone ureteral stent procedure (Mohan et al., 2020). Other case reports of *L. jensenii* bacteremia within obstetrics and gynecology that have occurred after vaginal delivery (Marciniak et al., 2014) and elective abortion using dilatation and curettage (Suárez-García et al., 2012) with patients who presented with infective endocarditis.

Nevertheless, the use of lactobacilli as a biotherapeutic drug is still justifiable due to its lower risk of use as a "generally regarded as safe" (GRAS) microorganism and its positive benefit-risk ratio compared to antibiotic usage in patients suffering from recurrent vaginal infections. Likewise, such side effects and contraindications can be averted with a well-characterized LBP strain lacking pathogenic factors and a defined antimicrobial sensitivity spectrum. These LBP candidates should be further explored within the ongoing clinical trials. Nevertheless, same as other drugs, high doses should also be used with caution, and monitored carefully by the administering physician along with any presenting underlying illnesses.

Thus, vaginal lactobacilli developed as intravaginally administered LBPs are meant to undergo further trait selection and safety testing, including considerations of the proper dosing, delivery form and optimal administration frequency and route, therapeutic indications, and expected side effects. Further, strain-specific properties and treatments prior to LBP administration is crucial (Wu et al., 2022). Based on this, various treatment modalities have been considered, such as the use of single lactobacilli strains, multi-strain lactobacilli combinations, and vaginal microbiome transplantation (VMT), with varying levels of efficacy specifically against BV, with or without antibiotic therapy prior to LBP treatment.

Single strain LBPs vs. multi-strain LBPs in recurrent BV and VVC

Substantial research has explored LBPs for treating recurrent BV and VVC, but they are not yet a standard clinical approach due to differences in trial size, methods, and uses. Recurrent BV has been the most impactful in the health and economic sectors, especially with its associations with diseases such as HPV, HIV and *Trichomonas* infection. Thus, clinical trials utilizing strains native to vaginal microbiome are on their way for recurrent infections. Those with clinical trial IDs, are intravaginally administered, and have published results are summarized in Table 1.

Early phase 1 trials have shown that vaginal administration of *L. rhamnosus* GR1 and *L. reuteri* RC-14 can increase vaginal *Lactobacillus* and modulate the immune response via TLR2 (Bisanz et al., 2014). EcoVag® with *L. gasseri* DSM 14869 and *L. rhamnosus* DSM 14870 showed possibility for adjunct therapy during three menstrual cycles, demonstrating a higher BV-free rate (64.90%) in the lactobacilli group compared to placebo group (46.2%) (Larsson et al., 2008). EcoVag® also shows strong potential in recurrent VVC alongside fluconazole treatment, but with only a 6- or 12-month cure rate of 50 and 67%, respectively, alongside clindamycin/metronidazole treatment for BV, respectively (Pendharkar et al., 2015). *L. plantarum* WCFS1, *L. pentosus* KCA1 and *L. rhamnosus* GG administered as a probiotic gel against VVC also achieved 45% symptom relief in their interventional therapy (Oerlemans et al., 2020b). Lastly in a phase 2 trial, *L. rhamnosus* BMX54 showed long-term use benefits for HPV clearance, in the context of vaginal dysbiosis (Palma et al., 2018).

As far, it is still debatable if the effect may be dependent on *Lactobacillus* species or their natural ecological niche, as similar reference strain standards have been utilized. However, given that most studies are interventional with BV recurrence, larger clinical cohorts are still lacking. Further, long-term effects are only found in a few of these studies, and few have been done without the use of antibiotics; thus, the effect on varying vaginal microbiomes,

TABLE 1 List of published clinical trials of intravaginally-administered adjuvant probiotic therapy and LBP development.

Title	Year	Clinical Trial ID	Study type	Enrollment (Actual)	Time Frame	Composition	Dose	Inclusion criteria	Condition	Phase	Aim	Result	References
Exploratory Study to Evaluate the Effects of the Probiotics *L. rhamnosus* GR-1 and *L. reuteri* RC- 14	2012-2013	NCT02139839	DB, R, C, PC	14	40 days	*L. rhamnosus* GR-1/*L. reuteri* RC-14	2.5×10^9 CFU/strain/capsule	Age: 40-80 years, Other: in good general health, but excluding participants with a Nugent Score of 0 3 or greater than 6	BV, Menopause	Early Phase 1	The objective of this double blinded, placebo-controlled crossover study was to evaluate in 14 post-menopausal women with an intermediate Nugent score, the effect of 3 days of vaginal administration of probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on the microbiota and host response.	The probiotic treatment increased abundance of *Lactobacillus* and lactate levels in the vagina, along with decreased abundance of *Atopobium*. Microarray results showed probiotics modulate immune responses via TLR2, affecting epithelial barrier function.	Bisanz et al., 2014
A prospective, placebo-controlled, double-blind study for the investigation of the effect and safety of EcoVag® as adjuvant treatment after treatment with clindamycin against bacterial vaginosis	2004-2006	ISRCTN62879834	DB, R, PC	100	6 months	*L. gasseri* (Lba EB01- DSM 14869), *L. rhamnosus* (Lbp PB01- DSM 14870)	10^8-10^9 CFU/strain/capsule	Age: 18-60 years, Other: Amsel criteria for bacterial vaginosis	BV	NA	The primary objective of this study was to investigate if supplementary lactobacilli treatment could improve the initial cure rate after vaginal clindamycin therapy, and secondly, if lactobacilli as repeated adjunct treatment during 3 menstrual cycles could lengthen the time to relapse after initial cure.	In the lactobacilli group, the initial intent-to-treat analysis showed a one-month cure rate of 64%, comparable to the placebo group at 78% However, considering population changes and excluding non-compliant patients, the adjusted initial cure rate for the lactobacilli group was 77%. Over six menstrual cycles, lactobacilli-treated women exhibited a significantly higher BV-free rate (64.9%) compared to the placebo group (46.2%), with a significant difference in the time from cure to relapse favoring lactobacilli treatment. Adjuvant therapy with lactobacilli significantly contributed to relapse avoidance.	Larsson et al., 2008
Will *Lactobacillus* Increase Cure Rate After Treatment of Bacterial Vaginosis and Chronic Vulvovaginal *Candida*	2014-2015	NCT02295579	0	50	6 months	*L. gasseri* (DSM 14869) *L. rhamnosus* (DSM 14870)	10^8 CFU/strain/capsule	Age: 18-55 years, Other: Diagnosis of bacterial vaginosis according to Hay/Ison or with candidiasis diagnosed with wet smear.	BV, R-VVC	NA	The aim of this study was to investigate the colonisation by lactobacilli and clinical outcome in women with BV and recurrent-VVC (R-VVC) receiving antibiotic or anti-fungal treatment in combination with the probiotic EcoVag®capsules.	In trial I, BV was treated with clindamycin/metronidazol e followed by 5-day EcoVag® capsules. In trial II, three groups were included: BV with prolonged clindamycin/metronidazol e and EcoVag®, R-VVC with extended fluconazole and EcoVag®, and fluconazole- only treatment. In trial I, the 6-month cure rate for BV was 50%, and in trial II, both the 6- and 12-month cure rates were 67%. For VVC, women receiving fluconazole and EcoVag® had 100% and 89% cure rates at 6 and 12 months, while those with fluconazole only had rates of 100% and 70% *Lactobacillus* species were associated with BV cure in both trials,	Pendharkar et al., 2015

(Continued)

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TABLE 1 Continued

Title	Year	Clinical Trial ID	Study type	Enrollment (Actual)	Time Frame	Composition	Dose	Inclusion criteria	Condition	Phase	Aim	Result	References
												while EcoVag® strains were significant only in trial II. A change in sexual partner correlated with BV relapse.	
Study of the Vaginal Microbiota and the Potential of a Vaginal Probiotic Cream in Vaginal Candidosis	2016-2019	NCT03975569	INT, FT	20	4 weeks	*L. plantarum* WCFS1, *L. pentosus* KCA1 and *L. rhamnosus* GG	10^9-10^{10} CFU/g	Age: 18 50 years, Other: positive *Candida* microscopy and/or culture, at least two of the following vaginal symptoms: burning, itching, redness, fissure, discharge, vulvar edema, postcoital itching, lesions with partner	VVC	NA	Here, three strains were selected for further evaluation in patients based on *in vitro* anti-*Candida* effects. They showed their ability to inhibit *Candida albicans in vitro* and formulated them in a gel, **which was tested in a proof-of-concept study aimed at investigating their influence on the vaginal microbiome and highlighting relevant characteristics for future improvements for probiotic strategies against WC.	45% of women using the probiotic gel experienced symptom relief without needing additional fluconazole medication, and their fungal concentrations were comparable to those treated with fluconazole. Fluconazole alone reduced the abundance of vaginal lactobacilli.	Oerlemans et al., 2020b
Probiotic Implementation as Help in Solving Vaginal Infections	2015-2016	NCT03372395	R	117	9 months	*L. rhamnosus* BMX 54	10^4 CFU/tablet	Age: 18 years and older, Other: documented BV or yeast vaginitis associated with HPV-infection documented as PAP-smear abnormalities (ASCUS, L-SIL or H-SIL histologically demonstrated as CIN1) and/or positive for HPV-DNA	BV	Phase 2	The aim of this study was to confirm that vaginal lactobacilli long-lasting implementation in women with HPV-infections and concomitant bacterial vaginosis or vaginitis might be able to help in solving the viral infection, by re-establishing the original eubiosis.	Individuals in the long-term probiotic usage group (Group 2) had twice the likelihood of resolving HPV-related cytological anomalies compared to those in the short probiotic usage group (Group 1). A total clearance of HPV was observed in 11.6% of short-term probiotic users compared to 31.2% in long-term users based on a negative HPV-DNA test at the end of the study period.	Palma et al., 2018
Clinical and microbiological efficacy of new probiotics in	2016-2019	ISRCTN34840624	R, P	182	3 months	*L. crispatus* strains DSM32717, DSM32720,	10^{10} CFU/strain/capsule	Age: 18 50 years, Other: Recurrent-BV (R-BV)	R-BV, R- VVC	NA	The aim of the current study was to evaluate the activity of the evidence-based	Both oral and vaginal capsules effectively improved symptoms in patients with BV, including reducing discharge volume, odor, and itching/	Mändar et al., 2023

(Continued)

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TABLE 1 Continued

Title	Year	Clinical Trial ID	Study type	Enrollment (Actual)	Time Frame	Composition	Dose	Inclusion criteria	Condition	Phase	Aim	Result	References
vaginal discharge disturbances						DSM32718 and DSM32716		episodes (diagnosed with complaints and through Amsel criteria, confirmation with microscopy in 2 days) or recurrent VVC (R-VC) episodes (diagnosed with complaints and clinical signs confirmation with culture testing in 2 days)			probiotics (*L. crispatus* strains DSM32717, DSM32720, DSM32718 and DSM32716) on BV and VVC patients in the clinicaltrial in terms of both clinical and microbiological improvement.	irritation. In VVC patients, both types of capsules successfully alleviated the main symptoms of discharge volume and itching/irritation.	
Intravaginal LACTIN-V for Prevention of Recurrent Urinary Tract Infection	2006-2012	NCT00305227	DB, R, PC	100	10 weeks	*L. crispatus* CTV-05	10^8 CFU/mL	Age: 18 50 years, Other: current symptomatic uncomplicated cystitis, and the cystitis is treated with trimethoprim-sulfamethoxazole (TMP-SMX)	UTI	Phase 2	Major goals of the study were to assess the ability of the probiotic to reduce the incidence of rUTI, to evaluate whether the probiotic achieved vaginal colonization, to assess effects on the vaginal microbiota of women after treatment for UTI, and to confirm the safety of the probiotic.	Women receiving Lactin-V experienced a lower recurrence of UTIs compared to those on placebo. Colonization with *L. crispatus* significantly reduced recurrent UTIs only in those treated with Lactin-V.	Stapleton et al., 2011
Safety and Efficacy Study of *Lactobacillus* Administered Vaginally in Women with Bacterial Vaginosis	2008-2009	NCT00635622	DB, R, PC	24	28 days	*L. crispatus* CTV-05	$2x10^9$ CFU/dose	Age: 18-40 years, Other: Untreated BV (asymptomatic or symptomatic) as diagnosed during the screening visit using Amsel criteria AND confirmed in	BV	Phase 2a	This phase 2a trial was designed to determine the colonization efficiency, safety, tolerability, and acceptability of LACTIN-V of 2 x 10° CFU/dose administered by vaginal applicator in	In the LACTIN-V group, 61% of women were colonized with *L. crispatus* CTV-05 at Day 10 or Day 28, with 78% colonization among those with complete adherence. Adverse events (AEs) were mostly mild or moderate (90%), evenly distributed between LACTIN-V and placebo, and predominantly of genitourinary origin (78%). No severe AEs occurred, and the product was well- tolerated without	Hemmerling et al., 2010

(Continued)

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TABLE 1 Continued

Title	Year	Clinical Trial ID	Study type	Enrollment (Actual)	Time Frame	Composition	Dose	Inclusion criteria	Condition	Phase	Aim	Result	References
								the laboratory using the Nugent scoring system (Nugent Score 7)			women treated for BV.	deep epithelial disruption during colposcopic evaluation.	
LACTIN-V Study for Recurrent Bacterial Vaginosis	2016-2019	NCT02766023	DB, R, PC	228	10 weeks	*L. crispatus* CTV-05	$2x10^9$ CFU/dose	Age: 18-45 years, Other: Untreated BV (asymptomatic or symptomatic) as diagnosed during the screening visit defined by >/=3 Amsel criteria, and as confirmed in the laboratory using the Nugent scoring system (Nugent Score >/= 4)	BV	Phase 2b	A randomized, double-blind, placebo-controlled, phase 2b trial to evaluate the ability of *L. crispatus* CTV-05 (Lactin-V) to prevent the recurrence of bacterial vaginosis.	The recurrence of BV by week 12 was lower in the Lactin-V group compared to the placebo group. *L. crispatus* CTV-05 was detected in 79% of Lactin-V participants at the 12- week visit, and adverse events were similar between Lactin-V and placebo groups.	Cohen et al., 2020

DB (Double Blind), RCT (R), C (Crossover), P (Parallel), PC (Placebo Controlled), O (Observational), INT (Interventional), FT (Fluconazole Treatment); BV (Bacterial Vaginosis), VVC (Vulvovaginal Candidiasis), UTI (Urinary Tract Infection); NA (Not applicable).

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retention, and mechanisms of action of these probiotic strains developed as potential LBP candidates may still need elucidation as the LBP drug functionality is still lacking in most studies.

Nevertheless, it has generally been accepted that native strains are more competitive and well-adapted to their ecological niche, such as in the use of glycogen in the vaginal microenvironment as a carbon source. Most lactobacilli are not capable of utilizing glycogen directly, but native vaginal strains have a generally efficient acquisition of glycogen-derived resources released by α-amylase production that break down glycogen in the vaginal tract (Nunn and Forney, 2016). Notably, LBPs containing native vaginal microbiome strains also stand out for their potential benefits in biofilm disruption and immunomodulation (Łaniewski and Herbst-Kralovetz, 2021). Exciting new prospectives for using natural, non-antibiotic pre-treatment alternatives, potentially in combination with LBPs, lie ahead (Latham-Cork et al., 2021).

Extensive research highlights *L. crispatus* as advantageous, providing protection against various pathogens and correlating with positive health and pregnancy outcomes (Ghartey et al., 2014; Argentini et al., 2022; Baud et al., 2023). Furthermore, compared to other dominant lactobacilli strains, pangenomic gene analyses have shown greater enrichment and evolutionary acquisition of key beneficial and protective functional genes (Bhattacharya et al., 2023). Moreover, although bacteriocin and organic acid biosynthesis are still strain-specific, *L. crispatus* seem to more ubiquitously carry key functionalities as compared to the heterogeneity found in either *L. iners*, *L. gasseri* or *L. jensenii* (Bhattacharya et al., 2023).

L. crispatus strains DSM32717, DSM32720, DSM32718 and DSM32716 which come from a collection of reproductive tract microorganisms, have been phenotyped and observed to be effective in reducing BV and VVC symptoms using both oral and vaginal capsules (Mändar et al., 2023). However, as far, Lactin-V based on the single strain *L. crispatus* CTV-05, has been the only one that has reached clinical phase 3, recently showing relative success in their clinical phase 2 trials for recurrent urinary tract infections in non-pregnant women (Stapleton et al., 2011), and in their phase 2a, and phase 2b trials for recurrent BV applications (Hemmerling et al., 2010; Cohen et al., 2020). In the studies, a 78% colonization rate in women treated for BV, and a lower BV recurrence after 12 weeks with Lactin-V has been demonstrated (Hemmerling et al., 2010; Cohen et al., 2020). Furthermore, Lactin-V has recently shown to be generally safe and well tolerated in pregnant women (Bayar et al., 2023), and is currently being investigated for use in reducing pre-term birth in women at high risk and enhancing *in vitro* fertilization success rates.

Vaginal microbiome transplantation

Aside from Lactin-V, exploratory studies using VMT have been performed for intractable and recurrent bacterial vaginosis in five women, with four achieving full long-term remission without adverse outcomes, despite a repeat VMT for three (Lev-Sagie et al., 2019). This provides promising results and opens more research opportunities based on VMT. In line with this, Freya Biosciences has started exciting work in LBPs based on VMT to improve reproductive outcomes. Two proof-of-concept intervention studies with their FB101 product have been completed, with published results in a patient presenting with severe vaginal dysbiosis and a history of recurrent pregnancy loss (Wrønding et al., 2023). In this antibiotic-free VMT approach, confirming donor strain engraftment followed by a successful pregnancy and delivery of the patient's vaginal microbiome from 90% *G. vaginalis* to 81.2% *L. crispatus* and 9% *L. jensenii* with just one VMT treatment. It remains, however, that post-VMT treatment, the patient received 75mg daily of acetylsalicylic-acid and low-molecular-weight heparin to resolve antiphospholipid syndrome that can have contributed to the successful pregnancy (Wrønding et al., 2023). The study demonstrates how VMT can be further utilized in other gynecological conditions resulting from abnormal vaginal microbiota and improving reproductive health outcomes. Large-scale randomized, placebo-controlled clinical studies are ongoing, but no results have been published.

The future applications of vaginal LBPs in influencing the cervical and endometrial microbiota have become a major focus in maternal and fetal health. Further understanding of the cervicovaginal microbiota can assist in guiding the treatment of vaginal dysbiosis relevant to endometrial function to improve reproductive health outcomes (Odendaal et al., 2024).

Regulatory pathways and challenges in the development of vaginally administered LBPs

The microbiome field has dramatically matured over the past decade, but the development and regulatory approval of life biopharmaceutical products pose several challenges, given the complexity of these live products. However, advancements like the approval of LBPs for preventing CDI recurrence (Mullard, 2022) marked a pivotal milestone that remains to be expanded to other indications.

In 2012, the Food and Drug Administration (FDA) was the first regulatory agency to create the category of LBP and to publish a guideline for industry stakeholders entitled "Early Clinical Trials with Live Biotherapeutic Products: Chemistry, Manufacturing, and Control Information" and recently updated in 2016 (FDA (Food and Drug Administration), 2016). The European Directorate for the Quality of Medicines and Healthcare (EDQM) accepted this new category of medical products on the European market in 2019 entitled "EDQM (European Pharmacopoeia) 3053E General Monograph on Live Biotherapeutic Products" (EDQM, 2019). However, LBPs currently have no separate status in the European regulatory framework. Therefore, developers in the EU need to rely on relevant regulatory concepts for biological medicinal products, but with guidelines from the European Medicines Agency based on information from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) regarding "ICH guideline E8 on General Considerations for Clinical Studies" in 2021 (European Medicines Agency, 2021) and the Committee for Medicinal Products for Human Use (CHMP) for

"CHMP guideline on Human Cell-Based Medicinal Products" for scientific guidelines in addressing development, manufacturing and quality control (European Medicines Agency, 2008) and a revised guideline on "First-in-Human Clinical Trials with Investigational Medicinal Products" (European Medicines Agency, 2017). All these regulations allow for comprehensive risk analysis and mitigation strategies, specifically with their intended use (Rouanet et al., 2020).

LBPs, such as individual strains of bacteria, defined consortia, or VMTs, involve intricate biological processes that are not fully known. Regulatory agencies must assess the complex mechanisms of action on both the human host and host microbiome, potential side effects, and long-term safety. The proposed mechanisms of action are generally to interfere with the growth of a pathogenic or potentially pathogenic microorganism in the vagina or to stimulate other potentially beneficial cellular processes because of transient persistence and/or long-term colonization with the microorganisms contained in the LBP (Wu et al., 2022; Lagenaur et al., 2021). Investigational objectives vary concerning prevention versus treatment and stand-alone therapy versus adjunct therapy to antimicrobial or other therapy. With this regard, newly developed and emerging *in vitro* and *in vivo* tools are becoming more available to support LBP risk documentation before early clinical trials. However, there are still challenges regarding quality control and manufacturing of LBP, as ensuring consistent quality and manufacturing processes for biopharmaceuticals is complex. The biological nature of these products potentially has unknown inherent risks and can lead to manufacturing variability. Thus, maintaining product integrity during large-scale production is one of the significant obstacles to overcome.

The early clinical phases are designed to determine tolerability, dosage range, and administration regimen, which depend on the formulation and delivery route. As of current, designing an effective dosage form and delivery system for vaginal administration poses challenges. The dosage form should provide a controlled release and adhere to the vaginal mucosa for optimal absorption. Safety and tolerability studies need to consider the unique environment of the vagina regarding potential irritation, allergic reactions, or other adverse effects. The potential for immune responses to LBP can impact both safety and efficacy, so when appropriate, an "optimal effective dose" and a "safe maximal dose" are necessary to determine the points at which the intended effect is achieved with no or acceptable adverse effects. Thus, developers must assess and manage immunogenicity risks through appropriate study designs and monitoring strategies in the target populations.

Intended study populations vary from healthy individuals who may be at risk for specific diseases, such as infertility or pregnancy complications, to individuals severely afflicted with vaginal infections or dysplasia. Given this, designing appropriate clinical trials for live biopharmaceuticals can be challenging due to unique patient populations, limited historical data, and the need for sensitive endpoints. Likewise, target populations may require repeat treatment or longer-term usage of the LBPs, where biobanking of samples from consenting participants in the different trials and clinical phases may also be necessary (Rouanet et al., 2020). Thus, rigorous, and well-designed clinical trials are crucial for demonstrating safety and efficacy, where confounding factors influential to the data should also be considered. In addition, patient knowledge, acceptance, and adherence to vaginally administered treatments may be influenced by cultural, social, and individual factors and norms. Ensuring user-friendly, comfortable, and discreet administration methods is crucial.

Finally, the rapid advancement of biopharmaceutical technologies may outpace the development of regulatory science. Addressing scientific gaps and uncertainties in evaluating these products is an ongoing challenge for regulatory agencies. Furthermore, LBP developers often seek global approval, requiring coordination with multiple regulatory agencies. Achieving global harmonization in regulatory requirements and standards can be difficult due to varying regional expectations. Addressing these challenges requires collaboration between regulators, industry stakeholders, and researchers. Ongoing dialogue, transparency, and a proactive approach to adapting regulations to scientific advancements are essential for the successful development, approval, and access to LBPs.

Conclusion and future directions

Understanding the mechanisms by which the vaginal microbiota can influence the progression of gynecological and obstetrical disorders will lead to the development of personalized approaches to shape the microbiota composition and function, aiding prevention, and treatment of diseases. High-quality studies, including functional genomics, are needed to define the composition of the vaginal microbiota and its mechanistic involvement to the different conditions. This includes a detailed mapping of the ecology of the vaginal microbiota in health and disease to understand the interactions between microbes, pathogens and potential treatments based on live biopharmaceuticals. Furthermore, the complex interplay between the human host and vaginal microbes requires a deeper understanding for successful development of vaginal microbiome-directed therapies.

Regulatory approval of VMT, isolated bacterial strains, or defined consortia of strains will depend on the documentation and demonstration of quality, safety, and efficacy to allow for an assessment of the benefit-risk ratio for an intended use. More refined clinical trials with larger cohorts, defined uses, standardized trials, and longer-term follow-ups will also be necessary for maturing the field and the pioneer LBPs in development. The registration of LBPs as drugs will require a close dialogue between developers and regulatory agencies as the regulatory requirements are not fully defined yet. With this regard, accurate use of terminologies can significantly improve the process.

Author contributions

VV: Conceptualization, Writing – original draft, Writing – review & editing, Investigation. EL: Writing – original draft, Investigation, Writing – review & editing. IH: Writing – review & editing, Investigation, Writing – original draft. YZ: Writing – review & editing, Writing – original draft. JD: Writing – review & editing,

Conceptualization, Investigation, Writing – original draft. IS: Supervision, Project administration, Conceptualization, Investigation, Writing – review & editing, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The ability of the Lab4 probiotic consortium to impact upon the functionality of serum deprived human keratinocytes *in vitro*

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Introduction: Dysfunction of keratinocytes contributes to a weakened skin barrier and impaired wound healing capability. Evidence suggests that probiotic supplementation can lead to improved skin function in vitro and in vivo. The Lab4 probiotic consortium comprises of two strains of Lactobacillus species and two strains of Bifidobacterium species.

Methods: Using serum deprived conditions to impair the functionality of immortalized human HaCaT keratinocytes, this study aimed to assess the impact of metabolites derived from the Lab4 probiotic consortium on keratinocyte function.

Results: A significant improvement in HaCaT metabolic activity and lower apoptotic activity was observed in tandem with a reduction in Caspase-3 gene expression and a lower Bax/Bcl2 ratio following the addition of Lab4. The probiotic also supported barrier integrity which was better maintained with a significant increase in Filaggrin gene expression. In damaged keratinocytes, Lab4 enhanced rates of re-epithelialization, which were associated with significantly increased gene expression of MMP-1 and enhanced secretion of IL-6 and IL-8.

Discussion: These results suggest that the Lab4 probiotic consortium may have the ability to benefit the functionality of skin.

KEYWORDS

keratinocyte, serum deprivation, probiotic, gut-skin axis, Lab4, conditioned media

1 Introduction

Skin provides a protective, frontline barrier against environmental agonists such as pathogens, allergens and chemicals. It also plays a role in facilitating permeability and moisture retention. The key structural component of skin that facilitates functionality is the outermost epidermal layer known as the stratum corneum, that is comprised of keratinocytes (Menon et al., 2012). Key functions of keratinocytes are to form a tight barrier and facilitate wound healing (Menon et al., 2012) and loss of function caused by numerous stress factors including aging, the environment and/or nutrient deficiency (poor diet) can lead to increased risk of infection and disease (Woo and Kim, 2024; Ahmed and Mikail, 2024). The HaCaT keratinocyte cell line (Boukamp et al., 1988) is extensively used to study epidermal homeostasis and pathophysiology *in vitro* (Blanchard et al., 2022).

It is becoming recognized that the gut microbiome - the trillions of micro-organisms residing in the intestinal lumen - contributes to skin health and function via the 'gut-skin axis' and alterations in the gut microbiome composition such as a lack of diversity and lower abundance of beneficial bacteria have been linked to a number of skin disorders including atopic dermatitis (Liu et al., 2023), psoriasis (Codoñer et al., 2018) and acne vulgaris (Yan et al., 2018). Oral supplementation with probiotic bacteria - defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014) - has been associated with beneficial skin effects. Strains of *Lactobacillus* species have shown an ability to reduce symptoms of atopic dermatitis in humans (Rather et al., 2021; Yamamoto et al., 2016), while *Bifidobacteria* have shown the capability to improve skin barrier integrity in mice (Kim et al., 2022) and ameliorate age-related skin damage in humans (Huuskonen et al., 2022). The underlying mechanisms are not yet understood but it is thought that these effects may be mediated, at least in part, by the translocation of bacterial metabolites from the gut to the skin via the circulatory system (Wilson et al., 2020).

The Lab4 probiotic consortium, comprising *Lactobacilli* and *Bifidobacteria*, has been shown to support the viability and function of numerous peripheral cell types *in vitro* (Michael et al., 2019, Davies et al., 2018; O'Morain et al., 2023), including serum deprived neurons (Webberley et al., 2023), and holistic benefits have been observed in human intervention studies (Mullish et al., 2024, 2023, John et al., 2024; Paduchová et al., 2024; Hepburn et al., 2013; Williams et al., 2009). The ability of Lab4 to support skin health and functionality is yet to be explored.

In this *in vitro* study, serum deprivation was used to impair the metabolic activity, barrier function and re-epithelialization capability (wound healing) of HaCaT keratinocytes in order to assess the potential of metabolites generated by the Lab4 consortium to support skin functionality during challenged conditions.

2 Materials and methods

2.1 Materials and reagents

All tissue culture and molecular reagents were purchased from ThermoFisher Scientific (Paisley, UK), unless otherwise stated. All microbiological reagents were purchased from Oxoid (Basingstoke, UK), unless otherwise stated.

2.2 HaCaT cell culture

Human derived immortalized HaCaT keratinocyte cells (gifted by Professor Ryan Moseley's group, Cardiff University, Wales) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin, in a humidified incubator (Heraeus, Hanau, Germany) at 37 °C and 5% CO_2. Cells of ~80% confluency were seeded into appropriate tissue culture plates at a density of 7.8 x 10^4 cells/cm² and incubated for 24 h to reach 100% confluency.

2.3 Preparation of Lab4 conditioned media

The Lab4 probiotic consortium comprises: *Lactobacillus acidophilus* CUL21 (NCIMB 30156), *L. acidophilus* CUL60 (NCIMB 30157), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *B. animalis* subsp. *lactis* CUL34 (NCIMB 30172). Each strain was grown anaerobically overnight (18 h) at 37 °C in MRS (de Mann Rogosa and Sharpe) broth for CUL21 and CUL60 and MRS supplemented with 0.25 g/L cysteine hydrochloride for CUL20 and CUL34. The viable numbers were determined according to the method of Tracey et al. (2023) and each culture was centrifuged (4000 x g for 10 min), washed in sterile phosphate buffered saline (PBS), re-centrifuged and re-suspended in PBS. The Lab4 probiotic consortium (1 x 10^8 Active Fluorescent Units (AFU)/ml) was constructed from these bacterial suspensions, mixed, centrifuged and the pellet was re-suspended in pre-reduced DMEM and incubated anaerobically (37 °C/18 h) to generate Lab4 conditioned medium (L4CM). There was no increase in bacterial numbers overnight. The L4CM was harvested, pH adjusted to 7.4, supplemented with 100 U/mL penicillin/streptomycin prior to filter sterilization (0.2 µm), aliquoted and stored at -70 °C. The L4CM was inoculated onto confluent HaCaT cells at 0.5 ml L4CM representing 5 x 10^7 bacterial AFU per 1.9 cm^2 of HaCaT cells (representing 1.85 x 10^5 cells) providing an application rate of probiotic to keratinocyte of ~270:1. For optimization experiments, doses of L4CM were created by mixing with DMEM and are expressed as volume to volume (v/v) dilutions.

2.4 Assessment of HaCaT metabolic activity

Confluent monolayers of HaCaT cells (grown for 24 h post-seeding) were exposed to decreasing levels of serum between 10% (representing 'healthy' conditions) to 0% (serum free conditions) and after a 48 h incubation (at 37 °C and 5% CO_2), metabolic activity (considered an indicator of viability) was assessed using the 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Michael et al., 2019). Metabolic activity was expressed as a percentage in relation to the 10% serum control that was designated as 100%.

2.5 Apoptosis/necrosis assay

HaCaT cells were seeded into black sided, clear bottomed 96-well microplates (Greiner, Kremsmünster, Austria) and incubated overnight to allow for adherence (at 37 °C and 5% CO_2). Levels of necrosis and apoptosis were measured in the HaCaT cells exposed to experimental conditions (delivered in phenol red-free media to eliminate interference) using the RealTime-Glo™ Annexin V Assay kit (Promega, Wisconsin, USA) in accordance with manufacturer's instructions (Kupcho et al., 2019). Levels of necrosis are presented as relative fluorescence units (RFU) and levels of apoptosis are expressed as relative luminescence units (RLU).

2.6 Gene expression analysis

Total RNA was isolated from the HaCaT cells using TRIzol™, quantified using a Qubit 2.0 fluorometer and cDNA generated from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit, all carried out in accordance with manufacturer's instructions. Realtime PCR was performed on 200 ng of cDNA according to Michael et al. (2016) using a QuantStudio™ 5 Real-Time PCR System. Oligonucleotide primer sequences are shown in Supplementary Table S1. Relative gene expression was calculated using $2^{-(\Delta Ct1 - \Delta Ct2)}$, where delta Ct represents the difference between the cycle threshold (Ct) for the target gene (Ct1) and the house keeping gene (β-Actin) (Ct2). Gene expression data were expressed as mean fold changes in comparison to the appropriate experimental controls that have been designated as 1.

2.7 Measurement of barrier integrity using trans epithelial electrical resistance

HaCaT cells were grown to confluency on 0.4 µm pore PET membrane trans-well inserts (Thincert™, Greiner, Kremsmünster, Austria) in 24 well plates. Trans epithelial electrical resistance (TEER) (ohms) was measured using an EVOM2 Epithelial Voltohmeter STX2 chopstick electrode set (World Precision Instruments, Hertfordshire, UK) in accordance with manufacturer's instruction (Srinivasan et al., 2015). Prior to experimentation, and between technical replicates, the electrode was sterilized by immersion in ethanol and then in sterile DMEM. TEER was calculated as ohms*cm^2 after correction for the background reading of a cell free trans-well insert and are presented as percentage change in comparison with the appropriate experimental control that has been designated as 100%.

2.8 Assessment of the impact of L4CM on scratch wounding

Confluent HaCaT monolayers in 24 well plates were scratched using a standard 200 µL pipette tip (method adapted from Vang Mouritzen and Jenssen (2018)), producing a single vertical lesion (approximately 550 µm in width) spanning the diameter of the well for migration assays (section 2.8.1) or 3 vertical and 3 horizontal lesions spanning the well for assessment of IL-6 and IL-8 protein levels (section 2.8.2) and gene expression (section 2.6). After scratching, cells were washed twice with PBS to remove debris before the addition of experimental conditions.

2.8.1 Re-epithelialization assay

Scratches were imaged over 48 h (at 4 X magnification) using a DMi1 Inverted Light Microscope with camera (Leica, Wetzlar, Germany). From the images, the area of the scratch (µm^2) was measured using the "Wound healing size tool" plug-in in ImageJ (Suarez-Arnedo et al., 2020). Rates of cell migration or 'wound healing' were calculated according to the equation (Jonkman et al., 2014):

$$Vmigration = \frac{[slope]}{2 \; x \; l}$$

Here, *Vmigration* is average velocity of cell movement. The *slope* value is area of the wound (µm^2) over time (h) and *l* refers to length of the region of interest (1671.82 µm for all images, as calculated by ImageJ). Values were calculated as µm/h and expressed as percentage change relative to the appropriate experimental control that has been designated as 100%.

2.8.2 IL-6 and IL-8 enzyme linked immunosorbent assay

Cell supernatants were collected 4 and 48 h after scratching and centrifuged at 2000 x g for 10 minutes, to remove cell debris. Supernatants were analysed for IL-6 and IL-8 protein concentration using the Human IL-6 SimpleStep Enzyme Linked Immunosorbent Assay (ELISA®) Kit (ab178013) and the Human IL-8 SimpleStep ELISA® Kit (ab214030) (both Abcam, Cambridge, UK) in accordance with manufacturer's instructions and as outlined in Lin et al. (2024). The remaining cell monolayers were processed for gene expression analysis as per section 2.6.

2.9 Statistical analysis

Normality of data was assessed using the Shapiro-Wilk test and by visual inspection of Q-Q plots to confirm normal distribution. Differences between groups were analysed using an unpaired parametric Student's t-test (for single comparisons), One-way

analysis of variance (ANOVA) with Dunnett's *post hoc* testing (for multiple comparisons) or Two-Way ANOVA, followed by Tukey's *post hoc* testing (for repeated measure multiple comparisons). Differences were determined to be significant where $p \leq 0.05$. All statistical analysis was performed using GraphPad (Prism, Version 10, Boston, USA). All data are presented as the mean ± standard deviation of at least 3 independent experiments, unless otherwise stated.

3 Results

3.1 L4CM preserves metabolic activity in serum deprived HaCaT cells

The metabolic activity of HaCaT cells exposed to decreasing concentrations of serum was assessed and indicated ~50% loss of activity after 48 h of serum-free (SF) conditions in relation to cells

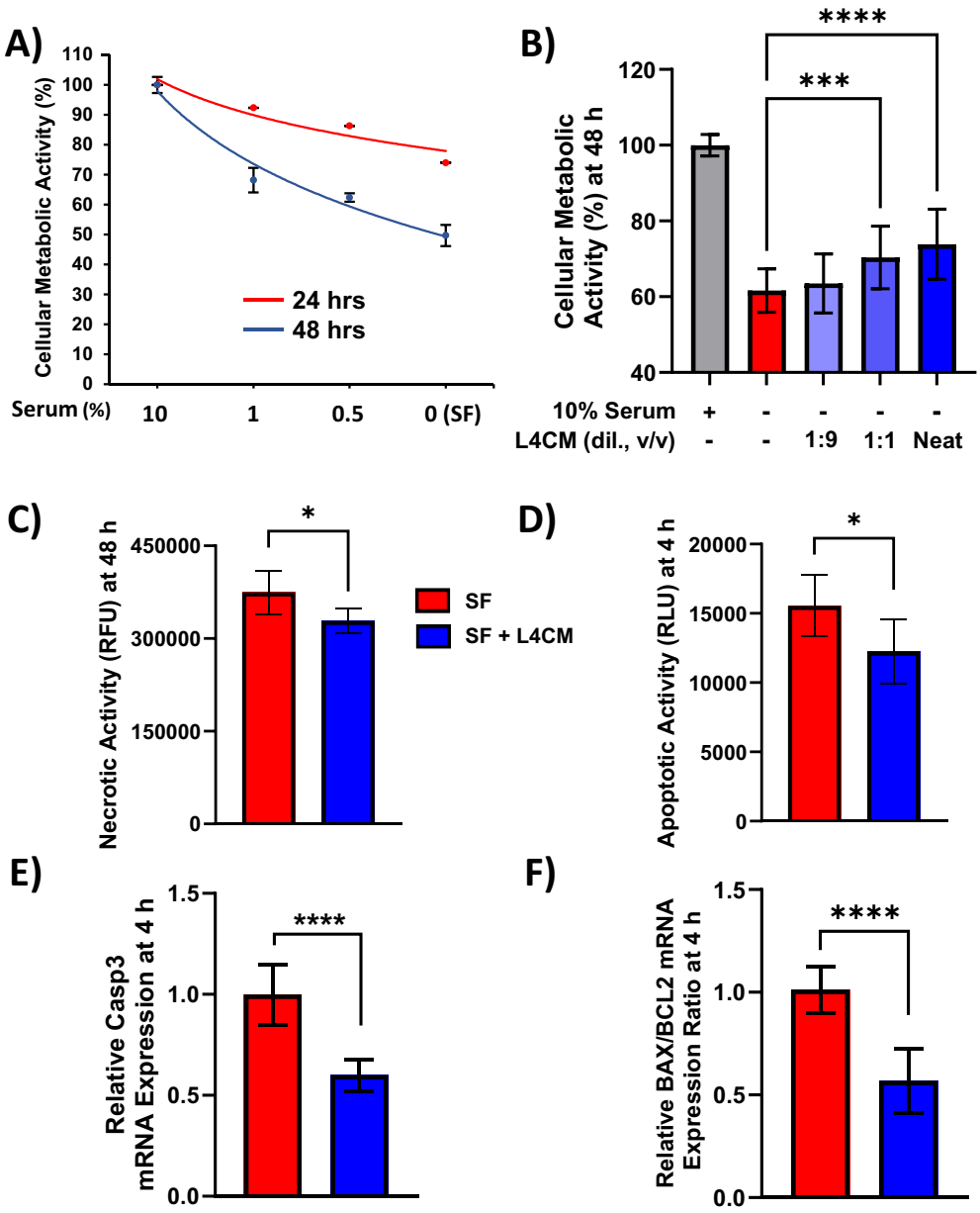

FIGURE 1
L4CM enhances metabolic activity and reduces apoptotic activity in serum free HaCaT cells. HaCaT cell metabolic activity with **(A)** decreasing concentrations of serum for up to 48 h. **(B)** HaCaT cells incubated with 10% (v/v) serum, without serum (serum free, SF) or SF with various dilutions (v/v) of L4CM for 48 h. Data are expressed as percentage in relation to 10% serum treated cells (set to 100%). **(C)** Necrotic activity in HaCaT cells after 48 h and **(D)** early apoptotic activity after 4 h of serum free HaCaT cells with or without neat L4CM supplementation. Data are expressed as the mean relative fluorescent units (RFU, for **C**) or mean relative luminescent units (RLU, for **D**). Relative gene expression levels of **(E)** Caspase-3 and **(F)** BAX/BCL2 in SF HaCaT cells supplemented with or without neat L4CM for 4 h. Data are expressed as fold change in relation to the SF control (set to 1). Values of *p* were determined using One-Way ANOVA followed by Dunnett's *Post Hoc* analysis (for **B**) or an unpaired two-way Student's t-test (for **C**–**F**) where *$p<0.05$, ***$p<0.001$ or ****$p<0.0001$. Bax, Bcl-2-associated X apoptosis regulator; Bcl-2, B-cell lymphoma 2 gene; Caspase-3, cysteine-aspartic protease 3.

incubated with 10% serum (Figure 1A). When SF cells were incubated with varying concentrations of Lab4 conditioned medium (L4CM) for 48 h there was a dose related increase in metabolic activity from 59% in the SF cells to 60% with the addition of a 1:9 dilution of L4CM, 67% (p=0.0007) with a 1:1 dilution of L4CM and 71% (p<0.0001) with neat (undiluted) L4CM (Figure 1B).

Metabolic activity is commonly used as an indicator of cellular viability and necrotic activity in cells exposed to neat L4CM for 48 h was 12% lower (p=0.0189, Figure 1C) compared to SF cells. For all subsequent experiments, L4CM was applied neat and is referred to as 'L4CM' hereafter. Necrosis is preceded by apoptosis and the highest levels of apoptosis were recorded in HaCaT cells after 4 h of serum deprivation (Supplementary Figure S1) when 21% less apoptotic activity was detected with L4CM compared to the SF control (p=0.0301, Figure 1D). The lower apoptotic activity observed at 4 h was associated with lower expression of the pro-apoptotic Caspase-3 gene (0.6-fold change, p<0.0001, Figure 1E) and a lower ratio of apoptotic markers Bax/Bcl-2 (0.57-fold change, p<0.0001, Figure 1F) when compared with SF cells.

3.2 L4CM preserves barrier integrity

Maximum HaCaT TEER was reached after 11 days of incubation as shown in Figure 2A. Subsequently, HaCaT cells

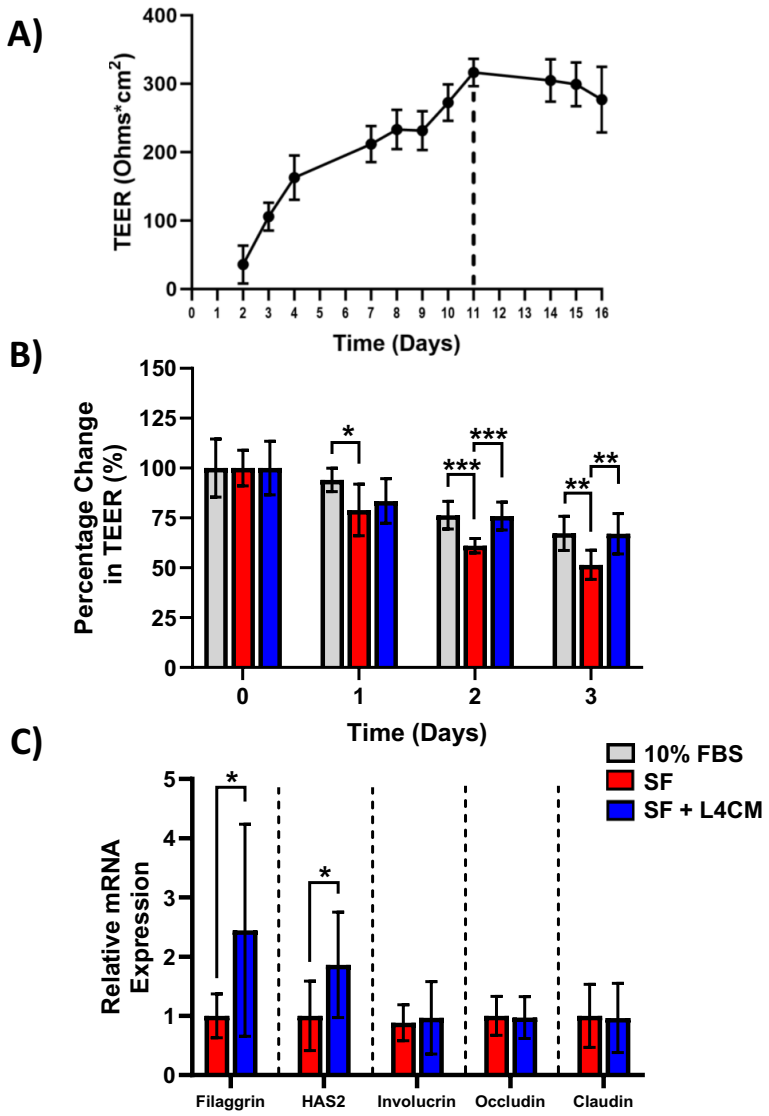

FIGURE 2
The impact of L4CM on barrier integrity in serum free HaCaT cells. **(A)** Temporal changes in TEER (ohms*cm^2) in HaCaT cells maintained with daily feeding for 16 days. Data represents mean ± SD of 2 independent experiments. **(B)** Changes in TEER of 11-day old HaCaT cells after exposure to 10% serum or serum free (SF) conditions with or without L4CM for 72 h. Data is expressed as mean percentage change compared to 10% serum (set at 100%). **(C)** Relative gene expression levels of filaggrin, HAS2, involucrin, occludin, and claudin in SF HaCaT cells with or without exposure to L4CM for 48 h. Data are expressed as a fold change in relation to the SF control (set to 1). Values of p were determined using repeated measures two-way ANOVA with the Geisser-Greenhouse correction and Tukey's *post-hoc* (for **B**) or unpaired parametric students t-test (for **C**) where *p<0.01, **p<0.01 or ***p<0.001. Filaggrin, filament aggregating protein; HAS2, hyaluronan synthase 2.

were incubated for 11 days and the effect of serum deprivation on the cells was subsequently assessed with and without the addition of L4CM over 3 days (Figure 2B).

The barrier integrity, as measured by the TEER of the SF HaCaT cells, decreased more over time in comparison with cells supplemented with 10% serum with a significant reduction in TEER of 16% observed after day 1 (p=0.0378), 20% after day 2 (p=0.0004) and 23.5% after day 3 (p=0.0036) in comparison. Levels of TEER for the 10% serum control and the L4CM supplemented cells remained similar throughout the 3 days.

The barrier integrity of HaCaT cells was found to be significantly better than that of SF challenged cells with the inclusion of L4CM over the 3 days. The SF cells showed greater deterioration of barrier integrity over time in comparison with cells supplemented with L4CM with a 5.7% decrease in TEER observed after day 1, 24.4% (p=0.0005) after day 2 and 30.2% (p=0.01) after day 3 in comparison.

Assessment of the barrier integrity related genes Filaggrin, HAS2, Involucrin, Occludin and Claudin indicated significantly higher expression levels of Filaggrin (2.44-fold change, p=0.0124, Figure 2C) and HAS2 (1.86-fold change, p=0.0131) in L4CM supplemented cells compared to SF cells after 48 h. Levels of Involucrin, Occludin and Claudin showed no significant differences.

3.3 L4CM improves re-epithelialization

Confluent monolayers of HaCaT cells were damaged by "scratching" and the rate of cell migration to close the denuded area (to achieve re-epithelialization) was monitored over 48 h (Figure 3A). Compared to the 10% serum control, rate of migration in the SF cells was 49.5% lower (p<0.0001, Figure 3B). In L4CM supplemented cells, the rate of migration was 61% higher (p=0.0036, Figure 3B) than seen with the SF cells and was comparable to that measured in the 10% serum control (p=0.0646).

Gene expression analysis 4 h after scratching indicated significantly higher levels of MMP-1 (1.22-fold change, p=0.0089, Figure 3C) and HAS3 (2.1-fold change, p<0.0001) in the L4CM supplemented cells compared to the SF control, but no changes were observed in MMP-9 (p=0.0868). Expression of the proliferation marker Ki-67 was significantly lower (0.66-fold change, p<0.0001) in L4CM cells compared with the SF control.

3.4 L4CM impacts the inflammatory response of scratched HaCaT cells

Protein levels of the secreted inflammatory cytokines IL-6 and IL-8 were assessed 4 h and 48 h after scratching of HaCaT monolayers. Higher concentrations in the L4CM supplemented cells compared with the SF control were observed after 4 h for IL-6 (141 pg/mL vs 25 pg/mL, p<0.0001, Figure 4A) and for IL-8 (193 pg/mL vs 43 pg/mL, p<0.0001, Figure 4B). Concentrations of IL-6 and IL-8 were similar in L4CM and SF groups 48 h after scratching (825 pg/mL vs 840 pg/mL, p=0.9384 for IL-6 (Figure 4C) and 1571 pg/mL vs 2185 pg/mL, p=0.1243 for IL-8 (Figure 4D)).

Gene expression analysis of IL-6 (Figure 4E) and IL-8 (Figure 4F) in scratched HaCaTs showed that exposure to L4CM had no significant impact on expression levels at 4 h, but did reduce expression levels at 48 h of IL-6 (0.85-fold change, p=0.0593) and IL-8 (0.73-fold change, p=0.0062) in comparison with SF cells.

4 Discussion

In HaCaT keratinocytes challenged with serum deprivation, metabolites of the Lab4 probiotic consortium (L4CM) were able to prevent loss of metabolic activity, barrier integrity and enhance rates of re-epithelialization while also modulating the acute inflammatory response to damage. These data highlight the potential of Lab4 to support keratinocyte function and augments the growing body of evidence suggesting that supplementation with probiotics can impact upon skin homeostasis and function via the gut-skin axis (Gao et al., 2023).

The removal of serum from HaCaT cells resulted in clear reductions in metabolic activity and apoptosis in alignment with studies performed elsewhere (Lu et al., 2008; Alexaki et al., 2009). In the presence of L4CM, metabolic activity of the serum deprived cells was significantly increased indicating improvement in cellular viability. The improved metabolic activity changes were associated with reductions in apoptotic and necrotic activities. There were also reductions in the gene expression levels of pro-apoptotic Caspase-3 and the BAX/Bcl2 ratio, which in tandem support the anti-apoptotic activity observed with the probiotic. It has been found that GAPDH derived from *Lactobacillus gasseri* can reduce keratinocyte apoptosis via interference of the Caspase-3 cascade and this process may contribute to the mechanism of action for the results obtained with L4CM (Chen et al., 2024).

A robust skin barrier is required to protect the host from environmental insults and to minimize water loss from the body. We found that L4CM supported the maintenance of barrier integrity in serum deprived HaCaT cells (measured via TEER) and increased gene expression levels of the barrier related genes, Filaggrin and Hyaluronan-2 (HAS2). Filaggrin contributes to the formation and maintenance of epidermal barrier integrity (Furue, 2020) and HAS2 contributes to the natural turgidity of the skin barrier and low expression of these genes, as observed in aging skin, is associated with compromised barrier function (Rinnerthaler et al., 2013) and decreased elasticity (Terazawa et al., 2015) respectively. Increased expression levels of Filaggrin and HAS2 have also been observed in keratinocytes with other strains of *Lactobacilli* and *Bifidobacteria* (Kim et al., 2022; Fusco et al., 2023; Zhu et al., 2024).

In order to maintain a robust skin barrier, the epidermis must be able to regenerate and repair effectively after injury - another capability reduced by serum deprivation. It has already been shown that strains of *Lactobacilli* and *Bifidobacteria* can improve re-epithelialization rates in wounded HaCaT monolayers (Lombardi et al., 2019; Brandi et al., 2020) and that probiotic supplementation can enhance skin healing in rats (Tagliari et al., 2022). In this study, the rate of re-epithelialization in serum deprived HaCaT cells was significantly increased in the presence of L4CM and was associated

with elevated expression levels of the MMP-1 and HAS-3 genes; MMP-1 encodes for a matrix metalloproteinase enzyme that is vital for matrix remodelling and cleavage of native fibrillar collagens during normal wound resolution (Yeo et al., 2020; Bucekova et al., 2017) and HAS-3 is recognized for its role in the early inflammatory response to injury (Stern et al., 2006).

The wound healing process in skin is orchestrated, at least in part, by inflammatory mediators released by keratinocytes; IL-6 and IL-8 are key pro-inflammatory cytokines produced during the early stages of wound healing and facilitate keratinocyte migration, angiogenesis and recruitment of immune cells such as neutrophils to the wound site (Johnson et al., 2020; Jiang et al., 2012). To explore

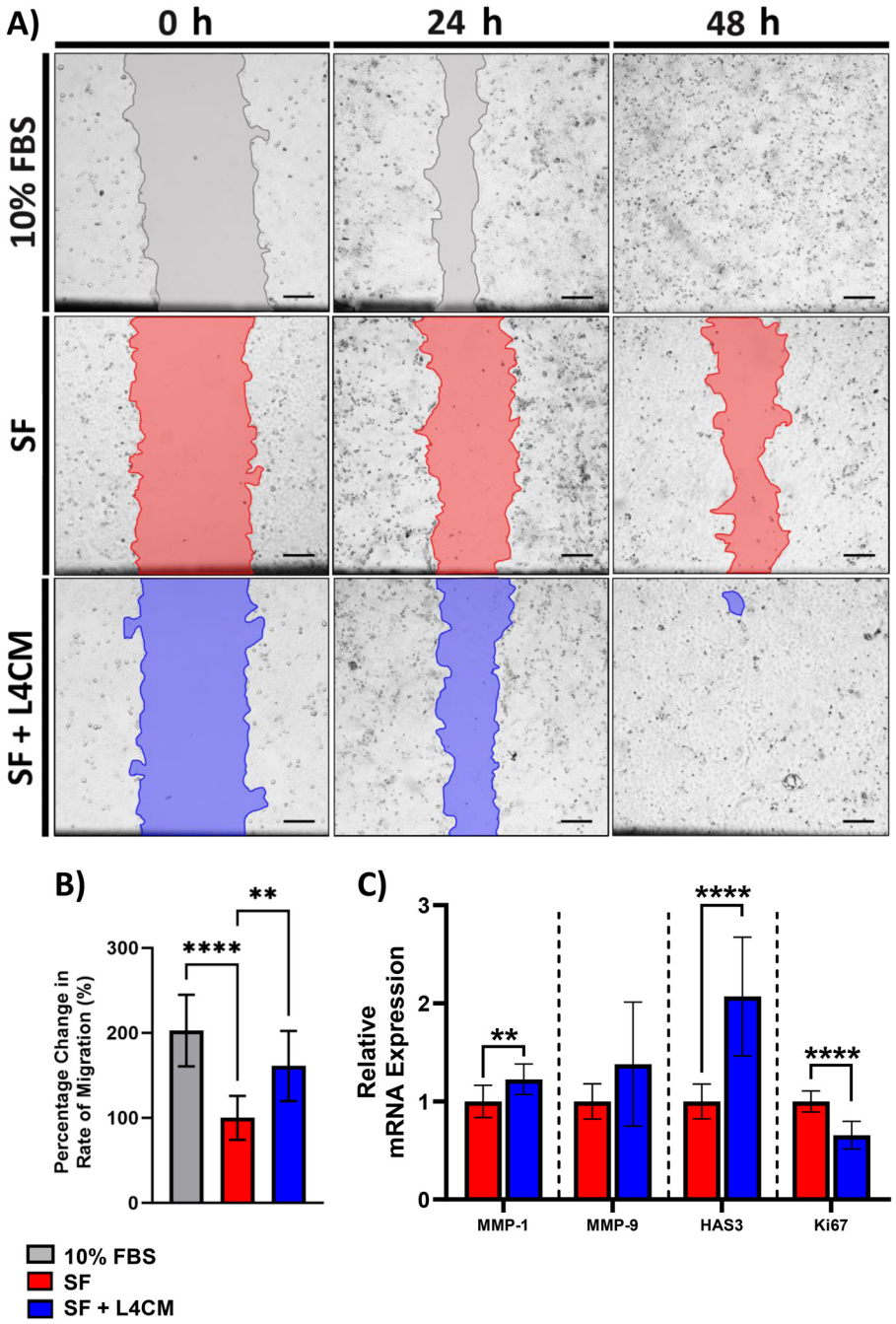

FIGURE 3
The impact of L4CM on HaCaT re-epithelialization under in serum free cells. **(A)** Representative images of scratched HaCaT monolayers incubated with 10% serum, or serum free (SF) conditions with or without L4CM supplementation for 48 h (4X magnification, scale bar = 150 μm). **(B)** Change in rate of HaCaT cell re-epithelialization in scratched HaCaT cells exposed to 10% serum or SF conditions with or without L4CM for 48 h. Data are expressed as a percentage change in relation to the SF control (set to 100%). **(C)** Relative gene expression of MMP-1, MMP-9, HAS3 and Ki-67 in scratched SF HaCaT cells with or without a 4 h incubation with L4CM. Data are expressed as fold change compared to SF cells (set to 1). Values of *p* were determined using an unpaired parametric Student's t-test where $^{**}p<0.01$ or $^{****}p<0.0001$. MMP-1, matrix metalloproteinase 1; MMP-9, matrix metalloproteinase-9; HAS3, Hyaluronan Synthase 3.

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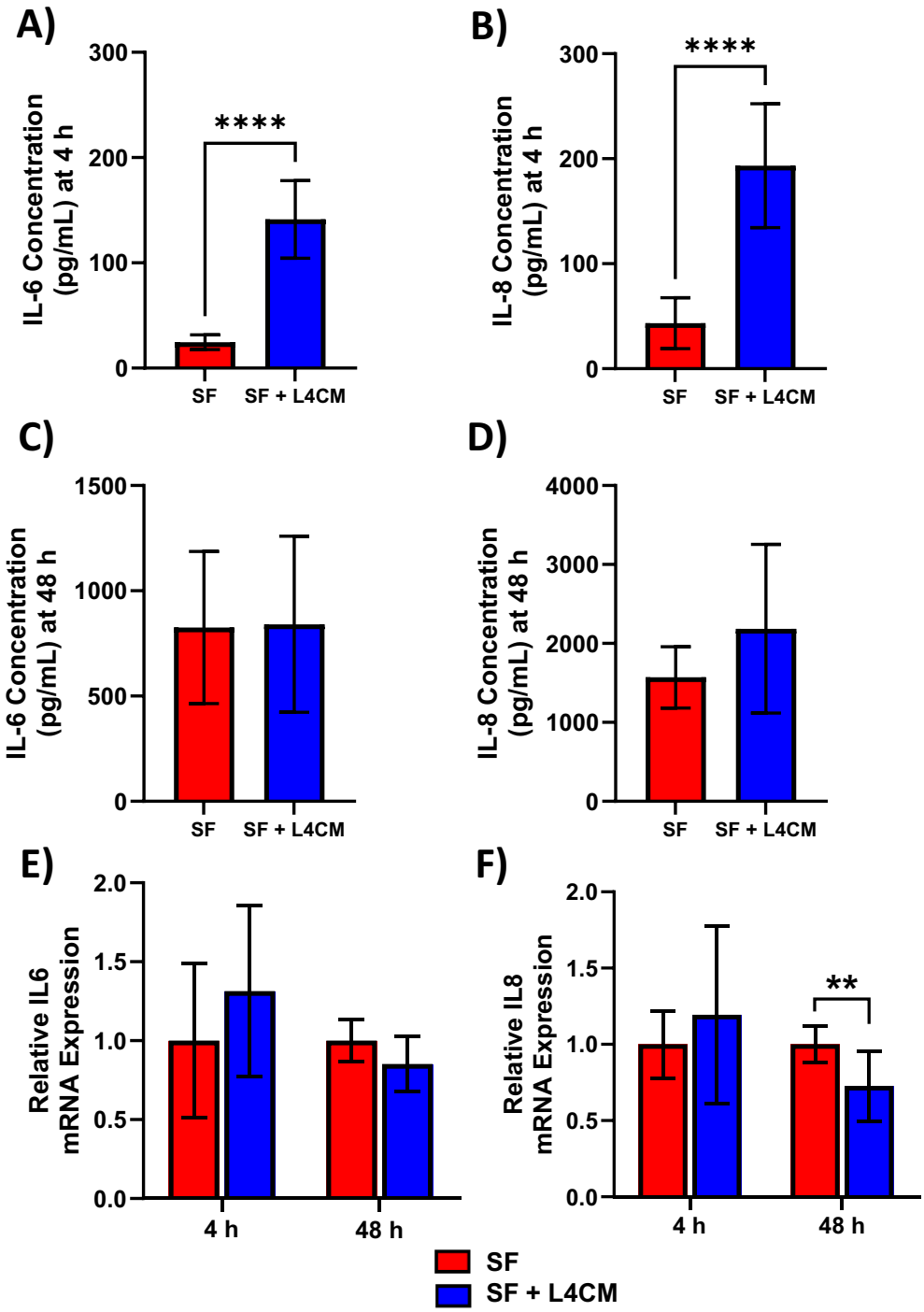

FIGURE 4
L4CM modulates IL-6 and IL-8 protein production in scratched, serum free HaCaT cells. Protein concentration of inflammatory cytokines IL-6 and IL-8 were determined via ELISA after **(A, B)** a 4 h and **(C, D)** 48 h incubation following scratching of the cell monolayer and the application of serum free (SF) conditions with or without the supplementation of L4CM. Relative gene expression of IL-6 **(E)** and IL-8 **(F)** in scratched SF HaCaT cells incubated for 4 h with or without L4CM. Data are expressed as fold change compared to SF cells (set to 1). Values of p were determined using an unpaired parametric Student's t-test where $^{**}p<0.01$ or $^{****}p<0.0001$. IL-6, Interleukin 6; IL-8, Interleukin 8.

the impact of L4CM on the inflammatory response to the induced injury the levels of IL-6 and IL-8 secreted by serum deprived HaCaT cells after wounding were measured. Significantly higher levels of IL-6 and IL-8 protein were secreted by cells exposed to L4CM in comparison with the serum free cells within 4 h of wounding suggesting enhancement of the acute inflammatory response. L4CM has been shown to improve the acute immune response of tissue macrophages to both viral and bacterial challenges (Davies et al.,

2018). Metabolites generated by *Lactiplantibacillus plantarum* have been shown to improve wound healing *in vitro* and *in vivo* with the latter associated with the upregulation of IL-6 during the early phase of wound healing (Dubey et al., 2021). By 48 h post-wounding the levels of IL-6 and IL-8 protein concentration were found to be similar in both the L4CM and serum free control groups and there were indications of lower expression levels of the IL-6 and IL-8 genes by the L4CM treated cells. Taken together, these data suggest that Lab4 mediated a stronger acute inflammatory response to wounding followed by quicker resolution of wound associated inflammation.

Our study has a number of potential limitations: Firstly, the inflammatory gene markers assessed during the scratch experiments were limited to IL-6 and IL-8 in this study but there are other relevant markers that should be considered for assessment in future work, including other pro-inflammatory cytokines that play a dynamic role in wound healing such as TNF-α (Ritsu et al., 2017), along with growth factors such as TGF-β (Liarte et al., 2020). Secondly, we did not assess the impact of the probiotic in the healthy control HaCaT cells. Finally, the active metabolites generated by Lab4 have not been determined. A recent *in silico* analysis of the Lab4 genome sequences identified the presence of genes involved in the generation of lactic acid, acetic acid and free amino acids (Baker et al., 2021) that have recognized skin benefits (Lew and Liong, 2013., Takaoka et al., 2019).

Future studies are likely to include work with *i*) other epidermal cell lines (Allen-Hoffmann et al., 2000; Baden et al., 1987) and *ii*) 3D skin models/organoids (Lombardi et al., 2024) in order to substantiate our preliminary findings and ultimately *iii*) an intervention study in human subjects. An area of particular interest is the role that probiotics might play in the prevention of skin aging (Waller and Maibach, 2005; Scioli et al., 2014; Bentov and Reed, 2015; Xiao et al., 2023).

In summary, this exploratory study shows that the conditioned media of the Lab4 probiotic consortium helps maintain metabolic activity and barrier integrity, improves the rate of re-epithelialization, and promotes an early immune response to injury in serum deprived HaCaT keratinocytes and therefore suggest that Lab4 may have the ability to support skin function *in vivo*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

ST: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. JK-S: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. SP: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. JB: Methodology, Writing – review & editing. DJ: Software, Writing – review & editing. EL: Investigation, Writing – review & editing. LP: Methodology, Resources, Writing – review & editing. JD: Investigation, Methodology, Supervision, Writing – review & editing. RM: Supervision, Writing – review & editing. DM: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

Authors ST, JK-S, DM, DJ, JB, JD and SP were employed by the company Cultech Ltd., Port Talbot, UK.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frmbi.2024.1488650/full#supplementary-material

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Postbiotics and their biotherapeutic potential for chronic disease and their feature perspective: a review

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Postbiotics, which are bioactive compounds derived from the metabolic processes of probiotics, are gaining recognition as a promising alternative for managing chronic diseases without the need for live microorganisms, positioning them as a valuable strategy in biotherapeutics that offers both curative and preventive techniques in modern medicine. This paper provides a comprehensive review of the potential health benefits of postbiotics, particularly concerning noncommunicable diseases like diabetes, cancer, obesity and cardiovascular conditions, which present significant global health challenges. We explore the various mechanisms by which postbiotics exert their beneficial effects, including immune modulation to enhance the body's immune response and reduce inflammation, as well as improving gut barrier function to maintain gut integrity and prevent increased intestinal permeability. Additionally, the antioxidant properties of postbiotics play a critical role in neutralizing oxidative stress, which is linked to the progression of chronic diseases. Despite the encouraging insights into their health benefits, we highlight the urgent need for further research to clarify the specific roles of different postbiotic components. A deeper understanding of these mechanisms is essential for developing targeted preventive healthcare applications, and by advancing this knowledge, we aim to create innovative strategies that could significantly enhance health outcomes for at-risk populations. Ultimately, integrating postbiotics into health interventions has the potential to improve preventive care and contribute to the overall well-being of affected individuals and communities.

KEYWORDS

biotherapeutic, biofunctional, microbiome, chronic disease, bioactive

1 Introduction

Postbiotics are bioactive compounds that are derived from the metabolic byproducts of probiotics through the fermentation process. They are non-viable bacterial products or metabolic byproducts from probiotic microorganisms that have biologic activity in the host. It includes short-chain fatty acids (SCFAs), exopolysaccharides (EPS), bioactive peptides (BAPs), cell components, organic acids, cell fragments, and vitamins, have potential health benefits such as anti-inflammatory, antioxidant, anti-cancer, and antihypertensive properties (Fattahi et al., 2020; Kamiloglu et al., 2022; Du et al., 2024). The term "postbiotic" is relatively new in the field of microbiome research and is used to describe these substances that confer health benefits similar to or distinct from those associated with probiotics. Postbiotics, as defined by the International Scientific Association of Probiotics and Prebiotics (ISAPP), are "preparations of inanimate microorganisms and/or their components that confer a health benefit on the host" (Salminen et al., 2021). A key aspect of this definition is that the final postbiotic product must include inactivated microbial cells or their components, with or without associated metabolites. Importantly, the definition excludes substantially purified metabolites in the absence of cellular biomass. For example, isolated compounds such as butyric acid or lactic acid should be referred to using their chemical nomenclature rather than being classified as postbiotics. They differ from probiotics, live microorganisms, and can include inactivated microbial cells or their metabolites. The concept of postbiotics gained attention as potential alternatives to probiotics, as they could potentially overcome some of the limitations associated with the use of live microorganisms' probiotics. Probiotics are live microorganisms that confer health benefits when consumed in adequate amounts, have been extensively studied and applied in various areas of health. They are often used to alleviate symptoms associated with irritable bowel syndrome and to rebalance the gut microbiome following antibiotic use (Boyte et al., 2023; Shi et al., 2024). Although significant health benefits, there are some limitations associated with their viability and stability of the live microorganisms during storage and transit through the gastrointestinal tract (Shah, 2010). The viability of probiotics is a critical factor, as they need to be alive to confer health benefits. This requirement makes them sensitive to storage conditions, including temperature and pH levels, which can affect their efficacy over time (Scarpellini et al., 2021; Zhang et al., 2022).The beneficial effects of probiotics are not solely attributed to the live microorganisms themselves, but also to the metabolites, cellular components, and other byproducts produced by these microbes during fermentation. These non-viable microbial components and their metabolites i.e. postbiotics are generally more stable and resilient than live probiotics, as they are not affected by environmental factors or the host's gastrointestinal conditions (Scarpellini et al., 2021; Zhang et al., 2022). Postbiotics can be more easily standardized and incorporated into various food, beverage, and pharmaceutical products, compared to the challenges of maintaining the viability of probiotic strains.

Noncommunicable diseases (NCDs) are a significant global public health issue, contributing to an estimated 74% of deaths in 2020, as noted by Jastrząb, Graczyk (Jastrząb et al., 2021) and Park, Joung (Park et al., 2022). Cardiovascular diseases, cancer, chronic kidney diseases, obesity, and diabetes are common NCDs, and their burden is expected to rise in the coming years due to urbanization, population aging, and lifestyle changes (Behera, 2020; Stasi et al., 2022). In 2020, cardiovascular disease was the primary cause of death with 17.9 million deaths, followed by cancer with 9.3 million deaths (Thorakkattu et al., 2022). In low- and middle-income countries, the high cost of treatment hinders access to care for NCDs. The treatment cost varies depending on the disease's severity, type, and healthcare resources available in the country, making the cost of treating NCDs in LMICs much higher than in high-income countries, which creates significant barriers to treatment (Kundu et al., 2018; Njuguna et al., 2020). The cost of treating hypertension and diabetes in LMICs ranges between US $100 and US$500 and US$200 and US$1000 per year, respectively, while cancer treatment can cost up to US$10,000 per year, making access to care a major challenge for people in LMICs (Kundu et al., 2018; Njuguna et al., 2020).

2 Current chronic disease management and postbiotics

Postbiotics have the potential to enhance the efficacy of conventional therapies by modulating inflammation, improving gut health, and providing synergistic effects with medications (Scott et al., 2022; Wang S. et al., 2024). Chronic diseases are often associated with low-grade inflammation, and postbiotics can help regulate inflammatory responses by promoting the production of anti-inflammatory cytokines and inhibiting pro-inflammatory markers (Koshiyama, 2010; Mundula et al., 2022). This modulation can improve patient outcomes when postbiotics are administered alongside traditional treatments, such as pharmacotherapy.

Many conventional therapies disrupt gut microbiota balance, resulting in gastrointestinal side effects and dysbiosis. Postbiotics offer a promising solution by restoring gut microbiome, alleviating symptoms associated with antibiotics or other medications, and enhancing overall treatment adherence (Zhang et al., 2018). Beyond mitigating side effects, postbiotics play a critical role in modulating gut microbiota composition and influencing drug pharmacokinetics by enhancing absorption, metabolism, and therapeutic efficacy. For instance, microbial metabolites have been shown to increase the bioavailability of omeprazole by 269.9% through the modulation of cytochrome P450 enzymes (Zhang et al., 2024). Additionally, short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate lower intestinal pH, which improving drug solubility and absorption (Blaak et al., 2020; Liu et al., 2021). This mechanism has been demonstrated to enhance the bioavailability of drugs like lurasidone by 4.3-fold (Collins et al., 2024). Moreover, in cancer treatment, postbiotics exhibit anti-proliferative and anti-inflammatory properties that can moderate the effectiveness of conventional therapies while reducing adverse effects (Rad et al., 2020). These highlight the potential of postbiotics to enhance drug efficacy and minimize therapy-related complications.

Dietary interventions play a critical role in managing chronic diseases such as obesity, diabetes, and cardiovascular disease. Incorporating postbiotics into dietary strategies can significantly enhance their effectiveness by promoting a balanced gut microbiome and improving gut barrier function (Ozma et al., 2022; Li et al., 2024). Postbiotics can also influence the metabolism of dietary components by modulating gut microbiota composition.

The diversity of gut microbiota is linked to the fermentation of dietary fibers, which is essential for SCFA production, particularly butyrate, acetate, and propionate (Salamone et al., 2021; Maiuolo et al., 2024). This synergy between postbiotics and dietary approaches underscores the need for a holistic strategy in chronic disease management.

Fermented foods, such as yogurt, kefir, sauerkraut, kimchi, miso, tempeh, and kombucha, are among the richest sources of postbiotics (Darwish et al., 2022; Gill and Staudacher, 2023; Gurunathan et al., 2023). These foods not only provide beneficial microorganisms but also bioactive compounds generated during fermentation, supporting gut health and overall well-being (Beshkova and Pavlov, 2012; Darwish et al., 2022). Incorporating fermented foods into the diet can be a valuable strategy for enhancing postbiotic intake.

While postbiotics complement traditional therapies, they also hold considerable potential as standalone treatments for promoting human health and managing chronic diseases (Nagarajan et al., 2022; Freitas et al., 2023; Sorrenti et al., 2023). Certain microbial metabolites, such as SCFAs, bioactive peptides, and exopolysaccharides, positively influence metabolic health and reduce the risk of conditions like obesity, type 2 diabetes, and cardiovascular disease (Wu et al., 2023; Eslami et al., 2024). Postbiotics improve metabolic health by regulating lipid profiles, enhancing insulin sensitivity, and modulating immune responses, offering a balanced approach that reduces reliance on pharmacological interventions.

In gastrointestinal health, postbiotics have demonstrated efficacy in managing disorders like irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) by restoring gut barrier function and promoting the synthesis and assembly of tight junction proteins, which are essential for maintaining the structural integrity of the gut epithelium (Scott et al., 2022) and reducing inflammation ((Valera et al., 2009). Additionally, they bolster immune function, particularly in individuals with compromised immune systems or chronic inflammatory conditions (Mehta et al., 2023).

2.1 Components of postbiotics

Postbiotics, comprising various constituents produced during the fermentation process or molecules that are found in the cell walls of some bacteria like teichoic acid, EPS, BAPs), and SCFAs such as butyrate, acetate, and propionate, are organic acids produced during the fermentation of dietary fibers by probiotic bacteria. Antimicrobial bacteriocins, BAPs, teichoic acids, and vitamins have demonstrated bioactive properties, including immunomodulation, anti-inflammatory, anti-microbial, anti-oxidant, and anti-proliferation (Scott et al., 2022; Vinderola et al., 2022). Studies by Mayorgas, Dotti (Mayorgas et al., 2021) and Thorakkattu, Khanashyam (Thorakkattu et al., 2022) have also shown that postbiotics contain essential vitamins, including vitamin B12, vitamin B2, vitamin B6, folic acid (vitamin B9), and vitamin K, which can be produced by some probiotic strains during fermentation of prebiotics.

2.1.1 Bioactive peptides and their biotherapeutic potential for chronic disease management

BAPs are short chains of amino acids that are derived from proteins through enzymatic hydrolysis, fermentation, or digestion. BAPs typically consist of 2 to 20 amino acids and the specific sequence of amino acids in the peptide chain is crucial for determining its biological activity (Karami and Akbari-Adergani, 2019; Rizwan et al., 2023). Essential and non-essential amino acids mostly glycine, Isoleucine, leucine, proline, arginine, Valine, Tyrosine, and lysine are common in BAPs.

ACE inhibitors peptides work by preventing the conversion of angiotensin I to angiotensin II, a strong vasoconstrictor, which helps to lower blood pressure (BP) and offers vascular protection Table 1. Additionally, these inhibitors enhance the availability of bradykinin, a substance known for its vasodilatory effects Table 1. Bioactive peptides derived from *L. amylovorus* have shown promising effects in modulating lipid metabolism and preventing obesity-related disorders Table 1. These peptides can reduce lipogenesis by inhibiting lipogenic enzymes and gene expression in hepatocytes and adipocytes (Udenigwe and Rouvinen-Watt, 2015).

The production of BAPs through fermentation is a complex process influenced by microbial strains, fermentation conditions, and type of protein substrate. Probiotic strains, particularly lactic acid bacteria (LAB), play a crucial role in this process. LAB possess proteolytic enzymes that break down food proteins into smaller peptides. During fermentation, LAB also metabolize carbohydrates, producing lactic acid, which lowers the environmental pH. This acidic condition enhances the activity of proteolytic enzymes, facilitating the hydrolysis of proteins into BAPs (Hati et al., 2014; Ter et al., 2024). The specific profiles of BAPs produced depend on the protein source and the LAB strains used, as the amino acids released during proteolysis vary across different substrates and microbes.

In addition to fermentation, enzymatic hydrolysis using specific proteases can be employed to produce BAPs. This technique can be used independently or in combination with fermentation to increase peptide yield and diversity (Wen et al., 2023; Ter et al., 2024).

Once produced, BAPs exhibit a wide range of health benefits. They include antimicrobial peptides such as nisin, pediocin, and plantaricin, which combat microbial infections (Wen et al., 2023; Setiarto and Anshory, 2024). Antioxidant peptides like Leucine-Leucine-Proline (LLP) and Valine-Tyrosine-Proline (VYP) scavenge free radicals, reducing oxidative stress (Abdulhussain Kareem and Razavi, 2020; Setiarto and Anshory, 2024). Anti-inflammatory peptides, such as Valyl-Prolyl-Proline (VPP) and Isoleucine-Proline-Proline (IPP), help reduce inflammation (Ahansaz et al., 2023; Setiarto and Anshory, 2024). Furthermore, anti-inflammatory peptides such as Valyl-Prolyl-Proline (VPP) and Isoleucine-Proline-Proline (IPP) contribute to inflammation reduction (Ahansaz et al., 2023; Setiarto

TABLE 1 Bioactive peptides and other postbiotic components biofunctional properties at *in vitro* and *in vivo* studies.

Type of probiotics	Postbiotics type	Type of Study	Biofunctional role	References
L. brevis, L. helveticus, and L. paracasei	Angiotensin-converting enzyme (ACE) inhibitory peptides	*In vitro*	Lowering blood pressure	(Ahn et al., 2009; Gonzalez-Gonzalez et al., 2013)
L. amylovorus	Lipid metabolism modulators peptides, and Antioxidant peptides	*In vivo*	Anti-obesity, prevent and treat dyslipidemia	(Bhat and Bajaj, 2019)
Lactobacillus spp	Butyric, Propionic, and Acetic acids	*In vitro*	Reduce obesity and diabetes	(Ibrahim et al., 2021)
B.adolescentis, L.casei,	Butyrate, Acetate	*In vitro*	Protect obesity and Type 2 diabetes	(Arora and Tremaroli, 2021) (Saravanakumar et al., 2021)
B.adolescentis	Butyrate	*In vitro*	Anti-inflammatory improve the integrity of the gut barrier	(Kim et al., 2022) (Singh et al., 2023)
L.casei, L.fermentum, B.adolescentis	Butyrate, Acetate, Propionate	*In vitro*	Inhibits cardiovascular disease and protect colorectal cancer, Maintain gut microbiota.	(Facchin et al., 2024)
L. plantarum, B. longum	Exopolysaccharides	*In vitro*	Antioxidant effect, anti-inflammatory and anti-type 2 diabetes, Anti-cancer	(Kwon et al., 2020) (Liang et al., 2024) (Inturri et al., 2017)
L. plantarum	Bacteriocins	*In vitro*	Anti-bacterial, Anti-inflammatory, Anti-obesity & anti-diabetes	(Dias, 2023)
L. casei, L. fermentum B. adolescentis, B. Longum.	Superoxide Dismutase, Catalase, Glutathione Peroxidase	*In vitro* and *In vivo*	Antioxidant, Reducing intestinal inflammatory	(Ahmad et al., 2022; Song et al., 2023)

and Anshory, 2024), Notably, VPP and IPP also act as antihypertensive agents by inhibiting angiotensin-converting enzyme (ACE), which prevents the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor. This action lowers blood pressure and enhances vascular protection (Paramithiotis et al., 2022; Ter et al., 2024). Both VPP and IPP are significant for managing blood pressure and inflammation, making them valuable in dietary interventions for cardiovascular health.

Additionally, ACE inhibitory peptides improve the availability of bradykinin, a vasodilatory substance, further contributing to blood pressure regulation and cardiovascular health (Table 1). Bioactive peptides derived from *Lactobacillus amylovorus* have shown potential in modulating lipid metabolism and preventing obesity-related disorders. These peptides reduce lipogenesis by inhibiting lipogenic enzymes and suppressing the expression of lipogenic genes in hepatocytes and adipocytes (Udenigwe and Rouvinen-Watt, 2015).

2.1.2 Shorts chain fatty acids and their biotherapeutic potential

SCFAs are fatty acids with fewer than six carbon atoms, primarily produced during the fermentation of dietary fibers by gut microbiota, particularly probiotics as shown in Figure 1. The most common SCFAs include acetate, propionate, and butyrate, which play significant roles in gut health and overall metabolic functions. Probiotics, ferment non-digestible carbohydrates (dietary fibers) into simpler sugars, which are then converted into SCFAs. Probiotic strains significantly impact the quantity and type of SCFAs produced during fermentation. The study of (Fernando et al., 2018) and (Farooq et al., 2013) showed that *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* have been shown to effectively produce acetate and butyrate from fermentation of dietary fibers. The type of dietary fiber used as a substrate for fermentation has also affects SCFAs type and yields. The study of (Farooq et al., 2013) indicate that total dietary fiber (TDF) generally leads to higher SCFA production compared to soluble or insoluble fibers.

SCFAs, are crucial for gut health as they serve as a primary energy source for colonocytes, helping to maintain the integrity of the gut barrier and promoting the production of mucin, which protects the intestinal lining (LeBlanc et al., 2017; Chang et al., 2021). Additionally, SCFAs exhibit anti-inflammatory effects by modulating immune responses and reducing inflammation in the gut, with butyrate specifically down-regulating pro-inflammatory cytokines (Asarat et al., 2015). They also play a significant role in metabolic regulation, influencing energy metabolism, appetite, and fat storage, which can aid in weight management and overall metabolic health (LeBlanc et al., 2017). Furthermore, SCFAs contribute to microbiota modulation by inhibiting pathogenic bacteria and fostering the growth of beneficial species (Chang et al., 2021; Marnpae et al., 2024), thereby supporting a healthy gut microbiome. The type of probiotic, the substrate used, and fermentation conditions significantly influence SCFA production, which in turn offers various health benefits, particularly for gut health and metabolic regulation.

Bacterial lipids are integral to human health, significantly influencing gut microbiota and metabolic processes. Notably, sphingolipids and polyunsaturated fatty acids (PUFAs) serve essential physiological functions and offer various health benefits, underscoring their relevance in dietary and therapeutic settings (Sugawara, 2022; Wang X. et al., 2024). Bacterial sphingolipids, which are characterized

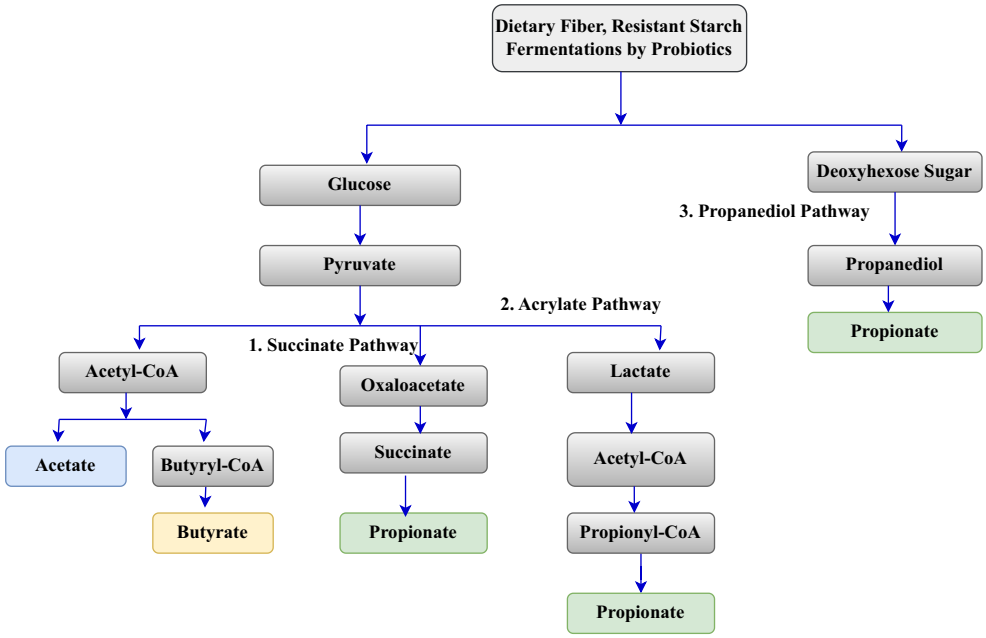

FIGURE 1
SCFAs production pathway.

by odd chain lengths, play a role in cell differentiation and immune responses, with the potential to migrate from the gut to other organs (Bai et al., 2023). Their interaction with dietary sphingolipids highlights a complex relationship that enhance host health. PUFAs, such as eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), are crucial for their anti-inflammatory and anti-tumor properties (Yamashita et al., 2021), and microorganisms can produce these beneficial fatty acids, providing a sustainable source for dietary supplements (Béligon et al., 2016).

In addition to these lipid classes, the influence of bacterial outer-membrane vesicles (OMVs) and extracellular vesicles (EVs) on host health is an emerging area of research with implications for various diseases. Both are nanosized vesicles released by bacteria, containing bioactive molecules that can interact with host cells (Meng et al., 2024; Razim et al., 2024). OMVs is a nanoscale phospholipid bilayer particles released by bacteria, encapsulate a variety of biomolecules, including lipids, proteins, and nucleic acids (Zwarycz et al., 2020; Ruiz-Moreno et al., 2024). These vesicles play a pivotal role in intercellular communication, immune modulation, and the delivery of bioactive lipids to host cells (Ghadami and Dellinger, 2023). For instance, OMVs can transport bacterial sphingolipids and other lipid mediators, potentially influencing host immune responses and gut homeostasis. They have also shown potential in cancer treatment by delivering therapeutic agents directly to tumor sites, minimizing systemic toxicity, carry specific antigens and immunomodulatory compounds, and enhancing immune responses against cancer cells (Meng et al., 2024).

Similarly, EVs, which are secreted by both Gram-positive and Gram-negative bacteria, are enriched in lipids that contribute to their structural integrity and functional roles in signaling (Melo-Marques et al., 2024; Razim et al., 2024) and nutrient exchange (Leiva-Sabadini et al., 2024; Melo-Marques et al., 2024). The lipid composition of these vesicles, including their unique lipid bilayer organization, is essential for their stability and ability to interact with host cells (Xavier et al., 2020).

The interplay between bacterial lipids and vesicles underscores their multifaceted role in human health. By facilitating the transport of bioactive lipids and mediating host-microbe interactions. OMVs and EVs expand the scope of bacterial lipid functions beyond their structural and metabolic roles. This emerging area of research highlights the potential of bacterial vesicles as therapeutic tools and diagnostic biomarkers in the context of gut health and metabolic diseases (Jalalifar et al., 2023).

2.1.3 Exopolysaccharides and their biotherapeutic potential

EPS are complex, high-molecular-weight carbohydrates produced by various microorganisms. They can be produced through the fermentation of dietary fibers by probiotic bacteria, particularly LAB species. During this process, probiotics break down complex carbohydrates into simpler sugars, which are then converted into EPS. The type of probiotic strain, substrate composition, and fermentation conditions can influence the quantity and composition of the produced EPSs (Maftei et al., 2024; Manoharan et al., 2024).

EPS have promising results in modulating the immune-inflammatory response in IBD patients. Studies have shown that EPSs produced by Streptococcus mutans and *Lactobacillus acidophilus* can affect the metabolic activity and viability of human gingival fibroblasts, which are crucial for the progression of chronic periodontitis (Szkaradkiewicz-Karpińska and Szkaradkiewicz, 2021). The research conducted by Kwon et al. (2020) demonstrated that EPS derived from *Lactobacillus plantarum* may serve as a natural therapeutic agent for inflammatory diseases. This is achieved by

inhibiting pro-inflammatory mediators such as IL-6, TNF-α, and COX-2, suppressing TLR4 expression and its activation by LPS, and regulating the MAPK and NRF2/HO-1 pathways, which ultimately reduces oxidative stress.

EPS regulate pro-inflammatory cytokines while promoting the production of anti-inflammatory mediators (Zampieri et al., 2020; Manoharan et al., 2024), thereby influencing the activity and differentiation of immune cells, including T cells and regulatory T cells, to maintain immune homeostasis (Manoharan et al., 2024).

Additionally, EPS inhibit the growth of pathogenic microorganisms, which helps prevent infections and supports a healthy gut microbiome (Maftei et al., 2024; Manoharan et al., 2024). Furthermore, they serve as a vital food source for beneficial gut bacteria, promoting their growth and proliferation (Maftei et al., 2024). Their anti-inflammatory, immunomodulatory, and antimicrobial properties make them attractive alternative for biotherapeutic of chronic disease.

EPSs are produced by microorganisms as a protective layer, aiding in biofilm formation and providing resistance to environmental stresses. They are composed of various monosaccharides and can exhibit diverse structures and properties depending on the sugar unit and the producing microorganisms' strain as well as environmental conditions (Nemati and Mozafarpour, 2024). EPS) are classified into homo-EPS, which are large (greater than 1000 kDa) and composed of a single type of sugar residue, and hetero-EPS, which are smaller (ranging from 100 to 1000 kDa) and consist of various types of sugar residues (Lu et al., 2023). Probiotics can utilize various substrates, including lactose, sucrose, and inulin, to synthesize EPS and addition of some substrate like inulin as fermentation substrate has been shown to enhance EPS biosynthesis (Guan et al., 2023).

3 Mechanisms of action of postbiotics

One of the key mechanisms by which postbiotics exert their effects is through the enhancement of gut barrier function. They achieve this by modulating tight junctions and promoting mucin production. Postbiotics regulate the expression of critical tight junction proteins, such as occludin and claudin, via the activation of signaling pathways like the PI3K/Akt pathway (Table 2). This process strengthens the intercellular connections within intestinal epithelial cells, thereby fortifying the integrity of the gut barrier (Tavalali et al., 2001). Additionally, they stimulate the production of mucins, protective glycoproteins secreted by goblet cells, by enhancing the expression of the MUC2 gene via the NF-κB signaling pathway. This increased mucin production contributes to a robust gut barrier, offering protection against pathogens and inflammation (Om et al., 2024).

They modulate the immune system, which is closely linked to gut barrier function. They activate Toll-like receptors (TLRs), their activation leads to the production of both pro-inflammatory and anti-inflammatory cytokines, thereby maintaining immune balance and gut homeostasis (Mosca et al., 2019; Chen et al., 2024). Furthermore, postbiotics interact with G-protein-coupled receptors (GPCRs), such as FFA2 and FFA3, which mediate anti-inflammatory effects, contributing to a healthier gut environment (Liu et al., 2024; Wang X. et al., 2024).

Postbiotics demonstrate considerable biotherapeutic potential, particularly in inhibiting inflammatory pathways. A key mechanism involves the modulation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, a critical regulator of inflammation (Table 2). Postbiotics can suppress this pathway by preventing NF-κB translocation to the nucleus, thereby reducing the expression of pro-inflammatory cytokines. This anti-inflammatory action is especially relevant in managing chronic inflammatory conditions such as inflammatory bowel disease (IBD) and metabolic disorders (Liang and Xing, 2023).

As shown in Table 2, postbiotics play a significant role in modulating the gut microbiome, restoring and enhancing the diversity of gut microbes within the gastrointestinal tract (Nagpal et al., 2018; Bianchi et al., 2019). Postbiotic bioactive compounds such as SCFAs, vitamins, BAPs, and other bioactive compounds, serve as substrates or signaling molecules that selectively support the growth of beneficial microbial species. This microbial community can be disrupted by various factors, including poor dietary habits, antibiotic use, and certain diseases (Huang et al., 2021; Popov et al., 2024). Furthermore, postbiotics promote the

TABLE 2 Common postbiotics biotherapeutic mechanisms of actions.

Mechanism of Action	Effects	References
Activation of PI3K/Akt pathway	Increased expression of tight junction proteins (occludin, claudin)	(Tavalali et al., 2001)
Activation of NF-κB pathway	Increased mucin production (MUC2 gene expression)	(Om et al., 2024)
Activation of Toll-like receptors (TLRs)	Balanced production of pro- and anti-inflammatory cytokines	(Mosca et al., 2019; Chen et al., 2024)
Interaction with G-protein-coupled receptors (GPCRs) (FFA2, FFA3)	Anti-inflammatory effects	(Liu et al., 2024; Wang X. et al., 2024)
Inhibition of NF-κB pathway	Reduced inflammation, particularly relevant in IBD and metabolic disorders	(Liang and Xing, 2023)
Modulation of gut microbiome	Increased SCFA production, restored and enhance microbial diversity, promoted beneficial bacteria	(Zhong et al., 2022; Liu et al., 2024)
Support of *Akkermansia muciniphila*	Enhanced gut barrier function, reduced inflammation, improved metabolic health	(Wang et al., 2021; Cheng et al., 2022; Wade et al., 2023; Zheng et al., 2023)

growth of beneficial bacteria, such as *Bifidobacterium* spp. and *Lactobacillus* spp., which are recognized for their positive impact on gut health (Nagpal et al., 2018; Bianchi et al., 2019; Mao et al., 2019). These bacteria contribute to the enhancement of the gut barrier and the production of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (Nagpal et al., 2018; Bianchi et al., 2019; Mao et al., 2019).They can also modulate immune responses by promoting the differentiation of regulatory T cells (Tregs) and influencing cytokine production. A study by Xu, Wu (Xu et al., 2023) demonstrated that postbiotics derived from *Saccharomyces boulardii* significantly modulated inflammatory responses in a mouse model of ulcerative colitis. The administration of these postbiotics resulted in increased levels of anti-inflammatory cytokines (such as IL-10) and decreased levels of pro-inflammatory cytokines (including IL-1β, IL-6, and TNF-α), highlighting their role in restoring immune balance and reducing inflammation.

As shown in Figure 2, postbiotic exert their influence on chronic diseases through two primary mechanisms. Firstly, they directly contribute to biotherapeutic functions, such as anti-inflammatory and antioxidant effects. Secondly, they indirectly modulate the gut microbiota, promoting a favorable composition and diversity, which in turn, ameliorates chronic disease management. Additionally, postbiotics strengthen the integrity of the intestinal epithelial barrier, a critical factor in preventing pathogen translocation and maintaining gut health. An *in vitro* study by Liu, Jiang (Liu et al., 2024) demonstrated that postbiotic administration in alcohol-induced chronic liver disease significantly increased the expression of tight junction proteins, reducing intestinal permeability and preventing the systemic circulation of harmful substances. Additionally, research by Hijová (2024) indicated that postbiotics derived from *Lactobacillus plantarum* exhibit significant antioxidant activity, which is particularly beneficial in chronic diseases characterized by oxidative damage, such as cardiovascular diseases and neurodegenerative disorders.

Postbiotics can provide SCFAs and other metabolites that serve as energy sources for intestinal epithelial cells and gut microbiota. This can support intestinal mucosal healing and overall gut health. The study by Hosseini, Abbasi (Hosseini et al., 2023) demonstrated that *in vitro* produced SCFAs, such as butyrate, can reduce inflammation, improve gut health, and inhibit the activation of inflammatory pathways, thereby contributing to their protective effects in chronic diseases. Furthermore, research by Ying, Mao (Ying et al., 2023) on postbiotics in rheumatoid arthritis indicated that they could modulate inflammatory pathways and reduce the expression of inflammatory mediators. This suggests that postbiotics may serve as a viable adjunctive therapy for rheumatoid arthritis by influencing immune processes and bone metabolism.

3.1 Major challenges to utilize postbiotics to improve human health

Utilizing postbiotics for the enhancement of human health, particularly in the context of chronic diseases, presents some critical challenges. Despite growing interest, the body of research on postbiotics is still relatively small compared to probiotics and prebiotics. More extensive clinical trials are needed to establish the health benefits, human clinical trials, and optimal dosages of

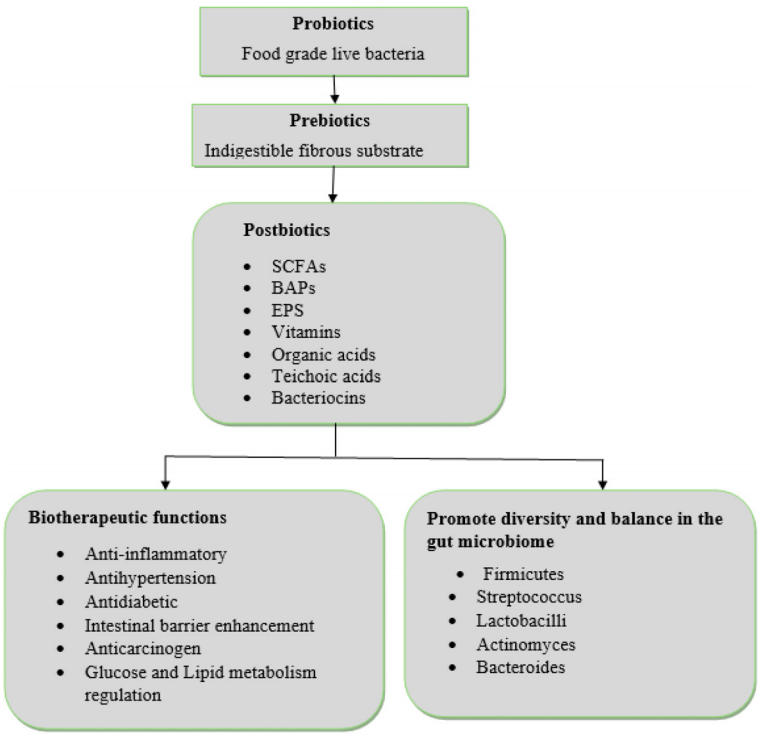

FIGURE 2
Postbiotics mechanisms of actions. SCFAs, Short-Chain Fatty Acids; BAPs, Bioactive Peptides; EPS, Exopolysaccharides.

postbiotics. This scarcity of human-focused research restricts the ability to generalize findings and comprehensively assess the full potential and safety of postbiotics applications in human populations (Vallianou et al., 2020; Eslami et al., 2024). Without extensive clinical trials, it is difficult to establish effective treatment protocols or understand the long-term impacts of postbiotic consumption.

The variability in study designs is another major hurdle. There exists significant inconsistency in the formulations, doses, and types of postbiotics utilized across different studies. This heterogeneity complicates the comparison of results and limits the ability to draw definitive conclusions regarding the efficacy and safety of postbiotics (Vallianou et al., 2020; Mehta et al., 2023). As a result, creating standardized protocol for its utilization face challenges.

While postbiotics are generally regarded as safe, there is a pressing need for more comprehensive safety evaluations, particularly concerning their long-term use and effects in specific population groups, such as allergic, immunocompromised individuals (Vallianou et al., 2020; Mehta et al., 2023). Ensuring that postbiotics do not pose any adverse effects in vulnerable populations is crucial for their broader acceptance and use in clinical settings.

A significant challenge lies in the limited information and understanding on the mechanisms by which postbiotics exert their beneficial effects. More research is needed to elucidate how various components of postbiotics interact with human physiology and contribute to health improvements (Li et al., 2021; Wu et al., 2023). A deeper understanding of these mechanisms is essential for optimizing postbiotic formulations and enhancing their therapeutic applications.

The lack of standardized guidelines for the production and quality control of postbiotics poses a barrier to their widespread adoption. Inconsistent product quality and efficacy can undermine trust among healthcare providers and patients (Scarpellini et al., 2021; Mehta et al., 2023). Establishing clear regulatory frameworks and quality assurance protocols is necessary to ensure that postbiotic products meet safety and efficacy standards.

Translating findings from preclinical studies into clinical practice presents a substantial challenge. There is a pressing need for high-quality, large-scale randomized controlled trials to validate the efficacy of postbiotics in the treatment of chronic diseases and to determine optimal dosing regimens (Wu et al., 2023; Eslami et al., 2024). Such rigorous investigations are crucial for establishing the clinical relevance of postbiotics and for enabling their incorporation into standard treatment protocols.

4 Future perspective

The investigation into the therapeutic potential of postbiotics is currently in its nascent phase, highlighting the need for extensive research to determine their efficacy, optimal dosages, bioavailability, storage stability, and long-term health effects. Future studies should prioritize elucidating the specific mechanisms through which different postbiotic compounds exert their beneficial effects, as well as exploring their potential applications in a variety of chronic conditions, such as obesity, diabetes, cancer and cardiovascular diseases. This deeper understanding could pave the way for more targeted and effective therapeutic strategies. Furthermore, incorporating postbiotics into functional foods and dietary guidelines represents a promising avenue for enhancing public health initiatives. By promoting the consumption of postbiotic-rich foods, health authorities could encourage preventive care and ultimately improve health outcomes, particularly among populations at heightened risk for noncommunicable diseases. Such integration could foster a proactive approach to health management, emphasizing the importance of diet in disease prevention and overall wellness.

5 Conclusions

Postbiotics are bioactive compounds generated from the metabolic processes of probiotics, offering health benefits without the need for live microorganisms. This characteristic makes them particularly appealing, as they retain the advantages of probiotics while alleviating concerns about the stability of live bacteria. The rise of chronic diseases, such as cardiovascular diseases, diabetes, and obesity, poses significant health challenges globally, especially in low- and middle-income countries, where they contribute to high morbidity and economic burdens. In this context, postbiotics present a promising biotherapeutic option for managing chronic diseases through mechanisms like immune modulation, gut barrier enhancement, and antioxidant activity. Key components of postbiotics include SCFAs, BAPs, and EPS, which play essential roles in metabolic health, inflammation regulation, and gut health. Collectively, these attributes highlight the potential of postbiotics in disease prevention and promoting overall metabolic wellness.

Author contributions

ZA: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. AB: Supervision, Writing – original draft, Writing – review & editing. EW: Supervision, Writing – original draft, Writing – review & editing. MH: Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chemistry, manufacturing, and controls for live microbial products: addressing classification challenges and enhancing process validation

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Traditional process validation life cycles need to be tailored to the specific needs of live microbial products (LMPs). LMPs can be divided into subcategories, and the product characteristics are the basis for the regulatory category and thereby the applicable guidelines. All LMPs fall under regulations related to GMP-compliant manufacturing; however, there are live microbial specific challenges. Both the FDA and the EMA do not have a regulatory framework for LMPs administered by injection. Full adherence to general guidelines for injectables is technically not feasible for LMPs, as sterility is required, which stands in conflict with living organisms as a product. Safety-related critical quality attributes (CQAs) of such LMPs typically include the absence of contaminants and proof of monoseptic condition of the product. This paper aims to holistically outline and compare LMP-relevant guidelines while highlighting different subcategories. Additionally, the status of the field is captured by collecting all LMP-related clinical trials to resolve specific challenges in LMP development. Taken together, this overview will aid in bringing future LMPs from development to commercialization.

KEYWORDS

live microbial product (LMP), injection, regulatory, classification, live biotherapeutic product (LBP), live bacterial therapeutic (LBT), monoseptic, commercialization

1 Introduction

To quote Cordaillat-Simmons and colleagues, "Regulatory and market success for a live biotherapeutic product (LBP) will depend on the quality of the development involving the credible demonstration of safety and efficacy in the intended population" (Cordaillat-Simmons et al., 2020). Beyond the aspect of clinical trials aimed at demonstrating safety and efficacy, it is crucial, as with any other finished product, to demonstrate quality—in terms of "ensuring and

providing documentary evidence that processes (within their specified design parameters) are capable of consistently producing a finished product of the required quality" (European Medicines Agency, 2016). To demonstrate that processes can consistently produce products of the required quality, it is essential to pair the appropriate analytical panel with effective process development and characterization for live microbial products (LMPs). This approach ensures a robust and consistent manufacturing process, which is ultimately validated through a successful process performance qualification, as is expected for any biological medicinal product.

This paper will focus on how the traditional process validation life cycle for biological products needs to be tailored to the specific needs of LMPs used as drugs, including, but not limited to, LBPs, for successful market access in Europe and the United States. First, stakes will be taken regarding the currently approved live microbial products, and the ambiguities in the regulatory landscape surrounding drugs consisting of living microbials (Microbiome Therapeutics Innovation Group and Barberio, 2024) will be highlighted. Special emphasis will be given to LMPs administered by injection, which do not fall under the umbrella of the LBP framework but seem to get traction when looking at the clinical trials that are currently ongoing. Of all the trials with a known route of administration, 22.5% are identified as injectables (Figure 1B). Then, the basics of process validation (for biologics) will be summarized, before focusing on the specific needs for those LMPs administered by injection. The paper will address the question of whether the regulatory category of a specific LMP has an impact on the development and validation of the associated manufacturing processes of those life-saving "bugs as drugs" that are currently in the clinical pipeline.

1.1 Important milestones in terms of market access for live microbial products

An important year for the live microbial world was 2022, as Rebyota and Vowst (formerly SER-109) were approved by the U.S. Food and Drug Administration (FDA) as the first two LBPs for commercial use. Rebyota is a live, fecal microbiota administered via rectal enema, and Vowst is an orally taken capsule of live microbiota spores. These first two products reached the market approximately 10 years after the FDA provided the first guideline for LBPs.

While classified as LBPs in several publications (Monday et al., 2024), both Rebyota and Vowst are often also called fecal microbial transplants (FMTs). There is an expectation that more such LBPs or FMTs will receive market authorization soon, given that in 2020, there were 134 active clinical trials involving such donor-derived LBPs or FMTs, of which 18 FMTs were in clinical phase III (Servetas et al., 2022).

Vowst differs from other FMTs because it is an oral, capsular formulation of approximately 50 species of Firmicutes spores (Jain et al., 2023) compared to the traditional naso-jejunal tube, colonoscopy, or retention enema approaches.

Compared to Vowst and Rebyota, which are both donor-derived products, the so-called "designed consortia" rely on a specific combination of (commensal) strains (McChalicher and Auniņš, 2022). A good example is Vedanta Biosciences' LBP product, consisting of eight strains of non-toxic, non-pathogenic *Clostridia* manufactured from clonal cell banks (Louie et al., 2023).

1.2 The origin of live microbial products

Before the FDA was even established, scientists Busch and Coley experimented with the use of *Serratia* and *Streptococcus* strains in the fight against terminal cancer in the late 1800s, sometimes with fatal outcomes (Brevi and Zarrinpar, 2023). Beyond this example, the use of FMTs dates back over 2,000 years according to certain sources (Monday et al., 2024).

Since 1989, 27 countries have been administering the FDA-approved Ty21a (typhoid vaccine live oral) Vivotif® (Ty21a), and in June 2016, the FDA approved CVD 103-HgR (VAXCHORA™),

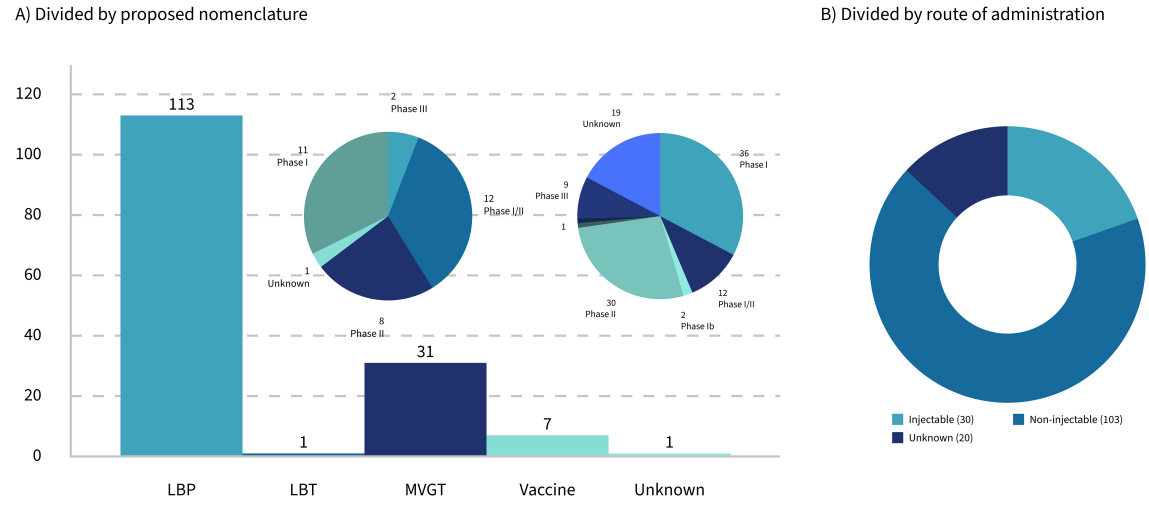

FIGURE 1
Breakdown of 153 LMP clinical trials by nomenclature **(A)** and delivery methods **(B)**.

a single-dose, oral cholera vaccine containing an attenuated form of *Vibrio cholerae* developed by SSVI Bern and eventually marketed by Pax-Vax (Levine et al., 2017). With this background, one can argue that neither Rebyota nor Vowst has been the first two LBPs to be approved by the FDA, and given the definition of an LBP by the FDA that specifically excludes vaccines, one can even argue that none of the abovementioned drug products qualify as LBPs.

1.3 Exploring the niches of bugs as drugs

Given the ambiguity around the terminology for LMPs, it is crucial to start with the definition of this niche category of medicinal products that is composed of a multitude of niche products. There are vaccines based on live organisms: traditional, orally administered LMPs; other engineered LMPs; microbiome- or microbiota-based products: delivery GMOs; and sometimes even pharmabiotics—basically a whole cohort of products that can be defined as "bugs as drugs" or LMPs as they will be named throughout this article. It seems that in this niche market of LMPs, every single entity has invented a new name for their type of product and maybe rightfully so, as every LMP seems to have a different approach, a different mode of action, a different approach with regard to CMC activities, and hence a distinct approach to regulatory approval and commercialization.

With the approval of the first "LBPs," the path to market access seems set, except that it is not. The approval of the first LBP highlights just more than ever that there is no clear regulatory framework for LMPs in general, just as there is no clear classification or commonly accepted nomenclature. There are a few guidelines that are specific to LMP categories, and there is a huge mesh of gray areas in between the different regulations, which might or might not apply to LMPs.

2 Live microbial products: regulatory landscape, clinical trials, and CMC-related aspects

2.1 Categorizing live microbial products

Any product that contains living microbials can be categorized by the broad term "live microbial product" (LMP—Table 1), which includes products intended as food, food supplement, or drug. Despite the regulatory guidelines related to LMPs, which focus on more specific product types, in this paper, we will focus on products used as a human drug. These are also sometimes referred to as pharmabiotics (Franciosa et al., 2023) or microbiome-based medicinal products (MMPs) (Rodriguez et al., 2025). MMPs or pharmabiotics differ from other live microorganism-based products, such as probiotics. The latter are used for several other purposes, such as (medical) food or derivatives (supplements) or cosmetics; therefore, topics related to probiotics are purposely excluded from the scope of this paper.

There is a key distinction between probiotics and pharmabiotics: probiotics are associated with a "health claim," while pharmabiotics are linked to a "medicinal claim" (Pot and Vandenplas, 2021; Grilc et al., 2023). In essence, probiotics focus on promoting general health benefits, whereas pharmabiotics aim to address or prevent specific medical conditions (Cordaillat-Simmons et al., 2020). In the United States, both types of microbial products—those with health claims and those with medical claim—are evaluated and regulated by the FDA, albeit under different guidelines. In Europe, however, the regulatory framework differs, as probiotics with health claims fall under the jurisdiction of the European Food Safety Authority (EFSA), while pharmabiotics with medicinal claims are overseen by the European Medicines Agency (EMA) (Pot and Vandenplas, 2021; Cordaillat-Simmons et al., 2020).

Another relevant category, which is on the edge of the definitions, is fecal microbiota transplants (FMTs). FMT is a group of products related to the screening, collection, storage, and administration of fecal material. This can be derived from both a direct donor and through centralized manufacturing from cell banks. FMTs are defined differently and follow different guidelines per country, which can make it confusing in some cases whether a product is qualified as FMT or LBP. One such example is the approved products from Ferring (Rebyota) and Seres (Vowst). These products are derived from qualified stool donors, but they are qualified as LBP. The reason is that they follow a standardized manufacturing process under good manufacturing practices (GMP) methods and are thoroughly screened for the absence of at least 29 pathogens (according to FDA requirements). Drugs derived from stool donors, which are not characterized, follow an unstandardized manufacturing process and/or study design and therefore are classified as FMT following guidance according to safety for organs and substances of human origin (SoHO) (Gonzales-Luna and Tillotson, 2023). Hence, the method of manufacturing and the associated level of control determine the classification of the final drug, rather than its application or characteristics. Due to different manufacturing processes and specificities of regulation around FMTs compared to other LMPs, FMTs that are not considered as LBPs will be out of scope for this paper. A detailed overview of the spectrum of microbiome-based therapies and MTs (microbiota transplants), as well as FMTs in particular, has recently been published by Rodriguez et al. (2025).

Conventional probiotics consist of lactic acid bacteria or bifidobacteria or a combination of those two, whereas next-generation probiotics (NGPs) consist of strains that are identified based on comparative microbiota analysis, showing health benefits. The following strains are commonly used as NGPs: *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Eubacterium hallii*, and *Roseburia* spp (Martin and Langella, 2019; Huanchang et al., 2022). With probiotics, NGPs, and FMTs, or MTs as more recently referred to (Rodriguez et al., 2025) set aside, LMPs can be separated into four main categories (Table 2): LBPs, MVGTs, vaccines, and LBTs.

A live biotherapeutic product (LBP) is an official category which is widely used and defined in the FDA guideline: "*Early Clinical Trials with Live Biotherapeutic Products: Chemistry, Manufacturing, and Control Information*" (Food and Drug Administration, 2016),

TABLE 1 Description of terms and abbreviations.

Term	Abbreviation	Description	Manufacturing under GMP required
Fecal microbial transplant	FMT	Group of products related to the screening, collection, storage, and administration of fecal material	No, but SoHO regulation applies[1]
Live bacterial therapeutic	LBT	Products consisting of living organisms, which are not orally administered (like LBPs) and are not engineered (like MVGTs)	Yes
Live biotherapeutic product	LBP	Product that contains live organisms and is applicable to the prevention, treatment, or cure of a disease or condition of human beings, but is not a vaccine. In addition, an LBP should not be administered by injection and is not an oncolytic bacteria	Yes
Live microbial product	LMP	Term overarching all prescriptive and non-prescriptive drugs consisting of living microorganisms	No
Microbial vector for gene therapy	MVGT	Products consisting of genetically modified microorganisms	Yes
Microbiome-based therapy	MBT	Therapy to reshape the composition of resident microbial communities and thereby restore health (https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2022.1046472/full) (Md Zahidul et al., 2023)	Yes[2]
Microbiome-based medicinal products	MMP	Medicinal products containing live microbial organisms (bacterial or yeasts) for human use (Rodriguez et al., 2025)	Yes[2]
Microbiota transplants	MT	See FMT	No
Next-generation probiotics	NGP	Products containing strains isolated from the human gut, showing potential health benefits based on comparative analyses (Martin and Langella, 2019)	No
Pharmabiotics	N/A	Products consisting of living microorganisms with a medical claim aiming to address or prevent specific medical conditions	No
Postbiotics	N/A	Probiotic-derived biologically active metabolites	No
Prebiotics	N/A	Foods that act to simulate the existing human microflora	No
Probiotics	N/A	General term for non-prescriptive products containing living microorganisms associated with a health claim and includes a.o., synbiotics, and postbiotics (1)	No
Synbiotics	N/A	Food ingredients and dietary supplements combining probiotics and prebiotics	No

[1]SoHO regulation: "Regulation on standards of quality and safety for substances of human origin intended for human application" (2024).
[2]Exception can apply. See section 2.1.

first published in 2012, as a product that contains live organisms and is applicable to the prevention, treatment, or cure of a disease or condition of human beings, but is not a vaccine. In addition, an LBP should, as a general matter, not be administered by injection and is not an oncolytic bacterium. Since 2019, the European Directorate for the Quality of Medicines & HealthCare (EDQM), cooperating with the EMA, has provided LBP-specific quality guidance in a dedicated monograph: "*3053E General Monograph on Live Biotherapeutic Products*" (European Pharmacopoaei, 2019). Ph. Eur. monograph 3053 specifically focuses on the production method including the removal of impurities or adventitious agents and on the used microorganism and its characterization (Franciosa et al., 2023).

Drugs that are used as a microbial vector for gene therapy (MVGT) are described as a separate category, with their own guideline for the US market (Food and Drug Administration, 2016). Whereas natural LMPs are ideal for general health applications and conditions where naturally occurring strains are sufficient, the engineered category differs from other categories, as the microbial product is genetically modified, typically to deliver some kind of payload for some form of cancer therapy. Additionally, other complex diseases, such as autoimmune disorders or metabolic diseases, can be targeted with genetically modified organisms (GMOs) to offer greater flexibility and precision. However, GMOs may raise safety concerns, such as the potential for horizontal gene transfer and unintended off-target effects or immune responses. Hence, a more extensive safety testing of the organism compared to natural strains is required, as well as additional CMC activities such as a strict containment and the need to confirm retention of the genetic modifications, ensuring stability. Thus, the development of genetically engineered LMPs is generally more complex and expensive than naturally occurring LMPs, and the general concerns about GMOs might shape public perception and foster greater resistance to adoption. Nevertheless, GMO LMPs offer unparalleled potential for treating complex diseases and conditions that require targeted or engineered solutions.

Following the LBP definition, vaccines consisting of live (attenuated) strains form a separate group entirely, where different guidelines apply for injectable or other administration forms of these vaccines.

TABLE 2 Overview of different live microbial product types and relevant guidelines in the EU and US applicable for these types of products (V = covered by the guideline, X = not covered by the guideline).

	LMP				
	LBP	MVGT	LBT	Vaccines	Others (e.g., probiotics)
Properties					
Live/inactivated microbials	Live	Both	Both	Both	Live
Engineered	Both	Yes	No	Both	Both
Route of administration	Non-injectable	Injectable + non-injectable	Injectable	Injectable + non-injectable	Non-injectable
Guidelines					
FDA LBP guideline[1]	V	V (if under LBP definition)	X	X	V (if falling under LBP definition)
FDA MVGT guideline[2]	V (if also falling under MVGT)	V	X	X	V (if falling under MVGT)
FDA guidance environmental assessment for microbial products[3]	V	V	V	V	V
EudraLex Volume 4, Annex I[4]	X	V (if injectable)	V	V (if injectable)	X
Directive 2001/83/EC in the EU[5]	V	V	V	V	X
EMA ATMP[6]	V (if genetically modified)	V	X	V (if genetically modified strain)	X
EMA Monograph on Live Biotherapeutic Products[7]	V	V	V	V	X
ICH Q11	V	V	V	V	V
FDA Guidance for Industry – Process Validation	V	V	V	V	V

1. FDA (CBER) Early Clinical Trials With Live Biotherapeutic Products: Chemistry, Manufacturing, and Control Information (2016) (https://www.fda.gov/files/vaccines,%20blood%20%26%20biologics/published/Early-Clinical-Trials-With-Live-Biotherapeutic-Products–Chemistry–Manufacturing–and-Control-Information–Guidance-for-Industry.pdf).
2. Recommendations for Microbial Vectors used for Gene Therapy (September 2016 (https://www.fda.gov/files/vaccines,%20blood%20&%20biologics/published/Recommendations-for-Microbial-Vectors-Used-for-Gene-Therapy–Guidance-for-Industry.pdf).
3. FDA's guidance (CBER) "Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products.
4. EudraLex Volume 4, Annex I (https://health.ec.europa.eu/document/download/e05af55b-38e9-42bf-8495-194bbf0b9262_en?filename=20220825_gmp-an1_en_0.pdf).
5. https://eur-lex.europa.eu/legal-content/ENG/TXT/PDF/?uri=CELEX:32001L0083.
6. https://www.ema.europa.eu/en/human-regulatory-overview/advanced-therapy-medicinal-products-overview/guidelines-relevant-advanced-therapy-medicinal-products
7. European Pharmacopoeia 3053E General monograph on Live Biotherapeutic Products for human use (EDQM, 2019).
8. ICH Q11 Development and manufacture of drug substances (chemical entities and biotechnological/biological entities) - Scientific guideline, 2012.
9. FDA Guidance for Industry – Process Validation: General Principles and Practices, R1, published January 2011.

There is a remaining group of drug products that are officially not categorized. This group consists of living organisms that are not orally administered (unlike LBPs) and are not engineered (otherwise they fall under the MVGT definition) and, in general, are not a vaccine. This group of products is here classified as live bacterial therapeutics (LBT).

According to the FDA definition, an LBP should contain living microbial organisms. For all other categories, the product can also contain inactivated microbials. In such cases, the word "live" does not apply. Manufacturing process steps are similar for products containing live or inactivated microbials, with an inactivation step as an additional activity for the latter product group. For the analytics, there are some minor differences between a product containing live or inactivated microbials, for instance, the need for an "inactivation assay" confirming the inactivation of the microbe prior to the final formulation.

Although single- and multistrain products differ from an analytical and manufacturing perspective, regulatory frameworks do not distinguish between them. As a result, this characteristic is not factored into the classification of microbial products.

To summarize the general approach to categorization of all different microbial products, distinctions are made based on five main criteria: 1) the level of control over the manufacturing process (distinguishing FMTs from other LMPs), 2) the physiological state of the microorganism (live or inactivated), 3) the genotype of the microorganism (engineered or wild-type strain), 4) the route of administration (oral, topical, or by injection), and 5) the overarching application (therapeutic or preventive). As for any other (investigational) medicinal product, the latter two categories will determine which regulatory guidelines apply for the development, manufacturing, and finally market authorization as we will see below.

2.2 Current regulatory landscape

Now that the properties of the different products as well as the nomenclature have been clarified (Section 2.1), it is important to look at the regulations and regulatory guidelines that are distinctive for each category (Table 2).

As illustrated in Table 2, there is no overarching guidance document encompassing all categories of LMPs, except for the general principles and practices that apply to the manufacturing of any medicinal product (Food and Drug Administration, 2016; European Medicines Agency, 2016). A genuine FMT product must adhere to the "Enforcement Policy Regarding Investigational New Drug Requirements for Use of Fecal Microbiota" for the US market and the so-called SoHO guideline (European Union, 2024) for the European market. In contrast, donor-derived LBPs (such as Rebyota and Vowst) are primarily governed by the "Early Clinical Trials With Live Biotherapeutic Products" guideline in the US and the "3053E General Monograph on Live Biotherapeutic Products" in the European Union.

Vaccines, whether they are orally or otherwise administered, are covered by the Code of Federal Regulation (Title 21) and approved by the FDA. In Europe, both are regulated via EudraLex Volume 4. In the first case, they will need to comply with Annex II and in the latter with Annex I (European Commission, 2022).

Engineered LMPs fall under the guidelines for MVGT in the United States. Furthermore, in specific cases, the LBP guideline might apply as well. In Europe, the situation is slightly different. Depending on the route of administration, the Directive 2001, EMA Monograph, and/or EudraLex Volume 4, Annex I might apply.

In any case, as stated above, for both vaccines and engineered LMPs, the Code of Federal Regulation is applicable for products for the US market. In Europe, EudraLex Volume 4, Annex II applies as these products are "by nature considered biological medicinal products as the active substances are live microorganisms, which are biological substances" (Cordaillat-Simmons et al., 2020). The FDA has provided guidance for LBPs via the guideline for "Early Clinical Trials With Live Biotherapeutic Products." In a similar way, the EMA has provided a monograph. However, none of the regulatory frameworks specifically address LMPs administered by injection. This is precisely where the topic gets challenging. In the European Union, a drug product intended for administration by injection would commonly fall under the scope of EudraLex Volume 4–Annex 1—"production of sterile medicinal products." However, inherent to the nature of LMPs, those products are not sterile. They might be considered "monoseptic" in the case of single-strain products or even contain a multitude of different live bacterial strains in the case of multistrain or consortium-based products. On the other hand, the fact that LMPs are administered into the bloodstream, injected directly into solid tumors, or applied to open wounds in patients who might inherently be considered "vulnerable" requires that these LMPs are free of any microbial or viral contaminations. Bioburden-controlled production processes, typically governed by EudraLex Volume 4–Annex 2, are not deemed sufficient to ensure patient safety. On the other side, a strict adherence to EudraLex Volume 4–Annex 1 might not be technically feasible, due to the nature of the typical production processes of live biological products. The remaining question is whether regulatory bodies, sponsors, and service-providing companies are aligned on this topic, given the lack of currently approved LMPs administered via injection. Hence, this paper intends to provide a basis for approval of such injectable LMPs in the future by considering the current landscape of clinical trials and regulatory guidelines.

3 Overview of current clinical trials involving live microbial products

In 2020, according to Servetas et al. (2022), there were 134 active clinical trials involving LMPs. In 2025, we retrieved 153, excluding all clinical trials that we considered involving true FMTs (Supplementary Table 1). The increasing interest in using LMPs as therapeutic agents results in experiences that can be used not only to further refine this niche regulatory landscape but also to learn from experience.

A clinical trial database—ClinicalTrials.gov—was searched using the keywords "Anaerobic" (excluding "Exercise," "Muscle," and "Performance"), "Microbial AND LBP," and "Solid Tumor AND Microbial." The datasets retrieved were manually searched for relevant trials, excluding all trials that were of "observational intent," focused on FMTs, or involved probiotics. Finally, the dataset was completed by searching for specific products known from the literature, resulting in a total of 153 clinical trials (Supplementary Table 1). To simplify categorization and allow for clustering, detailed target descriptions were replaced by "cancer/solid tumors" or "infections/chronic disease" where possible. Bacterial strain nomenclature was also simplified. The curated result of the search (Supplementary Table 1) is a non-exhaustive list of the current clinical trial landscape.

Only 32% of the trials analyzed are currently active, while 16% have been terminated, suspended, or withdrawn (Figure 2A). These figures highlight significant challenges faced by the trials and their sponsoring companies, such as feasibility issues, funding shortages, recruitment difficulties, and unforeseen complications that hinder progress or completion. The low percentage of active trials suggests that many have either concluded or failed to progress. This can contribute to a biased perception of success, particularly given that the progress and outcomes of the trials are often underreported.

Seventy-eight percent of the trials identified in this study were between phase 1 and phase 2 (Figure 2B), reflecting the early stage of development in the field of LMPs. This predominance of early-phase trials highlights the experimental nature of these products and the challenges associated with their development. However, it is important to note that the lack of updates on trial progress in databases such as ClinicalTrials.gov may have influenced the dataset, potentially underestimating the number of trials that have advanced to later phases.

If we look at the number of trials in categories as per Section 2.1, the majority of products in clinical trials are LMPs; only four different products involve inactivated organisms (inactivated,

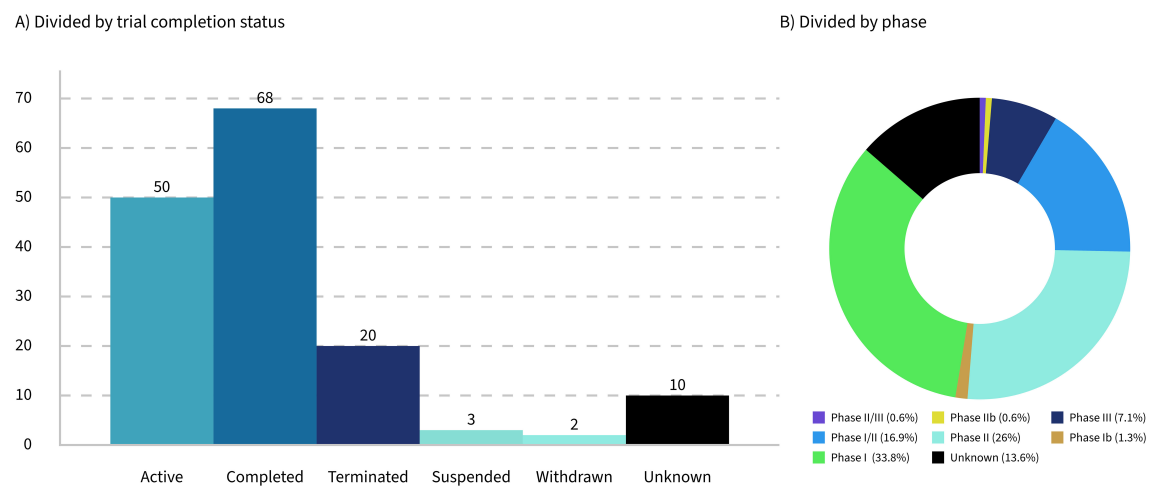

FIGURE 2
Breakdown of 153 LMP clinical trials by completion status **(A)** and phase **(B)**.

non-pathogenic, gram-negative bacteria in one case; *Prevotella histicola* in the other case; and *Mycobacterium obuense* in the last two cases). Of those four, only the *P. histicola* product is orally administered.

Thirty-six trials were identified as clearly involving engineered strains. Of these, only 11 are administered orally, while the remainder are either applied topically or administered via injection (Figure 1B).

Out of the 153 clinical trials in the present dataset, 103 trials are based on products that are either orally or topically (including vaginally or rectally) administered. Thirty trials[1] are based on products that are injected intravenously, intramuscularly, intratumorally, and intradermally or involved any other kind of injection or infusion. Among those that are injectable, only three trials, or 10%, were categorized as vaccines. Products that are intended for administration by injection seem to be prevalently aiming at cancer, particularly solid tumors, with only four known clinical trials involving treatments of other diseases.

Only a very small number of those trials are of a preventive nature (<6%), as the majority of the current clinical trials focus on therapeutic aspects. While most applications of LMPs still focus on different recurrent infections, inflammation, and metabolic disorders (Heavey et al, 2022), 39% of the clinical trials target a form of cancer. This is not surprising for two reasons. First, there are unmet needs as traditional cancer therapeutics do not sufficiently address heterogeneous tumors (Sieow et al., 2021). Combination therapies where LMPs are one component can be an approach. Second, LMPs are perfectly suited as anticancer agents, specifically for solid tumors, as LMPs can survive and act in a specific tumor microenvironment (Steidler et al., 2003; Sieow et al., 2021). LMPs, specifically engineered ones, have the advantage of being naturally tumor-targeting while inherently displaying proinflammatory properties (Charbonneau et al., 2020). This combination gives them a huge potential for future cancer treatments.

Cancer-targeting LMPs are often engineered strains that are administered by injection. The regulatory landscape for this kind of product is the most complex one. However, regardless of whether the microbial product is live or inactivated, non-modified, or engineered, the actual mode of action (Table 3) might not always be completely known or fully "deciphered" as phrased by Paquet et al. (2021), rendering not only the clinical study design more challenging but also the establishment of the right testing regime for the manufacturing process difficult. Taken together, this paper proposes a set of classifications for LMPs that clarifies the (regulatory) communication and discussion in the field going forward. If the proposed classification was applied to the dataset at hand, the landscape of the current clinical trials would reflect the previous sections: a large majority of LBP, a minority of vaccines and LBTs, and a growing part of engineered LMPs, represented by the category "MVGT," often targeting tumors (Figure 1A).

4 Life cycle for live microbial products: from development to market access

Regardless of the classification, all microbial products for human use fall under regulations related to GMP-compliant manufacturing. This includes the EMA's directive 2001/83/EC, ICH Q11, and the

TABLE 3 Possible mode of action of therapeutic live microbial products.

1. Microbiological (e.g., competitive exclusion)
2. Physiological (e.g., production of short-chain fatty acids)
3. Metabolic (e.g., cross-feeding with other member of the microbiota or production of antimicrobial metabolites)
4. Immunological: • Stimulation (e.g., stimulation of natural killer cells, secretory IgA, or regulatory T cells) • Payload delivery and expression (e.g., CD47, nanobodies, activation of the innate immune system)

FDA's guidance on process validation (European Commission, 2022; Official Journal of the European Communities, 2001). In this regard, the manufacturing of microbial products is equal to the manufacturing of biological products. Nevertheless, there are live microbial specific challenges that arise in the manufacturing process development when taking these guidelines into account.

While the guidelines and common perception often foresee a linear trajectory—where a process is first developed and characterized, then scaled up to provide material for all clinical phases in the same manner before undergoing process performance qualification—the reality looks slightly different. Indeed, often there are several years between the GMP production of the material for clinical phase I and the process performance qualification batches. During these years, process characterization at a small scale, adaptation of the production process at the final scale, and further development of analytical methods take place. Such data and knowledge, sometimes even arising from deviations encountered at the production scale, can be leveraged to reduce the uncertainty around the linkage between process variables and critical quality attributes. It is important to define early on—and continually refine over the course of the clinical studies—the critical quality attributes (CQAs) via a quality target product profile (QTPP). By linking these CQAs to process variables, their impact can be ranked in terms of severity. Additionally, the initial likelihood of these attributes falling outside of the control space should be assessed, along with the detection mechanisms in place to detect such failure events. This assessment can be further refined throughout the course of process characterization, eventually leading to a defined control strategy that can be put to the test in the process performance qualification stage (stage 2 of process validation according to the FDA guideline), a pivotal step prior to regulatory filing and approval of a new product. This approach has proven to be suitable for a variety of large-molecule products, such as recombinant proteins, conjugated vaccines, or mRNA-based products, and can be translated as such to LMPs, particularly products intended for administration via injection.

When looking at the expected QTPP of an LMP, typical safety-related CQAs include the absence of contaminants and proof of monoseptic condition of the final product. Based on the CBER Guidance for Industry: Early Clinical Trials with Live Biotherapeutic Products: Chemistry, Manufacturing and Control Information (February 2012) and Recommendations for Microbial Vectors used for Gene Therapy (September 2016) as well as the European Pharmacopoeia monograph 3053, the approach to testing LMPs (in terms of sterility) intends to demonstrate a monoseptic product by multiple purity tests (e.g., microbial limit test or absence of specified organisms as defined in the Pharmacopeia), rather than by a single sterility test. It is important to highlight that the development of methods is difficult, as the LMP itself, typically present in high concentration, may interfere in many ways, for instance, with the detection of other organisms.

1 For the remaining 20 products on trial, no clear information could be retrieved.

4.1 Manufacturing and analytical challenges for monoseptic products

The aim during development is to go from a typical discovery stage process to a large-scale manufacturing process. In contrast to biological products, the master cell bank is already the API. A typical LMP drug substance manufacturing process has a very simple downstream process focusing mainly on polishing and buffer exchange of the LMP intermediate substance compared to the biologics field where there is a need to isolate a specific biological entity from the cell debris (Figure 3). Hence, a more thorough characterization is expected from a regulatory point of view for LMPs' starting material, as these cell banks might directly influence the quality of the final product by means of aspects related to safety and efficacy (Paquet et al., 2021). It is usually expected that the origin of the strain(s) is documented, and the passage history leading to the creation of a research cell bank (RCB), master cell bank (MCB), or working cell bank (WCB) is described (Paquet et al., 2021). Clinical phase I trials can still be carried out with MCBs, but it is advised to start using and characterizing WCBs as early as possible to preserve the MCB stock. A change in cell bank after clinical phase I will result in a more extensive comparability exercise than for biologics manufacturing (Levine et al., 2017).

The manufacturing process itself also requires special attention. It needs to be developed into a process where every unit operation is performed in a closed system, seamlessly integrating with the next unit operation to prevent the introduction of viral or bacterial contamination. This is especially important for LMPs, as it is impossible for these samples to undergo holistic microbial evaluations. Therefore, the manufacturing control strategy often relies on different single-use disposables or confirmed clean multipurpose equipment together with closed systems (such as, for instance, a Thermo Fisher eSUF) that are connected to each other via different aseptic connections. Those can be either commercially available systems (such as AseptiQuik® or ReadyMates®), welds of different tubes, or via a controlled piping system. Additionally, the contamination control strategy often contains monitoring and product-specific testing. Inactivated LMPs might bear the inherent advantage of the possibility to perform sterility testing; however, they require the development of a specific inactivation assay to testify that the organism is indeed inactivated at the end of the relevant unit operation. Altogether, the contamination control strategy ensures a monoseptic end product by prevention and good design, as well as controls and monitors the manufacturing process supplemented by analytical evaluations (Figure 3).

Another important aspect to consider during the LMP manufacturing process development and scale-up is the formulation of the cell harvest. Formulation provides stability over the unit operations, such as filling, freezing, and lyophilization, as well as over the intended shelf life. Although non-frozen storage, either chilled or at room temperature, can be considered, freezing of the LMP is often required for longer-term shelf life. The impact of freezing and thawing needs to be explored during process characterization. Indeed, viability is likely a CQA of any LMP. As such, any aspect affecting viability can potentially be

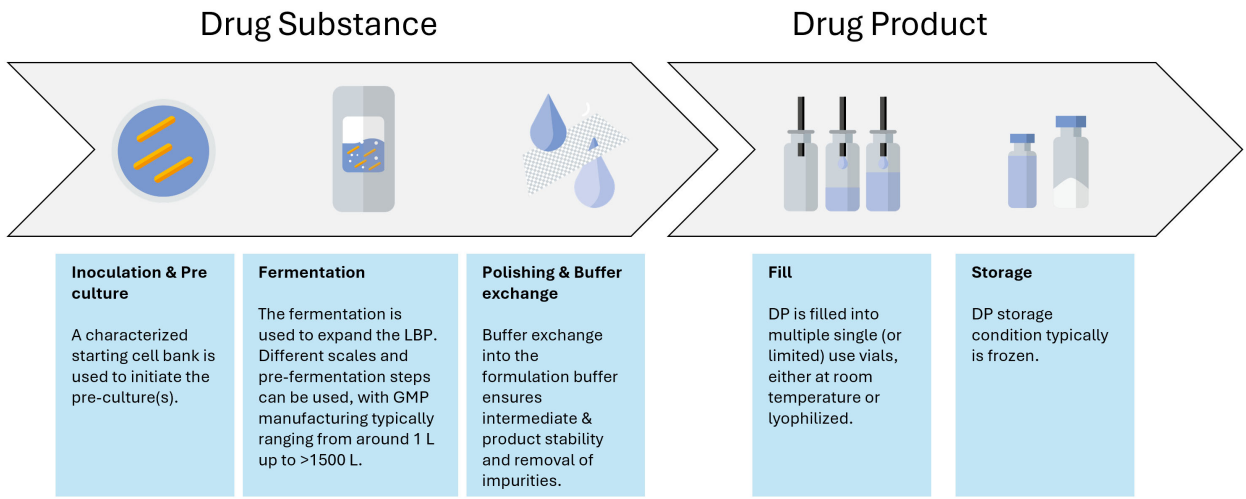

FIGURE 3
Manufacturing process flow as typically used for the manufacturing of LMPs.

considered a critical process parameter (CPP). In a commercial process, the control strategy associated with any CPP needs to be robust and reliable to yield an efficacious product. Lyophilization can be one of the options for controlled freezing associated with drying. Controlled freezing to −20°C or −70°C using controlled freezing systems such as the RoSS systems from SUS or the Celsius CTF systems from Sartorius can also be options, while other products that are currently in clinical trials are based on simple freezing of the drug product in conventional freezers as it is known from the biologics field. Regardless of the approach to freezing, the physiological state of the cells prior to the unit operation, in combination with the right formulation during the unit operation, will influence the viability of the cells upon reconstitution and hence directly impact the CQA "content" (Carvalho et al., 2004; Muñoz-Rojas et al., 2006).

The use of a suitable cryoprotectant is particularly important as it plays a vital role in minimizing the loss of viability when freezing the cells. Commonly used cryo- or lyoprotectants include saccharides, polyols, and amino acids or proteins (Grilc et al., 2023). As disaccharides are known as effective cryo- or lyoprotectant (Muñoz-Rojas et al., 2006), it is not unexpected that trehalose is one of the most often chosen cryoprotectants. Non-penetrating cryoprotectants such as trehalose reduce both intra- and extracellular ice formation (Bircher et al., 2017) by influencing osmotic effects, whereas polyol cryoprotectants such as glycerol reduce intracellular ice formation by acting on the properties of the cell membrane (Hubálek, 2003; Fowler and Toner, 2005; Bircher et al., 2017). LMPs contain live cells; hence, it is important to use non-fermentable sugars to prevent cell growth and acidification.

Whether orally or otherwise administered, choosing the right formulation is critical for all types of LMPs. The survival of the active ingredient (the living cells) and hence the clinical efficacy of orally administered LMPs depend not only on the cells' intrinsic, natural resistance to the potentially harsh manufacturing (e.g., harvest) and storage conditions but also on the matrix in which the cells are formulated (Pot and Vandenplas, 2021). These do not only have to pass the gastrointestinal tract but must also be able to survive and proliferate in those environments, competing with the microbiota already present (Heavey et al, 2022). In the case of oral LMPs, there is often a capsular formulation, adapted to the needs of maintaining the viability of cells throughout the passage through the gastrointestinal tract. Those capsular formulations are also often carbohydrate-based (Pot and Vandenplas, 2021).

For LMPs administered via injection, liquid formulations (or reconstituted liquid formulations) must be designed to prevent aggregate formation and maintain a homogeneous suspension while protecting against harsh conditions of lyophilization. This ensures shelf-life stability of the products.

As those cryo- and lyoprotectants, as well as any other formulation components, are considered excipients, it is important that they comply with the guidelines regulating excipients. In this context, it is important to control the endotoxin content of the excipients, as endotoxin testing can be complicated by the presence of the LMP API. In addition to freezing, cooling can also be considered, potentially in combination with reconstitution after freeze-drying of the API.

Assessing viability is crucial for in-process testing to gain process knowledge during development. One of the challenges associated with freezing is the loss of viability observed upon reconstitution or thawing. This loss of viability presents two challenges. First, while ideally minimized, loss of viability is often an uncontrolled and poorly characterized phenomenon, making it difficult to manage in a robust and reliable manner. Second, the assumption that the loss of viability is understood, characterized, and controlled to be reproducible adds another layer of complexity. Additionally, it is widely recognized in the industry that bulk lyophilization, a common unit operation in the manufacturing of LMPs, poses significant challenges in terms of sampling.

The challenge is further amplified by the absence of meaningful and reliable analytical methods for in-process control. Often, total

cell count, a fast and reliable method, is used as an indicator of in-process testing or in-process control. For LMPs, viability is, almost by definition, a critical quality attribute. Typically, viability is assessed via plate-based (spread or pour plate) or cell counter methods of cell enumeration. However, at the drug substance and drug product levels, total cell count is then often paired with any viable cell count method. Both the viable cell count and the total cell count methods, as well as dielectric spectroscopy or any other such method, are often used in the industry, while primary research and development use tools such as metabolic profiling (Kim et al., 2022) or genotypic analysis (Paquet et al., 2021). Although the industry might benefit from bringing such latter methods to a level where they can be used in routine quality control, the complexity of the methods and the difficulties with ensuring appropriate intermediate precision might currently still hinder their implementation.

Zaragoza and colleagues provided a clear overview of six key categories of analytical assays commonly used to evaluate the release profiles of microbial products. The first category involves growth curves, typically derived from cell count methods, which serve as a fundamental tool. The second category, flow cytometry, enables high-throughput analysis during the development phase. Similarly, the third category, colorimetric assays, also supports high-throughput workflows but may offer a more cost-effective alternative in terms of equipment investment. Spot assays, the fourth category, are appealing due to their simplicity, requiring neither specialized equipment nor highly trained personnel. However, their precision may fall short for later stages of development and manufacturing. The fifth and sixth categories, fluorescence microscopy and transcriptomics, provide valuable insights but are often limited by their high costs, time demands, and challenges in qualification (Zaragoza et al., 2024).

Once developed, the manufacturing process will be put to the test during the second stage of process validation. Assessing the consistency of the manufacturing process, particularly batch-to-batch consistency, is key to a successful process performance qualification (PPQ) campaign as required for market access. For example, variations in the quantity of live microorganisms between batches might be greater than variations expected in the production of biologics. The substantial variation in cell counts and viability evaluation methods makes it challenging to develop meaningful control strategies. Hence, it might be tempting to broaden the product specification (Paquet et al., 2021). However, most importantly, product specification in terms of cell counts (e.g., CQA "content") needs to be properly justified and fit the overall picture of manufacturing consistency and product requirements (also quote Cordaillat-Simmons et al., 2020).

Furthermore, depending on the physiological state, cells might not be able to divide and form colonies but may "nonetheless retain sufficient metabolic activity to perform some engineered functions *in situ*" (Charbonneau et al., 2020). Instead of pour or spread plates, enumeration using live/dead staining might be a more accurate alternative to the currently used cell count and viability testing. However, such methods are also more cumbersome to realize in routine GMP manufacturing. Real-time viability assessment, additionally compatible with single-use equipment, such as dielectric spectroscopy (Dabros et al., 2010), might be an interesting alternative that still has to find its way into routine GMP manufacturing. However, neither dielectric spectroscopy nor live/dead cell enumeration may be appropriate for monitoring the activity of cells in manufacturing processes, where active cell division serves as the actual indication or surrogate measurement of potency. It is important to note that in certain cases, viable cell count is used as a surrogate of potency, whereas a cell-based assay might be used to determine the efficacy.

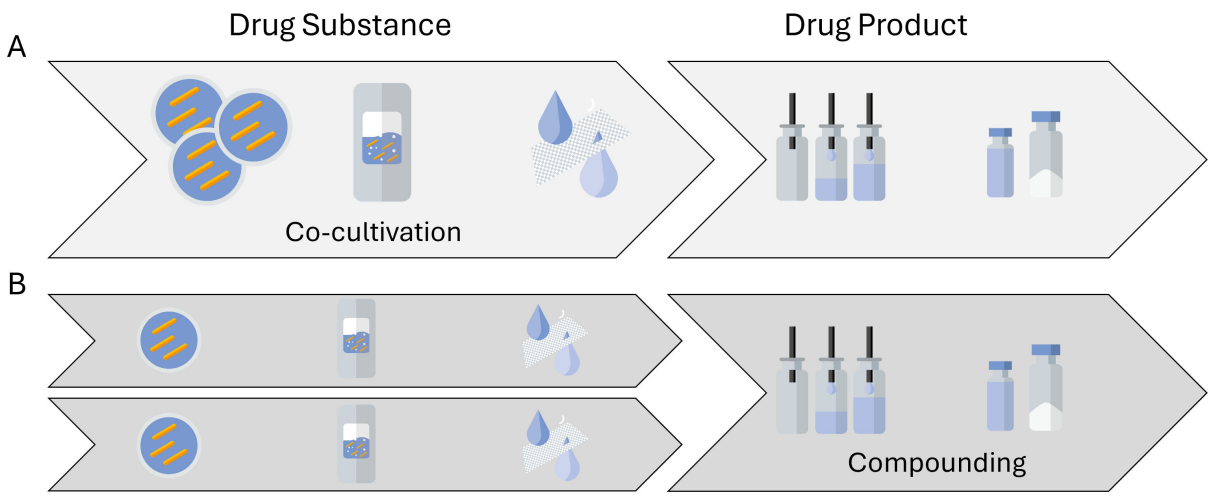

FIGURE 4
Possible routes to manufacture consortia-based, multistrain live microbial products. **(A)** A multistrain cell bank is used to generate a co-cultivated drug substance and subsequently a multistrain drug product material. **(B)** A single-strain drug substance material is manufactured, after which several different strains are compounded into a multistrain drug product.

4.2 Extrapolating those challenges to the manufacturing of consortia-based microbial products

The different challenges mentioned above are even greater when shifting focus from single-strain LMPs to consortia-based LMPs. Consortia-based products consist of multiple different strains in the same formulation, thereby increasing the efficacy or broadening the indication of such a therapeutic, if not both. The distinction needs to be made first between LMPs where strains are truly co-cultivated (Figure 4A) and those where the different strains are produced and harvested separately and then formulated into a single drug product (Figure 4B). True co-cultivation yields an end product in one manufacturing run but reduces control over the individual strain growth profiles. Hence, a co-cultivation approach might be challenging for products where tightly controlled therapeutic manufacturing is a must. It is hence not surprising that only a few entities are engaged in researching and developing true co-cultivation products. A more traditional approach is to grow single strains and thereafter compound them together in a predefined ratio. This is the approach chosen by most of the entities involved in the 46 clinical trials involving multiple strains. However, cultivating strains separately results in multiple batches, which also increases manufacturing time and costs. Furthermore, the metabolome of a co-cultivated consortia might differ from the metabolome of a formulated consortia, where the clinical benefits stem from the nature of manufacturing the next generation of consortia-based products.

In the compounding instance, analytical methods are often developed for the individual strains. The challenges then lie in ensuring that the purpose of the assays is still fulfilled for the product, where the strains are combined. As for the single-strain products, the challenge is often in finding purposeful methods or surrogate methods in the first place. Surrogates of potency and identity for LMPs are often methods around cell counts. However, such cell count methods, whether they are plate methods, optical density measurements, or dielectric spectroscopy-based measurements, are agnostic of the identity of the strains that they measure, meaning that they lack strain specificity to distinguish between a signal coming from a single-strain cell suspension or a multistrain cell suspension. While Magalhães et al. (2019) or Kallastu et al. (2023) are presenting research options to overcome the challenge of content or potency method surrogates for both single-strain and consortia-based live bacterial therapeutics, currently, there seem to be no viable options for routine quality control. Although not commonly applicable for consortia, in specific cases, identities could be distinguished by selective growth media, colony morphology, or other characteristics. Challenges, however, go beyond the appropriate quality control methods for consortia-based LMPs. As there are many advantages in consortia-based LMPs, it is not surprising that several research entities are engaged in developing the co-cultivation field.

5 Conclusion and perspectives

Bringing any medicinal product to the market entails going through the whole life cycle of process validation, starting with process development and characterization (also called stage 1 of process validation according to the FDA guidelines), followed by process performance qualification in order to provide documented proof that the process and associated control strategy that was developed are consistently delivering a product meeting predefined specifications suitable to the commercial production of the product. The traditional approach used for the development and characterization of manufacturing processes for biologics is ensuring that the relevant, phase-appropriate understanding is gained while that knowledge is leveraged across the different stages of process validation. The same approach is needed for LMPs, regardless of the category they fall into (Table 2). The development and characterization of a manufacturing process for an LMP are impacted by the regulatory category, as different regulations and guidelines need to be fulfilled. Nuances in the requirements for the manufacturing process, the application (therapeutic or preventive), the target (tumor or chronic infection), and the route of administration (injected or oral) are crucial in defining the specificities of the development and validation program.

Ultimately, overarching guidelines such as ICH Q11 and the FDA's guidance for process validation require an approach of continuously evolving documented process and product knowledge generation. This ensures that the process, developed in close collaboration with the authorities responsible for market authorization approval, can consistently produce products of appropriate quality. This is achieved through a data-driven control strategy, which is validated during a process performance qualification campaign.

Author contributions

RD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. MS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. RS: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. IC: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. NM: Data curation, Formal analysis, Methodology, Writing – review & editing. AJ: Writing – original draft.

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Supplementary material

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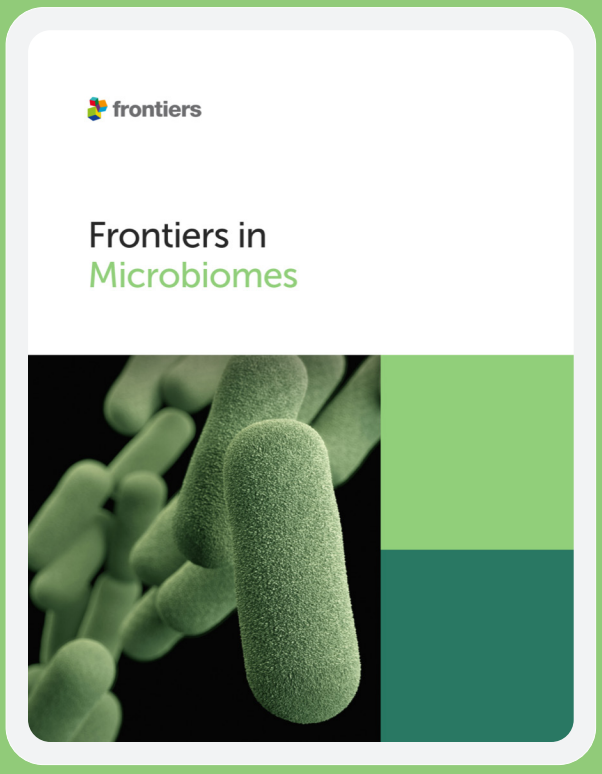

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