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Research review paper

Next generation probiotics: Engineering live biotherapeutics

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ABSTRACT

The population dynamics of the human microbiome have been associated with inflammatory bowel disease, cancer, obesity, autoimmune diseases, and many other human disease states. An emerging paradigm in treatment is the administration of live engineered organisms, also called next-generation probiotics. However, the efficacy of these microbial therapies can be limited by the organism's overall performance in the harsh and nutrient-limited environment of the gut. In this review, we summarize the current state of the art use of bacterial and yeast strains as probiotics, highlight the recent development of genetic tools for engineering new therapeutic functions in these organisms, and report on the latest therapeutic applications of engineered probiotics, including recent clinical trials. We also discuss the supplementation of prebiotics as a method of manipulating the microbiome and improving the overall performance of engineered live biotherapeutics.

1. Introduction

The human body harbors a wide range of microbes, and a large portion of them reside in the human gut. The complex interaction of gut microbes with the host can influence an individual's health and disease state (Fan and Pedersen, 2021). These microbes can affect many gut functions such as digestion, nutrient absorption, drug metabolism, control of pathogens and regulation of the host immune system (Valdes et al., 2018). Under ideal conditions most gut microbes are either commensal or beneficial to the host (Lozupone et al., 2012). However, the symbiotic relationship between gut microbes and the host can be influenced by various factors such as antibiotic treatment, stress, change in diet, pH, host genetics, and many others (Kurilshikov et al., 2021). In the case of host genetics, multiple variables control the invasion of pathogens. For example, an increase in bacterial diversity is related to increased gut immunity, which is influenced by host genetics, environment and diet (Bonder et al., 2016). With respect to pH, beneficial bifidobacteria like *Lactobacillus* and *Lactococcus* can break down complex carbohydrates to produce lactic acid. Under slightly acidic condition the growth of opportunistic pathogen like *Veillonella* and *Streptococcus* are inhibited (Yamamura et al., 2023). In contrast, neutral or alkaline pH can promote an environment suitable for invading pathogens and induce gut inflammation (Bäumler and Sperandio, 2016). The altered gut microbiota can be associated with chronic gastrointestinal intestinal diseases such as inflammatory bowel diseases which includes ulcerative colitis and Crohn's disease (Durack and Lynch, 2019). Further, they are also linked to several metabolic disorders like diabetes, obesity, fatty liver, cardiovascular and chronic kidney diseases (Boulangé et al., 2016).

Probiotic administration can potentially improve or reestablish the perturbed gut microbiota (Azad et al., 2018). According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are defined as "Live microorganisms which, when administered in adequate amounts, confer health benefits on the host" (Swanson et al., 2020). In recent years, more than 1,000 clinical studies have been registered with probiotics for the treatment of over 700 different disease conditions (Dronkers et al., 2020). Some clinical studies have shown the potential of probiotic administration for the treatment of various gastrointestinal, metabolic, and chronic diseases (Cheng et al., 2020; Quigley, 2022; Tenorio-Jiménez et al., 2020). However, successful therapeutic application of probiotics is often limited due to low colonization, strain to strain variation, poor dosage control, and dynamic interactions with the host (Suez et al., 2019).

To improve therapeutic outcomes, researchers are currently adapting synthetic biology tools to engineer and optimize the therapeutic efficiency of probiotics (Bober et al., 2018). Engineered probiotics can also be used as live diagnostic tools to sense and secrete therapeutic protein in response to environmental triggers (Koh et al., 2022; Riglar and Silver, 2018). Recent reports suggest great potential for bacteria engineered with therapeutic enzymes in treating metabolic diseases

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such as phenylketonuria (PKU), enteric hyperoxaluria, homocystinuria (HCU), hyperammonemia, and diabetes (Adolfsen et al., 2021; Le Barz et al., 2015; Lubkowicz et al., 2022; Majtan et al., 2023; Perreault et al., 2024; Prasad et al., 2023). These live biotherapeutics engineered with synthetic biology tools to enhance their therapeutic functions for the treatment of human diseases can be classified as next-generation probiotics (NGPs) as depicted in Fig. 1 (Lin et al., 2019). However, successful therapeutic application of NGPs is challenging for many reasons, including but not limited to reduced viability in the complex gut environment. Colonization resistance exerted by the commensal gut microbiota *via* competition for nutrients may also be an important obstacle to growth and survival of NGPs (Han et al., 2021).

To overcome these ecological disadvantages, the use of prebiotic supplementation may be considered as a method of uniquely proliferating the NGPs to maintain their therapeutic functions (Khalesi et al., 2019). ISAPP defines prebiotics as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Swanson et al., 2020). Often these substrates are complex carbohydrates, the utilization of which is privileged to certain microbes. In addition to proliferating beneficial bacteria in gut, various prebiotic substances like fructooligosaccharide (FOS), human milk oligosaccharides (HMO), inulin, galactooligosaccharide (GOS) and xylooligosaccharide (XOS) can also aid in the colonization of NGPs (Davani-Davari et al., 2019). Engineering probiotics with the ability to utilize privileged prebiotic substrates is indispensable for building next generation synbiotics. This work describes the genetic and molecular toolbox for engineered NGPs and the use of prebiotic supplementation towards successful translational applications of live biotherapeutics.

2. Probiotics

In 1965, Lilly and Stillwell coined the term "probiotic", *pro* meaning "for" and *biotic* meaning "life" to describe substances secreted by one organism promoting the growth of another (Lilly and Stillwell, 1965). Extensive research in the 20th century paved the way for the use of probiotics for direct intervention in human health. Recently, there has been a surge in interest among various research institutions and the biotechnology industry in genetically modifying probiotics as a live biotherapeutic agent for the treatment of human diseases (Charbonneau et al., 2020). These live biotherapeutics are intended to diagnose, cure, mitigate, treat, or prevent human disease. For this reason, from a regulatory standpoint, engineered probiotics for human health are considered as drugs, which require prior approval and clinical validation from the U.S. FDA (Hoffmann et al., 2014). In this review, we distinguish potential NGP candidates as "conventional", or "non-conventional" probiotics based on their history of human use. Lactic acid bacteria (LAB), *Escherichia coli* Nissle 1917 (EcN) and *Saccharomyces boulardii* (probiotic yeast) are classified as traditional probiotics, with more newly discovered probiotics including *Akkermansia muciniphila, Faecalibacterium prausnitzii, Bacteroides fragilis* and *Eubacterium hallii* treated as non-conventional (El Hage et al., 2017). While some wild-type probiotics do have clinical benefits, their ability to treat metabolic and gastrointestinal diseases is not well understood and can often be strain-specific (Dronkers et al., 2020). Understanding their interaction with the host and later engineering them for specific treatment will be essential in constructing future NGPs.

2.1. Lactic acid bacteria

Lactic acid bacteria (LAB) that are often classified as probiotics include *Lactobacillus* sp., *Lactococcus* sp., *Bifidobacterium* sp., and *Enterococcus* sp. (Ljungh and Wadström, 2006). These microbes utilize the sugars present in the gut and undergo fermentation to produce lactic acid as the major end product along with acetic acid, alcohol, and carbon dioxide (Wang et al., 2021). Reports suggest that LABs along with their fermented products are found to be effective for the treatment of

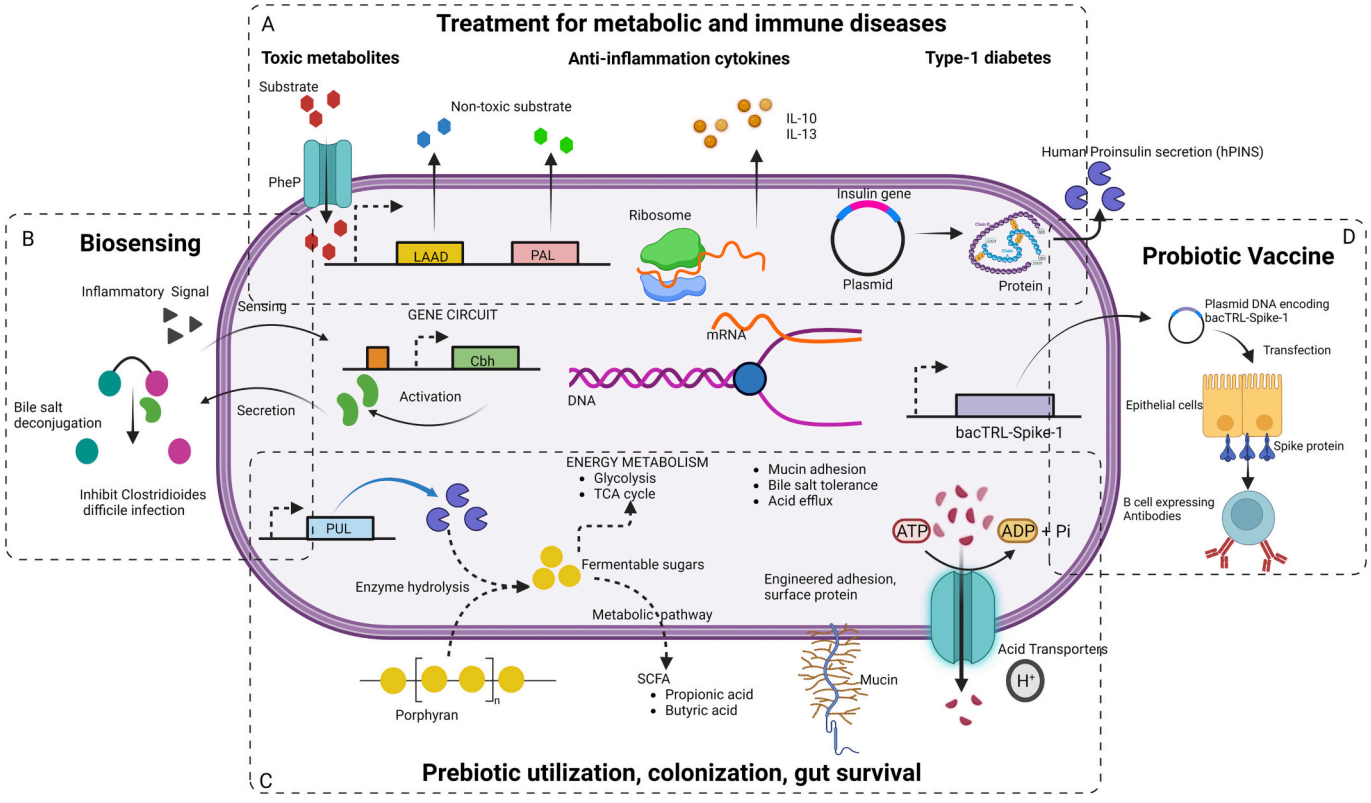

Fig. 1. Various synthetic biology approaches in engineering next-generation probiotics for the treatment of A) Metabolic and immune diseases, B) biosensing and diagnostics, C) prebiotic usage and gut colonization D) vaccine delivery.

inflammatory bowel disease (IBD) (Saez-Lara et al., 2015) and possess anti-inflammatory properties for cancer treatment (Lu et al., 2021). In addition, fermentation of lactose by LAB can also help in reducing lactose intolerance symptoms such as abdominal cramping, flatulence, diarrhea, vomiting and bowel inflammation (Bayless et al., 2017). Clinical investigations have shown that taking supplements of the DDS-1 strain of *Lactobacillus acidophilus* aids in lactose degradation (Pakdaman et al., 2016). Further, the anti-carcinogenic properties of LAB have also been investigated (Legesse Bedada et al., 2020). Several *in vitro* studies demonstrate the potential of LAB for cancer mitigation (Brevi and Zarrinpar, 2023). Reports suggest that probiotic treatment of *Lactobacillus paracasei* and *Lactobacillus rhamnosus* in HGC-27 gastric and DLD-1 colon cell lines are effective in cancer growth inhibition and induce apoptosis (Orlando et al., 2012). The anti-obesity effects of LAB are strongly correlated with gut microbiota composition (Tsai et al., 2014). Administering *Lactobacillus plantarum* LMT1–48 in high-fat diet mouse models significantly lowered body weight and abdominal fat, by down-regulating lipogenic genes (Choi et al., 2020). The above studies highlight the use of LAB for the treatment of IBD, cancer, obesity and potentially be engineered to increase their therapeutic functions.

2.1.1. Genetic tools for lactic acid bacteria

A key tool for genetically modifying LAB is the use of plasmids, small circular, double-stranded DNA molecules used for delivering genes of interest, recombinant protein production (*e.g.*, of human insulin), gene therapy and genome engineering (Wu et al., 2021). The use of plasmids in LAB is often limited by transformation efficiency. LAB are gram-positive bacteria possessing a thick peptidoglycan layer which acts as a barrier for the transfer of exogenous DNA into the cells (Wang et al., 2020). Other factors like strain-to-strain variation, low plasmid copy number, and presence of endonuclease activity inside the cells limit the use of plasmids in LAB (Di Giacomo et al., 2022). To tackle these challenges, shuttle vectors are designed to replicate in hosts with higher transformation and cloning efficiency such as *E. coli* (Spangler et al., 2019). The shuttle vector pTRKH2 was constructed by cloning the *E. coli* p15A replication origin into pAMPl-derived vectors to increase the plasmid copy number in *E. coli*, and later transformed into various *Lactococcus* and *Lactobacillus* species (O'Sullivan and Klaenhammer, 1993). Similarly, the pLEM415-ldhL-mRFP1 shuttle vector was designed with the f1 and pBR322 origins of replication to replicate in *E. coli* and LAB (Bao et al., 2013). For example, the above plasmid was cloned with red fluorescent protein (RFP) under the control of constitutive lactate dehydrogenase promoter to visualize the distribution of the LAB in the gastrointestinal tract of neonatal rats (Campbell et al., 2002). However, most plasmids require selection pressuresuch as antibiotic resistance genes to maintain replication in the face of the metabolic burden associated with DNA synthesis and heterologous protein production (Glick, 1995). Antibiotic stress could also induce mutations to the genes of interest present in the plasmid that favor cell growth at the cost of reduced replication or expression, along with broad-spectrum activity creating disruption of the host microbiome (Foster, 2007; Willing et al., 2011).

Alternatively, using genome engineering tools to insert the gene of interest in the chromosome of LAB increases the genetic stability of these constructs (Rothstein et al., 2020). Plasmid pTRK327 has an insertion sequence which was found to randomly integrate in the chromosome of *Lactobacillus gasseri* with limited homology, aided by Rec-independent recombination (Walker and Klaenhammer, 1994). To direct successful chromosomal integration, a homology sequence was added to the upstream and downstream of the *gusA* gene in the pTRK685 plasmid and co-transformed with a helper plasmid pGK12 expressing $RepA^{+}$ for assisting pTRK685 replication (Russell and Klaenhammer, 2001). The cre-lox system is another useful tool for genetic recombination in LAB (Enyeart et al., 2013). It offers flexibility and high recombination efficiency, allowing for specific gene deletion or insertion in any region of the bacterial chromosome. The *cre* recombinase recognizes the *loxP* site, whose direction determines whether a gene is inserted or deleted. The above system was successfully adopted for deleting thymidylate synthase genes in *L. lactis* (Zhu et al., 2015). Further, the same system was used to integrate multiple copies of *gfp* in six different chromosomal sites, and the functional redundancy increased fluorescence by 3.7-fold (Xin et al., 2019). The limitation of using the cre-lox system includes high false positive colonies, which necessitates iterative screening procedures, off-target effects and genomic instability (Song and Palmiter, 2018).

Alternatively, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas systems have been developed to provide a high throughput screening and genome editing platform (Jinek et al., 2012). The Cas enzyme makes lethal double-stranded breaks in the targeted region to eliminate false positive or wild-type colonies during screening (Hidalgo-Cantabrana et al., 2017). The van Pijkeren group was the first to target the genome of *Lactobacillus reuteri* using a gRNA-Cas9 complex (Oh and van Pijkeren, 2014). Using a single-stranded or double-stranded DNA template the cell repairs the damage and enables high-throughput screening and genome engineering of bacteria via homologous recombination (Reisch and Prather, 2015). The intense selective pressure can promote escape from CRISPR killing *via* mutation (Künne et al., 2018). In the case of the CRISPR-Cas system, many researchers use two-plasmid systems (harboring Cas9 nuclease and gRNA separately) which can cause metabolic burden to cells. To overcome challenges with plasmid maintenance, the pNZDual plasmid was designed consisting of both gRNA and Cas9 in a single plasmid (Els et al., 2018). The expression of these genes is induced by nisin, assisting single plasmid systems for CRISPR-Cas9/CRISPRi gene regulation in *Lactococcus lactis* NZ9000 (Berlec et al., 2018). The CRISPR-Cas system has been adapted for deleting proteolytic genes from LAB and to optimize the strain for expressing numerous heterologous proteins (Kieliszek et al., 2021). The NICE (NIsin Controlled gene Expression) system has been highly characterized for protein expression as well as to construct metabolic pathways in *Lactococcus lactis* NZ9000. Depending on the protein, recombinant proteins could be expressed either intracellularly by pNZ8148 or extracellularly by pNZ8123 (Mierau and Kleerebezem, 2005). A comprehensive list of genetic tools for engineering LAB is listed in Table 1.

2.1.2. Engineering lactic acid bacteria for probiotic outcomes

The use of genetic tools to modulate specific genes in LAB can unravel the mechanism of action and study the dynamic interaction with the host (Lebeer et al., 2008). Currently, several engineered LABs are in the early stages of clinical trials showing promising results. For example, AG019 ActoBiotics is an oral capsule consisting of *L. lactis* engineered to deliver the autoantigen human Proinsulin (hPINS) and human interleukin-10 (IL-10) for the treatment of Type 1 diabetes mellitus (Steidler et al., 2000). The drug showed promising results in both phase 1b as monotherapy and in phase 2a in combination with teplizumab (Mathieu et al., 2024). After AG019, ActoBio Therapeutics released their second antigen-specific investigational immunotherapy drug, AG017, for the treatment of celiac disease. AG017 is an engineered *L. lactis* expressing gliadin peptide and cytokines regulating the immune response to reverse gluten sensitivity (Huibregtse et al., 2009). The FDA has approved the Investigational New Drug (IND) application for Actobiotics AG017 and the drug is currently undergoing phase 1b/2a clinical studies (Lamousé-Smith et al., 2021). Prior to these two drugs, Actobio Therapeutics introduced AG013, a mouth rinse formulation of engineered *L. lactis* expressing human TFF1 (trefoil factor 1) (Caluwaerts et al., 2010). The TFF peptide helps in the protection of gastrointestinal tract against mucosal damage and with subsequent repair mechanisms (Taupin and Podolsky, 2003). However, after failing to demonstrate efficacy in phase 2 clinical studies, the drug was discontinued (Limaye et al., 2013). Similar to previous drugs, Second Genome's SG-5-00455, is also an oral capsule containing engineered *L. lactis* expressing a novel therapeutic protein which binds to the plasminogen activator inhibitor (PAI)-1/2 to block its effect, reducing fibrosis and improving mucosal

Table 1
List of genetic engineering tools for building next generation probiotics.

Organism	Genetic tools	Reference
Lactic acid Bacteria		
Lactococcus, Enterococcus, Streptococcus and Lactobacillus sp.	pTRKH2- Shuttle vector for *E. coli* and gram-positive bacteria.	(O'Sullivan and Klaenhammer, 1993)
Lactobacillus sp.	pLEM415-ldhL-mRFP1, pCOR48 Shuttle vector for *E. coli* and *Lactobacillus species.*	(Bao et al., 2013)
Lactobacillus gasseri & *Lactobacillus acidophilus*	pTRK685- Vector for homologous recombination. pTRK327- Vector for Rec - independent genome integration	(Russell and Klaenhammer, 2001; Walker and Klaenhammer, 1994)
Lactobacillus casei	Cre-lox-based system for multiple gene deletions.	(Xin et al., 2019)
Lactobacillus reuteri	CRISPR-Cas9-aided recombineering	(Oh and van Pijkeren, 2014)
Lactococcus lactis	CRISPR-Cas9/CRISPRi gene regulation, pNZ8148- Intracellular protein expression. pNZ8123- Extracellular protein secretion	(Berlec et al., 2018; Mierau and Kleerebezem, 2005)
Escherichia coli Nissle 1917		
EcN	pMUT1 and pMUT2- native vectors used for genome engineering.	(Kan et al., 2021)
	Bacterial conjugation for gene delivery and markerless genome integration in EcN.	(Seco and Fernández, 2022)
	Curli-operon for extracellular protein secretion in EcN.	(Praveschotinunt et al., 2019)
	In situ secretion of therapeutic nanobodies in the gut	(Lynch et al., 2023)
	CRISPR-based curing and analysis of metabolic burden of cryptic plasmid	(Zainuddin et al., 2019)
	CRISPR-based kill switches	(Rottinghaus et al., 2022)
	EcN as a drug delivery system for expressing anti-inflammatory molecule	(Chen et al., 2023)
	Colorectal cancer detection and treatment with engineered probiotics	(Gurbatri et al., 2023)
Saccharomyces boulardii		
S. boulardii	Plasmid pYC440 - Protein expression	(Douradinha et al., 2014)
	pGEM-ura3, Uracil-auxotrophic Strain for recombinant protein production	(Hamedi et al., 2013)
	Tunable transactivation system for gene expression	(Kwak et al., 2022)
	CRISPR-Cas assisted Genome engineering.	(Liu et al., 2016)
	Engineered G-protein coupled receptor to sense environmental cues and study quorum sensing, cell communication and sexual reproduction.	(Jensen et al., 2022)
	Biocontainment strategies	(Hedin et al., 2023)
Non-conventional Probiotics		
Akkermansia muciniphila	pSAM_Akk- transposon mutagenesis for gene editing.	(Davey et al., 2023)
Faecalibacterium prausnitzii	GutMIcrobiome (GuMI) physiome platform to study human-microbiome crosstalk.	(Zhang et al., 2021a)
Bacteroides fragilis	CRISPR/Cas system for gene expression, DNA repair and ability to survive exposure to antibiotics.	(Tajkarimi and Wexler, 2017)
Clostridium butyricum	CRISPR-Cas systems for genome editing	(Zhou et al., 2021)

healing (Shilova et al., 2022). Currently this drug is in pre-clinical studies and Second Genome is expected to file an IND application for the treatment of IBD. In addition to disease treatment, engineered LAB can act as a whole cell biosensor in detecting disease pathogenesis. Researchers constructed a quorum sensing (agrQS) circuit in *L. reuteri* to detect autoinducing peptide (AIP-1), a molecule expressed during *Staphylococcos aureus* infection (Lubkowicz et al., 2018). Similarly, *L. lactis* was also engineered to detect the quorum sensing signal from *Vibrio cholerae* and suppressed cholera in mice (Mao et al., 2018). As many LABs are generally regarded as safe (GRAS), they are used as a vaccine delivery system for antigenic display (Fig. 1 D) and cancer immunotherapy (Tarahomjoo, 2012; Zhu et al., 2022). For example, *L. lactis* was engineered to produce spherical polyhydroxybutyrate (PHB) inclusions which aid in displaying hepatitis C virus core (HCc) antigen (Parlane et al., 2011). *In vivo* studies suggest that engineered *L. lactis* triggers immune response upon antigen presentation with significant release of interferon gamma (IFN-γ) and interleukin-17 A (IL-17 A) (Wells and Mercenier, 2008). The above reports show the tremendous potential of next-generation lactic acid bacteria for the treatment of diseases like Type 1 diabetes mellitus, celiac disease, oral mucositis, IBD, gut dysbiosis (Cubillos-Ruiz et al., 2022) and other therapeutic applications of engineered LAB are listed in Table 2.

2.2. *E. coli Nissle 1917*

The discovery of *Escherichia coli* Nissle 1917 (EcN) dates to a shigellosis outbreak during World War I, during which German researcher Alfred Nissle identified an *E. coli* strain from the feces of a soldier who was resistant to the diarrhea outbreak (Nakkarach et al., 2020). Later it was found that the isolated EcN possesses various health benefits such as immunomodulation, anti-inflammatory effects, and inhibition of pathogenic bacteria (Sonnenborn, 2016). EcN is currently sold under the brand name Mutaflor, manufactured in Germany and available in several countries in Europe and Asia as a prophylactic against traveler's diarrhea (Bae, 2018). Clinical studies suggest the use of EcN in infants and toddlers suffering from acute diarrhea was found to be safe, well-tolerated, and significantly reduced the symptoms when compared to the placebo (Henker et al., 2007). Similarly, EcN administration was also found to decrease the disease symptoms of UC patients similarly to gold standard treatment with mesalazine (Kruis et al., 2004). The ability of EcN to induce anti-inflammatory cytokines aids in activating various signaling pathways involved in cellular maintenance, growth, and the fight against pathogens (Helmy et al., 2021). The above properties suggest that EcN might be useful for patients suffering from UC (Scaldaferri et al., 2016). Another beneficial trait of EcN is its ability to fight against invading pathogens (Huebner et al., 2011). EcN co-cultures with enterohemorrhagic *E. coli* (EHEC) demonstrate microcin H47 secretion, which aids in inhibiting the growth of gram-negative competitors (Sassone-Corsi et al., 2016). Overall, its general health benefits, versatile genetic toolbox, and long history of safe human consumption make EcN an attractive engineered biotherapeutic chassis (Deane, 2023).

Table 2
List of engineered probiotics and live biotherapeutics for the treatment of human diseases.

Organism	Treatment for	Characteristics	Clinical status	Reference
Lactic acid Bacteria				
Lactococcus lactis (AG019-ActoBio Therapeutics)	Type 1 Diabetes Mellitus	Expresses the autoantigen human Proinsulin (hPINS) and human Interleukin-10 (hIL-10).	Phase 1b/2a (completed)	(Mathieu et al., 2024)
*Lactococcus lactis (*AG017-ActoBio Therapeutics)	Celiac disease	Expresses gliadin peptide and cytokines regulating immune response to reverse gluten sensitivity.	Phase 1b/2a (under investigation)	(Huibregtse et al., 2009)
*Lactococcus lactis (*AG013-ActoBio Therapeutics)	Oral Mucositis	A mouth rinse formulation of engineered *L.Lactis* expressing human TFF1 (Trefoil Factor 1)	Phase 2 (Discontinued)	(Caluwaerts et al., 2010)
*Lactococcus lactis (*AG011-ActoBio Therapeutics)	Ulcerative colitis	Genetically modified L. *lactis* to secrete human Interleukin-10	Phase 2 (completed)	(Steidler et al., 2000)
SG-5-00455- *Lactococcus lactis (*Second genome)	Inflammatory bowel disease (IBD)	Expresses therapeutic protein for promoting mucosal healing.	Pre-clinical studies	(Shilova et al., 2022)
*Lactococcus lactis (*Vaccine delivery systems)	Hepatitis C	Engineered *L. lactis* producing polyhydroxy butyrate for displaying hepatitis C virus core (HCc) antigen.	*In vivo* mice studies	(Parlane et al., 2011)
*Lactococcus lactis (*Vaccine based immunotherapy)	Cancer immunotherapy	Engineered *L. lactis* secreting Flt3L and OX40 ligand	*In vivo* mice studies	(Zhu et al., 2022)
Lactococcus lactis	Antibiotic induced dysbiosis	Engineered *L. lactis* degrades broad spectrum antibiotics β-lactams	*In vivo* mice studies	(Cubillos-Ruiz et al., 2022)
Escherichia coli nissle 1917				
EcN SYNB1618 (Synlogic)	Phenylketonuria (PKU)	Engineered EcN encoding phenylalanine metabolizing enzyme.	Phase 2 (completed)	(Isabella et al., 2018)
EcN SYNB1020 (Synlogic)	Hyperammonemia	Engineered EcN converting ammonia to L-Arginine.	Phase 1b/2a (Discontinued)	(Kurtz et al., 2019)
EcN SYNB1353 (Synlogic)	Homocystinuria	Engineered EcN metabolizing excess methionine	Pre-clinical	(Perreault et al., 2022)
EcN SYNB1934 (Synlogic)	PKU	Evolved strain of SYNB1618 with 2-fold increase activity in phenylalanine degradation	Phase 3 (discontinued)	(Vockley et al., 2023)
EcN (Synlogic)	Enteric hyperoxaluria (EH) (chronic kidney stones and kidney failure)	Engineered EcN designed to consume oxalate in the gut and lower urinary oxalate as a potential treatment for EH.	Phase 1 (Under investigation)	(Lubkowicz et al., 2022)
EcN SYNB2081 (Synlogic)	Gout	Engineered to lower uric acid.	Pre-clinical	(Dalbeth et al., 2019)
EcN SYNB1891 (Synlogic)	Tumors	Engineered to produces cyclic di-AMP (CDA), a stimulator of the STING (STimulator of INterferon Genes) pathway	Phase 1 (completed)	(Luke et al., 2023)
EcN (Institute for Research in Biomedicine (Switzerland) & Synlogic	Tumors (Immunotherapy)	Engineered EcN to produce high levels of L-Arginine activates efficient antitumor T cell response.	*In vivo* mouse studies	(Canale et al., 2021)
EcN	Neurotransmitter disorders	Engineered EcN 1917 derivative by CRISPR/Cas9 to produce gamma aminobutyric acid (GABA) in antibiotic free system.	*In vitro*	(Lan et al., 2021)
EcN	Salmonella infection	Engineered EcN for sensing tetrathionate and utilizing it for microcin H47 production, which helps in salmonella inhibition.	*In vitro*	(Palmer et al., 2018)
EcN	acute liver injury	Genetically modified EcN expressing alcohol dehydrogenase gene, acetaldehyde dehydrogenase gene, NAD synthase gene, and NADH oxidase gene.	*In vitro*	(Cao et al., 2021)
EcN	*Clostridioides difficile* infection	Engineered EcN to metabolize bile salt limits the germination of endospores and the growth of vegetative cells of *C. difficile*	*In vivo* mouse studies	(Koh et al., 2022)
Saccharomyces boulardii				
Saccharomyces boulardii	Vitamin A deficiency	Engineered *S. boulardii* for producing vitamin A precursor (β-carotene).	*In vivo* mouse studies	(Durmusoglu et al., 2021)
Saccharomyces boulardii	*Clostridioides difficile* infection	Engineered probiotic Saccharomyces boulardii to constitutively secrete a single tetra-specific antibody against TcdA and TcdB toxins.	*In vivo* mouse studies	(Chen et al., 2020)
Saccharomyces boulardii	Cholera	Recombinant expression of *TcpA* protein in *S. boulardii*, as a vaccine delivery platform.	*In vitro*	(Awad et al., 2020)
Saccharomyces boulardii	DSS-induced colitis	Engineered *S. boulardii* producing SCFA	*In vivo* mouse studies	(Deleu et al., 2023)
Non-conventional Probiotics				
NOV-001- *Bacteroides* (Novome biotechnology)	Enteric hyperoxaluria	NB1000S, genetically engineered to degrade oxalate, and NB2000P, a seaweed-derived prebiotic. polysaccharide, which acts as a privileged carbon source for NB1000S.	Phase 1 (Under investigation)	(Shepherd et al., 2018)
Bacillus subtilis ZB183™ (ZBIOTICS)	Alcohol detoxification	Genetic engineering of *B. subtilis* for converting acetaldehyde (toxic byproduct of alcohol consumption) to SCFA (acetate).	*In vivo* rat studies	(Appala Naidu et al., 2019)
Bifidobacterium longum (Symvivo Corporation)	COVID-19 (probiotic vaccine)	Engineered *B. longum* to deliver a DNA vaccination expressing SARS-CoV-2-S into intestinal cells.	Phase 1 (Under investigation)	(Taghinezhad-S et al., 2021)

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Table 2 (*continued*)

Organism	Treatment for	Characteristics	Clinical status	Reference
RVB-001 (ResVita Bio) Organism unknown	Netherton Syndrome	Genetically engineered probiotic expressing Lympho-epithelial Kazal-type-related inhibitor (LEKTI)	Pre-clinical	(Chavanas et al., 2000)
Multistrain approach				
VE303 (Vedanta Biosciences)	*Clostridioides difficile* infection	Consists of 8 human commensal bacteria strains providing colonization resistance to *C. difficile*.	Phase 2 (completed)	(Louie et al., 2023)
VE416 (Vedanta Biosciences)	Food allergy	Consists of a defined bacterial consortium inducing immunoregulatory responses and reduced anaphylaxis and allergic symptoms.	Phase 2 (under investigation)	(Bunyavanich and Berin, 2019)
VE800 (Vedanta Biosciences)	Metastatic Cancer	Consists of 11 clonal human commensal bacteria strains manufactured under cGMP conditions and selected for their ability to induce CD8+ T cells	Phase 2 (completed)	(Davar et al., 2020)
VE202 (Vedanta Biosciences)	Inflammatory bowel disease (IBD)	Consists of 16 beneficial Clostridia strains that help in colonic T_{reg} cell induction.	Phase 1 (completed)	(Silber et al., 2022)
SER-109, VOWST™ (Seres therapeutic)	*Clostridioides difficile* infection	An oral microbiome therapeutic candidate consisting of a consortium of highly purified Firmicutes spores	Phase 3 (completed) First FDA-approved oral microbiome therapy	(Feuerstadt et al., 2022)
RBX2660, Rebyota (Ferring)	*Clostridioides difficile* infection	Rectally administered fecal microbiota product (FMP) prepared from stool donated by qualified individuals.	Phase 3 (completed) First FDA-approved FMP	(Khanna et al., 2022)

2.2.1. Genetic tools for E. coli Nissle 1917

The complete genome sequence of EcN has been annotated, providing the necessary information to engineer EcN as a potential biotherapeutics agent (Reister et al., 2014). While engineering *E. coli* cells, it has been highlighted that cloning genes of interest and expressing them in a host could potentially cause plasmid instability and metabolic stress (Silva et al., 2012). EcN harbors two cryptic plasmids, pMUT1 and pMUT2 (Blum-Oehler et al., 2003), which can be used for heterologous protein expression because they stably replicate without the need for selection (Kan et al., 2021). To study the stability of these plasmids individually, first they were cured from EcN using an engineered counterselection CRISPR-Cas9 system. Later, the pMUT2 was transformed and was found to be stable for over 90 generations without antibiotic selection (Zainuddin et al., 2019). Further, the pMUT1/2 plasmids have been engineered with additional features like antibiotic resistance genes, fluorescent markers, temperature sensitive promoters and curli fiber expression for extracellular protein secretion (Lynch et al., 2023). Expressing heterologous proteins of interest *via* the pMUT1/2 system could potentially increase the rate at which gene mutation occurs (Hall et al., 2021). As an alternative approach, integrating the protein of interest into EcN's chromosome via homologous recombination helps in increasing gene stability (Saleski et al., 2021). To increase the transformation efficiency, Seco et al. adapted a bacterial conjugation system to deliver the gene of interest in EcN. The donor strain, *E. coli* MFD, encodes the RP4 conjugation machinery, and when co-cultured with EcN, delivered the required gene of interest into the cell to improve the transformation efficiency for genome engineering (Seco and Fernández, 2022).

A key regulatory concern in the design of live engineered biotherapeutics is biocontainment, *i.e.*, preserving therapeutic efficacy when needed and removing the strain from the system without contaminating the surrounding environment. Under environmental stress, the cells undergo adaptive evolution, either to gain undesirable traits or lose beneficial function and could escape from the host to release into the environment (Zhao et al., 2019). These challenges raise concerns for their application in the biomedical industry (Lee et al., 2018). Researchers used genetically stable CRISPR-Cas9-based kill switches triggered by either single or two-input chemical inducers, followed by temperature-sensitive switches to increase the biosafety aspects of engineered probiotics (Rottinghaus et al., 2022). These kill switches address the biosecurity of live bacterial cells and promote the use of engineering EcN for therapeutic application (Sonnenborn and Schulze, 2009). Further applications of engineered EcN include drug delivery (Chen et al., 2023), heme-sensitive probiotic biosensing (Mimee et al., 2018), colorectal cancer detection (Gurbatri et al., 2023), and display of trefoil factors (TTF) to reduce inflammatory bowel diseases (Praveschotinunt et al., 2019). The above platforms along with genetic tools listed in Table 1 provide the flexibility to engineer EcN as NGPs to treat human diseases.

2.2.2. Engineering E. coli Nissle 1917 for probiotic outcomes

Recently, a significant number of research labs and biotechnology companies are exploiting the potential use of EcN as a novel therapy for immunological and metabolic disorders (Fig. 1 A). For example, Synlogic has developed engineered EcN (Table 2) with the goal of treating diseases such as phenylketonuria (PKU), hyperammonemia, homocystinuria, tumors and enteric hyperoxaluria. The first promising drug candidate SYN1618 was used for the treatment of PKU. SYN1618 was engineered to express genes encoding for phenylalanine metabolism. *In vivo* studies in the PKU mouse model highlight significant reduction of blood phenylalanine by 38% (Isabella et al., 2018). To increase the activity of SYN1618, Synlogic collaborated with Zymergen Inc., to develop a biosensor based high throughput approach for the screening of phenylalanine ammonia lyase (PAL) mutants (Adolfsen et al., 2021). The elite candidate from the screen was used for the construction of SYN1934 and it had two-fold increase in PAL activity when compared to SYN1618 (Puurunen et al., 2021). The Phase 2 clinical trial of above potential drugs was completed and found to reduce plasma phenylalanine by approxmately 20% for SYNB1618 and 34% for SYNB1934 (Vockley et al., 2023). The drug SYNB1934 advanced to phase 3 clinical studies in 2023. Synlogic's next potential drug candidate SYN1020 for the treatment of hyperammonemia, aimed to decrease the levels of ammonia in the bloodstream (Kurtz et al., 2019). However, SYN1020 failed to demonstrate its efficacy during the Phase 1b/2a clinical study and was therefore discontinued. Other potential drug candidates include SYNB1353 for homocystinuria (Perreault et al., 2022), SYNB2081 for gout (Dalbeth et al., 2019), SYNB8802 for enteric hyperoxaluria (Lubkowicz et al., 2022). The above drug pipeline involves treating patients suffering from metabolic disorders *in situ* in the gut and is an exciting area in live biotherapeutic development but failures in clinical trials indicate the significant challenges in implementation in humans.

Synlogic also developed EcN as an immunotherapy for suppressing tumor growth associated with ammonia (Canale et al., 2021). Engineered EcN helps in metabolizing ammonia to L-arginine, which in turn modulates T-cell metabolism and anti-tumor activity (Geiger et al., 2016). Recently, Synlogic, in a strategic partnership with Roche, developed synthetic biotic medicine addressing an undisclosed novel target in IBD (Zhu et al., 2022). Further, they have expanded their

pipeline in tumor suppression by engineering EcN to produce cyclic di-AMP (CDA), a stimulator of the STING (STimulator of INterferon Genes) pathway (Leventhal et al., 2020; Luke et al., 2023). Further, researchers have also engineered EcN for the treatment of neurotransmitter disorders, *Salmonella* infection, and acute liver injury. For example, Lan et al. overexpressed the gadB gene to produce high titers of gamma-aminobutyric acid (GABA) from EcN (Lan et al., 2021). For the treatment of *Salmonella* infection, scientists have engineered EcN to detect tetrathionate, a molecule produced during inflammation. Upon detection, EcN was designed with a feedback mechanism to produce microcin H47, which can inhibit the growth of *Salmonella* (Palmer et al., 2018). Similarly, patients receiving antibiotic treatment are prone to increased *Clostridioides difficile* infection (CDI) causing diarrhea and colon inflammation (Mullish and Williams, 2018). The pathogenicity of CDI is related to its ability to shuttle between active vegetative cells and endospore germination. Researchers hypothesized that microbiome derived bile salt deconjugation limits the germination of endospores and reduces CDI. The bile salt hydrolase Cbh of *Clostridium perfringens* was introduced in EcN to deconjugate taurocholate (Fig. 1 B) into cholate to limit the pathogenesis of CDI (Koh et al., 2022). Engineered EcN can also potentially help in reducing liver damage caused due to excessive alcohol consumption. Researchers have engineered EcN to express genes (alcohol dehydrogenase gene, acetaldehyde dehydrogenase gene, NAD synthase gene, and NADH oxidase) involved in alcohol detoxification. *In vivo* mouse studies suggest that engineered EcN can reduce serum alcohol and restore homeostasis in the gut environment (Cao et al., 2021). Based on the above studies, it is suggested that engineered EcN represents a promising platform for the treatment of human diseases. Many of the live biotherapeutics represented in Table 2 showed promising results in early to late clinical trials, in anticipation of delivering the first FDA-approved engineered EcN in the near future.

2.3. Yeast probiotics: Saccharomyces boulardii

Saccharomyces boulardii (*S. boulardii*) is a probiotic yeast capable of providing various health benefits to the host (Pais et al., 2020). *S. boulardii* is recognized as safe, well tolerated, and effective in various clinical studies (Sen and Mansell, 2020). It has been found to be effective in eliminating pathogenic *Clostridium difficile,* responsible for causing diarrhea (Smits et al., 2016). Individuals receiving a combination of *S. boulardii* with antibiotics have been found to have reduced recurrence of *C. difficile* infection (McFarland et al., 1994). As they are naturally resistant to bacterial antibiotics, they can be administered for the treatment of antibiotic-associated diarrhea (AAD) (Surawicz et al., 1989). Initial stage clinical results for the prevention of AAD with *S. boulardii* showed that treatment was effective and found to eradicate pathogenic *Helicobacter pylori* (McFarland, 2010). To further study the effect of *S. boulardii* in preventing AAD, phase 3 clinical investigations were conducted. The study involved 477 participants in total, 246 in the *S. boulardii* group and 231 in the placebo group. Nine serious adverse events were recorded in the *S. boulardii* group and 3 in the placebo group. Overall, no significance was observed to investigate the effectiveness of *S. boulardii* in preventing AAD (Ehrhardt et al., 2016). Another distinguishing feature of probiotic yeast over other bacteria is its ability to produce glycosylated protein (Celik and Calık, 2012). They could be engineered to express various therapeutic products like antibody-based drugs, Fc fusion proteins, anticoagulants, blood factors, bone morphogenetic proteins, therapeutic enzymes, interferons, interleukins and other proteins, whose function depends on glycosylation (Durmusoglu et al., 2023; Hudson et al., 2014). Given the eukaryotic nature of *S. boulardii,* it could potentially be engineered to perform complex functions and express glycosylated proteins to treat human diseases.

2.3.1. Genetic tools for Saccharomyces boulardii

The molecular and physiological characterization of *S. cerevisiae* and *S. boulardii* shows high genetic similarity, which potentially means that tools available to engineer *S. cerevisiae* could be applied to *S. boulardii* (Fietto et al., 2004). For example, the plasmid pYC440 used for yeast protein expression was successfully transformed into *S. boulardii* for expressing genes of interest (Douradinha et al., 2014). In terms of plasmid construction, most of the conventional antibiotics used in bacterial colony screening are not effective in yeast, with exceptions like G418 and Zeocin (Sunga et al., 2008). Even though antibiotics are helpful in designing genetic constructs, the use of antibiotic resistance genes in engineered live biotherapeutics needs to be avoided for public health reasons (Ben et al., 2019). Auxotrophic mutants present an alternative approach to antibiotic selection (Lv et al., 2015). Hamedi et al., inactivated the *URA3* gene present in the genome of *S. boulardii* by UV mutagenesis. External supplementation of uracil in growth media or transformation of the *URA3* gene into this strain's genome is essential for its proliferation in uracil-free media (Hamedi et al., 2013). Additional auxotrophic mutants like *leu2*, *his3*, and *trp1* (mutation/deletion) were also developed using CRISPR-Cas9 tools. Later, researchers used those auxotrophic mutants for selecting engineered organisms and constructed metabolic pathways in *S. boulardii* for xylose utilization. In addition, they also secreted human lysozyme in the gut by applying *S. cerevisiae* genetic tools in *S. boulardii* (Liu et al., 2016).

Another important tool for therapeutic production in synthetic probiotic yeast is precise control of transcriptional regulation. Kwak et al. constructed a synthetic promoter with a scaffold scRNA target sequence and core *GAL7* promoter sequence in which the transcription activator PCP was fused to VP64 and targeted to the synthetic promoter. The reporter was activated when the scRNA with PCP-VP64 matched the target sequence in the promoter (Kwak et al., 2022). This system enables precise control of gene expression via inducible promoters for expressing therapeutic proteins in *S. boulardii*. For biomedical applications, it is essential to track the progress of engineered *S. boulardii* in the GI tract to its therapeutic efficacy. Marker-free and efficient genome integration of multiple biosensors helps in monitoring various intracellular parameters like pH, ATP levels, oxidative stress, glycolytic flux, cell-cell communication (Jensen et al., 2022) and ribosome production to analyze the metabolic state of the organism (Torello Pianale et al., 2021). Adapting the above-mentioned tools and biocontainment strategies (Hedin et al., 2023) helps in genetic modification of *S. boulardii* to express therapeutic proteins for biomedical applications.

2.3.2. Engineering Saccharomyces boulardii for probiotic outcomes

Reports suggest that this yeast has been engineered for the treatment of vitamin A deficiency, CDI and cholera. CRISPR-Cas9 mediated gene editing tools have been adapted to construct the metabolic pathways in *S. boulardii* to synthesize β-carotene (vitamin A precursor) and violacein (anticancer drug). Engineered *S. boulardii* was colonized in the germ-free mice for 14 days, and the β-carotene recovered from the feces was found to be 56-fold higher than the wild type (Durmusoglu et al., 2021). This study demonstrated the application of *S. boulardii* to treat vitamin deficient patients and possess anti-bacterial, anti-viral, anti-fungal and anti-tumor activity of violacein (Choi et al., 2015). Further, adapting the tools shown in Table 1, *S. boulardii* was engineered to produce SCFA which in turn reduced DSS-induced inflammation in colitis mouse models (Deleu et al., 2023). Similarly, *S. boulardii* has also been used to treat people suffering from mild to severe diarrhea infected with *C. difficile* (Tung et al., 2009). In general, antibodies are used to reduce the gut inflammation caused by *C. difficile*. *S. boulardii* has been successfully engineered to constitutively express a single tetra-specific antibody to neutralize the virulence factors TcdA and TcdB for the treatment of CDI (Chen et al., 2020). Further, *S. boulardii* was used as a live biotherapeutic vaccine by engineering the organism to express recombinant TcpA protein against cholera (Awad et al., 2020).

Several factors including cell washout, low diffusion rate, weak target binding and proteolysis could limit therapeutic efficacy. To tackle these challenges, researchers have engineered *S. boulardi* to express

C. difficile Toxin A neutralizing peptide (NPA) with secretion tags (pre-OST1-proαMF) to increase their expression. Finally, the strain was engineered with knockouts including quadruple protease deletion to increase the NPA production from 76 mg/L to 5045 mg/L (Durmusoglu et al., 2023). These studies highlight the potential development of next generation *S. boulardii* as a vaccine and therapeutic protein expression host for treating various diseases.

2.4. Rising stars in probiotics

Non-conventional probiotic candidates including *Akkermansia muciniphila, Faecalibacterium prausnitzii, Bacteroides fragilis, Eubacterium hallii* have recently been explored for the treatment of various inflammatory and metabolic disorders. These organisms help in maintaining gut homeostasis by producing SCFAs like acetate, propionate, and butyrate (El Hage et al., 2017). *In vivo* studies show that *A. muciniphila* increases the production of a gut hormone, glucagon-like peptide-1 (GLP-1), which reduces metabolic disorder in mice (Yoon et al., 2021). Analyzing the genome sequence of *A. muciniphila* has revealed multiple copies of mucinase. These enzymes could aid in unravelling mucin metabolism and aid in gut colonization (Davey et al., 2023). *F. prausnitzii,* is a key butyrate-producing microorganism found in the human gut (Lopez-Siles et al., 2017). Butyrate produced from *F. prausnitzii* reduces the mucosal inflammation by inhibiting the activation NF-κB transcription factor (Liu et al., 2017). Similarly, *E. hallii* is a key propionate producing bacteria found in the gut, which regulates the key metabolites produced by glucose fermentation (Engels et al., 2016). Commensal *Bacteroides* species like *B. fragilis, B. thetaiotaomicron, B. vulgatus, B. uniformis* (Wexler, 2007) express myriad enzymes to utilize complex carbohydrate substrates, making them a competitive colonizer in the gut (Rios-Covian et al., 2013). However, their presence in the gut can also be detrimental due to bacteriocin production and some toxins produced by *B. fragilis* are correlated to the formation of colon cancer (Matsumiya et al., 2022). To overcome this limitation, deleting toxic genes and engineering them with carbohydrate utilizing enzymes could find its application as a better gut colonizer. Developing novel genetic tools to engineer rising probiotics also presents challenges that are unique to each organism. In parallel, screening existing tools listed in Table 1 would pave the way for high throughput engineering of non-conventional probiotic candidates.

2.4.1. Genetic tools for non-conventional probiotics

The use of engineered non-conventional probiotics as biotherapeutic agents is limited by the availability of genetic tools. Various synthetic biology tools used for engineering standard probiotics could also be applied for non-conventional species. For example, *A. muciniphila* is a promising candidate for its ability to utilize mucin as a carbon source and undergo fermentation to produce SCFA (Li et al., 2021). The standard Himar1c9 transposase used as a carrier for integrating foreign genes of interest, when codon optimized and cloned into a pSAM vector, was found to be functionally active in *E. coli* (Chen and Wang, 2019; Wiles et al., 2013). The above system was adapted in building a transposon mutant library for *A. muciniphila* to understand the mechanisms involved in mucin degradation. Further, the researchers also identified that the degradation of mucin and related proteoglycans was significant for the colonization of *A. muciniphila* in the GI tract (Davey et al., 2023). The production of butyric acid in *C. butyricum* was increased by knocking out genes involved in competing pathways *via* CRISPR-Cas9 (Jang et al., 2014; Yoo et al., 2020). However, the efficiency of the gene editing system (adapted from *Streptococcus pyogenes*) was limited by Cas9 toxicity (Zhang et al., 2020). Adapting the endogenous Type I-B CRISPR-Cas system found in *C. butyricum* eliminates the toxicity of Cas9 and improves plasmid uptake for genome engineering. Zhou et al. successfully deleted *spo0A* and *aldh* genes to increase butyrate production by 60% (Zhou et al., 2021). *Bacteroides* species are prospective gut colonizers, accounting for 25% of the human gut microbiome. However, they possess toxic genes in their genome (Zafar and Saier, 2021). To knock out these genes researchers have identified prominent CRISPR systems in *Bacteroides* including Type I-B, Type III-B, and Type II-C (Tajkarimi and Wexler, 2017). The discovery of multiple CRISPR-Cas systems in this genus could contribute to the control of pathogenic gene expression. In addition, tools to study the interaction between the microbes and the host are also limited in NGPs. Their interaction with the gut microbiome was studied by co-culturing them on human mucosa using a gut microbiome (GuMI) physiome platform. This work studied the anti-inflammatory effects of butyrate in downregulating TLR3 and TLR4 (Zhang et al., 2021a). Future work in developing genetic toolboxes will advance genetic engineering of non-conventional probiotics such as NGPs to express anti-inflammatory molecules.

2.4.2. Next generation non-conventional probiotics

Non-conventional commensal probiotics like certain *Bacillus subtilis* strains have been isolated from the human gastrointestinal tract (Kruse et al., 2021). Several clinical studies have shown that they are safe, tolerable and possess immunomodulatory effects (Garvey et al., 2022; Patch et al., 2023). Recently, Zbiotics have engineered *B. subtilis* with acetaldehyde dehydrogenase gene to reduce the toxic byproduct acetaldehyde to acetate (Appala Naidu et al., 2019). Non-conventional probiotics are also used as orally administered drug secreting antigens to induce mucosal and systemic immune response. The Symvivo Corporation has developed a BacTRL-Spike platform for oral delivery of engineered *Bifidobacterium longum* encoding spike protein for SARS-CoV-2 (Taghinezhad-S et al., 2021). Recently, non-conventional RVB-001, a skin probiotic, was developed by Resvita bio to treat Netherton Syndrome. A mutation caused on chromosome 5q32 in SPINK5 (serine protease inhibitor, Kazal type-5), encodes a serine protease called Lympho-epithelial Kazal-type-related inhibitor (LEKTI), corresponds to skin inflammation (Chavanas et al., 2000). Through synthetic biology and metabolic routes, RVB-001 could be engineered to express protease inhibitor LEKTI to treat inflammatory skin diseases like Netherton Syndrome, atopic dermatitis, psoriasis, acne, and rosacea.

All drug candidates discussed so far include a single-strain approach for the treatment of human disease. Alternatively, Vedanta Bioscience uses a multi-strain approach (listed in Table 2) consisting of rationally defined bacterial consortium (Tanoue et al., 2019). The leading drug candidate VE303 is an orally administered bacterial consortium consisting of eight *Clostridium* strains. They help in the establishment of commensal microbial communities by providing colonization resistance to recurrent *Clostridioides difficile* infection (rCDI) (Dsouza et al., 2022). Recently, they published promising Phase 2 clinical trial data and are set to advance for phase 3 in 2024 (Louie et al., 2023). Similarly, VE202 is a bacterial consortium designed for inducing the regulatory T cells in the gut mucosa for the treatment of inflammatory bowel disease (IBD) (Atarashi et al., 2011; van der Lelie et al., 2021). VE202 was generally regarded as safe and well-tolerated in healthy volunteers in Phase 1 clinical studies (Silber et al., 2022). VE416 is another potential drug candidate used for treating patients with peanut allergy, which is currently being investigated in a phase 2 clinical trial (Bunyavanich and Berin, 2019). VE800 consists of 11 human commensal bacteria strains possessing the ability to induce $CD8^+$ T cells, which help in tumor suppression. This drug is used in combination with Bristol-Myers Squibb's checkpoint inhibitor OPDIVO® (nivolumab) whose effects are currently under investigation in a Phase 2 clinical trial (Davar et al., 2020). Similarly, Seres Therapeutics also developed an oral drug candidate SER-109, using a bacterial consortium with highly purified Firmicutes spores designed to prevent the recurrence of CDI (Feuerstadt et al., 2022). The phase 3 clinical trial results of SER-109 show significant effectiveness in curing the disease when compared to placebo (Sims et al., 2023). Recently the company has filed a biological licensing application to FDA, if approved, SER-109 would potentially become a novel FDA-approved oral microbiome product. However, the race to the first FDA approved microbiome drug was won by Rebyota, which

designed the consortium RBX2660 (a fecal microbiota product) for the treatment of CDI (Khanna et al., 2022; Kwak et al., 2020). This opens up the future microbiome market to design novel drugs (live biotherapeutics) in the biopharma industry.

3. Challenges in probiotic supplmentation

While probiotics are intended to benefit the host, many studies also suggest that some so-called probiotics can have no effect or even be detrimental (Merenstein et al., 2023). In one of the most infamous studies, a double blinded clinical trial was conducted in Dutch patients suffering from acute pancreatitis. Administration of multispecies probiotics led to several patients developing bowel ischemia, with some fatal outcomes which were not observed in the placebo group (Sheldon, 2008). While several administrative shortcomings were noted in the study's design, it set back the clinical exploration of probiotic administration substantially (Kothari et al., 2019; Ritchie and Romanuk, 2012). In addition, LAB administration has been shown to elevate the levels of lactic acid to cause D-lactic acidosis, which is associated with neurocognitive symptoms like brain fogginess and memory loss (Rao et al., 2018). Similarly, individuals under antibiotic treatment to eliminate bacterial infection suffer from disrupted healthy gut microbiota which favors antibiotic-resistant organisms (Larsson and Flach, 2022). Probiotic therapies have been hypothesized to mitigate the development of antibiotic resistance. However, studies conducted by Suez et al. reveal probiotic consumption may impair or delay the re-establishment of the gut mucosal microbiome after antibiotic treatment (Suez et al., 2018). Additional concerns with probiotics include risk of sepsis, horizontal gene transfer and transferral of resistance to antibiotics (He and Shi, 2017; Kochan et al., 2011; Lerner et al., 2017). Mitigating unfavorable outcomes of probiotic supplementation should be a key strategy in improving clinical outcomes and patient compliance in this emerging technology. Increased throughput and efficiency of genetic engineering has the potential to reduce probiotic side effects and enable safe use of NGPs (Charbonneau et al., 2020).

Many engineered probiotic drug candidates show promising therapeutic results under *in vitro* laboratory conditions or even in early preclinical studies. However, when translated to animal and human studies, many failed to deliver efficacy especially in late-stage clinical trials (Eliaz, 2020). Possible reasons include diminished microbial stability, residence time, and colonization resistance of these probiotics in the human gut. To tackle these challenges, co-supplementation of prebiotic substances could increase the nutrition availability in the gut environment for the survival of next generation biotherapeutic (Glowacki and Martens, 2021). Novome biotechnology, a company co-founded by Stanford researchers, has established a metabolic niche by supplementing nutritional elements, which aids in the proliferation of specific strains. A marine polysaccharide, porphyran, was administered to promote the growth of *Bacteroides*, which was genetically engineered with 60-kb porphyran utilization locus (Hehemann et al., 2010). NOV-001 is a novel synbiotic, with a combination of NB1000S, a recombinant live biotherapeutic product, and NB2000P, a botanically derived polysaccharide, whose safety and efficacy are currently under investigation in phase 1 clinical studies (Shepherd et al., 2018). These research findings ignite the idea of next generation synbiotics by engineering probiotic strains with hydrolyzing enzymes for gut colonization, along with the supplementation of prebiotic substances as show in Fig. 1 C. The next section discusses potential prebiotic substrates to promote the growth and colonization of NGPs.

4. Prebiotics for microbiome manipulation

Prebiotics are nutrient sources that are selectively utilized by probiotic organisms which helps in the modulation of gut microbes (Enam and Mansell, 2019). Usually, they are complex carbohydrates which includes human milk oligosaccharides (HMOs), fructooligosaccharide (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), marine polysaccharides, and other polysaccharides used as food additives, whose representative structures appear in Fig. 2 (Davani-Davari et al., 2019). Among them, HMOs are an important component of breast milk, aiding in the development of the infant's microflora and immune system (Bode, 2012). Various combinations of glucose, galactose, fucose, *N*-acetylglucosamine and sialic acid make three major types of HMOs: Fucosylated HMOs, sialylated HMOs and non-fucosylated neutral HMOs (Kellman et al., 2022). Though HMOs cannot be directly utilized by humans, they can be metabolized by specific organisms present in the gut and help in controlling the microbial population dynamics (Akkerman et al., 2019). Among them 2′-fucosyllactose (2′FL), 3′-fucosyllactose (3′FL), 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), lacto-N-neotetraose (LNnT) and lacto-N-fucopentaose II (LNFP II) are the most abundant human milk oligosaccharides (Zhang et al., 2021b).

The prebiotic potential of human breast milk and its oligosaccharides was explored in the following research studies. The natural prebiotic source was supplemented to 59 healthy infants. Results demonstrated that breast milk helps in increasing *Bifidobacterium* species which in turn increases the lactic acid in infant gut (increasing infant gut immunity) (Laursen et al., 2021). *In vitro* gut models suggest that 2′-FL supplementation helps in increasing the relative abundance of *Bifidobacterium adolescentis* and butyrate-producing bacteria (Salli et al., 2021). In addition, 3′-FL supplementations also promote the relative abundance of *Bifidobacterium* species (Rousseaux et al., 2021). Similarly, the supplementation of sialylated oligosaccharide (3′-SL and 6′-SL) also aids in the growth of *Bifidobacterium* species to reduce gut inflammation (ten Bruggencate et al., 2014). For example, 3′-SL helps in inducing regulatory T-cell (Treg) differentiation and downregulates AD-related cytokines to maintain immunological homeostasis and exhibits anti-inflammatory properties (Kang et al., 2020). Similar properties were observed with 6′-SL supplementations in weaned pigs challenged with enterotoxigenic *E. coli*. 6′-SL decreased the serum concentrations of inflammatory cytokines and increased the relative abundance of *Lactobacillus*, *Bifidobacterium*, and *Bacillus* (Duan et al., 2022). Clinical studies were conducted in children who either received 2′-FL or 2′-FL in combination with LNnT (Zhang et al., 2022). Both 2′-FL and the mix help in increasing the population of beneficial bacteria in the gut, providing evidence to show a combination of HMOs are also effective, safe and well tolerated (Fonvig et al., 2021). Recently scientists from Prolacta Bioscience used a synbiotic approach by supplementing a pool of structurally diverse HMOs made from breast milk with live biotherapeutics. By feeding HMOs, they were able to successfully engraft up to 25% of the total bacterial population with *B. infantis*. This co-dosing strategy helps in significantly increasing the butyrate concentration and inhibits the growth of enteropathogens and has been proven safe in ameliorating gut dysbiosis (Button et al., 2022).

FOS are prebiotic fructans composed of linear subunits of fructose linked through a β (2 → 1) carbohydrate linkage. Clinical studies in humans were conducted in 80 patients to investigate the effect of FOS on human gut microflora. Increasing FOS dosage was positively correlated with the relative abundance of *Bifidobacteria* and *Lactobacillus*. Additionally, FOS also increased the population of butyrate-producing microbes like *Faecalibacterium, Ruminococcus* and *Oscillospira* conferring health benefits to the host (Tandon et al., 2019). As the population of beneficial organisms increases upon FOS supplementation, they are also found to reduce inflammation in the gut. For example, when FOS was co-administered with adalimumab, significant reduction of inflammation was observed in patients suffering from Crohn's disease (Borruel et al., 2018). Inulin is closely related polymeric fructan with complex and branched structure, whereas FOS are shorter and linear chains of fructose moieties. Clinical studies were conducted to explore the prebiotic effect of inulin in patients with type 2 diabetes. Results suggest that inulin supplementation significantly increases *Bifidobacterium adolescentis* and *Bacteroides sp.* population and also elevated the

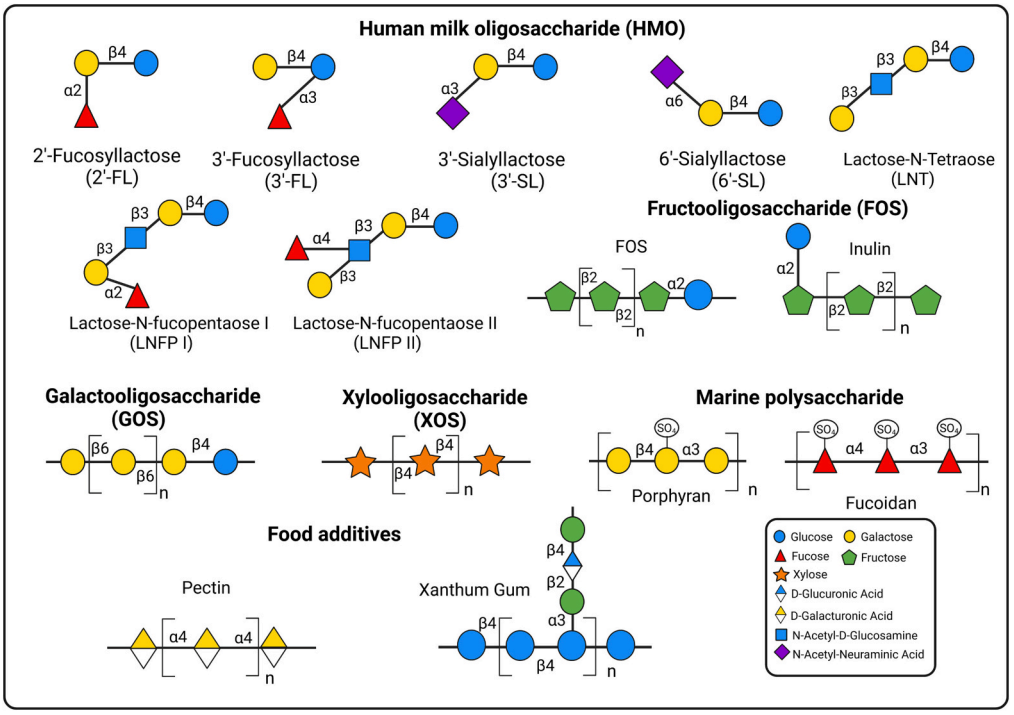

Fig. 2. Schematic representation of various prebiotic structure and their linkages.

concentration of fecal SCFA compared to placebo (Birkeland et al., 2020). Inulin's potential to change gut microbial composition is influenced by the degree of polymerisation (DP). Rats fed with lower degree of polymerisation DP inulin (DP4 > DP8 > DP16) were found to be efficient in increasing the *Lactobacillus* strains. Further analysis revealed, lower DP (DP4) helps in reducing the inflammation by triggering $CD4^{+}$T cells to produce IFN-γ and IL-10 (Ito et al., 2011).

Galactooligosaccharides (GOS) are made up of β-linked galactose subunits ending with either a glucose or galactose moiety. Clinical studies were conducted to observe the effect of GOS in younger and elderly adults. Reports suggest that GOS supplementation increased the relative abundance of *Bifidobacteria* in younger than the older adults and also reduced the microbial diversity in both the groups (Wilms et al., 2021). The prebiotics effects of rhamnogalacturonan were studies in colitis induced mice. Research findings demonstrated that rhamnogalacturonan supplementation reduced colitis severity by protecting the mice from weight loss, macroscopic damage and reduction of colon length (Maria-Ferreira et al., 2018). In most cases administration of prebiotic substances promotes increased SCFA concentrations. However, higher levels of GOS supplementation reduced butyrate-producing bacteria and were shown to downregulate beneficial genes causing an increase in blood glucose and leads to the development of diabetes (Cheng et al., 2018). Similar results were observed when high levels of xylooligosaccharide (XOS) were administered in healthy adults. XOS significantly increased the bifidobacterial population but failed to increase the SCFA (Finegold et al., 2014). These reports suggest that the prebiotic dosage needs to be optimized or modified to improve prebiotic activity. Identifying prebiotic metabolizing enzymes (listed in Table 3) will be key for the conversion of substrate into beneficial metabolites (Rogowski et al., 2015). The XOS utilization was enhanced by xylanase treatment of crude lysate of the yeast-like fungus *Aureobasidium pullulan*. The XOS hydrolysate increased the *Bacteroides, Megamonas* and *Limosilactobacillus* population and increased the production of SCFA (Victoria Gautério et al., 2022). Unlike simple XOS, complex XOS structures like glucuronoarabinoxylans may require a multi-enzyme cascade for their utilization (Lin et al., 2023). A combination of (1 → 4)-*β*-d-arabinoxylan arabinofuranohydrolase (AXH) and *endo*-(1 → 4)-*β*-d-xylanase (Xyl), both from *Aspergillus awamori*, were successfully found to degrade the complex XOS (Verbruggen et al., 1998). These complex substrates can potentially act as prebiotics substrate for NGP. Further, engineering the probiotics species with AXH and Xyl enzymes helps in utilizing complex substances, leading to the development of next generation synbiotics.

Marine polysaccharides including porphyran (typically found in red algae) and fucoidan (typically found in brown algae) are potential prebiotic substances (Lopez-Santamarina et al., 2020). The effect of fucoidan in healthy adults was studied by feeding them in combination with wheat peptide. Fucoidan supplementation helps in increasing the population of *Bifidobacterium pseudocatenulatum, Eubacterium siraeum* and *Bacteroides intestinalis* as well the amount of SCFA in fecal samples (Kan et al., 2020). Most of the prebiotics discussed so far increase the relative population of *Bifidobacteria*, *Bacteroides, Lactobacillus* species. There exists a competition among these gut microbes for prebiotic utilization and potentially limits the growth of specific bacteria which are engineered to treat various gastrointestinal and metabolic diseases. To tackle these challenges, *in vivo* experiments were conducted to create an exclusive metabolic niche by supplementing prebiotic porphyran and orally supplementing *Bacteroides* species (Kearney et al., 2018). The *Bacteroides* was genetically engineered, carrying porphyran utilizing genes for prebiotic utilization, and was successfully engrafted in the gut environment. The population dynamics of engineered *Bacteroides* was controlled by intermittently feeding the mouse with porphyran which demonstrates successful implementation of reversible engraftment in a manner similar to the enabling technology employed by Novome biotechnologies (Shepherd et al., 2018).

Apart from marine polysaccharides, polymeric food additives like pectin and xanthan gum are also potential prebiotic substances (Ndeh et al., 2017; Ta et al., 2021). Randomized clinical studies found out that pectin supplementation helps in restoring microbial diversity and improves SCFA production (Wei et al., 2016). Similarly, xanthan gum is also a polysaccharide widely used as a stabilizer and thickening agent in the food industry. Recent studies were conducted to identify the change in human microbiota upon consuming xanthan gum. 16S rRNA sequencing results revealed that *Ruminococcaceae* and *Bacteroides* OTU represent more than 20% of the total population. Here, *Ruminococcaceae*

Table 3
List of prebiotics and potential enzymes for increasing the colonization of next generation probiotics.

Prebiotic candidate	Enzymes required for utilization	Changes in microbiome	Reference
Human Milk Oligosaccharide			
Donor-derived human milk oligosaccharides (HMO) concentrate	NA	↑ *Bifidobacteria species* & Lactic acid (*In vivo*)	(Laursen et al., 2021)
2′-fucosyllactose (2′FL)	1,2-α-L-fucosidase EC 3.2.1.63	↑ *Bifidobacteria species.* (*In vitro*)	(Salli et al., 2021)
3′-fucosyllactose (3′FL)	1,3-α-L-fucosidase EC 3.2.1.111		
3′-Sialyllactose (3′SL)	Exo-α-sialidase EC 3.2.1.18	↑ *Bifidobacteria* (*In vivo*)	(Kang et al., 2020)
6′-Sialyllactose (6′SL)		↑ *Bifidobacteria* ↑ *Lactobacillus* ↑ SCFA (*In vivo*)	(Duan et al., 2022)
Lacto-N-neotetraose (LNnT)	Lacto-N-biosidase EC3.2.1.140	↑ *Bifidobacteria*	
Lacto-N-fucopentaose II (LNFP II)		↑ Anti-inflammatory effect (*In vivo* & *In vivo*)	(Zhang et al., 2022)
2′FL + LNnT	NA	↑ *Bifidobacteria* (*In vivo*)	(Fonvig et al., 2021)
Fructans: inulin and fructooligosaccharide (FOS)			
FOS	Fructan β-fructosidase EC 3.2.1.80	↑ *Bifidobacteria* & *Lactobacillus* ↑ Shannon index ↑ Butyrate producers (*In vivo*)	(Tandon et al., 2019)
FOS		↑ *Faecalibacterium Prausnitzii* (*In vivo*)	(Borruel et al., 2018)
Inulin	Inulinase EC 3.2.1.7	↑ *Bifidobacteria* ↑ SCFA (*In vivo*)	(Birkeland et al., 2020)
Inulin (different degree of polymerisation)		↑ *Lactobacillus* (*In vivo*)	(Ito et al., 2011)
Galactooligosaccharides (GOS)			
GOS	Galactan 1,3-β-galactosidase EC 3.2.1.145	↑ *Bifidobacteria* (*In vivo*)	(Wilms et al., 2021)
Rhamnogalacturonan	Rhamnogalacturonan Endolyase EC 4.2.2.23	NA	(Maria-Ferreira et al., 2018)
Xylooligosaccharides (XOS)			
XOS	Xylanase EC 3.2.1.156	↑ *Bacteroides* ↑ SCFA ↓ *Eubacteriales* ↓*Desulfovibrionales* (*In vitro*)	(Victoria Gautério et al., 2022)
XOS		↑ *Bifidobacteria* ↑ *Bacteroides* (*In vivo*)	(Finegold et al., 2014)
Glucuronoarabinoxylans	Endo-(1 → 4)-*β*-d- arabinoxylan arabinofuranohydrolase EC 3.2.1.55 & *endo*-(1 → 4)-*β*-d-xylanase 3.2.1.8	↑ *Bacteroides* ↑ Butyric acid (*In vitro*)	(Lin et al., 2023)
Marine Polysaccharides (porhypran, fucoidan, *etc.*)			
Porphyran	β-porphyranase EC 3.2.1.178	↑ *Bacteroides* (*In vivo*)	(Shepherd et al., 2018)
Fucoidan	endo-(1 → 4)-fucoidanase EC 3.2.1.212	↑ *Bifidobacterium* ↑ *Eubacterium* ↑ *Bacteroides* ↓ *Prevotella* (*In vivo*)	(Kan et al., 2020)
Food additives: Pectin, Xanthum gum, gum arabic, *etc.*			
Pectin	Pectin lyase EC 4.2.2.10	↑ Shannon index ↓ Decrease in *E. coli* (*In vivo*)	(Wei et al., 2016)
Xanthan gum	Xanthan lyase EC 4.2.2.12	↑ *Bacteroides* ↑ *Ruminococcaceae* (*In vivo*)	(Ostrowski et al., 2022)
Synthetic glycan			
Kaleido Bioscience constructed 653 Synthetic glycan	Glycoside hydrolases including GH-5,13,42,148,159 acting on synthetic glycan.	↑ Taxonomic diversity ↓ model pathogen (*In vivo*)	(Tolonen et al., 2022)

was identified as primary xanthan gum degrader releasing oligosaccharide which in turn increases the *Bacteroides intestinalis* population (Ostrowski et al., 2022). The above results show promising prebiotic activity of food additives to increase the colonization of beneficial microbes in the gut environment. Next-generation live biotherapeutics will need to be robust to withstand the competition from other gut microbes. Engineered orthogonal substrate usage is a potential path to increased robustness in the nutrient-limited environment of the gut. Table 3 lists some potential prebiotic-enzyme pairs that could potentially be used for niche engineering.

5. Conclusions and future perspectives

Over the past few years, the Food and Drug Administration (FDA) has

approved a substantial quantity of biologic drugs, including monoclonal antibodies, therapeutic proteins, and vaccines for clinical applications. Most of these products are genetically engineered, expressed through biological routes, and used for the treatment of various human diseases. However, the cost of manufacturing (especially in downstream processing), short half-life, and delivery limitations of drugs limits their therapeutic application (Chen and Zhang, 2021; Mitragotri et al., 2014). Engineered live biotherapeutics as an alternative could potentially secrete therapeutic proteins, deliver antigens, sense the gut environment, eliminate pathogens, modulate the immune system, and metabolize harmful substances to overcome above challenges (Charbonneau et al., 2020; Roslan et al., 2023). Choosing the optimum chassis for engineering live biotherapeutic can have many criteria. The ease of genetic manipulation, GRAS status, history of human use, robustness in the gut environment, necessity of post-translational modifications, and many other factors can influence this choice. For example, if a protein of interest requires eukaryotic translation machinery like disulfide bonds or glycosylation for its function, probiotic yeast like *S. boulardii* may be the appropriate choice. LAB and other Gram-positives may be better secretors of protein. The primary advantage of *E. coli* is the readily available nature of its genetic toolkit. Emerging strategies in this organism include rapid recombineering and the existence of genome-scale libraries to probe genotype-phenotype linkages to explore complex phenotypes such as colonization, tolerance, and secretion. Another important fundamental consideration is the ability of the chassis of interest to colonize the gut. Many of the *Bacteriodes* species mentioned in section 2.4 (Rising Stars in Probiotics) are native human gut microbes and as such are good colonizers, while EcN and *S. boulardii* generally fail to colonize under normal gut conditions. When considering an engineered strain of interest, researchers must balance the need for abundance (by colonization) with control (repeated doses of non-colonizing bacteria) and with the current state of the art of the genetic toolbox of the organism. As genetic tool discovery advances, the palette of available chassis organisms will continue to grow, but engineered workhorse organisms like EcN and *Lactococcus lactis* will still play a part because of our greater understanding of synthetic biology, heterologous expression, and secretion in these well-known organisms.

The success of various preclinical discoveries has accelerated the field of engineered probiotics for the treatment of cancer and metabolic disorder (Aggarwal et al., 2020). Currently, there are also promising preclinical results for the use of live biotherapeutics as cancer treatment. For example, clinical trials are underway for the use of FMT in treatment of solid tumors (Park et al., 2023). In addition, naturally tumor-colonizing bacteria such as *Salmonella sp.* can either kill tumor cells or activate the immune system against them (Aganja et al., 2022). Similarly, reprogramming and engineering probiotic strains could also be a promising avenue towards cancer prevention (Legesse Bedada et al., 2020). The treatment of cancer presents a host of unique challenges and the state of the art of live biotherapeutic cancer treatment has recently been reviewed in detail by Brevi and Zarrinpar (Brevi and Zarrinpar, 2023). The use of live biotherapeutics has myriad promising clinical results, especially for the treatment of metabolic disorders. However, they are experiencing mixed results in their pipeline because they discontinued SYNB1020 (for treatment of hyperammonemia), which displayed less efficacy in Phase 1b/2a clinical studies. In a large setback for engineered live biotherapeutic development, in February 2024, Synlogic announced the discontinuation of the Synpheny-3 Phase 3 clinical trial (SYNB1934) and decided to cease operations, an outcome that highlights the significant challenges faced in demonstrating therapeutic efficacy. Single-strain engineered live biotherapeutics are efficient when they are tested in laboratory conditions, however, when introduced into the human gut, these potential bacterial drugs interact with complex gut microbial populations (a source of person-to-person variation) which limits their efficacy. To tackle these challenges, we hypothesize that prebiotic supplementation could potentially help in increasing the growth of engineered live biotherapeutic agents in a mixed population. Various prebiotics like HMOs, FOS, GOS and XOS are found to be efficient in increasing beneficial gut microbes like bifidobacteria, bacteroides, and SCFA producers. However, more can be done to ensure that a prebiotic of interest is utilized directly by the engineered live biotherapeutic with sugar utilization enzymes listed in Table 3, which can increase the proliferation of NGPs. Tailoring prebiotic usage to the engineered organism is likely to improve the overall effect of bacterial therapy.

Engineered live biotherapeutic strains must compete with other bacteria for nutrition in the complex gut environment. Adapting Novome biotechnology strategy to increase the population of specific strains *via* specific nutritional enrichment could potentially increase therapeutic efficacy. The above platform opens the idea of building novel synthetic prebiotics with structural modifications to make them highly privileged (or protected). As a proof of concept, Kaleido Bioscience has designed synthetic glycans (SG) to control gut microbiota population. SG are found to be highly specific in increasing beneficial organisms thereby reducing pathogen growth when compared to reference glycan (Tolonen et al., 2022). Engineering NGPs with novel glycoside hydrolase enzymes to utilize SG could reduce competition from other bacteria and proliferate the growth of therapeutic probiotics. The above research work opens the field of synthetic prebiotics to modulate gut microbes. For example, adding a functional group *via* chemical and/or enzymatic methods including sulfation, phosphorylation, methylation, sialylation, fucosylation *etc.* to the substrate, might aid in protecting the prebiotic from utilization. Engineered NGPs would express specific enzymes to remove these functional groups for their utilization as a nutrient source.

Further, adapting the genetic tools listed in Table 1. can be leveraged to advance the development of robust probiotic strains to increase their fitness in the complex gut environment. Development of a genome-scale library for probiotics using CRISPRa/i will help in mapping genotype-phenotype relationships (Cui et al., 2018). For example, Crook et al. used a metagenome-scale strain library to discover that glycoside hydrolases increased cell viability in the gut (Crook et al., 2019). Hence, building novel prebiotic-probiotic pairs *via in vitro* prebiotics protection and *in vivo* deprotection of engineered probiotics could serve as a next-generation synbiotic platform for biotherapeutic applications. In addition, Biocontainment is also a regulatory issue that will be an important criterion in live biotherapeutics manufacturing and a clever strategy to ensure therapeutic effects *in situ* while minimizing their release in the environmental. Looking forward, a multitude of microbiome-based products are currently being investigated in various clinical stages. Recently, the FDA approval of RBX2660 for the treatment of CDI has opened the market for microbiome-based products. In upcoming years, a substantial number of startups, established biotechnology companies, and research institutions are expected to invest heavily in research and development for building live biotherapeutic products for the treatment of human diseases and diversify their pipeline with bacterial therapy.

CRediT authorship contribution statement

Sanjeeva Kumar Murali: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Thomas J. Mansell:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

TJM is an inventor on a provisional patent related to the engineering of *E. coli* Nissle 1917. Otherwise, the authors declare no competing financial or non-financial interest.

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