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Design and *in situ* biosynthesis of precision therapies against gastrointestinal pathogens

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Gastrointestinal pathogens employ a variety of mechanisms to damage host tissue, acquire nutrients, and evade treatment. To supplement broad-spectrum antimicrobials, there has been increasing interest in designing molecules that target specific taxa and virulence processes. Excitingly, these antivirulence therapies may be able to be synthesized by gut-resident microbes, thereby enabling delivery of these drugs directly to the spatial and temporal site of infection. In this review, we highlight recent progress in our understanding of small molecules that inhibit specific virulence mechanisms. We additionally discuss emerging methods to discover pathogen-specific and mechanism-specific peptides and small proteins. Finally, we cover recent demonstrations of probiotics engineered to produce antimicrobials in response to pathogen-specific cues in the gut. Collectively, these advances point to an emerging integrative approach to treatment of gastrointestinal diseases, comprising microbiologists, peptide chemists, and synthetic biologists.

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Introduction

Microbial pathogens cause significant suffering, mortality, and economic cost as a consequence of infection. Antimicrobials that inhibit processes essential to broad groups of bacteria have greatly improved quality of life, life expectancy, and agricultural productivity. These molecules have largely eliminated the threat of infection that once loomed over everyday life and common procedures, such as dental work, surgeries, and childbirth. Unfortunately, extended antibiotic use has led pathogens to evolve resistance [1,2], and many antibiotics cause collateral damage to the commensal bacteria inhabiting our body [3], the beneficial effects of which are only beginning to be unraveled [4]. This poses a strong need for new strategies to combat pathogens without affecting beneficial microbes.

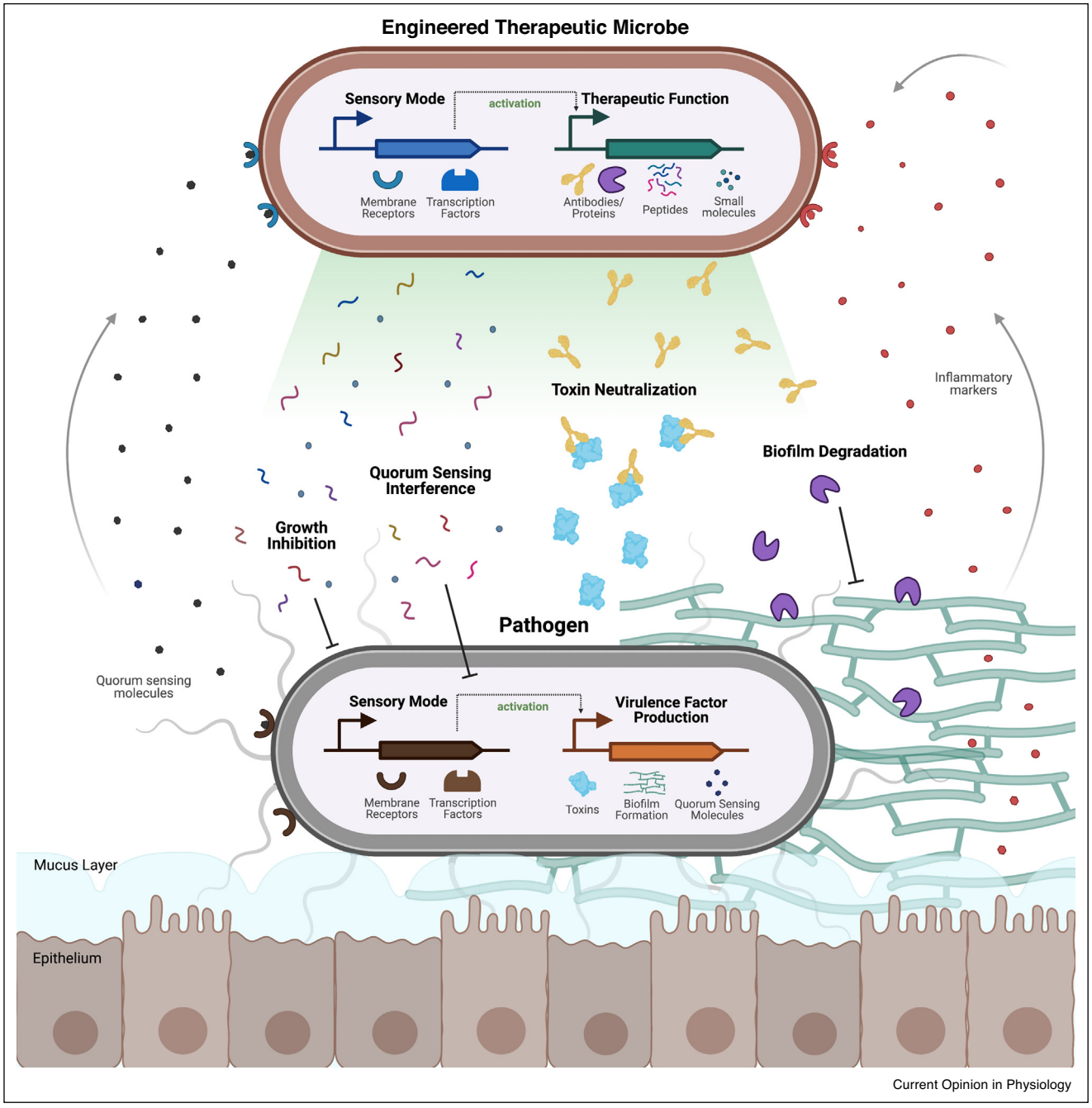
Decades of basic pathology research, coupled with more recent techniques including genomics and proteomics, has disclosed the molecular effectors of pathogenesis and detailed how these 'virulence factors' operate. Elucidation of these mechanisms has suggested approaches to therapy based on targeted inhibition of virulence factors, also known as 'antivirulence' therapies. By inhibiting virulence factors, antivirulence therapies synergize with traditional small-molecule antibiotics, or can be effective at treating disease on their own. Antivirulence therapeutics encompass a wide biochemical diversity, from small molecules to peptides and large proteins. A significant challenge with tackling GI infections, however, is achieving targeted delivery. This is because the gut is protected by the highly acidic stomach and has the highest concentration of proteases in the human body. Therefore, *in situ* biosynthesis of antivirulence drugs in the gut by engineered microbes is a promising strategy (Figure 1). In this review, we survey recent developments in the discovery of antivirulence small molecules, peptides, and small proteins, and our ability to engineer commensal microbes that secrete antivirulence therapeutics directly at the site of gut infections.

Small molecule antivirulence therapies

Pathogenic bacteria and fungi employ a multitude of strategies to infect their host. Common mechanisms of pathogenesis include quorum sensing (QS), biofilm formation, and toxin production (Figure 1). Targeting these virulence factors allows for much greater precision when fighting infections. Anti-virulence strategies in general have been covered in several excellent reviews [5••]; here, we provide examples of anti-virulence strategies that can be deployed by engineered probiotics as a new paradigm for treating infectious diseases in the gut.

QS systems are of particular interest, as they often regulate the other virulence pathways. The biology of QS systems has recently been reviewed [6]. Pathogens sense when they have attained a high abundance in their host

Figure 1



Mechanisms employed by gastrointestinal pathogens and engineered therapeutic microbes.
Pathogens utilize several virulence mechanisms including quorum sensing, biofilm formation, and toxin synthesis. Engineered microbes can target these virulence factors and the pathogen itself by synthesizing therapeutic molecules. Synthesis and release of the therapeutics can be regulated by environmental signals associated with infection (i.e. QS molecules derived from the pathogen, inflammatory and disease markers).

via chemical messengers known as autoinducers, which activate virulence pathways at a sufficient concentration. By interfering with these systems, it may be possible to hinder virulence and render the pathogen harmless. There are two primary methods that have been used to inhibit QS. First, QS-disrupting small molecules, which bind to the autoinducer receptors on the pathogen cell surface are highly effective [7]. Recently, Ahmed *et al.* showed that the plant-derived phenolics *trans*-cinnamaldehyde and salicylic acid disrupt quorum sensing in

Pseudomonas aeruginosa infection due to their structural similarity to acyl homoserine lactones, whose primary activity is to downregulate the expression of *P. aeruginosa*'s virulence factors [8]. As an alternative strategy, autoinducer-degrading enzymes can be employed [9]. Although both strategies show promise, QS inhibition-resistant organisms may arise through selective pressure, as the pathogens that can communicate and organize themselves have a greater chance of survival than the lone pathogen [10]. In fact, some evidence suggests that QS inhibitor resistance arises rapidly in *P. aeruginosa*, where mutations in the *mexR* gene, encoding a repressor of multidrug efflux pumps, led to substantially increased efflux of the quorum quenching molecule brominated furanone C-30 [11].

Some pathogens form biofilms to aid in colonization and act as a barrier to the immune response of the host [12]. Biofilms provide another potential drug target, as their disruption effectively reduces the colonization capacity of pathogens. Sicard *et al.* showed that *N*-acetyl-glucosamine, a mucin sugar, inhibits biofilm formation in the invasive LF82 strain of *Escherichia coli* by inactivating the NagC transcriptional regulator [13]. Dwivedi *et al.* showed that L-fucose, another sugar found in human mucin, reduces *Campylobacter jejuni* biofilm formation by, perhaps counterintuitively, reducing stress on the organism, as environmental stress can trigger biofilm formation as a protective mechanism [14•]. A reduction in biofilm formation and virulence factor production by *Candida albicans* was observed following exposure to morin, a small molecule commonly found in medicinal plants [15]. Additional studies have shown similar results with a variety of small molecules and bacterial pathogens [16]. Research on fungal biofilm disruption is less extensive, but patents on small molecules with fungal anti-biofilm activity have been reviewed by Serafini *et al.* [17].

Toxins are responsible for most of the observable signs of illness during infection due to their ability to damage host cells. Therefore, it is also possible to combat GI pathogens by inhibiting the toxins they produce. Komiazyk *et al.* showed that a variety of plant-derived small molecules strongly inhibit *Vibrio cholerae* enterotoxins and growth without inhibiting growth of a beneficial organism, *Lactobacillus rhamnosus*, at high extract concentrations [18]. Another example of toxin neutralization comprised an engineered λ phage, which expressed repressor genes that neutralized *E. coli*'s Shiga toxin at the genetic level, allowing the potentially pathogenic bacteria to be shed from the gastrointestinal tract before toxin production could take place [19].

Together, the examples presented here build a compelling case for the effective treatment of infectious diseases of the gut using small molecules that inhibit virulence. Since many of these molecules are naturally occurring, there is potential for their biosynthetic pathways to be placed in an engineered probiotic, enabling drug synthesis and delivery directly at the site of infection.

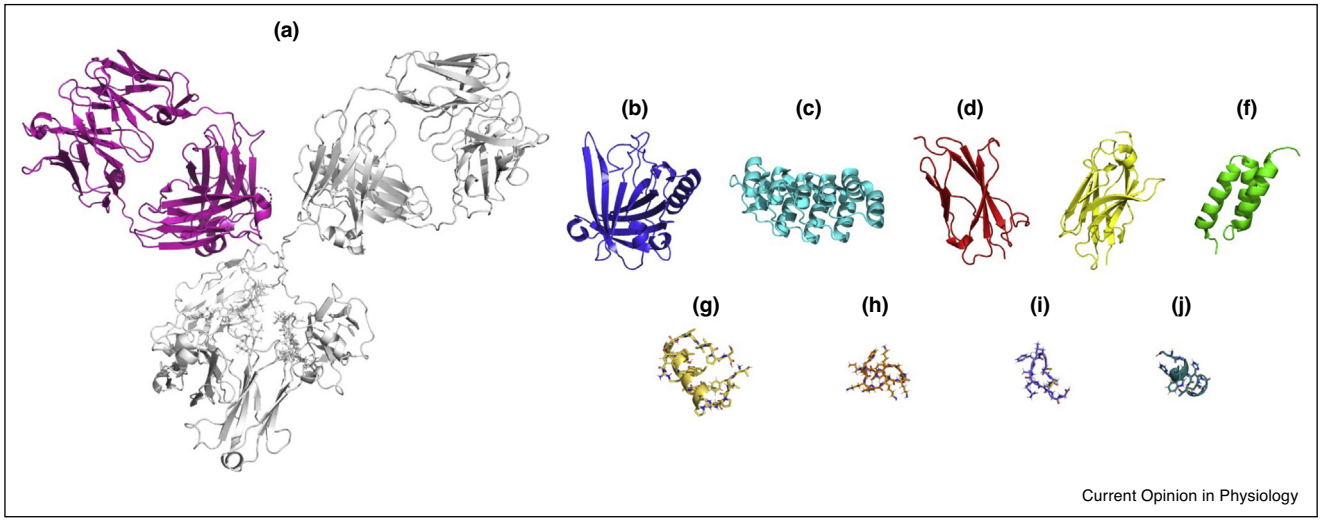
Design of pathogen-targeting peptides and small proteins

Treatment of gut pathogens has traditionally relied on small molecules. As an alternative, polypeptides are diverse in their mechanisms of action, structure, and specificity. AMPs can target narrow or broad taxonomic groups, and can also exhibit immunomodulatory or anti-biofilm activities [20,21]. Additionally, AMPs often work synergistically and are slower to drive resistance [22–24]. Traditionally, AMPs have been discovered from natural sources, either from organisms that exhibit antimicrobial activity, or from panning of natural AMP reservoirs such as unculturable soil and marine bacteria. However, more recently, screening of synthetic peptide libraries and rational design have gained popularity. The potential for AMPs as therapeutics was extensively reviewed by Magana *et al.* [24], and their clinical efficacy was evaluated by Koo and Seo [25]. Here, we focus on methods for AMP discovery, with emphasis on targeting specific virulence mechanisms. Advances in peptide design and synthesis have created a variety of scaffolds and design motifs to build upon, leading to the development of short linear peptides, cyclic peptides, DARPins, affibodies, anticalins, adnectins, and nanobodies that target gut pathogens [26] (Figure 2). A comprehensive list of peptides and small proteins targeting gut pathogens is presented in Table 1.

Linear peptides can be easily produced by synthetic chemistry or biological machinery. Their large chemical diversity facilitates the identification of peptides with high affinity and specificity for a desired target. Among the various *de novo* AMP development strategies, phage display is the most utilized. AMPs have been identified via phage display to target the virulence factors of *Clostridium difficile* [27] and *Helicobacter pylori* [28], as well as the cell wall biosynthesis enzymes of *P. aeruginosa* [29]. Using the same method, Pini *et al.* developed a non-natural antimicrobial peptide, M33, that limits lipopolysaccharide (LPS)-mediated cytokine release associated with *P. aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. As a tetra-branched peptide, M33 features high resistance to proteolytic degradation, serum stability over 24 hours, and an EC_{50} of 3.8×10^{-8} M for prevention of TNF-alpha release by LPS-activated macrophages [30••].

Cyclic AMPs feature a higher therapeutic efficacy compared to their linear counterparts, for their constrained structure enhances their biorecognition activity and prevents degradation by peptidases [31]. A number of promising candidates have been designed via sterical constraining of linear AMP precursors and by modifying native cyclic defensins [25]; examples include

Figure 2



Peptide and small protein scaffolds utilized to target GI pathogens.
3D renderings (to scale) of **(a)** Fab fragment of the *Clostridium difficile* Toxin B-targeting monoclonal antibody bezlotoxumab (4NP4), **(b)** Anticalin (Siderocalin) targeting Anthrax Bacillus' Petrobactin (6GQZ), **(c)** Ankyrin repeat protein (DARPin) (2QYJ), **(d)** Adnectin (4OV6), **(e)** Llama nanobody targeting *Archaeoglobus fulgidus*' protease (5G5R), **(f)** Affibody targeting ZTaq (2B89), **(g)** Peptide TFLPQPRCSALLRYLSEDGVIVPS (inhibitor of *Helicobacter pylori* urease holoenzyme), **(h)** Peptide TWIKKKKWKKAK, **(i)** Peptide CRVFLCGCGGG, and **(j)** Peptide EGWHAHTGGGC (inhibitor of *C. difficile* Toxin A). Peptides (g)–(j) were obtained via atomistic molecular dynamics simulation in explicit solvent.

peptidomimetics based on antimicrobial peptide protegrin I against *P. aeruginosa* [32•], and a cyclic mini-beta-defensin analog against *P. aeruginosa*, *E. coli*, and *Enterococcus faecalis* [33]. A popular target of cyclic AMPs is QS-mediated virulence. Auto-inducing peptides perform similar roles in quorum sensing as the small-molecule autoinducers mentioned above. Cyclic auto-inducing peptide (AIP) mimetics were therefore designed to inhibit quorum sensing in *Staphylococcus aureus* [34,35].

Increasing in size and complexity from linear and cyclic peptides are a variety of engineered protein scaffolds that blend the high affinity and selectivity of antibodies with the ease of production and stability of small peptides. The most promising scaffolds are based on natural ligand-binding proteins and include DARPins ('designed ankyrin repeat proteins', 14–17 kDa), affibodies (based on the Z domain of *S. aureus* protein A, 7 kDa), anticalins (based on lipocalins, 18 kDa), and adnectins (based on the fibronectin type III domain, 10 kDa) [26]. DARPins have high affinity, stability, and low immunogenicity. Ultra-potent DARPins against *C. difficile* virulence factor TcdB were found via phage display and functional screening [36]. Affibodies have excellent tissue penetration properties, high stability, and solubility, which makes them ideal for diagnostic and purification applications [26]. There have been recent advances in therapeutic affibodies, though to our knowledge no affibodies targeting gut pathogens have been described to date. It is interesting to note that anti-TNF-alpha affibodies have been secreted by engineered *L. lactis* in an *ex vivo* model for IBD — bridging the gap between designer protein scaffolds and engineered probiotics [37]. Anticalins differ from other protein scaffolds in that their parent structure, lipocalin, is naturally found circulating in human plasma [26]. Anticalins that inhibit *P. aeruginosa* and *Bacillus anthracis* siderophore activity, which aids in obtaining iron during infection, were discovered through combinatorial protein design based on human siderocalin [38•]. A number of adnectins have performed well in pre-clinical and clinical trials, largely targeting extracellular or cell surface displayed targets [26]. As with affibodies, adnectins represent a potent class of peptide scaffolds that have been underutilized in targeting gut pathogens. Yang *et al.* [39] used yeast surface display to engineer a bispecific neutralizing adnectin against perfringolysin O (PFO), a bacterial pore-forming protein that is involved in the delivery of exogenous proteins. While their primary objective was to increase the therapeutic window of PFO-mediated delivery of large therapeutic molecules, their work has interesting implications for treatment of *Clostridium perfringens* infections, where PFO is a key virulence factor [40].

Intermediate between small peptides and large proteins are nanobodies, which are single domain antigen-targeting proteins derived from camelid antibodies. Their limited size (15 kDa) compared to traditional antibodies (150 kDa) lends to easier production, higher solubility, and enhanced tissue penetration. Nanobody libraries can

Table 1

Peptides, peptidomimetics, and small proteins developed as inhibitors of gut pathogen virulence factors

Type of peptide	Discovery platform	Pathogen targeted	Molecular target	Peptide structure	Reference
Linear	Phage display	*Clostridioides difficile*	TcdA and TcdB	EGWHAHTGGGC HQSPWHHGGGC	[27]
Linear	Phage display	*Helicobacter pylori*	Urease Holoenzyme	TFLPQPRCSALLRYLSEDGVIVPS YDFYWW	[28]
Linear	DIPPS (direct in-gel profiling of protease specificity assay)	*Helicobacter pylori*	HrtA	2-Abz-AQRVAF-Y(NO2)	[45]
Linear	Phage display, consensus	*Pseudomonas aeruginosa*	MurF	TMGFTAPRFPHY	[29]
Branched	Phage display, rational modifications	*Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii*	LPS	KKIRVRLSA	[30••]
Linear amphiphile, nanotube self assembly	Rational design of secondary structure	*Pseudomonas aeruginosa, Candida albicans*	Biofilms	WGIRRILKYGKRSAAAAAAK (C19)-CONH2	[46]
Cyclic	Rational design based on CagL exposed loop structure	*Helicobacter pylori*	CagL binding to human integrins	Cyclic-RGDL-dA Cyclic-RGD-dL-A Cyclic-RGD-dF-A*	[47]
Cyclic peptidomimetic	Peptidomimetic library screening based on protegrin I	*Pseudomonas aeruginosa*	LptD	Cyclic-AS-dP-PTWIX-Orn-dX-XWXX* Cyclic-TWIXKXXWXXAX Cyclic-TWLKKRRWKKAK	[32•]
Cyclic	Intracellular cyclic peptide library SICLOPPS (split intein-mediated circular ligation of peptides and proteins) *in vivo*	*Staphylococcus aureus*	Replisome beta-sliding clamp	Cyclic-CRVFLCGC Cyclic-CRSQGLFK	[48]
Cyclic	Modifications of truncated native auto-inducing peptide	*Staphylococcus aureus*	ArgC AIP binding site	Cyclic-CSSLF	[35]
Cyclic peptidomimetic	Modifications of simplified scaffold from Muir lab	*Staphylococcus aureus*	ArgC AIP binding site	Cyclic-Phac-C-n7O-Cpa-3fF	[34]
Cyclic	Rational design based on beta-defensins	*Pseudomonas aeruginosa, Escherichia coli*	N/A	Cyclic-CPIFTKIQGTC-GGRRKK	[33]
DARPin	Phage display	*Closridioides difficile*	TcdB CROP domain		[36]
Anticalin	Phagemid display	*Pseudomonas aeruginosa*	Pyoverdine types I-III, and pyochelin	'Tetra-calin', 4 anticalins fused together	
Anticalin	Phagemid display, combinatorial protein design based on human siderocalin (Scn/Lcn2, NGAL)	*Bacillus anthracis*	Petrobactin	'Petrocalin'	[38•]
Adnectin	Yeast surface display	*Clostridium perfringens*	PFO		[39]
Nanobody	Immunized Camel phagemid library	*Acinetobacter baumannii*	Bap		[49]
Nanobody	Immunized Camel phagemid library	*Enterohemorrhagic Escherichia coli*	TirM		[50]
Nanobody	Immunized Llama phage display library	*Closridioides difficile*	SLP		[42]
Nanobody	Immunized Alpaca phage display libraries	*Closridioides difficile*	TcdA and TcdB	Tetra-valent fusion of ABBA nanobodies	[43,44••]
Nanobody	Semi-synthetic Llama phage display library	*Staphylococcus aureus*	SEB		[51]
Nanobody	Naive Camel phage display library	*Vibrio cholerae*	LPS		[52]
Nanobody	Semi-synthetic Llama phage display library	*Vibrio cholerae*	Cholera toxin		[51]

Cyclic-RGD-dF-A*, * = known affinity ligand for integrin alpha-V-beta-3.
Cyclic-AS-dP-PTWIX-Orn-dX-XWXX*, * = Murepavadin (POL7080) has FDA Qualified Infectious Diease Product designation for several indications, where X = L-2,4-diaminobutyric acid.

be generated synthetically or sourced from immunized or naive camelids. Phage and yeast display are the most popular methods for *de novo* discovery of anti-microbial nanobodies [41]. A number of nanobodies that neutralize the virulence factors of *C. difficile* have been identified by phage display [42,43,44••].

Taken together, the potency of antimicrobial peptides and proteins motivate their use for the treatment of gastrointestinal infections. Furthermore, the simplicity with which they can be encoded in DNA (i.e. a single open reading frame) make them ideal candidates for production in engineered probiotic microbes.

Engineered probiotic delivery of precision antimicrobials

Advances in synthetic biology have enabled the development of next-generation probiotics (microbes that are engineered to confer health benefits once administered) as a new therapeutic modality against gastrointestinal pathogens (see Figure 1 and Table 2) [53,54•]. These microbes can be engineered to sense molecules associated with infection, and in response synthesize therapeutics, such as the antivirulence compounds described above, directly in the gut from unused dietary material.

Targeting virulence factors such as toxins, surface proteins, and morphological structures (flagella, pilli, hyphae) has proven to be a powerful antipathogen strategy in engineering next-generation probiotics for treating infections [55]. In this context, probiotics can be programmed to inhibit the transcription of the virulence genes by interfering with quorum sensing. For example, *E. coli* Nissle 1917 was engineered to interfere with *V. cholerae (Vc)* QS and restrict the transcription of virulence genes by synthesizing cholera autoinducer 1 (CAI-1) [56], as high concentrations of CAI-1 inhibit the expression of cholera toxin and pilus [57]. Furthermore, QS molecules involved in inter-species interactions can be synthesized to achieve the same effect. For example, commensal *E. coli* NGF-1 was programmed to secrete the *Burkholderia cenocepacia* QS molecule cis-2-dodecenoic acid (BDSF) to block *C. albicans*' (*Ca*) hyphae formation, as *Ca* uses hyphae to adhere to and invade epithelial cells [58,59].

Probiotics can also be engineered to neutralize the toxins secreted by GI pathogens. This strategy may be favored over inhibition of quorum sensing, as the availability of QS molecules can regulate processes other than pathogenesis, and can be shared among different bacterial species [60]. Initial examples of toxin neutralization have recombinantly expressed host cell receptors for toxins on the surface of engineered probiotics, thereby creating a 'toxin sponge' [61,62]. In addition to displaying toxin-binding molecules, engineered probiotics can secrete them. For instance, the probiotic yeast *Saccharomyces boulardii* has been programmed to secrete a tetra-specific nanobody that binds and inactivates both of the *C. difficile* toxins TcdA and TcdB [44••].

Another promising strategy is blocking the pathogen's adhesion proteins, since these proteins facilitate the pathogen's attachment to host epithelial cells [63]. This can be achieved by competitive binding of the engineered probiotics to host cells, thus excluding the pathogen and mimicking a natural mechanism of anti-pathogen activity performed by un-engineered probiotics [64,65]. To this end, display of the pathogen's surface layer proteins on the surface of engineered probiotics is a successful strategy [66••,67]. However, care must be taken as bacterial surface layer proteins also have impacts on the immune system and cell proliferation [68,69].

By inhibiting biofilm formation, engineered probiotics can improve the delivery of drugs to biofilm-forming organisms. For instance, *E. coli* and *Lactobacillus plantarum* were engineered to produce biofilm dispersal agents, such as endonucleases (DNAse I) and glycoside hydrolases (Dispersin B, PelAh), which degrade the extracellular DNA and polysaccharides that comprise *P. aeruginosa* biofilms [70,71•,72,73]. One of these systems exploited *P. aeruginosa*'s QS mechanism to achieve pathogen-activated synthesis of the anti-biofilm components, augmenting the efficacy and the specificity of the therapeutic activity through localization with the target [70]. Some *E. coli* designs were programmed to synthesize multiple therapeutic compounds (i.e. an antibiofilm agent and an antimicrobial peptide), thereby increasing efficacy [70,71•].

Engineered probiotics may also inhibit the growth of pathogens through production of antimicrobial peptides (AMPs) and proteins. These agents have various modes of action, including pore formation, or inhibition of DNA replication, transcription, translation and cell wall synthesis [74]. Using engineered probiotics as a delivery system allows localized and sustained release of antimicrobial agents, and are increasingly viewed as powerful weapons to defeat gut pathogens. In this context, several *E. coli* strains have been engineered to synthesize antimicrobial peptides against several pathogens, including *Salmonella enterica*, shiga-like toxin producing *E. coli* (STEC) and vancomycin-resistant *Enterococci* spp. and *V. cholerae* [75–78]. In addition to vegetative bacterial cells, bacterial spores and yeast cells have been engineered to display and synthesize antimicrobial peptides, respectively, showing promising inhibitory activity [79,80]. Using bacterial spores or yeast cells could be beneficial, as it would avoid potential self-toxicity of the AMP to a vegetative bacterial producer. Furthermore, the simplicity of AMP synthesis pathways enables simultaneous expression of multiple antimicrobials from a single probiotic, thereby targeting different mechanisms and/or pathogens at once, which is particularly advantageous for polymicrobial

Table 2

Engineered microbial therapies developed against gastrointestinal pathogens

Engineered therapeutic microbe	Target pathogen	Expression mode	Therapeutic molecule	System details	Models tested	Results	Reference
Escherichia coli R1	*Escherichia coli* O157:H7	Surface display	Gb3 receptor	Lactose inducible promoter: pLac	*In vitro, in vivo*	Up to 99% toxin neutralization (*in vitro*). >14 days survival (*in vivo*).	[62]
Escherichia coli R1	*Vibrio cholerae* (*Vc*)	Surface display	GM1 receptor	Lactose inducible promoter: pLac	*In vitro, in vivo*	Up to 99% toxin neutralization (*in vitro*). Increased survival rate with multiple gavage (*in vivo*).	[63]
Escherichia coli Nissle 1917	*Vibrio cholerae* (Vc)	Production	Cholera autoinducer 1 (CAI-1)	Constitutive promoter: pfliC	*In vivo*	Upto 82% survival rate. Reduction in toxin and *Vc* cell load.	[56]
Escherichia coli NGF-1	*Candida albicans* (*Ca*)	Sense-respond production and secretion	cis-2-dodecenoic acid (BDSF)	QS (HPA) sensing: 4-HPA transporter (HpaX), 4-HPA transcription factor (HpaA) and pBC promoter	*In vitro* (Caco-2 cells co-cultured with *Ca* cells)	∼20% decrease in cytotoxicity, ∼80% decrease in hypha formation when incubated with bacterial supernatant (engineered *E. coli* NGF-1).	[58]
Sacchromyces boulardii MYA-796	*Clostridioides difficile* (*Cdiff*)	Production and secretion	Tetravelant antibody	Constitutive promoter: pTEF1 secretion tag: alpha mating factor signal	*In vivo* (primary CDI, recurrent CDI)	Prophylactic and therapeutic activity. Reduced mortality (upto ∼100% survival in prevention study), decreased inflammation and tissue damage, reduced *Cdiff* load and toxin titers in feces.	[44••]
Lactobacillus casei	*Listeria monocytogenes* (*Lm*)	Surface display	Listeria adhesion protein (LAP)	Constitutive promoter: pAmy secretion tag: alpha-amylase signal	*In vitro, in vivo*	Increased survival rate (∼upto 90%), decreased *Lm* adhesion, invasion and translocation (*in vitro* and *in vivo*), improved intestinal barrier function and immunomodulatory action.	[66••]
Escherichia coli UU2685	*Pseudomonas aeruginosa* (*Pa*)	Sense-respond production and secretion	DNaseI, Microsin S	QS (AHL) sensing: LasR transcription factor and LasR-AHL induced promoter: pLasI motility: CheZ. Degradation tag: YBaQ secretion Tag: YebF	*In vitro*	∼60% reduction in *Pa* biofilm and ∼40% reduction in biofilm viable cells.	[70]
Escherichia coli Nissle 1917	*Pseudomonas aeruginosa* (*Pa*)	Sense-respond production and self-lysis	Dispersin B, pyocin S5	QS (AHL) sensing: LasR transcription factor and LasR-AHL induced promoter pLasI self-lysis: E7 lysin alanine auxotrophy for plasmid stability	*In vitro, in vivo* (*C. elegans* and mice)	∼80% reduction of mature biofilm mass, reduction in biofilm viable cells (*in vitro*). Prophylactic and therapeutic activity. Clearance of *Pa* cells in mice model (∼77% of the initial load), 98% inhibition of *Pa* infection.	[71•]
Lactobacillus plantarum, Lactobacillus rhamnosus	*Pseudomonas aeruginosa* (*Pa*)	Production and secretion	EngZ cellulase, NucA nuclease, PelAh glycoside hydrolase	Pheromone inducuble promoter: pSppA secretion tag: Lp_3050 signal	*In vitro*	Reduction in *Pa* biofilm mass by PelAh-secreting *L. plantarum.*	[73]
Escherichia coli Top10	*Pseudomonas aeruginosa* (*Pa*)	Sense-respond production and self-lysis	Pyocin S5	QS (AHL) sensing: LasR transcription factor and LasR-AHL induced promoter: pLuxR self-lysis: E7 lysis protein	*In vitro*	Upto ∼90% *Pa* biofilm formation inhibition. ∼99% *Pa* cell growth inhibition.	[72]
Escherichia coli K-12 MG1655	*Pseudomonas aeruginosa* (*Pa*)	Sense-respond production and secretion	Chimeric bacteriocin (CoPy)	QS (AHL) sensing: LasR transcription factor and LasR-AHL induced promoter: pLasI secretion tag:FlgM signal	*In vitro*	∼10^5 Ec cells inhibited the growth of planktonic *Pa* cells. Limitations in secretion levels were observed (i.e. only 5% of the intracellular CoPy was secreted).	[75]

Table 2 (*Continued*)

Engineered therapeutic microbe	Target pathogen	Expression mode	Therapeutic molecule	System details	Models tested	Results	Reference
Escherichia coli Nissle 1917	*Salmonella enterica* (*Se*)	Production and secretion	Microcin J25	Constitutive promoter: σ70-like promoter and inducible synthetic hybrid-promoter: pON	*In vivo*	∼10-fold reduction in *Se* load in cecum. Higher efficiency than enroflocaxin. No significant alteration in the native microbiota by produced microcin.	[76]
Escherichia coli Nissle 1917	*Salmonella typhimurium* (*St*)	Sense-Respond production and self-lysis	Microcin H47	Tetrathionate sensing: TtrR-TtrS two-component system and constitutive promoter: pJ23119	*In vitro*	Effective *St* killing on solid media assays. Reduction in *St* fitness on competition assays in liquid media.	[77]
Escherichia coli Nissle 1917	Vancomycin resistant *Enterococcus* spp.	Production and secretion	Enterocin A, enterocin B, hiracin JM79	Inducible synthetic hybrid-promoter: pON Secretion tag: Vsp tag	*In vitro, in vivo*	Three-peptide system reduced *Enterecocci* growth (*in vitro*). Decreased *Enterecocci* load in feces.	[81]
Escherichia coli Nissle 1917	*Vibrio cholerae* (*Vc*)	Sense-respond production and secretion and self-lysis	Artilysin 85	QS (CAI-1) sensing: CqsS/LuxU-LuxO two component system and CRISPRi system arabinose inducible promoter: pBAD secretion tag: YepF signal Self-lysis: Artilysin 85	*In vitro*	Artilysin produced by ∼10^8 *E. coli* Nissle 1917 cells was sufficient for effective killing of ∼10^8 *Vc* cells.	[82]
Bacillus subtilis	*Listeria monocytogenes* (*Lm*)	Spore surface display	*Lactobacillus rhamnosus* p75 protein (peptidoglycan hydrolase)	Constitutive promoter: pCotG anchor protein: CotG	*In vitro*	∼Upto 100% activity of displayed p75. Reduction in viable *Lm* cells after incubation with CotG-p75. Surface deformation on *Lm* cells.	[79]
Saccharomyces boulardii CNCM I-745	*Listeria monocytogenes* (*Lm*)	Production and secretion	Leucocin C	Constitutive promoter: pTEF1 secretion tag: alpha mating factor signal	*In vitro*	93% killing of the *Lm* cells (initial load 6.8 × 10^8 CFU/mL).	[80]
Lactobacillus lactis NZ9000	*Enterococcus faecalis* (*Ef*)	Sense-respond production and secretion	Enterocin A, hiracin JM79, enterocin P	QS (cCF-10) sensing: PrgZ :receptor, PrgX: repressor, PrgX-cCF-10 regulated promoter: pPgQ reporter: lacZ secretion tag: usp45	*In vitro*	Decrease in *Ef* cell viability when cocultured with three-peptide system.	[78]

infections [78,81]. Similar to QS-activated antibiofilm designs, sensors for QS molecules or inflammation biomarkers can be integrated into AMP-producing probiotics, conserving the probiotic's metabolic resources until they are needed. In some cases, this sensory-based approach was also utilized to trigger the release of intracellularly accumulated therapeutic molecules through self-lysis [82]. Using self-lysis in lieu of secretion facilitates biocontainment and can increase the dose of difficult-to-secrete molecules.

In addition to the proteins described above, clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated genes (Cas) are a promising alternative strategy for fighting pathogens [83]. As an innate bacterial defense mechanism against bacteriophages and other mobile genetic elements, CRISPR-Cas systems can be repurposed to target gastrointestinal pathogens [84]. Guide RNAs can be designed to direct Cas endonucleases to cleave virulence genes or other essential genes, eliminating pathogenesis or pathogen survival in the gut. These large RNA-protein complexes can be delivered to pathogenic bacteria via engineered phages. Recently, a CRISPR-Cas13 system targeting antimicrobial resistance genes (*blaIMP-1*, *metA*) was delivered to carbapenem-resistant *E. coli* and methicilin-resistant *S. aureus* in the M13 phage capsid [85•]. The targeting specificity and efficiency of this CRISPR-Cas13a system was demonstrated on a mixed microbial population [85•]. Furthermore, engineered probiotics can be engineered to deliver CRISPR-Cas systems into pathogens via horizontal gene transfer. Various CRISPRi [86,87] and CRISPR-Cas9 [88] systems targeted against mobile integrins, virulence genes, and antimicrobial resistance genes were implemented in conjugation-proficient *E. coli* strains. Conjugation with pathogenic bacteria enabled transfer of the therapeutic DNA cargo, reducing antibiotic resistance, growth, and toxicity.

Outlook

The studies collected in this review highlight the need for synergistic teams of microbiologists, immunologists, chemists, synthetic biologists, and clinicians in developing next-generation 'living' therapies against GI infections. To realize the promise of these therapies, there are several obstacles that remain, including fundamental unanswered questions regarding gut pathogenesis, antivirulence therapies, and features of engineered commensal microbes. Regarding pathogenesis, mathematical models of GI infections can provide mechanistic and biological insights into infection progression, allowing efficient design of therapeutics [89]. In addition, studying the ecological dynamics between the gut pathogens and probiotics through the use of culturomics and genomics will reveal the molecular interactions between the pathogen and the probiotic, informing which probiotic chassis is most suitable for delivering the therapeutic cargo to a particular pathogen [90,91]. Regarding antivirulence therapies, elucidating the roles of mixing, diffusion, and degradation of drugs in the gut is critical to better define the localization of therapeutic microbes and improve the biomolecular design of antivirulence therapeutics. Furthermore, the ability of microbes to resist antivirulence therapies is severely understudied and warrants further research into their safety profile and off-target effects. Regarding the engineered microbe itself, additional knowledge on the kinetics of infection biomarkers is needed to tailor the sustained release of therapeutics by microbes [71•,92] in terms of both volume and rate of secretion [93]. Similarly, model-based approaches can be harnessed in the design of engineered therapeutic functions to maximize the synthesis of therapeutic cargo meanwhile minimizing the metabolic burden introduced by the engineered functions [94,95]. Functional and combinatorial screening of transcriptional regulators in the probiotic chassis will enable graded therapeutic production by engineered microbe [96,97]. Furthermore, probiotics can be engineered with both diagnostic and therapeutic functions to facilitate 'point-of-care' drug synthesis by the living therapy [98,99]. In addition, genetic modifications can be introduced to the engineered microbial cells to facilitate their perseverance in the infection site, for example leading to their binding to pathogen cells, mucus lining and/or inflamed tissue [100,101]. Finally, high-throughput assays to measure the inhibition of virulence activity *in vitro* and *in vivo*, in tandem with rational design approaches, are needed to accelerate the identification and characterization of therapeutic leads. Responding to these exciting challenges will demonstrate the effectiveness of scalable, easy-to-administer probiotics against a broad host of pathogens and establish them as next-generation therapeutics.

Conflict of interest statement

Nothing declared.

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