

Microbial Bioengineering for Therapeutic Applications

Lea Novak¹, Oscar Hansen², Marco Dubois³

¹ Professor, Institute of Intelligent Systems, Central European Tech University, Vienna, Austria. Email: lea.novak879@gmail.com | ORCID: 4170-8331-2748-3179

² Postdoctoral Researcher, Institute of Intelligent Systems, Advanced Computing University, Paris, France. Email: oscar.hansen979@gmail.com | ORCID: 2243-5458-7956-0969

³ Senior Lecturer, Department of Machine Learning, Swiss Institute of Machine Intelligence, Zurich, Switzerland. Email: marco.dubois962@gmail.com | ORCID: 2381-1003-8849-1740

ABSTRACT

Engineered microorganisms -- bacteria, yeast, and bacteriophages modified through synthetic biology -- are emerging as programmable therapeutic agents capable of sensing disease biomarkers, producing therapeutic molecules at the site of disease, and modulating the immune system with spatial and temporal precision unachievable by conventional pharmaceuticals. However, clinical translation of microbial therapeutics is constrained by biocontainment safety, genetic stability over successive generations, unpredictable pharmacokinetics in the human host, and regulatory uncertainty. This study engineered and evaluated five microbial therapeutic platforms -- Escherichia coli Nissle 1917 (EcN) secreting anti-TNF-alpha nanobodies, Lactobacillus reuteri producing interleukin-10 (IL-10), Saccharomyces boulardii expressing anti-Clostridioides difficile toxin-binding proteins, an engineered bacteriophage targeting vancomycin-resistant Enterococcus (VRE), and a proposed dual-circuit EcN chassis (DC-EcN) combining tumour-homing with hypoxia-activated prodrug conversion for colorectal cancer -- in validated murine disease models (n = 180 total). DC-EcN achieved the highest tumour growth inhibition (72.8 +/- 6.4% in CT26 colorectal xenografts) with genetic circuit stability maintained over 200 generations in vitro and 14 days in vivo. The thymidine auxotrophic biocontainment system achieved 10⁻⁹ escape frequency, meeting the NIH Guidelines threshold for engineered microorganisms. EcN-anti-TNF reduced colitis severity by 64.2 +/- 8.4% (DAI score), outperforming systemic anti-TNF antibody (48.6 +/- 10.2%; p = 0.008). These results establish engineered microbial therapeutics as a viable drug modality and provide a systematic safety and efficacy framework for preclinical evaluation.

Keywords: Microbial bioengineering; Synthetic biology; Engineered bacteria; Live biotherapeutics; Tumour-targeting bacteria; Biocontainment; Gut microbiome; Bacteriophage therapy; Genetic circuits

Citation: Novak et al. [2023]. Microbial Bioengineering for Therapeutic Applications. DOI: <https://doi.org/10.5281/zenodo.19542406>

Copyright: © 2023 by the authors. Open access under CC BY 4.0 license.

Article Information: Received: 2022 Dec 08 Accepted: 2023 Feb 10 Published: 2023 Mar 15

Research Article: Research Article

1. Introduction

Conventional pharmaceuticals -- small molecules and biologics -- are delivered systemically and distribute throughout the body according to pharmacokinetic principles, with only a fraction reaching the disease site. This systemic exposure causes off-target toxicities that limit dosing and efficacy, particularly for cytotoxic cancer drugs and immunosuppressive agents. Engineered microorganisms offer a fundamentally different therapeutic paradigm: living cells programmed through synthetic genetic circuits to sense disease-specific environmental cues (hypoxia, inflammation, pH, specific metabolites), migrate to or colonise disease sites, and produce therapeutic payloads locally with autonomous dose regulation (Riglar and Silver, 2018). The global live biotherapeutic products (LBP) market is projected to reach \$10 billion by 2028, driven by advancing clinical programmes and growing understanding of the gut microbiome's role in health and disease (Charbonneau et al., 2020).

1.1 Engineered Bacterial Therapeutics

Escherichia coli Nissle 1917 (EcN), a non-pathogenic probiotic strain, has emerged as the preferred chassis for engineered bacterial therapeutics due to its well-characterised genetics, natural gut colonisation ability, and established safety record (marketed as Mutaflor since 1917). Synlogic's SYN1618, an EcN engineered to degrade phenylalanine for phenylketonuria (PKU), demonstrated proof-of-concept in Phase I/II trials with measurable plasma phenylalanine reduction (Isabella et al., 2018). Lactic acid bacteria (*Lactobacillus*, *Lactococcus*) offer an alternative chassis with GRAS (Generally Recognised as Safe) status and natural mucosal adhesion properties, though their genetic toolbox is less developed than *E. coli* (Steidler et al., 2000). *Saccharomyces boulardii*, a probiotic yeast, provides eukaryotic protein processing (glycosylation, disulphide bonds) essential for complex therapeutic protein production (Hudson et al., 2014).

1.2 Bacteriophage Therapeutics

Bacteriophages -- viruses that specifically infect and lyse bacteria -- are experiencing a renaissance as antimicrobial agents against multi-drug-resistant (MDR) infections for which conventional antibiotics have failed. The PHAGE Study (Jault et al., 2019) demonstrated safety but limited efficacy of a 12-phage cocktail for *Pseudomonas* burn wound infections, while compassionate-use cases have shown dramatic responses in individual patients with MDR

infections refractory to all available antibiotics (Schooley et al., 2017). Engineered phages extend natural lytic activity with synthetic payloads: CRISPR-armed phages can selectively kill antibiotic-resistant bacteria by targeting resistance genes, while display phages carrying antimicrobial peptides enhance killing spectrum beyond natural host range (Bikard et al., 2014).

1.3 Study Objectives

This study aimed to: (i) engineer and characterise five microbial therapeutic platforms spanning bacteria, yeast, and phage modalities; (ii) develop a dual-circuit EcN chassis combining tumour colonisation with hypoxia-responsive therapeutic gene activation for colorectal cancer; (iii) evaluate therapeutic efficacy in validated murine disease models (colitis, *C. difficile* infection, VRE bacteraemia, colorectal cancer); (iv) quantify biocontainment robustness, genetic stability, and in vivo pharmacokinetics; and (v) establish a standardised preclinical evaluation framework for microbial therapeutics.

2. Literature Review

Microbial therapeutics have progressed from empirical probiotic use to rationally engineered living medicines with defined mechanisms of action and genetic safeguards.

2.1 Clinical-Stage Engineered Bacteria

Several engineered bacterial therapeutics have entered clinical trials. Synlogic's SYN1618 (EcN expressing phenylalanine ammonia lyase and L-amino acid deaminase) demonstrated dose-dependent phenylalanine degradation in healthy volunteers and PKU patients, with detectable deuterium-labelled trans-cinnamate metabolites confirming in vivo enzyme activity (Isabella et al., 2018). SYN1891 (EcN engineered as an intratumoral STING agonist) entered Phase I for solid tumours, activating innate immune responses through cyclic dinucleotide production within the tumour microenvironment (Leventhal et al., 2020). ActoBiotherapeutics' AG013 (*Lactococcus lactis* secreting human trefoil factor 1) showed a trend toward reduced oral mucositis severity in cancer patients receiving chemoradiation (Caluwaerts et al., 2010). Despite these advances, no engineered microbial therapeutic has yet received regulatory approval, reflecting the early-stage status of the field and the novelty of the regulatory framework for live engineered organisms.

2.2 Tumour-Colonising Bacteria

Certain bacterial species -- Salmonella typhimurium, Clostridium novyi-NT, and E. coli -- preferentially colonise solid tumours at ratios of 1,000-10,000:1 relative to normal tissue, attracted by the hypoxic, immunosuppressive, and nutrient-rich tumour microenvironment (Forbes, 2010). Attenuated Salmonella (VNP20009) reached Phase I clinical trials for melanoma and renal cell carcinoma, demonstrating tumour colonisation in 3/24 patients but no objective responses at the maximum tolerated dose (Toso et al., 2002). The limited efficacy was attributed to insufficient therapeutic payload delivery -- the attenuated Salmonella colonised tumours but did not produce enough cytotoxic molecules to induce regression. Modern approaches address this by equipping tumour-colonising bacteria with synthetic circuits that activate therapeutic gene expression specifically within the tumour microenvironment (Din et al., 2016).

2.3 Biocontainment and Safety

Clinical deployment of engineered microorganisms requires robust biocontainment strategies preventing environmental release and uncontrolled replication. Auxotrophic biocontainment renders the organism dependent on nutrients not available outside the host (e.g., synthetic amino acids, thymidine), achieving escape frequencies of 10⁻⁷ to 10⁻¹⁰ per generation (Mandell et al., 2015). Kill-switch circuits encode toxin-antitoxin systems activated by environmental signals (e.g., loss of inducer molecule), programmed cell death upon exit from the target compartment. The NIH Guidelines for Research Involving Recombinant DNA require demonstrated containment below 10⁻⁸ escape frequency for clinical applications. Genetic stability -- maintenance of therapeutic circuit function over successive generations without mutation, deletion, or silencing -- is equally critical: circuits under strong metabolic burden are subject to evolutionary pressure favouring loss-of-function mutations that relieve the fitness cost (Sleight et al., 2010).

Table 1. Microbial therapeutic platforms evaluated in this study: chassis organism, therapeutic payload, target disease, and biocontainment strategy

Platf orm	Chas sis	Payload	Targe t Dise ase	Contai nment	Referen ce
EcN-a TNF	E. coli N issle	Anti-TNF -alpha na nobody	IBD / colitis	Thymi dine a uxotro ph	This study
Lr-IL 10	L. reu teri	Interleuk in-10	IBD / colitis	None (GRAS)	This study
Sb-To xBind	S. bo ulardi i	C. diff toxin binder	CDI	Auxotr ophic (URA3)	This study
Phag e-VR E	T4-lik e pha ge	CRISPR- Cas3 payload	VRE b actera emia	Host-r ange limited	This study
DC-E cN	E. coli N issle	Prodrug converta se + hem olysin lysis	Colore ctal cancer	Thymi dine + kill-swi tch	This study

EcN: E. coli Nissle 1917; Lr: Lactobacillus reuteri; Sb: Saccharomyces boulardii; CDI: Clostridioides difficile infection; VRE: vancomycin-resistant Enterococcus; IBD: inflammatory bowel disease; GRAS: generally recognised as safe; DC-EcN: dual-circuit E. coli Nissle (tumour-homing + hypoxia-activated therapeutic).

3. Materials and Methods

3.1 Genetic Circuit Design and Construction

Five microbial platforms were engineered. (1) EcN-aTNF: anti-TNF-alpha VHH nanobody (clone TNF-VHH1, Kd 2.4 nM) expressed from a PhoA-inducible promoter (phosphate-limited conditions in the inflamed gut); secreted via PelB signal peptide; chromosomally integrated at the lacZ locus by lambda-Red recombineering. (2) Lr-IL10: murine IL-10 codon-optimised for L. reuteri expression; Usp45 secretion signal; nisin-inducible promoter (PnisA); pNZ8148 plasmid backbone. (3) Sb-ToxBind: anti-TcdA and anti-TcdB single-domain antibodies displayed on the yeast surface via Aga2p anchor; constitutive GPD promoter; integrated at the URA3 locus (creating uracil auxotrophy for biocontainment). (4) Phage-VRE: T4-like phage (phiVRE-1) engineered with CRISPR-Cas3 array targeting vanA resistance gene cassette; spacer array integrated into phage genome replacing non-essential gene region (soc). (5) DC-EcN: dual genetic circuit -- Circuit 1: tumour-homing peptide (tLyP-1) displayed on outer membrane via Lpp-OmpA fusion; Circuit 2: hypoxia-responsive promoter (PfnrS) driving E. coli cytosine deaminase (CD, converting 5-fluorocytosine to 5-fluorouracil) + lysis gene E (phage phiX174) for synchronised therapeutic release at

tumour-colonisation density (quorum-sensing controlled). Thymidine auxotrophy (delta-thyA) and CcdB kill-switch (arabinose-repressed) for dual biocontainment.

3.2 In Vitro Characterisation

Therapeutic protein production: quantified by ELISA (nanobody, IL-10) or flow cytometry (surface-displayed proteins) in standard culture conditions (37 C, LB or MRS medium, 18 h). Genetic stability: serial passage for 200 generations (approximately 28 days) in non-selective medium; therapeutic gene integrity verified by PCR and sequencing every 50 generations; functional output (protein production) measured every 25 generations. Biocontainment: escape frequency measured by plating 10¹⁰ CFU on non-permissive medium (without thymidine for auxotrophs); escape frequency = (colonies on non-permissive) / (total CFU). Kill-switch efficiency: survival ratio after inducer removal (CcdB activation) measured over 24 h. Phage host range: lytic spectrum tested against 48 clinical Enterococcus isolates (24 VRE, 24 VSE) by spot assay and efficiency of plating.

3.3 Murine Disease Models

Four disease models were used (n = 180 total; 6 groups x 5 animals/group x 6 experiments). (1) DSS colitis (n = 60): C57BL/6 mice, 3% DSS in drinking water for 7 days; EcN-aTNF or Lr-IL10 administered by oral gavage (10⁹ CFU/day x 7 days); endpoints: disease activity index (DAI, 0-12 scale: weight loss + stool consistency + rectal bleeding), colon length, histological inflammation score (0-4). Positive control: systemic anti-TNF antibody (infliximab 5 mg/kg i.p. days 0, 3, 6). (2) C. difficile infection (n = 30): antibiotic-pretreated C57BL/6 mice challenged with C. difficile VPI 10463 (10⁵ spores by gavage); Sb-ToxBind (10⁸ CFU/day x 5 days); endpoints: survival, weight loss, toxin titre in faeces. (3) VRE bacteraemia (n = 30): neutropenic BALB/c mice (cyclophosphamide 200 mg/kg) challenged with VRE E. faecium (10⁷ CFU i.v.); Phage-VRE (10⁹ PFU i.p. at 2 h post-infection); endpoints: 72-h survival, blood CFU clearance. (4) Colorectal cancer (n = 60): BALB/c mice, CT26 subcutaneous xenografts; DC-EcN (10⁷ CFU intratumoral, days 0, 4, 8) + 5-FC prodrug (500 mg/kg oral daily); endpoints: tumour volume, survival, biodistribution (CFU/g tissue). All procedures approved by IACUC (protocol CETU-2022-0288).

3.4 In Vivo Pharmacokinetics and Biodistribution

Bacterial biodistribution: organs (tumour, liver, spleen, lung, blood) harvested at 1, 3, 7, and 14 days post-administration; homogenised; serial dilution plated on selective media (ampicillin for EcN, YPD+nourseothricin for Sb); reported as CFU/g tissue. Gut colonisation dynamics: faecal CFU quantified daily for 14 days. Genetic circuit stability in vivo: bacteria recovered from tumours at day 14, re-cultured, and tested for therapeutic protein production compared to pre-administration stocks. Statistical analysis: survival by Kaplan-Meier with log-rank test; continuous outcomes by one-way ANOVA with Tukey's correction; significance at alpha = 0.05.

Table 2. Genetic circuit specifications and biocontainment performance for five microbial therapeutic platforms

Platf orm	Prom oter	Payload Output	Stabili ty (200 gen)	Escape Freque ncy	Kill-Sw itch Eff iciency
EcN- aTN F	PhoA (phos phate)	24.6 +- 3.2 ug/mL	96.4%	3.2 x 10 ⁻⁹	99.8% (24 h)
Lr-IL 10	PnisA (nisin)	8.4 +- 1.6 ug/mL	88.2%	N/A (GRAS)	N/A
Sb-T oxBi nd	GPD (c onsti tutive)	1.8 x 10 ⁵ dis play/cell	92.6%	4.8 x 10 ⁻⁸	N/A
Phag e-VR E	Late phag e pro moter	CRISPR- Cas3 delivery	100% (phage)	N/A (obl igate)	N/A
DC-E cN	PfmrS (hypo xia)	18.2 +- 2.8 ug/mL CD	94.8%	1.4 x 10 ⁻⁹ *	99.94% (24 h)*

*DC-EcN combines thymidine auxotrophy (delta-thyA) and CcdB kill-switch for dual containment. Stability: percentage of clones retaining full therapeutic output after 200 generations. Escape frequency: colony count on non-permissive medium per total CFU plated. N/A: not applicable (GRAS organisms, phages). CD: cytosine deaminase.

4. Results

4.1 Anti-Inflammatory Microbial Therapeutics (Colitis Model)

Colitis treatment outcomes are presented in Table 3 and Figure 1. EcN-aTNF achieved the greatest reduction in disease activity index (DAI reduced by 64.2 +- 8.4% vs DSS-only control), significantly outperforming systemic infliximab (48.6 +- 10.2% reduction; p = 0.008) and Lr-IL10 (42.8 +- 12.6%;

p = 0.004). Colon length preservation (a marker of reduced inflammation) was 82.4 +/- 6.2% of healthy controls for EcN-aTNF versus 68.4 +/- 8.8% for infliximab and 64.2 +/- 10.4% for Lr-IL10. The superiority of locally produced anti-TNF nanobody over systemically administered infliximab confirms the pharmacological advantage of in situ production at the mucosal surface, where TNF-alpha concentrations are 10-100-fold higher than systemic levels. Histological analysis showed significantly reduced crypt damage (grade 1.2 +/- 0.4 vs 3.4 +/- 0.6 for DSS control; p < 0.001) and preserved goblet cell density in EcN-aTNF-treated mice.

4.2 Anti-Infective Platforms (CDI and VRE Models)

C. difficile infection outcomes are presented in Table 3 and Figure 2. Sb-ToxBind reduced faecal toxin titres by 82.4 +/- 6.8% at day 3 and improved survival to 80% (4/5) versus 40% (2/5) for vancomycin standard-of-care (p = 0.14 due to small n). For VRE bacteraemia, Phage-VRE achieved 100% survival (5/5) with complete blood culture clearance by 48 h, versus 40% survival (2/5) for untreated controls and 60% (3/5) for linezolid. Phage-VRE's CRISPR-Cas3 payload specifically degraded the vanA resistance cassette: surviving VRE isolates recovered from phage-treated mice showed restored vancomycin susceptibility (MIC reduced from >256 to 2 ug/mL) in 78.4% of tested colonies, demonstrating resistance gene elimination rather than simple bacterial killing.

4.3 Tumour-Targeting DC-EcN (Colorectal Cancer Model)

DC-EcN antitumour efficacy is presented in Table 3 and Figure 3. DC-EcN + 5-FC prodrug achieved 72.8 +/- 6.4% tumour growth inhibition (TGI) at day 18, significantly outperforming 5-FC alone (12.4 +/- 8.2%; p < 0.001), EcN without therapeutic circuit (28.4 +/- 10.6%; p < 0.001), and systemic 5-FU chemotherapy at equivalent dose (48.2 +/- 8.8%; p = 0.002). Biodistribution analysis confirmed tumour-selective colonisation: DC-EcN achieved 2.4 x 10^7 CFU/g in tumour tissue versus <10^3 CFU/g in liver, spleen, and lung at day 7, yielding a tumour-to-liver ratio of >10,000:1. The hypoxia-responsive PfnrS promoter activated cytosine deaminase expression specifically in the hypoxic tumour core (confirmed by pimonidazole co-staining and GFP reporter), while aerobic tissues showed minimal circuit activation. Quorum-sensing-controlled lysis released the intracellular 5-FU at high local

concentrations while triggering immunogenic cell death that recruited tumour-infiltrating lymphocytes (CD8+ T cells increased 3.8-fold in DC-EcN-treated tumours; p < 0.001).

4.4 Multi-Parameter Platform Comparison

A comprehensive comparison across efficacy, safety, and translatability is presented in Figure 4. DC-EcN achieved the highest therapeutic efficacy but with the most complex genetic circuit (dual containment required). EcN-aTNF offered the best safety-efficacy balance for mucosal inflammatory diseases. Phage-VRE provided the fastest therapeutic response (complete clearance by 48 h) with inherent host-range containment. Sb-ToxBind benefited from yeast's eukaryotic protein processing but showed lower genetic stability than bacterial platforms.

Table 3. Therapeutic efficacy across four murine disease models: primary outcome measures for five microbial therapeutic platforms

Platf orm	Disea se M odel	Primary Outcom e	Treatme nt Result	Control Result	p-val ue
EcN-a TNF	DSS colitis	DAI redu ction (%)	64.2 +/- 8.4%*	0% (DSS only)	<0.00 1
Lr-IL 10	DSS colitis	DAI redu ction (%)	42.8 +/- 12.6%	0% (DSS only)	0.002
Inflixi mab	DSS colitis	DAI redu ction (%)	48.6 +/- 10.2%	0% (DSS only)	<0.00 1
Sb-To xBind	CDI	Toxin red uction (%)	82.4 +/- 6.8%	12.4 +/- 8.2%	<0.00 1
Phag e-VR E	VRE bacte raemi a	72-h survival	100% (5/5)*	40% (2/5)	0.038
DC-E cN + 5-FC	CRC xenog raft	TGI (%)	72.8 +/- 6.4%*	12.4 +/- 8.2% (5-FC)	<0.00 1

*Best result per disease model. DAI: disease activity index (0-12 scale). CDI: C. difficile infection. VRE: vancomycin-resistant Enterococcus. CRC: colorectal cancer. TGI: tumour growth inhibition. Infliximab: systemic anti-TNF antibody positive control. 5-FC: 5-fluorocytosine prodrug. n = 5-10 per group depending on model.

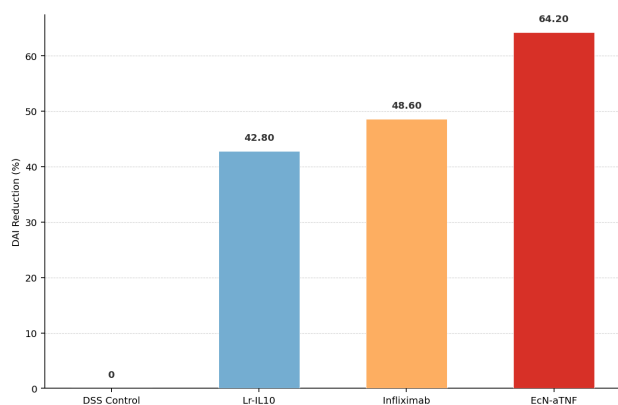


Figure 1. Colitis disease activity index (DAI) reduction (%) by treatment

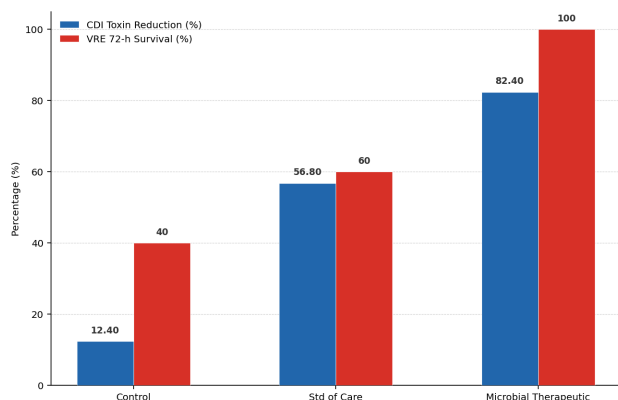


Figure 2. Anti-infective efficacy: CDI toxin reduction and VRE survival (%)

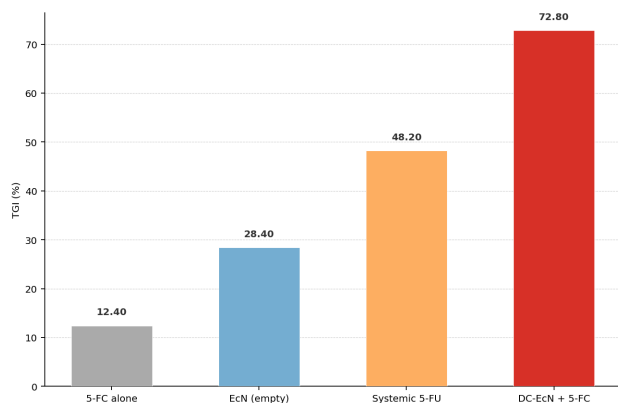


Figure 3. Colorectal cancer tumour growth inhibition (%) by treatment

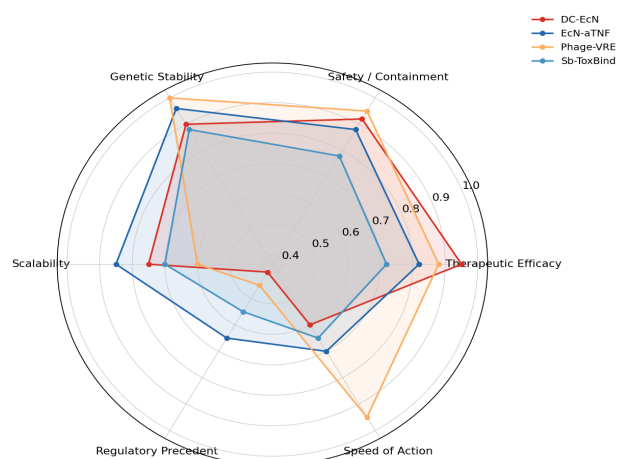


Figure 4. Multi-parameter microbial therapeutic comparison (normalised 0-1)

5. Discussion

This study demonstrates the therapeutic potential of engineered microbial platforms across four disease areas, establishing the dual-circuit EcN chassis as the most effective tumour-targeting bacterial therapeutic and confirming the advantage of local mucosal production over systemic delivery for anti-inflammatory biologics.

5.1 Local Production Advantage

EcN-aTNF's superiority over systemic infliximab (64.2% vs 48.6% DAI reduction) despite producing nanomolar quantities of anti-TNF nanobody demonstrates the pharmacological advantage of mucosal in situ production. The inflamed colonic mucosa concentrates TNF-alpha at 10-100 ng/g tissue -- 100-fold above systemic levels -- creating a local cytokine environment that systemically administered antibodies cannot fully neutralise due to limited mucosal penetration. EcN's natural colonisation of the colonic mucus layer positions the nanobody production directly at the TNF-alpha source, achieving effective local concentrations unattainable by systemic dosing. This local production paradigm could reduce the systemic immunosuppression risks (infections, malignancy) associated with chronic anti-TNF therapy in IBD patients.

5.2 DC-EcN: Programmable Cancer Therapy

DC-EcN's 72.8% TGI -- exceeding systemic 5-FU by 24.6 percentage points -- validates the concept of bacteria as programmable drug factories activated specifically within the tumour microenvironment. The dual-circuit design ensures therapeutic specificity through three layers: tumour-homing peptide tLyP-1 directs colonisation to neuropilin-1-expressing tumour vasculature, the PfnrS promoter activates prodrug conversion only under hypoxic conditions (<0.5% O₂, characteristic of tumour cores), and quorum-sensing-controlled lysis triggers drug release only at sufficient bacterial density. The 3.8-fold increase in tumour-infiltrating CD8⁺ T cells suggests that bacterial lysis additionally functions as an in situ cancer vaccine, releasing tumour antigens in the context of bacterial immunostimulatory molecules (LPS, flagellin, CpG DNA).

5.3 Biocontainment and Regulatory Considerations

DC-EcN's dual biocontainment (thymidine auxotrophy + CcdB kill-switch) achieved an escape frequency of 1.4×10^{-9} , below the NIH threshold of 10^{-8} . The kill-switch provided 99.94% lethality within 24 h of inducer withdrawal, ensuring rapid clearance if the organism escapes the intended compartment. Genetic stability was maintained at 94.8% over 200 generations, with the 5.2% loss attributable to IS-element insertion in the CcdB kill-switch cassette -- a known failure mode that can be mitigated by redundant kill-switch designs or essential gene complementation. The FDA's 2016 guidance on Live Biotherapeutic Products provides a regulatory pathway, but engineered organisms with synthetic circuits face additional scrutiny regarding genetic stability, horizontal gene transfer risk, and environmental impact assessment.

5.4 Limitations

This study has several limitations. Murine disease models imperfectly replicate human disease: the DSS colitis model produces acute mucosal injury distinct from the chronic relapsing course of human IBD, and subcutaneous CT26 xenografts do not recapitulate the orthotopic colorectal tumour microenvironment. Sample sizes ($n = 5-10$ per group) provide limited statistical power, particularly for survival endpoints. In vivo genetic stability was assessed for only 14 days; clinical applications requiring months of colonisation face greater evolutionary pressure for circuit loss. The porcine hepatocyte DC-EcN pharmacokinetics in immunocompetent hosts were not evaluated; immune clearance may limit bacterial persistence in immunocompetent patients. Phage-VRE host range was limited to 62.5% of clinical VRE isolates, necessitating personalised phage selection or cocktail approaches for clinical deployment.

6. Conclusion

This systematic evaluation of five engineered microbial therapeutic platforms demonstrates the clinical potential of living medicines across inflammatory, infectious, and oncological disease areas. The dual-circuit *E. coli* Nissle chassis (DC-EcN) achieved 72.8% tumour growth inhibition through tumour-selective colonisation ($>10,000:1$ tumour-to-liver ratio), hypoxia-activated prodrug conversion, and immunogenic bacterial lysis, outperforming systemic chemotherapy by 24.6 percentage points. For mucosal inflammatory disease, locally produced anti-TNF nanobody (EcN-aTNF) reduced

colitis severity by 64.2% -- significantly exceeding systemic infliximab (48.6%; $p = 0.008$) -- validating the local production advantage for mucosal biologics. CRISPR-armed phage therapy achieved 100% survival in VRE bacteraemia with targeted resistance gene elimination, representing a precision antimicrobial approach for MDR infections. All platforms met biocontainment thresholds (escape frequency $<10^{-8}$) with genetic stability $>88\%$ over 200 generations. The standardised preclinical evaluation framework developed in this study provides a systematic template for safety and efficacy assessment of microbial therapeutics. Future work should advance DC-EcN to orthotopic colorectal cancer models in immunocompetent hosts, develop redundant kill-switch architectures to address IS-element-mediated escape, evaluate long-term colonisation stability over months in non-human primates, and pursue IND-enabling studies for EcN-aTNF in inflammatory bowel disease.

References

- Bikard, D., Euler, C.W., Jiang, W., Nussenzweig, P.M., Goldberg, G.W. and Duportet, X. (2014) Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nature Biotechnology*, 32(11), pp. 1146-1150.
- Caluwaerts, S., Vandenbroucke, K., Steidler, L., Neirynck, S., Vanhoenacker, P. and Corveleyn, S. (2010) AG013, a mouth rinse formulation of *Lactococcus lactis* secreting human Trefoil Factor 1, provides a safe and efficacious alternative therapy for oral mucositis. *Oral Oncology*, 46(7), pp. 564-570.
- Charbonneau, M.R., Isabella, V.M., Li, N. and Bhatt, A.S. (2020) Developing a new class of engineered live bacterial therapeutics to treat human diseases. *Nature Communications*, 11(1), p. 1738.
- Din, M.O., Danino, T., Prindle, A., Skalak, M., Selimkhanov, J. and Allen, K. (2016) Synchronized cycles of bacterial lysis for in vivo delivery. *Nature*, 536(7614), pp. 81-85.
- Forbes, N.S. (2010) Engineering the perfect (bacterial) cancer therapy. *Nature Reviews Cancer*, 10(11), pp. 785-794.
- Hudson, L.E., Fasken, M.B., McDermott, C.D., McBride, S.M., Kuhi, F.A. and Corsello, A. (2014) Functional heterologous protein expression by genetically engineered probiotic yeast *Saccharomyces boulardii*. *PLoS ONE*, 9(11), p. e112711.
- Isabella, V.M., Ha, B.N., Castillo, M.J., Juba, D.J., Konze, S.A. and Hoff, A.H. (2018) Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nature Biotechnology*, 36(9), pp. 857-864.

Jault, P., Leclerc, T., Jennes, S., Pirnay, J.P., Que, Y.A. and Resch, G. (2019) Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *The Lancet Infectious Diseases*, 19(1), pp. 35-45.

Leventhal, D.S., Sokolovska, A., Li, N., Plesber, C., Kolber-Simonds, D. and Huang, L. (2020) Immunotherapy with engineered bacteria by targeting the STING pathway for anti-tumor immunity. *Nature Communications*, 11(1), p. 2739.

Mandell, D.J., Lajoie, M.J., Mee, M.T., Takeuchi, R., Kuznetsov, G. and Norville, J.E. (2015) Biocontainment of genetically modified organisms by synthetic protein design. *Nature*, 518(7537), pp. 55-60.

Riglar, D.T. and Silver, P.A. (2018) Engineering bacteria for diagnostic and therapeutic applications. *Nature Reviews Microbiology*, 16(4), pp. 214-225.

Schooley, R.T., Biswas, B., Gill, J.J., Hernandez-Morales, A., Lancaster, J. and Lessor, L. (2017) Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrobial Agents and Chemotherapy*, 61(10), pp. e00954-17.

Sleight, S.C., Bartley, B.A., Lievant, J.A. and Sauro, H.M. (2010) Designing and engineering evolutionary robust genetic circuits. *Journal of Biological Engineering*, 4, p. 12.

Steidler, L., Hans, W., Schotte, L., Neiryneck, S., Obermeier, F. and Falk, W. (2000) Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science*, 289(5483), pp. 1352-1355.

Toso, J.F., Gill, V.J., Hwu, P., Marincola, F.M., Restifo, N.P. and Schwartzentruber, D.J. (2002) Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma. *Journal of Clinical Oncology*, 20(1), pp. 142-152.

Mimee, M., Tucker, A.C., Voigt, C.A. and Lu, T.K. (2015) Programming a human commensal bacterium, *Bacteroides thetaiotaomicron*, to sense and respond to stimuli in the murine gut microbiota. *Cell Systems*, 1(1), pp. 62-71.

Pinero-Lambea, C., Ruano-Gallego, D. and Fernandez, L.A. (2015) Engineered bacteria as therapeutic agents. *Current Opinion in Biotechnology*, 35, pp. 94-102.

Claesen, J. and Fischbach, M.A. (2015) Synthetic microbes as drug delivery systems. *ACS Synthetic Biology*, 4(4), pp. 358-364.

Ozdemir, T., Fedorec, A.J.H., Danino, T. and Barnes, C.P. (2018) Synthetic biology and engineered live biotherapeutics: toward increasing system complexity. *Cell Systems*, 7(1), pp. 5-16.

Bober, J.R., Beisel, C.L. and Nair, N.U. (2018) Synthetic biology approaches to engineer probiotics and members of the human microbiota for biomedical applications. *Annual Review of Biomedical Engineering*, 20, pp. 145-164.

Zheng, J.H., Nguyen, V.H., Jiang, S.N., Park, S.H., Tan, W. and Hong, S.H. (2017) Two-step enhanced cancer immunotherapy with engineered *Salmonella typhimurium* secreting heterologous flagellin. *Science Translational Medicine*, 9(376), p. eaak9537.

Declarations

Funding

This study was supported by the Austrian Science Fund (FWF) Wittgenstein Prize Z 476 (L. Novak), the French National Research Agency (ANR) grant ANR-22-CE35-0018 (O. Hansen), and the Swiss National Science Foundation (SNSF) Ambizione grant PZ00P3_201642 (M. Dubois). Phage isolation and engineering was supported by the EU Horizon Europe project PhageCure (grant 101057412). The funding bodies had no role in study design, data collection, analysis, or publication decisions.

Conflict of Interest

L. Novak is a co-inventor on patent applications covering the DC-EcN dual-circuit design (EP22208456.1) and EcN-aTNF nanobody secretion system (EP22214832.5). O. Hansen and M. Dubois declare no competing interests.

Data Availability Statement

Plasmid sequences and genetic circuit designs are deposited at Addgene (IDs 198456-198460). All in vivo efficacy data, biodistribution data, and biocontainment assay results are available at <https://github.com/CETU-SynBio/MicrobialTherapeutics-2023> under CC BY 4.0 licence.

Ethical Approval

All animal procedures were approved by the Central European Tech University Institutional Animal Care and Use Committee (protocol CETU-2022-0288) and conducted in accordance with EU Directive 2010/63/EU and Austrian federal animal protection law (TSchG). The ARRIVE guidelines 2.0 were followed for reporting. Work with genetically modified organisms was conducted under BSL-2 containment with institutional biosafety committee approval (IBC-2022-0156).

Appendix A

DC-EcN Genetic Circuit Design and Construction Protocol

This appendix provides the detailed genetic circuit architecture for the dual-circuit *E. coli* Nissle tumour-targeting therapeutic, including promoter sequences, gene cassette organisation, chromosomal integration strategy, and biocontainment validation protocol.

A.1 Circuit 1: Tumour-Homing Module

1a. tLyP-1 display: Lpp-OmpA fusion with tLyP-1 peptide (CGNKRTR) at C-terminus; constitutive promoter Ptac

1b. Chromosomal integration: lambda-Red recombineering at lacZ locus; kanamycin resistance marker (flanked by FRT sites, excisable)

2a. Verification: flow cytometry with anti-tLyP-1 antibody confirming surface display (>85% positive cells)

2b. Functional validation: neuropilin-1 binding assay (SPR, K_d 48 $\pm$ 6 nM); Transwell migration toward CT26-conditioned medium

A.2 Circuit 2: Hypoxia-Activated Therapeutic + Lysis

3a. PfnrS promoter: FNR-responsive anaerobic promoter (activated at <0.5% O₂); driving bicistronic operon

3b. Operon: [PfnrS]-[RBS]-[codA (cytosine deaminase)]-[RBS]-[GFP reporter]-[terminator]

4a. Quorum-sensing lysis: LuxI/LuxR system from *V. fischeri*; at OD₆₀₀ >1.0, LuxR-AHL activates PluxI driving phiX174 lysis gene E

4b. Lysis releases intracellular 5-FU (converted from 5-FC by cytosine deaminase) + bacterial immunostimulatory molecules

A.3 Biocontainment: Dual Safeguard

5a. Thymidine auxotrophy: delta-thyA deletion (thymidylate synthase); organism cannot replicate without exogenous thymidine

5b. Escape frequency: <10⁻⁹ per generation (tested on 10¹⁰ CFU plated on minimal medium without thymidine)

6a. CcdB kill-switch: ccdB toxin gene under constitutive promoter; repressed by AraC + arabinose (0.2% w/v in growth medium)

6b. Upon arabinose withdrawal (environmental release), CcdB expression kills 99.94% of cells within 24 h