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Investigation of orally administrable bacteria for cancer therapy

Thesis presented by

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Table of Contents

Declaration 6
Acknowledgements 7
Abbreviations 8
Abstract 12
Chapter 1: Literature review 14
Section 1: Bacterial anticancer therapy 15
Abstract 16
Introduction 17
The bacterial biofactory: Bacteria as enzyme delivery vehicles 21
Constructing a successful BDEPT: Design principles 24
Design principle I: Small-molecule pharmacokinetics 26
Design principle II: Orientation of the enzyme 28
Design principle III: Optimisation of enzyme kinetics 31
Design principle IV: Toxicity of the prodrug/API to the bacterium 34
Design principle V: Choosing a bacterial carrier 37
Improving BDEPT R&D with tools for monitoring bacterial enzymatic activity 43
Barriers to the clinic 46
BDEPT prospects 51
Conclusion 55
Section 2: The current state of drug R&D and its effects on advanced therapies 56
Abstract 57
Introduction 58
Understanding the process flow; 'What' can be improved vs. 'Who' can be improved 60
Putting a face to the name: Who does what, and why? 64
Appreciating actor risk preferences and motivations 67
Conclusion 72
References 73
Chapter 2 – Optimised microbiome analysis of breast cancer patient tissue 97
Abstract 98
Introduction 100
Materials and Methods 104
Independent validation of clinical material 104
Clinical specimen collection, transportation, and storage 104
DNA extraction and 16S rRNA library preparation and sequencing 105

Bioinformatic analyses 106
Data analysis and visualisation 108
Results 109
Refined protocol produces bioinformatic data with increased stringency 109
Targeting different 16S rRNA gene regions of breast bacteria affects sequencing output 110
DNase treatment of human specimens does not significantly alter bacterial diversity 112
Hierarchical clustering suggests existence of distinct bacterial signatures in patient breast tumours 114
Differentially abundant bacterial species exist between breast tumour and normal adjacent tissues 115
Machine learning predictions based on bacterial signature are effective in differentiating malignant and non-malignant tissues and closely agree with the standard independent validation approach 117
Discussion 121
Conclusion 125
References 126
Chapter 3: Application of culturomics techniques to human breast tissue 131
Abstract 132
Introduction 133
Materials and Methods 136
Murine RENCA tumour induction and excision 136
Clinical specimen collection and transportation 136
Culture and Sanger-sequencing of bacterial isolates 137
16S rRNA gene quantitation of cancer patient breast tissues 139
Results 141
Development of a culture method using murine tissue 141
Culture of human specimens indicates low bacterial diversity and load 143
Culture-based assay has high sensitivity for the sample types analysed 146
Discussion 150
Conclusion 153
References 154
Chapter 4: Investigation of tumour targeting of orally administered probiotic bacteria in cancer patients 159
Abstract 160
Introduction 161

Materials and Methods 165
Assessment of probiotic bacterial translocation ex vivo 165
Assessment of the viability and variability of 'Mutaflor' and 'Alflorex' 165
Patient recruitment and specimen collection 166
Transformation of EcN 167
Animals, mammalian cell culture, and tumour induction 168
Bacterial preparation and oral/IV administration 169
Viable bacterial recovery from mice 170
Identification of viable bacteria recovered from fresh murine tissues 170
FFPE tissue processing, embedding, and DNA extraction 171
Human and murine FFPE tissue DNA extraction 171
Human fresh tissue DNA extraction 173
Human and murine stool DNA extraction 173
IF staining of murine FFPE tissues 174
Gram-staining of murine FFPE tissues 175
Determination of limits of detection of probiotic bacterial DNA against a mammalian DNA background 175
qPCR of bacterial DNA in FFPE and fresh tissues, stool samples, and spiked samples 176
Results 178
1. Work-up in vitro assays and detection strategy optimisation 178
B. breve UCC2003 does not affect gut permeability ex vivo 178
Probe-based qPCR is superior to dye-based qPCR for amplification of bacterial DNA in mammalian tissues 178
Background mammalian DNA at relevant quantities has little effect on amplification of bacterial DNA 179
Proprietary probiotic capsules have consistent bacterial quantities 181
2. Clinical study 183
qPCR for test bacteria in patient tumour tissue 183
Persistence in human stools varies between bacterial species 185
3. Post-clinical study murine studies 185
Murine model of oral administration of test bacteria to tumour-bearing mice . 185
Murine GIT colonisation by probiotic bacteria reflects pattern observed in patients 186
Murine model of IV administration of test bacteria to tumour-bearing mice 189
Bacterial DNA is amplifiable in murine FFPE tissue blocks with low limits of detection 189

IV-administered probiotic bacterial species are visualisable in murine FFPE tumour sections 191
Discussion 194
Conclusion 198
References 199
Chapter 5 - Tumour-directed evolution of bacteria 204
Abstract 205
Introduction 207
Materials and Methods 210
Bacterial and mammalian cell culture 210
Animals and tumour induction 210
Bacterial administration to mice 211
Bacterial recovery from murine tumour and liver tissues 211
Identification of bacteria recovered from murine tissues 212
Whole genome sequencing and bioinformatic analysis 212
Biofilm formation assessment using crystal violet staining and population assays 214
Biolog EcoPlate assay 214
Formalin-fixed tissue processing, embedding, and IF staining 215
Results 217
Repeated bacterial cycling through tumours can affect trends in intratumoural bacterial quantity and persistence 217
In vitro biofilm formation is significantly affected in tumour-recycled bacteria 223
Tumour recycling of bacteria promotes discrete shifts in bacterial metabolism 227
Tumour-to-tumour recycling does not result in significant changes to the bacterial genome sequence 230
Discussion 233
Conclusion 237
References 238
Chapter 6: Prospects 243
Conclusion 248
References 249

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.



Glenn Hogan

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Abbreviations

ACV	Acyclovir
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
ASV	Amplicon sequence variant
ATMP	Advanced therapy medicinal product
AUC	Area under the curve
AWCD	Average well colour development
BCG	Bacillus Calmette-Guerin
BDEPT	Bacteria-directed enzyme prodrug therapy
β-G	β-glucuronidase
BLA	Biologics license application
BL35624	*Bifidobacterium longum* ssp. *longum* 35624
bp	Base pair
CBRD	Chemical, biological, and radiopharmaceutical drug
CD	Cytosine deaminase
CDER	Center for Drug Evaluation and Research
CFU	Colony-forming unit
CHMP	Committee for Medicinal Products for Human Use
Cm	Chloramphenicol
cm	Centimetre
CNOB	6-chloro-9-nitro-5-oxo-5Hbenzo(a)phenoxazine
CPG2	Carboxypeptidase G2
CRO	Contract research organisation
Cys-HCl	L-cysteine hydrochloride
Da	Dalton
DGAM	Diluted Gifu anaerobic medium
DNA	Deoxyribonucleic acid
dpi	Days post-injection
EcN	*E. coli* Nissle 1917
EDTA	Ethylenediaminetetraacetic acid
Em	Erythromycin

EMA	European Medicines Agency
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FFPE	Formalin-fixed, paraffin-embedded
FIAU	2'-fluoro-1-beta-D-arabino-furanosyl-5-iodo-uracil
GAM	Gifu anaerobic medium
GCV	Ganciclovir
GDEPT	Gene-directed enzyme prodrug therapy
GIT	Gastrointestinal tract
h	Hour
HRP	Horseradish peroxidase
HSV-1	Herpes simplex virus type 1
HTS	High-throughput sequencing
IF	Immunofluorescent
IMP	Investigational medicinal product
Isc	Short-circuit current
IV	Intravenous
kg	Kilogram
kV	Kilovolt
l	Litre
LB	Luria Bertani
LBEm300	LB medium containing erythromycin at a concentration of 300 μg/ml
LPS	Lipopolysaccharide
M	Molar
m	Metre
MAA	Marketing authorization application
MCHB	9-amino-6-chloro-5H-benzo(a)phenoxazine-5-one
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
MRI	Magnetic resonance imaging

mRNA	Messenger RNA
MRS	de Man, Rogosa, and Sharpe
M&A	Merger and acquisition
M9G	M9 medium containing 0.4% glucose
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NDA	New drug application
ng	Nanogram
NGS	Next-generation sequencing
nM	Nanomolar
NME	New molecular entity
NTB	New therapeutic biologic
NTR	Nitroreductase
OD_{590}	Optical density at 590 nanometres
OD_{600}	Optical density at 600 nanometres
OI	Optical imaging
PBS	Phosphate-buffered saline
PBS-TS	PBS plus 0.1% tween 20 and 0.05% saponin from quillaja bark
PCR	Polymerase chain reaction
PET	Positron emission tomography
pmol	Picomole
PNP	Purine nucleoside phosphorylase
PSRI	Public sector research institution
qPCR	Quantitative polymerase chain reaction
RCA	Reinforced clostridial agar
RCM	Reinforced clostridial medium
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
R&D	Research and development
Sec	Second

SME	Small and medium-sized enterprise
SNP	Single nucleotide polymorphism
SPF	Specific-pathogen-free
TBS	Tris-buffered saline
TEAE	Treatment-emergent adverse event
TK	Thymidine kinase
TTO	Technology transfer office
UPRT	Uracil phosphoribosyl transferase
VDEPT	Virus-directed enzyme prodrug therapy
WGS	Whole-genome sequencing
5-FC	5-fluorocytosine
6MeP	6-methylpurine
6MeP-dR	6-methylpurine-2-deoxyriboside
9-AC	9-aminocamptothecin
9-AG	9-aminocamptothecin glucuronide
ºC	Degrees Celsius
µF	Microfarad
µg	Microgram
µl	Microlitre
µM	Micromolar

Abstract

Diminished progress in improving prognoses and treatment outcomes of several cancers, using conventional drugs, is being countered by investigations into a range of new, advanced formulations, of which bacteria-based anticancer therapies are one. Bacteria-based treatments hold an especially intriguing place within this group of disruptive medical technologies for the well-documented ability of bacteria to selectively colonise tumours following IV administration and replicate therein. Based on this property, bacteria have been studied both clinically and preclinically to evaluate their potential to promote tumour regression and confine noxious effects to the malignant tissue. Some of these studies have attempted to engineer the bacteria genetically to reduce their virulence and/or accentuate their capability to exert tumour-selective damage, but efforts to date have failed to penetrate clinical barriers and produce a safe, effective, and marketable bacteria-based product for cancer therapy.

This thesis examines ways in which bacteria that are truly optimised for cancer treatment might be acquired and/or developed, using data derived from patient (Chapters 2, 3, and 4) and preclinical studies (Chapters 3, 4, and 5). The thesis can be divided into three main hypotheses: i) if naturally occurring bacteria can be identified in human tumour tissues, then these may be better tumour-targeting agents than the laboratory and probiotic strains that are routinely used in tumour-targeting studies (Chapters 2 and 3); ii) if bacteria that are administered orally to cancer patients can successfully colonise their tumours, then these may be safer and equally effective alternatives to intravenously administered microbes (Chapter 4); and iii) if bacteria are continually recycled through tumours via IV administration, then this may result in strains with improved tumour selectivity over their parental counterparts (Chapter 5). A prevailing concept throughout this work is that exposure to tumours, either via purposeful human intervention or natural colonisation, and the resultant evolutionary effects could adapt bacteria to the intratumoural environment more effectively than what genetic engineering would allow.

This thesis contributes considerably to several bodies of knowledge, including the human tumour microbiota and the feasibility of administrable bacteria as vehicles for tumour-selective transmission of therapeutics. Chapters 2 and 3 use sequencing and culture techniques to develop an accurate and comprehensive picture of the human

breast tumour microbiota. A culture-based assay was able to define the microbial diversity and community structure of breast tumours, when placed in the context of refined bioinformatic analyses. Despite a lack of evidence for viable, endogenous, tumour-selective bacteria, breast tumours contained a characteristic microbiological signature, as determined by deep sequencing, that warrants further exploration in varying tumour types to establish the diagnostic potential of this finding.

Chapter 4 reports clinical data in relation to the translocation of probiotic bacteria from the GIT to distal tumours, using both FFPE specimens and fresh tissues. Results of this study, and corresponding animal experiments, shed light on the viability, or lack thereof, of this route of administration for prospective bacterial tumour-targeting studies in the clinic.

Lastly, Chapter 5 is an account of the effects of tumour-to-tumour cycling on probiotic bacterial strains. The data in this chapter indicate that recycling bacteria through murine tumours can alter intratumoural growth dynamics and metabolic capacity, which was reflected in enhanced tumour persistence and biofilm formation. This work emphasises tumour recycling as a method by which bacteria can be adapted to the complex, heterogenous environment of cancerous tissue in a relatively simplistic way, which may have implications for the success of this treatment modality as it gains more clinical traction.

Chapter 1: Literature review

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Section 1: Bacterial anticancer therapy

Abstract

Persistently high mortalities for several forms of cancer have shifted research efforts towards advanced therapies, including those based on bacteria. In the first section of this literature review, the therapeutic anticancer potential of bacteria is illustrated via the well-studied example of BDEPT. BDEPT is a biphasic variant of gene therapy in which an administered bacterium, armed with an enzyme that can convert an inert prodrug into a cytotoxic compound, induces tumour cell death following tumour-selective prodrug activation. BDEPT combines the innate ability of bacteria to selectively proliferate in tumours post-administration, with the capacity of prodrugs to undergo contained, compartmentalised conversion into active metabolites *in vivo*.

Although bacterial anticancer therapies, including BDEPT, have been tested clinically, they have received limited clinical exposure overall, and have yet to achieve regulatory approval. Here, BDEPT is reviewed from the system designer's perspective, and detailed commentary on how the designer should strategize its development *de novo* is provided. Contemporary advancements in this field, that aim to enhance BDEPT in terms of safety and efficacy, are reviewed. Finally, clinical and regulatory barriers facing BDEPT are discussed, and promising approaches through which these hurdles may best be tackled are considered.

Introduction

Putting aside the many benefits imparted by the industrial revolution, one of its most inconvenient drawbacks is captured well by the term “disease of civilisation”, which refers to illnesses, such as certain types of cancer, that have an increased incidence in countries with relatively high levels of economic development (Lindeberg, 2012). Despite technological advancement in the West, medical science has yet to reach a level of sophistication where it can mitigate satisfactorily the adverse health consequences of this disease. The difficulty in shouldering the burden of cancer is due largely to its complexity: it has several built-in features that contribute to its diversity, multiplicity, and ability to spread to distant organs in the body from its point of origin, including a capacity to negate apoptotic pressure and bypass cellular signals that suppress its growth (Hanahan and Weinberg, 2011). Its diversity is such that different cancers show different incidence patterns when recent trends are analysed – for example, thyroid cancer and lung cancer show increased and decreased incidence rates, respectively, while renal cancer incidence rates have stabilised (Noone et al., 2017).

Given the high incidence and mortality of this disease globally, from low-income to high-income countries, and the likelihood that incidence will continue to increase for at least some cancers over the coming decades (Bray et al., 2018), the identification of alternative therapeutic, diagnostic, and preventative measures, that exceed the current standard, is a priority. Off-target toxicity is a key limitation in cancer chemotherapy, where the concentration of drug usually required for efficacy is too toxic to the patient, inducing unacceptable side effects and preventing tumour clearance. To overcome this, several substitute strategies have been developed over the years that aim to localise treatment to the site of the tumour, thus sparing healthy tissue. The clinical armamentarium for anticancer treatment has shifted, from exclusive use of indiscriminate cytotoxic chemicals, to more specific, small-molecule drugs (Hoelder et al., 2012) and the inclusion of targeted biopharmaceuticals as first-line agents in, for example, colorectal cancer (Parikh et al., 2016) and chronic lymphocytic leukaemia (Zhou et al., 2014). Even so, most anticancer drugs that achieved regulatory approval in Europe between 2009 and 2013 lacked evidence of prolonging survival in patients, or improving their quality of life over already-established treatments (Davis et al., 2017).

The emergence of cancer cells that are refractory to multiple cytotoxic drugs has mobilised efforts to combat multi-drug resistance, such as the investigation of novel phytopharmaceuticals (Kuete et al., 2016) and the use of nanocarriers to enhance the activity of long-standing cytotoxic agents (Wang et al., 2016). The interest that gene therapies, and other ATMPs, have sustained by their performance in clinical oncology trials (Ginn et al., 2018) comes at a time when there are rising concerns regarding the long-term viability of anticancer drugs in the clinician's standard repertory. Gene therapy involves engineering of drug delivery vehicles to carry nucleic acids to tumour cells, encouraging the production of anticancer biomolecules. Early gene therapies relied heavily on viruses to serve as gene carriers (Wirth et al., 2013, Rogers and Pfuderer, 1968), but the scope of what can be regarded as a carrier has since expanded beyond viral vehicles to include bacteria. The use of bacteria in treating cancer, however, predates discussion of gene therapy by decades. It was initially pioneered over a century ago by William Coley, after he observed that some cancers of hospitalised patients regressed following accidental *Streptococcus pyogenes* infections, and by Friedrich Fehleisen, who had begun treating oncology patients with the same microbe during the same period (Cummins and Tangney, 2013, Pawelek et al., 2003). These accounts prompted further research on bacteria in the treatment of cancer, although, as this method was not at the time curative, it was gradually abandoned.

In the mid-20th century, it became apparent that bacteria can preferentially accumulate within tumour tissue. For example, the systemic injection of *Clostridium tetani* spores into mice led to the exclusive localisation of their vegetative form in murine tumours (Malmgren and Flanigan, 1955). It has since emerged that some bacteria possess inherent anticancer attributes, without the requirement for ‘arming’ them with heterologous genes. Intracellular replication of invasive bacteria (e.g., salmonellae) within cancer cells can induce apoptosis, while certain species-specific toxins have been identified, such as with cutibacteria, which can induce apoptosis in colorectal carcinoma cells with fatty acids that are naturally produced by this genus (Jan et al., 2002). Similarly, unmodified *Lactococcus lactis* is cytotoxic to cancer cells and impedes proliferation in several cancer cell lines (Han et al., 2015). With the birth of molecular biology in the late 20th century, interest in bacteria as anticancer agents has reignited in earnest. The field is continually evolving, and different preclinical

approaches exist that investigate bacteria as tumour-selective entities (Morrissey et al., 2010). Rather than simply viewing bacteria as unmodifiable agents for treating cancer, as they were recognised during Coley's and Fehleisen's time, we have since re-considered their flexibility and have postulated their usability in conjunction with other, more modern types of cancer treatment.

Notwithstanding therapeutic potential, the usefulness of any treatment is limited in some cancers by the complications involved in providing a timely diagnosis. For example, the lack of specific biomarkers in pancreatic cancer is identified as a significant challenge in its management, and has been attributed to its persistently high mortality (Kleeff et al., 2016). Thus, diagnostics that employ technology that is as disruptive as gene therapy are the subjects of sincere research efforts. Remarkably, bacteria have been investigated for their potential as next-generation diagnostics for cancer (Danino et al., 2015). However, the first section of this literature review will focus chiefly on the therapeutic applications of bacteria with reference to cancer, using BDEPT, also referred to as bacterial prodrug therapy, as its primary example. BDEPT is a modification of GDEPT, which uses gene therapy techniques to enzymatically convert inactive prodrugs to their active drug counterparts (Zhang et al., 2015). GDEPT is a growing discipline that was initially conceived to invoke differential expression of enzymes in tumour tissue compared with healthy tissue for medicinal purposes. It essentially encapsulates the tumour-selective expression of a gene, coding for a prodrug-converting enzyme, which logistically leads to the tumour-selective activation of an administered prodrug. Different manifestations of GDEPT exist, characterised by gene delivery that is assisted by carriers predisposed to tumour entry, such as VDEPT (Padma, 2015). Gene expression can be controlled by ensuring that transcription occurs via a promoter operating only within tumour cells (Nettelbeck et al., 2000). This promoter can be either a constituent of the carrier's genome or part of the tumour cell's default machinery.

BDEPT constitutes a two-step procedure for treating cancer using bacteria as *in-situ* sources of prodrug-converting enzymes (Lehouritis et al., 2013). In the first phase of treatment, bacteria, that have been armed with a gene coding for an enzyme that can convert an inert prodrug into a cytotoxic API, are administered to the patient. The bacteria travel to the tumour site, preferentially colonise it, and then begin to proliferate. Once intratumoural bacterial numbers are adequate, and enzyme

production amplified, the second step is initiated by administering the prodrug. Following administration, the prodrug spreads systemically throughout the body, but is primarily activated in the tumour by the locally produced enzyme. The API diffuses then throughout the malignancy, relieving healthy tissue of any damage. As the active drug is generated locally inside the tumour, this approach can be thought of as *in situ* chemotherapy (Figure 1).

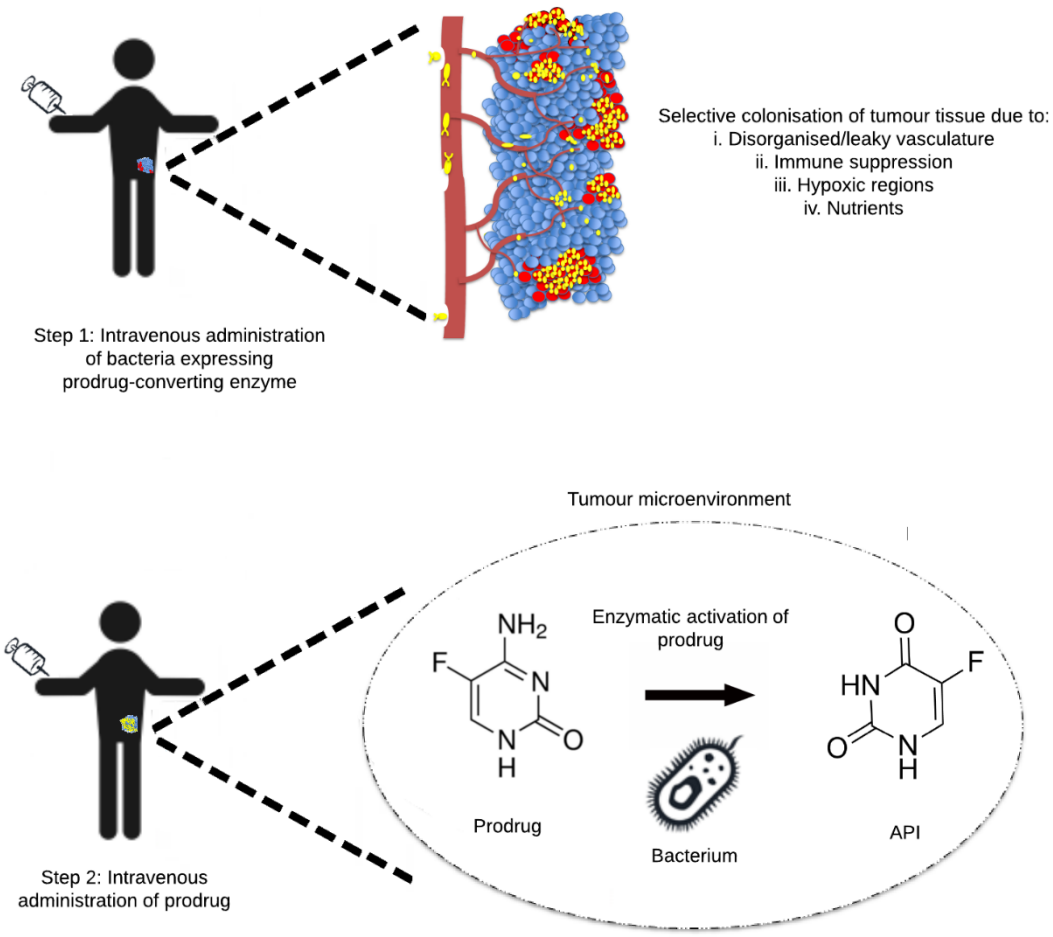


Figure 1. BDEPT. *The two-step procedure of BDEPT is illustrated, wherein bacteria expressing a prodrug-converting enzyme are administered to an oncology patient (step 1), followed by systemic administration of a corresponding prodrug when the tumour is adequately colonised (step 2). The in situ bacteria-mediated conversion of 5-FC (a prodrug) to 5-FU (an API) is illustrated in step 2.*

The administration of a prodrug has enormous benefits over administering a drug pre-formulated as an API. Ideally, prodrugs are synthesised with certain molecular features that conceal an underlying pharmacodynamic mechanism of action (Clas et al., 2014). These features often bestow upon the drug improved pharmacokinetic qualities, augmenting its bioavailability *in vivo*. Described as a "chemistry-enabled drug delivery tool", prodrugs are metabolised to their active form, following administration, by either endogenous or exogenous enzymes (Rautio et al., 2008). As the core therapeutic objective that BDEPT seeks to accomplish revolves around the confinement of tissue damage to tumours, the use of prodrugs activated by endogenous enzymes is inadvisable. This is because the prodrug is liable to encounter the enzyme en route to the tumour, resulting in extra-tumoural toxicity (Zawilska et al., 2013). Contrastingly, a prodrug that is activated by an exogenous enzyme gives the clinician significant control regarding the calibration of the patient's treatment, allowing them to time the site-specific initiation of therapy, if the enzyme is guided to the tumour appropriately.

The bacterial biofactory: Bacteria as enzyme delivery vehicles

Bacteria have many characteristics that favour them as tumour-targeting agents over other transduction and transfection methodologies. Firstly, they are naturally tumour-selective following administration to an organism. A diverse panel of bacteria, including Gram-positive, Gram-negative, aerobic, and anaerobic strains, has illustrated the selective colonisation of various tumour models by all intravenously administered microbes under study (Yu et al., 2008). Secondly, bacteria proliferate post-delivery, unlike replication-defective viruses, such as adenoviruses, and non-viral carriers (Zhang et al., 2010, Schepelmann and Springer, 2006, Baban et al., 2010). Thirdly, the genomic capacity of bacteria is far greater than that of viruses, facilitating sophisticated and complex bioengineering via synthetic biology, permitting the design of 'intelligent' production cell systems (Forbes, 2010, Flores and Tangney, 2017). In fact, a range of therapeutic biomolecules has been used in conjunction with harmless or mildly toxic bacteria, ranging from cytokines to antigens (Bermudez-Humaran et al., 2003, Ganai et al., 2009, Loeffler et al., 2008, Bruhn et al., 2007). Finally, in the

case of emergency, bacteria can be cleared from the patient by antibacterial agents (Toso et al., 2002) - something that is impossible with other gene therapy carriers.

The nature of tumour-selective colonisation is dependent on a multitude of factors, many of which relate to passive features of malignant tissue. The abnormal, porous vasculature of the tumour facilitates penetration of bacteria, in which they lodge and begin to proliferate, protected naturally from immune surveillance (Yu et al., 2004). Immunity serves to quickly clear extra-tumoural bacteria from the bloodstream and non-malignant tissue following administration to humans (Toso et al., 2002, Heimann and Rosenberg, 2003) and animals (Cronin et al., 2012b, Whelan et al., 2010, Zhao et al., 2005), but it also complicates tumour-selective colonisation of the carrier. Threshold administration quantities of microbes to induce colonisation are apparent, and it is likely that once a threshold is exceeded, host innate immune defences are insufficient to neutralise all microbes before they encounter the malignancy (Yu et al., 2008, Kimura et al., 1980). Indeed, while even obligate anaerobic bacteria, such as *Bifidobacterium breve* UCC2003, have been recovered from healthy organs distal to tumours, tumour-to-liver colonisation ratios in the region of 10000:1 have been reported for these microbes (Cronin et al., 2010). Some bacterial genera have been shown to exclusively colonise tumour areas characterised by hypoxia or anoxia, while others are capable of colonising both necrotic and normoxic tissues (St Jean et al., 2008, Ganai et al., 2011). Growth usually occurs in multiple groups of colonies inside the tumour (Forbes et al., 2003, Cronin et al., 2012a). The proliferation of bacteria *in vivo* is contingent on the availability of certain nutrients such as iron (Marx, 2002). This dependence can be exploited by host immune defences by curtailing the availability of such nutrients, to counteract the accumulation of bacteria (Chlosta et al., 2006).

Not only do tumours protect microbes within it from host defences, but tumour tissues hold a wealth of nutrients, such as amino acids, that provide vital sustenance for bacteria, especially in the case of auxotrophic mutants (Ganapathy et al., 2009, Zhao et al., 2005). There is some evidence suggesting that bacteria are attracted to tumours (or their intratumoural spread is increased) chemotactically by compounds present in quiescent and necrotic cancer cells (Kasinskas and Forbes, 2006), propelled there by their own motility (Toley and Forbes, 2012). It has also been purported, contrarily, that motility has a negligible effect on bacterial migration to tumours (Stritzker et al.,

2010). Colonisation correlates positively with tumour size (Mei et al., 2002), probably because large, advanced tumours contain marked hypoxia, amenable to more extensive colonisation (Shannon et al., 2003). However, some tumours as small as 20 mm^3 have proven to be colonisable by bacteria (Yu et al., 2004).

The unification of bacterial systems and enzyme-mediated prodrug activation is ideal. Commensal bacterial genes typically code for enzymes, and not toxins, and thus are unlikely to be lethal to the host (Gilmore and Ferretti, 2003). Furthermore, in the event of bacterial leakage from the tumour into the bloodstream, iatrogenic effects of the API are minimised, as these microbes will be rapidly and systemically eliminated from non-malignant tissue by the immune system. Pathogenic bacteria, such as *Clostridium difficile*, having an innate ability to kill healthy host cells through exotoxin production, can kill tumour cells when in proximity (Kushnaryov et al., 1992). Despite the tumour-selective growth of administered bacteria, the non-specific toxicity of pathogenic microbes does not provide a therapeutic index wide enough for safe and effective BDEPT (Malmgren and Flanigan, 1955). Modified strains with reduced virulence, or inherently non-pathogenic bacteria, present an opportunity to circumvent this. For example, *Clostridium novyi*, heat-treated to abolish one of its lethal toxins, brought about tumour regression in mice following administration, probably due to the release of oncolytic products from the bacterium (Dang et al., 2001). The attenuated strain *C. novyi*-NT has been explored preclinically in combination with other tumour-targeting entities, such as liposomally encapsulated chemotherapies, for its natural facility to enzymatically release the contents of liposomes (Cheong et al., 2006). This strain has also been combined with more conventional forms of cancer treatment, including radiation therapy, as a means of selectively targeting hypoxic tumour regions, against which radiation is known to be less effective (Bettegowda et al., 2003). In 2019, a phase I clinical trial was completed involving administration of *C. novyi*-NT spores to patients with cancers refractory to standard treatment (clinicaltrials.gov identifier: NCT01924689) (Roberts et al., 2014). Between 1×10^4 spores to 3×10^6 spores were injected intratumourally across six cohorts of patients to identify potential dose-related toxicity issues. Administration unfortunately resulted in TEAEs in all 24 patients involved in the study, 13 of whom experienced serious TEAEs, and 4 of whom experienced life-threatening TEAEs.

Constructing a successful BDEPT: Design principles

Putting aside everything which makes BDEPT promising, it has yet to achieve a place in the standard anticancer repertory of the clinic, and it is worth examining why this is so. BDEPT is not by any means a ‘traditional’ kind of treatment, and it is unlike any form of anticancer therapy currently in clinical use. Non-targeted chemotherapies and monoclonal antibodies, for instance, are comprised of chemical functional groups and amino acids, and are not reliant on living delivery vehicles for conveyance to their sites of action. BDEPT designers, having no blueprint of what a truly effective BDEPT really looks like yet, are compelled to feel their way as they search for successful therapies of this kind. It is therefore imperative to have a solid understanding of all design variables and how best to tailor them for optimisation of safety and efficacy of this platform.

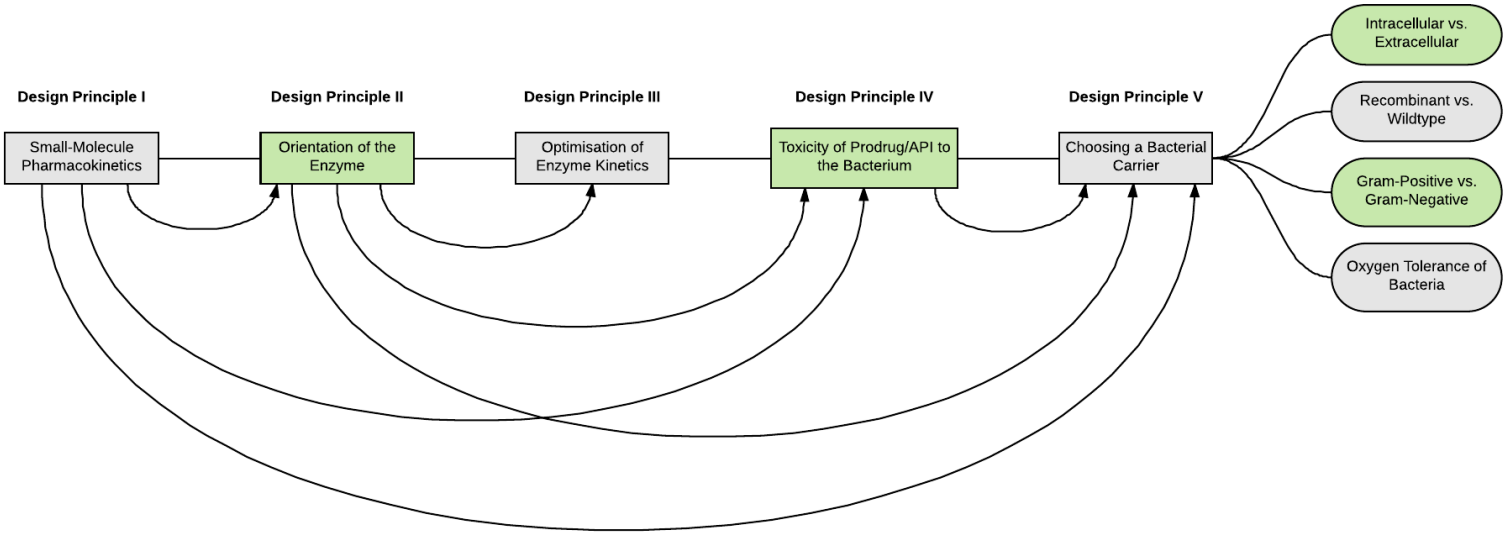


Figure 2. BDEPT design process flow. *An illustration of the order in which the de novo design of BDEPT should be approached. The process is divided into five distinct design principles. Curved arrows pointing from one design principle to another indicate a relationship in which an understanding of the design principle being pointed to is dependent on a familiarity with the design principle from which the arrow comes.*

Design principle I: Small-molecule pharmacokinetics

The most pertinent questions to pose when designing a BDEPT concern the pharmacology of the prodrug and its corresponding API. Fundamental questions about the distribution pharmacokinetics of both drug and prodrug should be considered. It is crucial that these behaviours are adequately defined from the outset, because this will guide many of the ensuing steps of BDEPT design, including the correct alignment of the bacterial enzyme (Design Principle II), management of toxicological issues (Design Principle IV), and the selection of an appropriate bacterial carrier (Design Principle V) (Figure 2). The designer must know how easily their compound can infiltrate a cell by crossing the cell membrane. A prodrug will be converted to an API by the intracellular enzyme of a bacterium only if it can enter the bacterial cytoplasm. Furthermore, an API, which relies on direct engagement with intracellular tumour elements to function, must be present within a tumour cell for its mechanism of action to take hold. For example, 5-FU, an API commonly employed in BDEPT, acts as an inhibitor of DNA and RNA synthesis only when it is processed intracellularly by mammalian enzymes (Longley et al., 2003).

Pharmacokinetic properties can vary considerably between drug and prodrug. They can change so markedly upon activation that a prodrug that is capable of crossing cell membranes with ease can become impeded from doing so once enzymatically metabolised, thereby negating or reducing its capacity as a chemotherapeutic agent (Rautio et al., 2008). If an API is generated external to a tumour cell, and its cell membrane is impenetrable to the API, the drug will be unable to initiate its anticancer modality. In these scenarios, prodrug conversion must occur within tumour cells for therapy to be effective. Conversely, an API that crosses cell membranes easily can undergo activation extracellularly while retaining therapeutic efficacy.

Fortunately, the properties of small-molecule drugs that alter their ability to cross cell membranes are well known. The ‘Rule of 5’ is a popular predictive tool for determination of a drug’s permeability *in vivo* based on its physicochemical attributes (Lipinski et al., 2001). Solubility affects a drug’s partition coefficient (Waring, 2010), while molecular size and charge can similarly influence its movement (Lipinski, 2000). The chemical properties of the ideal small-molecule drug are delicately balanced and it must be ensured that no one property is disproportionate. For instance,

lipophilicity encourages drug movement across cell membranes, while hydrophilicity enhances drug diffusion around cells (Minchinton and Tannock, 2006), and although it might seem that lipophilicity is thus preferable for moving a drug inside a cell, an overly lipophilic compound is associated with a poor toxicological profile (Waring, 2009). It is acknowledged that making an informed prediction of the path a compound is likely to take *in vivo* is highly complicated; thus, the use of various mathematical and computational techniques are advocated to combat this challenge (Minchinton and Tannock, 2006). The 'Rule of 5', for example, sets upper limits on a compound's lipophilicity (a logP no greater than 5) and molecular size (no more than 500 Da) (Lipinski, 2000). Additionally, physiologically based pharmacokinetic modelling attempts to improve prediction accuracy by accounting for the variability of living systems (Nestorov, 2007).

There are several examples of BDEPT enzymes which introduce modifications to their prodrug substrates that disfavour their passage across cell membranes. UPRT for instance, catalyses the conversion of uracil to uridine monophosphate, and converts 5-FU to 5-fluorouridine monophosphate which then disrupts RNA and DNA synthesis (Schwartz et al., 1985). The activated form of 5-fluorouridine is phosphorylated and therefore its charge will impede membrane permeability. Similarly, TK is a phosphotransferase that is utilised in BDEPT with the prodrugs GCV and ACV, phosphorylating them to inhibit DNA synthesis. This likewise results in a charged API, reducing transport across lipid membranes. However, phosphotransferase-based BDEPT has not proven to be ineffectual. *Bifidobacterium infantis* has been engineered to express TK, derived from the HSV-1, and significantly arrested the growth of murine bladder tumours following administration of GCV (Tang et al., 2009).

On the other hand, some enzymes can introduce favourable changes. For example, CD converts 5-FC to 5-FU via the pyrimidine salvage pathway. 5-FU has lipophilic properties that are conducive to its movement across cell membranes. Following the first report of CD expression in *Bifidobacterium longum* (Nakamura et al., 2002), CD-BDEPT has since been tested preclinically with various bifidobacterial species. *B. longu*m expressing CD was used to localise prodrug conversion to autochthonous mammary tumours in rats, with corresponding antigenicity studies in guinea pigs confirming low immunogenicity (Sasaki et al., 2006). *B. breve* was later found to express the enzyme in vitro more stably, showing 3- to 6-fold greater enzymatic

activity than that of *B. longum* (Hidaka et al., 2007). Non-bifidobacterial species have also been used to express CD. Preclinical *in vivo* studies in rhabdomyosarcoma-bearing rats showed that intratumoural injections of *Clostridium acetobutylicum* expressing CD, led to the conversion of 5-FC to 5-FU in the injected tumours (Theys et al., 2001).

Design principle II: Orientation of the enzyme

Bacteria, being living entities, may be subject to the same drawbacks of chemotherapeutic agents as human cells, including their accessibility to small-molecule compounds. This limitation can be addressed by manipulation of the enzymology of the BDEPT. The enzymatic reaction is the central event by which drugs are modified chemically in BDEPT. If chemical modifications of drugs bring about their activation, and impact their movement between extracellular and intracellular compartments, the designers of BDEPT must therefore understand the enzymology of their design, and how it could alter the pharmacodynamics and pharmacokinetics of a compound.

The biochemistry of GDEPT enzymes is known in detail (Malekshah et al., 2016, Karjoo et al., 2016). The design of GDEPT prodrugs and the pharmacology of their active forms are also well known (Tietze and Schmuck, 2011). Despite the extensive range of enzymes used in GDEPT (Duarte et al., 2012), the variety of BDEPT enzymes is comparatively low (Table 1). Presumably, this is because GDEPT, and its various offshoots such as antibody-directed enzyme prodrug therapy are older styles of gene therapy (Bagshawe, 1987). It could also be the case that because many GDEPT enzymes are mammalian, without prokaryotic counterparts, their application in bacterial carriers is more complex.

Table 1. Examples of enzyme-prodrug strategies, their mechanisms of action, and the bacterial carriers with which they are associated.

Bacterial Carrier	Enzyme	Prodrug	Mechanism of Action	Reference
Bifidobacterium longum	**CD**	5-FC	Prevention of DNA synthesis by inhibition of multiple enzymes	(Nakamura et al., 2002, Sasaki et al., 2006)
Clostridium sporogenes	**NTR**	CB1954	DNA cross-linking	(Minton et al., 1995, Theys et al., 2006)
Salmonella enterica **serovar Typhimurium VNP20009**	**CPG2**	Range of aromatic nitrogen mustard prodrugs	DNA cross-linking	(Friedlos et al., 2008)
Escherichia coli **DH5α**	**β-G**	9-AG	Topoisomerase inhibitor	(Cheng et al., 2008)
Attenuated ***Salmonella*** **SL7207**	**TK**	GCV	DNA polymerase inhibitor	(Zeng et al., 2012)
S. enterica **serovar Typhimurium VNP20009**	**PNP**	Fludarabine	Prevention of DNA synthesis by inhibition of multiple enzymes	(Chen et al., 2013)
Listeria monocytogenes	**CD/PNP/UPRT**	5-FC	Prevention of DNA synthesis by inhibition of multiple enzymes	(Stritzker et al., 2008)

The placement of the selected enzyme within a bacterial carrier is critical. If the enzyme requires reducing conditions or cofactors (e.g., NTR (Johansson et al., 2003)), it should be expressed in the cytosol. However, a bacterium expressing a cytoplasmic, prodrug-modifying enzyme can activate a prodrug only if both are simultaneously present in the bacterial cell. An API without an aptitude for crossing the cell membrane may become trapped inside the bacterium after it is activated. If the prodrug itself is hydrophilic, it may not be able to enter the cell in the first place. This problem can be negotiated by tethering the enzyme to the cell wall of Gram-positive bacteria, or the

outer membrane of Gram-negative bacteria using leader peptides and transmembrane sequence systems (Lehouritis et al., 2013), or by re-routing it to the periplasm – a protein rich boundary found in the bacterial cell wall (Silhavy et al., 2010). There are numerous examples of enzymes which have been subjected to this method in Gram-negative bacteria, including CPG2 and β-G (Figure 3).

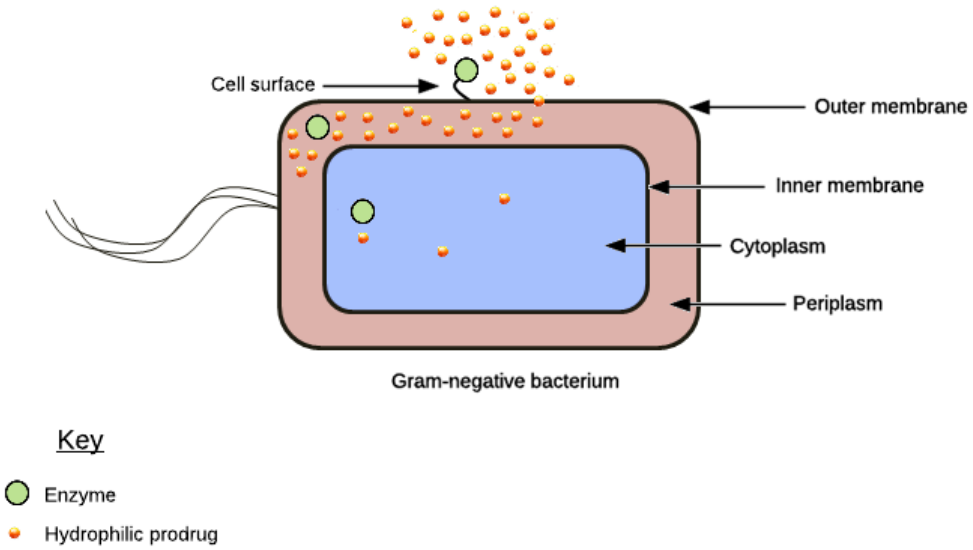


Figure 3. Enzymatic alignment strategies in BDEPT. *An illustration of the various enzymatic conformations that have been investigated in BDEPT. The activation of hydrophilic prodrugs has been enhanced by coupling BDEPT enzymes to the surface or directing them to the periplasm of Gram-negative bacteria (e.g., E. coli BL21 and Salmonella enterica serovar Typhimurium VNP20009, respectively).*

CPG2 is a dimeric protein, derived from *Pseudomonas* sp. RS-16, that can remove a deactivating glutamate moiety from a range of mustard prodrugs (Goda et al., 2009). This results in the formation of potent DNA cross-linking agents with high cytotoxicity differentials between drug and prodrug. Cytoplasmic CPG2 has not proven to be very efficacious, most likely because the prodrugs relevant to CPG2 are hydrophilic, and the enzyme naturally resides in the periplasm of *Pseudomonas* sp. RS-16. Periplasmic CPG2 has thus been investigated to confront this issue. CPG2 has been directed to the periplasm of *Salmonella enterica* serovar Typhimurium

VNP20009 via the *ompA* signal peptide, and was effective in activating many mustard prodrugs. This construct was tested with human breast and colon carcinoma cell lines, resulting in retarded tumour growth (Friedlos et al., 2008).

The hydrolytic activity of β-G can render several drugs active, including phenol mustards and anthracyclines (Melton and Knox, 2012). The enzyme has been experimentally expressed in *E. coli* DH5α cells, with results indicating inferior activation of irinotecan, a topoisomerase inhibitor, by the bacterial carrier compared with an adenoviral carrier expressing the same enzyme (Hsieh et al., 2015). One of the reasons put forth to explain this observation was that the enzyme of *E. coli* DH5α was placed in its periplasmic space, and was therefore less accessible than the adenoviral enzyme which was delivered to the membranes of virally infected cells. β-G has been directed to the outer membrane of *E. coli* BL21 by fusing it with an autotransporter protein, demonstrating activation of the p-hydroxy aniline mustard β-D-glucuronide and inducing toxicity in experimental human colon cancer cells (Cheng et al., 2013).

Augmenting an enzyme's exposure to the prodrug by relocating it closer to the cell surface could possibly impact the stability of the enzyme itself. Some enzymes exhibit contrasting biochemistry when their expression is switched from cytoplasmic to periplasmic to surface-bound conformations. β-galactosidase, for example, is rendered toxic when exposed to the periplasm of *E. coli* (Snyder and Silhavy, 1995). On the other hand, the reduced quantity of proteases in the periplasm compared with the cytoplasm (Baneyx and Mujacic, 2004) may favour enzymatic stability in the environment of the former.

Design principle III: Optimisation of enzyme kinetics

A central goal of BDEPT is to design a therapy with a high k_{cat} / K_M ratio. This ratio is commonly used to compare the kinetics of a single enzyme across multiple substrates, or to compare the kinetics of single substrate against multiple enzymes (Eisenthal et al., 2007). With BDEPT, both applications are relevant. Unmodified, the kinetics of several BDEPT enzymes leave much to be desired and this requires reworking of either the enzyme or its substrate to ameliorate overall reactivity. Early efforts of enzyme optimisation include the mutagenic adjustments made to the HSV-1 TK (Black et al., 2001). Optimisation of this enzyme is needed because its unaltered

form has a high K_M for ACV, and because GCV is immunosuppressive at therapeutic doses. A library of mutated TK enzymes was screened for improved kinetics, identifying three mutants which, when tested *in vitro* in a rat glioma C6 cell line, greatly increased the sensitivity of the cell line to GCV and ACV compared with native HSV-1 TK.

Enzyme kinetics are relevant also to CD. CD is not found in mammalian cells but it is found naturally in the cytoplasm of bacteria (Nishiyama et al., 1985). Thus, bacteria that inhabit the human intestinal tract, and express the enzyme, can activate prodrug substrates with which they come into contact (Fuchita et al., 2009). Wildtype CD has a relatively high K_M value for its prodrug substrate 5-FC than it does for cytosine (Mahan et al., 2004), which warrants high doses of prodrug for BDEPT to be efficacious. This elicits undesirable, off-target toxicity.

The kinetics of CD have been revamped via mutagenic alterations. Amino acid substitution of regions lining the active site of CD has resulted in the identification of a CD variant with improved kinetics over the native enzyme in a xenograft tumour model (Fuchita et al., 2009). Similarly, a shuttle plasmid incorporating CD with a mutation in its active site has demonstrated a 10-fold improvement in its kinetics over prior attempts (Hamaji et al., 2007). Ideally, enhanced CD kinetics will help to overcome the impediments that are obstructing CD-BDEPT from the clinic, such as the rapid elimination of 5-FC from the systemic circulation and insufficient gene delivery.

The NTR family of enzymes has likewise been scrutinised for improved kinetics. NTR was among the first enzymes implicated in BDEPT, in conjunction with *Clostridium beijerinckii* (Lemmon et al., 1997, Minton et al., 1995). NTR activity is evident in anaerobic bacteria commonly found in the GIT (Rafil et al., 1991); thus, as with CD, prodrug substrates for NTR may cause non-specific toxicity when they are administered. The native NTR enzyme NfsB of *E. coli* can reduce the weak, alkylating 5-(aziridine-1-yl)-2,4-dinitrobenzamide prodrug CB1954 to potent DNA-intercalating agents, using NADH or NADPH as a cofactor (Prosser et al., 2010). NfsB was originally the most widely studied prodrug-activating NTR (Denny, 2002). However, wildtype NfsB is kinetically unimpressive with multiple prodrugs (Christofferson and Wilkie, 2009, Green et al., 2013b) and its high K_M has inspired research into

alternative enzymes with better kinetic properties, such as NfsA. NfsA has demonstrated a higher k_{cat} / K_M ratio *in vitro* compared with NfsB for CB1954 (Vass et al., 2009).

As CB1954 is hepatotoxic when metabolised by the liver (Tang et al., 2007), setting tighter upper limits on doses of this prodrug is desirable. Other strains of bacteria, apart from *E. coli*, have been searched for the presence of NTR enzymes with improved kinetics that could allow dose reductions of CB1954. A fluorogenic probe was used to identify the flavin reductase MsuE, from *P. aeruginosa*, which exhibited a kinetically similar aptitude to the NfsA of *E. coli in vitro* for the reduction the next-generation DNA cross-linking 3,5-dinitrobenzamide-2-mustard prodrug PR-104A, and the next-generation nitrochloromethylbenzindoline prodrug CBI-DEI (Green et al., 2013a). Furthermore, the NfsB enzyme of *P. aeruginosa* has recently been described for its improved kinetics *in vitro* over the NfsB enzyme of *E. coli* for reduction of CBI-DEI (Green et al., 2013b). The newly discovered enzyme NmeNTR, derived from *Neisseria meningitidis*, has the advantage over the NfsB enzyme of *E. coli* in that it reduces CB1954 purely to the toxic 4-hydroxylamine derivative, whereas NfsB produces equimolar concentrations of the 4-hydroxylamine derivative, and the less toxic 2-hydroxylamine derivative (Heap et al., 2014).

Screening of NTR enzymes has led to the finding of more kinetically advanced NTR-BDEPT systems, which may allow dose reduction of CB1954. An oxioreductase library has been generated and screened using *E. coli* SOS reporter strains to quantify activation of CB1954 and PR-104A by NTR enzymes (Prosser et al., 2013). The *in-vitro* sensitisation of *E. coli* cells to the activated prodrugs was used to evaluate enzyme efficiency. This led to the discovery of YcnD_Bs, which has a higher k_{cat} / K_M ratio than NfsA. It also led to the discovery of 14 reductases with higher k_{cat} / K_M ratios than NfsB_Ec, which up until that point was the most active PR-104A reductase.

As with CD, an effort has been made to forge mutated versions of NTR with better kinetics (Jaberipour et al., 2010). An *E. coli* SOS reporter strain was used to identify another flavin reductase, FRaseI from *Vibrio fischeri*, which displays better kinetics than NfsB *in vitro* in HCT-116 human colon carcinoma cells lines, and was further improved via site-directed mutagenesis (Swe et al., 2012). Directed evolution, again using an *E. coli* SOS reporter system, has produced an NfsA variant with a higher

affinity for PR-104A than the native NfsA of *E. coli,* inducing higher drug sensitivities in human cell lines and mouse xenografts (Copp et al., 2017). Moreover, a codon optimisation strategy was implemented with *C. sporogens* to heighten its ability to stably express NTR enzymes derived from *E.* coli (Liu et al., 2008). Resultantly, doses of CB1954 and PR-104A, which were sub-therapeutic prior to codon optimisation, exhibited therapeutic potential thereafter in tumour-bearing mice.

Another approach to increase the k_{cat} / K_M of an enzyme is to manufacture better substrates for that enzyme. Novel prodrugs have been synthesised in the interest of developing better substrates for NTR. For example, CNOB is a prodrug that can be converted by the enzyme ChrR6 to the cytotoxic and fluorescent agent MCHB (Thorne et al., 2009). MCHB has been used with the non-virulent *Salmonella* strain SL7838 to treat murine tumours. Impressive remission rates were seen in mice implanted with tumours endogenously expressing ChrR6, following administration with CNOB. Unfortunately, only 10% of the mice treated with SL7838-ChrR6 survived after 60 days of treatment, implying that a defect was present in some facet of the BDEPT, possibly related to unsatisfactory availability of the bacterial enzyme to the prodrug.

Design principle IV: Toxicity of the prodrug/API to the bacterium

Although BDEPT is designed to mitigate off-target toxicity to healthy tissues, the toxicology of the activated drug is still relevant. A chemotherapeutic drug that damages healthy and cancerous human tissue potentially poses a threat to the viability of bacterial cells. However, not all drugs will affect bacterial survival (Lehouritis et al., 2016). Studies with VNP20009 suggest that hydrophilic APIs are only harmful to the bacterium if, following activation outside the cell, the cell membrane is destabilised and the drug leaks into the bacterial cytoplasm (Lehouritis, 2006). If the drug is activated in the periplasm of the bacterium and is too hydrophilic to cross its inner membrane, the API will not harm bacterial DNA even if the enzyme is over-expressed.

The molecular differences between prokaryotic and eukaryotic cells are significant in that they may allow the mechanisms of action of chemotherapeutic cancer drugs to solely target mammalian cell machinery (the same principle holds in reverse with

many antibacterial therapies, like penicillin, which relieve patients of unpleasant side effects by homing in on prokaryotic cellular peculiarities). The topoisomerase I inhibitor 9-AC, for example, targets a topoisomerase enzyme chiefly expressed in mammalian cells (Nitiss et al., 2012, Pommier, 2006). Hence, it should not pose a toxicological threat to bacteria. *E. coli* DH5α has been employed to deliver β-G to experimental tumours (Cheng et al., 2008). Here, the prodrug 9-ACG was administered, which is hydrolysed by β-G to 9-AC. Importantly, 9-AC did not destroy the bacterial vehicle, and *in vivo*, the combined treatment hampered CL1-5 human lung tumour growth in a murine model.

Some chemotherapies, however, can damage bacteria. A recent study catalogued the effects of various chemotherapeutic agents on lactic acid and bifidobacterial strains, indicating the sensitivity these strains have towards antimetabolite drugs like 5-FU and doxorubicin (Florez et al., 2016). 2-fluoroadenine, a metabolite of fludarabine (Figure 4), has antibacterial properties (Blount and Breaker, 2006). PNP, the enzyme that catalyses the conversion of fludarabine, can break down non-toxic deoxyadenosine molecules into highly toxic adenine analogues (Hughes et al., 1995). The endogenous PNP enzyme of *Salmonella* (genetically homologous to the PNP of *E. coli*) has been exploited to convert 6MeP-dR, a substrate for bacterial PNP but not human PNP, to 6MeP. *In vivo*, murine B16-F10 tumour growth was diminished following this regimen (Chen et al., 2013). Though the inhibition of DNA synthesis instigated by these toxic molecules results in tumour cell death, bacterial viability too may be affected. This may partly explain why adenoviral PNP delivery has enjoyed success in phase I clinical trials (Rosenthal et al., 2015), while PNP-BDEPT has yet to be tested in patients.

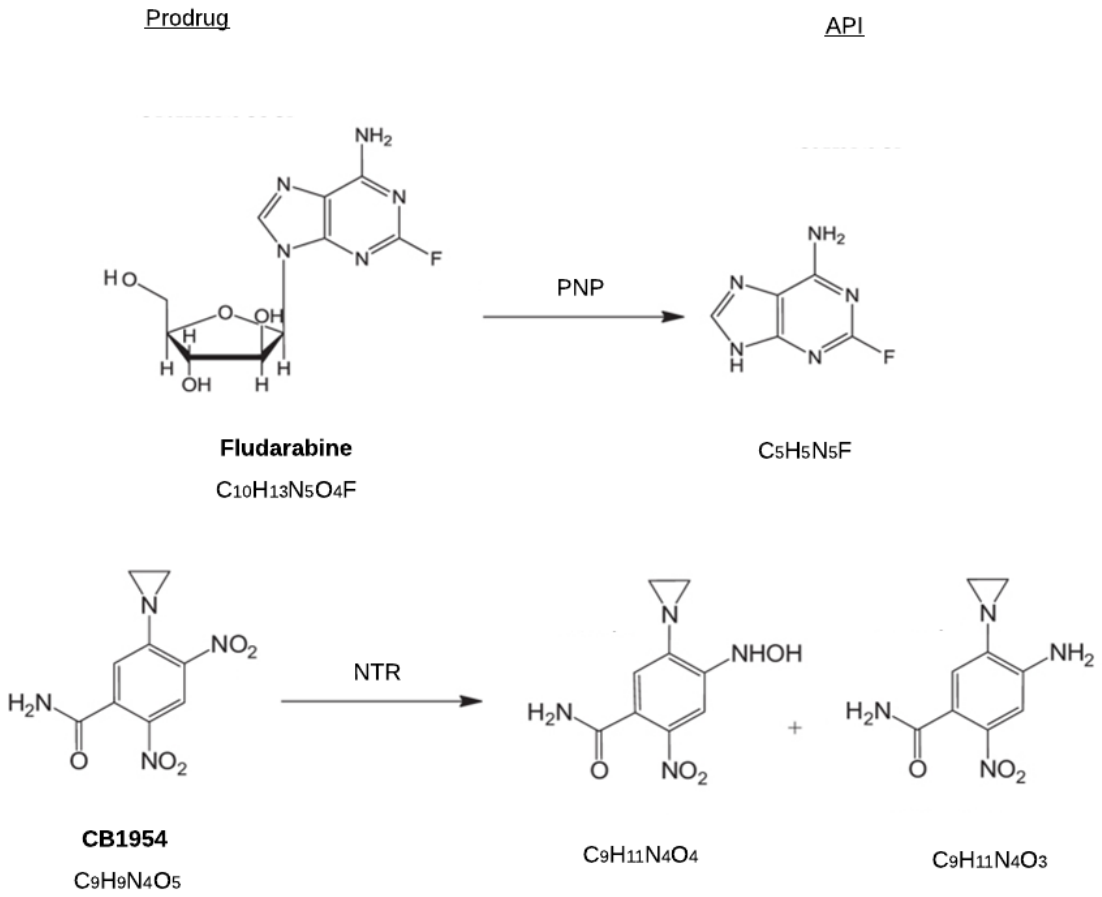


Figure 4. Toxic metabolite production via BDEPT enzymes. *Fludarabine is converted by PNP to 2-fluoroadenine ($C_5H_5N_5F$), while CB1954 is converted by NTR to DNA cross-linking agents, all of which are potentially deleterious to bacteria via inhibition of DNA, RNA, and protein synthesis.*

Studies of CB1954 revealed that bifidobacterial strains and certain *E. coli* strains (e.g., MG1655) are highly sensitive to the prodrug, whereas other *E. coli* strains (e.g., EcN) and *Lactococcus* spp. are more resistant. Western blot studies of NTR suggest that high concentrations of NfsaA and NfsB are responsible for the toxicity of CB1954 to bacteria. The activated forms of CB1954 are a mixture of 2- and 4-hydroxylamines, of which the latter is the more effective DNA cross-linker (Figure 4). The hydroxylamine compounds to which CB1954 is metabolised are mutagenic and are inhibitory to bacterial DNA, RNA, and protein synthesis (Gross, 1985, Marczylo et al., 1999).

Toxicity issues can be worked against by enzymological adjustment of a BDEPT. While it is a feasible tactic for enriching the activation of a hydrophilic prodrug, re-orientation of an enzyme such that it is closer to the surface of the bacterial cell can also be applied to circumvent toxicological threats to bacteria. Again, the implications of engineering the enzyme and possibly affecting its activity, as well as the distribution pharmacokinetics of the API, should be borne in mind. A lipophilic, toxic API, despite being activated in a non-cytoplasmic region, may nevertheless cross into the cytoplasm of the bacterial cell, exerting a toxic effect. It is vital at this point for the designer to understand both the toxicity and membrane permeability of their compound, because this will clarify whether re-calibration of the enzyme will be truly beneficial to BDEPT efficacy.

Design principle V: Choosing a bacterial carrier

At this stage, having defined the pharmacological and toxicological traits of both drug and prodrug, and having gained an appreciation of the biochemistry of their chosen enzyme, the designer should be able to deliberate on an appropriate bacterial carrier. This decision should integrate four separate considerations: the level of invasiveness desired of the bacterium, the appropriateness of a wildtype carrier versus a recombinant carrier, the advantages offered by Gram-positive versus Gram-negative bacteria, and facultative versus obligate anaerobic bacteria.

(i) Intracellular vs. extracellular bacterial carriers

In BDEPT, the bacterium must be positioned such that it is capable of enzymatically activating its prodrug target. If prodrug metabolism is to occur within the tumour cell, the bacterium will require intracellular access to supply its enzyme, but the requirement is waived if the drug can enter the cancer cell following activation. ‘Bactofection’ is the bacterial equivalent of transfection, whereby an invasive bacterium is engineered with a plasmid featuring a transgene under the control of a mammalian promoter which, following administration, invades the host, lyses, and releases the plasmid within the host’s cells (van Pijkeren et al., 2010). The plasmid migrates to the host cell’s nucleus where the transgene can be transcribed. This procedure typically relies on disease-causing bacterial strains, presenting safety

concerns in terms of off-target toxicity and immune stimulation (Baban et al., 2010). An example of bactofection involves exploitation of attenuated *L. monocytogenes* administered to murine tumours *in vivo*, and human breast tumour tissues *ex vivo* (van Pijkeren et al., 2010). The microbial cells were sensitive to ampicillin and were ruptured following systemic administration of the antibiotic. This caused the release of plasmid DNA, and the subsequent expression of a lysin transcribed by a mammalian promoter. Additional cell lysis occurred due to heightened expression of the lysin itself.

In BDEPT, bactofection can be applied if the API has difficulty crossing cell membranes, if the enzyme cannot be expressed inside bacterial cells optimally, or in a 'host cell conditional' strategy involving expression of genes from a mammalian promoter. Non-invasive and non-pathogenic bacteria have been engineered to confer upon them invasive qualities. For example, the bacterial designer strain *Escherichia coli* BM2710 was engineered to express two genes to formulate an invasive strain: the *inv* gene from *Yersinia pseudotuberculosis* which facilitates invasion of mammalian cells that express the β1-integrin, and the *hly* gene from *Listeria monocytogenes*, which induces phagosomal escape and the release of bacterial cytoplasmic proteins and plasmid DNA into the cytoplasm of mammalian cells (Grillot-Courvalin et al., 1998).

Bactofection has been incorporated into several BDEPT designs to internalise drug activation in the target cell. This overcomes the drawback of enzymes like PNP and TK, which phosphorylate their targets, thereby impeding their movement into cells. *L. monocytogenes*-mediated bactofection has delivered PNP into several tumour cell lines, allowing efficient cytotoxicity (Stritzker et al., 2008), while attenuated *Salmonella* has been employed to deliver TK to experimental B16 melanoma cells *in vivo* in which, following GCV administration, significant suppression of tumour growth was observed (Zeng et al., 2012).

Anticancer therapies that rely on intracellular agents to deliver genes to tumours generally fall drastically short of depositing their payload in the entirety of the malignancy (Zhang et al., 2015, Dachs et al., 2009). The bystander effect is critically important for these intracellular agents. The bystander effect hinges on the idea that tumour cells that do not acquire the gene of interest may nevertheless be affected by

downstream events resulting from its expression in other cells. Within the context of BDEPT, these downstream events refer to the passage of prodrugs into tumour cells containing the gene of interest, and the passive or active translocation of cytotoxic metabolites into local cells or distant tumours (Duarte et al., 2012). An understanding of the bystander effect is an essential part of predicting the efficacy of BDEPT mediated by intracellular bacteria. The importance of the bystander effect is evident in a study in which an attenuated strain of *S. enterica s*erovar Typhimurium was used to deliver PNP *in vitro* to murine Lewis lung carcinoma and melanoma cells (Fu et al., 2008). Significant reductions in cell survival were observed, even in cases where only 1% of cells tested positive for the carrier.

The impact of the bystander effect can be magnified, often by using kinetically superior, mutated (Fuchita et al., 2009) or naturally occurring enzymes (Vass et al., 2009), or alternative prodrugs (Wilson et al., 2002, Green et al., 2013b). The *in-vitro* measurement of the bystander effect is sensitive to the type of cell line analysed (Dachs et al., 2009). Multilayer and spheroid cell lines are advocated for their stronger resemblance to the tissue densities of tumours compared with monolayer cell lines. Most importantly, the bystander effect is intertwined with therapeutic efficacy; thus, an increase in its magnitude will permit dose reductions of administered prodrugs.

(ii) Recombinant vs. wildtype bacterial carriers

It is recommended that bacterial recombination of enzyme-coding genes should take place within chromosomal DNA rather than within plasmid DNA (Heap et al., 2014). Not only does chromosomal integration increase the stability of transgene expression, but chromosomes are not considered ‘mobile genetic elements’, and are not known to partake in horizontal gene transfer from one bacterium to another, unlike plasmids (de la Cruz and Davies, 2000). In this way, translocation of genetically engineered material between microorganisms *in vivo* is reduced, which is of great significance to the regulators of recombinant IMPs (see later).

Bacteria that have been engineered to express heterologous genes at high levels can synthesise enzymes in large quantities (Glick, 1995). However, recombinant bacteria are liable to suffer from a metabolic burden resulting from gene overexpression. This burden originates from a divergence of the microbe’s cellular resources so that transgene expression can be maintained. This can overload the carrier and disrupt its

targeting capacity due to loss of its transgene and compromised viability (Jones et al., 2000). Transgene expression can be negated due to spontaneous loss of plasmids (Corchero and Villaverde, 1998, Overton, 2014). In this case, the survival of genetically unencumbered microbes which have lost their transgene is favoured, while recombinant bacteria are eventually eliminated by natural selection. However, there are ways of stabilising plasmid expression by, for example, keeping plasmid-encoded transgenes inside bacterial cells without the possibility of plasmid loss *in vivo* by use of chromosomally integrated plasmids (Riedel et al., 2007), or through post-segregational killing of bacteria that have lost their plasmid by use of toxin-antitoxin systems (Fedorec et al., 2019).

If recombinant gene overexpression is a potential threat to the integrity of the bacterial carrier, this problem could be alleviated by exercising stricter control over bacterial enzyme production. Burdensome recombinant gene expression reduces the pliability of BDEPT carriers, as it complicates the production of appropriate levels of enzyme. *E. coli*, for instance, possesses natural proteolytic facilities to counteract the overexpression of proteins that do not benefit its survival (Sorensen and Mortensen, 2005). This problem can be manoeuvred by using protease-deficient or heat-shock-deficient strains, for example. An excessively taxed bacterium, deprived of amino acids due to transgene expression, could also be supplemented with exogenous amino acids to correct the depletion.

Putting genetically engineered, recombinant bacteria aside, the BDEPT designer could also entertain the option of using wildtype bacteria which naturally express prodrug-converting enzymes. Wildtype bacterial cells, by definition, tax themselves according to what is required by their default cellular needs, and are not expected to mobilise additional resources to produce foreign biomolecules. This means that the expression of prodrug-converting enzymes by wildtype bacteria could overstep the disadvantages of using recombinant strains, because natural expression would be less likely to affect the integrity of the carrier. Wildtype bacteria have been used to activate different prodrugs. The prodrug fludarabine has been successfully employed in conjunction with the endogenous activity of EcN to induce cancer cell death in multiple cell lines (Lehouritis et al., 2016, Lehouritis et al., 2015). It has been reported that several species of bacteria can activate CB1954 with a natural enzymatic complement, and have shown that it is possible to activate multiple prodrugs concurrently (5-FC,

CB1954 and fludarabine), presenting opportunities for treating refractory tumours with more than one prodrug. Interestingly, prodrugs can be activated without a known corresponding enzyme, suggesting that wildtype bacteria have an 'enzymolome' that could be harnessed to activate drugs for cancer therapy. For instance, the drug AQ4N can be activated by EcN *in vitro* to kill cancer cells, although the mechanism of activation is unknown.

While the notion of delivering a carrier with a diverse enzyme reservoir to a tumour appears auspicious in terms of prodrug activation and therapeutic efficacy, a range of prodrugs frequently used in mainstream chemotherapy has been reported that can be deactivated by bacterial enzymes, including etoposide phosphate, cladribine, and gemcitabine (Lehouritis et al., 2015). This is not unexpected, as a bacterium's enzymolome is vast and may harbour enzymes that deactivate certain drugs while activating others. This clinically relevant phenomenon ought to be addressed by the designer, irrespective of whether they integrate a recombinant or wildtype strain into their design.

(iii) Gram-positive vs. Gram-negative bacterial carriers

When selecting a bacterial carrier, it is helpful for the designer to contemplate the influence, both physical and biochemical, of inner and outer bacterial membranes on prodrug activation. This necessitates discussion of Gram-positive and Gram-negative bacteria, which differ from each other in the constitution of their cell envelopes. The cytoplasm of both Gram-negative and Gram-positive bacteria is separated from the extracellular environment by a cell envelope. In Gram-negative bacteria, this consists of an outer membrane and an inner membrane, separated by a cell wall containing the periplasmic space (Silhavy et al., 2010). Most importantly, in Gram-positive bacteria, an outer membrane is lacking, and the existence of a Gram-positive periplasm is less noted, although it has been described (Merchante et al., 1995, Matias and Beveridge, 2006, Shen and Chou, 2007). This should be taken into consideration when mapping-out a BDEPT design. The differences between Gram-positive and Gram-negative cell envelopes have implications for bacteria in terms of the way they interact with their host, and their flexibility as a BDEPT carrier.

Examinations of VNP20009 have revealed the affliction that Gram-negative bacteria can visit upon their host. Lipid A, which forms part of the LPS component of the outer

membrane, can provoke toxic shock syndrome in the organisms they inhabit (Hedley et al., 2007). A convenient workaround for this obstacle involves deleting the bacterial gene *msbB*, which abolishes the toxicity of lipid A (Somerville et al., 1996, Low et al., 1999). LPS plays another role by decreasing the permeability of the outer bacterial membrane, although mutations in *msbB* do not appear to affect the structural integrity of this barrier (Vaara and Nurminen, 1999).

With Gram-negative bacteria, the outer membrane represents a supplementary blockade against the admission and escape of prodrug and API to and from the cell, limiting the passage of large, charged molecules, but letting hydrophilic molecules in through porin channels (Pages et al., 2008). Lipophilic molecules are less prone to crossing the outer membrane than the inner membrane, probably because of the comparatively disordered arrangement of the inner membrane's lipid content (Plesiat and Nikaido, 1992). Overall, the multi-layered, multi-functional barrier that is unique to Gram-negative bacteria complicates the migration of both hydrophilic and lipophilic drug molecules due to the inner and outer bacterial membranes, respectively.

(iv) Oxygen tolerance of bacteria

Differences in oxygenation between tumour tissue and healthy tissue, and even between different parts of the tumour itself (Kizaka-Kondoh et al., 2003), is a foundational aspect of tumour-selective colonisation by administered bacteria. Selectivity occurs because the hypoxia that is evident in tumours does not materialise as severely in healthy tissues (Forbes, 2010). The selective colonisation of necrotic, hypoxic tissue by administered anaerobic bacteria is viewed as advantageous, because it permits access to parts of tumour tissue that are notoriously hard to penetrate using standard chemo- and radiotherapy (Dang et al., 2001).

There are drawbacks of exclusive necrotic tissue colonisation that ought to be noted. An obligate anaerobe that colonises only necrotic tissue will be farther removed from incoming prodrugs that are chiefly delivered to oxygenated tissues (Kizaka-Kondoh et al., 2003). Alternatively, a flexible facultative anaerobic bacterium, which can acclimatise to varying grades of oxygenation, will be better placed than an obligate anaerobe to promote maximal prodrug activation at its intended site of action. One might expect this flexibility to come at the price of potential colonisation of more

oxygenated extra-tumoural tissues and consequential systemic toxicity; however, both obligate and facultative anaerobic bacteria display similar tumour-to-liver colonisation ratios (Cronin et al., 2014, Cronin et al., 2010).

While facultative anaerobic bacteria are, in theory, more likely to inhabit more diverse tumour regions than their obligate anaerobic brethren, their adaptability is not assured. *S. enterica* serovar Typhimurium, for example, has been shown to colonise only necrotic regions of murine tumours, despite being a facultative anaerobe (Forbes et al., 2003). An auxotrophic variant of this same bacterium has since been engineered and observed to grow in both viable and necrotic tissue (Zhao et al., 2005). It is important to be cognisant that different auxotrophic modifications can be used to influence such bacterial platforms in different ways; for example, in the case here, the mutation increases the range of tumour tissue within which the bacterium can grow (due to oxygen-related metabolic activity) while in other cases, auxotrophic mutations restrict where a bacterium may survive.

Improving BDEPT R&D with tools for monitoring bacterial enzymatic activity

The defining reason for failure of oncological medicines in late-stage clinical trials is a lack of therapeutic efficacy (Arrowsmith and Miller, 2013). One of the causative factors may include insufficiencies in the technologies available for therapeutic drug monitoring *in vivo*. These technologies could help familiarise investigators with the *in-vivo* agency of elusive, novel medicines like BDEPT. For gene therapies in particular, the Recombinant DNA Advisory Committee of the National Institutes of Health recognises the need for reliable assays of transgene expression, and quality control of gene therapy carrier trafficking (Hollon, 2000). Blood sampling and tissue biopsies are invasive, periodic, and retrospective, and introduce convolution into the procedure (Gambhir et al., 1999). Contemporary standards therefore demand non-invasive, real-time facilities for continual assessment of transgene expression and therapeutic efficacy. The availability of a high-calibre facility for monitoring the progress of a medical intervention is correlated with an accelerated bench-to-bedside timeframe (Amir-Aslani and Mangematin, 2010), so it is worthwhile reflecting on how BDEPT can be satisfactorily monitored to mobilise its translation into the clinic.

For the BDEPT designer, real-time monitoring of the impact of the bacterial carrier and prodrug administration on tumour regression is an invaluable way of evaluating responsiveness to a medical intervention. The unique features of BDEPT offer the designer a special and convenient vantage point for surveillance of the course of treatment. The selective colonisation of tumour tissue by administered bacteria, and the resultant enzyme expression, can be utilised to fashion a prodrug-like compound into an imaging probe. In this setting, the probe is attached to a signal quencher that, when enzymatically cleaved, emits a detectable signal that can be observed *in vivo* using imaging techniques. Thus, a prodrug that is visually detectable when activated by a bacterial enzyme is indicative of transgene expression. A further potential benefit of this strategy involves the generation of data indicating the location and size of a malignancy.

Readouts can be produced via molecular imaging techniques, such as OI, PET, and MRI (Brindle, 2008). For preclinical imaging, OI represents the most feasible method for researchers in terms of accessibility, however, PET and MRI are more informative approaches for determination of clinical activity, and aid the formation of a complete diagnostic picture (Tangney, 2012, Collins et al., 2012). Several imaging modalities exist for non-invasive visualisation of bacterial enzymatic activity (Byrne et al., 2013, Tangney and Francis, 2012, Cronin et al., 2012a). Critical proof-of-concept issues can be tackled by adoption of these technologies, including quantification of bacterial growth, assessment of intratumoural growth patterns, and confirming whether tumour-selective growth has occurred at all post-administration.

Various dyes exist that are reduced to their fluorescent form by NTR. These include the redshifted, quenched fluorophore, CytoCy5S, which is reduced to its fluorescent form by NTR (Bhaumik et al., 2012, McCormack et al., 2013). This probe is useful in tracking the location of tumour-targeting bacteria *in vitro* and *in vivo*. (Stanton et al., 2015, Vorobyeva et al., 2015) VNP20009, expressing TK, has been engineered to enable its visualisation in C38 colon and B16-F10 melanoma tumours in mice (Soghomonyan et al., 2005, Tjuvajev et al., 2001). The radiolabelled nucleoside analogue FIAU, following VNP20009-TK-mediated phosphorylation to sequester it within tumours cells, allowed visualisation of bacteria *in vivo* by PET and autoradiography. Bacterial proliferation in tumours could be easily monitored, as radioactivity levels correlated with the numbers of bacteria present. Similarly, CPG2

activity has been monitored *in vivo*, in a xenograft murine tumour model, using the ^{19}F magnetic resonance spectroscopy reporter molecule 3,5-DFBGlu (Jamin et al., 2011).

While CytoCy5S, FIAU, and 3,5-DFBGlu are advantageous in that they permit monitoring of enzymatic activity, they are not therapeutic with respect to tumour growth inhibition. Several compounds have been synthesised that function as both therapeutic and monitoring agents. CNOB, for example, is converted into MCHB, which is both fluorescent and cytotoxic (Thorne et al., 2009). Moreover, the conversion of ^{19}F-labelled 5-FC into cytostatic 5-FU by VNP20047 expressing CD as a reporter gene has enabled visualisation of murine tumours *in vivo* in a human HCT116 colon tumour xenograft by MRI (Dresselaers et al., 2003).

BDEPT enzyme activity may also be examined in *ex-vivo* samples through indirect methods that assess the presence of activated probes within the sample (Figure 5). Such strategies have also been proposed as potential cancer diagnostics, in that they may reveal the presence of a tumour within the body due to their acting as a readout for administered bacteria that have colonised malignant tissue. For example, *E. coli* expressing a regulated *lacZ* gene was designed to detect experimental liver metastases (Danino et al., 2015). Following bacterial colonisation of hepatic tumours in mice, the bacterial *lacZ* device was induced by systemically administered isopropyl β-D-1-thiogalactopyranoside. Subsequently, a caged luciferin probe was administered, cleaved, and activated by lacZ, and renally excreted. Urine samples were quantified by emission of light for the presence of luciferin, facilitating quick and non-invasive cancer detection. In a similar fashion, an inducible biomarker module was created that can be detected in blood samples using a type of enzyme-linked immunosorbent assay (Panteli et al., 2015). The biomarker, ZsGreen, was expressed by *Salmonella* in a preclinical model to detect experimental colon carcinoma tumours.

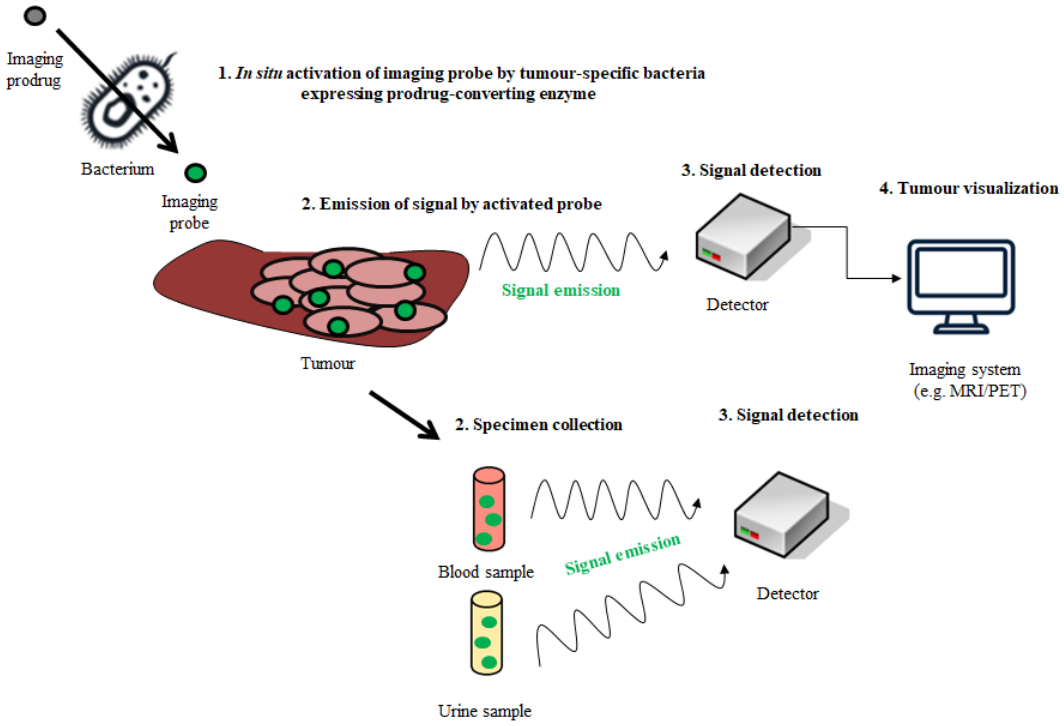


Figure 5. BDEPT enzymatic activity detection. *Therapeutic enzymatic activity may be detected in situ, or ex vivo. Direct in-vivo detection can be accomplished by activation of a light-emitting probe by bacteria-expressed enzymes at the site of the tumour, followed by MI, to acquire an image of the probe. Indirect detection may be achieved by analysis of blood or urine samples for probes that have been activated by bacterial enzymes in the tumour and secreted into the circulation.*

Barriers to the clinic

Several examples of clinically trialled BDEPT exist; however, the regulatory requirements necessary to make a breakthrough, regulated medicine of this kind have yet to be satisfied. It may be insightful to review the clinical progress achieved thus far with this therapy. The reasons for the absence of BDEPT from the clinic are probably multi-faceted, and the way in which designers comprehend and confront the barriers to the clinic will dictate how readily access to it is granted.

CD-BDEPT has been tested in phase I clinical trials with intravenously administered VNP20009 (Toso et al., 2002). Data were collected for 25 patients – 24 patients with melanoma, and one patient with metastatic renal cell carcinoma. Despite using an attenuated strain, doses above 3×10^8 CFUs/m^2 resulted in low levels of tolerance in

patients, and only three patients had tumours in which the administered bacterium was detected. Tumour regression did not occur in any patient. Trial investigators noted that preclinical studies in mice were not reflective of what was witnessed clinically, and speculated about host-specific differences between murine and human tumour composition that could affect colonisation of bacteria in malignant tissue. Following publication of the data for the phase I clinical trial, an update was issued describing the intravenous administration of VNP20009 in four additional melanoma patients (Heimann and Rosenberg, 2003). Results mirrored those of the pivotal study, with no patient achieving a significant clinical response.

After the phase I clinical trial of VNP20009, a pilot clinical study of CD-BDEPT was conducted with the same strain. This was narrower in scope than the pivotal study, recruiting three patients with head and neck cancer. (Nemunaitis et al., 2003). Notably, the gene for CD was chromosomally integrated into VNP20009 to increase therapeutic efficacy. Intratumoural injections of VNP20009-CD resulted in persistence of bacteria in two of these patients, bringing about the conversion of 5-FC to 5-FU at the patients' tumour sites. No significant measurements of 5-FU were made systemically. Even so, no tumour regression was observed in either patient and the trial was discontinued. This was a disheartening conclusion to encouraging preclinical data. King *et al.* had previously demonstrated efficacy in murine tumours using VNP20009 harbouring CD in which an 88-96% inhibition of tumour growth was observed in mice treated with VNP20009-CD and 5-FC compared with 38%-79% inhibition achieved with VNP20009-CD alone (King et al., 2002).

The results of the pilot and pivotal clinical trials of VNP20009-CD call for an explanation as to why tumour colonisation patterns differ between murine models and human subjects. Taken together, two factors appear to be common to both studies: poor enzymatic activation of 5-FC and poor colonisation of tumour tissue by VNP20009. It cannot be overlooked that the lacklustre kinetics of wildtype CD, referred to earlier, may explain why human trials of VNP20009-CD have so far returned with disappointing results in terms of inadequate tumour regression in tumour-colonised patients. Furthermore, as mentioned previously, colonisation of VNP20009 within tumours tends to be compartmentalised within necrotic regions, which are distinct from the viable zones of tumour tissue into which prodrugs are primarily delivered. A review of the literature reveals various efforts that endeavour

to address these problems. The identification of auxotrophic VNP20009 mutants and more efficient CD variants, referenced above, are clear attempts to improve tumour colonisation and tumour growth inhibition, while reducing the bacterial burden on the patients to whom the therapy is administered.

Recently, a transformed strain of *B. longum* (APS001F), containing a point mutation in its CD gene, was described as having 10-fold greater enzymatic activity *in vitro* than its wildtype counterpart (Sasaki et al., 2010). It subsequently demonstrated significant inhibition of tumour growth in murine models following 5-FC administration, and is the subject of a phase I/II clinical trial presently enrolling oncology patients (clinicaltrials.gov identifier: NCT01562626). Results from this trial are eagerly awaited, and may shed light on the effects of enzyme optimisation on the therapeutic efficacy of BDEPT.

Beyond therapy-associated parameters potentially responsible for inconsistencies between clinical and preclinical findings, external causes of disparities could be traced to the properties of the animal model used. A ‘quick win, fast fail’ approach is advised in R&D, by which the viability of a product is determined at the earliest possible stage (Paul et al., 2010). Acquiring insights into product inadequacies at the preclinical stage allows for expedited curtailment of failing R&D projects, thereby improving R&D productivity. Of course, the choice of an appropriate preclinical model that is the most illustrative of the clinical reality is an indispensable part of this process.

There has been a recent reallocation of interest to non-murine animal models, such as canine models, which may be more representative of a human response to cancer treatment (Paoloni and Khanna, 2008, Ranieri et al., 2013). The use of naturally occurring tumours in companion dogs has been examined for the benefits it offers over artificially induced tumours in mice for predicting clinical response. The genetically heterogenous character of spontaneous tumours in canines keeps more closely to the disparate nature of human malignancies than the artificial tumours of inbred mice, which have more a more homogenous make-up. Additionally, the circulatory system of dogs more closely resembles that of humans in terms of blood volume. The more expansive blood supplies of humans, compared with mice, can reduce bacterial colonisation within tumours. Tumours themselves within larger animals also tend to be comparatively smaller. Studies in another relatively large animal - the pig - are also

anticipated to have good translatability to human studies (Neff, 2019). Studies in this animal model are currently limited by the lack of experience with it in the cancer research setting, however – spontaneous tumours are not as commonly studied in pigs as they are in dogs, perhaps because the pig's role as livestock abbreviates its lifespan to a point where spontaneous tumours are significantly less common. Still, the resemblance that pigs share with non-human primate models in terms of their metabolism, genetics, pathology, and other comparators has led to sincere discussions of the role these animals can play in cancer studies (Schachtschneider et al., 2017).

The higher financial costs of carrying out canine tumour experiments compared with murine experiments are apparent (Paoloni and Khanna, 2008); however, dose-finding studies in mice may underestimate the dose necessary to achieve tumour colonisation in humans, due to incompatible physiologies. This may lead to more dissatisfactory patient responses in the clinical setting, ultimately leading to greater losses of time and money overall. Indeed, a canine study that compared IV and intratumoural administration with *C. novyi*-NT reported a significant anti-tumour response when the bacterium was administered directly into the tumour, with no animal achieving a complete response when administration was IV (Roberts et al., 2014). Had such a study been conducted in mice, it is possible that IV administration may have been sufficient for tumour colonisation, giving perhaps a more distorted reflection of what might occur in human subjects. A further study in dogs injected intravenously with *C. novyi*-NT spores reported a high frequency of noxious events resulting from treatment (Krick et al., 2012). This is reflective of the recent results of intratumoural injection of *C. novyi*-NT spores into cancer patients, detailed above, which uncovered similar toxicity issues, supporting the claim that the results of canine tumour models map neatly onto those of human cancer studies.

Gene therapy medicinal products are increasingly navigating their way into clinical trials, overseen by regulatory authorities that deliberate on whether the product makes it from one stage to the next. Regulatory authorities set stringent criteria against which the safety and efficacy of medicinal products are judged. This applies, too, to gene therapies, for which the use of live carriers to transmit therapeutic material warrants special regulatory consideration. Therapies containing live bacteria, up until now, have had a relatively weak foothold in the clinical armamentarium. BCG, a treatment for non-muscle invasive bladder cancer, is the only live bacterial therapy that is

currently approved by the FDA (Olle, 2013), and apart from being attenuated, is not genetically engineered (Zheng et al., 2015, Morgan et al., 2011). Safety assurance is key with bacterial treatments. Microbiological testing is a fundamental part of sound safety assessment of cancer gene therapies, and contamination of bacterial therapies with fungi, or indeed other bacteria, is a pressing regulatory concern (Husain et al., 2015). In fact, recent shortages of BCG have been attributed to possible fungal contamination (Bross et al., 2015). This underlines the importance of quality control and quality assurance in the manufacture of therapies like BDEPT.

Genetically engineered bacteria intended for *in vivo* applications face an atypical kind of regulatory hurdle in terms of safety assurance. The transference of resistance genes from genetically engineered bacterial therapies to host microbiota and pathogenic agents could result in the genesis of antibiotic-resistant disease, compromising patient safety. Thus, bacteria must be rid of antibiotic resistance genes before administration to clinical trial subjects can take place. Even innocuous probiotic bacteria, when formulated as feed additives, must be devoid of antibiotic resistance genes before they can be approved for use in Europe (Wassenaar and Klein, 2008). Microbes, even those that have been genetically weakened, are at risk of being shed from the initial host and spread from person-to-person (Husain et al., 2015). Thus, antibiotic-resistant disease could manifest at both the intra- and inter-individual level. Bacteria should ideally be disabled from replication in the final product, and in case of their release into the environment, where they could be easily acquired by other individuals, their growth should be negated. Heap *et al.* have recently developed a technology that obviates the need for inclusion of antibiotic selection markers in bacterial strains (Heap et al., 2012). They thereafter reported the first example of a bacterium, *C. sporogenes*, with a chromosomally encoded prodrug-converting enzyme that does not require antibiotic resistance genes (Heap et al., 2014). The strain was also rendered auxotrophic by inhibiting its ability to synthesise pyrimidines, meaning that it would not proliferate if it were released into the environment. In doing so, they have invented a strain that potentially has a far more attractive safety profile for patients, which could help further ease regulators' worries about the propagation of antibiotic resistance.

BDEPT prospects

The ingredients necessary for transportation of BDEPT into the clinic may already be available, but their correct management and coordination by the designer will prove instrumental in building a meaningful product. As the bacterial carrier is the defining feature of this type of DEPT, a bacteria-centric focus stands to illuminate the way forward in terms of tackling deficiencies in BDEPT designs.

There is much to learn from the technical shortcomings of recent preclinical investigations of BDEPT that can be instructive for prospective design attempts. It has been reported that lesser prodrug activation using BDEPT, compared with VDEPT, could be accounted for by disparities between viral and bacterial carriers that lead them to concentrate in different types of tissue (Hsieh et al., 2015). Viruses, such as those derived from the murine leukaemia virus, gather only in viable, proliferating cells (Schepelmann and Springer, 2006), while bacteria colonise both viable and necrotic tissues to different degrees, depending on their ability to thrive in oxygenated and deoxygenated environments. It may be constructive to bear this in mind at the design stage. The ideal bacterial carrier will colonise diverse types of tumour tissue. Poor colonisation of viable cells will diminish prodrug activation, while poor colonisation of hypoxic, necrotic tissue may affect therapeutic potential to reduce tumour growth. It would be prudent to clarify the intratumoural growth patterns of the bacterial carrier prior to its inclusion in the BDEPT design.

Other bacterial properties that merit further investigation include the bacterial cell envelope. The Gram-positive periplasm is smaller than that of Gram-negative bacteria (Malanovic and Lohner, 2016), which may explain why only the Gram-negative periplasm has been exploited in BDEPT to improve enzymatic activity; however, an unfamiliarity with the Gram-positive periplasm in general could be limitational. Given that Gram-positive bacterial cell envelopes omit an obstructive outer membrane, an enzyme incorporated into the Gram-positive periplasm will be more accessible to a circulating prodrug. The Gram-negative bacterial periplasm, although more familiar to the designer, is sheathed in greater membranous complexity. It may therefore be productive for BDEPT designers to investigate the use of the Gram-positive periplasm as a means of improving BDEPT efficacy.

Both Gram-positive and Gram-negative bacteria contain membrane-associated efflux pumps that can export drugs from the bacterium (Piddock, 2006, Blair et al., 2015). Efflux pumps are conventionally studied in the context of multidrug bacterial resistance against antibiotics, so not much is known about them in relation to chemotherapies. Nonetheless, there is speculation that they may be responsible for ejecting chemotherapeutic drugs from bacteria (Florez et al., 2016). While this may have a hand in contributing to bacterial resistance to chemotherapeutic toxicity, efflux pumps could be seen alternatively as passageways through which an API may make its way outside a cell. Future BDEPT designs might benefit from considering the potential for efflux pumps to act as escape hatches for drugs that would otherwise be trapped by their unfavourable pharmacokinetics. Research into the effects of bacterial efflux pumps on chemotherapeutic drugs would be welcomed in this regard.

Stimulating productivity in R&D is contingent on researchers keeping abreast of emerging technologies that could help expedite their products into the clinic. Synthetic biology is a novel scientific discipline that offers a profound design tool, giving its practitioners the means to bioengineer molecular circuits, enabling the design of 'intelligent' cellular systems (Flores and Tangney, 2017). It is a valuable discipline from a BDEPT perspective, because it allows its designers to assemble 'parts' (e.g., promoters and secretion tags) into 'devices' (e.g., a plasmid or chromosome) within host systems called 'chassis' (i.e., the bacterial carrier), permitting fine-tuning of enzyme expression and function (Figure 6). Enzymatic constants like K_M and k_{cat} are relatively easy to predict. However, these constants change inside the chassis, making it difficult to calculate the kinetics of the enzyme. Synthetic biology can be utilised to fine-tune the chassis and the device together for optimal performance.

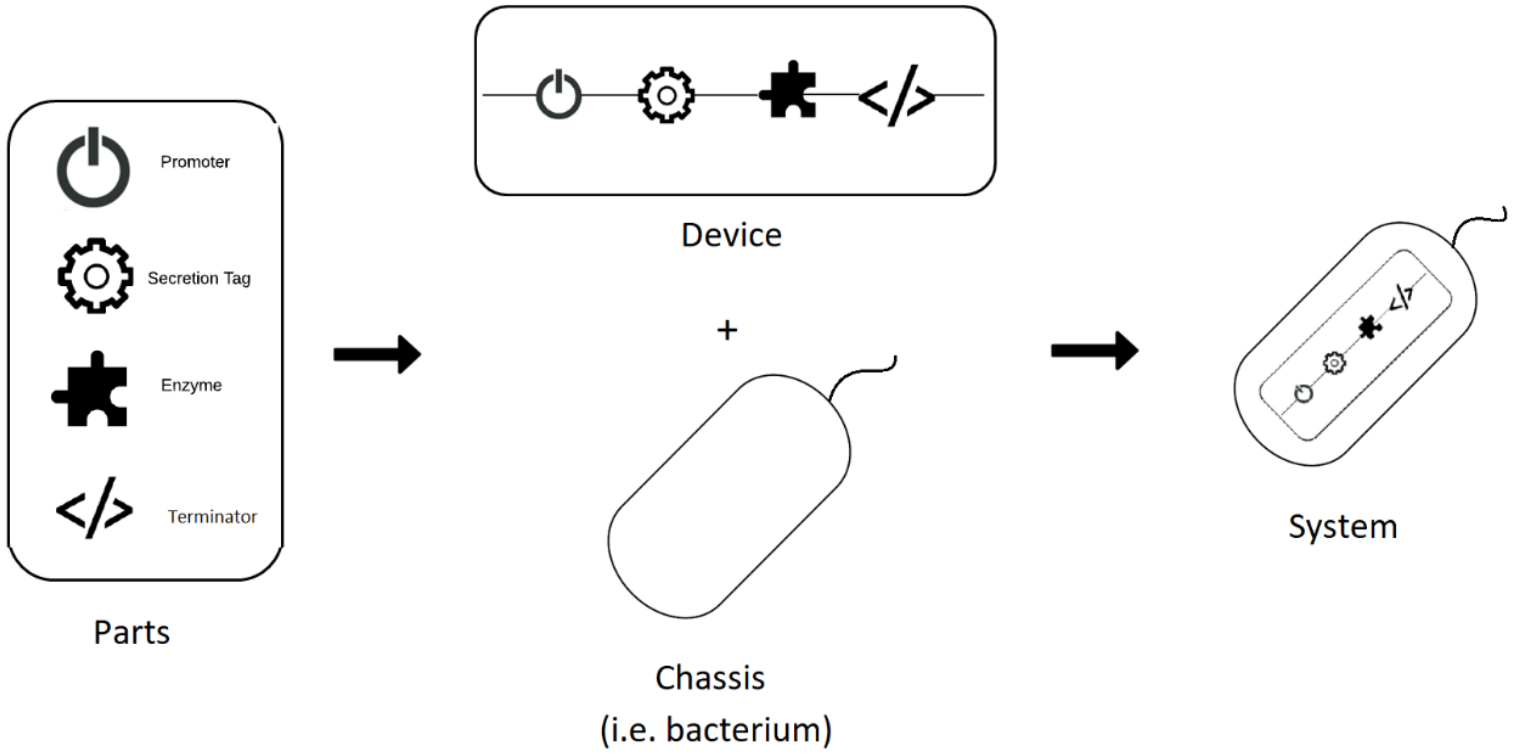


Figure 6. The application of synthetic biology to BDEPT. *Synthetic biology rationalises BDEPT systems design by reducing its holistic structure to well-characterised, functional "parts" which, in concert with one another, are built into a modular "device" to carry out a predefined role. The device, when embedded in a chassis, forms a "system" that is fit to complete a task in accordance with its design. Within BDEPT, bacteria act as chassis for devices in the form of plasmids and chromosomes. These devices are equipped with various parts, e.g., secretion tags, that aim to coordinate the expression of a prodrug-converting enzyme.*

Synthetic biology has been investigated for its applicability in perfecting bacterial homing to tumours. While bacteria are indeed tumour-selective following IV administration, clinical experience thus far underlines the reality that human tolerance of administered bacterial loads places restrictions on BDEPT dosing regimens (Heimann and Rosenberg, 2003, Toso et al., 2002). In fact, there are instances where even commensal bacteria have had dosage limitations placed upon them in preclinical studies (Cronin et al., 2010). From synthetic biology principles, synthetic adhesins have been assembled based on the β-barrel domain of intimin - an adhesin derived from pathogenic *E. coli* strains - and the variable immunoglobulin domain of heavy-chain-only antibodies (Pinero-Lambea et al., 2015). When affixed to bacteria, these adhesins raise the tumour-targeting capacity of the chassis, and reduce the bacterial titre required for tumour colonisation.

Gene therapies in general have had scattered clinical fortune, and even those that are therapeutically efficacious and safe in clinical trials have ultimately been unusable due to commercial incompatibilities (Touchot and Flume, 2017). This means that BDEPT can undergo translation from bench-to-bedside, and its developers are still liable to be short-changed by additional blockades. It is therefore wise for the BDEPT designer to subject their product to wide-angled scrutiny so weaknesses in their product can be uncovered at an early stage.

This review makes clear that there is a complex line of questioning the BDEPT designer must pursue to create a workable product, yet this kind of therapy holds much promise to become a breakthrough mainstay of cancer treatment. Aside from potential design flaws of BDEPT keeping it from the clinic, its momentum could be hampered by external forces, such as R&D culture, which is a major consideration of section 2 of this literature review. The novelty of BDEPT makes it an especially lucrative avenue of R&D to explore, and its advent comes at a time of concern and precariousness in the pharmaceutical industry. A decline in R&D efficiency is evident, and it is speculated that it is becoming harder for novel medicines to reach the clinic, partly because of increasingly demanding regulatory authorities and the extremely high expectations of healthcare standards set by existing treatments (Scannell et al., 2012). BDEPT is opportunely positioned to overstep these hurdles. There is a need in oncology for safe, targeted medicines, whereas for other indications such as hypertension and asthma, standards of care are already impressive and tough to

improve on. This creates an entry point for novel oncological medicines, like BDEPT, which does not exist as obviously with medicines for other indications, for which the bar might be set much higher.

An indulgence in a field of drug discovery that has not yet begun to flourish may feel to the drug designer to be an overly risky endeavour. However, it is pertinent to recall that risk and prosperity in R&D go hand-in-hand (Munos and Chin, 2011). Designers of BDEPT are potentially on the verge of forging a new, ground-breaking form of cancer therapy. How adeptly they will engage with this prospect will depend on their familiarity with the realities of R&D, and on their mastery of BDEPT design principles.

Conclusion

BDEPT possesses singular characteristics that differentiate it from other forms of gene therapy and directed enzyme prodrug therapy. These assets have placed it in the company of other promising treatments that aspire to control and cure malignant disease while minimising off-target damage. However, there are still obstacles that must be traversed before BDEPT can occupy a *de facto* position in the clinician's anticancer repertory.

BDEPT has experienced relatively little exposure to clinical testing thus far, but, to the extent that it has, it is notable that human subjects respond to treatment in a way that is sometimes inconsistent with preclinical findings. This disparity raises the question of how R&D practices should be strategized and enhanced to bridge the gap between clinical and preclinical observations.

Bacteria are undeniably adept at preferential accumulation in malignant tissues, yet clinical reports have pointed out that the targeting potential of these microbes may be restricted by dosage limitations that apply even to attenuated bacteria. This concern is reflected in the myriad studies that seek to reduce bacterial titres needed for tumour colonisation by, for example, the optimisation of enzyme kinetics. The co-option of synthetic biology to engineer superior tumour-targeting facilities into bacteria is also indicative of this concern.

Section 2: The current state of drug R&D and its effects on advanced therapies

Abstract

The healthcare industry is experiencing a significant decline in R&D productivity, casting doubt on its sustainability, and provoking considerable inquiry into the prevailing forces at the root of the issue. General inquiry has focussed on optimisation of technical, product-related R&D practices, with only brief consideration given to the people ("actors") by whom the institutionalisation of these practices is ultimately considered.

The shape of actor participation in early-stage R&D can vary wildly depending on the 'historical novelty' of a medicine. Prior analyses have demonstrated that drug discovery in general is dominated by large pharmaceutical companies, while new data presented here show that the discovery of disruptive pharmaceutical technologies is avoided in big companies. It is suggested in this literature review that this trend does not result from a technical inability of big companies to discover historically novel drugs, but from human attitudes and behaviours that may be overcome in a people-centric way.

The impact of current R&D culture on discovery and development of advanced therapies, including bacterial anticancer therapies, is considered here and big pharma's disinclination towards historically novel drug discovery is questioned in the context of an increasingly unproductive industry. A case is made for why R&D efficiency is dependent on an understanding of the way inter-group relationships manifest within the field of drug discovery and development, and a basis is provided for why the involvement of actors of all types from the earliest stages of R&D can enhance R&D productivity and help to restore the industry's viability. It is also explained how confined, stereotypical perceptions of big pharma, small biotechnology companies, and academia can be disruptive to this effort.

Introduction

R&D within the pharmaceutical industry is undergoing a well-catalogued decline. Success stories are rare, as the number of marketed medicines that can be found on the hospital trolley or the pharmacy shelf is dwarfed by the sheer volume of products lost along the way in the drug pipeline (DiMasi et al., 2016, Cook et al., 2014). The concept-to-product path for a commercial therapeutic is a long and costly one, with total capitalised expenses approaching three billion dollars (DiMasi et al., 2016). Assuming the compound in question is not among those that are to be weeded out, it will take 10 to 15 years on average for it to complete its bench-to-bedside journey (Field and Lo, 2009). A product entering a phase 1 clinical trial, following preclinical evaluations, has a 10.4% chance of having its NDA BLA approved for a particular indication (Hay et al., 2014), but some variability exists across different product types and indications. For instance, systemic anti-infective agents have been shown to have higher clinical approval success rates than cardiovascular drugs – 23.9% and 8.7%, respectively (DiMasi et al., 2010). In addition, a biological medicine has an almost two-fold greater chance of proceeding from a phase 1 clinical trial to regulatory approval than an NME – 11.5% and 6.2%, respectively (Thomas et al., 2016). These statistics overall reflect poorly on R&D efficiency, and the need to improve on these numbers is widely acknowledged.

The capital required to conduct R&D (8-10%) is slightly greater than the internal rate of return for small-molecule drugs (7%), while biologics have a marginally more prosperous return rate of 13% (Meier et al., 2013), and more substantial investments in R&D do not result in a corresponding improvement in regulatory approval rates (Munos, 2009). It should come as no surprise then that big pharmaceutical companies are becoming more reticent about investing as much in R&D as they once did (LaMattina, 2011). The same industry that was once praised for embracing high-quality, disruptive technology is now criticised for investing its time in developing medicines with marginal benefits over their predecessors (Munos, 2013). The industry is of course responsible for recently making available important treatments for diseases both rare and common, such as the 2017 approval by the FDA of the first drug treatment for a form of Batten disease (Johnson et al., 2019) as well as the approval of PD-1 and PD-L1 inhibitors over the past decade for urothelial cancer immunotherapy (Hargadon et al., 2018). Nevertheless, attrition rates have been

steadily increasing across all clinical trial phases over the past two decades, attracting scrutiny of conventional R&D practices in an effort to unearth potential flaws (Mignani et al., 2016).

There is ongoing debate on the extent to which various obstacles come to bear on the unfavourable development times, costs, and clinical success rates for medicines. Different authors can even part company in their assessment of what constitutes an obstacle in the first place. For example, some authors suggest that a more restrictive regulatory presence can be counterproductive, reasoning that its austerity is rarely alleviated once it is imposed (Scannell et al., 2012), while others note that heightened regulation can help to concentrate a drug company by forcing it to be choosier about which products it develops, thereby making it more selective with its resources (Munos, 2009). An increase in the size and intricacy of clinical trials is apparent, which is possibly a consequence of more stringent regulations, and might be implicated in R&D diminishment (Getz et al., 2008). Impediments to successful R&D programmes have recently been scrutinised in anticipation of contemporary problems that challenge the healthcare industry. Patent expiration of products like Plavix, Lipitor, and Seroquel threatens to dry-up drug company income markedly (Mignani et al., 2016). Coupled with the elusive forces that compromise the integrity of the contemporary drug development framework, the patent cliff quandary has re-directed the attentions of drug companies, economists, and academics towards alternative ways of reducing costs, yet amplifying output.

One review provides a summary of the numerous avenues of thought that other reviewers have pursued, such that plausible resolutions to the drug development dilemma could be identified (Scannell et al., 2012). Most of these proposals suggest adjustments of process-related parameters like outsourcing R&D activity to more affordable countries, or paying more attention to facets of clinical research that can enlarge the R&D budget, such as the over-powering of a clinical trial (David et al., 2009). Indeed, while we have a broad awareness of the mechanistic factors and how they might impinge on the economics and functionality of R&D, more consideration to the human side of the issue is owed. Healthcare products are conceived, crafted, and judged by humans, and thus a comprehensive knowledge of drug discovery and its pitfalls is achieved by understanding not only technical aspects of R&D, but also by understanding the people who influence triage from idea through to market. This

concept is of increasing importance to scholars who attempt to provide an evidence-based rationale for advancing public and private scientific policy (e.g., Science of Science and Innovation Policy).

Understanding the process flow; 'What' can be improved vs. 'Who' can be improved

To frame this review, a people-centric R&D model is illustrated here, populated by "actors" who serve different functions in the idea-to-product timeline (Figure 1). Healthcare products are effectively carried through obstacles, or "gates", from concept-to-market by individuals or groups who vouch for them (referred to as "promoters"). Any actor who takes an active, pioneering role in the conveyance of a medicine from concept-to-product, by building value into it such that it can oppose whichever forces keep the gate locked, can be considered a promoter. During stage (a) for example, a Principal Investigator in a university, or other PSRI, could be the actor in charge of this value-building exercise, or creating the value in the first place through foundational research (Dahlin et al., 2015, Frearson and Wyatt, 2010). In the wider, non-academic R&D sphere, biotechnology and big pharmaceutical companies assume the role of promoter and use industrial-grade resources to prepare a medicine to cross its gates (Kaitin, 2010).

Gates are maintained by "gatekeepers" – individuals or groups who deliberate on whether the product makes it past a checkpoint. Gatekeepers can have diverse profiles and objectives. They can range from public (government) and private (venture capitalist) investors who may approve or disapprove of a product on commercial or philanthropic grounds (Cavalla and Minhas, 2010, Dixon et al., 2009), to regulatory authorities who hold a product up against strict efficacy and safety criteria. The "forces" referred to previously that prevent the gate from opening, and with which the promoter must grapple, are therefore equally diverse and are reflective of the gatekeeper's motivations - the desire to invest in a viable, profitable thing in the investor's case, and the obligation to ensure patient safety and healthfulness in the regulatory authority's case. This diversity necessitates wide-ranging expertise on the promoter's part to permit a smooth transition from one gate to the next. This often requires the product to change hands several times between early-stage bench research

and regulatory approval. However, promoters may not always work alone in discrete pockets of R&D, and there is greater advocation for the integration of different kinds of promoters (referred to as "open innovation") as collaborative research becomes more attractive (Mignani et al., 2016). Gatekeepers represent a key stumbling block for promoters. A venture capitalist's categorical decision not to invest, a regulatory authority's dismissal of an IMP, or big pharma's rejection of lower-tier companies following phase II clinical trials (Moore, 2003) are all illustrative of the harsh realities of R&D for any actor looking to promote a medicine. Market-approved products are brought closer to the end-user (i.e., the patient), where payers weigh up the financial consequences of including the therapy in their budget, and physicians compare the clinical value of the product with the current standard of care.

Finally, at the peripheries, but still crucial, are the "facilitators". These are distinguished from promoters in that, while they may be similarly proactive and focussed on product progression, their role is more ancillary. They are necessary for product progression, but are not decision makers. For example, TTOs position themselves at the threshold between academia and industry with the aim of progressing the output of an academic institution (Dahlin et al., 2015, Smith, 2011). Similarly, CROs and hospitals play host to clinical trials, and are commissioned by promoters to provide a supportive function, rather than conduct an independent, unsolicited act of scientific creativity and entrepreneurship (Kaitin, 2010).

The predominant perception in the literature is that small biotechnology companies are "innovation-driven" but "cash-starved" (Khanna, 2012, Malik, 2009); that wealthy big pharma is reliant on them and "need[s] their wares" (Moore, 2003); that universities are an "important source of commercially-exploitable technology for pharmaceutical companies"; and that "most basic research occurs outside the walls of pharmaceutical laboratories" (Wang et al., 2015). Big pharma is increasingly viewed as a generalist with a creativity deficit, and is often noted for its predilection for M&As that help it profit from the labour of smaller, more inventive companies (Gautam and Pan, 2016, Moore, 2003, Wang et al., 2015). Sometimes it is acknowledged that drug discovery can in fact take place in big pharmaceutical corporations, but even in these instances the phrase "discovery research" is omitted from a list of their strengths (Fishburn, 2013). There is a sense that an overarching, linear flow proceeds from discovery-oriented academia and small-scale firms, to development-oriented big

pharma (Edwards et al., 2003), to the physicians who make the final judgment call on whether the medicine gets to be used in a patient, wholly removed from the context of a study.

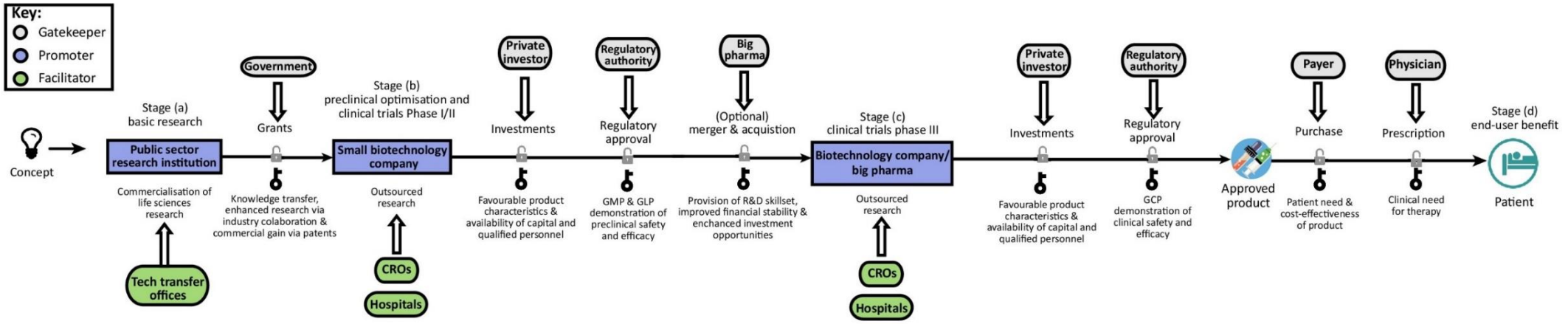


Figure 1. The actors in drug discovery and development. *A depiction of the array of individuals and organisations involved in bringing a medicinal product from concept-to-market, colour-coded based on their typical functionality within the R&D framework. Promoters (blue) and facilitators (green) are assigned to the stages of R&D with which they are commonly associated. The auxiliary role that facilitators provide to promoters is indicated by an arrow pointing from the facilitator. Each stage is divided from the next by gates, each represented by a lock and key. Gates are overseen by gatekeepers (grey). Actions that unlock each gate are indicated by an arrow pointing from the gatekeeper. Conditions that favour the movement of a medicinal product from stage-to-stage are described underneath each lock and key.*

Putting a face to the name: Who does what, and why?

Evidently, the success of a healthcare product hinges on many different determinants, and a great deal of effort is spent pinning down these determinants and ensuring they are fine-tuned. To the extent that the actors of drug discovery and development are explored, discussions mostly concern the "discovering organisation" (or "originator") of an IMP. A comprehensive review of drug discovering organisations concludes that, in Europe, big pharmaceutical companies are the biggest source of drug origination in general, and they comprise almost half of all originators in the USA (Kneller, 2010). It also underlines that big pharma is the biggest contributor to the discovery of drugs that are expected to "provide substantial therapeutic benefits" as defined by the CDER in the USA. The review makes clear that different promoters are attracted to different sub-types of drug discovery, given that big pharmaceutical companies are the strongest originators by far of follow-on, or "me-too" drugs (referred to as "old"). However, drugs with novel chemical structures or modes of action (referred to as "new") are noticeably overtaken by biotechnology companies and universities. Here, newness is discussed in the context of a drug's structural and functional ("scientific") novelty, but a different kind of novelty is not considered, namely the 'historical novelty' of a medicine.

The pharmaceutical industry's history with small-molecule drugs predates the advent of biological drugs by decades. The first successful double-blind, randomised controlled trial for a chemical compound occurred in 1946 with streptomycin (Bhatt, 2010), four decades prior to the regulatory approval of insulin, in 1982 (Walsh, 2005), and the first monoclonal antibody, muromonab, in 1986 (Liu, 2014). It may be the case that pharmaceutical companies are prolific originators of follow-on drugs for two reasons: i) they are being guided by the blueprints of the innovative medicines on which they are based (i.e., they have little structural and functional novelty, and therefore are 'known' to them), and ii) most of these are small-molecule drugs, which are a relatively old group of medicines (i.e., they have little historical novelty). It is known that big pharmaceutical companies are disinclined to discover drugs with structural and functional novelty (Kneller, 2010), and this bias may also exist against the discovery of historically novel drugs, such as NTBs. Big pharma discovered 66% of all NMEs approved by the FDA between 1998 and 2007, but only 11% of NTBs. Thus, big pharma tends to become more hesitant about its participation in drug

discovery the more historically novel a medicine is, and its less impressive input into biological drug discovery may be attributable primarily to the historical novelty of compounds like muromonab.

Two further sets of analyses outline how the actors who initiate and take ownership over an R&D programme may have a bearing on the outcome of the project (Lincker et al., 2014, Munos, 2009). In the former article, data are presented on the originators of CBRDs approved by the EMA from 2010 to 2012. No distinction is made between scientifically novel, historically novel, and follow-on discoveries, and CDER classifications are not considered. Once again, however, the largest companies emerge as the most prolific originators, having discovered 28% of all CBRDs approved within this period. The latter article follows suit and reports big pharma to be the prevailing source of NMEs approved by the FDA in the past half century.

If it is true that the historical novelty of a medicine is inversely proportional to big pharma's willingness to get involved at the discovery level, then this has serious implications for R&D efficiency in general and the strength of big pharma's drug pipelines in particular. The output of the biotechnology industry is interwoven with the economical profile of many countries (Carlson, 2016). Additionally, the sustainability of the pharmaceutical industry itself relies on investment in high-risk, innovative R&D (Meier et al., 2013, Munos and Chin, 2011) which is characteristic of historically novel drugs. The idea that unfamiliarity may be precluding big pharma from engaging with the discovery of historically novel drugs is countered by the volume of resources are available to bridge this gap. In Europe for example, rich archives of information for small-molecule drugs and biologics are made available by the EMA via public assessment reports (Papathanasiou et al., 2016), post-marketing surveillance schemes (Johnson and Hutchinson, 2015), and the disclosure of scientific opinions (Permanand et al., 2006), among other sources. These archives can help to define patterns of success and failure, and probe the question of how to enhance target hits and reduce misses. Additionally, an abundant understanding exists of the physicochemical properties of small-molecule drugs and biologics, in terms of what makes a chemical soluble, what makes a protein safe and stable, and so on (Manning et al., 2010, Moynihan and Crean, 2009).

Yet, there are new forms of medicine for which this quantity of information is not available, including ATMPs, that may suffer more significantly from lack of attention from big pharma at the discovery stage. To put their novelty into perspective, a Committee for Advanced Therapies was established only recently by the EMA, in 2007, to coordinate the ATMP review process of the CHMP (Hughes-Wilson and Mackay, 2007). ATMPs comprise gene therapy medicinal products, somatic cell therapy medicinal products, and tissue-engineered products, and thus bacterial anticancer therapies would be categorised under the ATMP heading. The insubstantial number of ATMPs that have been examined by the EMA to date restricts the scope through which they may be analysed. As of January 2017, only 12 ATMP MAAs have been received by the CHMP. Contrastingly, in the USA, 43 NDAs and BLAs were received by the FDA in 2012 alone (Mullard, 2013), and in Europe, 48 MAAs for CBRDs were received by the CHMP in 2009 alone (Eichler et al., 2010). The novelty of ATMPs calls for an examination of the promoters who are bringing these medicines out of obscurity. As originator profiles, approval rates, and development costs change from small-molecule drugs to biologics, it is reasonable to presume that ATMPs similarly have their own distinctive trends. Furthermore, as the quantity of raw clinical and preclinical data for ATMPs is scant, and will only be published slowly over time, an inspection of originators of ATMPs may be the most lucrative and informative way of learning more about them.

An original analysis of the discovering organisations of ATMPs revealed that, since 2009, the EMA has granted marketing authorisations for 10 ATMPs, of which three have already been either withdrawn or suspended (Chondrocelect, MACI, and Provenge) and one has expired without renewal (Glybera) (Hogan and Tangney, 2018). Public statements issued by the EMA highlight that commercial factors resulted in the withdrawal of Chondrocelect and Provenge, the absence of an authorised manufacturing facility prompted the suspension of MACI, and lack of demand resulted in the non-renewal of Glybera. Each of these ATMPs followed a common track in which discovery and development were overseen by SMEs with collaborative input from large firms occurring long after clinical trials commenced, if at all. Glybera, for example, was discovered and developed by the SME Amsterdam Molecular Therapeutics and first trialled in patients in 2005 (Bryant et al., 2013), eight years prior to Chiesi Farmaceutici's involvement with the product. Chondrocelect and Provenge

were discovered and developed solely by SMEs (TiGenix and Dendreon Corporation, respectively), and MACI (discovered and developed by the SME Verigen AG) was acquired by Genzyme in 2005, seven years after the product was made available in several European countries, Australia, and parts of Asia.

The revocation of ATMP marketing authorisations for primarily commercial reasons, and not due to post-marketing surveillance detecting safety and efficacy concerns not observed in clinical studies, is revealing. Small biotechnology companies are often reliant on larger firms for marketing and manufacturing expertise (Yang et al., 2013), and delayed evaluation of late-stage issues like these even at the discovery phase could have dramatic effects downstream. One might reason that the expansive budgets and strong footholds big pharmaceutical companies typically have would encourage them to partake in ATMP discovery, and that the exorbitant costs of clinical and preclinical R&D would appeal more to the organisations that would not take such a hit were their efforts to fail, notwithstanding the novelty of the research being undertaken. The reality does not reflect this, however. Biotechnology companies are advised to be wary of aspiring to big pharma's business model, because organisations tend to become less innovative and less agile the bigger they are (Douglas et al., 2010, Moore, 2003). It is also reasoned that biotechnology companies are not meeting the mark expected because they may have already adopted big pharma's model (Munos, 2009), and the bureaucracy that is characteristic of big companies could stifle their inventiveness (Booth and Zemmel, 2004).

Appreciating actor risk preferences and motivations

The absence of large pharmaceutical companies from early-stage ATMP R&D could be due to multiple reasons. It may indicate a lack of confidence that these companies have in ATMPs as a commercially dependable therapeutic class. Its reluctance may also partially derive from a technical inability to discover historically novel drugs, as the fundamental platforms necessary for this type of R&D are lacking in pharmaceutical companies by default. Big pharma is increasingly entertaining the prospect of acquiring the platforms of large biotechnology companies to combat this problem (Malik, 2009, Rafferty, 2007), and this may be the primary reason why it has been able to tap into biological drug discovery at the low level it has to date. These

platforms unfortunately have not yet translated into significant success in biological drug discovery by big pharma, which suggests that they are probably being used as developmental tools and not discovery tools. It also suggests that technical inability is not the overriding impediment to historically novel drug discovery in big organisations.

If mechanistic factors are not at play here significantly, it is again worth regarding the problem from a people-centric perspective; big pharma's absence among historically novel drug originators may stem from the manifestation of human attitudes that do not materialise in universities and small biotechnology companies to the same degree. It is proposed here that R&D projects fail at least in part due to the risk appetites of the various actors being at odds with one another, further causing the actors to sequester themselves in different parts of the R&D timeline (Figure 2). Alongside this, there may be a tendency in the literature to confine the R&D actors prematurely and unjustifiably to specific roles. Actors of R&D are often placed into isolated boxes based on how they are perceived to operate, allocating small biotechnology companies and academia to drug discovery, and big pharmaceutical companies to drug development. Each R&D actor is accustomed to asking questions addressing problems pertinent to their immediate circumstances, causing those questions to be answered diffusely across the entire timeline. Ideally, questions that reveal salient information about a therapy should be answered at the beginning of drug development projects. The fact that different actors tend to concentrate at discrete parts of the R&D timeline means that each one is fortified with valuable drug development insights resulting from their unique vantage points; however, this specialisation comes at the cost of a sequestered, myopic perspective of the wider picture. It can be proposed therefore that the sooner that actor coordination occurs, the greater the benefit to R&D productivity.

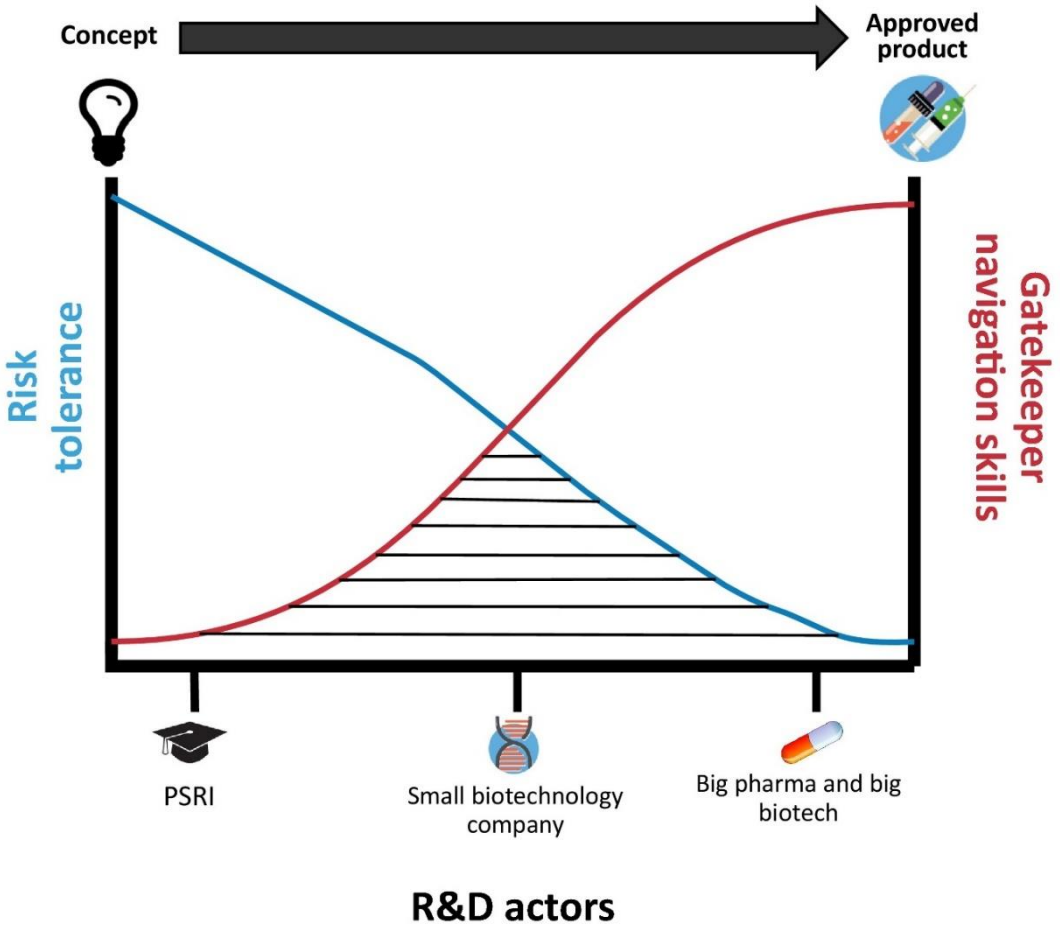


Figure 2. Actor aptitudes across the R&D timeline. *The degree to which fundamental R&D attributes (gatekeeper familiarity and risk tolerance) are expressed in the R&D timeline by the R&D actors. In being stationed at the extremities, PSRIs and big drug companies have developed a skewed skillset in favour of one attribute over the other. An ideal balance of both attributes coincides with the Small Biotechnology Company, found within the middle ground of the R&D timeline.*

Novel therapies are likely to be discovered in a PSRI or small biotechnology company outside of a collaborative context, and later acquired by larger corporations, primarily because big drug companies are required to cope with the extraordinary costs of late-phase clinical testing (Lipton and Nordstedt, 2016). Such sequential transfer of technology creates 'distance' between early and late-stage actors, resulting in under-informed design of the product and a fragmented knowledge of the technology as it changes hands, ultimately diminishing success rates. Although fledgling biotechnology companies have tremendous flexibility, their futures are uncertain as

SMEs sparingly have the occasion to engage multiple unsuccessful projects before they become financially exhausted (Löfsten, 2016), leaving them with little room to 'get it wrong'. Incentivising the R&D actors to collaborate before a product's design is finalised would be supportive in this respect. This is an important point, and it comes on the heels of a purposeful push in the literature for more collaboration in R&D (Fishburn, 2013, Khanna, 2012, Mignani et al., 2016). There is a corresponding increase in R&D output when more actors pitch into drug discovery and development (Munos, 2009), while M&As tend to have the opposite effect. They are seen as growth immobilisers (LaMattina, 2011), and signify a reduction in the number of promoters available to add value to an R&D project. Collaboration would thus mitigate some of the risk that big pharma typically tries to evade.

There is evidence that big drug companies already possess a sophisticated approach to R&D in terms of the way they form collaborative networks between themselves and external partners (Mignani et al., 2016). While large firms have indeed made progress interfacing with R&D programmes at earlier stages through collaboration, the above analysis of market-approved ATMPs suggests that this has not translated into critical, early-stage collaborations in high-risk, innovative zones of drug discovery. The disconnect between the R&D actors is at its core governed by human attitudes towards risk and can only be truly appreciated by probing the motivating factors behind institutional behaviours Big corporations with up-and-running pipelines are often comfortable with sticking to what they know best, and the emergence of a new technology with which the company is unfamiliar can signal a disturbance to its viability (McNamee and Ledley, 2012). Additionally, given the austere regulatory environment and considerable financial risk that pervades the pharmaceutical industry, it is understandable that these groups appear reluctant to commit to novel R&D projects in their early stages, however detrimental it may be to R&D productivity. Furthermore, if the stereotypical view is accepted that big pharma's domain lies in developing or sponsoring the development of drugs, and not discovering them, it will wrongly be dismissed out-of-hand when early-stage collaboration is discussed. If big pharma itself submits to the domineering perceptions of it – that it is possessive of proprietary data and awkward to work with (Douglas et al., 2010) – it will be ill-equipped to pursue collaboration, precompetitive or otherwise.

This raises the question of how a quid pro quo relationship should be drawn between large drug companies, PSRIs, and SMEs to bring them together at the outset of R&D projects. Large drug companies, being more intimately at the frontier between regulated and unregulated medicines than any other R&D actor, are ideal counsellors on the selection of appropriate patient cohorts, providing advice on adverse drug reactions and adverse events, and clarifying the existence of a clinical need for the therapy, while universities are criticised for being out-of-touch with industrial standards (Whitty, 2011). This may be because different promoters gauge the success of a product using different metrics. Pharmaceutical companies measure success by the commerciality of their output, whereas in academia, the publication of research papers is the benchmark (44). An academic scientist who invents something, upon publication of their research, might reason that what they have invented will be successful because the criterion on which they base their success has been fulfilled. This may lead to deficient products that are overvalued by academics because their research is publishable, and poorly received by industry because their products are not marketable.

If industrial companies delay their participation and instead opt to inherit the half-baked fruits of academic institutions downstream of the discovery stage, it may be too late for their input to correct any design flaws. Successful universities are adept at both basic research and entrepreneurial activity (D'Este and Perkmann, 2011), and the output of a university will have a greater likelihood of being marketable if its research programmes are market-informed. This should encourage academics and big pharma to seek out connections with each other at the earliest opportunity, so these differences can be negated. These partnerships would hugely benefit not only big pharma, but small biotechnology companies also, which depend on universities to create successful products (Bstieler et al., 2014). Furthermore, big pharmaceutical companies provide less established promoters with the tools to overstep several gatekeepers and facilitators in the early stages of the people-centric tapestry (TTOs, CROs, public and private investors, etc.) that would otherwise be time-consuming and cumbersome to interact with.

Navigation of the various gatekeepers, an unavoidable reality of drug R&D, can arguably be accomplished only by actors in the later stages of the R&D timeline. However, late-stage actors are risk averse, while early-stage actors are risk tolerant.

As successful R&D projects are low-probability events (as outcomes are highly uncertain, especially at the concept phase), the early-stage actors are arguably better placed to undertake drug discovery projects as their high risk tolerance allows them to initiate and manage R&D projects at critical points. Academic drug discovery is largely directed towards historically novel drugs because they represent a happy hunting ground for breakthrough scientific findings. Similarly, the risk tolerance and agility of young biotechnology companies allow them to carve out a path using disruptive technologies that are relatively inaccessible to conservative, less adaptable larger companies, thereby giving them an entry point into the R&D arena. PSRIs and small biotechnology companies could therefore be said to have a duty to incorporate gatekeeper preferences into their activity. Small biotechnology companies, possessing the optimal blend of risk tolerance and gatekeeper navigation skills, may be the best actor to 'curate' the drug development cycle.

Conclusion

Were the actors at all stages to more fully appreciate each other's risk preferences and motivations, it may permit more optimal project initiation, design and progression processes. Prescribing a single Standard Operating Procedure to facilitate such interactions is a naïve aspiration, and optimal success in this context is likely to be derived from a portfolio of approaches appropriately targeted at actors. For example, innovative approaches to bestowing an understanding of actor behaviours on individuals (educational vehicles on 'human-practices') could play an important role here, in addition to a focus on funding mechanisms that promote outsourcing of industrial research to PSRIs (technical practices).

One of the surest ways of tackling declining R&D efficiency may involve reconsidering our own attitudes and instigating a cultural shift in the industry. If the intellectual ideas of humans serve as the raw material for fully formed therapies, these ideas will be optimised if the people from whom they come are likewise optimised.

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Chapter 2 – Optimised microbiome analysis of breast cancer patient tissue

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European Journal of Cancer as "Biopsy bacterial signature as a predictor of host tissue malignancy" Glenn Hogan, Sidney P. Walker, Neegam Narayanen, Julia Eckenberger, Marcus J. Claesson, Mark Corrigan, Deirdre O'Hanlon, Mark Tangney

ABSTRACT

Background: High-throughput 16S rRNA sequencing has recently been used to describe the microbial communities of *in vivo* compartments that were, up until that point, described as "sterile". Among these is the human breast, the microbiome of which has been described in patients with and without breast cancer. However, despite publication of several reports on this topic since 2014, doubt exists over whether a putative breast microbiome has been adequately described, considering the concerns raised retrospectively regarding sample contamination and bioinformatic integrity. The gravity of these concerns, and the implications they have for tumour microbiome research, necessitate refinement of sample collection and processing methodologies such that a more accurate perspective of any breast microbiome can be obtained.

Aim: The first aim of this study was to improve breast microbiome research at all points of sample retrieval and processing by designing a comprehensive workflow, from bedside-to-bench, to minimise the undue influence of external factors on tissue samples. Using this advanced approach, this chapter also aimed to determine if using the bacterial signature of tissue samples could help identify the malignancy status of the tissue.

Materials and Methods: Fresh tissue samples and environmental controls from 28 female and 2 male patients were subjected to 16S rRNA DNA sequencing and bioinformatic analyses. Stringent contamination control practices were imparted at all points of specimen handling and bioinformatic manipulation.

Results: Here, a more informed and accurate depiction of the breast microbiome is offered than that of previous studies. This advanced approach allowed several novel observations to be made about bacteria found in breast tissue. These data clarify that the V1-V2 hypervariable region is more appropriate than the V3-V4 region for profiling of bacterial DNA found in breast tissues, and that the DNA isolated largely originates from intact bacterial cells residing in the breast specimens. Breast tumour and normal adjacent tissue bacterial profiles proved sufficiently different to provide a bacterial signature specific to tumour tissue.

Conclusion: The present work outlines a workflow for optimal collection and analysis of human specimens that will lead to a more realistic representation of their microbial profile. It may be valuable to emulate this approach in prospective microbiome studies,

especially in the case of specimens in which the anticipated microbial load is low or unknown. This work also illustrates the potential for intratissue bacteria to act as a cancer biomarker.

INTRODUCTION

It has been reported that several body sites, that were previously thought to be bacteria-free, harbour a microbiome. Perhaps the most well-studied of these sites is the human breast, of which the fresh, frozen, healthy-tissue microbiome was described between 2014 and 2016 in patients with and without breast cancer (Urbaniak et al., 2014, Urbaniak et al., 2016), and subsequently by other groups (Hieken et al., 2016). The microbiome of fresh breast tumours was described also (Lehouritis et al., 2016, Wang et al., 2017, Costantini et al., 2018). Additionally, several studies have profiled the microbial communities of FFPE breast tumour tissues (Banerjee et al., 2018, Banerjee et al., 2015, Xuan et al., 2014, Nejman et al., 2020).

Such studies have prompted questions regarding whether these microbes are incidental colonisers of tumours, or if they are themselves tumourigenic. Although its mortality is decreasing, breast cancer remains the second most common cause of death in women after lung cancer, and invasive breast cancer will afflict 1 in 8 women over a lifetime (DeSantis et al., 2019). Breast health is therefore still a key concern, and this is reflected in the myriad publications that aim to mobilise efforts to improve screening and diagnoses of breast cancer and, indeed, define its microbiome.

Microbiome research is continually advancing, bringing with it pushes for increased refinement and standardisation in the way data are collected and analysed (Sinha et al., 2017). Part of the ground that needs to be covered in this field concerns analysis of low-biomass samples, which are especially prone to undue influence by contamination (Eisenhofer et al., 2019). There are relatively old reports that indicate that the viable bacterial fraction of human breast tissue meets the definition of “low biomass” (Thornton et al., 1988). A more recent report corroborates this finding, noting that, at the lower end of the range, 75 CFUs are recovered per gram of breast tissue (Urbaniak et al., 2014). This latter study also notes that up to 2000 CFUs per gram of tissue were recovered from breast tissues, but even this quantity may fall under the definition of “low biomass”, which some authors claim can be applied when the specimen in question contains less than 10000 microbial cells (Eisenhofer et al., 2019). Additionally, it is commonly accepted, in discourse on both microbiome and microbiota, that most live bacteria are unculturable using contemporary culture methods (Pande and Kost, 2017), meaning that breast microbiome work may currently

be the most feasible approach for accurate definition of the bacterial content of breast tissue.

The apparent low biomass of breast tissue has created ambiguity in breast microbiome studies, given the limitations that deep sequencing techniques have in relation to low-biomass samples. The revelation that DNA extraction kits contain bacterial DNA (Salter et al., 2014) reinforces concern that low-biomass samples may be especially influenced by kit contaminants if samples are not handled properly. Furthermore, the sequencing of low-biomass patient samples is often challenged by the overwhelming proportion of human reads that surface compared with those of bacterial origin (Marotz et al., 2018). These factors have stifled research in this field, as some studies analysing breast tissue, and low-biomass material in general, have been criticised for taking insufficient precautions in limiting the effect that environmental contamination may have on the data (Eisenhofer et al., 2019).

A review of the sample collection protocols of breast microbiome studies reveals that inconsistencies arise here. Some studies utilise negative controls that aim to capture environmental contamination of samples that might arise in the operating theatre, while omitting controls that would indicate contamination originating from the patient's skin (Urbaniak et al., 2014, Wang et al., 2017, Costantini et al., 2018), while other studies control for the reverse (Hieken et al., 2016), and one study utilised both skin and environmental contamination controls (Urbaniak et al., 2016). Additionally, investigators of the breast microbiome often report relatively high levels of sample manipulation prior to DNA extraction, including excision of the breast specimen, followed by further handling in a pathology laboratory (Hieken et al., 2016, Wang et al., 2017). Acknowledging the proneness to contamination that breast specimens may have, minimising human interaction with them prior to analyses is apt, and this issue is approached in this chapter. Such realisations invite reassessment of best practices with respect to sample collection and processing in microbiome research, and aligning these with sound bioinformatic analyses, which is at the forefront of this chapter's considerations. Thus, the approaches described below aim to approach with greater sensitivity the potential sources of contamination that can come to bear at many points of specimen collection and processing.

A further factor that may confound breast microbiome research is the specimen type on which analyses are conducted. Much of what has been written on the breast microbiome to date has rested on data pulled from FFPE specimens, such as the above-referenced studies. FFPE tissues may have a markedly different targeted-amplicon-sequencing output compared with their fresh, frozen counterparts due to the DNA damage that these specimens sustain during formalin fixation at room temperature (Koshiba et al., 1993), as clinical specimens traditionally are (Bass et al., 2014). An exclusive focus on fresh specimens is therefore appropriate, especially while concerns exist concerning the validity and reproducibility of results drawn from specimen types, like FFPE tissues, that undergo more extensive processing.

Further measures adopted in this study to improve the intelligibility of the output included varying DNase treatment of samples, as well as targeting different hypervariable regions of the 16S rRNA gene. DNase treatment was both incorporated in, and withheld from, fresh, frozen-tissue DNA extractions to determine the extent to which 16S reads were contributed both by naked DNA and DNA deriving from intact, cellular bacteria. In terms of selection of 16S rRNA hypervariable regions, past investigation of the most appropriate 16S rRNA region for characterisation of the breast microbiome concluded that the V3 region was most suitable (Costantini et al., 2018). However, while this study was extensive, it omitted the V1 region from its scope. Thus, in the current study the V3 region was chosen for most sequencing analyses, but a subset of analyses probed the V1 region. This was further motivated by the claim that the V1 region facilitates amplification of low-biomass samples, due to its relatively short length (Lauder et al., 2016).

Recently, a comprehensive microbiome analysis across 33 cancer types suggested that differences in bacterial diversity exist between malignant and healthy tissues, as well as between different cancer types (Poore et al., 2020). This presents the possibility of exploiting such differences diagnostically. However, these findings are based on microbiome data drawn from The Cancer Genome Atlas project. These specimens are potentially unsuitable for analysis of microbial DNA, due to a high likelihood of contamination, a lack of negative controls, and DNA extraction techniques that are incompatible with bacterial cells. While robust bioinformatic contamination control was applied to these microbiome data, no method exists that can decontaminate samples completely *in silico*. Nonetheless, the findings of this study present the

potential for a new class of bacteria-based oncological biomarker. To expand on this, this chapter examines if microbiome-based detection of malignancy is still effective when the confounding factors listed above have been accounted for, and in an 'in practice' setting (biopsies).

Overall, considering the inconsistencies in breast microbiome work that have affected this research area to date, this chapter and the next chapter aim to derive high-quality bacterial profile data from fresh biopsy specimens, collected specifically for this purpose, so that important applicational aspects of this field might be explored (Figure 1).

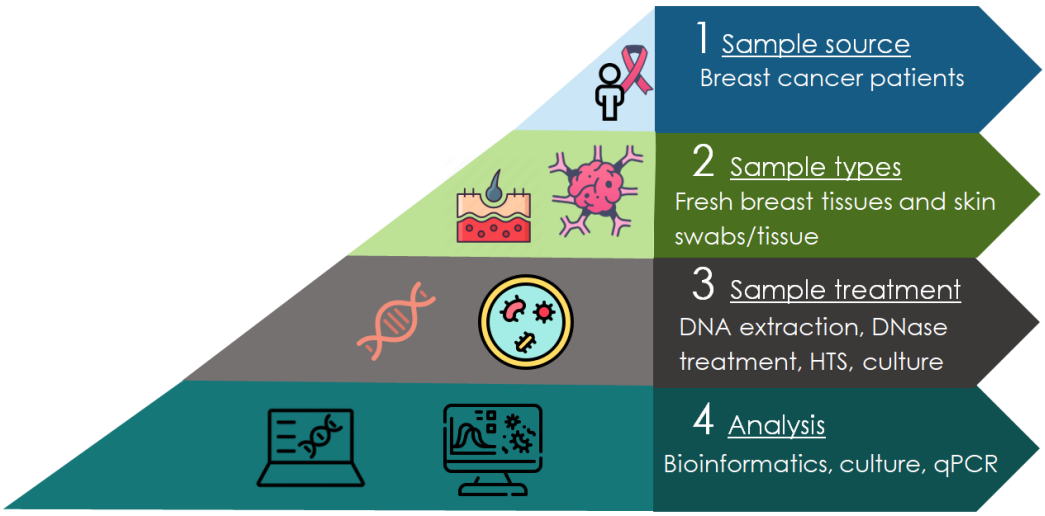


Figure 1. Overview of optimised protocol for analysis of the breast tumour microbiome *(relevant to Chapters 2 & 3 of this thesis).*

MATERIALS AND METHODS

Independent validation of clinical material

Clinical research was approved by the Clinical Research Ethics Committee of University College Cork, Cork, Ireland. Breast cancer was confirmed in each patient using a 'triple assessment' approach (Karim et al., 2020). This protocol is the gold standard for breast cancer diagnosis, incorporating physical examination, imaging (e.g., mammography), and core biopsy. When used individually, each of these modalities is associated with an appreciable degree of unreliability, especially when compared with their use in concert. When combined, triple assessment yields a positive predictive value of 100%, as well as a sensitivity and specificity of 94.7% and 100%, respectively. Following a positive diagnosis, it was ensured that tumour biopsies retrieved only tissue from within the patient's lump by working with palpable masses only (i.e., tumours were not biopsied if they were not palpable). Normal adjacent tissue was biopsied by removing tissue at least 1 cm from the primary tumour margins.

Clinical specimen collection, transportation, and storage

28 female patients and 2 male patients with breast cancer were enrolled in this study. Each patient was interviewed prior to surgery regarding their antibiotic history over the past month, their use of probiotic products and, where appropriate, if they ever breastfed their children. The patient's skin was disinfected at their surgical site with ChloraPrep with Tint (CareFusion) and was subsequently swabbed with a sterile gauze pad prior to surgical incision. The gauze pad was then left exposed to the operating theatre's environment until all samples were collected. Breast tumour tissue was extracted from each patient using a 14-French core biopsy needle (ACHIEVE programmable automatic biopsy system, Merit Medical) and normal adjacent tissue was excised from each patient using a diathermy needle (Smoke Pencil, SafeAir). Breast tissues were divided and placed into 30-ml universal containers (Thermo Fisher Scientific) that were either dry (for HTS purposes) or that contained 500 µl RCM (for culture purposes – please see Chapter 3). Skin swabs were stored and transported in 1 ml RCM alone. Samples were transferred from the operating theatre to the laboratory within 20 min of collection. Tubes containing skin swab samples were vortexed,

followed by removal of the gauze pad with a sterile forceps (Servoprax). Specimens that were kept dry, and some volume of RCM from the skin swab samples, were flash-frozen and stored in a -80ºC freezer for subsequent bacterial DNA extraction.

DNA extraction and 16S rRNA library preparation and sequencing

DNA from 30 patient tissues and skin swabs was subjected to 16S rRNA sequencing. DNA was extracted from flash-frozen, patient breast tissue and skin swab samples using the Ultra-Deep Microbiome Prep kit (Molzym). Skin swabs and tissue samples were processed per 'Protocol 1' and 'Protocol 2' of the kit manual, respectively. For a subset of patient samples (4 tumour samples, 10 normal adjacent samples, and 4 skin swab samples), treatment with DNase was both included in, and excluded from, the protocol to determine whether the genomic output derived from cellular bacteria, or if it originated from naked DNA within the samples. 1 ml Buffer SU was run through the kit as a negative control, per 'Protocol 1'. Steps requiring use of a thermomixer were performed using a T-Shaker (EuroClone) at 1000 rpm at the recommended temperature.

Eluted DNA was quantified using a Qubit fluorometer (Invitrogen) using the 'High Sensitivity' assay, and PCR-amplified using primers targeting the V3-V4 region of the 16S rRNA gene (forward primer 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and reverse primer 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3'). Breast tumours from 8 patients were sequenced also with primers targeting the V1-V2 region of the 16S rRNA gene (forward primer 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGM GTT YGA TYM TGG CTC AG-3' and reverse primer 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGC TGC CTC CCG TAG GAG T-3'). 35-µl reactions were set-up per the following recipe: 17.5 µl NEBNext Ultra II Q5 Master Mix (New England Biolabs), 1.75 µl forward and reverse primers (final concentration: 0.5 µM), and 14 µl template DNA. Reactions were run in a Mastercycler Gradient per the following protocol: 98ºC for 30 sec, followed by 25 cycles of 98ºC for 10 sec, 60ºC for 30 sec, and 72ºC for 40 sec, followed by a final extension step of 72ºC for 5 min. Amplification was verified, as

above; however, samples proceeded to sequencing irrespective of amplification being observed. The product was approximately 460 bp.

Reactions were cleaned per the '16S Metagenomic Sequencing Library Preparation' protocol (Illumina), with the exception that samples were dried for 90 sec following removal of ethanol, rather than for 10 min. Additionally, samples were eluted in 30 µl Buffer EB (Qiagen). Purified DNA proceeded to index PCR per the Illumina protocol, with the exception that 15 µl template was used, while PCR-grade water was omitted from the recipe. Index PCR products were cleaned per the Illumina protocol, reducing the drying time, as above. DNA quantification was performed using a Qubit fluorometer, as above. Samples were normalised separately by pooling 40 ng DNA per sample. Samples that were too dilute to be normalised to these quantities had their total volume added to their respective pool. A paired-end, 300-bp run was subsequently completed on an Illumina MiSeq, at GENEWIZ, Inc., USA.

Bioinformatic analyses

The quality of 2 x 300-bp, paired-end sequence data was initially visualised using FastQC (v0.11.6), and then filtered and trimmed using Trimmomatic (v0.36) to ensure a minimum average quality of 25. The remaining high-quality reads were then imported into the R environment (v3.6.2) for analysis with the DADA2 package (v1.8.0). DADA2 was used to build an error model that was used to collapse raw sequences into ASVs which were then filtered to remove chimeric reads and human-aligning sequences. ASVs were classified to the genus level using the classify.seqs function within the Mothur suite of tools.

The bioinformatic contamination control tools Decontam (v1.0.0) (Davis et al., 2018) and SourceTracker (v1.0) (Knights et al., 2011b) were used, according to published guidelines (Walker et al., 2020b, Walker et al., 2020a), to retrospectively assess and remove contamination, based on sequencing data from negative controls. Numbers of samples deemed suitable for further analysis are tabulated (Figure 2).

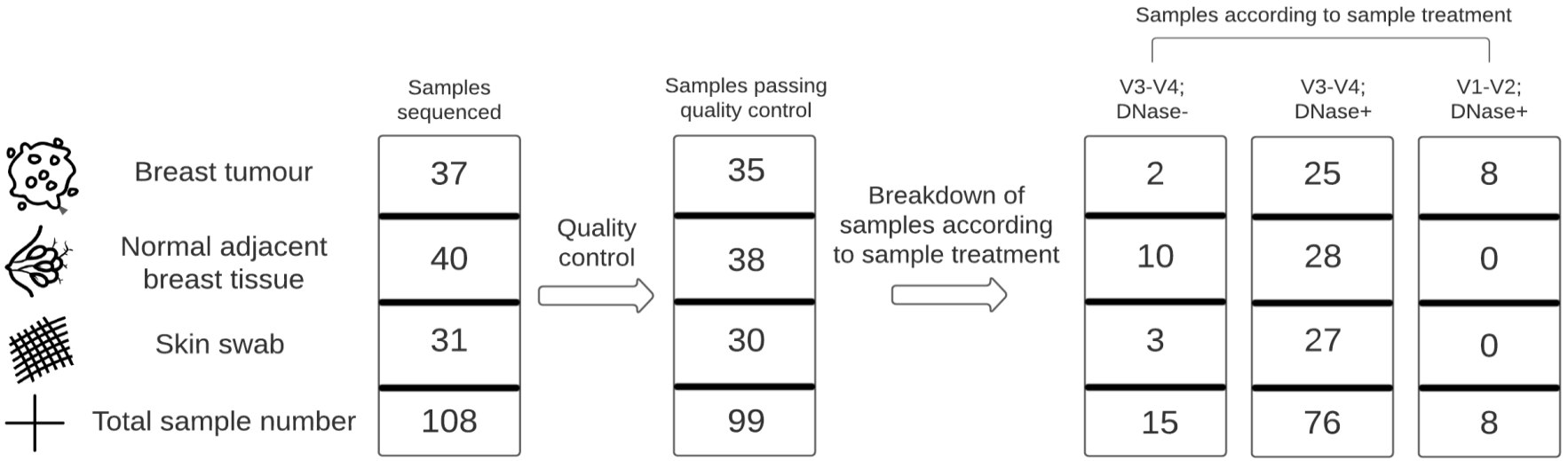


Figure 2. Numbers of total bacterial DNA extractions, from patient breast tissues and skin swabs, sequenced and bioinformatically analysed. Final sample numbers bioinformatically analysed are tabulated in terms of the bacterial 16S rRNA region sequenced, and DNase treatment status. Quality control entailed removal of samples from final analyses if less than 500 reads remained after bioinformatic contamination control and removal of non-microbial reads.

Data analysis and visualisation

All statistical analyses were performed in the R environment (v3.6.2), with relevant statistical tests specified in figure legends. Hierarchical clustering was performed using the made4 package (v1.54). Alpha and beta diversity were calculated using the vegan (v2.5.6) and phyloseq (v1.3.2) packages and visualised using ggplot2 (v3.3.2).

To distinguish between tissue sampling sites, *n* leave-one-out gradient-boosted tree models were generated, using xgboost (v1.2.0.1) in the R environment, to predict the class of the n^{th} sample. Optimal model parameters were determined with bootstrapping of 100 iterations and five-fold cross-validations. Feature importance was determined by the 'gain' that an included bacterial species added to a model and the frequency with which each species was used for a model. The performance of the classification was measured by the area under the ROC curve, utilising the pROC package (v1.16.2).

RESULTS

Refined protocol produces bioinformatic data with increased stringency

As the biopsies under study are low-biomass specimens, first it was ensured that any biological signal was not distorted by environmental contaminants or by inter-patient variation. SourceTracker indicated low-to-moderate levels of contamination. For only four samples, more than half the sequencing data comprised contaminants; however, contamination was subsequently removed with Decontam. The strong correlation between the numbers of ASVs before and after contamination removal (Figure 3A) reinforces the deduction, facilitated by the SourceTracker algorithm, that the biological signal of these samples has not been significantly distorted during collection and processing, increasing the likelihood of obtaining meaningful insights from additional analyses. Analysis of beta diversity confirmed that, despite paired samples being present in the dataset, the patient of origin had minimal effect on the between-sample diversity (Figure 3B). Overall, 25 breast tumour samples, 28 normal adjacent tissue samples, and 27 skin swab samples were processed, subsequently passed quality control tests, and proceeded to downstream analyses.

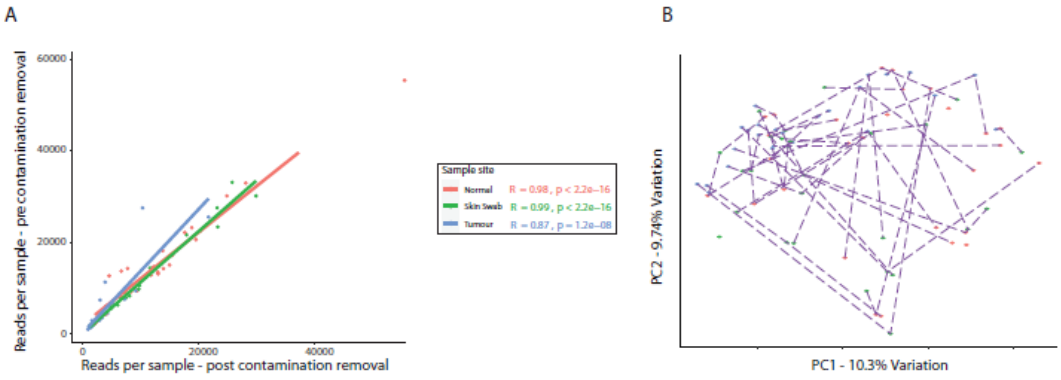


Figure 3. Investigation and mitigation of the effects of confounding factors on sequencing data. (A) *The effect of contamination removal on numbers of bacterial reads, measured by correlation of read numbers per sample before and after bioinformatic contamination removal.* ***(B)*** *Investigation of clustering dynamics of paired patient samples, using principal coordinates analysis ordination of a Bray-Curtis dissimilarity matrix.*

Targeting different 16S rRNA gene regions of breast bacteria affects sequencing output

To determine the advantages of sequencing unexplored 16S rRNA hypervariable regions of breast tumour bacteria, both the V1-V2 and V3-V4 hypervariable regions were sequenced and compared using DNA extracted from 8 breast tumours. Primers targeting the V1-V2 region of the 16S rRNA gene displayed significantly higher numbers of reads generated per sample, when compared with the sequencing output of primers targeting the V3-V4 region, after removal of contaminant and non-bacterial reads (Figure 4). Sequencing of the V1-V2 region also led to a decreased loss of read numbers after contamination removal, when compared with the loss of read numbers after sequencing of the V3-V4 region. More than 90% of non-bacterial, V3-V4 reads were classified as human in origin by the Basic Local Alignment Search Tool. Primers targeting the V1-V2 region result in, on average, 80% less human-genome-aligning reads than the V3-V4 region, allowing for more statistically significant analysis of the bacterial communities residing in these samples. Despite significant differences in read numbers generated per sample between sequencing of the V1-V2 and V3-V4 regions, the community structure in samples amplified with V1-V2 primers was visually similar to those amplified with V3-V4 primers, and no difference in alpha diversity was observed between the two regions, indicating that primer choice did not have any adverse effect on downstream results.

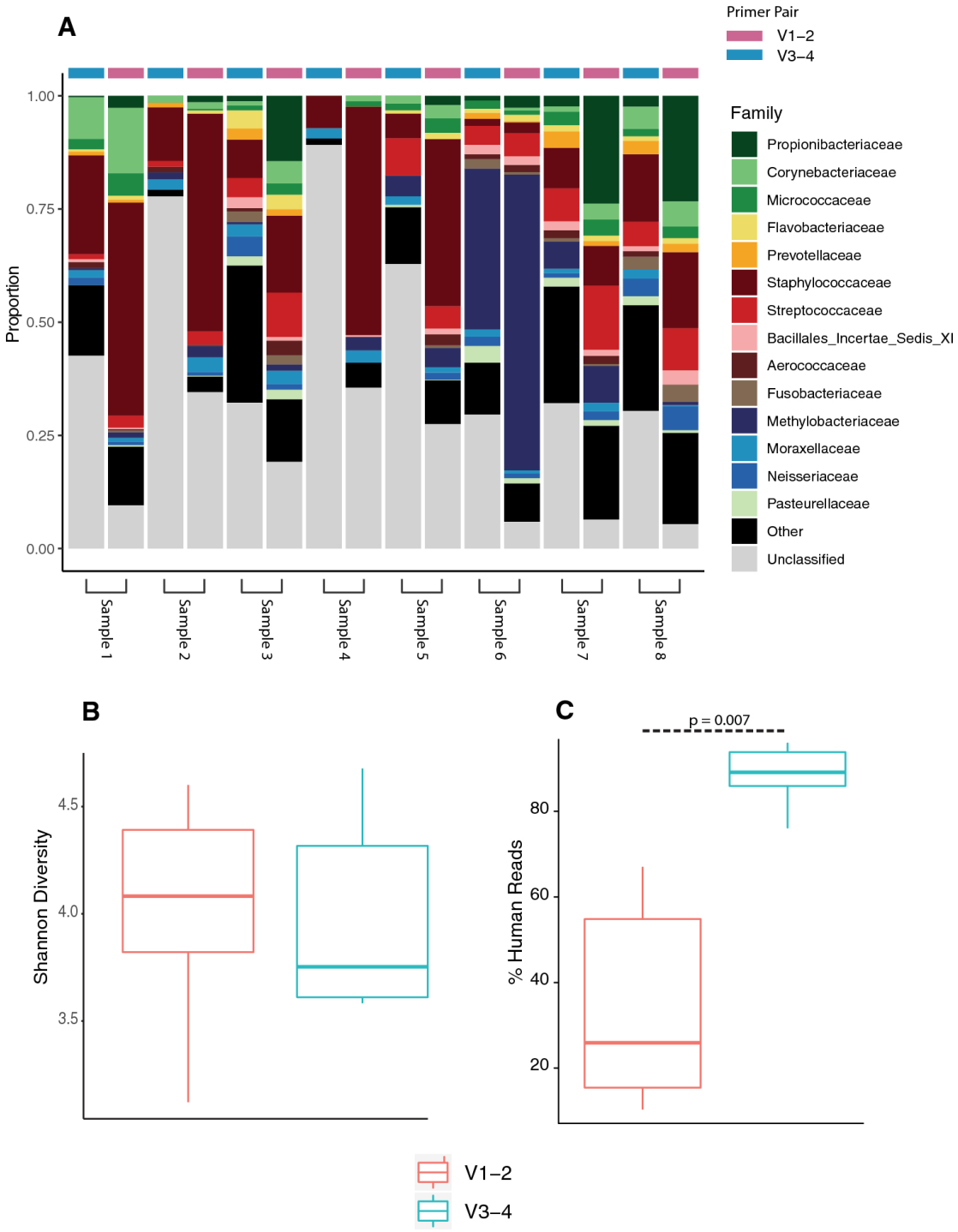


Figure 4. Pairwise comparison of samples using primers targeting the V1-V2 and V3-V4 regions of the 16S rRNA gene fragment. (A) *Family-level composition of paired samples.* ***(B)*** *Average Shannon diversity comparison between samples amplified using V1-V2 primers (red) and V3-V4 primers (blue).* ***(C)*** *Percentage of total sequencing reads aligning to the human genome using V1-V2 and V3-V4 primers. Statistical testing in (B) and (C) was performed using the Wilcoxon signed-rank test.*

DNase treatment of human specimens does not significantly alter bacterial diversity

To generate information on whether sequencing data were derived from intact bacteria (at the time of sampling) or free DNA, a subset of samples was processed for sequencing +/- DNase. Beyond family-level bacterial community structure, analysis of alpha and beta diversity also indicated no significant differences between samples treated with and without addition of DNase (Figure 5). Chao1 species richness did not differ significantly between paired samples ($P = 0.085$). and significant separation of paired samples, in terms of Bray-Curtis dissimilarity, did not occur ($P = 0.980$). This indicates that DNA in these patient samples existed inside intact bacterial cells prior to extraction, rather than as free DNA.



Figure 5. Pairwise comparison of patient samples with and without DNase treatment. ***(A)*** *(i) Sequencing read numbers generated per patient sample, post-contamination-removal. (ii) Family-level composition of paired (DNase +/-) patient samples.* ***(B)*** *Bray-Curtis dissimilarity between DNase + and DNase – patient samples, showing no significant difference between groups per PERMANOVA (P = 0.980). Lines indicate paired samples* ***(C)*** *Alpha diversity of patient samples with and without DNase treatment, in terms of Chao1 species richness. Significance was calculated using the Wilcoxon signed-rank test.*

Hierarchical clustering suggests existence of distinct bacterial signatures in patient breast tumours

The potential for a machine learning model to predict tissue malignancy was first explored by using unsupervised hierarchical clustering to detect discrete differences between sample types in terms of their bacterial signature. When the resulting dendrogram was cut 50% of total height, five clusters formed, with 23/25 (92%) tumour samples located in the large, third cluster (Figure 6). Normal adjacent breast tissues and skin swabs had a more stochastic distribution.

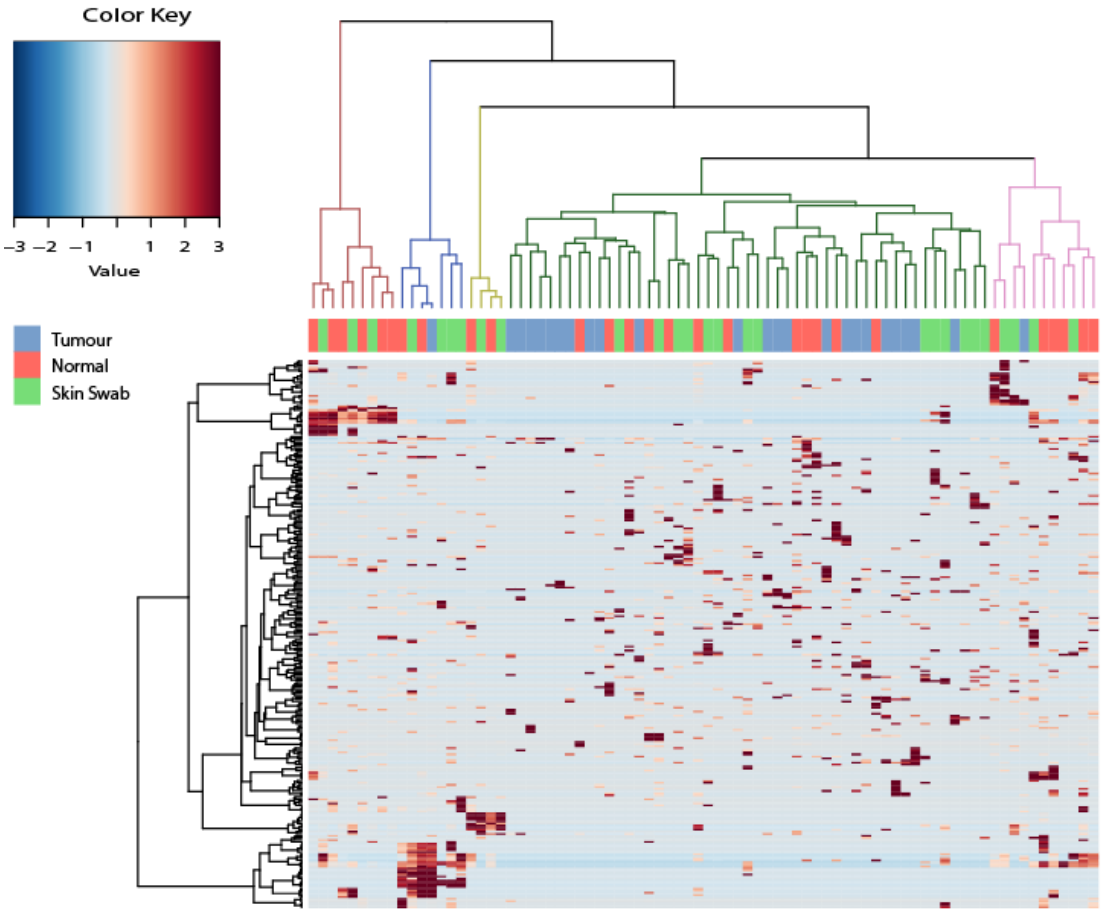


Figure 6. Hierarchical clustering of patient breast tissues and skin swabs. *Each column in the heat map represents a sample and each row represents a unique ASV. Unsupervised hierarchical clustering was performed using Ward's method, and 1-Pearson correlation distance was used to calculate the distance between rows and columns. A dendrogram at the top of the figure illustrates five logical clusters, separated by colour, which are formed by cutting the tree at h/2.*

Differentially abundant bacterial species exist between breast tumour and normal adjacent tissues

Examination of the differentially abundant taxa implied by the clustering heatmap (Figure 6) was carried out at the species level using the Wilcoxon rank-sum test. Six bacterial species were confirmed to be differentially abundant in breast tumour tissue (Figure 7). This, along with the hierarchical clustering analysis above, form a strong basis for the development of a predictive tool for tissue malignancy based on a distinct bacterial readout.

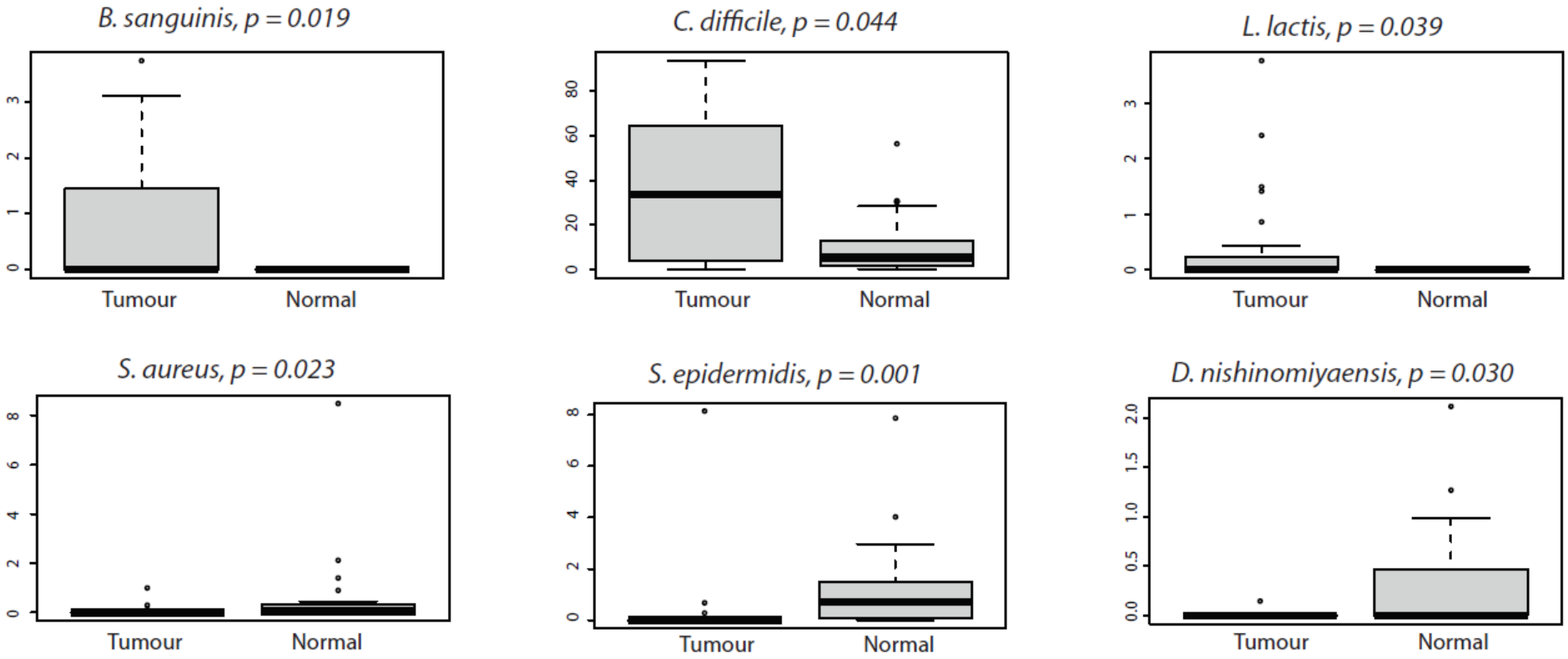


Figure 7. Differentially abundant bacterial species between breast tumour and normal adjacent biopsies. *Differential abundance was calculated using the Wilcoxon signed-rank test, with type I errors controlled for using the 'false discovery rate' method.*

Machine learning predictions based on bacterial signature are effective in differentiating malignant and non-malignant tissues and closely agree with the standard independent validation approach

The distinctiveness of different patient sample types, in terms of their bacterial profile, was determined using the 'Extreme Gradient Boosting' machine learning technique, including bacterial species present in at least 5% of all samples, and proportionally normalised. The classifier was able to distinguish between breast tumour and normal adjacent tissues (as validated as described in Materials and Methods) with an AUC of 0.87 and accuracy of 87.3%, which was significantly higher than the corresponding no-information rate of 54.6%, (P = 2.26e-7) (Figure 8).

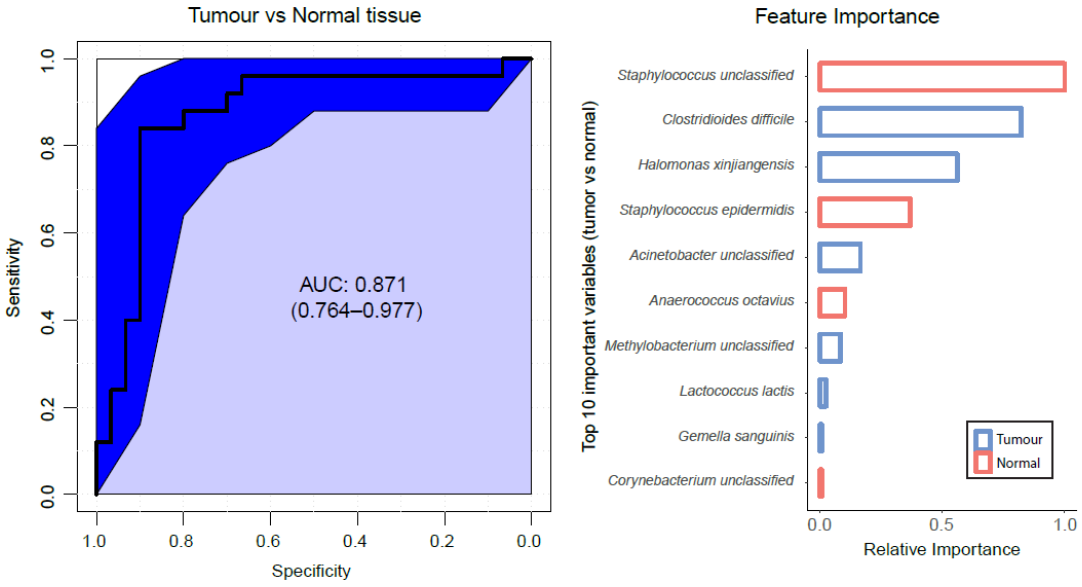


Figure 8. Machine-learning-based classification of malignant vs non-malignant breast tissues. *A ROC curve for boosted tree models is illustrated to the left, with a corresponding 'feature importance' plot illustrated to the right. An area-under-the-ROC of 0.5 indicates that the model cannot discriminate between malignant and non-malignant tissue, whereas a score of 1 indicates separation of samples by malignancy status.*

Less surprisingly, this machine-learning-based classification of sample origin was also able to differentiate between both tumour and skin swab samples (AUC = 0.914,

accuracy = 0.8846) and normal adjacent tissue and skin swab samples (AUC 0.985, accuracy = 98.2%) (Figure 9).

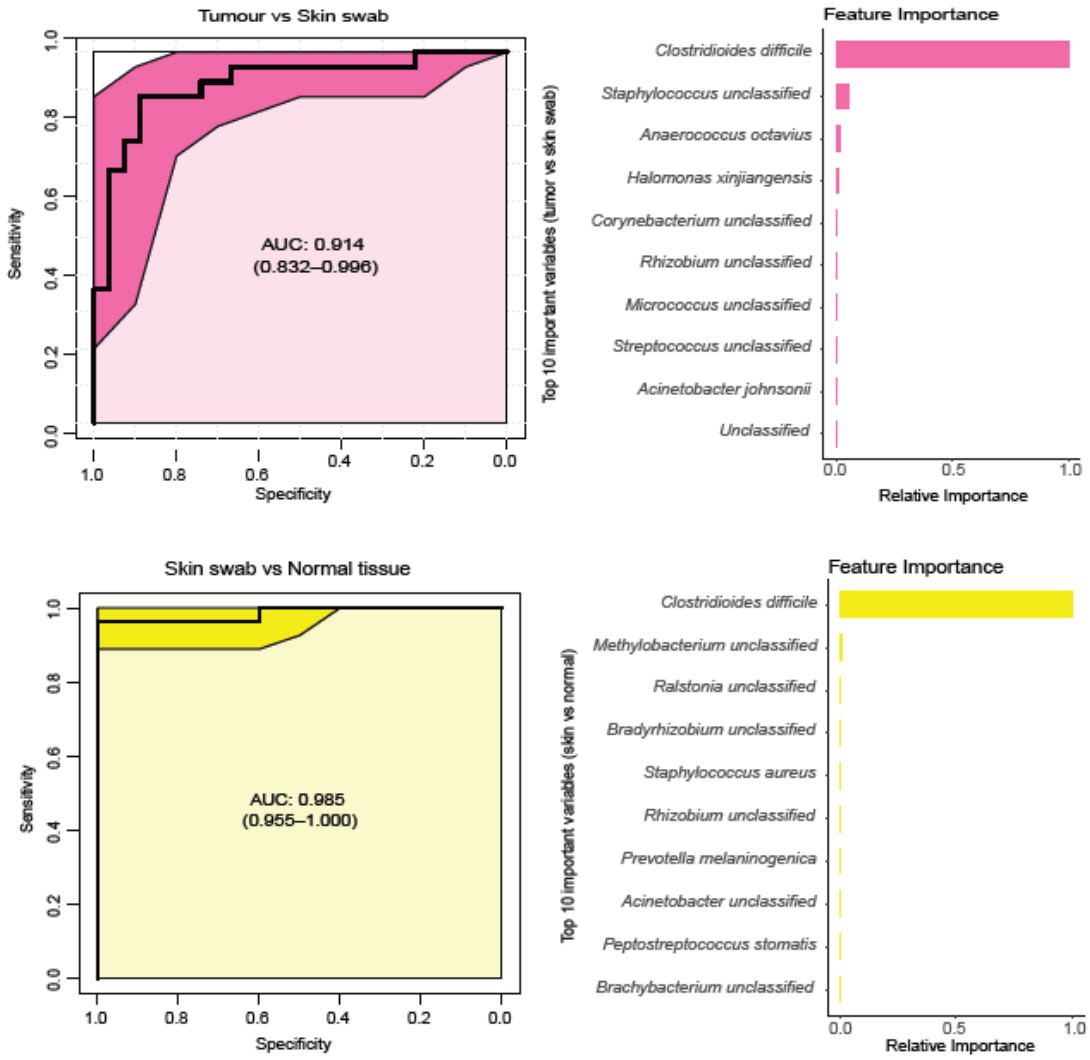


Figure 9. Machine-learning-based classification of tumour and normal adjacent tissue vs. skin swabs. *The ROC curve for boosted tree models (left), with a corresponding feature importance plot (right). An area under the ROC of 0.5 indicates that the model cannot discriminate between malignant and non-malignant tissue, whereas a score of 1 indicates perfect classification of test samples.*

The effect of selected patient characteristics, such as breast histology and tumour size, on tumour bacterial diversity was also examined (Table 1). When plotted apart from normal adjacent breast tissues, patient breast tumour tissues disperse more markedly

in terms of beta diversity. The only patient-specific characteristic examined that accounted for this observation related to whether patients reported a recent antibiotic history (Figure 10).

Table 1. Significance (*p*-values) of changes in alpha and beta diversity of breast tumours, depending on breast histology, medical and surgical history, and age.

Patient feature	Chao1	Bray-Curtis
Surgery type	*0.431*	*0.714*
Tumour grade	*0.459*	*0.803*
Recent probiotic use	*0.919*	*0.578*
Antibiotic use	*0.019*	*0.507*
Tissue necrosis	*0.683*	*0.683*
Presence of metastases	*0.773*	*0.091*
Skin involvement	*1*	*0.707*
Age	*0.805*	*0.988*
Tumour size	*0.592*	*0.902*

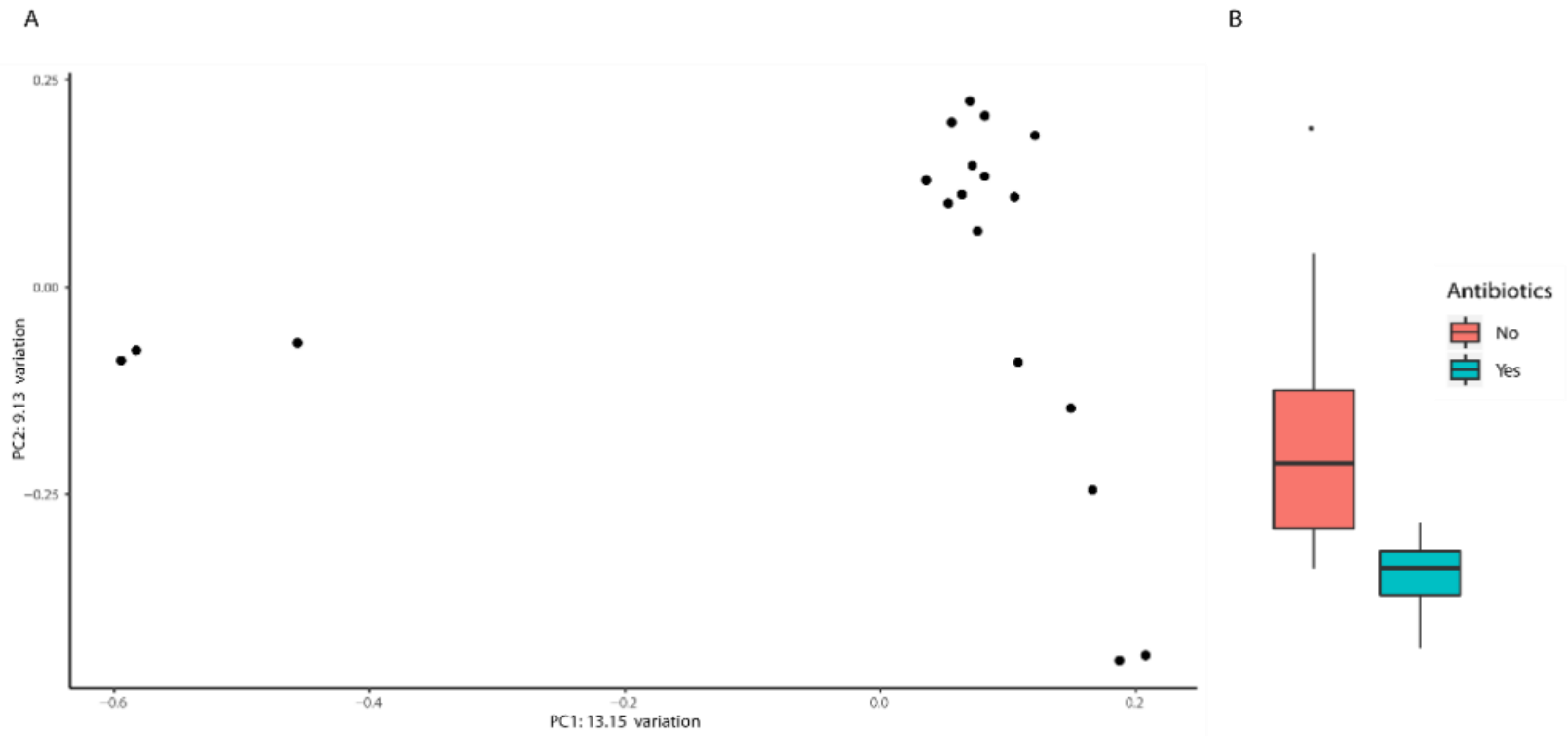


Figure 10. Beta and alpha diversity of patient breast tumour specimens. *(A) Principal coordinates analysis of patient breast tumour specimens in terms of Bray-Curtis dissimilarity. (B) Alpha diversity, in terms of Chao1 species richness, of breast tumour specimens derived from patients either confirming or denying a recent antibiotic history.*

DISCUSSION

Considering the justifiable concerns that are raised regarding contamination potentially affecting experimental output in low-biomass microbiome studies, the workflow followed in this study was calibrated to minimise the probability of contamination in both a wet laboratory and bioinformatic context using several approaches. First, all patient samples were provided directly by the breast surgeon, from the operating theatre, to laboratory personnel during the patient's surgery. This was a favourable truncation of the traditional procedure, which involves handling of the specimen by pathology staff before transfer to the laboratory. In this way, patient tissues were handled by less people over a shorter timeframe and were exposed to less potential environmental contamination that might arise in a pathology department. Second, all patient samples were provided by a single surgical team under one consultant breast surgeon, providing a more consistent and reliable foundation for sample collection. Bioinformatic contamination control strategies were also sound, as described above, and incorporated recently described decontamination programs such as Decontam (Davis et al., 2018) and SourceTracker (Knights et al., 2011a).

Variations in DNase treatment of tissue specimens in this study aimed to partially mitigate the limitation of microbiome data that disallows verification of the alive-or-dead status of a microbial community. Although this extra measure does not fully allow determination of this, DNase treatment of breast tissues does suggest that their microbial content derives primarily from bacterial cells and not from free DNA. This approach has proven useful in other microbiome studies. One study of the lung microbiome in pigs derived important insights from DNase +/- treatment of samples, allowing authors to support the hypothesis that the lung is sterile despite the sequencing of bacterial DNA therein, on the basis of widespread bacterial DNA sensitivity to DNase I (Pezzulo et al., 2013). Yet, a microbiome that comprises DNA exclusively may not necessarily be ineffectual, as it has been shown that probiotic activity can, at least in part, be mediated by DNA alone (Lammers et al., 2003). Despite the insights it can provide, however, the use of DNase to clarify NGS data is still not standardised in microbiome research. Routine use of DNase +/- sample treatment in studies may allow for a more holistic evaluation of NGS data in this context.

An extra analytical insight was achieved by targeting different hypervariable regions of the 16S rRNA gene of breast bacteria. Sequencing of the V1 region did not significantly alter the structure of the microbial communities sequenced, nor their alpha diversity, compared with sequencing of the V3 region. However, the V1 region yielded significantly higher numbers of reads than did the V3 region, following bioinformatic quality control. This supports earlier assessments that sequencing of the V1 region is appropriate for low-biomass samples (Lauder et al., 2016) and its overall resemblance to V3 sequencing data is reassuring, given the preference already established for this region in breast microbiome work (Costantini et al., 2018). Most loss of read numbers from V3 sequencing was attributed to the removal of reads aligning with the human genome. This highlights a problem that is adequately reported in WGS (Marotz et al., 2018), but that is perhaps underreported in targeted amplicon sequencing, in which amplification of human DNA can occur in samples with a disproportionately high ratio of human DNA to bacterial DNA. The work detailed in this chapter suggests that this issue can be overcome by use of primers that target different hypervariable regions. As the microbiome data in this study are of a relatively low complexity compared with those of, for example, the GIT, high sequencing depth is probably not required for extensive coverage of this environment; thus, the V3 sequencing data in this study are not invalidated by the above drawback.

One confounding factor potentially affecting the diagnostic aspect of this study included a lack of comparison between tumour tissues and matched tissues taken from non-cancer patients. While the precedent investigation on this topic also did not acquire these data (Poore et al., 2020), the diagnostic authenticity of the approach is likely unaffected by this, given that the ability to distinguish between tumour and normal adjacent tissues within the same patient is probably more powerful than the ability to distinguish between corresponding tissues in cancer and non-cancer patients. Indeed, some microbiome studies have employed normal adjacent tissues as substitutes for tumour tissues, due to their anticipated similarities in terms of their microbial communities (Hieken et al., 2016, Urbaniak et al., 2016). The predictive, machine learning model used in this study is accurate when used to determine the malignancy status of human tissue, strongly suggesting that intratumoural bacteria may have the facility to act as cancer biomarkers. The classification accuracy of 87.3% is impressive and compares favourably with established clinical cancer diagnostics

that are known to underperform. An example of this is the high false positive rate observed (between 30-87%) when attempting to differentiate ductal carcinoma *in situ* from benign breast disease (Grimm et al., 2019). However, it may be premature to pronounce on the true diagnostic utility of this technology, due to the variability to which sequence-based analyses of bacterial communities can be prone (He et al., 2018). Despite this, with the increasing, widespread availability of bacterial DNA sequence data, from this and other tumour microbiome studies, a sufficiently varied training data set will soon be publicly available to bridge this gap.

Prospective work on this topic should investigate alternative tumour types to establish how broadly a cancer diagnostic approach that incorporates microbial profiling can be applied. It is reported that malignancies beyond breast cancer are associated with a microbiome, and these are being explored for various microbiome-based medical applications. For example, it has been proposed that the pancreatic ductal adenocarcinoma microbiome has the capacity to generate oncogenic signals via tumour immunosuppression, that could be potentially intercepted to disrupt disease progression (Pushalkar et al., 2018). Given that diagnostic algorithms for pancreatic adenocarcinoma are poorly defined (Grossberg et al., 2020), the exploration of microbiome data as a diagnostic tool for this cancer is a worthy pursuit.

Considering the sensitivity of HTS, and as the human breast tissues analysed in this study are low biomass, thorough consideration of the potential for contamination is an essential part of determining whether this biomass truly originates from the breast. The probable source of this contamination, if it exists, is almost assuredly the skin. Given that bold claims rooted in HTS data have recently been attributed solely to the presence of contaminant DNA, it is urged that contamination is seriously considered as a possible source of exogenous bacteria in microbiome data (Lusk, 2014). It is worthwhile, when gauging the potential for skin contamination in microbiome studies, to consider how the skin is sampled. It is known that different skin sample formats have different quantities of microbial DNA, with punch biopsies containing significantly higher quantities than skin swabs (Grice et al., 2008). However, as NGS can only calculate relative abundance, and as skin swabs and biopsies have a similar microbiome composition, the use of skin swabs alone to assess skin contamination of NGS samples is probably reasonable for the sake of convenience. On the other hand, when assessing skin contamination in microbiota studies, where absolute CFUs can

be calculated (and thus quantifiable differences between bacteria recovered from both the skin and the tissue of interest can be assessed), skin swabs alone may be insufficient, as these may not capture the full extent of contaminant bacteria present.

The breast microbiome defined in this study resembles that of the patient skin swabs sampled. However, while similar, breast tumours were shown to have a distinct microbiological signature from that of the skin and normal adjacent breast tissue. The significance of this must be considered alongside the fact that breast tumour tissues were harvested using sterile core biopsy needles that were unpackaged immediately before biopsies were taken, while normal adjacent breast tissues were harvested using a diathermy needle that was in use from the outset of the surgery. Core needle biopsies have already been validated as a relatively non-invasive alternative to surgical excision biopsies for the purpose of breast microbiome characterisation (Costantini et al., 2018). In the present study, core needle biopsies are further shown to be advantageous in reducing the extent to which samples are handled by surgical and dissection tools. Despite this benefit, the lack of contact with the skin that the core biopsy needles in this study had, compared with the diathermy needles, may account for apparent differences in microbiological signals that are observed in this study, which complicates interpretation of the data. This issue is discussed in further detail in Chapter 3.

The breast microbiome data presented here differ markedly from those of previous studies, which is certainly accounted for, at least partially, by the refinements in study design and analyses described in this chapter. Given its similarity to the skin microbiome, the existence of a breast microbiome can only be appropriately considered in conjunction with culture data. However, data from this study support the claim that past study designs were incomplete, and this justifies structuring breast microbiome research protocols in a similar way to this study, so that meaningful, high-quality data can be obtained more consistently.

CONCLUSION

The results of this work have implications for microbiome studies in general, and the microbiome of low-biomass samples especially, given the significant differences in output that occur when more stringent quality control measures are applied. The author advocates and rationalises the inclusion of a suite of practices described above in prospective microbiome studies, if consistency, quality, and insight are to be achieved.

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Chapter 3: Application of culturomics techniques to human breast tissue

ABSTRACT

Background: Bacteria have attracted much attention in recent years for their potential as next-generation tumour-targeting agents. Furthermore, several DNA sequencing studies have recently confirmed the presence of bacteria in a variety of human tumours. It is hypothesised in the present study that, were natural bacteria cultured from patient tumours, these may have enhanced tumour-targeting abilities over those routinely used in clinical and preclinical tumour-targeting studies.

Aim: The aim of this study was to develop a comprehensive, bespoke culturomics strategy, to facilitate:

i) generation of data on viable, intratumoural bacteria to support DNA sequencing studies indicating that tumours support bacterial growth, and

ii) isolation of clinically appropriate bacterial strains with tumour-targeting potential.

Materials and Methods: A culture-based assay was validated preclinically in murine tissues before application to human specimens. Consent was obtained from 42 female patients and 2 male patients with breast cancer, and progressive contamination control practices were imparted at all points of specimen handling. Absolute bacterial numbers were quantified using qPCR.

Results: No cultured bacteria were identified as tumour selective. Bacteria were found to be infrequent colonisers of breast tumours, compared with those isolated from normal adjacent tissues, as determined by the culture-based assay and qPCR. Tumour-isolated bacteria were almost invariably skin-associated, and were not verifiable as having originated within the tumour.

Conclusion: Bacteria recovered from breast tumours in the present study are probably not suitable for adoption as tumour-targeting agents, given their quantitative and qualitative similarities to those recovered from normal adjacent breast tissues.

INTRODUCTION

The administration of exogenous bacteria to tumour-bearing organisms has indicated a remarkable propensity for microbes to colonise selectively a wide range of tumour types. This phenomenon has been demonstrated both in animal models (Cronin et al., 2012) and human subjects (Toso et al., 2002), across several administration routes, including IV and intratumoural. Several tumour-specific features have been suggested to account for these observations, including defective tumoural immunity and a porous vasculature that facilitates bacterial entry (Baban et al., 2010). Such tumour selectivity has generated many initiatives investigating bacteria as genetically customisable vectors for anti-tumour treatment, such as BDEPT (Lehouritis et al., 2017). Despite this selectivity, a key drawback noted of tumour-targeting bacteria deployed in the clinical setting is captured in reports that detail, in the case of *Salmonella enterica* serovar Typhimurium, for example, that colonisation of patient tumours is minimal even when administration is intratumoural (Nemunaitis et al., 2003) and that off-target toxicity can occur even when sub-optimal IV doses are administered (Toso et al., 2002). These clinical studies underscore the value of identifying new strategies for producing bacteria with refined tumour-targeting potential.

Apart from tumours, it is well known that bacteria are adept at colonising other anatomical locations even in the absence of purposeful clinical intervention, such as the skin at birth via contact with the birth canal (Bourgeois-Nicolaos et al., 2010), and the gut via ingestion of foods (Adlerberth, 2008). It is hypothesised in this chapter that the generous quantities of endogenous microbial cargo that humans typically carry naturally may lead to tumour colonisation in patients with cancer. It is further reasoned that, were endogenous bacteria cultured from human tumours, they may outclass the laboratory strains that are routinely used. If such microbes existed, then this would be welcome information for researchers who are selecting bacteria at the clinical level for oncology trials, as they may provide the means of circumventing the unsatisfactory clinical outcomes experienced to date in this field. Thus, this chapter investigates the presence of viable bacteria in the breast, about which some information is already available regarding its relationship with bacteria – both in healthy and malignant tissues.

Early effort to characterise the breast microbiota suggests that the viable, culturable portion of healthy breast tissue has low biomass (Thornton et al., 1988); however, this must be considered in the context of the relative antiquity of this work, which was published in the same decade that the phrase "great plate count anomaly" was coined (Staley and Konopka, 1985). Then, bacteriologists were setting out to define and understand the problem of unculturable bacteria, addressing the reality that the true bacterial load within a sample may in fact be much higher than what a culture-based assay might imply. More recently, advancements in 16S rRNA gene sequencing technology have helped researchers interpret the microbiome from a different, more high-throughput angle (Ju and Zhang, 2015). Deep sequencing has been presented as a means of uncovering unculturable microbes in many specimen types, to the extent that studies describing the bacterial content of breast tissue thus far have relied almost exclusively on deep sequencing techniques for microbial profiling of both healthy (Hieken et al., 2016, Urbaniak et al., 2014, Urbaniak et al., 2016) and malignant (Lehouritis et al., 2015, Wang et al., 2017) breast tissues, with far less reliance on culture-based assays, creating its own set of challenges. While NGS can help to access the culturally unreachable areas of a bacterial community, it is disadvantageous in that it cannot confirm the viability of the microbes it detects (Møretrø and Langsrud, 2017). Thus, data derived from potentially non-viable microorganisms may lead to an incomplete impression of the breast tumour microbiota, and are thus unsuitable for ascertaining the presence of live, tumour-associated microbes.

The aims in this study were to i) generate data that could help to validate DNA sequencing studies indicating that tumours support bacterial growth and ii) isolate clinically appropriate bacterial strains with tumour-targeting potential. These aims were approached via development of a culture-based assay, capable of capturing as wide an array of viable bacteria as possible from tumour and normal adjacent tissues of the human breast, with the intention of identifying live microbes that proliferate naturally within breast tumour tissues more extensively than in healthy breast tissues. Various methodologies were built around this culture-based assay (i.e., HTS and machine learning approaches) that would help to clarify the tumour as the probable origin of these bacteria. 16S qPCR was used to absolutely quantify total bacterial numbers in fresh, frozen human samples to complement the 16S metabarcoding data of Chapter 2, which quantified bacterial communities only in terms of relative

abundance. Past studies have used qPCR to quantify absolute numbers of total bacteria in FFPE tumour tissues (Nejman et al., 2020) and in fresh pancreatic ductal adenocarcinomas (Geller et al., 2017), but so far this has not been accomplished for fresh breast tumours, to the author's knowledge. Development of the culture-based assay was guided by HTS data from past breast microbiome work. Preclinical validation was completed in murine tumour (4T1 and RENCA) and liver samples before translation to the clinical setting.

MATERIALS AND METHODS

Murine RENCA tumour induction and excision

Animal experiments were approved by the Health Products Regulatory Authority (Dublin, Ireland) and the Animal Experimentation Ethics Committee of University College Cork (Cork, Ireland). 4T1 tumours were induced in five 5-8-week-old female BALB/c mice by subcutaneous injection, of 5 x 10^5 4T1 tumour cells suspended in 200 µl serum-free RPMI media, into the right flank. RENCA cells were grown in RPMI media (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific) in a Thermo Forma Series II Water-Jacketed CO_2 incubator (Thermo Fisher Scientific) at 37ºC at a CO_2 concentration of 5%. Cell counts were calculated using a NucleoCounter (Chemometec). 4T1 RENCA tumours were induced in ten 6-8-week-old female BALB/c mice by subcutaneous injection of 1 x 10^5 RENCA cells suspended in 200 µl serum-free RPMI media into the right flank. Tumour induction was performed with a 25-gauge needle (BD Microlance 3, Becton-Dickinson). RENCA tumours were measured every 2-6 days with a Vernier caliper. Tumour volume was approximated using the formula $V = \alpha\beta^2 \, x \, \pi/6$, where α = the longest diameter of the tumour, and β = the diameter perpendicular to diameter α (Ueha et al., 2017). When RENCA tumours were approximately 500 mm^3, and when 4T1 tumours were between 65 mm^3 and 1750 mm^3, mice were culled by cervical dislocation following percussive blow to the head.

Clinical specimen collection and transportation

Clinical research was approved by the Clinical Research Ethics Committee of University College Cork (Cork, Ireland). Please refer to Chapter 2 for a more detailed description of patient surgery and specimen collection. In addition to the methods detailed in Chapter 2, the following was performed for the purposes of Chapter 3: Healthy breast ductal tissue was extracted from three patients using a 14-French core biopsy needle (ACHIEVE programmable automatic biopsy system, Merit Medical). For 12 patients, a full-thickness skin biopsy was excised from the breast using a diathermy needle immediately after excision of tumour and normal adjacent tissues. Ductal tissue and skin biopsies were placed into 30-ml universal containers (Thermo Scientific) containing 500 µl RCM (Oxoid).

Culture and Sanger-sequencing of bacterial isolates

Five 4T1 murine tumours and ten RENCA murine tumours were cultured, and 38/44 patients had their tissues and skin swabs cultured. Tissues were transferred to a 70-µm nylon cell strainer (Corning) (Figure 1) and homogenised using the rubber end of a 10-ml syringe (Syringe Without Needle, Terumo). Cell strainers were washed with 500 µl RCM and liquid adherent to the bottom of the cell strainers was aspirated and added to the total filtrate. 100 µl tissue homogenate/skin swab sample were then spread-plated onto the following types of agar: Brain Heart Infusion (Oxoid); MRS (Oxoid); GAM (HiMedia); and Columbia sheep blood agar (VWR). For murine specimens, 100 µl tissue homogenates were also plated on DGAM agar (a one-tenth dilution of GAM agar). Agar plates were placed in a Thermo Forma Series II Water-Jacketed CO_2 incubator and were incubated at 37ºC at a CO_2 concentration of 5% for 2 to 5 days.

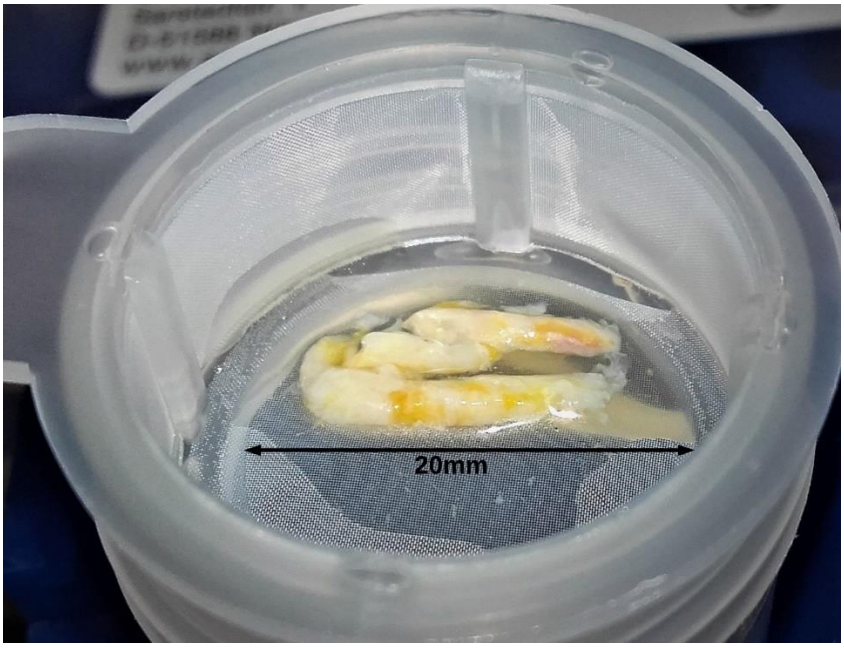


Figure 1. Core biopsied breast tumour tissue from a female breast cancer patient. *Tumour tissue was biopsied using a 14-French ACHIEVE programmable automatic biopsy system. Specimen is characterised by off-white tumour tissue, admixed with amber-orange benign tissue. Tissue has been placed into a 70-µm nylon cell strainer prior to homogenisation.*

DNA from bacterial isolates was PCR-amplified by using primers targeting the V3-V6 region of the 16S rRNA gene (forward primer 5'-CCA GAC TCC TAC GGG AGG CAG-3' and reverse primer 5'-ACA TTT CAC AAC ACG AGC TGA CGA-3'). 25-µl reactions were set-up per the following recipe: 12.5 µl Taq 2X Master Mix (New England Biolabs), 0.5 µl both primers (final concentration: 0.2 µM) (Integrated DNA Technologies), 11.5 µl nuclease-free water (Qiagen), and the colony of interest. Reactions were run in a Mastercycler Gradient (Eppendorf) per the following protocol: 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 sec, 55ºC for 45 sec, 72ºC for 1 min, followed by a final extension step at 72ºC for 7 min. Amplification was confirmed by running 5 µl PCR product on a 1.5% agarose gel at 70 volts for 80 min, followed by imaging on a Gel Doc EZ System (Bio-Rad). The product was approximately 735 bp.

PCR-positive samples were cleaned by running 15 µl PCR product through a MinElute PCR Purification Kit (Qiagen) per the kit protocol. Purified samples were quantified

by analysing 2 µl purified PCR product on a NanoDrop ND-1000 UV-Vis Spectrophotometer (Mason Technology). Samples were then diluted to 20-80 ng/µl with nuclease-free water, and 5 µl of each sample were added to 5 µl forward primer (final concentration: 2.5 µM). Samples were Sanger-sequenced by Eurofins Genomics Sequencing GmbH, Cologne, Germany. Sequences were analysed using the Microbial Nucleotide Basic Local Alignment Search Tool hosted by the National Center for Biotechnology Information of the National Institutes of Health. Representative genomes were searched using an algorithm optimised for highly similar sequences. Search results were excluded if Expect Values were greater than 0. Search results with the highest Max Score, Total Score, Query Cover, and Percent Identity were used to assign strain-level identities to the original bacterial isolates.

16S rRNA gene quantitation of cancer patient breast tissues

A probe-based qPCR was used to determine 16S rRNA gene copy numbers in DNA extractions derived from malignant and benign patient breast tissues. A standard was first made by inoculating BL35624 into RCM and incubating anaerobically overnight at 37ºC. The next day, 1500 µl bacterial culture were processed using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich), incorporating lysozyme from chicken egg white (Sigma-Aldrich), per the product protocol. DNA was eluted in 150 µl Elution Solution. A species-specific probe set was designed, targeting a chromosomal glycosyl transferase gene of this bacterial strain (probe 5'-/56-FAM/ CGCATCATC/ZEN/CAGTCAGGGAAGGTC/3IABkFQ/-3', forward primer 5'-GTG TGA TTG TGA TTA CCG AGG A-3', reverse primer 5'-GAC AAA TGT GTG CGA GCA ATA G-3').

To calculate the copy number of this gene in the elution, a 20-µl qPCR was set-up per the following recipe: 1 µl probe mix (final concentrations: 500 nM forward and reverse primers and 250 nM probe) (Integrated DNA Technologies), 10 µl Luna Universal Probe qPCR Master Mix (New England Biolabs), 3 µl ROX reference dye (final concentration: 375 nM) (Biotium), 1 µl Buffer EB, and 5 µl template DNA. No-template controls were run in parallel by replacing 5 µl template DNA with 5 µl Buffer EB. No-amplification controls were also made per the following recipe: 1 µl probe mix, 3 µl ROX reference dye, 14 µl Buffer EB, and 2 µl gBlock (4×10^7 gene copies)

(Integrated DNA Technologies). All samples were plated in triplicate. qPCR amplification proceeded in an AriaMX (Agilent) per the following protocol: 95ºC for 1 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 30 sec.

Gene copy numbers per cell, of the glycosyl transferase and 16S rRNA genes, were determined bioinformatically for BL35624. From this, the 16S rRNA gene copy number per µl in the BL genomic DNA elution was calculated. A 16S probe set was then designed, based on previously published sequences (probe 5'-/56-FAM/CAGCAGCCG/ZEN/CGGTA/3IABkFQ/-3', forward primer 5'-CCT ACG GGD GGC WGC A-3', reverse primer 5'-GGA CTA CHV GGG TMT CTA ATC-3') (Liu et al., 2012). 20-µl qPCRs were set-up per the following recipe: 1 µl probe mix (final concentrations: 500 nM forward and reverse primers and 250 nM probe), 10 µl Luna Universal Probe qPCR Master Mix, 3 µl ROX reference dye (final concentration: 375 nM),4 µl Microbial DNA-Free Water (Qiagen), and 2 µl template DNA. The protocol used for qPCR amplification was adapted from that used in a previous study employing the same probe set: 95ºCfor 1 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min (Stefan et al., 2018).

RESULTS

Please refer to Chapter 2 for HTS data.

Development of a culture method using murine tissue

Agar media were selected for application to murine specimens based on published culture data indicating compatibility of the media with human specimens (Rettedal et al., 2014). Selection was also guided by published bioinformatic data, choosing agars that would cover bacteria that comprised a relatively large proportion of the output (e.g., lactobacilli and MRS agar) (Urbaniak et al., 2016). DGAM agar was used for murine specimen culture based on evidence that diluted media can sometimes capture broader bacterial diversity than conventional media (Sun et al., 2019).

Of the 12 RENCA tumours cultured, 8 were culture-positive and 4 were culture-negative (Figure 2). 23 different bacterial species from 12 genera were identified across all media types and atmospheric conditions, excluding the two Gram-negative bacteria that were unidentifiable. Most of the isolates were anaerobic (facultative and obligate), Gram-positive bacteria. *Bacillus* spp. were the only strictly aerobic bacteria identified, and *Alistipes* sp., *Bacteroides* spp., and *Shigella dysenteriae* were the only Gram-negative bacteria identified. No bacterial isolates were recovered from murine 4T1 tumour tissues. Murine culture data were used as justification for removing DGAM agar from the set of media used to culture human specimens, based on the lesser bacterial numbers isolated using this medium, compared with conventional GAM agar. Similarly, these data were used to justify the omission of aerobic culture conditions for culturing human specimens due to its inferiority compared with the other atmospheric conditions in terms of CFUs recovered. Total bacterial numbers ranged from 1-44 CFUs per tumour. Numbers did not correlate with tumour volume.

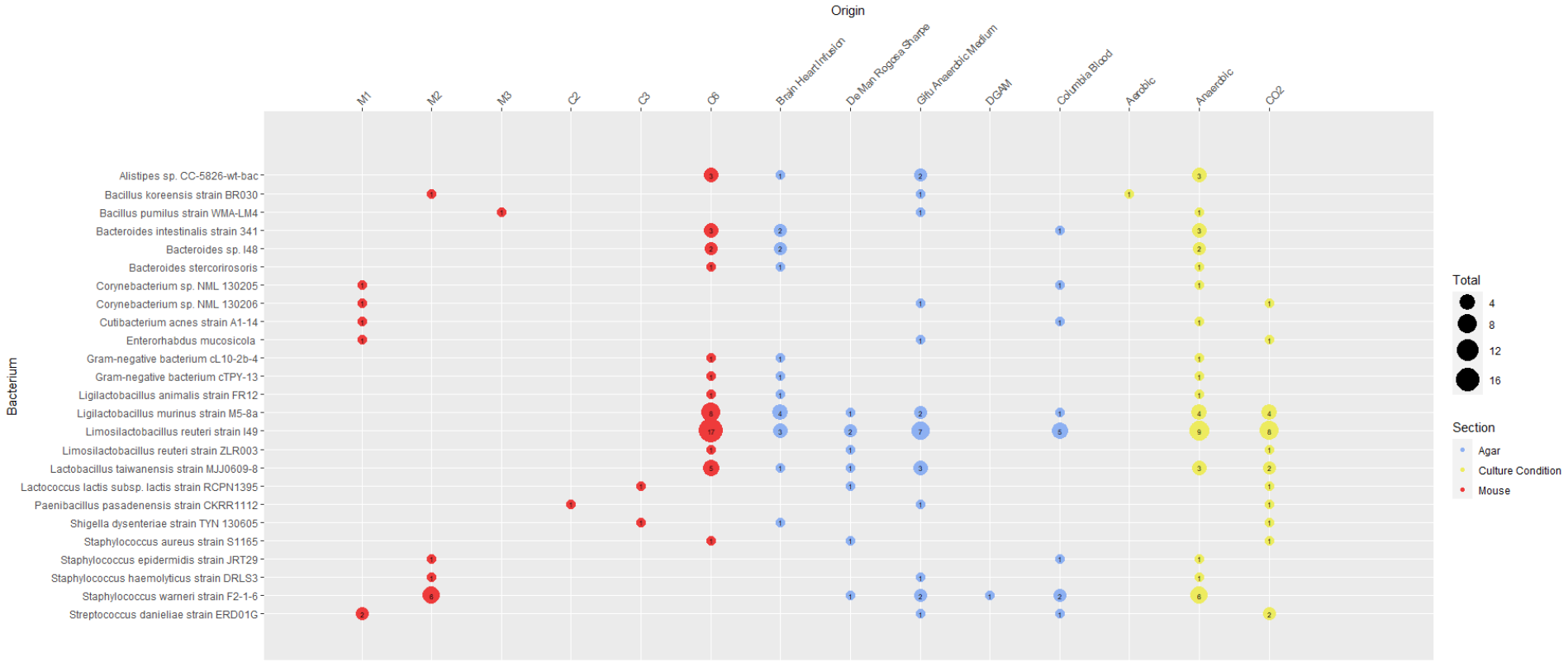


Figure 2. Bubble chart of viable bacteria recovered from murine tumour tissues. *Bacteria were plotted according to the mouse from which they grew (brilliant red), the medium type on which they grew (light cobalt blue), and the type of specimen from which they grew (brilliant yellow). Numbers inside the bubbles represent absolute CFUs of bacteria cultured. Tumour tissue weight range = 65 mg - 509 mg; median weight = 363 mg. Weights were calculated by assuming that 1 cm^3 = 1000 mg.*

Culture of human specimens indicates low bacterial diversity and load

All isolates cultured from breast tumour and normal adjacent tissues and skin swabs were sequenced. Viable culture data reflected relatively low viable bacterial diversity in all patient samples plated (Figure 3). 15 bacterial species, from four genera, were isolated across all tumour and normal adjacent specimens, the most frequent and numerous of which were coagulase-negative staphylococci – in particular, *Staphylococcus epidermidis* (isolated from 16/21 culture-positive patients) and *Staphylococcus capitis* (isolated from 12/21 culture-positive patients). *Corynebacterium* spp. were also numerous, but were largely drawn from one patient, whereas staphylococcal growth was more evenly spread from patient to patient. Some breast tumour tissue isolates were not found in normal adjacent breast tissue, and vice versa: *Corynebacterium imitans* and *Cutibacterium avidum* were found only in breast tumour tissue, and *Actinomyces neuii*, *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis*, and *Staphylococcus pettenkoferi* were found only in normal adjacent breast tissue.

Skin biopsy culture data resembled those of breast tissues in that bacterial isolates were exclusively coagulase-negative staphylococci (Figure 4). Again, *S. epidermidis* and *S. capitis* were among the most frequently isolated species. Skin swab culture data also bore a resemblance, with some scattered exceptions; for example, one skin swab, culture-positive for *Bacillus thuringiensis*, was taken from a patient that would otherwise have been culture-negative across all specimen types. Absolute numbers of CFUs recovered from all specimen types were low. All breast tissues grew less than 25 CFUs per agar plate, and 14/38 patients were in fact culture-negative across all specimen types. Additionally, culture-positive rates varied depending on specimen type: 7/38 breast tumour specimens, 18/38 normal adjacent breast specimens, 9/12 skin biopsies, and 5/38 skin swabs were culture-positive. All three ductal specimens were culture-negative.

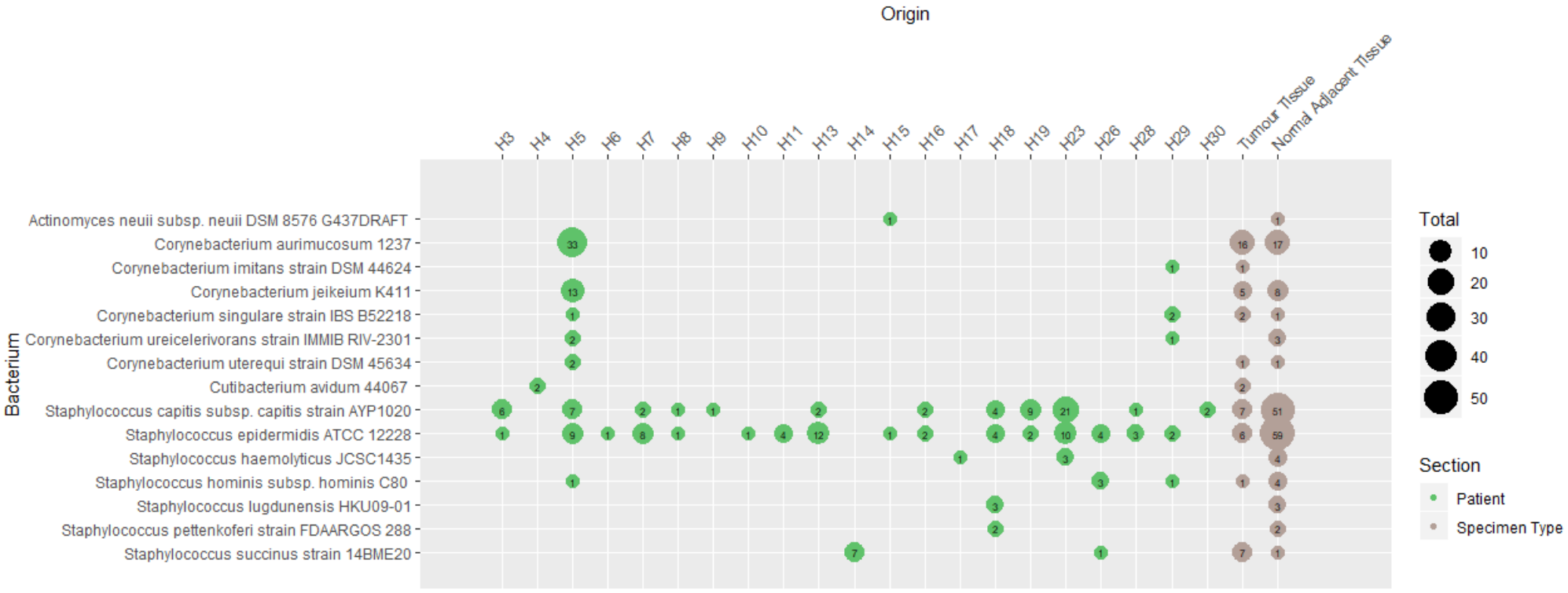


Figure 3. Bubble chart of viable bacteria recovered from cancer patient breast tissues. *Bacteria were plotted according to the patient from whom they grew (emerald green), and the type of specimen from which they grew (mocha). Numbers inside the bubbles represent absolute CFUs of bacteria cultured. Tumour tissue weight range = 20 mg - 140 mg; median weight = 60 mg. Normal adjacent tissue weight range = 30 mg - 1200 mg; median weight = 280 mg.*

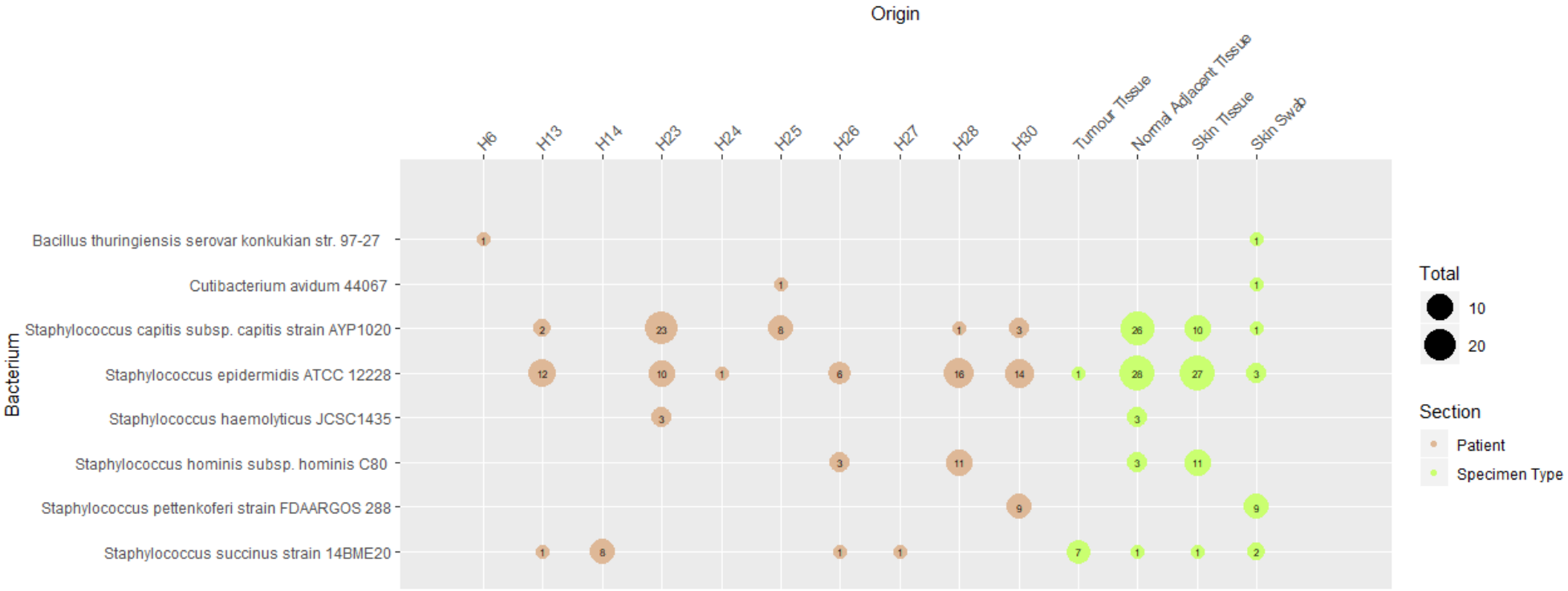


Figure 4. Bubble chart of viable bacteria recovered from cancer patient skin biopsies and swabs and corresponding culture data from breast tumour and normal adjacent tissues. *Bacteria were plotted according to the patient from whom they grew (ivory), and the type of sample from which they grew (olive green). Numbers inside the bubbles represent absolute CFUs of bacteria cultured. Each patient included in the graph had a skin swab taken prior to surgery. Patients H6, H13, and H14 had no skin biopsies cultured. Tumour tissue weight range = 20 mg – 100 mg; median weight = 60mg. Normal adjacent tissue weight range = 30 mg – 770 mg; median weight = 140 mg. Skin tissue weight range = 20 mg – 490 mg; median weight = 188.2 mg.*

Culture-based assay has high sensitivity for the sample types analysed

Differences in sensitivities of the culture-based assay and NGS analysis are reflected in the fact that bacteria were detected in 24/108 samples by the former method and 99/108 samples by the latter method, after removal of contaminant and human reads. To validate the results of Sanger sequencing, culture data for culture-positive samples were compared with their corresponding NGS data to determine if the same isolates detected by the culture-based assay were also present in the deep sequencing output (Figure 5). 23 culture-positive patient samples also had corresponding NGS bacterial reads, and 21 of these samples had strain-level commonalities across both datasets. As the culture-based assay was less sensitive than the NGS, it was calculated the degree to which Sanger-sequenced bacteria from cultured specimens populated the output of their corresponding, deep-sequenced samples. Sanger-sequenced isolates constituted greater than 90% of the NGS reads for 10/21 samples with strain-level commonalities in both culture and NGS data.

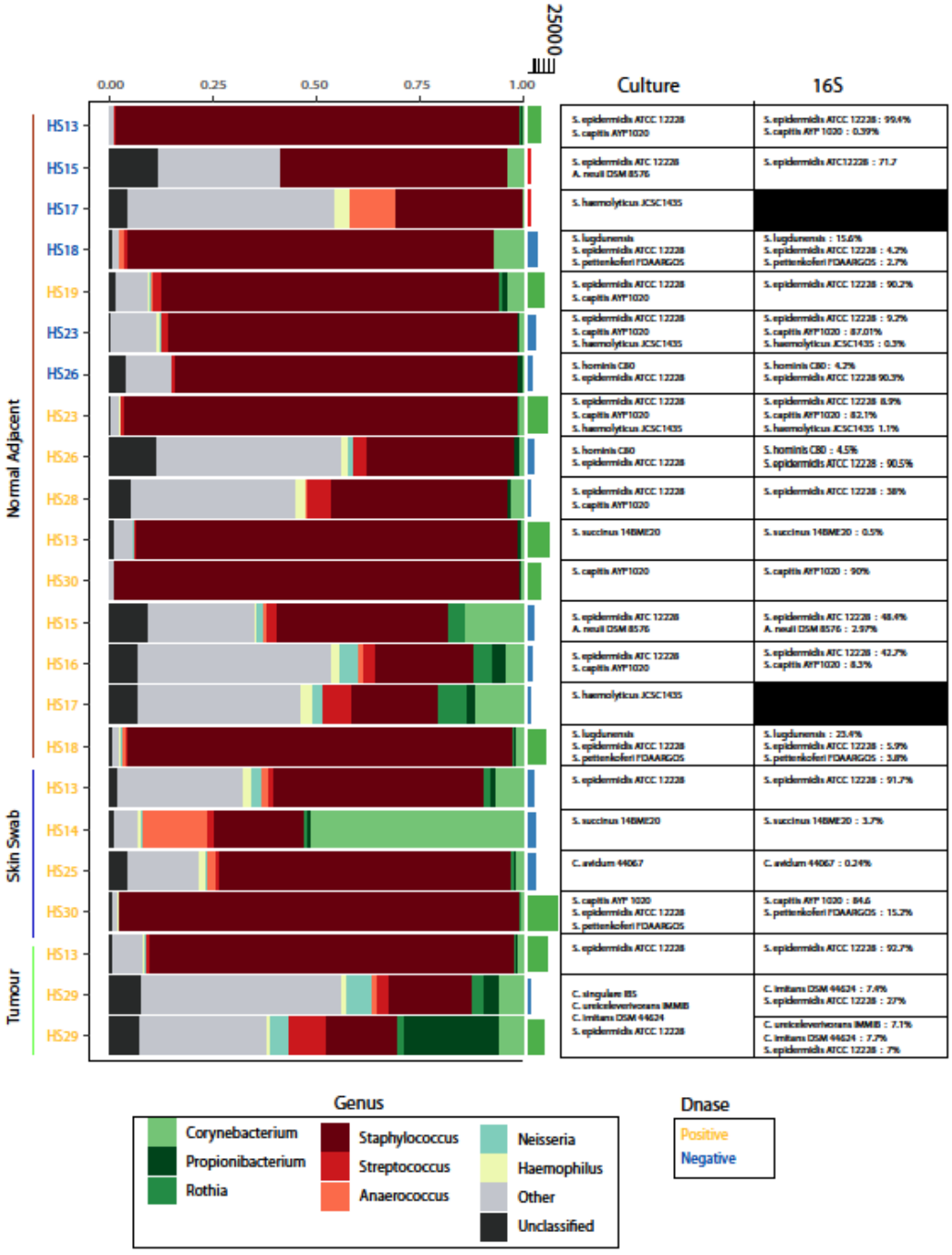


Figure 5. Agreement between culture-based assay isolate data and deep sequencing data derived from cancer patient breast tumour and normal adjacent tissues, and skin swabs. *Viable bacterial strains cultured from human breast tissues and skin swabs are tabulated next to metabarcoding data, derived from the same patient specimen, showing cases in which the same strains were, or were not, detectable*

across both data sets. Overall high-throughput bacterial composition at the genus level is indicated to the left of the tabulated strains. These data demonstrate that the culture-based assay has high sensitivity in relation to the sample types cultured, as it can capture most of the microbes identified via HTS.

The bacterial load found in patient breast tumour tissues is largely undetectable by qPCR (90% of cases) (Figure 6). In cases where a bacterial load is detectable, there were, on average, 7.8×10^2 16S gene copies per gram of tissue. Quantities in patient normal adjacent breast tissues were more variable, ranging from no detectable 16S gene copies (approximately 40% of cases), to, on average, 1.2×10^3 gene copies per gram of tissue. As high-throughput and culture data were compared to gauge analytical consistency, so too were qPCR and culture data. There were scattered cases in which qPCR-negative breast specimens were culture-positive – four in total; however, all of these were cases of low viable bacterial counts (1-9 CFUs per specimen). There were more instances in which qPCR-negative patient breast specimens were also culture-negative – 10 in total. Corresponding deep sequencing read number data and culture data for qPCR-positive patient breast specimens are indicated in Figure 7 - seven qPCR-positive patient breast specimens were culture-negative, and seven qPCR-positive patient breast specimens were culture-positive. These data also indicate that the culture-based assay is sensitive to the sample types under study, as in many cases qPCR-positive breast specimens are associated with successful bacterial isolation via culture.

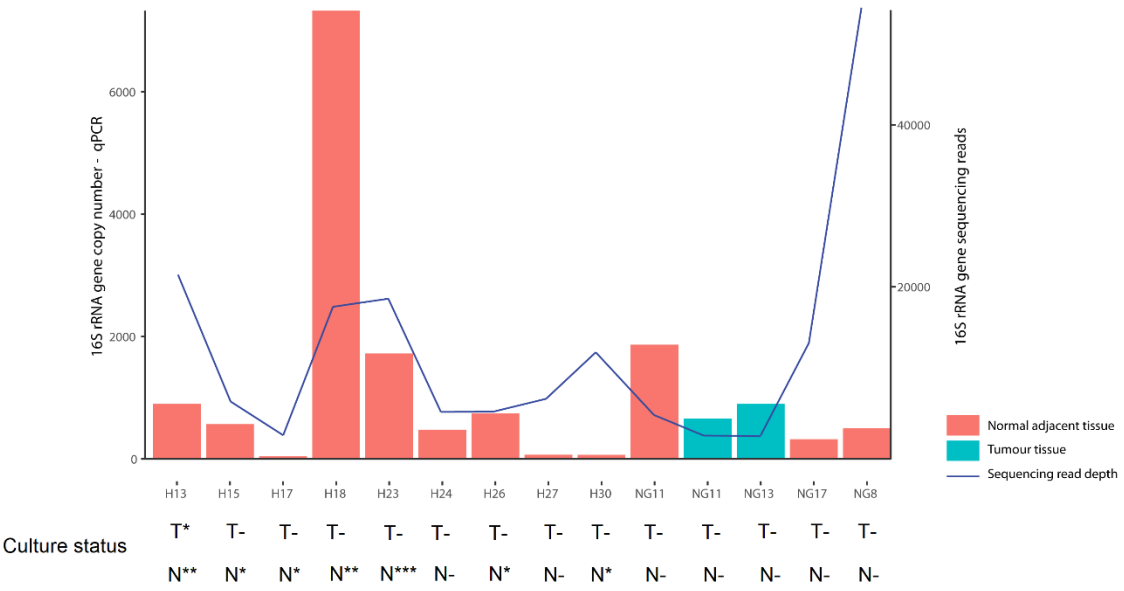


Figure 6. 16S rRNA gene copy quantities in cancer patient breast tissue, with corresponding culture and deep sequencing data. *qPCR-recovered 16S rRNA gene copy numbers per gram of breast tumour and normal adjacent tissue are indicated on the left y-axis and HTS read numbers are indicated on the right y-axis. Each patient's culture status is represented below each bar by asterisks, indicating CFUs isolated per specimen (* 1 – 9 CFUs, ** 10 – 19 CFUs, *** 20 – 29 CFUs). A minus sign indicates a culture-negative patient sample. The limit of detection by qPCR was 16 copies.*

DISCUSSION

Tumour-selective bacteria were not identified in the current study based on the recovery of similar, low bacterial quantities from breast tumour and normal adjacent tissues – both in terms of gene copy and CFU numbers. The two bacteria that were found only in breast tumour tissues – *C avidum* and *C. imitans* – were found in only one patient each, at low numbers. The viable bacterial profile of patient breast tumour and normal adjacent tissues was narrow in scope, as virtually all bacteria identified were skin-associated commensals.

The integrity and sensitivity of the culture-based assay were validated both by its correlation with qPCR and HTS data, and due to its ability to isolate fastidious bacteria, such as the lipophilic *Corynebacterium jeikeium* (Ferrer et al., 2004). Often, the results of culture-based assays are reported tentatively on the basis that most bacteria are "unculturable" and are therefore not capturable by standard culture techniques (Lok, 2015); this makes the degree to which "unculturable" bacteria populate breast tumour and normal adjacent tissues a critical topic worth exploring. It is noteworthy that many of the bacterial genera described in past breast microbiome studies were not isolated successfully from human specimens using the culture-based assay described in this study, despite using culture conditions that others report can routinely grow those genera, including *Lactobacillus* spp. (Jiang et al., 2015), *Gardnerella* spp. (Kidane et al., 2017), and *Micrococcus* spp. (Putonti et al., 2017) and a more recent 2020 study (Nejman et al., 2020) likewise reported the presence of various genera in breast tumour tissue that were culturable, but not cultured, by the method described in this chapter – e.g., *Pseudomonas* sp. (Young and Armstrong, 1972, Daly et al., 1984) and *Acinetobacter* spp. (Cai et al., 2011, Wang et al., 2003).

Recently, researchers cultured bacteria from five patient breast tumours, with a large variation in the CFUs recovered between specimens (Nejman et al., 2020). This study did not attempt to culture normal adjacent breast tissues, nor did it use any controls to detect potential skin contamination. Additionally, a Canadian cohort of 43 female patients yielded culture-positive results across all plated normal adjacent breast specimens, despite using a less comprehensive culture-based assay (Urbaniak et al., 2014). Variability in bacterial recovery from breast tumour and normal adjacent tissues in the current study compared with past studies may be reflective of different

levels of aseptic stringency in tissue collection. Notwithstanding the progressive practices adopted in this study to combat contamination, the impact of contamination on sample composition is important. Recovery of viable bacteria from 9/12 patient skin biopsies, post-disinfection, invited revaluation of the effects that skin bacteria surviving surgical disinfection may have on patient breast biopsies. It is known that bacterial quantities vary significantly in human skin depending on the sample type analysed, with punch biopsies collecting more bacteria than skin scrapes, and more still than skin swabs (Grice et al., 2008). This accords with recovery rates observed in this study for skin biopsies and swabs, which were higher in the former sample type.

Skin swabs have the benefit over skin biopsies of being non-invasive and more rapid, making them ideal for extensive profiling of the skin microbiome. In terms of assessing the probability of contamination by the skin microbiota of other body sites, however, the author suggests that the culture of skin swabs alone is insufficient, and that full-thickness sections are required, due to the increased recovery of skin bacteria from the latter sample type noted here. Despite disinfection, it is still likely that bacteria present underneath the skin's surface, that may not receive as much exposure to surgical disinfectants as those colonising the epidermis, could reach breast tissue, or indeed any internal body site, by contamination of surgical instruments when the skin is incised and the tissue of interest handled. Thus, this advice applies to any microbiota study in which surgical bypassing of the skin is necessary to access the tissue of interest, and is particularly relevant when the strain cultured most frequently from the tissue of interest is also cultured from skin swabs and skin biopsies, as was the case here.

The predominant genus cultured and sequenced from the current patient cohort, *Staphylococcus* spp., is an easy-to-culture genus (Breitkopf et al., 2005), yet it was culture-negative in most breast specimens, despite appearing prominently in the accompanying NGS data. Thus, it is unlikely that live, unculturable staphylococci were present in these breast tissues and it is more plausible that the disparity between culture and NGS data exists due to the sequencing of either naked staphylococcal DNA, or DNA originating from dead staphylococci. As there was no detectable intra-sample variation based on DNase treatment of samples pre-lysis, it is apt to postulate that this DNA derives from non-viable, cellular microbes that were killed during

surgical disinfection of the skin, rather than from bacteria pre-existing in a more permissive internal environment.

One of the hypotheses put forward to explain the existence of skin-associated bacteria in breast tissues is that bacteria migrate from the skin's surface to the breast parenchyma via milk ducts of the breast. However, this is at odds with the culture-negative nature of the ductal breast tissues cultured in this study. Taken together, this work indicates that breast tumour and normal adjacent tissues have either a vanishingly small, endogenous microbiota, or one that comprises a significant and unavoidable, yet minimised, level of skin contamination originating from standard surgical practices.

While the breast tumour microbiota does not appear to provide a fruitful foundation for the study of endogenous, tumour-selective bacteria for clinical exploitation, there are still paths to explore. The drastic contrast in the microbiota profiles of murine 4T1 and RENCA tumours described in this report calls for application of a similar culturomics approach to different human tumour types. The study of pancreatic cancer, for example, has revealed that the pancreatic ductal adenocarcinoma microbiome has the capacity to generate oncogenic signals via tumour immunosuppression, that could be potentially intercepted to disrupt disease progression (Pushalkar et al., 2018). As with the breast microbiome, however, attempts to understand the pancreatic cancer microbiome have been guided primarily by deep sequencing approaches and would likely be enhanced by adoption of a culturomics-based workflow.

CONCLUSION

The impression of the mammary microbiota to date has been informed primarily by 16S rRNA metabarcoding data. Moreover, the limited information available on the tumour microbiota in general is obtained almost exclusively from HTS analyses. Consequentially, until now, the breast microbiota has been incompletely described, providing an insecure foundation for discussion of its potential applications. The data arising from the current study do not indicate the presence of culturable bacteria appropriate for use in clinical and preclinical tumour targeting studies.

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Chapter 4: Investigation of tumour targeting of orally administered probiotic bacteria in cancer patients

ABSTRACT

Background: The increasing prevalence of clinical studies that probe the feasibility of using bacteria as genetically tractable, tumour-targeting drug delivery agents justifies further study of the most tenable and practical routes of administration available. Prior work has shown that tumour-bearing mice that have been orally administered probiotic bacteria experience tumour-selective colonisation by the administered microbes.

Aim: A pilot clinical study was implemented, examining the potential for GIT-to-tumour transmission of orally administered probiotic bacteria in cancer patients.

Materials and Methods: Consent was obtained from 14 patients with renal cancer and 38 patients with lung cancer. Either fresh tumour and normal adjacent tissues, or FFPE tumour tissues, were obtained and processed for each patient. A mouse model was utilised to verify the efficiency of bacterial DNA extraction from FFPE tissues, and confirm limits of detection.

Results: A qPCR strategy for detection of test bacteria in FFPE patient tumour samples was developed and validated. Tumour colonisation of ingested bacteria was not evident by qPCR in human subjects. Stool sample analysis of a subset of patients confirmed GIT colonisation. A subsequent mouse trial produced results in line with those of the clinical study. Limits of detection ($1 \times 10^2 - 1 \times 10^3$ gene copies) were determined to be sufficient to detect what would be expected in the case of colonisation.

Conclusion: This trial is, to the author's knowledge, the first attempt to transfer the findings of previous, related murine findings to a clinical setting. The clinical outcomes were negative – no tumour colonisation following oral administration of test bacteria was evident - and in direct contrast to previously published murine studies. Subsequent murine studies here also disagreed with the previous murine studies, correlating with the current clinical study, suggesting recent increased study stringency may highlight study inadequacies in the older studies carried out in the previous decade.

INTRODUCTION

The aim of cancer therapy is to maximise cancer cell death while minimising the death of normal cells. To achieve tumour selectivity, a novel therapeutic approach involving bacteria has been identified. Bacteria represent unique tumour-targeting agents, as they can preferentially colonise and proliferate within tumours following systemic administration (Baban et al., 2010, Cummins and Tangney, 2013). Many bacterial genera have been shown to accrue within tumours, and some have been investigated as anticancer agents, including *Salmonella, Escherichia, Clostridium, Bifidobacterium,* and *Listeria* (Cummins and Tangney, 2013, Roberts et al., 2014).

Several oncological traits have been suggested to enable tumour-selective colonisation by bacteria following their administration, including low intratumoural oxygen levels, which set malignant tissues apart from healthy tissues, local immunosuppression, which prevents bacterial elimination by the host immune response, and a distinguished nutrient composition within necrotic tumour regions (Lehouritis et al., 2017). Cumulative reports detailing the array of bacteria that can preferentially grow within tumours has led to them being placed among other, modern drug delivery modalities that are touted for their potential for targeted cancer treatment, such as liposomes (Cao et al., 2016) and antibodies (Sharma and Bagshawe, 2017). Furthermore, the genetic tractability of certain bacteria makes them amenable to the bioengineered expression of compounds which, when administered to patients, may have an exclusively anticancer effect (Bernardes et al., 2013). This technology can be based on the use of non-disease-causing "good bacteria" (e.g., probiotics) that do not enter cancer cells, but grow to high concentrations within tumour tissue, where they can release therapeutics or locally active agents, making this applicable to a wide range of therapeutic and diagnostic strategies (Byrne et al., 2014, Cronin et al., 2014, Lehouritis et al., 2013). While these bacteria, when not engineered, have no effect on tumour growth, it has been shown that non-engineered probiotics can mediate effective tumour therapy in conjunction with prodrug chemotherapies (Lehouritis et al., 2015, Lehouritis et al., 2016). Taken together, these qualities have encouraged clinical investigation of bacterial administration in cancer patients; however, all clinical studies thus far investigating bacteria as potential carriers for anticancer therapies have done so in the context of either IV (Heimann and Rosenberg, 2003) or intratumoural (Nemunaitis et al., 2003) administration.

Bacterial translocation refers to trafficking of bacteria from the GIT to extraintestinal sites, and investigations of this phenomenon are normally confined to bacterial sepsis, associated with various conditions (Balzan et al., 2007). Relatively little information is available on translocation of indigenous bacterial species; however, sampling from humans has indicated that bacterial translocation may occur in healthy individuals and may be a normal physiological event without deleterious consequences (Sedman et al., 1994). Additionally, clinical studies, in which probiotic bacteria were taken by healthy subjects, report that severe disease is rarely caused by the bacteria, even when they are shown to translocate from the GIT (Liong, 2008). Published evidence suggests that gut bacteria naturally resident in the GIT may have the ability to penetrate intact, intestinal epithelia and migrate from the gut to the circulatory system in cancer patients receiving chemotherapy (Ota et al., 2020). It has been previously shown in several labs, via viable, culture-based recovery, that oral administration of probiotic bacteria to tumour-bearing mice results in bacterial translocation to, and selective colonisation of, tumours (Cronin et al., 2010a, Danino et al., 2015). Mice remained healthy throughout these experiments. The introduction of orally administered, probiotic bacteria into the systemic circulation, and resultant tumour colonisation, has the potential to influence the design of clinical tumour-targeting studies, were this phenomenon demonstrated in cancer patients. Due to the presumed low bacterial biomass of the systemic circulation, and concerns that culture-based assays may be ill-equipped to recover the full complement of gut-associated bacteria within a sample (Rajilic-Stojanovic and de Vos, 2014), data to date on microbial gut translocation have been drawn largely from non-culture-based methods, like qPCR, on the basis of greater analytical sensitivity.

Preclinical experience with oral administration of bacteria to tumour-bearing animals invites exploration of this alternative administration route in cancer patients. Oral medicines have several advantages over other dosage forms, especially those necessitating invasive administration, in terms of patient acceptance and convenience for healthcare professionals. The oral route is a more attractive pathway by which medicines may be administered to patients compared with, for example, the IV route, due to improved patient compliance and reduced constraints on healthcare systems resulting from a reduced need for specialist technique (Banna et al., 2010). Considering the low levels of endogenous gut bacteria detected in the systemic

circulation of cancer patients, oral administration of bacteria also has the potential benefit of producing lower levels of systemic bacteria than if they were intravenously administered, which could place a more stringent limit on side effects experienced by the patient. This could be particularly advantageous if relatively low systemic levels of bacteria are all that is required for seeding a tumour and providing a foundation for exponential bacterial growth.

The aim of this study was to examine trafficking of orally administered probiotic bacteria to tumours in cancer patients, to establish if these bacteria might be clinically applicable as orally administrable, tumour-selective vehicles for therapeutic and/or diagnostic purposes. The development of a strategy for rapid detection of administered probiotics in patient samples was also sought. Here, new findings are reported from a pilot clinical study in which patients, recently diagnosed with renal or lung cancer, were invited to take either one of two commercially available probiotic supplements – either 'Mutaflor' or 'Alflorex'. Mutaflor contains EcN and has shown clinical efficacy in the management of a wide range of gastrointestinal conditions, including treatment of idiopathic, chronic constipation (Chmielewska and Szajewska, 2010) and maintaining remission in ulcerative colitis (Rembacken et al., 1999). 'Alflorex' ('Align' in the USA) contains BL35624 and has similarly shown to be beneficial in the treatment of intestinal diseases like irritable bowel syndrome (Brenner et al., 2009). Mutaflor was selected due to the facility of *E. coli* to be manipulated relatively easily via genetic engineering (Kamionka, 2011), which would make it easier to render it as an orally administrable anticancer agent were its translocation to tumours via the oral route demonstrated. Alflorex was chosen due to the contrasts between BL35624 and EcN, the former being a Gram-positive obligate anaerobe and the latter a Gram-negative facultative anaerobe; it is known, for example, that facultative and obligate anaerobes colonise tumours differently (St Jean et al., 2008) and thus the deployment of bacterial strains with varied mechanisms with respect to tumour colonisation could increase the probability of detecting intratumoural growth.

Upon surgical resection, renal and lung tumour tissues were tested to verify translocation of the relevant probiotic bacteria. The primary sample type collected and analysed was FFPE tissue. FFPE tissue forms an expansive repertory of patient material that can be retrieved relatively flexibly by laboratory researchers, compared with fresh tissues (Kalmar et al., 2013). Additionally, FFPE tissue is advantageous due

to its suitability for bulk analyses, the non-requirement for specimen retrieval immediately after surgical resection, and its stability at ambient temperatures. Despite its strengths, this specimen type is often noted for the deleterious impact it can have on nucleic acids, especially RNA but also DNA (Hall et al., 2008). Calculations of the mutagenic potential of formaldehyde fixation estimate that one base per every 500 bases is affected. Considering the anticipated downstream consequences of this on nucleic acid amplification, some fresh specimens were collected in addition to FFPE tissues. Stool samples were collected also in a subset of patients to calculate probiotic gene copy numbers in the gut and/or patient compliance with the probiotic regimen.

A positive control was established by producing FFPE tissue blocks from a murine tumour model. Probiotic bacterial strains were visualised in these blocks via IF and Gram staining. Due to the commercial unavailability of an extraction method for bacterial DNA in FFPE tissues, an approach that was developed in-house for this purpose was utilised. DNA extraction and qPCR protocols applied to human FFPE specimens were validated by testing these methodologies on positive-control FFPE tissue blocks.

MATERIALS AND METHODS

Assessment of probiotic bacterial translocation ex vivo

MF1 nu/nu female mice were gavaged orally with either 100 µl PBS (n = 6) or 100 µl of 10^9 *B. breve* UCC2003 (n = 6) prepared as previously described (Cronin et al., 2010b) for three consecutive days. At necropsy, distal ileum and colonic segments were immediately immersed in oxygenated Kreb's buffer, stripped of external muscle and myenteric plexus, and adjacent segments mounted into Ussing chambers. The chamber exposed 0.5 cm^2 of tissue surface area to 8 ml circulating oxygenated Kreb's buffer at 37°C.

The chambers contained agar-salt bridges to monitor the potential difference across the tissue and to inject the required short circuit current (Isc) to maintain a zero potential difference, as registered via an automated voltage clamp. Tissue conductance, representing passive permeability to ions, was calculated by Ohm's law. Baseline values for Isc, indicating net ion secretion, and conductance, as an inverse measure of tissue resistance, were recorded at equilibrium 1 h after mounting of the intestinal segments.

Mucosal-to-serosal transport of macromolecules was assessed by measuring transepithelial flux of HRP, used as a model protein antigen. After mounting the tissues for 15 min, HRP (type VI; Sigma-Aldrich) was added to the luminal buffer. Serosal samples (0.5 ml) were obtained after 2 h. HRP activity was determined by a modified Worthington method, and mucosal-to-serosal fluxes of HRP expressed as pmol/h/cm^2.

Assessment of the viability and variability of 'Mutaflor' and 'Alflorex'

It is recommended by the manufacturer that the proprietary probiotic product 'Mutaflor' is kept refrigerated; however, data were not available quantifying the reduction in viability, if any, that would ensue from ambient storage. As patients randomised to the 'Mutaflor' arm of the pilot study were requested to take this probiotic over a maximum period of two months, it was important to determine if a significant decrease in the viability of EcN would result over this period, were the product stored unrefrigerated. To determine these effects, 24 Mutaflor capsules were stored at 2-8ºC

and 22 Mutaflor capsules were stored at room temperature, and two capsules per storage condition were cultured every 3-10 days over a 60-day period. Capsules were opened using a scalpel (Swann-Morton), the contents added to LB media (Sigma-Aldrich), and serial dilutions performed. Serial dilutions were spread-plated onto LB agar and agar plates were incubated overnight at 37ºC. In the case of Alflorex, capsules were stored at room temperature alone, and two capsules were cultured every 3-11 days over 34 days. Capsules were opened and added to RCM, serially diluted, and spot-plated in triplicate on RCA. RCA plates were incubated anaerobically for one day in a BD GasPak EZ container (Becton-Dickinson) by adding an AnaeroGen 3.5 l sachet (Oxoid) at 37ºC. The viability of EcN and BL35624 and capsule-to-capsule variability under recommended storage conditions, were determined via colony counts.

Patient recruitment and specimen collection

Clinical research was approved by the Clinical Research Ethics Committee of University College Cork (Cork, Ireland). All patients consented were scheduled for surgery for resection of either renal or lung tumours. Patients with renal cancer were interviewed in Mercy University Hospital (Cork, Ireland) and patients with lung cancer were interviewed in Cork University Hospital (Cork, Ireland). Clinical notes for each patient were reviewed prior to each interview to establish whether exclusion criteria applied: patients with gastrointestinal disease that might affect bacterial colonisation of the GIT (e.g., Crohn's disease), patients with clinically significant immunodeficiency, patients over 75 years' old, patients with an antibiotic history within the past month, patients likely to require antibiotics during the study, and patients deemed unlikely to comply with the probiotic regimen were excluded from the study. Patients were also queried during the interview regarding their antibiotic history. Patients randomised to the 'Mutaflor' arm of the study were asked if they would have access to a refrigerator during the trial period.

Patients were requested to take either one Alflorex capsule or one Mutaflor capsule daily until tumour resection. A smaller subset of patients was given no probiotic so that their tissues could be compared quantitatively with 'treated' patients, to gauge the potential for cross-contamination, and/or assess natural tumour colonisation by the

probiotic bacteria, of patient tumours. Several patients provided a stool sample on the day of surgery to estimate numbers of the probiotic bacteria in the GIT by qPCR. Stool samples were placed in faecal collection (Sarstedt) and stored in a -80ºC freezer until bacterial DNA extraction. Fresh tumour and normal adjacent specimens were collected for 8 patients on the day of surgery. Two further patients had fresh normal adjacent specimens alone collected from their surgeries. Fresh tissues were snap-frozen and stored in a -80ºC freezer for subsequent DNA extraction. FFPE tumour tissues were collected for 46 additional patients. An overview of the numbers of patients consented, in terms of the probiotic with which they were provided, and the specimen type processed, is given in Table 1.

Table 1. Breakdown of lung and renal cancer patient numbers consented for this study, according to the probiotic they took, and the specimen type analysed.

Patient tissue type	Probiotic supplied			
	Mutaflor	**Alflorex**	**None**	**Unknown**
Fresh lung	1	1	1	0
FFPE lung	20	9	2	3
Fresh kidney	2*	1	3*	0
FFPE kidney	4	5	3	0

*one of these patients had only normal adjacent tissue available for analysis

Transformation of EcN

EcN was transformed with the plasmid p16S*lux*, which confers both antibiotic resistance and luminescent properties onto the bacterium via the plasmid's Em resistance gene and *lux* operon, respectively. Firstly, the strain was rendered electrocompetent: EcN was sub-cultured by adding 100 µl overnight culture to 25 ml LB media. Upon reaching an OD_{600} of 0.6, the culture was placed on ice for 10 min to arrest bacterial growth, followed by centrifugation at 3220 x *g* for 10 min in a centrifuge pre-cooled to 4ºC. After this, the supernatant was discarded, and cells were washed with 10 ml ice-cold ultrapure water. Cells were centrifuged and washed an additional time, as above, and resuspended in 40 µl ice-cold ultrapure water.

Following addition of 150 µg plasmid DNA, cells were incubated on ice for 15 min. The entire suspension was then transferred to a 2-mm, 400-µl electroporation cuvette (VWR) and electroporated using a Gene Pulser (Bio-Rad) at the following settings: 2.5 kV, 25 µF, 200 ohms. The suspension was then added to 950 µl LB media and incubated for 3 h at 30ºC, followed by spread-plating onto LB agar supplemented with 300 µg/ml Em (Sigma-Aldrich). Serial dilutions were performed using PBS (Sigma-Aldrich). Agar plates were incubated overnight at 30ºC.

Recovered colonies were imaged the next day using an *In-Vivo* Imaging System (Xenogen), with exposure time set to 'Auto'. Luminescent colonies were sub-cultured, as above, followed by spread-plating the next day onto antibiotic-selective LB agar at 42ºC to encourage chromosomal integration of the plasmid. Luminescent colonies were continually sub-cultured at 42ºC for 17 days, spread-plating from liquid culture on alternate days.

Animals, mammalian cell culture, and tumour induction

All *in vivo* experiments were approved by the Health Products Regulatory Authority (Dublin, Ireland) and the Animal Experimentation Ethics Committee of University College Cork (Cork, Ireland). RENCA and 4T1 cells were maintained in RPMI media (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific) in a Thermo Forma Series II Water-Jacketed CO_2 incubator (Thermo Fisher Scientific) at 37ºC at a CO_2 concentration of 5%. Cell counts were performed using a NucleoCounter (Chemometec). RENCA tumours were induced in 8-week-old, female BALB/c mice by subcutaneous injection of 1 x 10^6 RENCA tumour cells, suspended in 200 µl serum-free RPMI media, into the right flank. 4T1 tumours were induced in 5-8-week-old, female BALB/c mice by subcutaneous injection of 5 x 10^5 4T1 tumour cells, suspended in 200 µl serum-free RPMI media, into the right flank. Tumour induction was performed with a 23-gauge needle (BD Microlance 3, Becton-Dickinson). Tumours were measured once daily with a vernier caliper. Tumour volume was approximated using the formula $V = \alpha\beta^2 \times \pi/6$, where α = the longest diameter of the tumour, and β = the diameter perpendicular to diameter α (Ueha et al., 2017).

Bacterial preparation and oral/IV administration

Bacteria were prepared for administration once murine RENCA tumours were approximately 5 mm x 5 mm. Mice scheduled for gavage were provided either with drinking water supplemented with 5 mg/ml streptomycin (Sigma-Aldrich) *ad libitum* for one week prior to the first oral administration, or with potable water alone *ad libitum*. Transformed EcN was initially inoculated into LB media, supplemented with 300 µg/ml Em, overnight at 37ºC. The following day, the strain was sub-cultured by adding 100 µl overnight culture to 10 ml fresh LB media supplemented with 300 µg/ml Em. The culture was harvested at an OD_{600} of 0.8 by centrifugation at 3220 x *g* for 5 min. The supernatant was removed, and cells were washed with 10 ml PBS. Cells were centrifuged and washed three times in total. Serial dilutions were performed in PBS.

Bifidobacterium breve UCC2003 and BL35624 were initially inoculated, under anaerobic conditions, into RCM at 37ºC. The following day, the strains were sub-cultured by adding 500 µl overnight culture to 50 MRS media (Oxoid), supplemented with 0.05% cys-HCl (Sigma-Aldrich). Cys-HCl was filter-sterilised before use with a 0.2-µm filter (Filtropur). The following day, cultures were harvested and washed as with EcN, with the exception that PBS was supplemented with 0.05% cys-HCl.

For IV administration, RENCA-tumour-bearing and tumour-free mice were administered either transformed EcN (1 x 10^6 CFUs), *B. breve* UCC2003 (1 x 10^6 CFUs), or PBS, in a 100-µl dose via injection of the lateral tail vein. IV administration was accomplished using a 30-gauge needle (BD Micro-Fine Plus Demi 0.3 ml Insulin Syringe, Becton-Dickinson). For oral administration, RENCA-tumour-bearing and tumour-free mice were gavaged with either transformed EcN, *B. breve* UCC2003, BL35624, or PBS, on three consecutive days with a 20-gauge x 38-mm oral dosing needle (Vet Tech). 1 x 10^9 CFUs of each bacterium were administered in a 200-µl dose on each occasion.

Cultures were retrospectively spot-plated in triplicate, either onto LB agar supplemented with 300 µg/ml Em (in the case of EcN), or onto RCA (in the case of *B. breve* UCC2003 and BL35624). RCA plates were incubated anaerobically for two days at 37ºC. LB agar plates were incubated overnight at 37ºC.

Viable bacterial recovery from mice

When tumour sizes were between 5 mm x 5 mm and 15 mm x 15 mm, mice bearing 4T1 tumours were euthanised by cervical dislocation, following percussive blow to the head. Between 7 to 11 days after oral/IV administration of bacteria/PBS, mice bearing RENCA tumours and tumour-free mice were euthanised as above. 4T1 tumours were aseptically excised, flash frozen, and stored in a -80ºC freezer. RENCA tumours and livers from each RENCA-tumour-bearing mouse, and livers from each tumour-free mouse, were aseptically excised and halved. One half of each specimen was placed in at least 15 volumes of 10% buffered formalin (KB Scientific) and fixed for 24 h at room temperature, and the other half was placed in 1 ml PBS. PBS was always supplemented with 0.05% cys-HCl in the case of *B. breve* UCC2003 and BL35624. Non-formalin-fixed specimens were homogenised by pushing them through a 70-µm nylon cell strainer (Corning) with the rubber end of a 10-ml syringe (Syringe Without Needle, Terumo). Cell strainers were washed with 1 ml PBS, and liquid adherent to the bottom of the cell strainer was aspirated and added to the total filtrate. Serial dilutions were performed in PBS and were spot-plated in triplicate onto LB agar supplemented with 300 µg/ml Em (for specimens derived from mice that were administered transformed EcN) and RCA supplemented with 50 mg/l mupirocin (Sigma-Aldrich) (for specimens derived from mice that were administered *B. breve* UCC2003 and BL35624). Specimens derived from mice that were administered PBS were plated on antibiotic-selective RCA and LB agar. Agar plates were incubated as above.

Identification of viable bacteria recovered from fresh murine tissues

Bacterial colonies, spot-plated onto LB agar plates, were IVIS-imaged to verify the identity of EcN via luminescence. *B. breve* UCC2003 was identified using primers targeting the chromosomal *apuB* gene of this strain (forward primer 5'-CGC TCG CGT CAA GGA AAT G-3' and reverse primer 5'-GAG CCG TTG GTC AGG TTG-3'). 22 colonies grown on RCA (12 isolated from murine RENCA tumour tissues and 10 isolated from murine liver tissues) were randomly selected, across three mice, and PCR-amplified in 25-µl reactions comprising: 12.5 µl Taq 2X Master Mix (New England Biolabs), 0.5 µl both primers (final concentration: 0.2 µM) (Integrated DNA

Technologies), and 6.5 µl nuclease-free water (Qiagen). PCR amplification proceeded in a Mastercycler Gradient (Eppendorf) per the following protocol: 95ºC for 30 sec, followed by 30 cycles of 95ºC for 20 sec, 54ºC for 30 sec, and 68ºC for 30 sec, followed by a final extension step at 68ºC for 5 min. Amplification was confirmed by running 5 µl PCR product on a 1.5% agarose gel at 70 volts for 80 min, followed by imaging on a Gel Doc EZ System (Bio-Rad). The product was 174 bp.

FFPE tissue processing, embedding, and DNA extraction

8 x 11" Formalin Neutraliser Pads (CellPath) were used whenever formalin was being handled. FFPE murine tissues were processed using a LOGOS J Hybrid Tissue Processor (Milestone). Specimens were placed between two biopsy pads (Kaltek) inside a histology cassette. The following processing conditions were used: flushing for 25 min at 37ºC, rinsing (reagent #1) for 1 h at 37ºC, rinsing (reagent #2) for 1 h 10 min at 37ºC, ethanol for 1 h 25 min at 37ºC, xylene (Ocon Chemicals) for 2 h 20 min at 37ºC, isopropanol (EMD Millipore) for 50 min (30 min at 37ºC, then 20 min at 60ºC), and wax (CellWax Plus, CellPath) impregnation for 3 h 46 min at 62ºC. Following this, tissues were embedded in paraffin wax using a Tissue-Tek TEC (Sakura).

Human and murine FFPE tissue DNA extraction

FFPE tissues were sectioned using a HM 355 S Automatic Microtome (Thermo Fisher Scientific). 8-x-10-µm sections were processed for each specimen. Samples were subsequently processed with a QIAamp DNA FFPE Tissue kit (Qiagen) per the standard protocol, with the following exceptions: two xylene washes were used instead of one, samples were incubated in Buffer ATL and proteinase K at 56ºC for 1 h 45 min instead of 1 h, and samples were subsequently incubated at 90ºC for 59 min instead of 1 h. When drying the membrane of the QIAamp MinElute column after washing it with Buffer AW2, samples were centrifuged at 17,000 x *g* for 5 min instead of at 20,000 x *g* for 3 min. Finally, samples were eluted in 35 µl Buffer ATE by centrifuging at 17,000 x *g* for 2 min instead of at 20,000 x *g* for 1 min.

DNA extractions were also performed using the following protocol:

450 µl deparaffination solution (Qiagen) were added to each FFPE tissue sample. Samples were incubated at 56ºC for 2 min, and centrifuged at 1,000 x *g* for 1 min. The filtrate was then discarded and 450 µl 100% ethanol were added to the sample, followed by vortexing and centrifugation at 1,000 x *g* for 1 min. After discarding the filtrate, 450 µl 80% ethanol were added to each sample followed by vortexing and centrifugation at 1,200 x *g* for 1 min. 300 µl TBS (50-mM tris-HCl, 150-mM NaCl, pH 7.6) were added to each sample, followed by vortexing and centrifugation at 1,200 x *g* for 1 min.

250 µl tissue dissociation solution (250 µl TBS supplemented with 20 µg proteinase K) were added to each sample and samples were mixed by pipetting. Samples were incubated at 56ºC for 5-8 min on a shaking platform set to 800 rpm. Samples were then centrifuged at 1,500 x *g* for 1 min. The filtrate was discarded and 300 µl host depletion solution (375 U benzonase, 0.2% saponin, 0.1% Tween-20 in 300 µl TBS plus $MgCl_2$ (20-mM tris-HCl, 20-mM NaCl, 2-mM $MgCl_2$), pH 8) were added to each sample. Samples were mixed by pipetting and incubated at 37ºC for 15-20 min on a shaking platform set to 650 rpm. Benzonase activity was halted by adding 20 µl EDTA (500-mM) and mixing samples by pipetting. Samples were then centrifuged at 1,800 x *g* for 1 min, and the filtrate was discarded. Samples were washed with 400 µl PBS, and mixed by pipetting. Samples were centrifuged at 2,000 x *g* for 1 min and the filtrate was discarded.

Samples were resuspended in 200 µl bacterial lysis solution (PBS supplemented with 150-200 µg MetaPolyzyme (Sigma-Aldrich)) and mixed by pipetting. Samples were incubated at 35ºC at 4 h on a shaking platform set to 460 rpm. 30 µl proteinase K were added to separate 2-ml centrifuge tubes during incubation. After incubation, samples were centrifuged briefly and 160 µl decrosslinking solution (2-M guanidine hydrochloride, 1.25% Triton X-100, 1.25% Tween-20, 75-mM EDTA (pH 8.0), 100-mM tris-HCl (pH 8.0)) were added to each sample and mixed by pipetting. Each sample was then transferred to a 2-ml tube with 30 µl proteinase K. Samples were incubated at 56ºC for 18 h on a shaking platform set to 700 rpm. After this, samples were centrifuged briefly and 20 µl proteinase K were added, followed by another 18 h of centrifugation at 56ºC on a shaking platform set to 700 rpm.

Samples were incubated at 80ºC for 1 h, followed by cooling for 5 min and brief centrifugation. 700 µl column binding buffer (1:1 mixture of 100% ethanol and Buffer AL (Qiagen)) were added then, followed by inverting sample tubes 3-4 times and brief centrifugation. 650 µl of the contents of each tube were added to QIAamp MinElute columns, followed by centrifugation at 8,000 x *g* for 1 min. The filtrate was discarded and the rest of the sample was added to the column, followed by centrifugation at 8,000 x *g* for 1 min. Samples were washed with 600 µl Buffer AW1, and then 600 µl Buffer AW2, centrifuging samples at 8,000 x *g* for 1 min after addition of each buffer. Samples were then centrifuged at 18,000 x *g*. 50 µl tris-HCl, warmed to 50ºC, were added to the column, followed by incubation for 5 min at room temperature. After this, samples were eluted by centrifugation at 18.000 x *g* for 1 min.

Human fresh tissue DNA extraction

Fresh renal and lung tumour and normal adjacent tissues were processed using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), per the product protocol, with the exception that 30 µl proteinase K were added to samples instead of 20 µl, and samples were incubated overnight at 55ºC following addition of proteinase K. A rotary evaporator was used to decrease sample volume of all samples to approximately 50 µl following DNA extraction. All patient specimens were extracted in duplicate, excepting the two normal adjacent renal tissue specimens with no corresponding tumour specimen, and the tumour and normal adjacent tissue specimens of one unfed renal cancer patient.

Human and murine stool DNA extraction

Stools sampled from mice that were gavaged with EcN, BL35624, *B. breve* UCC2003, and PBS, and stools sampled from patients provided with Mutaflor and Alflorex, were processed with a DNeasy PowerSoil Kit (Qiagen). Stools were processed per the product protocol, with the exception that disruption and homogenisation were accomplished using a FastPrep FP120 instrument (MP Biomedicals) set to 6.0 m/s for 40 sec, which has been used successfully to isolate bacterial DNA from stool samples with the same kit (Sinha et al., 2016).

IF staining of murine FFPE tissues

FFPE RENCA tumour tissues, derived from mice that were IV-administered either EcN or PBS, were cut into 4-µm sections using a HM 355 S Automatic Microtome. Each specimen was sectioned in duplicate; additionally, two extra sections were cut for one FFPE RENCA tumour specimen derived from a mouse that was IV-administered EcN, to serve as a negative control for the primary antibody staining step. Sections were applied to SuperFrost Plus Adhesion slides (Thermo Fisher Scientific) and incubated at 37ºC overnight. The next day, sections were deparaffinised in two changes of xylene for 10 min each. Sections were then rehydrated in two changes of 100% ethanol for 10 min each, followed by two changes of 95% ethanol for 3 min each, followed by one change of 80% ethanol for 3 min, followed by 2 changes of PBS plus 0.1% Tween 20 (Sigma-Aldrich) and 0.05% Saponin from quillaja bark (Sigma-Aldrich) for 3 min each. Hydrophobic borders were then drawn around each section using a Super PAP Pen Liquid Blocker (Newcomer Supply).

Sections were blocked with PBS-TS plus 10% normal donkey serum (Sigma-Aldrich) and placed into a humidifying chamber for 30 min. Blocking buffer was then removed and sections were incubated with an anti-*E. coli* antibody (Abcam), diluted 1:50 in blocking buffer, in a humidifying chamber for 1 h (sections serving as a negative control for this step were incubated with blocking buffer alone). After removal of the primary antibody/blocking buffer, sections were washed twice using PBS-TS for 10 min each on a shaking platform (Shake 'n' Stack Hybridization Oven, Thermo Fisher Scientific). PBS-TS was then removed, and sections were incubated with a donkey anti-rabbit IgG Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch), diluted 1:100 in blocking buffer, in a humidifying chamber for 1 h; slides were protected from the dark using aluminium foil. After removal of the secondary antibody, sections were washed twice using PBS-TS for 10 min each on a rocker, again, protected from light. Following removal of excess liquid, slides were mounted using ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific) and incubated overnight in the dark at 2-8ºC. Sections were imaged using an EVOS FL Auto Imaging System (Thermo Fisher Scientific).

Gram-staining of murine FFPE tissues

FFPE RENCA tumour tissues, derived from mice that were IV-administered either *B. breve* UCC2003 or PBS, were cut into 4-µm sections using a HM 355 S Automatic Microtome. Each specimen was sectioned in duplicate. Sections were applied to SuperFrost Plus Adhesion slides and incubated at 37ºC overnight. The next day, sections were deparaffinised in two changes of xylene for 10 min each. Sections were then rehydrated in two changes of 100% ethanol for 5 min each, one change of 95% ethanol for 2 min, one change of 70% ethanol for 2 min, and two changes of deionised water for 2 min each. Sections were then Gram-stained and dehydrated using the Richard-Allan Scientific Gram Stain (Tissue) Kit (Thermo Fisher Scientific) per the product protocol. Slides were mounted using DPX new non-aqueous mounting medium for microscopy (Merck), and imaged using an Olympus BX51 microscope.

Determination of limits of detection of probiotic bacterial DNA against a mammalian DNA background

DNA was extracted from flash-frozen murine 4T1 tumours using the Ultra-Deep Microbiome Prep kit (Molzym) per the product protocol, with the exception that DNase treatment was excluded. Overnight cultures of wildtype EcN and BL35624 were inoculated in antibiotic-free LB and RCM, respectively, and incubated overnight at 37ºC aerobically and anaerobically, respectively. The next day, 1500 µl of both bacterial cultures were processed using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich), incorporating lysozyme from chicken egg white (Sigma-Aldrich), per the product protocol, for extraction of DNA from BL35624. DNA was eluted in 150 µl Elution Solution. Eluted mammalian and bacterial DNA were quantified using a Qubit fluorometer (Invitrogen) using the 'High Sensitivity' assay. Overnight cultures of EcN and BL35624 were spread-plated on LB agar and RCA, respectively, and incubated as above, to determine CFU counts.

4T1 tumour DNA and probiotic bacterial DNA were serially diluted in Buffer EB (Qiagen). Various quantities of probiotic bacterial DNA, from both strains, were added to various quantities of 4T1 tumour DNA, and qPCR-amplified to determine the inhibitory effect of mammalian DNA, if any, on the detectability of probiotic bacterial DNA. Additionally, the limits of detection of EcN and BL35624 DNA in patient

samples were quantified by spiking qPCR-negative patient FFPE tumour DNA extractions with various quantities of probiotic bacterial DNA and performing qPCR.

qPCR of bacterial DNA in FFPE and fresh tissues, stool samples, and spiked samples

Species-specific probe sets were designed for EcN, *B. breve* UCC2003, and BL35624 to facilitate amplification of bacterial DNA in patient and murine tissues and stool samples.

The primer and probe sets for EcN were designed to target the cryptic plasmid pMUT2 of this strain (probe 5'-/56-FAM/AGCCATCAA/ZEN/ATCGGCATCATCCTCGGT/3IABkFQ/-3', forward primer 5'- GGG CTC GGG GAT CAA ATT CAA G-3', reverse primer 5'-AAC ACT GGA ATA TGT GGC CCA AAG -3'). The set for *B. breve* UCC2003 was designed to target a gene coding for a short-chain dehydrogenase (probe 5'-/56-FAM/AGCAGTTGG/ZEN/CGAAGATCACCGA/3IABkFQ/-3', forward primer 5'-TCA TAC GGT GCC CAA AAG G-3', reverse primer 5'-GCA ATG GAT CAA GAC GTT CG-3'). The set for BL35624 was designed to target the glycosyl transferase gene of this strain (probe 5'-/56-FAM/ CGCATCATC/ZEN/CAGTCAGGGAAGGTC/3IABkFQ/-3', forward primer 5'-GTG TGA TTG TGA TTA CCG AGG A-3', reverse primer 5'-GAC AAA TGT GTG CGA GCA ATA G-3'). 20-µl dye-based qPCRs were set-up per the following recipe: 10 µl Luna Universal qPCR Master Mix (New England Biolabs), 0.5 µl forward and reverse primers (final concentration: 0.2 µM) (Integrated DNA Technologies), 7 µl Buffer EB (Qiagen), and 2 µl template DNA. 20-µl probe-based qPCRs were set-up per the following recipe: 1 µl probe mix (final concentrations: 500 nM forward and reverse primers and 250 nM probe) (Integrated DNA Technologies), 10 µl Luna Universal Probe qPCR Master Mix (New England Biolabs), 3 µl ROX reference dye (final concentration: 375 nM) (Biotium), 4 µl Buffer EB, and 2 µl template DNA. No-template controls were run in parallel with test samples by replacing 2 µl template DNA with 2 µl Buffer EB. No-amplification controls were also made per the following recipe for dye-based qPCR: 0.5 µl forward and reverse primers (final concentration 0.2 µM), 17 µl Buffer EB, and 2 µl template DNA (4 x 10^6 gene copies), and the

following recipe for probe-based qPCR: 1 µl probe mix, 3 µl ROX reference dye, 14 µl Buffer EB, and 2 µl template DNA (4 x 10^6 gene copies). Fresh and FFPE patient tissue extractions and FFPE murine tissue extractions were plated in duplicate. Human and murine stool extractions, and human and murine tissue extractions spiked with probiotic bacterial DNA, were plated in triplicate. qPCR amplification proceeded in an AriaMx (Agilent) per the following protocol: 95ºC for 1 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 30 sec.

RESULTS

1. Work-up in vitro assays and detection strategy optimisation

B. breve UCC2003 does not affect gut permeability ex vivo

The Ussing chamber study did not indicate any generic increased gut permeability due to *B. breve* UCC2003, as evidenced by a lack of HRP translocation (Figure 1).

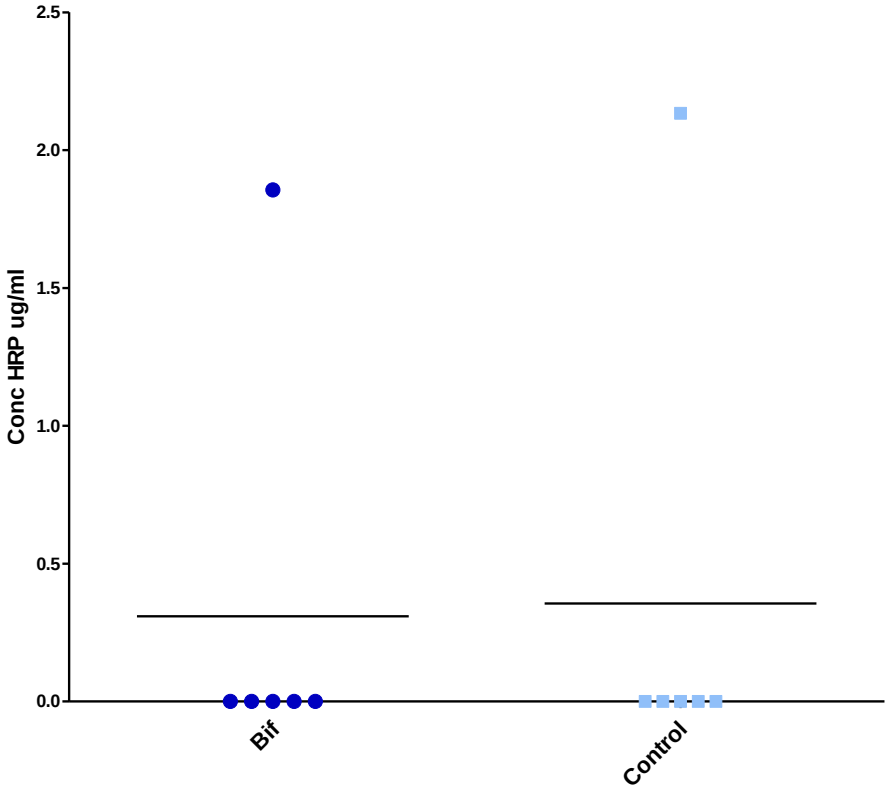


Figure 1. Effect of B. breve UCC2003 on HRP flux across the murine distal ileum and colon.

Probe-based qPCR is superior to dye-based qPCR for amplification of bacterial DNA in mammalian tissues

Probe-based and dye-based qPCR were compared to determine if there were a significant difference in amplification of bacterial DNA using either method. There was a greater-than-one log-fold difference in bacterial DNA recovery, in favour of

probe-based qPCR, compared with dye-based qPCR (Figure 2). A paired, two-tailed t-test indicated that this was a significant difference ($P = 0.032$).

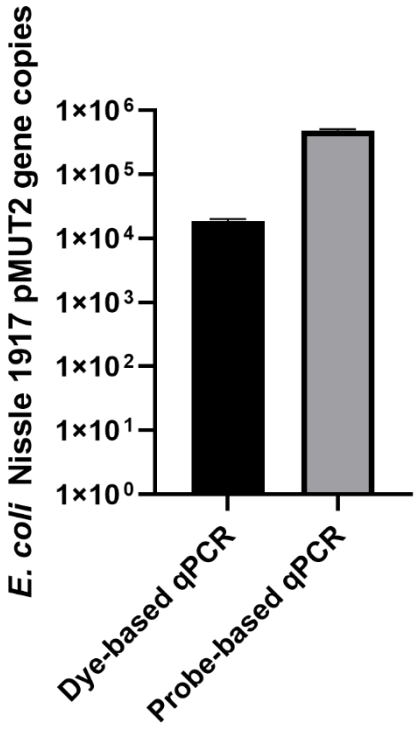


Figure 2. Amplification of DNA using dye-based and probe-based qPCR. *DNA that was isolated from a RENCA FFPE tumour, derived from a mouse administered EcN, was amplified using dye-based and probe-based qPCR. Probe-based qPCR was significantly better at amplifying bacterial DNA than dye-based qPCR (P = 0.032).*

Background mammalian DNA at relevant quantities has little effect on amplification of bacterial DNA

To determine the effects of mammalian DNA on the amplification of bacterial DNA, various concentrations of DNA extracted from fresh murine tumour tissues were spiked with various concentrations of probiotic bacterial DNA. Additionally, probiotic patients who were qPCR-negative for EcN and BL35624 were similarly spiked with probiotic bacterial DNA.

A one-way ANOVA was used to test if there were significant differences in bacterial DNA amplification across different concentrations of mammalian tumour DNA. In

the case of EcN, there were no significant differences in amplification of DNA derived from relatively high (P = 0.074), medium (P = 0.671), and low (P = 0.221) numbers of CFUs in relatively high, medium, and low concentrations of mammalian DNA (Figure 3). In the case of BL35624, there were no significant differences in amplification of DNA derived from medium (P = 0.280) and relatively low (P = 0.536) numbers of CFUs in relatively high, medium, and low concentrations of mammalian DNA (Figure 4). Furthermore, while there was a significant difference in amplification of DNA derived from relatively high numbers of BL35624 CFUs (P = 0.008) in relatively high, medium, and low concentrations of mammalian DNA, amplification was in fact greater in relatively high concentrations of mammalian DNA.

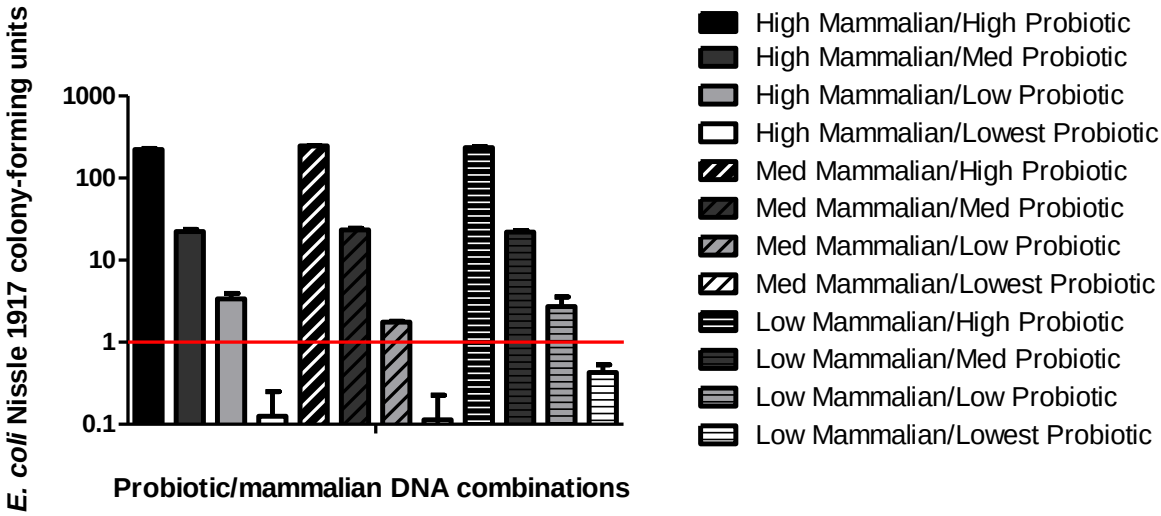


Figure 3. Effect of varying concentrations of mammalian DNA on qPCR-amplification of varying concentrations of EcN DNA. *Reactions with high, medium, and low concentrations of mammalian DNA contained 12.944 ng, 1.294 ng, and 0.130 ng mammalian DNA per 20-µl reaction, respectively. Reactions with high, medium, low, and lowest concentrations of probiotic bacterial DNA contained DNA derived from 2.34 x 10^2 CFUs, 2.34 x 10^1 CFUs, 2.34 x 10^0 CFUs, and 0 CFUs per 20-µl reaction, respectively. The limit of detection is indicated by a red, horizontal line.*

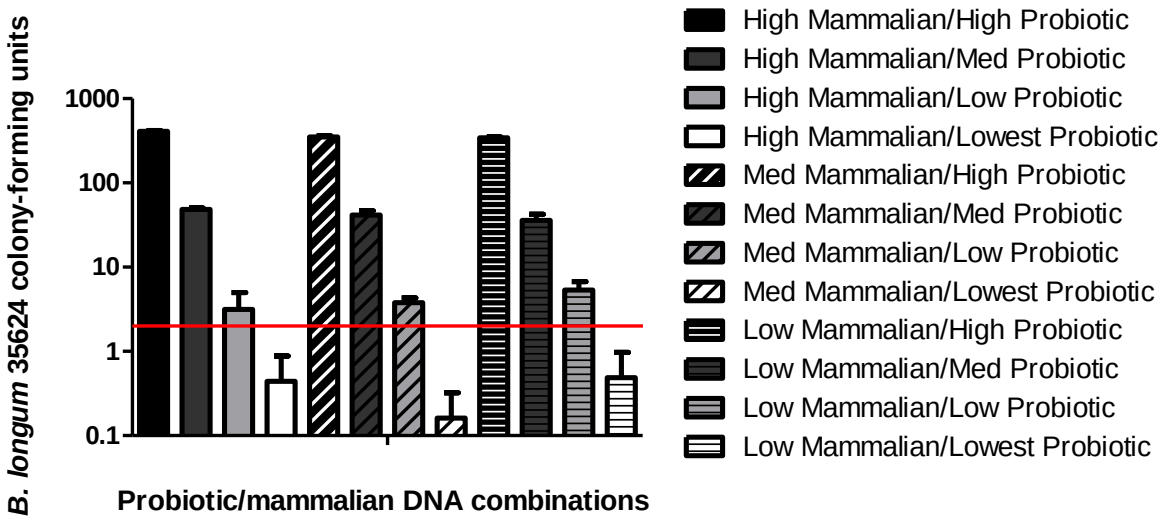


Figure 4. Effect of varying concentrations of mammalian DNA on qPCR-amplification of varying concentrations of BL35624 DNA. *Reactions with high, medium, and low concentrations of mammalian DNA contained 12.384 ng, 1.238 ng, and 0.124 ng mammalian DNA per 20-µl reaction, respectively. Reactions with high, medium, low, and lowest concentrations of probiotic bacterial DNA contained DNA derived from 3.34 x 10^2 CFUs, 3.34 x 10^1 CFUs, 3.34 x 10^0 CFUs, and 0 CFUs per 20-µl reaction, respectively. The limit of detection is indicated by a red, horizontal line.*

In terms of determining the limits of detection of bacterial DNA in patient samples, four qPCR-negative patient samples were selected such that their final concentrations were between 83.2-94.4 ng per 20-µl reaction, and samples were spiked with either EcN or BL35624 DNA. The limits of detection of EcN and BL35624 were 2.34 x 10^1 CFUs and 3.34 x 10^1 CFUs, respectively.

Proprietary probiotic capsules have consistent bacterial quantities

Mutaflor capsules, stored in accordance with manufacturer's instructions, had low capsule-to-capsule variability (Figure 5). Between 1.5 x 10^9 CFUs – 5 x 10^9 CFUs were recovered per refrigerated capsule. A paired, two-tailed t-test indicated a significant difference between the bacterial viabilities of capsules stored under

refrigerated and ambient conditions ($P = 0.001$) due to a 4-log reduction in EcN colony counts after 60 days of room-temperature storage.

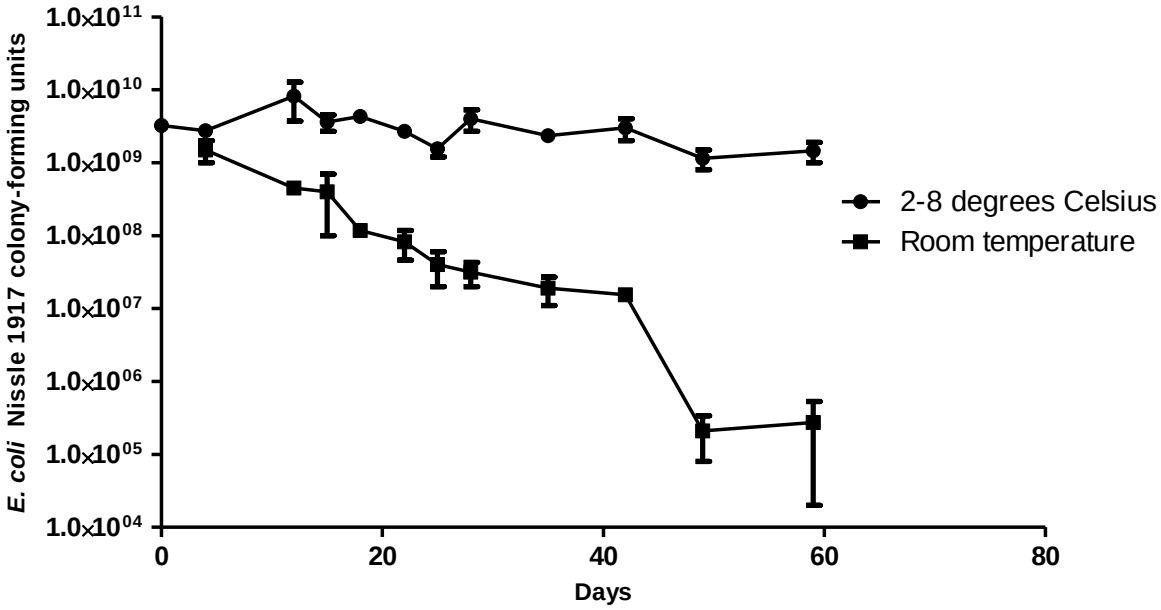


Figure 5. Quality control testing and viability of the proprietary probiotic product 'Mutaflor'. *Capsule-to-capsule variability is conveyed by CFUs cultured from 24 refrigerated 'Mutaflor' capsules over approximately two months. The effect of ambient storage on 22 capsules over the same period is also shown in terms of CFUs. Error bars represent the standard error of the mean.*

CFUs cultured from Alflorex capsules were slightly lower than the 1 x 10^9 CFUs claimed to be contained within each capsule at manufacture, where between 3 x 10^8 to 8 x 10^8 CFUs were recovered from each capsule over the 34 days on which culture was performed (Figure 6). No downward trend in CFUs cultured from each capsule over time was observed.

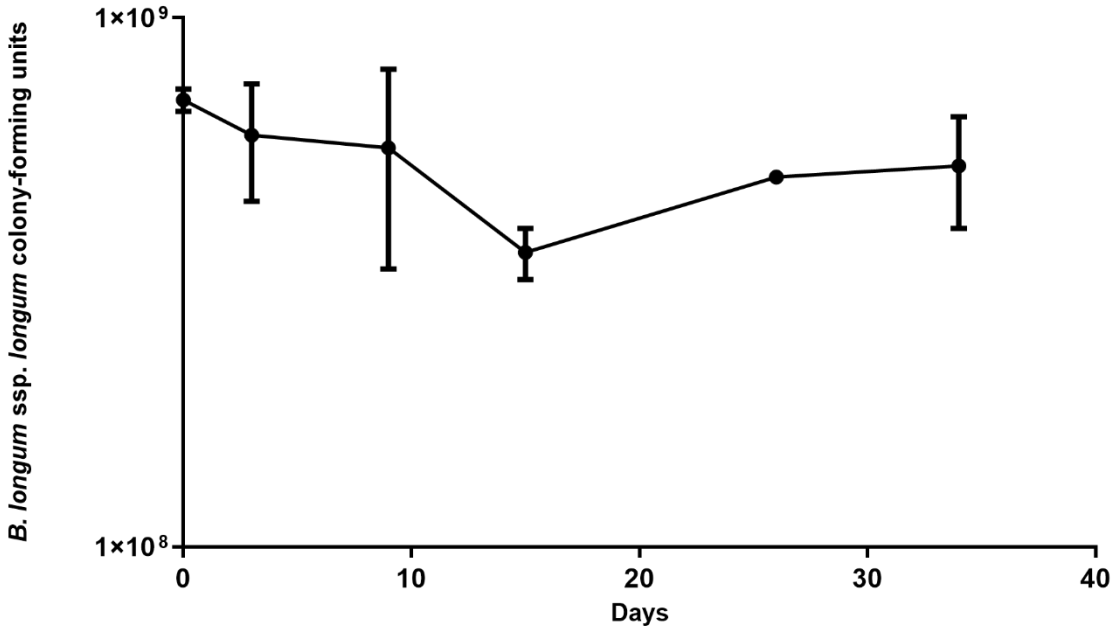


Figure 6. Quality control testing and viability of the proprietary probiotic product 'Alflorex'. *Capsule-to-capsule variability is conveyed by CFUs cultured from 12 'Alflorex' capsules stored ambiently over approximately one month. Error bars represent the standard error of the mean.*

2. Clinical study

qPCR for test bacteria in patient tumour tissue

To determine whether probiotic bacteria ingested by cancer patients resulted in translocation of the bacteria from the GIT to tumour tissue, FFPE and fresh tumour tissues from these patients were analysed by test-bacterium-specific qPCR. 9 lung cancer patients took Alflorex capsules for 38 days on average (maximum 69 days, minimum 20 days) and 19 lung cancer patients took Mutaflor capsules for 46 days on average (maximum 90 days, minimum 19 days). Five renal cancer patients took Alflorex capsules for 30 days on average (maximum 53 days, minimum 18 days) and four renal cancer patients took Mutaflor capsules for 59 days on average (maximum 84 days, minimum 21 days).

One lung cancer patient provided with Alflorex capsules, five lung cancer patients provided with Mutaflor capsules, and two renal cancer patients provided with Mutaflor

capsules were not supplied with enough capsules to allow them to take one capsule daily between enrolment in the study and their surgery, assuming perfect compliance with the dosing regimen. There was no evidence of gut translocation of BL35624 and EcN in human subjects, as evidenced by qPCR-negative fresh and FFPE tissues. Limits of detection for FFPE tissue DNA extractions were based on calculations from published data (Flores Bueso et al., 2020). As EcN contains approximately 8 copies of the pMUT2 plasmid per cell, a limit of detection of 5.6×10^2 gene copies means that the limit of detection in terms of bacterial cells was 70. Similarly, as the copy number of the glycosyl transferase gene in BL35624 is four, a limit of detection of 2.36×10^2 gene copies means that the limit of detection in terms of bacterial cells was 59.

Persistence in human stools varies between bacterial species

Stool samples were collected from patients where feasible = 8 patients. Probe-based qPCR of patient stool samples demonstrated EcN was detectable in 1/4 patients and BL35624 was detectable in all 4 patients, as evidenced by qPCR-positive stool samples analysed (Figure 7). This higher detection rate of BL35624 compared with EcN resembles the higher rate observed in this strain in murine stool samples.

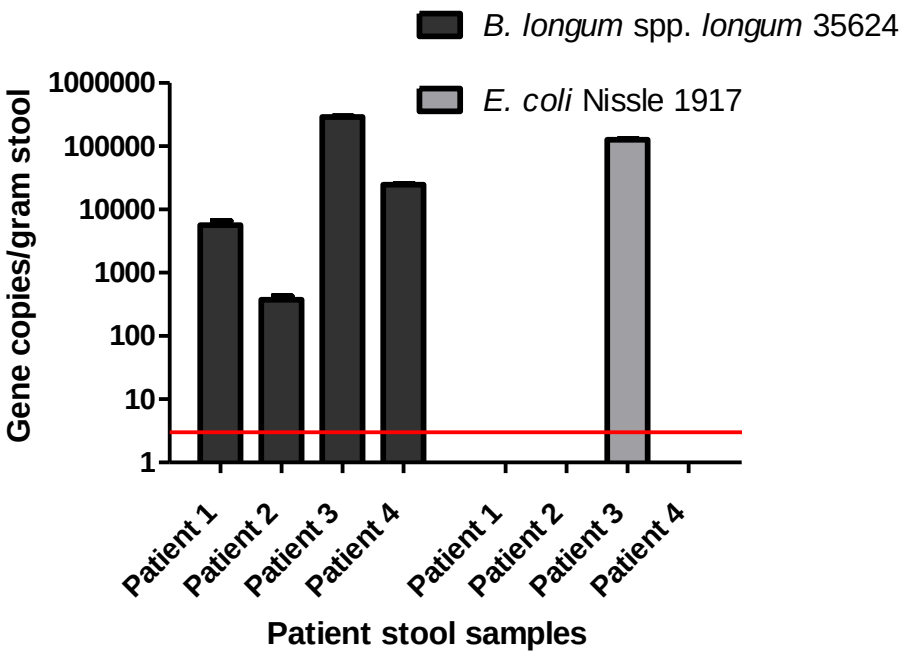


Figure 7. Recovery of BL35624 and EcN from stools of renal and lung cancer patients. *Gene copy numbers were quantified by qPCR of purified DNA extractions of human stools. Stool sample weight range =75.8 mg – 245.9 mg; median weight = 238.9 mg. The limit of detection is indicated by a red, horizontal line.*

3. Post-clinical study murine studies

Murine model of oral administration of test bacteria to tumour-bearing mice

To validate the results of previously published murine studies that showed that probiotic bacteria administered *per os* resulted in subcutaneous tumour colonisation,

tumour-bearing and tumour-free mice were gavaged with *B. breve* UCC2003, BL35624 or EcN and their tumours cultured 7-11 days post-administration.

Fresh tumours of mice, gavaged with *B. breve* UCC2003, BL35624, and EcN that were cultured on selective agars were culture-negative. Fresh livers were also culture-negative for *B. breve* UCC2003 and BL35624; however, small numbers of EcN colonies (< 25 CFUs) were cultured from the liver of one gavaged mouse; this mouse was not pre-treated with streptomycin and was the only mouse examined the stool of which was qPCR-positive for EcN (see below).

Murine GIT colonisation by probiotic bacteria reflects pattern observed in patients

Murine GIT target bacteria gene copy numbers were variable, with varying detection rates between *B. breve* UCC2003 (Figure 8), BL35624 (Figure 9), and EcN (Figure 10), as evidenced by qPCR of murine stools. Mice gavaged with both bifidobacterial species had higher frequencies of qPCR-positive stool samples than those gavaged with EcN, with 3/7 mice and 4/6 mice containing detectable levels of *B. breve* UCC2003 and BL35624, respectively, while only 1/6 mice had detectable levels of EcN. The lone stool that was qPCR-positive for EcN came from a mouse the liver of which was colonised by EcN (see above). Of the two mice the stools of which were qPCR-negative for BL35624, one was pre-treated with streptomycin, and one was not. The stool that was qPCR-positive for EcN was sampled from a mouse that was not pre-treated with streptomycin. The gene copy numbers recovered also varied between bacterial species: For qPCR-positive stools, average gene copy numbers recovered for BL35624, *B. breve* UCC2003, and EcN were 28042 copies, 1567 copies, and 439 copies, respectively, per gram of stool.

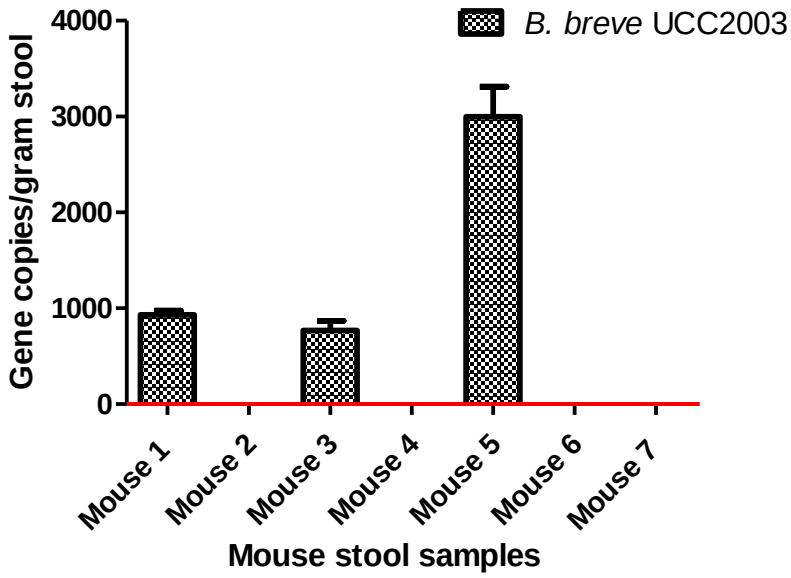


Figure 8. Recovery of B. breve UCC2003 gene copies from stools of gavaged mice. *Gene copy numbers were quantified by qPCR of purified DNA extractions of murine stools. All mice were provided with water supplemented with 5 mg/ml streptomycin ad libitum for one week, prior to gavage. Stool sample weight range = 18.7 mg – 37.3 mg; median weight = 20.7 mg. The limit of detection is indicated by a red, horizontal line. Stools were collected 11 days after initial gavage. All mice were tumour-bearing, except Mice #6 and #7.*

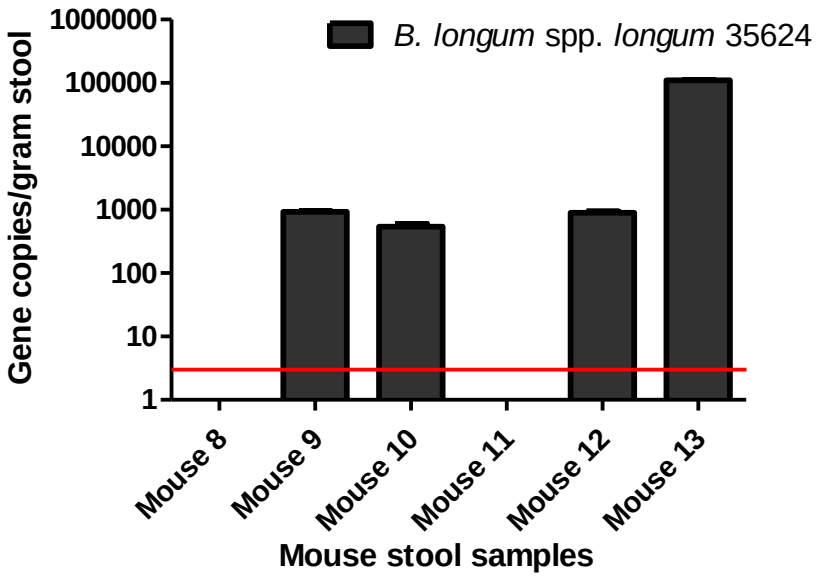


Figure 9. Recovery of BL35624 gene copies from stools of gavaged mice. *Gene copy numbers were quantified by qPCR of purified DNA extractions of murine stools. All mice, except Mouse #8, were provided with water supplemented with 5 mg/ml streptomycin ad libitum for one week, prior to gavage. Mouse #8 was provided with potable water ad libitum. Stool sample weight range = 20.4 mg – 36.3 mg; median weight = 24.25 mg. The limit of detection is indicated by a red, horizontal line. Stools were collected 11 days after initial gavage, except for Mouse #8, the stool of which was collected 10 days after initial gavage. All mice were tumour-bearing, except Mice #12 and #13.*

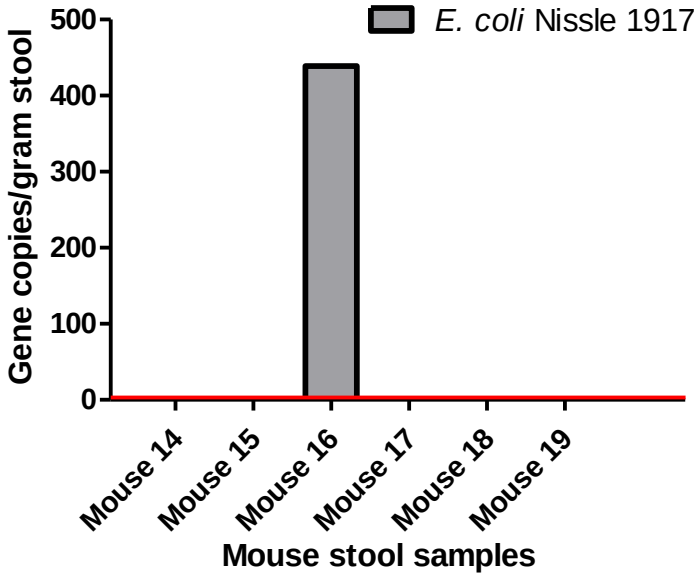


Figure 10. Recovery of EcN gene copies from stools of gavaged mice. *Gene copy numbers were quantified by qPCR of purified DNA extractions of murine stools. Mice #17, #18, and #19 were provided with water supplemented with 5 mg/ml streptomycin ad libitum for one week, prior to gavage. Mice #14, #15, and #16 were provided with potable water ad libitum. Stool sample weight range =11.6 mg – 47.8 mg; median weight= 30.6 mg. The limit of detection is indicated by a red, horizontal line. The stool for all mice were collected 11 days after initial gavage, except for Mouse #17, the stool of which was collected 10 days after initial gavage. All mice were tumour-bearing, except Mice #15 and #16.*

Murine model of IV administration of test bacteria to tumour-bearing mice

To validate the methodology used for detection of test bacteria *in vivo*, FFPE tumour and liver tissues, obtained from mice that were IV-administered these bacteria, were processed to extract bacterial DNA, followed by qPCR.

Bacterial DNA is amplifiable in murine FFPE tissue blocks with low limits of detection

DNA from EcN and *B. breve* UCC2003 was amplifiable from murine FFPE tumour tissues taken from mice that were IV-administered these strains (Figure 11 and Figure

12). EcN was also detectable in murine livers. The highest gene copy numbers recovered for both EcN and *B. breve* UCC2003 were obtained from murine specimens that were culture-positive for the same species. No CFUs were isolated from tumour and liver tissues taken from tumour-free mice nor from mice administered PBS.

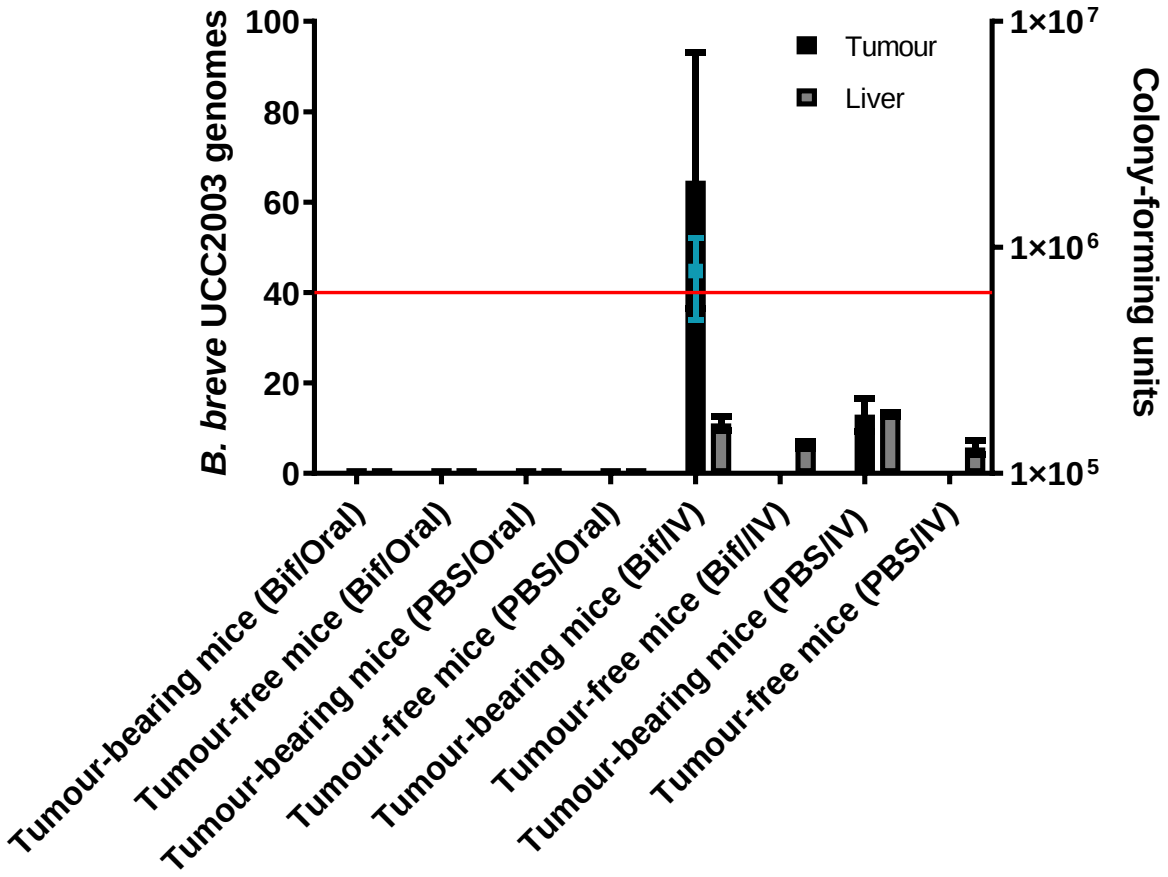


Figure 11. Recovery of B. breve UCC2003 from murine RENCA tumour and liver FFPE tissues retrieved from mice IV-administered either B. breve UCC2003 or PBS. *Gene copy numbers were quantified by qPCR of purified DNA extractions of murine FFPE tissues (black and grey bars, left y-axis). CFUs isolated from fresh RENCA tumour and liver tissues deriving from the same mice are superimposed over the bars (blue squares, right y-axis). The limit of detection for gene copies is indicated by a red, horizontal line.*

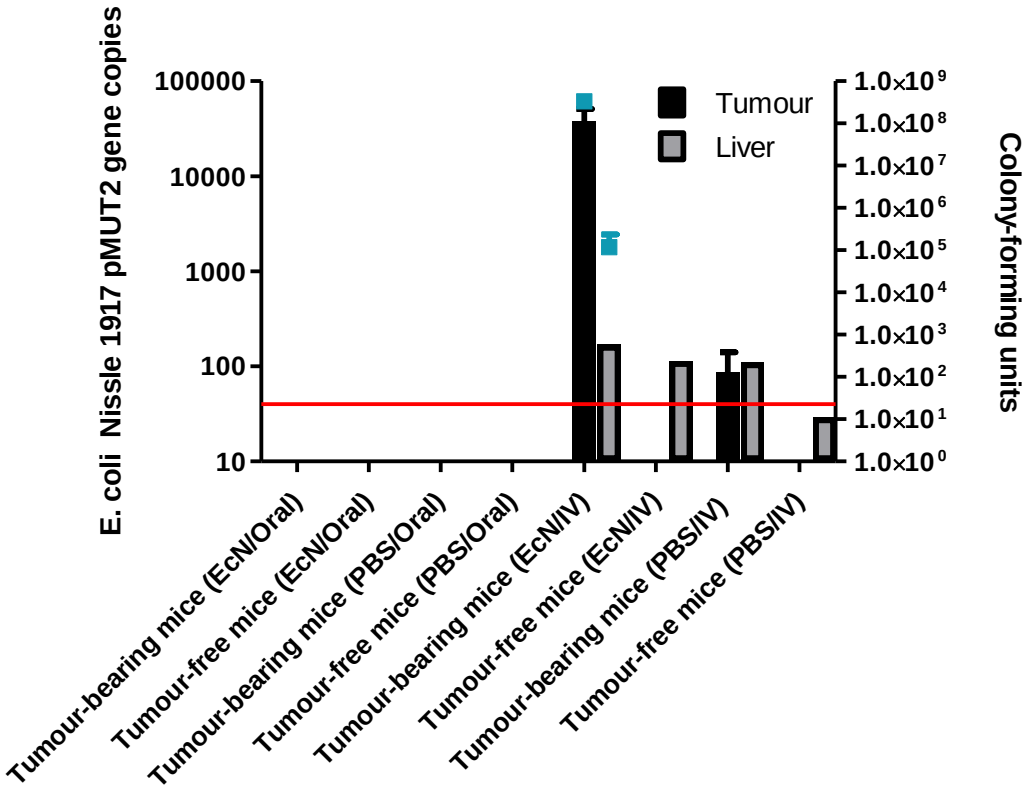


Figure 12. Recovery of EcN from murine RENCA tumour and liver FFPE tissues retrieved from mice IV-administered either EcN or PBS. *Gene copy numbers were quantified by qPCR of purified DNA extractions of murine FFPE tissues (black and grey bars, left y-axis). CFUs isolated from fresh RENCA tumour and liver tissues deriving from the same mice are superimposed over the bars (blue squares, right y-axis). The limit of detection for gene copies is indicated by a red, horizontal line.*

IV-administered probiotic bacterial species are visualisable in murine FFPE tumour sections

To provide further confirmation that probiotic bacteria IV-administered to tumour-bearing mice were indeed present within their tumour tissues, FFPE RENCA tumours obtained from these mice were Gram-stained (for visualisation of *B. breve* UCC2003) or IF-stained (for visualisation of EcN).

EcN and *B. breve* UCC2003 were observable in murine FFPE tumours, retrieved from mice that were IV-administered these bacteria, using IF staining (Figure 13) and Gram staining (Figure 14), respectively. No immunofluorescence was seen in murine tumours that derived from mice that were IV-administered PBS, nor in murine tumours that derived from mice that were IV-administered EcN from which treatment with primary antibody was withheld. Similarly, *B. breve* UCC2003 was not observable in murine tumours that derived from mice that were IV-administered PBS. Tissues in which bacteria were detected by either staining method were devoid of bacteria across large areas of each section; instead, bacteria were concentrated in pockets scattered sparsely across each tumour. Both bacteria invariably occupied spaces between mammalian cells, rather than appearing to localise within them.

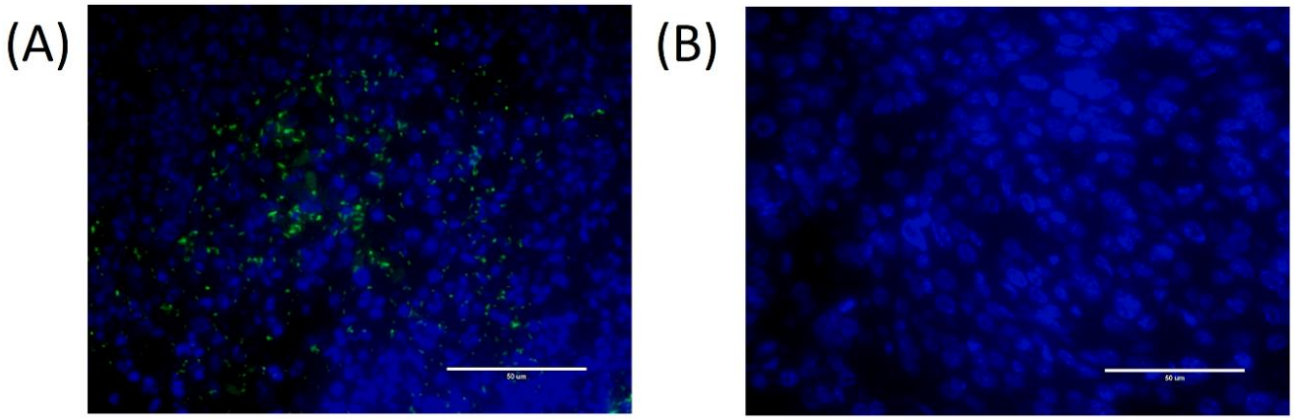


Figure 13. IF staining of FFPE RENCA tumour tissues derived from mice IV-administered EcN. *Murine RENCA cells are stained with DAPI (blue); EcN cells are stained with an anti-E. coli antibody (green). Tissues were stained with either (A) primary antibody or (B) blocking buffer.*

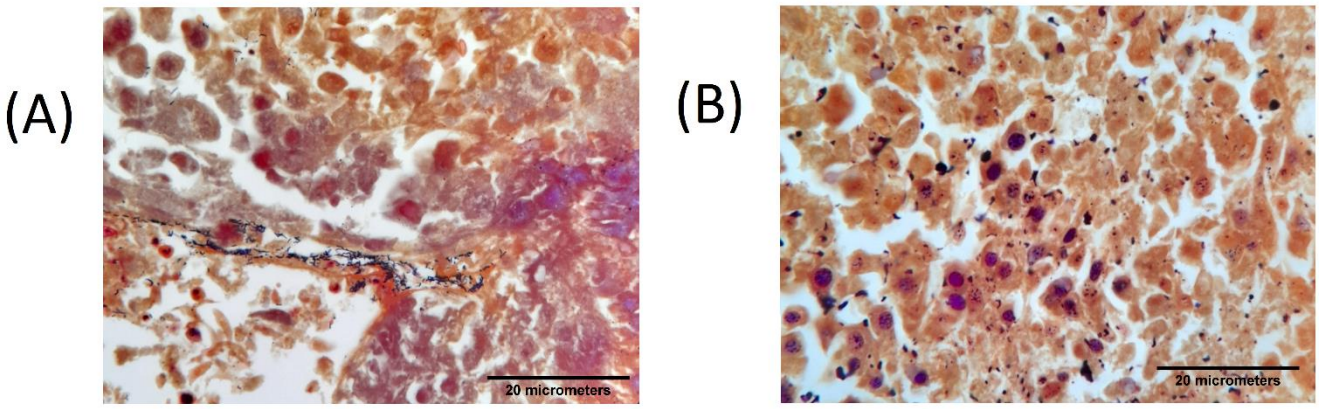


Figure 14. Gram-staining of FFPE RENCA tumour tissues derived from mice IV-administered either (A) B. breve UCC2003 or (B) PBS. *Murine RENCA tissues are stained with tartrazine (yellow); B. breve UCC2003 cells are stained with crystal violet (blue); elastic fibres are stained black.*

DISCUSSION

The results of oral probiotic administration to humans mirror those of the murine gavage experiment in this chapter, with respect to GIT-to-tumour translocation, in terms of the absence of detectable test bacteria in tumour samples examined. Murine data differ from results obtained in similarly structured murine studies, in which mice gavaged with *B. breve* UCC2003 yielded tumours that were culture-positive for this strain (Cronin et al., 2010a), with similar findings in another study using EcN (Danino et al., 2015). Murine tumour cell lines in this prior study and the current study differed; however, this is likely not the source of variability in gut translocation ability of administered bacteria, given that oral translocation to murine tumours was demonstrated in multiple subcutaneous tumour cell lines in the past, including B16 melanoma cells and Lewis lung carcinoma cells.

A key difference between the current and previous mouse studies is the SPF status of the mice. Unlike the older studies, SPF mice were utilised in the present study. The impact on bacterial gut translocation imposed by a potentially inflamed GIT, caused by non-excluded pathogens, is feasible. Increased permeability of the GIT, for instance, may facilitate bacterial movement across the gastrointestinal barrier in such circumstances. Concerns about viral and bacterial pathogens confounding preclinical data interpretation have led to the increasing elimination of “dirty” mice, that have foregone screening for specific pathogens, from preclinical research (Ericsson et al., 2017). The SPF mice used in the present study were screened for microbes that are known pro-inflammatory pathogens of the murine gut, such as *Salmonella* spp., *Helicobacter* spp., and *Clostridium piliforme* (Baker, 1998). The inclusion of non-SPF mice for comparative purposes in the present study was not possible.

There are concerns that a preoccupation with using the “cleanest” mice in preclinical research may confound data interpretation in other ways, such as the non-uniform exclusion of pathogens across different producers, which may cause inconsistencies in the animals' gastrointestinal physiology, among other issues (Ericsson et al., 2017). The elimination of certain bacteria from mice, for which evidence of adverse health consequences is limited, such as *Pseudomonas aeruginosa*, reinforces these concerns.

It has been shown that translocation by multiple species of probiotic bifidobacteria in SPF mice (including *B. longum*) does not appear to occur, even when administered

oral doses are high (approximately 1 x 10^{12} CFUs/kg/day for 7 days) (Abe et al., 2010). Conversely, intragastric administration of *B. longum* to germfree mice, at nearly a four-log-fold decrease in dose, does result in translocation (Yamazaki et al., 1985a), perhaps indicating the influence of microbial populations, resident in the gut, on this phenomenon. Additionally, head-to-head comparisons of SPF mice and gnotobiotic mice (including germfree mice) have shown that translocation of *E. coli* is inhibited in SPF mice following intragastric administration of this species, while translocation in gnotobiotes is sustained (Berg and Garlington, 1979). These comparisons have shown also that gnotobiotic mice, that have been inoculated with the entire caecal microflora of SPF mice, do in fact experience bacterial translocation, which may suggest that the microbiota of SPF mice exert inhibitory effects on translocation (e.g., priming the mouse's immune system) only after a relatively long time '*in situ*'. Therefore, this inhibition may not be instantly reversible upon elimination of the microbiota (as was attempted in this study using streptomycin). It may be the case that SPF mice are at a halfway point between the permissive effects of inflammation and immunological naivete, to which “dirty” and germfree mice are prone, respectively, that gives SPF mice a more formidable barrier against translocation. More recent studies of bacterial translocation in SPF and gnotobiotic mice have shown that there exist differences between different *E. coli* strains that affect their ability to translocate *in vivo* (Gronbach et al., 2010). EcN, for example, was only able to translocate in germfree mice that were immunocompromised, and was unable to translocate in immunocompromised SPF mice, suggesting that there may be a multiplicity of factors, on the part of the host and the bacterium, that govern the probability of translocation occurring.

Other authors have reported varying degrees of success in achieving tumour colonisation in mice, following bacterial GIT translocation. The oral administration of EcN, for instance, was shown to result in the colonisation of murine hepatic metastases, but did not result in the successful colonisation of subcutaneous tumours across multiple cell lines using the same bacterial dose (Danino et al., 2015). Thus, while gut microbiota composition may play some role in initialising bacterial gut translocation and setting limits on its efficiency, the proximity of the organ (to which bacteria are to translocate) to the GIT may also be significant. This point is supported by the low-level recovery of EcN from the liver of one gavaged SPF mouse in the

present study. Non-recovery of bifidobacteria from the livers of gavaged SPF mice is remarkable, given that *B. longum* in fact has demonstrated the ability to translocate to extraintestinal sites of germ-free mice, including the liver, following oral administration and sustain colonisation at these sites, albeit transiently (Yamazaki et al., 1985b), perhaps indicating that the germfree/SPF nature of the mouse has a greater influence on gut translocation than organ proximity to the GIT. It may be the case that bifidobacterial translocation efficiency is in fact higher than that of *E. coli*; however, its long-term survival in hepatic tissues may be compromised by its stringent metabolic requirements as an obligate anaerobe, that are not a concern with *E. coli*.

Concerning the detection of target bacterial DNA in murine stool samples, variability was observed between mice orally administered the same microbe, as well as between mice administered different microbes. It is known that long-term GIT colonisation by exogenously administered bacteria is likely dependent on a range of individualistic, host-specific factors like the presence of related and unrelated taxa in the gut, or the availability of a niche (such as with bifidobacterial), as well as taxon-specific factors (Maldonado-Gomez et al., 2016), which may account for the variability seen in the present study. For depletion of naturally resident murine microflora, a subset of mice was administered streptomycin prior to bacterial gavage. This has been found to be particularly conducive to the gut colonisation of *E. coli* (Spees et al., 2013), although the lone mouse, the stool of which was qPCR-positive for EcN, was not provided with streptomycin-supplemented water. While acknowledging the limited number of patients from whom stool samples were obtained, the resemblance of their stool qPCR data to those of the mice suggests that compliance with the probiotic regimen was acceptable among this selection.

A further reassuring aspect of this study was the reproducibility of the probiotic capsules examined, in terms of the CFUs cultured from them over time. The high-quality control that was observed for their manufacture was fortunate, given the concerns expressed by other authors regarding inconsistencies between labelled quantities and actual quantities (Kolacek et al., 2017). The slight decrease in CFUs retrieved from Alflorex capsules, compared with the numbers purported to be present within the capsules at manufacture, may be due to a slight decrease in viability over time following manufacture, or may genuinely reflect the original numbers included

in each capsule at manufacture. This decrease is unlikely to have affected translocation in patients taking this probiotic.

The integrity of the patient data in this study - especially those originating from FFPE tissues, which were the primary human specimen type analysed - depends on the validity of the analytical techniques employed, as verified by testing in murine FFPE tissues that were confirmed to be colonised by the bacteria of interest, via staining techniques, prior to downstream analyses. Although molecular analyses of FFPE tissues are typically cautioned due to the effects of formalin fixation on DNA quality, it has been shown that FFPE specimens can yield precise data, even when compared with their same-patient, fresh-tissue counterparts, despite discrepancies in quality between the two specimen formats (Matsukuma et al., 2006). Moreover, the impact of DNA damage on downstream analyses is minimalised if primer design is appropriate (Hall et al., 2001). Accordingly, primers in this study were designed such that qPCR amplicons were no more than 170 bp. Probe-based amplification was also chosen, given the greater sensitivity of probe-based detection of low-copy-number templates, compared with other methods, such as dye-based amplification (Yin et al., 2001). Comparative qPCRs on murine FFPE tissue extractions resulted in marked inhibition of bacterial DNA amplification when the DNA-binding dye SYBR Green I was used, compared with a 5' nuclease probe, presumably due to non-specific intercalation of the dye to the mostly-mammalian DNA template.

The ability of bacteria to translocate to extraintestinal body sites is evident, but the implications of this for tumour colonisation in both humans and mice are ambiguous due to variability observed between different organisms and tumour types. The fact that even IV doses of bacteria that are as high as 1×10^4 CFUs fail to colonise subcutaneous tumours (Danino et al., 2015) implies that the low levels of systemic bacteria that might result from oral administration would be inadequate to colonise this type of tumour. While orally administrable, genetically modifiable bacteria may have some utility as tumour-targeting anticancer agents, their applicational potential is probably limited to specific cancer types, particularly those more proximal to the gut.

CONCLUSION

While bacterial colonisation of tumours in cancer patients via oral administration remains a possibility, the phenomenon is, at best, limited to tumour types or bacterial species that remain unexplored. The IV route, therefore, remains for now the most faithful route of administration for bacterial tumour targeting studies in the clinic.

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Chapter 5 - Tumour-directed evolution of bacteria

ABSTRACT

Background: Advancements have led to appreciable deployment of bacteria in oncology patient trials, but a lack of breakthrough on this front, so far, calls for a reassessment of the aspects of this technology that may cap its potential. Among these are the bacteria themselves, which are often predefined strains that are selected based on their historical use or convenience. In place of this approach, it may be possible instead to first tailor bacterial qualities in a manner that optimises their selectivity for tumours post-administration and accentuates their growth within this type of environment. The precise mechanism behind bacterial survival and replication within the complex tumour environment is poorly understood.

Aim: The aims of this study were to obtain bacterial strains with improved intratumoural growth and to examine bacterial properties involved in this phenomenon.

Materials and Methods: Two probiotic bacterial strains were intravenously administered separately to tumour-bearing mice, subsequently recovered from their tumours, and re-administered to tumour-bearing mice three more times. These "recycled" strains were compared with their parental counterparts by examining i) growth patterns, ii) metabolic indicators and iii) whole-genome sequences.

Results: Significant changes in intratumoural bacterial numbers were observed *in vivo* between parental and tumour-recycled EcN 13 dpi ($P < 0.0001$). Bacterial persistence of recycled strains was improved over their unrecycled variants, especially at later stages of tumour colonisation. An increase in tumour persistence was correlated with a decrease in tumour selectivity in tumour-recycled EcN during earlier stages of tumour colonisation compared with its parental strain, but not in later stages.

In vitro, several shifts in growth dynamics and metabolic activity occurred in one of two recycled bacterial strains compared with its corresponding parental strain. Carbon utilisation was altered across several organic compounds, and changes in biofilm formation were observed. These changes occurred despite no overt changes being evident at the DNA level.

Conclusion: These findings suggest that bacteria that are repeatedly exposed to the tumour environment experience changes that may impact on their growth characteristics.

INTRODUCTION

Many genera of bacteria have been shown to preferentially accrue within tumours post-administration, and some have been investigated as anticancer agents, including *Salmonella, Escherichia, Clostridium, Bifidobacterium*, and *Listeria* (Cummins and Tangney, 2013). Tumours are tissues that are notorious for their irregularity and heterogeneity (Meacham and Morrison, 2013), but they are purported to have several core features that give them a distinct relationship with bacteria and allow them to sustain bacterial reproduction *in vivo*, such as the availability of favourable nutrition and oxygen levels, while extra-tumoural bacterial growth is curtailed (Lehouritis et al., 2017). However, conclusive evidence that connects all these factors with tumour-selective bacterial growth is still lacking, and thus the precise reasons for why bacteria possess this aptitude may in fact be more complex than what is currently supposed. Nevertheless, bacteria are identified as potential next-generation agents for tumour-selective drug delivery and have been deployed clinically in cancer patients, with mixed results (Nemunaitis et al., 2003, Roberts et al., 2014, Toso et al., 2002).

There have been attempts already to genetically amend bacteria so their anti-tumour activity is accentuated by, for example, engineering them to express anticancer compounds only when exposed to the hypoxia that is characteristic of tumours (Ryan et al., 2009, Mansour et al., 2020). Additionally, efforts to improve the functionality of bacteria as tumour-targeting agents include attenuating bacterial virulence to limit the persistence of bacteria in healthy tissues (Frahm et al., 2015) as well as generating auxotrophic mutant strains that require tumour-specific nutrients for survival (Hoffman, 2011). However, even when these measures are taken, the long-term efficacy of bacterial anticancer treatment is not assured (Li et al., 2013). Thus, more extensive adaptation of bacteria may be required before they can become fit for long-term anticancer regimens.

Bacteria have long been a cornerstone of biopharmaceutical production, permitting, for example, the manufacture of significant quantities of recombinant proteins for medical applications (Overton, 2014). Their heavy use in this field has incentivised approaches to enhance bacterial production efficiency, such as by directed evolution. Directed evolution is, by definition, concerned with guiding proteins toward a point of improved activity (Turner, 2009), but there exist also approaches that aim to

understand how the bacteria themselves might be tailored more holistically. This concept of classical strain improvement can be accomplished by encouraging mutation in a population and screening for the desired mutant (Stroman et al., 2018). Alternatively, molecular techniques like genome shuffling can produce libraries of recombinant bacteria more rapidly and with greater diversity than what natural evolutionary processes might allow (Biot-Pelletier and Martin, 2014). In the case of both classical strain improvement and genome shuffling, there is often a clear phenotypic, metabolic, and/or functional change that is desirable in the bacteria, such as enhancing survival at a lower pH to facilitate fermentation (Patnaik et al., 2002). Therefore, the methodologies employed are often applicable only in settings that are relatively simple to understand, such as in a bioreactor, where processing conditions and their effects on the bacteria are usually known (Berlec and Strukelj, 2013). Conversely, when an optimised bacterial strain is required for use in a more complex, heterogeneous system (such as tumours), a different approach may be necessary.

There is a perception that, despite the capacity for targeted colonisation of tumours by bacteria, clinical studies of bacterial tumour targeting would benefit from the use of bacteria with an improved targeting capacity over those currently available. This invites consideration of how classical strain improvement might be applied to bacteria to enhance their activity with respect to the complex intratumoural environment, including honing their ability to find tumours in the body and proliferate within them. Rather than approach this through hypothesis and deliberate genetic modification of bacterial genes, the approach was taken to allow the tumour environment to produce selective pressure on introduced bacteria to direct selection of spontaneous mutants with optimised growth in the tumour environment.

This chapter investigates bacterial functions involved in tumour colonisation, for two purposes; firstly, to obtain a better understanding of the phenomenon, and, secondly, to obtain strains with improved targeting capabilities for future utilisation in bacterial anticancer therapy. There is evidence that a single passage of auxotrophic *Salmonella enterica* serovar Typhimurium through murine tumours increases its adherence to tumour cells *in vitro*, and that this may be responsible for improved anti-tumour efficacy *in vivo* (Zhao et al., 2006). A more recent study has shown that a single passage of *S. enterica* serovar Typhimurium causes some phenotypic changes, such as reductions in motility (Choe et al., 2014). It is known that evolutionary change is

accelerated in plants and animals with faster generation times, through mutation and phenotypic selection, and it has been demonstrated only recently that this applies to bacteria also (Weller and Wu, 2015). To date, intratumoural growth dynamics of tumour-recycled bacteria have not been investigated, and an evidence-based explanation of why bacterial strains passaged through murine tumours experience apparent changes in their affinity for tumour cells has not been provided. Additionally, different bacteria ought to be examined to ascertain whether the effects of tumour passaging vary across genera.

As a wide range of tumour types serve as permissive environments for a wide range of bacteria (Yu et al., 2008), continual re-exposure of bacteria to such conditions could instil progressive changes, both generally and by adapting bacteria more strongly to tumour-selective growth *in vivo*. In this study, the probiotic bacteria EcN and *Bifidobacterium breve* UCC2003 were administered intravenously to tumour-bearing mice and thereafter underwent several cycles of recovery from and re-administration to murine tumours. EcN was chosen due to its high tumour-targeting ability that has been demonstrated across numerous studies (Cronin et al., 2012, Stritzker et al., 2007), and *B. breve* UCC2003 was added due to its contrasting cellular and metabolic characteristics compared with EcN – *B. breve* UCC2003 being a Gram-positive obligate anaerobe (Ruiz et al., 2017) and EcN being a Gram-negative facultative anaerobe (Revelles et al., 2013) – which, it was speculated, would capture a wider variety of changes at the genotypic and phenotypic level as a result of their exposure to disparate intratumoural conditions, which would affect colonisation patterns. Recycled strains were compared with their corresponding parental strain across several parameters, including their ability to form biofilms. Trends in bacterial growth and metabolic activity were also compared between recycled and parental strains, and WGS was performed.

MATERIALS AND METHODS

Bacterial and mammalian cell culture

EcN, containing the integrated p16S*lux* plasmid (Riedel et al., 2007), was grown routinely in LB medium (Sigma-Aldrich) and M9 medium containing 0.4% glucose (Sigma-Aldrich) at 37°C in a shaking incubator (Labwit) at 200 rpm. Where noted, Em (Sigma-Aldrich) was added to LB medium at a concentration of 300 μg/ml. *B. breve* UCC2003, containing the pLuxMC3 plasmid, was grown in RCM (Oxoid) containing 4 μg/ml Cm (Sigma-Aldrich) statically and anaerobically at 37°C for 24 h. The strain was then sub-cultured on two consecutive days at 5% into MRS (Oxoid) containing 4 μg/ml Cm and 0.05% cys-HCl (Sigma-Aldrich), and incubated as above.

CT26 murine colon carcinoma cells were maintained in RPMI medium (Sigma-Aldrich) containing 10% FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37°C in a Thermo Forma Series II water-jacketed incubator (Thermo Fisher Scientific) in a humidified atmosphere of 5% CO_2. Cell counts were performed using a NucleoCounter (Chemometec).

Animals and tumour induction

All animal experiments were approved by the Health Products Regulatory Authority (Dublin, Ireland) and the Animal Experimentation Ethics Committee of University College Cork (Cork, Ireland). Mice were kept at a constant room temperature (22°C) with a natural day/night light cycle in a conventional animal colony. Standard mouse chow and potable water were provided *ad libitum*. Tumours were induced in healthy, 6-8-week-old, female BALB/c mice (Envigo) by subcutaneous injection of 5×10^4 CT26 cells, suspended in 200 μl serum-free and antibiotic-free RPMI medium, into the right flank. Tumour induction was performed with a 23-gauge needle (BD Microlance 3, Becton-Dickinson). Tumours were measured once daily with a Vernier caliper. Tumour volume was approximated by measuring tumours at their longest diameter, and at the diameter perpendicular to this.

Bacterial administration to mice

Overnight cultures of parental and tumour-recycled EcN were adjusted to an OD_{600} of 0.05 in 10 ml fresh LBEm300 medium and incubated with agitation at 37°C until an OD_{600} of 0.7 (approximately 1 x 10^7 CFUs/ml) was reached. Then, cultures were centrifuged at 3220 x *g* for 10 min, the supernatant removed, and cells washed twice in PBS (Sigma-Aldrich). Parental and tumour-recycled *B. breve* UCC2003 cultures were centrifuged similarly at 3220 x *g* for 10 min, followed by two wash steps using PBS containing 0.05% cys-HCl.

When murine tumours reached a volume of approximately 100 mm^3, mice were randomly divided into experimental groups and administered 1 x 10^6 CFUs of either EcN or *B. breve* UCC2003 in a 100-µl volume via injection of the lateral tail vein. The viable count of each inoculum was determined by retrospective plating onto LB agar containing 300 µg/ml Em for EcN, or RCA (Oxoid) containing 4 µg/ml Cm for *B. breve* UCC2003. LB agar plates were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. RCA plates were incubated anaerobically in a sealed BD GasPak EZ container (Becton-Dickinson) by adding an AnaeroGen 2.5-l sachet (Thermo Scientific). RCA plates were incubated at 37°C for up to 48 h.

Bacterial recovery from murine tumour and liver tissues

Animals were euthanised by cervical dislocation, following percussive blow to the head. Tumours and livers were removed aseptically and homogenised using a sterile plunger removed from a 10-ml syringe (Syringe Without Needle, Terumo). Homogenised tissue was pushed through the 70-µm pores of a nylon cell strainer (Corning). Cell strainers were washed with PBS (for tissues derived from mice administered EcN) or PBS containing 0.05% cys-HCl (for tissues derived from mice administered *B. breve* UCC2003). Bacteria were recovered following serial dilution of tissues and plating onto LB agar containing 300 µg/ml Em (for tissues derived from mice administered EcN) or RCA containing mupirocin (50 mg/l) (Sigma-Aldrich) (for tissues derived from mice administered *B. breve* UCC2003). LB agar plates were incubated overnight as above, and RCA plates were incubated for up to 48 h as above. Tumour-recovered bacteria were designated “R1”, “R2”, etc. according to the round of serial passage from which they were recovered, e.g., “EcN R4” refers to an EcN

isolate recovered from the fourth round of serial passage.

Identification of bacteria recovered from murine tissues

Following each round of serial passage through, and isolation from, tumours, bacterial isolates were PCR-verified using primers targeting the pMUT2 plasmid of EcN (forward primer 5'-GTA GCA GTC ATG CAG CTC TCG C-3' and reverse primer 5'-GAA TTT GAT CCC CGA GCC CTG AAG-3') and the 16S rRNA gene of *B. breve* UCC2003 (forward primer 5'-TAA GCG ATG GAC TTT CAC ACC-3' and reverse primer 5'-GGG TGG TAA TGC CGG ATG-3'). Individual colonies were first homogenised in 50 µl sterile water or PBS (containing 0.05% cys-HCl for isolates derived from tissues of mice administered *B. breve* UCC2003) and 1 µl of this was used as DNA template. Other reaction components were as follows: 12.5 µl Taq 2X Master Mix (New England Biolabs), 0.5 µl forward primer and 0.5 µl reverse primer (final concentration of both primers: 0.2 µM), and 10.5 µl nuclease-free water (Qiagen). PCRs containing primers for amplification of EcN DNA proceeded via an initial denaturation step of 95°C for 5 min, followed by 29 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 15 sec, followed by a final extension step of 72°C for 7 min. PCRs containing primers for amplification of *B. breve* UCC2003 DNA proceeded via an initial denaturation step of 95°C for 5 min, followed by 29 cycles of 95°C for 30 sec, 48°C for 30 sec, 72°C for 30 sec, followed by a final extension step of 72°C for 7 min. Genomic DNA, extracted from the parental strains of EcN and *B. breve* UCC2003 using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich), was added to separate PCR mixtures as positive controls. Sterile water was added to PCR mixtures in place of template DNA, as a negative control. PCR products were analysed on a 1% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen) and visualised on a Gel Doc EZ System (Bio-Rad) using Image Lab software. PCR amplicons were 138 bp and 441 bp, for EcN and *B. breve* UCC2003, respectively.

Whole-genome sequencing and bioinformatic analysis

RNA-free DNA was extracted from cultures of parental and recycled strains of EcN and *B. breve* UCC2003 using a GenElute Bacterial Genomic DNA kit. For EcN, an individual colony of the parental strain, and an individual colony recovered following

the 4th round of serial passage (R4) through murine tumours, were used to inoculate overnight cultures in LBEm300, from which DNA was isolated. This was similarly conducted *B. breve* UCC2003, with the additional step of first homogenising colonies in suitable media to facilitate dispersion of bacteria (*B. breve* produce a protective capsular polysaccharide), followed by inoculation of the parental and R4 isolates into RCM containing 4 μg/ml Cm for 24 h. Isolated DNA was tested for concentration and purity using a Nanodrop ND-1000 UV-Vis Spectrophotometer (Mason Technology). Only DNA with $A_{260/280}$ greater than 1.8 was deemed suitable for sequencing. The genome sequence of each strain was determined by GeneWiz (Germany).

HiSeq reads were filtered to ensure that only high-quality bases (Phred score > 30) were analysed, to minimise the risk of false-positive variants. Trimmomatic (v0.38) was used to remove all reads shorter than 60 bp, to reduce the likelihood of interference from contaminant bacterial genomes and to trim reads when the average per-base quality in a sliding window of size 4 dropped below 30. The average read length was 150 bp. The Burrows Wheeler alignment tool 'BWA-MEM' (v0.7.17) was used, as this is recommended when read length exceeds 70 bp. Default settings were used, except that alignments with a minimum score of 0 were allowed, rather than the default minimum score of 30. Given the stringent parameters used for read length and quality filtering, relaxing the minimum alignment score gave a greater possibility of detecting variants. All sequences were aligned with the original reference genomes.

Variant calling was performed with BCF tools, using the BCF call function. Variants were filtered using the norm and filter functions. Variants were removed when read depth fell below 10, when quality fell below 40, or when the variant identified was not supported by both the forward and reverse read of a read pair. Variant number was then normalised between samples based on the read coverage in the initial alignment BAM file. Using the Picard tool within the Genome Analysis Toolkit suite, reads were down-sampled to ensure that the SNP:coverage ratio remained constant when coverage was reduced to the lowest level present in samples.

Biofilm formation assessment using crystal violet staining and population assays

Examination of biofilm formation via crystal violet staining was conducted by inoculating overnight cultures of the EcN parental strain and three individual EcN R4 isolates routinely in LBEm300. The OD_{600} of overnight cultures was adjusted to 0.05 the following day in fresh LBem300 or M9G. Cultures were dispensed in 2-ml aliquots into individual 15-ml polypropylene tubes. Three technical replicates were aliquoted per strain examined. Cultures were incubated statically at 37°C for 24 h. After this, liquid media was removed and tubes were washed twice with PBS, to remove loosely attached cells. 2.5 ml 0.1% crystal violet were added to each tube and cells were stained at room temperature for 30 min. The crystal violet was decanted, and tubes were washed twice in a basin of dH_2O. All tubes were then inverted, patted on paper towels to drain excess liquid, and allowed to dry overnight. The next day, 1 ml 99% ethanol was added to each tube to solubilise the crystal violet. Individual samples were transferred to cuvettes and OD_{590} values were measured using a SpectraMax M2 microplate reader (Molecular Devices).

For biofilm population assessment, the method described above was followed up to and including the PBS wash steps, excepting that overnight cultures were inoculated into M9G only. 1 ml PBS was then added to each polypropylene tube and a sterile pipette tip was used to scrape the attached cells from the inside walls of the tube at the 2-ml mark. The PBS was used to wash scraped cells from the walls. Samples were then vortexed vigorously, to facilitate dispersion of attached cells, and serially diluted. Dilutions were plated on LB agar containing 300 µg/ml Em and plates were incubated overnight at 37°C in a 5% CO_2 incubator.

Biolog EcoPlate assay

EcN parental and pooled EcN R4 recycled isolates were grown overnight in LBEm300. The following day, the bacteria were diluted 1 in 100 in fresh LB and grown for 6 h. Bacteria were washed twice in PBS and diluted to OD_{600} 0.8. 100 µl were added to EcoPlates (Biolog) and incubated at 37°C overnight. Plates were read at OD_{590} every 24 h for 144 h using a SpectraMax M2 microplate reader.

Formalin-fixed tissue processing, embedding, and IF staining

Six murine tumours (three per mouse administered parental EcN, and three per mouse administered EcN R4) were formalin-fixed and paraffin-embedded for IF staining. 8 x 11" Formalin Neutraliser Pads (CellPath) were used whenever formalin was being handled. Formalin-fixed murine tissues were processed using a LOGOS J Hybrid Tissue Processor (Milestone). Specimens were placed between two biopsy pads (Kaltek) inside a histology cassette. The following processing conditions were used: flushing for 25 min at 37ºC, rinsing (reagent #1) for 45 min at 37ºC, rinsing (reagent #2) for 25 min at 37ºC, ethanol for 45 min at 37ºC, xylene (Ocon Chemicals) for 35 min at 37ºC, isopropanol (EMD Millipore) for 50 min (30 min at 37ºC, then 20 min at 45ºC), and wax (CellWax Plus, CellPath) impregnation for 5 h at 60ºC. Following this, tissues were embedded in paraffin wax using a Tissue-Tek TEC (Sakura).

FFPE tissues were sectioned using a HM 355 S Automatic Microtome (Thermo Fisher Scientific). 4.5-µm sections were retrieved in duplicate for each specimen. Sections were applied to SuperFrost Plus Adhesion slides (Thermo Fisher Scientific) and incubated at 37ºC overnight. The next day, sections were deparaffinised in two changes of xylene for 10 min each. Sections were then rehydrated in two changes of 100% ethanol for 10 min each, followed by two changes of 95% ethanol for 3 min each, followed by one change of 80% ethanol for 3 min, followed by 2 changes of PBS plus 0.1% Tween 20 (Sigma-Aldrich) and 0.05% Saponin from quillaja bark (Sigma-Aldrich) for 3 min each. Borders were then drawn around each section using an ImmEdge Pen (Vector Laboratories).

Sections were blocked with PBS-TS plus 10% normal donkey serum (Sigma-Aldrich) and placed into a humidifying chamber for 30 min. Blocking buffer was then removed and sections were incubated with an anti-*E. coli* antibody (Abcam), diluted 1:50 in blocking buffer, in a humidifying chamber for 1 h (sections serving as a negative control for this step were incubated with blocking buffer alone). After removal of the primary antibody/blocking buffer, sections were washed twice using PBS-TS for 10 min each on a shaking platform (Shake *'n' Stack Hybridization Oven, Thermo Fisher Scientific)*. PBS-TS was then removed, and sections were incubated with a donkey anti-rabbit IgG Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch), diluted 1:100 in blocking buffer, in a humidifying chamber for 1 h; slides were

protected from the dark using tin foil. After removal of the secondary antibody, sections were washed twice using PBS-TS for 10 min each on a rocker, again, protected from light. Following removal of excess liquid, slides were mounted using ProLong Diamond Antifade Mountant (Thermo Fisher Scientific) and incubated overnight in the dark at 2-8^{o}C. Sections were imaged using an EVOS FL Auto Imaging System (Thermo Fisher Scientific).

RESULTS

Repeated bacterial cycling through tumours can affect trends in intratumoural bacterial quantity and persistence

Unpaired, two-tailed t-tests were used to determine if statistically significant differences existed between CFU numbers recovered from murine tumours after administration of parental and recycled bacterial strains. At all timepoints examined, there was a trend in increased recovery of EcN from murine tumours when recycled strains were administered, compared with administration of the corresponding parental strain (Figure 1). There was an increase in recovery of EcN R2 from murine tumours 7 dpi compared with EcN R1 with, on average, 8.0×10^8 CFUs and 8.6×10^7 CFUs recovered respectively per gram of tumour tissue (Figure 2). This increase was not observed between EcN R2 and EcN R3, nor between EcN R3 and EcN R4, but there was a sustained increase in recovery, over EcN R1, of all variants cycled through tumours two times or more at 7 dpi, with 5.9×10^8 CFUs and 5.3×10^8 CFUs recovered, per gram of tumour tissue on average, for EcN R3 and EcN R4, respectively. This increase was statistically significant between EcN R1 and EcN R3 CFUs ($P = 0.015$) and between EcN R1 and EcN R4 CFUs ($P = 0.013$), but not between EcN R1 and EcN R2 CFUs ($P = 0.091$). The difference in mean EcN R3 CFUs recovered from tumours between 3-10 dpi, compared with EcN R1, was not statistically significant ($P = 0.083$). Similarly, the difference in mean EcN R4 CFUs recovered from tumours between 7-13 dpi, compared with EcN R1, was determined not to be statistically significant ($P = 0.137$). However, EcN R4 sustained significantly greater intratumoural growth than EcN R1 on day 13 alone, with 4.7×10^8 CFUs and 4.5×10^3 CFUs recovered respectively per gram of tumour tissue at this timepoint ($P < 0.0001$).

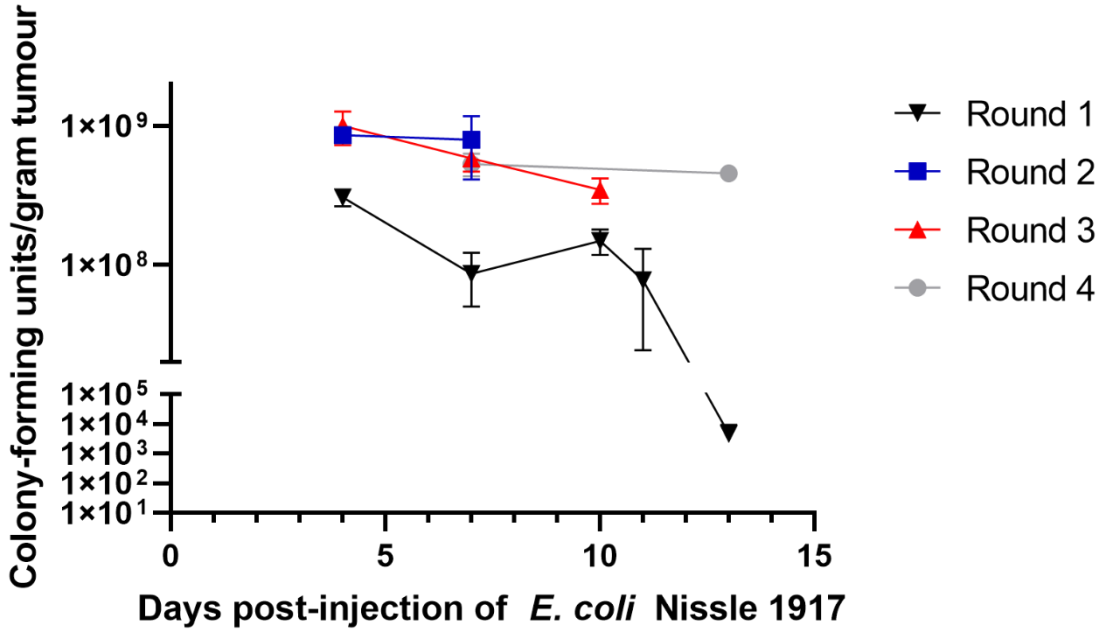


Figure 1. Trend in recovery of parental and tumour-recycled EcN from murine tumour tissue over time. *CFUs were recovered from murine CT26 tumours up to 13 dpi. The recycled strains were cycled through mice and recovered from their tumours between one time to four times. Each data point represents the mean CFUs derived from three murine tumours, except for EcN R2, for which the value at day 4 is derived from one murine tumour and the value at day 7 is derived from two murine tumours. Error bars represent the standard error of the mean.*

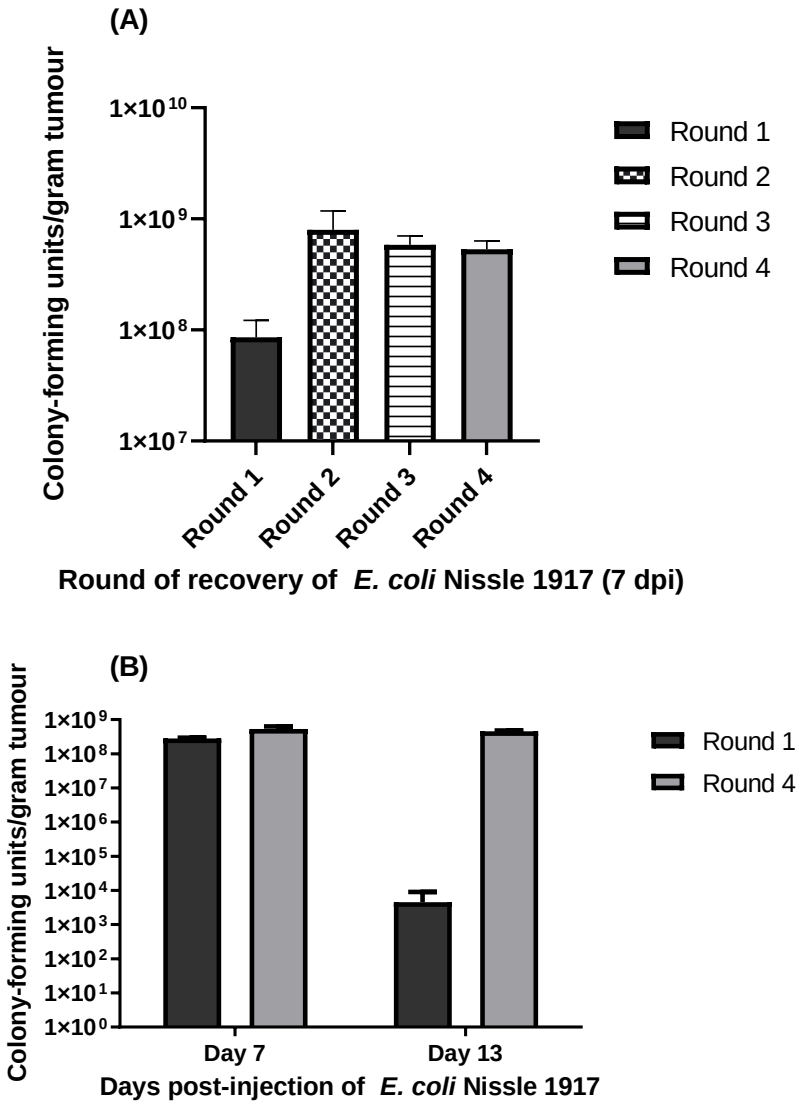


Figure 2. Recovery from murine tumours of tumour-recycled EcN and its parental (unrecycled) strain between 7-13 dpi of bacteria. *The recycled strains were cycled through mice and recovered from their tumours between one time to four times. Recovery of all strains (R1-R4) at 7 dpi* ***(A)*** *and recovery of EcN R1 and R4 at 7 dpi and 13 dpi* ***(B)*** *are illustrated. Each data point represents the mean CFUs derived from three murine tumours, except for EcN R2, the value of which is derived from two murine tumours. Error bars represent the standard error of the mean.*

A trend of increased intratumoural bacterial growth was not evident in *B. breve* UCC2003 when later-phase tumour-recycled variants were compared with *B. breve* UCC2003 R1 (Figure 3). There was no significant difference in tumour-recovered

CFUs of R1 and R4 variants between 7-14 dpi (P = 0.681) (Figure 4). Moreover, although R4 variants were able to sustain higher levels of intratumoural growth than R1 variants during the more advanced stages of tumour colonisation, given an average recovery of 4.5 x 10^5 CFUs and 4.9 x 10^4 CFUs respectively per gram of tumour tissue at 14 dpi, only one of three tumours was culture-positive for R4 variants, while two of three tumours were culture-positive for R1 variants. The difference was also not statistically significant (P = 0.424). This pattern was reversed in EcN, in which three of three tumours were culture-positive for R4 variants, while only one of three tumours was culture-positive for R1 variants at 13 dpi.

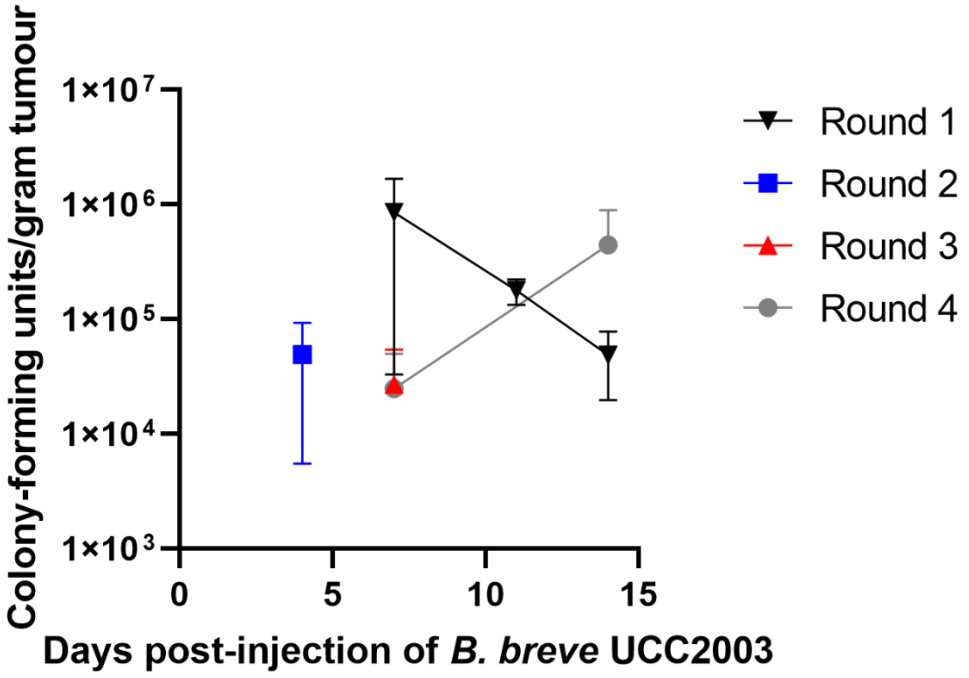


Figure 3. Trend in recovery of parental and tumour-recycled B. breve UCC2003 from murine tumour tissue over time. *CFUs were recovered from murine CT26 tumours up to 14 dpi. The recycled strains were cycled through mice and recovered from their tumours between one time to four times. Each data point represents the mean CFUs derived from three murine tumours, except for EcN R2, for which the value is derived from two murine tumours. Error bars represent the standard error of the mean.*

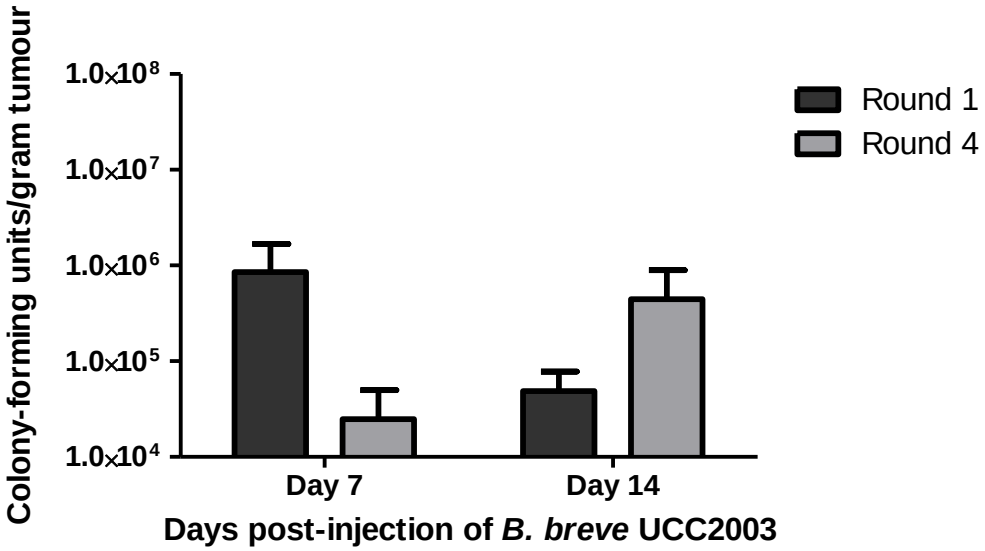


Figure 4. Recovery from murine tumours of tumour-recycled B. breve UCC2003 and its parental (unrecycled) strain between 7-14 dpi of bacteria. *The recycled strains were previously cycled through mice and recovered from their tumours either one time (Round 1) or four times (Round 4). Each data point represents the mean CFUs derived from three murine tumours. Error bars represent the standard error of the mean.*

The livers of most mice that were administered parental and recycled EcN strains (EcN R1-R4 inclusive) were culture-negative for these strains (27/36), and the livers of all mice that were administered parental and recycled *B. breve* UCC2003 strains were culture-negative. For mice that were administered EcN R1 and EcN R4, from which livers were cultured at 7 and 13 dpi (three livers per strain, per timepoint), only two livers were culture-positive (Figure 5). These were recovered at 7 dpi from mice that were administered EcN R4. The tumour-to-liver ratio for these mice was 4.59 x 10^5:1 CFUs. Two livers, processed between 4-13 dpi, were culture-positive for mice administered EcN R1. There was over a four-log-fold difference in CFUs recovered between these days, with lower recovery on day 13.

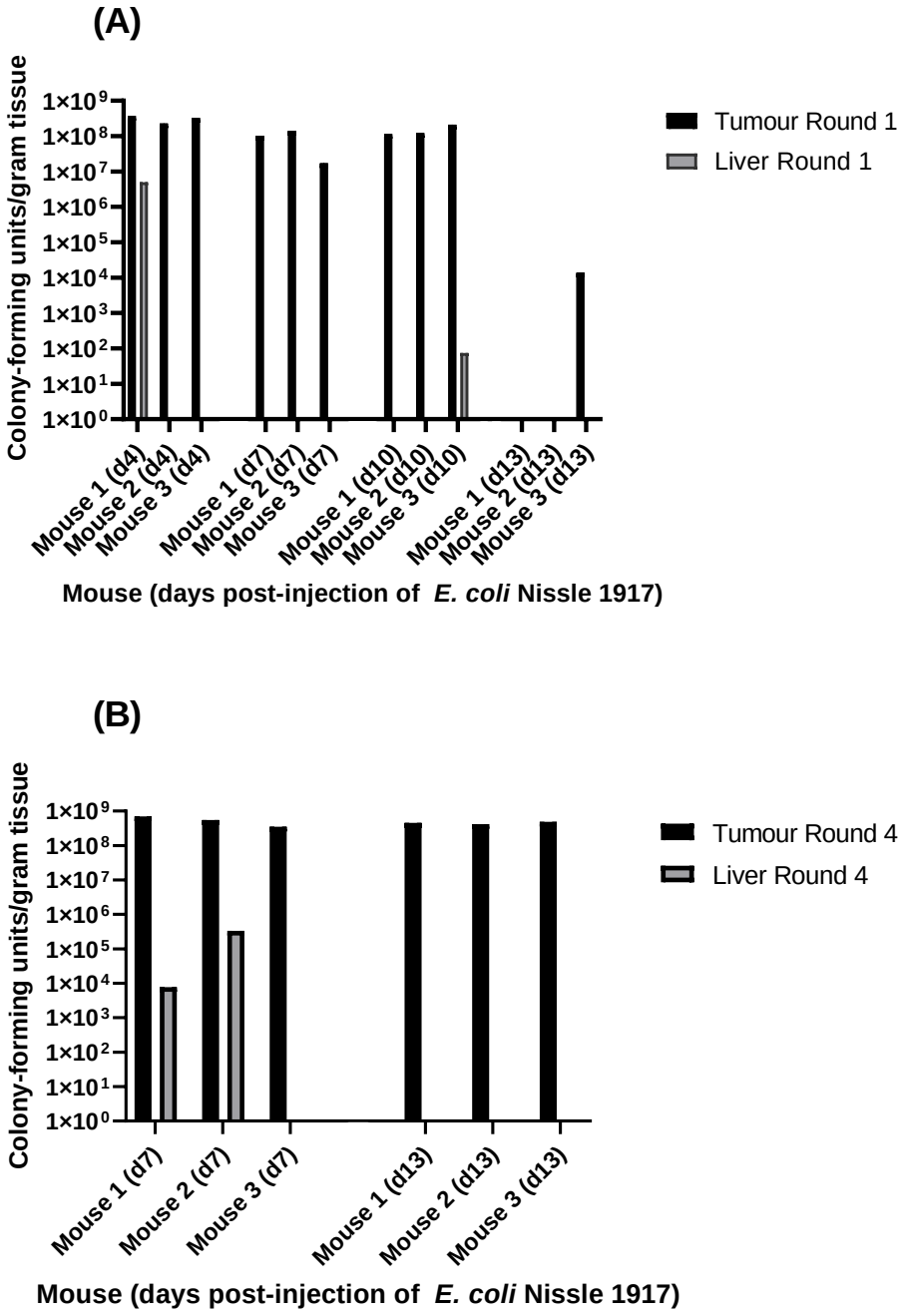


Figure 5. Bacterial colonisation by EcN R1 and EcN R4 in paired murine tumour and liver tissues. *Data points represent absolute numbers of CFUs derived from individual CT26 tumour and liver specimens. Graphs show CFUs recovered for EcN R1 between 4-13 dpi* ***(A)*** *and CFUs recovered for EcN R4 between 7-13 dpi* ***(B)****.*

IF staining indicates no differences in intratumoural growth patterns between EcN parental and recycled strains

Large areas of FFPE murine tissues were negative for the presence of both parental and recycled EcN. In areas where there was colonisation, no significant differences were noted in terms of the density or the pattern of colonisation (Figure 6).

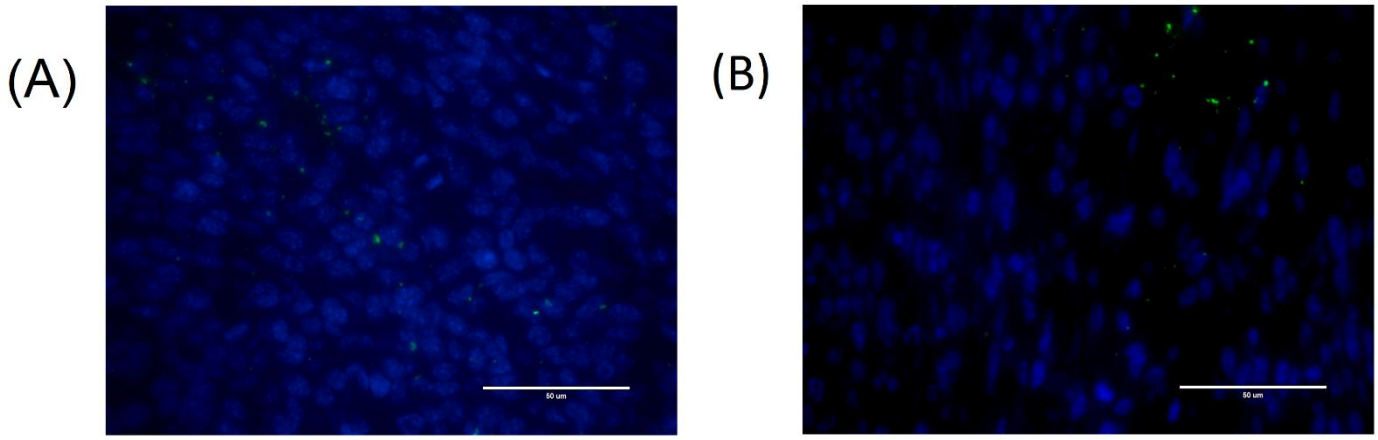


Figure 6. IF staining of parental (A) and R4 (B) EcN in FFPE murine CT26 tumour tissues. *CT26 cells are stained with DAPI (blue) and EcN are stained with an anti-E. coli antibody (green).*

In vitro biofilm formation is significantly affected in tumour-recycled bacteria

An ANOVA calculated that there was a statistically significant difference between the mean OD_{590} readings obtained from crystal violet staining of biofilms of EcN parental and R4 strains grown in LB and M9G media (P = 0.001) (Figure 7). A Bonferroni post-hoc test was subsequently performed and calculated significant differences between parental EcN vs. EcN R4 (both cultured in M9G media) (**), between EcN R4 (cultured in LB media) vs. EcN R4 (cultured in M9G media) (*), and between parental EcN (cultured in LB media) vs. EcN R4 (cultured in M9G media) (**). The non-detection of significance between parental EcN and EcN R4 cultured in LB media corroborates an earlier finding that minimal media (i.e., M9G) facilitates the formation of robust biofilms that is not permitted in nutrient-rich media (i.e., LB) (Fang et al., 2018), thereby assisting in comparative analyses of inter-strain biofilm production. Additionally, biofilm populations of tumour-recycled EcN contained nearly a one-log-

fold increase in CFUs compared with parental EcN biofilms, with an average CFU count of 4.54 x 10^7 CFUs/ml vs. 5.32 x 10^6 CFUs/ml, respectively (Figure 8). These changes in biofilm formation were unaccompanied by changes in growth kinetics (Figure 9).

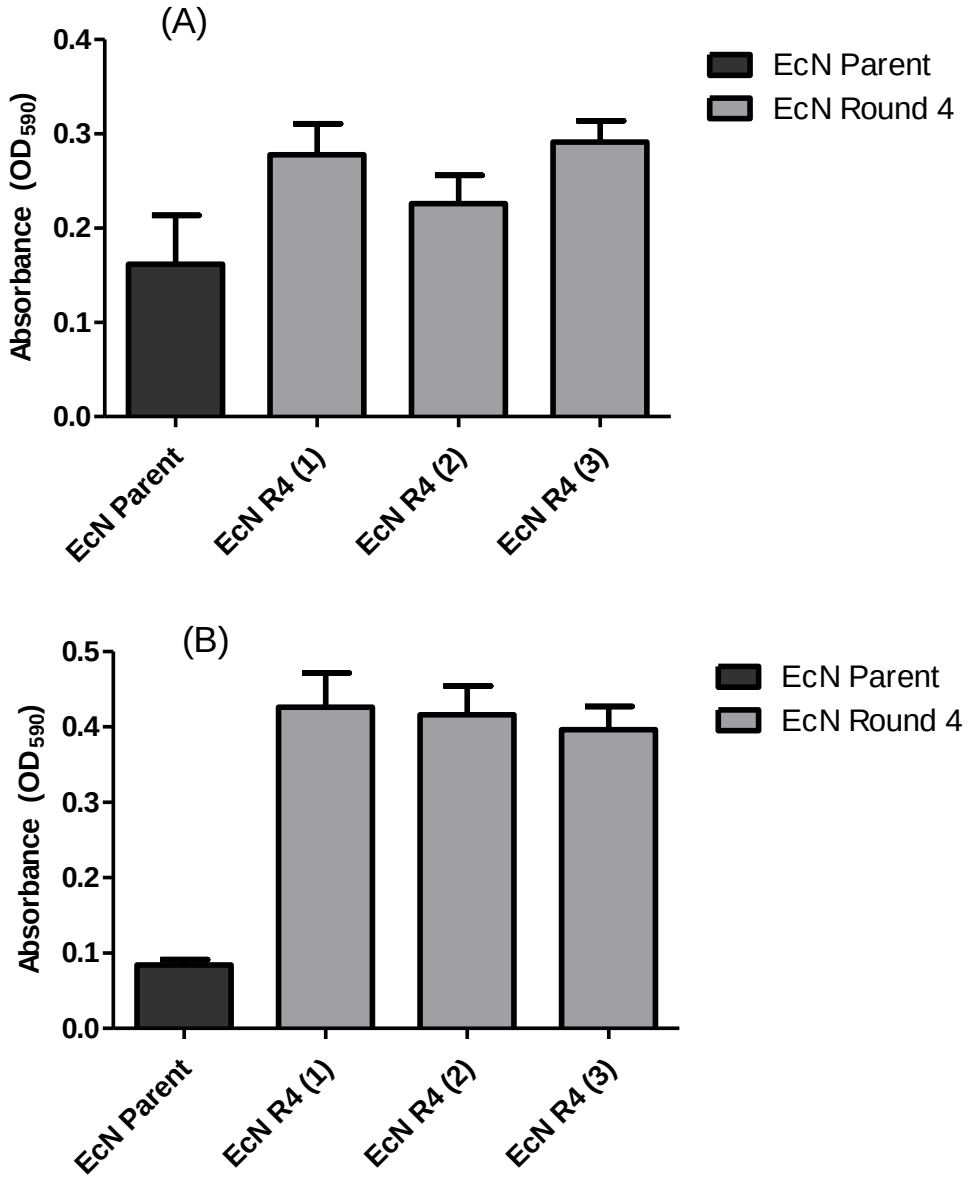


Figure 7. Biofilm-formation capacity of tumour-recycled EcN, compared with its parental (unrecycled) strain. *Biofilms formed by strains grown in (A) LB and (B) M9G media for 24 h were assessed by optical density readings of resuspended, crystal-violet-stained bacteria after removal of planktonic cells. Each data point derives from six OD_{590} readings taken from six cultures, three of which were analysed on one occasion and three of which were analysed on another occasion. Parental EcN cultures were derived from a single isolate. Each EcN R4 data point represents a unique tumour isolate. All tumour-recycled isolates derived from the same tumour. For statistical purposes, these EcN R4 isolates were combined according to the media type in which they were cultured. Error bars represent the standard error of the mean.*

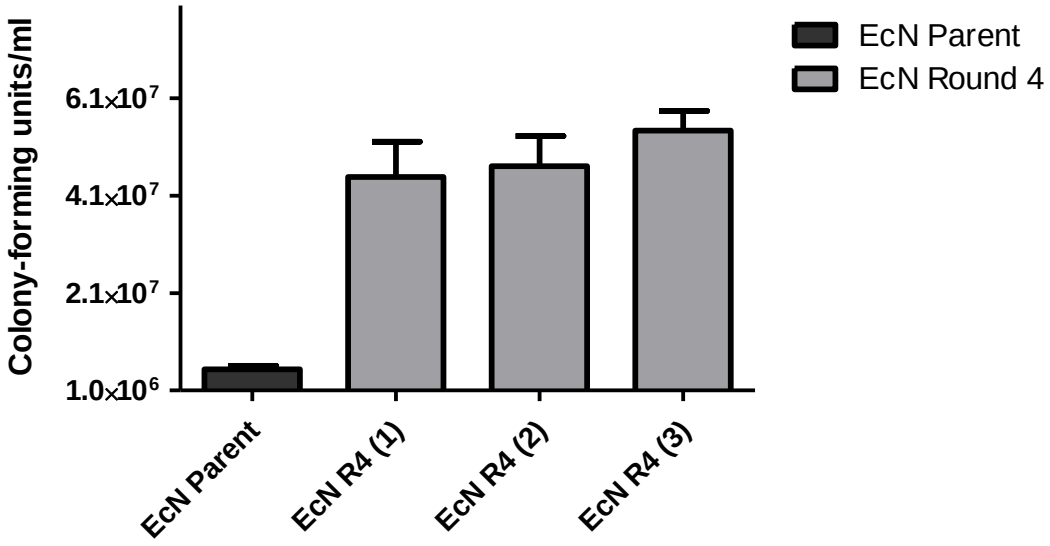


Figure 8. Biofilm population assay of tumour-recycled EcN, compared with its parental (unrecycled) strain. *Strains grown in M9G media for 24 h were scraped from biofilms, after removal of planktonic cells, and cultured to determine CFU counts. Each data point derives from three separate cultures. Parental EcN cultures were derived from a single isolate. Each EcN R4 data point represents a unique tumour isolate. All tumour-recycled isolates derived from the same tumour. Error bars represent the standard error of the mean.*

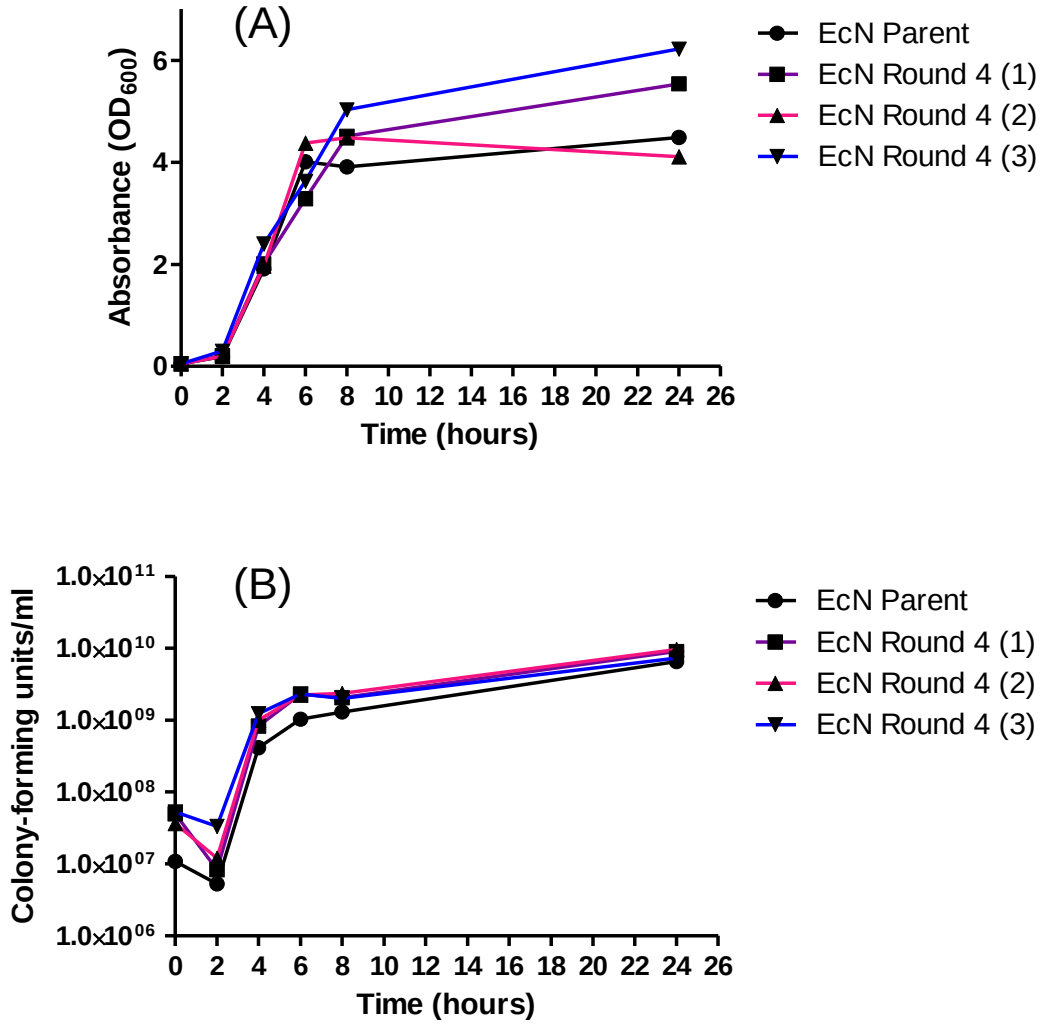


Figure 9. Growth kinetics of tumour-recycled EcN, compared with their parental strain. *Each growth curve representing EcN R4 (purple, pink, and blue) derives from a unique, tumour-recycled isolate. All tumour-recycled isolates derived from the same tumour. Parental (black) and recycled strains were inoculated into LB media with 300 μg/ml Em. (A) Growth curves were composed for parental and tumour-recycled EcN strains, from optical density readings taken over 24 h. (B) CFUs of parental and tumour-recycled EcN strains recovered from liquid culture over 24 h.*

Tumour recycling of bacteria promotes discrete shifts in bacterial metabolism

AWCD was measured to provide an overall, normalised indication of the capacity of parental and tumour-recycled EcN strains to metabolise organic substrates in

EcoPlates over time (Table 1). AWCD readings largely aligned between parental and recycled strains at each timepoint, excepting at the 72-h mark, at which time there was a relatively marked increase in the difference of AWCD values between the strains. Changes in bacterial responses to individual substrates over time were also calculated and aggregated (Figure 10). Calculations were guided by prior work examining bacterial utilisation of organic compounds (Garland, 1996). An unpaired, two-tailed t-test detected no significant difference between the recycled and parental strains in terms of their general ability to significantly metabolise the organic substrates tested over the 144-h period ($P = 0.493$). Once again, however, there was a relatively sharp increase in the carbon consumption of the parental strain at the 72-h mark, compared with the R4 strain, due to a significant response to 30 of the 31 substrates under test by the former strain, compared with 18 substrates by the latter strain.

A more detailed analysis of carbon utilisation in terms of substrate groups suggested that widespread metabolic change was not statistically significant between parental and recycled strains at the 24-h ($P = 0.879$), 48-h ($P = 0.990$), 72-h ($P = 0.224$), and 144-h ($P = 0.880$) marks, although there were discrete areas of significantly increased carbon consumption by the parental strain compared with the recycled strain, and vice versa (Figure 11). Most significant changes occurred at the 72-h mark: L-arginine, L-phenylalanine, α-ketobutyric acid, itaconic acid, α-cyclodextrin, all amines, and all phenolic compounds were significantly consumed by the parental strain at the 72-h mark ($OD_{590} > 0.25$), while no consumption of these compounds was evident in the recycled strain. The parental strain responded significantly also to D-cellobiose, γ-hydroxybutyric acid, D-glucosaminic acid, and polysorbate 80 at the 72-h mark, while the recycled strain exhibited a positive, yet insignificant, response. Outside of the 72-h mark, the only compound that elicited a significant response in the parental strain to a compound which did not elicit a significant response from the recycled strain was polysorbate 40 at 48 h. The only incidence in which EcN R4 exhibited a significant response to any compound, that its parental strain did not respond to significantly, was at the 24-h mark, at which point the former strain responded significantly to D-cellobiose, D-malic acid, and α-ketobutyric acid, while the latter strain exhibited a small, insignificant response to these compounds.

Table 1. AWCD of EcN parental and R4 tumour-recycled strains over 144 hours.

Time (hours)	AWCD EcN Parent	AWCD EcN R4
24	0.017	0.017
48	0.019	0.019
72	0.024	0.018
144	0.018	0.017

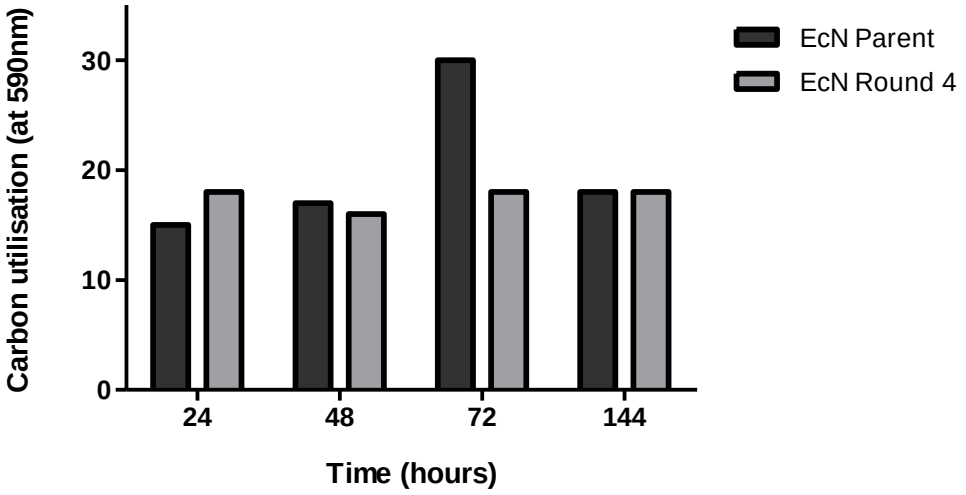


Figure 10. Capacity of tumour-recycled EcN, compared with its parental (unrecycled) strain), for metabolism of organic compounds. *Consumption of 31 organic compounds was analysed using an EcoPlate. Optical density readings were taken over 144 h of incubation. OD_{590} readings greater than 0.25 represented a significant, positive response to the substrate and resulted in a score of 1 (indicating "richness"). Scores for each substrate were added for each strain per timepoint.*

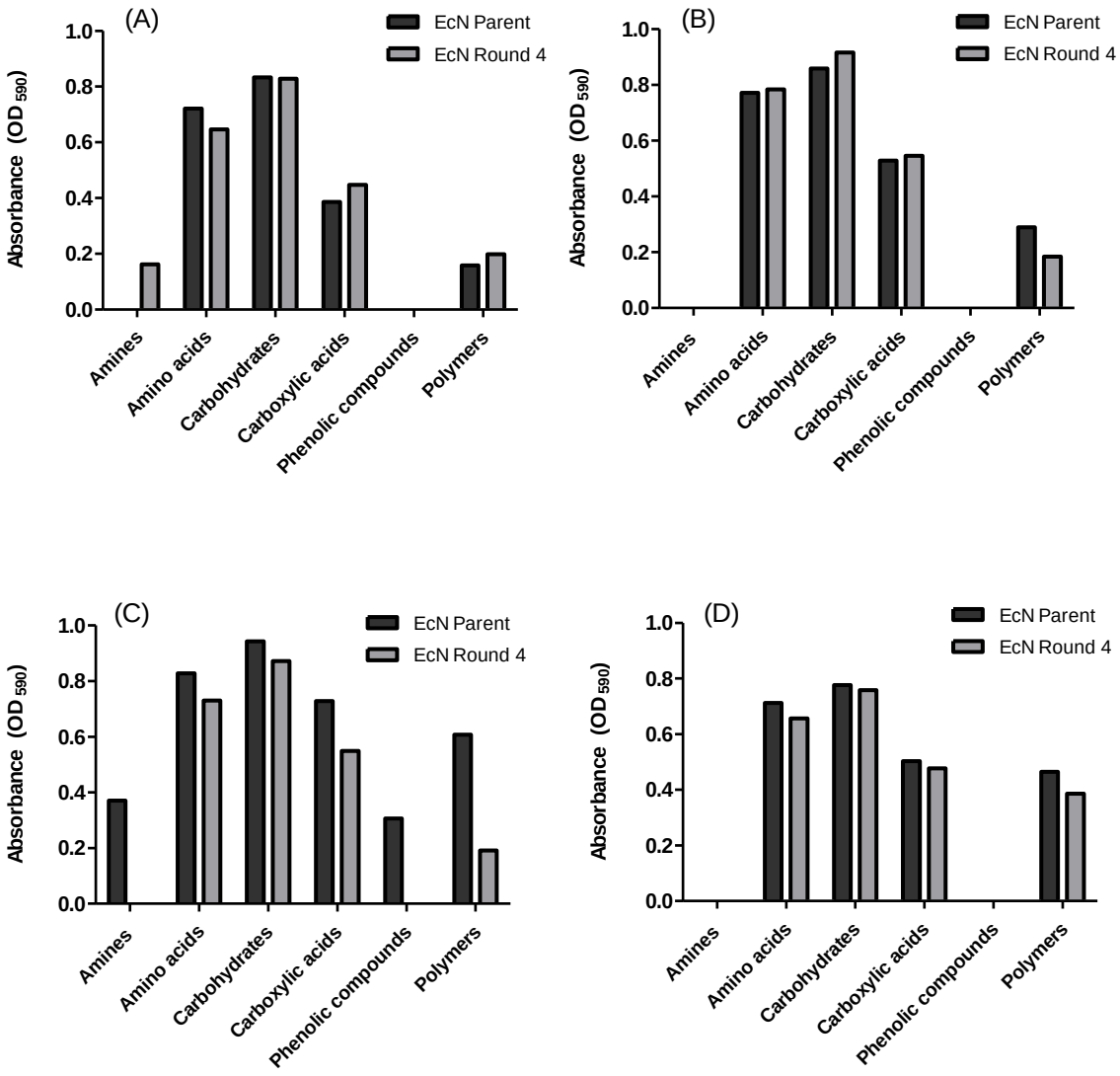


Figure 11. Carbon utilisation of tumour-recycled EcN, compared with its parental (unrecycled) strain, across various substrate groups. *Consumption of 31 organic substrates was quantified using an EcoPlate. Each organic substrate was allocated to its substrate group and optical density readings were taken after 24 h (A), 48 h (B), 72 h (C), and 144 h (D) of incubation. Each absorbance value is the average of triplicate, blank-corrected* OD_{590} *readings.*

Tumour-to-tumour recycling does not result in significant changes to the bacterial genome sequence

No significant differences were observed in WGS data between parental and recycled strains both in the case of EcN (Figure 12) and *B. breve* UCC2003 (Figure 13),

indicating that any phenotypic changes observed were not mediated by mutational changes in DNA sequence.

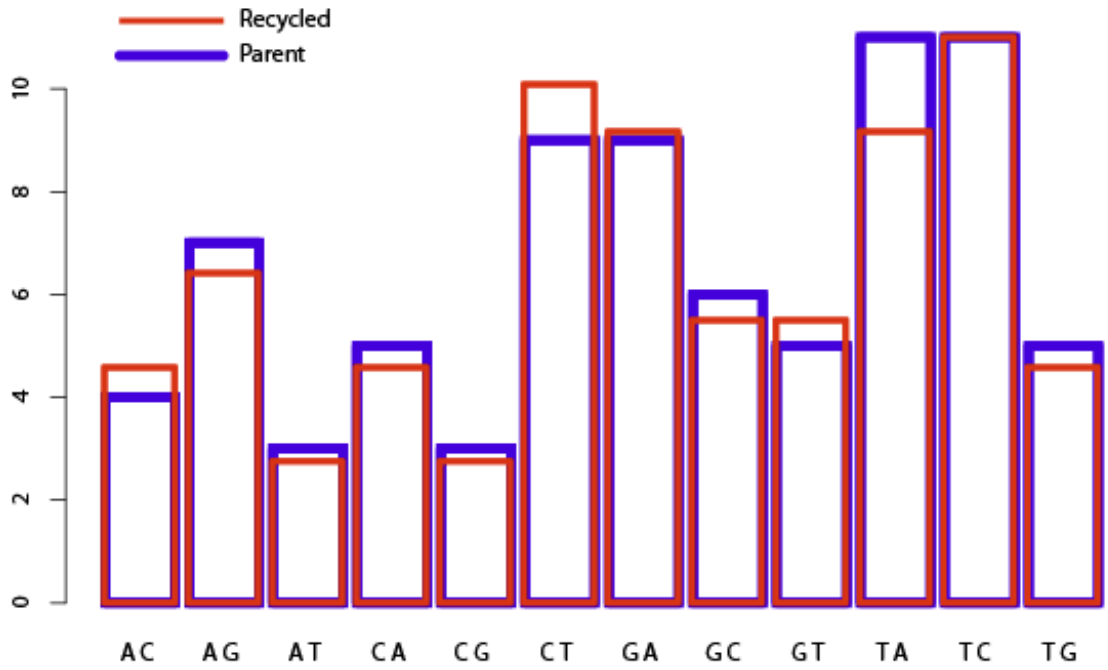


Figure 12. SNPs in EcN recycled through murine tumours, compared with its parental (unrecycled) strain.

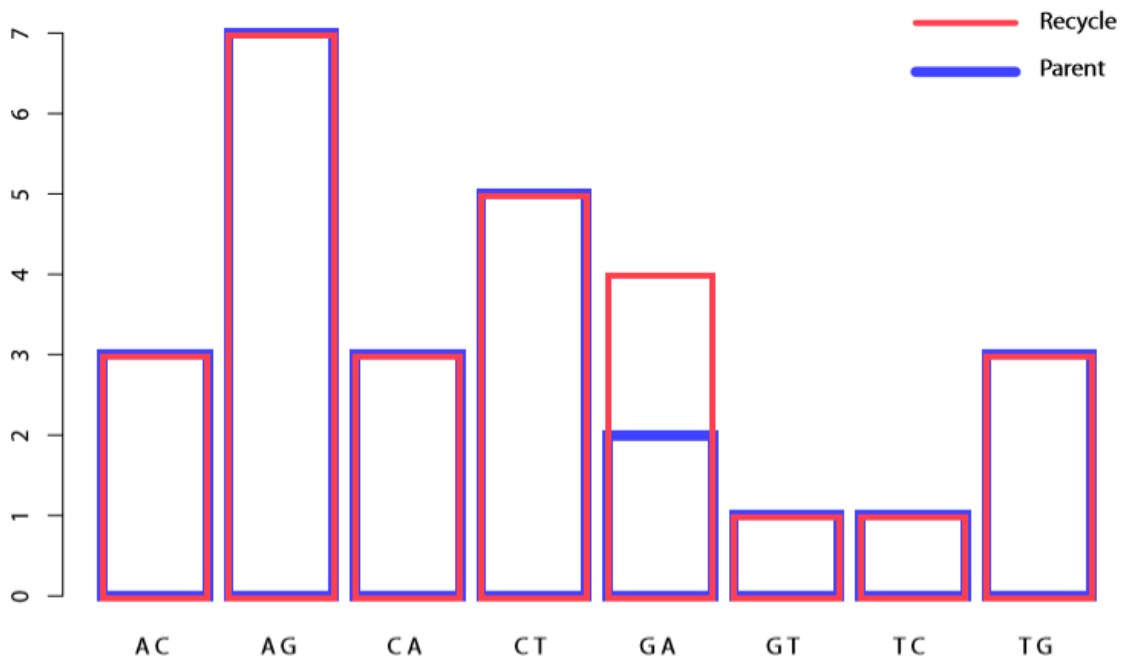


Figure 13. SNPs in B. breve UCC2003 recycled through murine tumours, compared with its parental (unrecycled) strain.

DISCUSSION

The approach undertaken in this study to produce fit-for-purpose bacteria was less interventional than traditional methods used to produce functionally refined strains, insofar as bacteria were introduced into a new environment (i.e., the tumour) and allowed to replicate without any deliberate modifications being made to the microbes by the researcher. This differs from techniques like genome shuffling and other classical strain improvement studies, which usually impose something on the bacteria to encourage mutations and changes in phenotype, such as ultraviolet irradiation (Stroman et al., 2018) or recombination strategies (Zhang et al., 2002). This more passive method was deemed to be the most logical way of progressively adapting administered microbes to the complex, irregular environment of the tumour.

The two strains examined in this study changed in response to recycling through tumours, in terms of their persistence, but not their tumour selectivity. Unrecycled EcN (i.e., R1) experienced a four-fold log reduction in CFUs after 13 dpi, compared with earlier timepoints, in culture-positive tumours. Moreover, it appeared to experience a decline in its ability to colonise tumours outright, having a 100% colonisation success rate at timepoints up to 10 dpi, followed by colonisation of two of three tumours at 11 dpi, and one of three tumours at 13 dpi. Contrastingly, EcN R4 persisted in all three tumours, at high levels, at both 7 and 13 dpi. This suggests that the use of tumour-recycled EcN (R4) is associated, beneficially, with a longer duration of tumour colonisation, as it more frequently colonises tumours successfully than EcN R1 (especially >10 dpi), without any obvious change in tumour selectivity. A similar outcome was observed with *B. breve* UCC2003, in that R1 appeared to persist less in tumours at 14 dpi. Conversely, the R4 variant improved in its ability to colonise tumours in the longer term. Differences in tumour selectivity could not be assessed in *B. breve* UCC2003, as no liver was culture positive for any parental or recycled strain.

There was a sustained increase in intratumoural growth of tumour-recycled EcN, over its R1 variant, after more than one cycle of tumour recovery. The shift in intratumoural growth dynamics of tumour-recycled EcN thus occurred over a relatively short timeframe, and without a corresponding change in genotype, as evidenced by WGS. This trend did not apply to *B. breve* UCC2003, which did not sustain increased intratumoural growth over its R1 variant, even after undergoing three additional cycles

of tumour recovery. These differences might relate to the varying generation times between *E. coli* and *B. breve*. Although it is difficult to provide general estimations of bacterial generation times due to inevitable variability in growth conditions (Weller and Wu, 2015), numerous studies often report short generation times for *E. coli* of under one hour (Eudy and Burrous, 1973, Tuomanen et al., 1986), and longer generation times for *B. breve* (Dubey and Mistry, 1996). The inconsistent impact of tumour recycling that was observed, in terms of the different intratumoural growth dynamics between the two bacterial genera in this study, suggests that the benefits of tumour recycling are probably not applicable to all bacteria, and may be more relevant to those with relatively fast generation times that help them adapt to environmental change more readily.

The amplified intratumoural bacterial numbers achieved for EcN, following multiple passages through tumours, suggest that bacteria can be progressively primed for tumour colonisation by tumour-to-tumour recycling. As noted above, once-off recovery of *S. enterica* serovar Typhimurium from murine tumours augmented bacterial adhesion to mammalian cells *in vitro*, compared with its parental strain (Zhao et al., 2006). Taken together, these results indicate that more extensive tumour passaging may be required for some changes in bacterial interactions with tumours to manifest *in vivo* compared with interactions *in vitro*. Another possibility is that different aspects of bacteria-tumour interactions may be affected at different rates due to serial passaging through tumours. Were this the case, then increased bacterial cell adhesion to mammalian cells, presumably due to improved biofilm formation, is a relatively early consequence of passaging bacteria through tumours, and heightened bacterial proliferation within tumours is a later change. Given that the two bacteria included in this study displayed contrasting intratumoural growth dynamics, the effects of tumour recycling are probably highly bacteria-specific. This is further supported by evidence that a single passage of *S. enterica* serovar Typhimurium through tumours in fact reduced bacterial fitness in terms of decreased motility and growth dynamics compared with its parental strain (Choe et al., 2014).

Following repeated exposure to tumours, phenotypic changes were observed in EcN, such as an altered capability of tumour-recycled EcN to metabolise various organic compounds, compared with its parental strain. While there were scattered examples of compounds to which a significant response was observed exclusively in tumour-

recycled EcN in the first 24 h of incubation (e.g., D-cellobiose), most changes occurred at more mature stages of bacterial growth, during which time tumour-recycled EcN displayed a subdued response to several compounds that its parental strain did not experience. This reduction in metabolic activity may represent an energy conservation strategy for reallocation of resources to other physiological processes that are more important for bacterial survival in tumours.

Tumour-recycled EcN did, in fact, acquire other phenotypic changes in areas that are known to play an important role in facilitating tumour colonisation, such as biofilm synthesis (Pawar et al., 2014). Biofilm formation by *S. enterica* serovar Typhimurium, for example, has been shown to be important in helping tumour-colonising bacteria resist phagocytosis (Crull et al., 2011). Furthermore, EcN is a proficient biofilm producer, and this has been linked with its ability to colonise the intestines and outperform other *E. coli* strains in this respect (Hancock et al., 2010). Thus, it is reasonable to postulate that upward and/or downward shifts in carbon utilisation may be at least partially linked to a heightened requirement for biofilm formation in EcN. Interestingly, it is suggested that cellobiose metabolism is involved in biofilm formation by *Klebsiella pneumoniae* (Wu et al., 2012), and that this substrate stimulates biofilm production in *Listeria monocytogenes* when it is provided as the sole carbohydrate (Kim and Frank, 1995), which may explain why tumour-recycled EcN is significantly more responsive to this substrate than its parental counterpart during relatively early phases of metabolic activity.

WGS analysis was used to examine differences in the frequency with which SNPs occur in tumour-recycled versus parental bacterial strains. While no significant differences were detected regarding frequency of mutations, there may exist specific genetic loci that are differentially mutated between parental and recycled strains that could indicate if certain genes are involved in tempering bacterial fitness with respect to their growth within tumours. To query if epigenetic or transcriptional changes were involved, DNA methylation analysis or mRNA analysis would also provide information. While it was beyond the time limit of this thesis to perform a mouse study to isolate bacterial RNA from tumours for RNA studies, DNA methylation analysis was pursued via PacBio sequencing. Isolated DNA was processed and shipped for sequencing. However, due to technical errors with the sequencing company, the data have not been provided by the company at the time of writing. These data, should they

demonstrate that bacterial adaptability to tumours is regulated epigenetically, could expound the mechanisms by which bacteria become primed for intratumoural growth, perhaps beyond enhanced biofilm formation.

The rate of evolutionary development in tumour-recycled bacteria is likely to significantly affect the magnitude of any phenotypic changes observed. It is known that mutations in the proofreading subunit of DNA polymerase III in *E. coli* are responsible for the emergence of "hypermutators" that display a 1000-fold increase in mutation rate compared with the parental strain (Ramiro et al., 2020). Alternatively, it has been shown that production of a bacterial strain with a defective mismatch repair system can increase its mutation rate from 100 to 200 times that of the parental strain (Lee et al., 2012). Although this has been examined *in vitro*, there is evidence that mutation frequencies can be similar between the *in vivo* and *in vitro* settings (Ramiro et al., 2020). By increasing the rate at which mutations in the bacterial genome occur by compromising polymerase fidelity and/or mismatch repair, the effects of tumour recycling on intratumoural bacterial growth and metabolism may become accelerated and more pronounced to a point where more radical quantitative and qualitive genomic changes are also observed, providing a wider scope through which the genes involved in bacterial tumour colonisation can be analysed.

The inclusion of *E. coli* strains in tumour targeting studies has several advantages over other genera due to its well-known biochemistry and its relatively pliable genetics (Paton et al., 2012). However, despite these advantages, and the non-pathogenicity of EcN, intraperitoneal injections of 5×10^6 EcN CFUs into tumour-bearing mice resulted in appreciable mortality (40%) (Stritzker et al., 2007), underlining the importance of reducing the bacterial doses required for tumour colonisation. It is hypothesised that a small proportion (< 0.1%) of EcN, in the initial IV dose, colonises the tumour, perhaps indicating that the proportion of these bacteria that are truly adapted for life within tumours is also small. Thus, in this study, a method is proposed for improving the adaptability of some bacteria for tumour colonisation, potentially allowing for refinement of bacterial tumour targeting and better therapeutic outcomes.

CONCLUSION

Recycling probiotic bacteria through murine tumours can alter certain aspects of bacterial biochemistry. These changes are interlinked with processes that are associated with tumour colonisation, such as biofilm formation. The genetic/epigenetic/transcriptional underpinnings that underlie these changes, if any, are yet to be defined. The variability that was observed between different tumour-recycled bacterial genera means that each bacterium should be tested on a case-by-case basis before it can be claimed that tumour recycling is beneficial to the strain.

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Chapter 6: Prospects

This thesis examines the current state of bacteria as a disruptive technology for the treatment of cancer, and explores microbes as potentially better clinical instruments than cancer therapeutics that are already clinically regulated. Several aspects of the bacterial relationship with cancer, and with human physiology in general, are explored, and data gathered from both clinical and preclinical investigations are used to justify focusing on certain approaches over others with a view to improving bacterial tumour targeting. This thesis describes novel investigations that build on past, more preliminary work, and does so using more refined and comprehensive methods. Some work presented here could thus be considered to some extent a quality control exercise that aims to identify the best ways for accelerating progress in this field.

Thematically, much of this work focuses on how bacteria might be best orientated to maximise their clinical applicability, in terms of i) isolating or producing tumour-selective bacteria, and ii) confirming the ideal route by which such bacteria can be administered once they are identified. These two points are addressed by both clinical and preclinical data in the thesis. The former point is approached by the attempted isolation of viable bacteria pre-existing within murine tumours and patient breast tumours (ultimately described by data in Chapter 3, and the context of which is framed bioinformatically in Chapter 2) and the production of tumour-adapted bacteria by tumour-to-tumour recycling in mice (Chapter 5). The latter point is approached by the oral and IV administration of probiotic bacteria to mice and the oral administration of probiotic bacteria to patients (Chapter 4).

The protocols arranged in Chapters 2 and 3 for collection and analysis of the microbiome and microbiota of patient breast samples is an advancement on those developed priorly, leading to decreased bacterial contaminants in collected specimens – assuredly at the bioinformatic level and almost certainly at the culture level. The apparently scant bacterial numbers in murine tumours and patient breast tissues examined in this thesis should not discourage further research into the existence of endogenous breast microbes and tumour-dwelling bacteria. Admittedly, this thesis can make claims only about the microbiome of one type of subcutaneous murine tumour tissue (4T1) and the microbiota of two types of subcutaneous murine tumour tissues (RENCA and 4T1). Future mouse work could be informative and more conclusive if different tumour models were sequenced and cultured – both in terms of the cell line

used and the site of tumour induction. For example, induction of 4T1 tumours at an orthotopic site (i.e., the mammary fat pad), rather than subcutaneous induction near the flank, could be insightful with respect to assessing the effect of tumour location on tumour colonisation by bacteria. Differences in culture data between 4T1 and RENCA tumours further justify branching out to different murine tumour types and surveying the bacterial communities present in these, if any.

Furthermore, from the work in this thesis, claims can be made about the microbiota and microbiome of only one type of human tumour tissue (breast). Additionally, despite the comprehensive volume of culture and sequencing data acquired for this sample type, they were sourced from a single-centre study. Therefore, more work could be done by investigating differences in the bacterial community structure, if any, between breast tumours sampled from patients in different geographical regions. Given the profound interregional differences in gut microbiome composition that can occur (He et al., 2018), it is reasonable to suspect that this may be the case with tumours, too. Of course, similar work could also be carried out on different tumour types altogether, paying strict attention to the possibility of contamination, particularly from the skin, that has the potential to significantly influence the tumour microbiome and microbiota. Considering the naturally low biomass of the tumours analysed in this thesis, it is probable that other tumour types, in both mice and humans, are low biomass too, and therefore subject to the same contamination risk.

The correct way of going about all these investigations would resemble the path advocated in Chapters 2 and 3 of this thesis, i.e., to design a culture-based assay guided by microbiome data, with appropriate contamination controls. Admittedly, the work in this thesis deviated from this flow by generating microbiome data alongside culture data, as the culture-based assay was originally designed around older microbiome data that was not known to be outmoded at the time of design. This likely had no impact on the validity of the culture-based assay, as the new microbiome data generated were less complex than those detailed in prior studies, and thus a culture-based assay derived from the higher-quality microbiome data would probably have been less comprehensive than the assay ultimately used. Nevertheless, the microbiome of most tumour types has not yet been examined and it would therefore be sensible to tackle these unknown areas in a more sequential manner, as described above. Beyond breast tumours, the microbiomes of murine H22 tumours (a hepatic carcinoma cell line) and

human liver tumours have been defined recently (Zhang et al., 2018). However, the way in which data were retrieved was either insufficiently described (in terms of sample collection) or improperly carried out (in terms of bioinformatic analyses), and thus new, higher-quality microbiome data are required before a culture-based assay could be applied to these tumour types.

In terms of deciding on the optimal route of administration for bacteria in clinical studies, this thesis adds evidence that tumour colonisation by orally administered bacteria may be a more delicate matter than previously thought. While clinical and preclinical data matched up in this thesis, as both murine and human tumours were negative for orally administered bacteria, deviations in the translocation potential of microbes administered to mice *per os* exist in the literature that preclude the author from dismissing the usefulness of this administration route in tumour targeting studies. Indeed, while some authors have noted the ability of orally administered bacteria to colonise hepatic but not subcutaneous murine tumours (Danino et al., 2015), other authors have reported successful colonisation of murine subcutaneous tumours by this route (Hoffman, 2011, Li et al., 2013, Cronin et al., 2010).

These inconsistencies point to a potential link between tumour location and/or bacterial strain and the colonisation of tumours following oral administration. For example, *Salmonella enterica* serovar Typhimurium although attenuated, probably retains a significant level of invasiveness (that probiotic bacteria like EcN inherently lack) to permit translocation. Prospective work could help to clarify these confounding factors by gavaging mice bearing diverse tumours with diverse bacterial strains. Results from these studies could be used to justify the orchestration of clinical trials that incorporate patients with more relevant types of cancer, and/or testing oral administration of more appropriate bacterial strains. Such trials would be limited by the range of commercial probiotics available for supply to patients, and this should be borne in mind when choosing bacteria for gavage studies in tumour-bearing mice. Nonetheless, the wider range of bacteria that can reliably achieve tumour colonisation across a wider range of tumours when intravenously administered (Yu et al., 2008) suggests that this route of administration will likely prevail over the intragastric route in prospective tumour targeting studies, at least until the variables governing tumour colonisation via the oral route can be elucidated.

The tumour-recycled bacteria produced during this thesis are also the subjects of multiple prospective experiments that could be informative. As noted in Chapter 5, microbial titres required for tumour colonisation should be low, preferably, to avoid toxicity issues that can occur even with probiotic strains (Stritzker et al., 2007). However, there is a threshold below which bacterial tumour colonisation fails (Yu et al., 2008), which necessitates investigation of therapeutic indices so that appropriate doses can be determined. Future experiments with these tumour-recycled bacteria should therefore involve dose-ranging studies in tumour-bearing mice. The results of these experiments would ideally clarify if the effects of tumour recycling allow for lower doses of bacteria to be intravenously administered (e.g., $< 1 \times 10^4$ CFUs in the case of *Escherichia coli*), while still resulting in tumour colonisation. Additionally, although WGS did not identify significant differences between parental and tumour-recycled bacterial strains, other types of NGS technologies could be used to uncover potential differences beyond the DNA level, of which RNA sequencing (Stark et al., 2019) and epigenetics studies targeting, for example, the methylome (Rhoads and Au, 2015) are examples.

CONCLUSION

This thesis presents a body of work that aims to establish ways in which bacterial anticancer therapies can be optimised to maximise the probability of their success in clinical trials. It provides novel information on the tumour microbiota, and advises on how naturally occurring, tumour-adapted bacteria might be acquired through refined approaches to microbiome research. It also examines the gut translocation efficiency of probiotic bacteria, at the clinical level most importantly, to assess the merits of the oral route for bacterial administration, compared with more common routes, such as intratumoural and IV administration. Finally, this thesis details more active ways by which tumour-adapted bacteria can be produced, via tumour-based classical strain improvement.

The relationship between bacteria and cancer has yet to be fully defined, and this limits the extent to which bacteria can be applied optimally as cancer therapeutics. The value of this work therefore lies in broadening the understanding of how bacteria interact with cancer, such that smarter steps on advancing this field can be taken.

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