The role of bile-metabolising enzymes in the pathogenesis of *Clostridioides difficile* infection, and the impact of faecal microbiota transplantation

Benjamin Harvey Mullish

Division of Integrative Systems Medicine and Digestive Disease, Department of Surgery and Cancer,
Faculty of Medicine

Imperial College London

2019

Thesis submitted for the degree of Doctor of Philosophy

Abstract:

The pathogenesis of *Clostridioides difficile* infection (CDI), and mechanisms of efficacy of faecal microbiota transplant (FMT) in treating recurrent CDI (rCDI), remain poorly-understood. Certain bile acids affect the ability of *C. difficile* to undergo germination or vegetative growth. Loss of gut microbiota-derived bile-metabolising enzymes may predispose to CDI via perturbation of bile metabolism, and restitution of gut bile-metabolising functionality could mediate FMT's efficacy.

Initially, human samples were analysed, i.e.: 1) biofluids collected from rCDI patients pre- and post-FMT (and their donors), and 2) stool samples from primary CDI patients, including both recurrers and non-recurrers. Analysis included: 16S rRNA gene sequencing; liquid chromatography-mass spectrometry for bile acid profiling; gas chromatography-mass spectrometry for short chain fatty acid (SCFA) quantification; bile salt hydrolase (BSH) enzyme activity; and qPCR of *bsh/ bai*CD genes involved in bile metabolism. Human results were validated in *C. difficile* batch cultures and a rCDI mouse model.

A reduced proportion of the stool microbiota of rCDI patients pre-FMT contained BSH-producing bacteria compared to donors or post-FMT. Pre-FMT stool was enriched in taurocholic acid (TCA; a potent trigger to *C. difficile* germination); TCA levels negatively correlated with bacterial genera containing BSH-producing organisms. Post-FMT stool demonstrated recovered BSH activity and microbial *bsh/ bai*CD gene copy number compared with pre-treatment (p<0.05), and recovery of SCFA including valerate (p<0.001). Dynamics of stool bile acids/ BSH activity differed in primary CDI patients with and without disease recurrence. In batch cultures, culture supernatant from engineered *bsh*-expressing *E. coli* reduced TCA-mediated *C. difficile* germination relative to supernatant from BSH-negative *E. coli*. *C. difficile* total viable counts were ~70% reduced in a rCDI mouse model after administration of BSH-expressing *E. coli* relative to mice receiving BSH-negative *E. coli* (p<0.05).

These data demonstrate that gut microbiota BSH functionality is a key mechanism influencing vulnerability to CDI and efficacy of FMT.

Declaration of Originality:

I certify that this thesis, and the research to which it refers, are the product of my own work. Any ideas or quotations from the work of other people, published or otherwise, are fully-acknowledged in accordance with the standard referencing practices of the discipline.

Benjamin H Mullish

London

April 2019

Acknowledgements and Dedication:

I am very fortunate to have had two supervisors as outstanding as Professors Mark Thursz and Julian Marchesi. They have both been consistently kind, good humoured and motivational, no matter whatever obstacles there were to progress or however spectacular the disasters I presented them with. I particularly remember the challenging times near the start of this project where I had no FMT service, no samples, no funding and no data, and they were totally unwavering in their support of me and belief in this project at all times, which is a testament to the character of them both. I owe them both a real debt of gratitude.

I would like to thank Dr Horace Williams for helping me set up the Imperial FMT service, and for joining me in the character-building exercise that was writing the UK FMT guidelines. Other Imperial Consultants have been fantastic role models and hugely supportive throughout, with particular thanks owed to Dr Ameet Dhar and Dr Pinelopi Manousou.

I also could not have asked for better collaborators than Dr Jessica Allegretti (Brigham and Women's Hospital/ Harvard Medical School, UK), Dr Dina Kao (University of Alberta, UK), and Dr Tanya Monaghan (University of Nottingham, UK). The three of them are all inspiring clinician scientists, full of enthusiasm and ideas, and have given me huge amounts of time (and samples!) towards making this project successful. Dr Cormac Gahan and Dr Susan Joyce (from SFI Research Centre APC Microbiome Ireland, University College Cork, Ireland) kindly supplied the engineered *bsh*-expressing *E. coli,* and were very helpful throughout in planning the mechanistic studies.

In the laboratory at Imperial, my greatest thanks must go to Dr Julie McDonald. Julie has been an amazing lab 'big sister' who has been a constantly supportive mentor and brilliant teacher, and I am very grateful for all her support throughout. Other thanks for friendship, assistance and camaraderie in the lab are owed to Grace Barker, Jesus Miguéns-Blanco, Dr Holly Jenkins, Dr Rohma Ghani, Dr Diya Kapila and Dr Fouzia Sadiq. I will miss the strong coffee, vegan baking and post-conference nights out that have helped to keep me sane during my research. Thanks are also owed to Dr Alex Pechlivanis, Dr Isabel Garcia Perez, Dr Zhigang Liu, Dr Jia Li and Jerusa Brignardello, who have all patiently endured many a nagging email or call from me

as I have very slowly learned metabonomics. Dr Tom Clarke and Izabela Glegola-Madejska both went beyond the call of duty to help me with the mouse experiments.

However, the greatest thanks I owe are to my family and friends; they have bravely survived more conversations about faecal manipulation over the past few years than anyone should rightfully endure. My mother, Sharon, has been – as ever - incredibly supportive throughout, despite having so much on her own shoulders to deal with. She has made so many sacrifices and worked so hard to support my training and my career. Hilary has always been there for me, and has been an amazing source of kindness, endless encouragement, and wise counsel. They are both very cherished.

This thesis is dedicated to the memory of my father, David Mullish.

Benjamin H Mullish
London
April 2019

Table of contents:

List of figures:

List of tables:

List of abbreviations:

ANOVA	analysis of variance
ATP	adenosine triphosphate
bai	bile acid-inducible (operon)
BCFA	branched chain fatty acid(s)
bp	base pair
BSH	bile salt hydrolase
BMI	body mass index
CA	cholic acid
CDCA	chenodeoxycholic acid
CDI	*Clostridioides difficile* infection
CFU	colony forming unit(s)
CMV	cytomegalovirus
CPE	carbapenemase-producing *Enterobacteriaceae*
CV	coefficient of variance
DCA	deoxycholic acid
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DSS	dextran sodium sulfate
DTT	dithiothreitol
EIA	enzyme immunoassay
EBV	Epstein-Barr virus
ESBL	extended-spectrum beta-lactamase(s)
ESI	electrospray ionisation
FDR	false-discovery rate
FGF	fibroblast growth factor
FISH	fluorescent *in situ* hybridisation
FMT	faecal microbiota transplant(ation)
FXR	farnesoid X receptor
GC MS	gas chromatography mass spectrometry
GCA	glycocholic acid

GCDCA	glycochenodeoxycholic acid
GDCA	glycodeoxycholic acid
GDH	glutamate dehydrogenase
GI	gastrointestinal
GTP	guanosine triphosphate
GTV	glycerol trivalerate
HCAI	healthcare-associated infection
HDAC	histone deacetylase(s)
IBD	inflammatory bowel disease
Ig	immunoglobulin
IL	interleukin
iNKT	invariant natural killer T (cells)
IPTG	isopropyl-β-d-thiogalactopyranoside
LCA	lithocholic acid
mCCEY	moxifloxacin-Braziers Cycloserine Cefotixin Egg Yolk
MCFA	medium-chain fatty acid(s)
MRSA	methicillin-resistant *Staphylococcus aureus*
NAAT	nucleic acid amplification test
NAP1	North American PFGE type 1
NK	natural killer (cell)
NMR	nuclear magnetic resonance
NPX	Normalised Protein eXpression
NTA	nitrilotriacetic acid
OCA	obeticholic acid
OD	optical density
OPLS-DA	orthogonal projections to latent structures discriminant analysis
OTU	operational taxonomic unit
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCA	principal component analysis
PERMANOVA	permutational multivariate analysis of variance
PLS	partial least squares

PMA	propidium monoazide
PPI	proton pump inhibitor(s)
ppm	parts per million
PSC	primary sclerosing cholangitis
QC	quality control(s)
(q)PCR	quantitative polymerase chain reaction
rCCA	regularised canonical correlation analysis
rCDI	recurrent *Clostridioides difficile* infection
RCT	randomised controlled trial
rRNA	ribosomal ribonucleic acid
sBHI	supplemented brain heart infusion
SCFA	short chain fatty acids
SDS	sodium dodecylsulfate
SOP	standard operating procedure
STAMP	statistical analysis of metagenomic profiles
T-RFLP	terminal restriction fragment length polymorphism
TCA	taurocholic acid
Tcd	toxin of *C. difficile*
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TGR5	Takeda G-protein-coupled receptor 5
TNBS	trinitrobenzenesulfonic acid
TNF	tumour necrosis factor
TVC	total viable count(s)
UC	ulcerative colitis
UDCA	ursodeoxycholic acid
UPLC-MS	ultra-performance liquid chromatography-mass spectrometry
VRE	vancomycin-resistant *Enterococci*

Chapter 1. Introduction:

1.1. Overview of *Clostridioides difficile* infection:

Clostridioides difficile (formerly named *Clostridium difficile* (Lawson *et al.*, 2016)) is a Gram-positive, spore-forming, obligate anaerobic bacillus. It was originally recognised as a member of the gut microbiota of healthy infants in 1935, and was given its name because of the difficulties experienced in isolating and culturing it on conventional media (Hall & O'Toole, 1935). The pathogenic nature of *C. difficile* was first recognised in the 1970s after investigations into the aetiology of pseudomembranous colitis, an often-severe diarrhoeal disease characterised by yellow-white plaques scattered over the colonic mucosa with characteristic histological appearances. The key link between the condition and *C. difficile* was made after observation of a toxin in the stool of patients with antibiotic-associated pseudomembranous colitis, and demonstration that this toxin was able to recapitulate a similar phenotype of disease in animal models (Larson *et al.*, 1977; Chang *et al.*, 1978). It was subsequently recognised that *C. difficile* is the cause for virtually all cases of pseudomembranous colitis, and approximately 20% of cases of antibiotic-associated diarrhoea (Wiström *et al.*, 2001).

The spectrum of clinical disease that occurs in *Clostridioides difficile* infection (CDI) is now recognised as being very marked, ranging from mild diarrhoea and occasional fever in the least severe cases, up to severe colitis, toxic megacolon (i.e. profoundly-dilated colon), colonic perforation and/or systemic inflammatory response syndrome, multi-organ failure and death in the worst cases (Mullish & Williams, 2018).

1.2. Epidemiology of *Clostridioides difficile* infection:

CDI represents a large and growing healthcare problem, with approximately 453000 cases of CDI (and 29000 directly-attributable deaths) caused by the condition in the USA alone in 2011 (Lessa *et al.*, 2015). Globally, severe CDI infections have an infection-related mortality of approximately 5%, and an all-cause mortality of up to 20% (Feuerstadt, Das & Brandt, 2014). CDI came to particular prominence as a major healthcare concern after an increase in incidence rates during the 1990s (Archibald, Banerjee & Jarvis, 2004), before marked further

increases a decade later with the arrival of new epidemic strains, discussed further in **Section 1.5.1.**

The greatest burden of CDI remains in hospitalised and institutionalised patients. Whilst approximately 3% of healthy adults are asymptomatically colonised with *C. difficile,* the rate of colonisation rises up to 10% in elderly patients at hospital admission, and as high as 50% of elderly inpatients after four weeks of hospital admission (Padua & Pothoulakis, 2016). *C. difficile* is the most common healthcare-associated infection (HCAI) in the USA, accounting for 12.1% of all HCAIs in the country as of 2011 (Magill *et al.*, 2014). The CDI incidence in hospitalised patients has increased from 31 cases per 100000 patients in 1996 up to 84 cases per 100000 patients in 2004 (Redelings, Sorvillo & Mascola, 2007). CDI is recognised as quadrupling the cost of hospitalisation and increasing annual health expenditure by approximately $1.5 billion in the USA (Leffler & Lamont, 2015).

It has also recently been recognised that a significant and growing proportion of the burden of CDI is in the community, with at least 25% of CDI cases (and often a somewhat higher percentage) within the USA being described as community-acquired (Lessa *et al.*, 2015; Khanna *et al.*, 2012). Patients with community-acquired CDI tend to be younger, have fewer co-morbidities, have had less recent exposure to antibiotics and/or proton pump inhibitors (PPI), and have less severe disease than patients with nosocomial CDI (Khanna *et al.*, 2012). Transmission of CDI through retail food products and/or via contact with domestic animals are postulated theories for the increasing levels of community-acquired CDI (Gould & Limbago, 2010).

Rates of CDI vary markedly by country (or even by region within them) and can change markedly over short periods of time. For example, while both England and the USA had almost identical CDI rates of approximately 108/100000 population in 2007, a cross-sectional retrospective study using national discharge databases identified a CDI rate of 19.3/100000 population in England in 2012, compared to a rate of 115.11/100000 population in the USA (King *et al.*, 2017) (**Figure 1.1**). Of note, the period between 2007 and 2012 was not associated with any co-ordinated US healthcare policy regarding reduction in CDI rates, whilst this was a public health priority within the UK within the same period. Specifically, over this period, the

UK government co-ordinated a mandatory national enhanced surveillance system and robust changes in infection control policy, including a focus on patient isolation and antibiotic stewardship (Dancer *et al.*, 2013). Whilst it is difficult to directly demonstrate that UK health policy explains the epidemiological differences between the UK and USA over this period, the close temporal relationship between policy change and decline in CDI rates clearly makes it highly plausible that this is a major contributory factor.

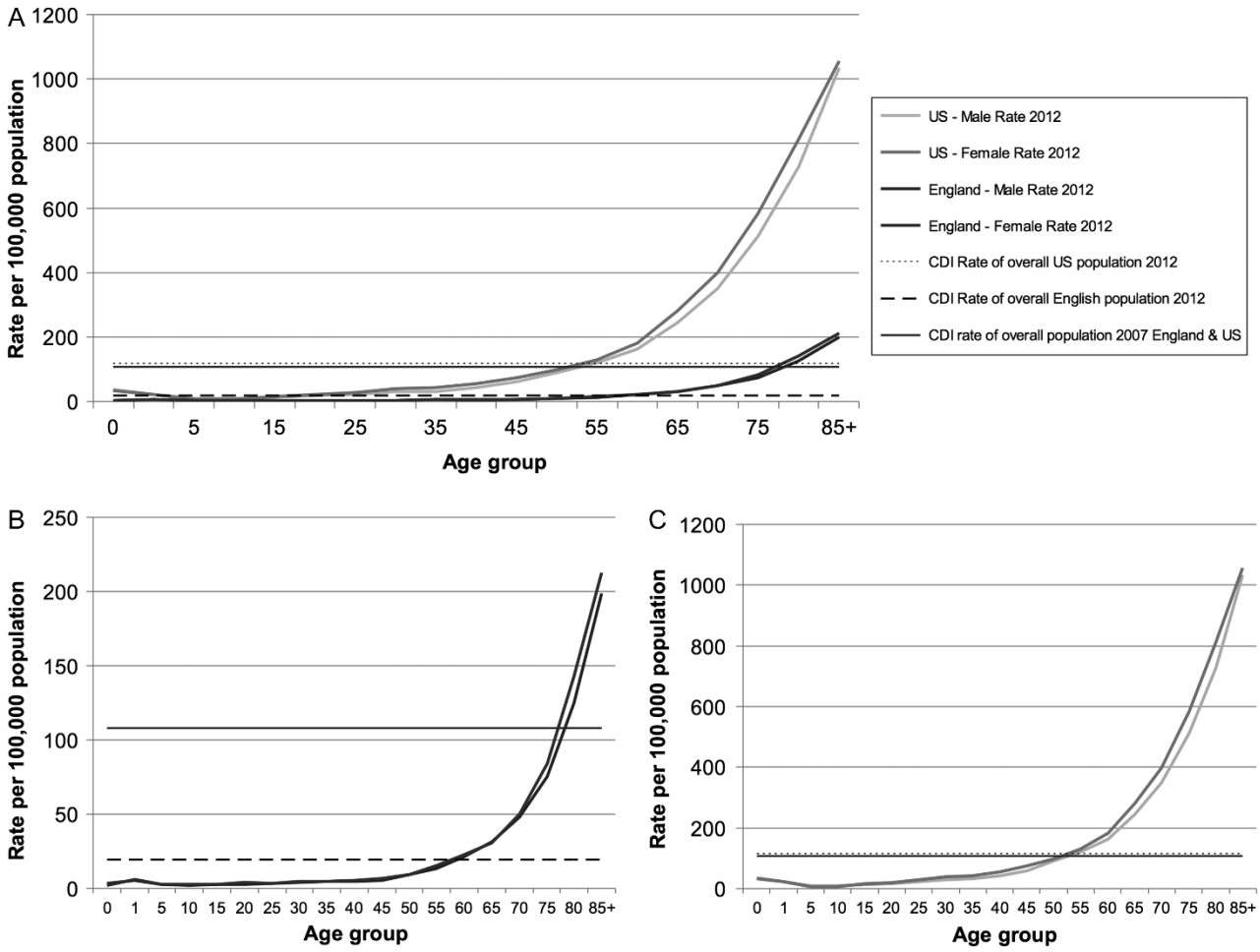


Figure 1.1: CDI by sex and age in England and USA in 2012. Rates are shown per 100000 population split by sex over five year age groups for both countries in 2012. Rate in 2007 (solid line) is also shown for reference when it was consistent in both countries. Overall rates per 100 000 population in 2012 are also shown in each country. A: rates of CDI by sex and age in England and USA in 2012; B: rates of CDI in England in 2012; C: rates of CDI in USA in 2012. Reproduced with permission from (King *et al.*, 2017).

Whilst most epidemiological data regarding CDI has focused on North America and Europe, it is important to note that the disease burden of CDI is recognised globally. For instance, CDI

caused by highly pathogenic strains of *C. difficile* has also been described in Central America, Asian-Pacific countries, and beyond (Clements *et al.*, 2010).

1.3. Aetiopathogenesis of *Clostridioides difficile* infection:

1.3.1. Risk factors:

A summary of established risk factors for the development of nosocomial CDI are presented in **Table 1.1**.

Table 1.1: Risk factors for nosocomial CDI.

Risk factor	Details
Age	• Rates of CDI in patients >65 years old are approximately 10-fold higher than in younger patients (Lessa *et al.*, 2015; Pepin, Valiquette & Cossette, 2005).
Recent antibiotic exposure	• Almost all antibiotics are associated with increased vulnerability to CDI, but clindamycin, cephalosporins, fluoroquinolones, carbapenems and certain penicillins (e.g. co-amoxiclav) are the strongest risk factors (Thomas, Stevenson & Riley, 2003).
Hospitalisation/ institutionalisation	• Recent hospitalisation, prolonged hospitalisation (>7 days) and/or prolonged antibiotic courses are all associated with an increased risk of CDI (Padua & Pothoulakis, 2016). • Being admitted to a hospital room where the previous patient had CDI (or even where they just received antibiotics) is a risk factor for infection in itself (Freedberg *et al.*, 2016). • Having a normal place of residence in a long-term care facility (e.g. nursing home) is also a CDI risk factor.
Acid-suppressing medications	• Both PPI and H2-receptor antagonist use increase risk, but particularly PPIs (Bourgault *et al.*, 2011; Howell *et al.*, 2010). • In a large Dutch cohort, PPI use was associated with a reduction in faecal microbiota alpha-diversity, and enrichment of bacterial taxa commonly found in the oral microbiota of patients, providing a possible mechanistic link between PPI use and increased rates of enteric infection (including CDI) (Imhann *et al.*, 2016).
Chronic metabolic, GI and/or liver disease	• There is an elevated risk of CDI in patients with inflammatory bowel disease, with risks highest in patients taking immunosuppressive therapy and/or with co-morbidities (Singh *et al.*, 2017). • Cirrhosis is also a risk factor for CDI, with rifaximin-resistant strains now detected in patients prescribed long-term rifaximin as treatment for hepatic encephalopathy (Reigadas *et al.*, 2018). • Obesity is also associated with an increased CDI risk (Mograbi *et al.*, 2013).

Immunocompromise	• A number of different forms of immunocompromise – including haematopoietic stem cell transplant and cancer chemotherapy (Anand & Glatt, 1993) – are associated with an increased risk of CDI.
Enteral feeding	• All forms of enteral feeding (but particularly jejunal feeding) appear to infer an increased risk of CDI (Bliss *et al.*, 1998).

Risk factors for the development of recurrence of CDI in particular include age≥ 75 years, ≥10 bowel movements within 24 hours, serum creatinine ≥1.2mg/dl (i.e. approximately ≥106 μmol/l), and previous recurrences of CDI (D'Agostino *et al.*, 2014).

1.3.2. Overview of transmission and pathogenesis:

C. difficile is highly transmissible via the faeco-oral route through the ingestion of spores. In clinical practice, the major concern related to *C. difficile* is its ability to undergo transition from spore to vegetative cell within the gut of vulnerable human subjects (referred to as germination). From here, *C. difficile* can colonise and rapidly-proliferate within the gastrointestinal tract and, where it is able, produce the toxins which cause the observed pathologies (*vide supra*). Spores are able to survive on inanimate objects, and are resistant to commonly-used decontaminants including heat, acid, bleach-free cleaning agents and antibiotics for long periods without any loss of potential viability (Gerding *et al.*, 1995). This resistance is a major contributory factor as to why *C. difficile* may be so rapidly-transmitted within a hospital environment; it has been shown that the organism can be readily cultured from the hands, clothing and stethoscopes of healthcare workers, as well as from commodes and electronic thermometers (Gerding *et al.*, 1995).

C. difficile is genetically-variable, with some strains producing protein exotoxins, whilst others do not; it is the majority of strains (70-90%) which produce toxins that are able to cause clinical disease (Liu *et al.*, 2013). The two toxins produced by *C. difficile* - TcdA (toxin A; 'enterotoxin') and TcdB (toxin B; 'cytotoxin') – act via at least two key mechanisms to severely disrupt the structure of the colonic mucosa and result in the death of colonic epithelial cells. Firstly, both toxins are able to inactivate members of the Rho and Ras family of guanosine triphosphatases (GTPases) that have important contributions to the maintenance of cytoskeleton structure. Secondly, both toxins markedly disrupt intercellular tight junctions

(Brito *et al.*, 2002; Hecht *et al.*, 1992). Furthermore, toxins have direct contributions to the colonic inflammation that characterises CDI; toxin A is able to stimulate neutrophil activity directly, toxin B induces production of reactive oxygen species (Farrow *et al.*, 2013), while both toxins are able to promote neutrophil migration into pseudomembranes, as well as the underlying colonic mucosa (Pothoulakis *et al.*, 1988; Souza *et al.*, 1997). Toxin B is essential for the virulence of *C. difficile,* and has been demonstrated to be more than tenfold as potent as toxin A on a molar basis for its ability to cause disruption of the colonic mucosa (Lyras *et al.*, 2009; Feil *et al.*, 2008).

A number of host factors are also relevant to the pathogenesis of CDI. One such factor is the ability to produce serum anti-toxin antibodies, which appears to be an important protective factor against the development of CDI. Specifically, asymptomatic carriers of *C. difficile* have been demonstrated to have higher serum levels of anti-toxin A immunoglobulin G (IgG) antibodies than patients who develop clinical CDI (Kyne *et al.*, 2002). Similarly, the ability to mount a serum anti-toxin A antibody response during primary CDI appears to confer relative protection against development of recurrent CDI (Kyne *et al.*, 2001a). A common polymorphism in the interleukin (IL)-8 gene promoter is also associated with an increased risk of both primary and recurrent CDI (Jiang *et al.*, 2007). Progressive impairments in innate immune system function that occur with age are believed to be one of the factors explaining the association between advancing age and increased CDI risk (Shin, High & Warren, 2016).

A major factor influencing the vulnerability of the host to *C. difficile* colonisation and subsequent infection is the *gut microbiota*, defined as the total assemblage of microorganisms presented within the gut mucosal environment (in contrast, the term *gut microbiome* refers to the entire gut ecological habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes and viruses), their genomes, and the surrounding environmental conditions) (Marchesi & Ravel, 2015). Antibiotics are one of the major factors responsible for perturbation of the composition and functionality of the gut microbiota, which is the key link between recent antibiotic use and development of CDI. This feature is discussed in further detail in **Section 1.5.4.1.**

1.4. Diagnosis of *Clostridioides difficile* infection:

A range of different laboratory tests are available to aid diagnosis of CDI, as summarised in **Table 1.2**.

Table 1.2: Diagnostic tests for CDI.

Test	Details
Glutamate dehydrogenase enzyme immunoassay (GDH EIA)	• GDH is an enzyme produced constitutively by a broad range of bacteria (Merrick & Edwards, 1995), with a NAD-dependent form of the enzyme found in all strains of *C. difficile*, both toxigenic and non-toxigenic. • This is a sensitive, quick and relatively cheap screening test, but lacks high specificity for toxigenic forms of *C. difficile.* In particular, the test may cross-react to the same enzyme present in other clostridial species (Lyerly *et al.*, 1988). As such, this is typically used as the initial screening assay in a multi-step diagnostic process (Goy *et al.*, 2007).
Toxin A and B EIA	• Highly-specific test (~99%), but somewhat lower sensitivity (~75%) (Swindells *et al.*, 2010). • Appreciable false negative rate, since may require up to 1ng of toxin for a positive test result (Bartlett, 2002).
Nucleic acid amplification tests (NAAT).	• These most typically take the form of polymerase chain reaction (PCR) for genes specific to toxigenic forms, and particular *tcdB.* • Often highly sensitive tests (Paquet-Bolduc *et al.*, 2012). • However, a positive result does not distinguish active toxin production and true CDI from asymptomatic carriage of toxigenic *C. difficile.*
C. difficile culture	• Rarely used clinically, because of requirements for strict anaerobic conditions and slow growth of *C. difficile.* • Culture is also unable to distinguish toxigenic from non-toxigenic disease.
Cell culture cytotoxicity assay	• This involves adding a prepared stool sample (i.e. post-dilution, buffering and filtration) on to a monolayer of cultured cells; the cytopathic effect of *C. difficile* toxin is recognised by examining for cell rounding (Kelly, Pothoulakis & LaMont, 1994). • This is widely-recognised as the reference standard test. However, the complexities in performing it mean that it is rarely performed in most clinical laboratories.

Adapted from (Mullish & Williams, 2018).

As described in the table, there is no single test that combines ideal sensitivity, specificity and practicality. As such, CDI diagnostic algorithms typically include at least two stages of testing, aiming to start with a more sensitive assay and follow-up with a more specific test (Department of Health, 2012; Crobach *et al.*, 2016; Surawicz *et al.*, 2013b; McDonald *et al.*, 2018b).

1.5. Conventional therapy for *Clostridioides difficile* infection, and emerging concerns:

1.5.1. Overview of therapy:

Treatment algorithms for CDI stratify disease based on first episode *vs* recurrent, as well as on the basis of the severity of condition. 'Severe CDI' is classified variably in different guidelines, but a recent consensus of international expert opinion defined it by white blood cell count > 15000 cells/ml and/or serum creatinine ≥ 1.5mg/dl (approximately 133 μmol/l) (McDonald *et al.*, 2018b). This same consensus defined 'fulminant CDI' (previously referred to as 'severe, complicated CDI') as CDI complicated by hypotension or shock, ileus, or megacolon (McDonald *et al.*, 2018b).

Between the 1970s and the start of this century, the mainstay of treatment of CDI was medical therapy using the bacteriostatic antibiotics, metronidazole and vancomycin. Metronidazole is a nitroimidazole prodrug, which is converted by anaerobic bacteria into nitroso radicals, which exert its antibacterial effect via inhibition of nucleic acid synthesis (Kedderis, Argenbright & Miwa, 1989). Vancomycin is a glycopeptide, which functions through inhibition of the synthesis of peptidoglycans required by bacteria for cell wall formation (Reynolds, 2005). Oral vancomycin is not systemically absorbed and, as such, reaches high levels in the distal gut (Fekety *et al.*, 1989); intravenous vancomycin is of no benefit, since it is not excreted into the colon at all. For mild to moderate CDI, conventional first-line treatment has been oral administration of metronidazole and vancomycin for at least 10 days (Public Health England, 2013; McDonald *et al.*, 2018b; Debast *et al.*, 2014). In severe/ fulminant cases of primary CDI, metronidazole may be administered intravenously and vancomycin orally ± retention enema. Intravenous immunoglobulin (IVIg) is another available option; however, whilst this appears to be efficacious in case series, it has never been assessed in a randomised controlled trial, and is therefore only cautiously recommended for clinical use (Public Health England, 2013).

In the case of recurrent CDI, oral vancomycin may be administered in a tapering regimen over at least six weeks (Public Health England, 2013; McDonald *et al.*, 2018b; Debast *et al.*, 2014). However, in light of recent data demonstrating that metronidazole appears to be less effective compared to vancomycin in the initial cure of CDI (Johnson *et al.*, 2014), new US guidelines recommend vancomycin in preference to metronidazole in the treatment of CDI in most scenarios, including even for mild cases and/or first recurrence (McDonald *et al.*, 2018b). Where cases of CDI are refractory to medical therapy, surgical intervention (i.e. via subtotal colectomy with rectal sparing) may be a life-saving intervention (Public Health England, 2013; McDonald *et al.*, 2018b; Debast *et al.*, 2014). However, surgical intervention in this scenario is associated with a high mortality, quoted as high as 80% (Neal *et al.*, 2011).

For around the past two decades, there have been several interrelated global changes in the pattern of CDI that have made it particularly challenging to treat:

Metronidazole failure: Firstly, there has been the emergence of strains of *C. difficile* with poor response to traditional antimicrobials, and particularly with regards to metronidazole. Whilst rates of *C. difficile* metronidazole failure were quoted at <5% prior to the year 2000, they are now regularly described as >25% (Kelly & LaMont, 2008), and even up to ~47% in certain populations (Valiquette *et al.*, 2005). The specific reasons for this rise in metronidazole failure remain poorly-defined, but do not appear to reflect true increases in antibiotic resistance *per se* (Hu *et al.*, 2008).

Rising rates of CDI recurrence: Secondly, there has been recognition of rising rates of *C. difficile* recurrence (i.e. re-emergence of the full spectrum of clinical disease within a short period of completion of apparently successful antimicrobial therapy). Recurrence may represent re-exposure to *C. difficile,* or reactivation of dormant spores within vulnerable patients. The risk of recurrence within eight weeks following treatment for primary CDI is 15-25%, and rises as high as 40-65% for patients experiencing further recurrences (Cornely *et al.*, 2012a; Johnson *et al.*, 2014).

Hypervirulent strains: A third difficulty has been the emergence of novel strains of *C. difficile*, and particularly the BI/NAP1/027 strain (McDonald *et al.*, 2005). Not only are such strains

typically less responsive than conventionally-described strains to antibiotic therapy, but they are often described as 'hypervirulent' (Pepin, Valiquette & Cossette, 2005), producing forms of protein exotoxin that cause particularly severe clinical disease. In particular, BI/NAP1/027 produces an additional toxin called binary toxin (Geric *et al.*, 2004), and may also produce larger amounts of toxins A and B than conventional strains (Warny *et al.*, 2005). BI/NAP1/027 has been associated with significant CDI outbreaks and epidemics throughout the UK, USA, Canada, and a large number of other territories (Pepin, Valiquette & Cossette, 2005). Fluoroquinolone use appears to have been a major precipitating factor to the emergence of this strain, with B1/NAP1/027 having a mutation conferring fluoroquinolone resistance (He *et al.*, 2013). This strain is also uniquely able to use low concentrations of the disaccharide trehalose as a carbon source (Collins *et al.*, 2018); trehalose was only widely-introduced into food supplies in Europe and North America approximately 25 years ago, and so this may have also contributed to the initial emergence of hypervirulent CDI epidemics. Whilst the incidence of BI/NAP1/027-related disease has started to fall in Western Europe, it persists as a problem in North America, and may even be increasing in certain regions, particularly Eastern Europe (Martin, Monaghan & Wilcox, 2016).

Such increasing complexities in treating CDI have been a major drive to identify novel antimicrobial strategies for tackling the condition. In general, these have taken a number of broad categories: novel antibiotics; immune modulation; and manipulation of the gut microbiota.

1.5.2. Novel antibiotics:

1.5.2.1. Fidaxomicin:

Fidaxomicin is a poorly-absorbed macrocyclic antibiotic with bactericidal properties. It has a much narrower spectrum than conventional anti-CDI antibiotics, limited to *C. difficile* and a relatively limited number of Gram-positive aerobes and anaerobes. It is administered orally, with very limited systemic absorption (Gerber & Ackermann, 2008). Its central mechanism of action appears to be through inhibition of the RNA polymerase of bacteria, although it appears to have anti-inflammatory properties in the intestine as well (Koon *et al.*, 2014).

Fidaxomicin has been compared to vancomycin for treatment of CDI in a number of randomised trials. In the initial Phase 3 trials, initial cure rates were comparable for both medications (Louie *et al.*, 2011; Cornely *et al.*, 2012a); however, the risk of subsequent CDI recurrence was much lower in patients treated with fidaxomicin as compared to vancomycin (15.4% *vs* 25.3%) (Louie *et al.*, 2011). Pooled analysis from both trials demonstrated that fidaxomicin and vancomycin appeared to have similar efficacy in causing remission from CDI for patients with a first CDI recurrence, but that rates of second recurrence were lower in patients treated with fidaxomicin than vancomycin (Cornely *et al.*, 2012b). A subsequent randomised trial demonstrated superiority of pulsed fidaxomicin to a standard vancomycin regimen in enacting sustained remission from CDI (Guery *et al.*, 2017).

Based on the results of these studies, fidaxomicin has now been incorporated as a treatment option for CDI into guidelines. Certain international guidelines have endorsed its use from the first episode of CDI (Debast *et al.*, 2014; McDonald *et al.*, 2018b), whilst current UK guidelines endorse its use only in the case of recurrent disease (Public Health England, 2013). However, there are a number of outstanding questions about the optimal use of fidaxomicin. On re-examination of the trial from Louie and colleagues, it was noted that the patients infected with the BI/NAP1/027 strain (more than one-third of the cohort) did not experience reduced recurrence following its use (Louie *et al.*, 2011). Whilst the data that exist suggest that fidaxomicin also has higher efficacy than vancomycin in treating severe/ fulminant CDI (Louie *et al.*, 2011; Cornely *et al.*, 2012a), these are derived from small subgroups as part of much larger trials, and are therefore difficult to generalise. Fidaxomicin is also expensive at present (with a ten day course costing approximately fourfold more than vancomycin and over a hundredfold more than metronidazole), and health economic analyses have questioned whether it is a cost effective alternative to other antibiotics (Bartsch *et al.*, 2013).

1.5.2.2. Other antibiotics:

A number of different antimicrobials which are also already licensed for non-CDI have also been evaluated for their efficacy in the treatment of CDI (including fusidic acid, rifaximin, teicoplanin and nitazoxanide), although none appear on current evidence to be superior to conventional anti-CDI antimicrobials in isolation (Nelson, Suda & Evans, 2017).

There are also several other antimicrobials with potent activity against *C. difficile in vitro* which are at different stages of clinical development, but progress has overall been limited. Ridinilazole has recently been demonstrated to be non-inferior to vancomycin in the treatment of CDI in a Phase 2 trial (Vickers *et al.*, 2017). The novel bactericidal cyclic lipopeptide surotomycin demonstrated non-inferiority to vancomycin in one Phase 3 trial (Daley *et al.*, 2017) but not another (Boix *et al.*, 2017), and failed to show superiority to vancomycin in either of these trials. Cadazolid, a novel quinoxolidinone antibiotic, had shown promise in Phase 2 trials, but failed to show non-inferiority to vancomycin in the treatment of CDI in two Phase 3 trials (Gerding *et al.*, 2019).

1.5.2.3. Other novel antibacterial strategies:

One approach of recent interest is the use of agents to inactivate any antibiotics where they are excreted in the gut, with the aim of minimising their exposure to the gut microbiota. For instance, in the scenario whereby a patient is receiving broad-spectrum intravenous antibiotics for sepsis, any intestinal excretion of these antibiotics may increase risk of CDI via perturbation of the gut microbiota (**Section 1.5.4.1**). One such agent is DAV132, an activated charcoal sorbent coated with a pH-dependent enteric polymer, that sequesters antibiotic residues within the distal gut (de Gunzburg *et al.*, 2018). An alternative approach is the use of ribaxamase (SYN-004); this is a recombinant β-lactamase enzyme designed to avidly degrade any β-lactam antibiotics being administered systemically that overspill into the gut (Bristol *et al.*, 2017). In a double-blind Phase 2b trial, patients receiving ceftriaxone as treatment for pneumonia were randomised to also receive either ribaxamase or placebo four times daily during, and for 72 hours after, treatment with ceftriaxone; rates of CDI were lower in the ribaxmase-treated group, but only with a one-sided *p* value of 0.045 (Kokai-Kun *et al.*, 2019).

Another strategy of interest has been the use of anion-binding resins to bind and remove *C. difficile* toxin. Experience with the most well-established anion-binding resins, colestipol and cholestyramine, is relatively-limited but disappointing, with apparent limited efficacy observed (Mogg *et al.*, 1980; Kreutzer & Milligan, 1978). Whilst initial trials with a resin designed specifically for CDI, tolevamer, were promising, the agent demonstrated inferiority

to both metronidazole and vancomycin in treatment of CDI in two randomised controlled trials (Johnson *et al.*, 2014).

1.5.3. Immune modulation:

There has been research exploring both active and passive immunisation as treatment strategies against *C. difficile.*

The focus of attempts at active immunisation has been the design of vaccines against TcdA and TcdB (although other components of *C. difficile*'s pathogenicity have been considered as vaccine candidates too, including S-layer proteins and flagellar proteins). The leading recent candidate for a possible vaccine had been developed by Sanofi; despite promising results from a Phase 1 trial, a Phase 3 trial was terminated early because of apparent low efficacy, and further clinical development of the vaccine was discontinued (Sanofi, 2017). However, there is still interest from other parties in pursuing this area, with a vaccine candidate from Valneva having completed Phase 2 trials (Valneva, 2019) and one from Pfizer in Phase 3 trials (Pfizer, 2019) at the time of writing.

There has been significant recent progress in the field of passive immunisation. The basis for this approach arises from the observation that the higher the level of serum antibodies that humans who have had recent primary CDI develop against TcdA (Kyne *et al.*, 2001b) and TcdB (Leav *et al.*, 2010), then the greater the apparent protection against the development of CDI recurrence. Based on the results of two double-blind, placebo-controlled randomised controlled trials (RCTs) (MODIFY I and II), together involving 2655 patients, the anti-TcdB human monoclonal antibody *bezlo*toxumab has been licensed for use as an agent to limit recurrence of CDI (Wilcox *et al.*, 2017). In these studies, adults with CDI received an infusion of bezlotoxumab or placebo together with standard oral anti-CDI antibiotics, and rates of CDI recurrence were significantly reduced within the bezlotoxumab arm. The response rate in patients receiving bezlotoxumab did not appear to be influenced by which antibiotics were co-administered, although the study had not been designed to specifically evaluate this. The addition of an anti-TcdA antibody, actoxumab, did not result in any additional benefit (Wilcox *et al.*, 2017), which may suggest that toxin B is the major determinant of virulence within recurrent CDI in humans. No comment was made in these trials upon the effect of actoxumab

or bezlotoxumab upon the development of endogenous anti-TcdA and/or -TcdB antibodies within humans following recent CDI.

There are still some remaining uncertainties related to bezlotoxumab. Firstly, the specific mechanism of action remains incompletely defined, since *C. difficile* toxin is found within the intestinal lumen, and circulating antibodies do not typically cross the intact intestinal barrier. It is presumed that in CDI with severe colitis, a disrupted gut barrier allows paracellular transport of antibodies into the intestinal lumen. Secondly, one of the most common side effects of the use of bezlotoxumab is diarrhoea (Wilcox *et al.*, 2017), which complicates assessment of its efficacy. Finally, the currently-limited data on its efficacy in special cases (e.g. severe and/or 027 disease) and minimal cost effectiveness analysis means that its optimal role in CDI treatment algorithms remains uncertain.

1.5.4. Manipulation of the gut microbiota:

1.5.4.1. The concept of colonisation resistance:

Achieving colonisation within the host is the first step taken by pathogenic bacteria in the establishment of infection. Since the 1950s (Bonhoff, Drake & Miller, 1954), there has been recognition of the concept that the healthy microbiota has the capacity to prevent colonisation of mucosal surfaces by pathogenic bacteria, and any insult that perturbs the microbiota may disrupt this functionality. The hypothesis that healthy host microbial communities are stable environments with the ability to prevent colonisation and/or outgrowth of pathogens is referred to as *colonisation resistance* (Lawley & Walker, 2013).

Recent advancements in culture-free characterisation of the gut microbiota, arising from developments in microbial sequencing technologies (see **Section 2.2.1**) has helped to define the specific changes in gut microbiota composition that occurs in response to antibiotics and which therefore characterises the gut microbiota in patients with CDI. Specifically, at the phylum level, this has been recognised for the last decade to include an almost total loss of *Bacteroidetes,* a clear reduction in *Firmicutes,* and a marked increase in the relative abundance of *Proteobacteria* (Khoruts *et al.*, 2009).

As such, the concept has arisen that CDI may be viewed as a primarily ecological disease defined by loss of colonisation resistance, whereby profound disruption of the composition and functionality of the gut microbiota in response to antibiotics (or other insults to the gut microbiota) establishes an ecological niche favouring *C. difficile* colonisation, spore germination and vegetative growth. Whilst antimicrobial therapy for CDI kills *C. difficile,* it does not in itself restore colonisation resistance; indeed, it may disrupt it yet further, given that no antimicrobial therapy has particular specific activity against *C. difficile* (or phylogenetically-similar bacteria) only.

By extension, the concept arose that restoration of the gut microbiota to a pre-morbid state may restore colonisation resistance, and as such may be a novel therapeutic approach to treating CDI. As such, one recent major area of interest in CDI therapeutics has been focused on the manipulation of the gut microbiota, and a number of different such approaches have been attempted.

1.5.4.2. Probiotics:

Probiotics are defined by the Food and Agriculture Organisation of the United Nations/ World Health Organisation as, 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Joint FAO/WHO Working Group Report, 2002). Most commonly-available probiotic preparations include a single strain or a combination of *Lactobacilli, Bifidobacteria*, and/ or other gut commensal bacteria. The probiotic yeast *Saccharomyces boulardii* is considered separately (see **Section 1.6.6.2.2**).

The aim of probiotic use is to administer microorganisms that can easily colonise the gut (albeit possibly transiently), and which restore a missing functionality of the disturbed gut microbiota. There are a number of postulated mechanisms by which probiotics may act in this way act treat CDI, including: by competition for the same ecological niche (e.g. competition for nutrition) (Wullt *et al.*, 2007); through the production of antimicrobial chemicals (e.g. acidic metabolites that lower gut pH) (Gill, 2003); via interference with the process of *C. difficile* toxins interacting with the colonic epithelium (Banerjee, Merkel & Bhunia, 2009); or via immunomodulatory actions (e.g. promotion of natural killer (NK) cell activity) (Gill, Rutherfurd & Cross, 2001).

There are a number of difficulties in performing and interpreting trials of probiotics for the prevention or treatment of CDI, particularly related to the formulation used. In particular, trials have been highly heterogenous in the particular strains used, their titre, the length of administration, the co-administration of acid-suppressing medication, etc. The clinical evidence of probiotics for the *prevention* of CDI is contradictory. For instance, a trial of 2941 patients aged at least 65 years old at risk of CDI randomised participants to 21 days of a multi-strain preparation of *Lactobacilli* and *Bifidobacteria,* or placebo; no reduction in CDI rates was seen in participants treated with probiotics compared to placebo (Allen *et al.*, 2013). In contrast, other trials have made the opposite conclusion. This includes a double-blind trial performed at Imperial College Healthcare NHS Trust/ Imperial College, where 135 hospitalised adult patients who were taking antibiotics were randomised to receive either a probiotic drink (containing *Lactobacillus casei, L. bulgaricus,* and *Streptococcus thermophilus*) or placebo (a sterile milkshake) twice daily (Hickson *et al.*, 2007). The probiotic or placebo was administered to patients during their antibiotic course and for one week after the course finished. No patients in the probiotic group and 9/53 (17%) in the placebo group developed (p=0.001), equating to an absolute risk reduction of 17% (7% to 27%), and number needed to treat of 6 (4 to 14) (Hickson *et al.*, 2007). Furthermore, two recent large meta-analyses have concluded that probiotic administration in people at risk of CDI may cause a reduction in relative risk of >50%, with risk reduction most marked in participants with the highest baseline risk of CDI (Shen *et al.*, 2017; Goldenberg *et al.*, 2017). Evidence for the use of probiotics in the *treatment* of CDI is weak, with any possible benefit only in the setting of recurrent CDI (Pillai & Nelson, 2008).

There remain outstanding concerns regarding the use of probiotics in this setting. One concern relates to a number of case reports of bacteraemia/ fungaemia attributable to probiotic use, with the strongest risk factors including immunosuppression, recent surgery, multiple comorbidities and prolonged hospitalisation (Boyle, Robins-Browne & Tang, 2006). The further major concern is related to a recent study investigating the influence of probiotic administration in antibiotic-treated mice upon gut microbiota dynamics. This study reported that antibiotic-treated mice who were subsequently administered probiotics experienced a delayed and persistently incomplete restitution of their stool and mucosal microbiota compared to the spontaneous recovery observed in mice not administered antibiotics (Suez

et al., 2018). Whilst there has been debate following this study about the appropriateness of the probiotic preparation used in mice and apparent marked disparities in outcome between administration of probiotics to mice and humans (Rackaityte & Lynch, 2018), this study has nevertheless added to the reservations regarding probiotics and CDI.

As such, based on the contradictory clinical data to date and outstanding concerns, probiotics are not recommended either as prevention or treatment for CDI within guidelines (Public Health England, 2013; Debast *et al.*, 2014; McDonald *et al.*, 2018b).

1.5.4.3. Non-toxigenic *Clostridioides difficile:*

It has been well-established that a significant proportion of hospital inpatients are colonised with non-toxigenic strains of *C. difficile,* and that such colonised patients are less likely than those who are not to become colonised/ infected with toxigenic forms of *C. difficile* and subsequently develop CDI (Shim *et al.*, 1998). The leading hypothesis explaining this was that toxigenic and non-toxigenic forms of *C. difficile* would compete for the same ecological niche within the gut, and hence prior colonisation with non-toxigenic forms of *C. difficile* limits the ability of toxigenic *C. difficile* to also colonise.

Based upon this, Gerding and colleagues investigated the impact of administration of spores of non-toxigenic *C. difficile* for the attempted prevention of recurrence of CDI (Gerding *et al.*, 2015). In a Phase 2, randomised, double-blind, placebo-controlled trial, patients with CDI (either primary episode or first recurrence), who had successfully completed CDI therapy with metronidazole or vancomycin, were administered spores of non-toxigenic *C. difficile* at one of three doses, or placebo. The specific strain of non-toxigenic *C. difficile* chosen, M3, was chosen on the basis that it appeared to easily colonise antibiotic-treated hospitalised patients. The major finding was a significantly reduced CDI recurrence rate in patients treated with non-toxigenic *C. difficile* spores compared to placebo (11% (*n*=14/125) *vs* 30% (*n*=13/43) respectively) (Gerding *et al.*, 2015).

A number of reservations have been raised about this approach. Colonisation of non-toxigenic *C. difficile* was relatively short-lived, but the authors argued that spore therapy may still have utility for providing transient protection during which more definitive anti-CDI

therapy could be administered. Another concern relates to the possibility of transfer of toxin genes from toxigenic *C. difficile* into non-toxigenic strains, especially since it has been demonstrated that it is possible for this to occur (Brouwer *et al.*, 2013).

1.5.4.4. Introduction to faecal microbiota transplant:

One other approach for reconstituting the disrupted gut microbiota in patients with CDI would be to take the entire gut ecological habitat of a healthy person – including all biotic and abiotic factors, ideally with as little perturbation as possible – and transplant this into a CDI patient, with the primary aim of restoring colonisation resistance. An easily-accessible means of sampling a healthy person's gut microbiota is through collection of a stool sample. As such, this has been the drive for *faecal microbiota transplant (FMT),* i.e. the process of taking a stool sample from a healthy, screened donor; processing this in a laboratory into a liquidised microbiota suspension; and administration of this into an affected patient (Mullish *et al.*, 2015). Despite the unusual nature of this intervention, it has recently become well-embedded in clinical practice as an efficacious treatment option for recurrent CDI. The next section discusses in detail the current clinical status of FMT.

1.6. Faecal microbiota transplant in clinical practice:

1.6.1. Demonstration of efficacy of faecal microbiota transplant as treatment for recurrent *Clostridioides difficile* infection:

While the basic concept associated with faecal transplantation has been long recognised in veterinary medicine, its recognition as a possible entity for the treatment of humans was not until the mid-Twentieth Century. Specifically, the first description of faecal transplantation in Western medical literature is from 1958, when the surgeon Eiseman (together with colleagues in Colorado, USA), reported on four patients with pseudomembranous enterocolitis (three of whom were critically ill), who all responded rapidly to treatment with faecal enemas (Eiseman *et al.*, 1958). From this point, a number of intermittent case reports and (generally small) case series consistently reported that FMT was apparently safe and effective in the treatment of CDI, but FMT remained very much on the fringes of medical interest.

The huge interest that currently surrounds FMT was precipitated by the publication of the first randomised controlled trial investigating the efficacy of FMT in the treatment of rCDI in 2013 (van Nood *et al.*, 2013). In this study, patients with multiple recurrences of CDI were randomised to one of three arms: FMT via nasoduodenal tube; vancomycin alone (the conventional standard of care), or vancomycin together with bowel purgative. FMT was freshly-prepared on the day of administration and collected from healthy unrelated screened donors. The primary end point was the resolution of diarrhoea without relapse at 10 weeks after FMT. The trial was stopped early after an interim analysis by the overseeing ethics board, because of the markedly higher proportion of people reaching the primary outcome in the FMT arm compared to those in both vancomycin arms. Specifically, 81% (*n*=13/16) of patients in the FMT arm reached the primary endpoint after the first FMT, with two of the three patients not responding to the first FMT going on to respond to a second administration. In contrast, only 31% (*n*=4/13) of patients in the vancomycin alone and 23% (*n*=3/13) in the vancomycin/ bowel purgative group reached the primary endpoint ($p<0.001$ for both vancomycin arms *vs* the FMT arm). Significantly, no serious adverse events were reported, with self-limiting gastrointestinal and constitutional symptoms being the main complications found.

From this study, another randomised trial soon followed, with researchers reporting the superiority of colonoscopic-administered FMT to vancomycin in treating rCDI (Cammarota *et al.*, 2015). Again, FMT was highly-effective, with no serious adverse events (albeit, as with the initial randomised trial, with relatively limited follow-up). Subsequent randomised trials have consistently demonstrated FMT to have a treatment efficacy of >80% (and superior efficacy to vancomycin) for the treatment of rCDI regardless of the route of administration used, including via nasogastric tube (Youngster *et al.*, 2014b) or as multiple enemas (Lee *et al.*, 2016) (but not superiority when only a single enema is used (Hota *et al.*, 2017)).

In response to criticisms that these early randomised trials had been largely open label, a randomised controlled double-blind clinical trial of FMT as treatment for rCDI was also performed (Kelly *et al.*, 2016). Patients with rCDI were randomised to colonoscopically receive either healthy donor stool, or their own stool re-transplanted ('autologous FMT'). The key finding was significantly higher rates of remission in the arm of patients receiving healthy

donor stool, but intriguingly, 62.5% (*n*=15/24) of patients in the autologous FMT also experienced resolution of their diarrhoea. Of further interest, rates of remission after autologous FMT were significantly higher in one of the recruiting sites than the other (Kelly *et al.*, 2016).

Until recently, no clinical data had been presented describing the relative efficacy of FMT or fidaxomicin as treatment for rCDI, with vancomycin conventionally used as the comparison arm in previous clinical trials. However, a recent RCT recruited patients with multiple recurrences of rCDI and randomised them to either vancomycin, fidaxomicin or FMT (administered either colonoscopically or via nasojejunal tube) (Hvas *et al.*, 2019). Results from this study noted significantly higher remission rates in patients treated with FMT in comparison to those treated with either fidaxomicin or vancomycin (with no significant difference in remission rates between patients in the fidaxomicin or vancomycin arms) (Hvas *et al.*, 2019).

Most trials to date investigating FMT as treatment for rCDI have prepared FMT in ambient air, and use a single donor's stool sample for each unit of faecal transplant. Of interest, however, of the four randomised trials published to date exploring FMT as treatment for ulcerative colitis (see **Section 1.6.4**), the highest rates of remission appear to occur in patients treated with FMT prepared anaerobically and derived from pooled healthy donor samples (Costello *et al.*, 2019, 2017).

1.6.2. Development of faecal microbiota transplant as a viable clinical treatment for *Clostridioides difficile* infection:

1.6.2.1. Frozen FMT:

One key factor limiting the practicality of use of FMT more widely in the clinical setting was the complexity of logistics in obtaining a fresh sample, performing the laborious process of homogenisation and filtration of stool to generate FMT, followed by administration of the slurry to the patient, all within a few hours. Hamilton and colleagues were the first to describe a standardised protocol for the preparation of FMT using 10% glycerol as cryopreservative; they further demonstrated comparable efficacy of FMT prepared fresh to that pre-prepared and kept in the freezer for a prolonged period, before being easily thawed shortly prior to

administration (Hamilton *et al.*, 2012). Further research using plate-based culture demonstrated that microbial communities cultured from a fresh FMT (*vs* that from frozen/ glycerol-containing FMT, cultured after six months at -80^{o}C) were comparable (Costello *et al.*, 2015), although no assessment of bacterial functionality/ metabolism was performed. Subsequent further simplification of protocols for the preparation of frozen FMT (Satokari *et al.*, 2015) again increased potential practicality for its more widespread use. Fresh and frozen FMT were also demonstrated as having similar efficacy for the treatment of rCDI in a randomised trial setting, both when administered colonoscopically (Jiang *et al.*, 2017) and when administered via multiple enemas (Lee *et al.*, 2016).

The widespread rapid acceptance of frozen FMT as an efficacious option as treatment for rCDI has been a key precipitating factor for the development of 'stool banks' in many areas of the world. These banks are typically organisations who recruit and maintain their own donor pools, and retain significant stocks of pre-prepared frozen FMT, which they can rapidly transport to healthcare facilities where required. The best-established of these is the US stool bank OpenBiome, which now supplies more than 1000 FMT preparations per month (OpenBiome, 2019).

1.6.2.2. Capsulised FMT:

There are a number of different patient groups for which conventional administration routes for FMT slurry present difficulties. For instance, the elderly, frail patients with multiple co-morbidities who are the typical patient cohort seen with rCDI may not be able to safely undergo lower GI endoscopy, or may not be able to retain the material for any significant period of time even where they can undergo the procedure. Similarly, upper GI administration of FMT may be unsuitable for patients at risk of regurgitation (e.g. large hiatus hernia, severe gastro-oesophageal reflux disease, etc), and/or those with swallowing disorders (Mullish *et al.*, 2018d). As such, there has been a drive for the establishment of less invasive administration routes.

One such possible route of interest has been the use of capsulised FMT. Following on from case series demonstrating promising efficacy data when capsulised FMT slurry was administered to patients with rCDI (Youngster *et al.*, 2016, 2014a) (as well as when lyophilised

FMT was capsulised instead of conventional frozen slurry (Hecker *et al.*, 2016)), the potential role of capsulised FMT was investigated in a randomised trial setting. In a recent large RCT, patients with rCDI were randomised to receive thawed frozen FMT either via colonoscopy or via capsules (one treatment of 40 capsules) (Kao *et al.*, 2017). On per protocol analysis, remission at 12 weeks after a single treatment occurred in 96% in both arms (*n*=51/53 by capsule, *n*=50/52 by colonoscopy).

Whilst capsulised FMT has grown in favour, unresolved issues remain regarding its use (Mullish *et al.*, 2018d). In particular, capsules are often large and cannot be chewed, and swallowing >30 capsules in a single day may be a significant undertaking for the typical CDI patient, who may be frail and elderly with an existing high pill burden.

1.6.2.3. Safety and efficacy in special groups, and complications of FMT:

Retrospective analysis has demonstrated FMT to have apparently comparable efficacy (and no specific additional safety concerns) when used as treatment for rCDI in patients with a wide range of immunocompromised states (including the use of immunosuppressive medications) (Kelly *et al.*, 2014; Cheng *et al.*, 2019). FMT also appears efficacious when administered to patients with inflammatory bowel disease (IBD) and super-added rCDI; whilst generally safe in this setting, there appears to be a potential small but significant risk (~5%) of an IBD flare when applied in this context (Qazi *et al.*, 2017).

In all the randomised trials assessing FMT as treatment for rCDI, second (or even further FMTs) have been shown to be a suitable option for patients not resolving after a single FMT. A consistent finding has been that patients with severe/complicated rCDI (and particularly those with pseudomembranous disease) are a group less likely to enter remission after a single FMT. Two cohort studies have shown that a course of several closely-interspersed FMTs (with short courses of vancomycin or fidaxomicin between FMTs) appears to have high efficacy in treating severe/complicated CDI, and/or pseudomembranous disease (Fischer *et al.*, 2015; Ianiro *et al.*, 2018). There have now been several small studies (including randomised trials) investigating the use of FMT (or similar 'bacteriotherapy') as treatment for first episodes of CDI, but collective results have not been strongly compelling for FMT's efficacy in this setting (Juul *et al.*, 2018; Lagier *et al.*, 2015; Camacho-Ortiz *et al.*, 2017).

The most common side effects associated with the use of FMT for rCDI are self-limiting constitutional and/or GI symptoms (Baxter & Colville, 2016; de Groot *et al.*, 2016). However, a number of more serious adverse events have also been described (including deaths) primarily related to endoscopic administration, including aspiration at the time of a colonoscopic FMT (Kelly *et al.*, 2014), and faecal regurgitation and vomiting when administered via the upper GI route (Baxter *et al.*, 2015; de Groot *et al.*, 2016). A further concern is that given the relative novelty of FMT as treatment for rCDI, it is unknown whether there are any long-term complications associated with its use; however, in a cohort of 84 recipients of FMT followed-up for a mean of 3.8 years, no adverse long-term health sequelae were observed (Jalanka *et al.*, 2018).

As such, FMT appears to be a safe and efficacious option for rCDI in almost all patient cohorts, although caution should still be exercised for those patients where there are theoretical risks of harm and limited clinical experience, including pregnancy, severe food allergies, etc (Mullish *et al.*, 2015).

1.6.3. Current status of faecal microbiota transplant as treatment for *Clostridioides difficile* infection, and outstanding concerns:

Given this clinical evidence supporting the safety and efficacy of FMT as treatment for rCDI (coupled with health economic analysis demonstrating its cost effectiveness (Konijeti *et al.*, 2014)), FMT has been assessed by national and international authorities for its inclusion within recommended algorithms for the treatment of rCDI. At the time of writing, FMT is recommended for this indication by, amongst others, US guidelines (McDonald *et al.*, 2018b; Surawicz *et al.*, 2013a), European recommendations (Debast *et al.*, 2014; Cammarota *et al.*, 2017), and by a number of authorities within the UK, including NICE (National Institute for Health and Care Excellence (NICE), 2014), Public Health England (Public Health England, 2013), and in joint British Society of Gastroenterology (BSG)/ Healthcare Infection Society (HIS) guidelines (Mullish *et al.*, 2018d, 2018c). While many key aspects of recommendations regarding FMT in such guidelines are, by the very nature of the intervention, based upon expert consensus opinion rather than experimental evidence (e.g. recommended protocol for laboratory screening of donors; see **Table 1.3A and B**), such guidelines nevertheless serve an important role in defining best practice and standardising care. Some countries have begun

to establish national registries of all FMTs performed within that territory to enable more formal identification of safety and efficacy, and factors which may influence success of the procedure (Kelly *et al.*, 2017; Peri *et al.*, 2019).

Table 1.3:

A

Pathogen screening:

- Hepatitis A IgM
- Hepatitis B (HBsAg and HBcAb)
- Hepatitis C antibody
- Hepatitis E IgM
- HIV -1 and -2 antibodies
- HTLV-1 and -2 antibodies
- *Treponema pallidum* antibodies (*TPHA, VDRL)*
- Epstein-Barr virus IgM and IgG*
- Cytomegalovirus IgM and IgG*
- *Strongyloides stercoralis* IgG
- *Entamoeba histolytica* serology

General/ metabolic screening:

- Full blood count with differential.
- Creatinine and electrolytes
- Liver enzymes (including albumin, bilirubin, aminotransferases, gamma-glutamyltransferase and alkaline phosphatase).
- C-reactive protein

B

- *Clostridium difficile* PCR
- *Campylobacter, Salmonella,* and *Shigella* by standard stool culture and/ or PCR
- Shiga toxin-producing *Escherichia coli* by PCR.
- Multi-drug resistant bacteria, at least carbapenemase-producing *Enterobacteriaceae* (CPE) and extended-spectrum beta-lactamases (ESBL)*.
- Stool ova, cysts and parasite analysis, including for *Microsporidia.*
- Faecal antigen for *Cryptosporidium* and *Giardia.*
- Acid fast stain for *Cyclospora* and *Isospora.*
- *Helicobacter pylori* faecal antigen.
- Norovirus, Rotavirus PCR.

Table 1.3 (page 45): Recommended laboratory screening protocol of potential stool donors from BSG/ HIS FMT guidelines. A: Recommended minimal blood screening for stool donors; B: Recommended minimal stool screening for stool donors. *EBV and CMV testing is only recommended where there is the potential that the FMT prepared from that donor will be administered to immunosuppressed patients at risk of severe infection if exposed to CMV and EBV; **Whilst CPE and ESBL are the only multi-drug resistant bacteria that are recommended to be screened for universally, consider testing for other resistant organisms (including vancomycin-resistant *Enterococci* (VRE) and/ or methicillin-resistant *Staphylococcus aureus* (MRSA)) based upon risk assessment and local prevalence. The guideline recommended that before FMT produced from a particular donor may be used clinically, the donor should have completed health questionnaires and laboratory screening both before and after a period of stool donation. Their FMT material should remain in 'quarantine' within a freezer until the second screen has been passed, and only be released for use at this point. Figure reproduced from (Mullish *et al.*, 2018c, 2018d).

However, a number of major concerns still exist regarding the use of FMT as treatment for rCDI in practice (Mullish *et al.*, 2018e). The idea of receiving FMT is clearly unpalatable, and the conventional invasive routes of administration have associated risks, as already discussed; whilst capsulised FMT may reduce these concerns, it clearly does not ameliorate it entirely (see **Section 1.6.2.2**). Despite careful donor screening, there is still at least a theoretical risk of transmission of infection via FMT. There are a large number of conditions (both GI and otherwise) which have been associated with perturbation of the composition and functionality of the gut microbiota (Marchesi *et al.*, 2016); as such, concerns exist that although a FMT may resolve rCDI, it may potentially be at the significant cost of transmission of a 'microbiota trait' associated with increased risk of the future development of a novel condition, such as inflammatory bowel disease. A further difficulty regarding the use of FMT relates to the complex regulation associated with its use (Mullish *et al.*, 2018e; Mullish & Williams, 2015). Within the UK, whilst the introduction of the recognition of FMT as a 'medicinal product' (and the regulatory structure that occurs in association with that) by the Medicines and Healthcare products Regulatory Authority (MHRA) ensures high-quality FMT production, this may have had the unintentional effects of restricting the number of centres with the capacity to meet the required standard of production, and limitation of patient access to FMT as a result (Quraishi *et al.*, 2017).

Whilst FMT is highly-effective at treating rCDI, there is evidently a small but appreciable failure rate even in patients treated with multiple FMTs. However, at present, it remains

unclear whether this reflects donor or recipient factors. Furthermore, there is currently no defined biological means by which donor and recipient are matched.

Collectively, these factors present a compelling case for further research to better understand the specific mechanisms by which FMT acts to cause remission from rCDI. Elucidating these mechanisms may allow more rational selection of stool donors, and might be exploitable for the development of novel, more targeted therapies for rCDI which avoid the drawbacks associated with FMT. At present, despite a number of theories (Khoruts & Sadowsky, 2016), such mechanisms are not well-defined.

1.6.4. Other potential directions for faecal microbiota transplant:

There are an ever-growing number of medical conditions that have been associated with distinctive perturbations of the composition and/or functionality of the gut microbiota; however, outside of CDI, it remains largely unclear whether such changes are cause, consequence or incidental to the disease in question (Marchesi *et al.*, 2016). However, given the marked success of FMT as treatment for rCDI – and the presumption that this acts through restoration of a critically-altered gut ecosystem back to a premorbid state – there is widespread interest in exploring the potential clinical efficacy of FMT in non-CDI states.

Non-CDI conditions in which FMT has been investigated either in case series or in a trial setting include inflammatory bowel disease (particularly ulcerative colitis (UC) (Moayyedi *et al.*, 2015; Rossen *et al.*, 2015; Paramsothy *et al.*, 2017; Costello *et al.*, 2019)), irritable bowel syndrome (Johnsen *et al.*, 2018; Halkjær *et al.*, 2018), refractory immune checkpoint inhibitor-related colitis (Wang *et al.*, 2018), obesity and metabolic syndrome (Vrieze *et al.*, 2012; Kootte *et al.*, 2017; de Groot *et al.*, 2019; Allegretti *et al.*, 2019b), intestinal decolonisation of multi-drug resistant organisms (Huttner *et al.*, 2019), autistic spectrum disorders (Kang *et al.*, 2017), hepatic encephalopathy (Bajaj *et al.*, 2017), alcoholic hepatitis (Philips *et al.*, 2017), and primary sclerosing cholangitis (PSC) (Allegretti *et al.*, 2019a). Despite some promising early efficacy data from some of these trials, FMT is at present not recommended for use in any condition other than CDI (Mullish *et al.*, 2018d).

As such, research elucidating the contribution of the gut microbiota to CDI and mechanisms of efficacy of FMT in treating rCDI may serve as a paradigm for future research focused on better understanding the pathogenesis of a wider-range of human diseases, and/or the 'mining' of the gut microbiota to aid the development of novel, targeted therapeutics. Given the high prevalence, significant morbidity – and potential mortality – associated with many of these non-CDI conditions in which FMT is being trialled, there are clearly significant potential clinical implications for this direction of research.

1.6.5. Refinement of faecal microbiota transplant:

A number of different case series and clinical trials of FMT as treatment for rCDI have applied microbial sequencing techniques to compare gut microbiota profiles (predominantly through stool analysis, although also through mucosal biopsy assessment) between pre- and post-FMT. A consistent observation has been the marked recovery in a number of ecological metrics after FMT (including increased microbial diversity and richness), as well as a change in gut microbiota profile to a pattern comparable to that of healthy stool donors, particularly with regards to recovery of the phyla *Bacteroidetes* and *Firmicutes* (Khoruts *et al.*, 2009; van Nood *et al.*, 2013) (discussed in detail in **Section 3.4.1**). Gut microbiota changes may be generally more variable and less marked after FMT for non-CDI indications, but are nonetheless observed with relative consistency in such studies, particularly those related to UC (Paramsothy *et al.*, 2018). As such, a major area of interest from both the research and pharmaceutical communities has focused on whether results from gut microbiota profiling studies such as this may be exploited to aid development of a refined, simplified and/or more standardised form of FMT, with the major focus at present being upon the treatment of rCDI.

One such approach has been to try and produce a 'broad spectrum' microbiota product derived from human stool, essentially a standardised form of FMT preparation. This has been the approach taken by one group of researchers allied to Rebiotix (a microbiota therapeutics company in the USA), who investigated the efficacy of enema administration of their product RBX2660 (a stool-derived product of donor stool diluted in saline) in a randomised trial (Dubberke *et al.*, 2018). While RBX2660 is described by Rebiotix as a 'standardised product', it is notable that full standardised operating protocols related to its preparation have not been made available, and the extent to which this differs from conventional FMT processing

remains unclear. A Phase 2B trial comparing three groups (two doses of RBX2660; two doses of placebo; or one dose of RBX2660 and one dose of placebo) did not show efficacy for the primary outcome, prevention of recurrent CDI at week 8 after two doses of RBX2660 (Dubberke *et al.*, 2018). As such, the likelihood of success of this strategy remains uncertain.

One alternative approach is the attempted generation of a 'narrow spectrum' microbiota product of well-characterised bacteria, sometimes referred to as a 'defined microbial community'. There is an evidence-base to suggest that this approach may be feasible, with one key piece of research in this area being the 'RePOOPulate' study (Petrof *et al.*, 2013). In this study, 33 different commensal bacterial species were cultured from the stool of healthy donors, and these were used to generate a 'stool substitution therapy' consisting of a mixture of purified bacterial cultures derived from these stool-derived bacteria. When administered colonoscopically to two patients with rCDI, both patients achieved a rapid and sustained remission (Petrof *et al.*, 2013). In another study from Khanna and colleagues (Khanna *et al.*, 2016), healthy donor stool was first treated with ethanol (to kill vegetative cells); the resulting spores were fractionated and capsulised, and delivered orally as a preparation named SER-109. In a cohort of 30 patients, 29 achieved clinical remission from rCDI after one or two administrations of SER-109 (Khanna *et al.*, 2016). However, despite this early promise, SER-109 produced negative results when administered in a Phase 2 clinical trial. There have been several postulated reasons for the negative results from this study, including the possibility that patients were colonised by (but not actually infected with) *C. difficile,* and/or inadequate spore selection and dosing (Seres Therapeutics, n.d.).

1.6.6. Mechanisms of efficacy of faecal microbiota transplant in the treatment of recurrent *Clostridioides difficile* infection:

1.6.6.1. Introduction:

Given that these above-described approaches for 'next generation' FMT have met with only guarded success, more emphasis has again been placed on the importance of improved understanding of the specific mechanisms of efficacy of FMT. Whilst the conclusion from these studies (and particularly from 'RePOOPulate') is that a bacterially-derived factor alone may be sufficient to mediate a cure from rCDI, there has until recently been few such candidate factors identified. One leading theory has been that a potential mechanism of

action may be competitive niche exclusion (e.g. out-competition of *C. difficile* by the restored gut microbial community for a similar nutritional niche) (Khoruts & Sadowsky, 2016).

Other recent related data of interest arose from a pilot study reported by researchers in Germany (Ott *et al.*, 2017). In this study, researchers prepared a sterile faecal filtrate by passing FMT slurry through progressively narrower pore filters, culminating in a 0.2μm pore filter. Sterile faecal filtrate was administered to five patients with rCDI via a nasojejunal tube; all five patients rapidly entered a sustained remission (>6 months) from their CDI, comparable to that degree of efficacy seen after administration of conventional FMT. The authors concluded that, rather than FMT directly requiring live intact bacteria for its efficacy, it was instead likely that one or more soluble factors associated with bacteria within the filtrate potentially mediated its mechanism of action (Ott *et al.*, 2017). Specific potential examples included bacteriophages, bacteriocins, other bacterially-derived proteins, and/or gut metabolites (either those fully-derived from bacteria, and/or host-derived metabolites which underwent downstream metabolism by gut microbiota community members) (Khoruts & Sadowsky, 2016; Baktash *et al.*, 2018).

The following section will summarise the leading theories related to mechanisms of efficacy of FMT in the treatment of rCDI. Whilst FMT-mediated changes in host immunological function are a related area of interest and starting to be defined (e.g. (Burrello *et al.*, 2018)), they are not considered in detail in this section, but considered further within the **Discussion** (**Section 6.3.2**).

1.6.6.2. Non-bacterial gut microbiota components - bacteriophages and fungi:

1.6.6.2.1. Bacteriophages:

Bacteriophages (also known as phages) are viruses which can replicate within bacteria and archaea. The key interaction between bacteriophages and bacteria is mediated by specific receptors on bacterial cells, and this also dictates the host range of bacteriophages. *Lytic* bacteriophages are defined as those which are able to infect, replicate within, and subsequently lyse bacterial cells. In contrast, *lysogenic* bacteriophages typically integrate their genome into host DNA and exploit host cellular machinery to multiply, or can establish their genome as a plasmid within bacterial cells, without immediate bacterial cell lysis (Ofir &

Sorek, 2018). The interaction between bacteriophages and the gut microbiota is complex and bidirectional, with phages able to directly influence both the composition and aspects of functionality (e.g. biofilm formation) of the microbiota (Mirzaei & Maurice, 2017). Bacteriophages are now recognised as the predominant component of the gut microbiota, and may be up to 20 times more abundant than bacteria in mucosal samples (Barr *et al.*, 2013).

Bacteriophages which able to infect *C. difficile* are well-recognised, and belong to the order *Caudovirales* and families *Myoviridae* and *Siphoviridae* (Hargreaves & Clokie, 2015). 'Phage therapy' – applying a combination of phages with established infectivity for *C. difficile* as potential anti-CDI treatment – has shown a potent killing effect against a range of *C. difficile* strains (including pathogenic strains) in a number of CDI models, ranging from batch fermentation (Nale *et al.*, 2018), to wax moths (*Galleria mellonella*) (Nale *et al.*, 2016a), as well as animals, e.g. in hamsters (Nale *et al.*, 2016b). Phages were also demonstrated to prevent *C. difficile*-associated biofilm forming, and to cause disruption of existing biofilms (Nale *et al.*, 2016a).

A number of studies have now evaluated the effect of FMT for rCDI on bacteriophage profiles. A study from Zuo and colleagues used ultra-deep metagenomic sequencing to evaluate both virome and microbiome changes in patients with CDI treated with FMT, patients with CDI treated with vancomycin, and healthy controls (Zuo *et al.*, 2017). CDI patients were noted to have a significantly higher abundance of *Caudovirales* and lower *Caudovirales* diversity, richness and evenness compared to healthy household controls (Zuo *et al.*, 2017). The abundance of *Caudovirales* was noted to significantly reduce after FMT, with FMT success more likely if donors had a higher fraction of *Caudovirales* colonising their stool virome. In contrast to the case with FMT, it was also noted that CDI treatment with vancomycin alone was associated with changes in the gut microbiome, but not in the virome (Zuo *et al.*, 2017). A further comparable study demonstrated that the gut viromes of rCDI patients receiving FMT rapidly resemble those of the donor, and this effect is maintained for at least 12 months (Draper *et al.*, 2018). It has also been established that colonisation of individual bacteriophages is dependent upon each particular donor-recipient pairing; specifically,

multiple recipients from a single donor were found to have widely-disparate phage colonisation patterns (Draper *et al.*, 2018).

1.6.6.2.2. Fungi:

Research into the fungal communities (or 'mycobiota') of mucosal surfaces within mammals has lagged behind that of the bacterial microbiota, but is beginning to gain momentum (Limon, Skalski & Underhill, 2017; Scanlan & Marchesi, 2008). Analysis of the stool of healthy people and rodents has demonstrated that the principles of colonisation resistance appear to apply to gut fungi as well as bacteria. Specifically, while the healthy gut microbiota appears to resist colonisation by *Candida albicans,* marked colonisation may rapidly occur in subjects treated with broad spectrum antibiotics prior to exposure to this fungal species (Limon, Skalski & Underhill, 2017).

Saccharomyces boulardii has long been recognised as a potential probiotic therapy for the treatment of intestinal pathogens including *C. difficile* (Kelesidis & Pothoulakis, 2012). There have been a number of postulated mechanisms as to why *S. boulardii* may have an anti-CDI effect, including the production of a protease which inhibits both toxin A and B (Castagliuolo *et al.*, 1999), the ability to stimulate intestinal immunoglobulin A (IgA) production (Kelly *et al.*, 2002), or influence upon gut bile acid metabolism (Kelly *et al.*, 2019). However, this agent is only infrequently used in the UK for a number of reasons. Firstly, it is not available as a licensed product within the UK. Secondly, the clinical data from human studies examining its efficacy has presented conflicting results (Public Health England, 2013). Thirdly, *S. boulardii* has been described as causing fungaemia in both immunocompetent and immunocompromised individuals (Enache-Angoulvant & Hennequin, 2005). As such, *S. boulardii* is not currently recommended in guidelines regarding the treatment of rCDI either in the UK or in most other territories.

Data regarding the relationship between FMT for rCDI and changes in the gut mycobiota have recently been described for the first time. In patients with rCDI, Zuo and colleagues observed decreased fungal diversity, richness and evenness, but overgrowth of *Candida albicans* (Zuo *et al.*, 2018). Successful FMT for rCDI was found to be associated with colonisation of donor-derived fungal taxa, in particular members of *Saccharomyces* and *Aspergillus*; however, high

relative abundance of *Candida* in donor stool was found to be associated with reduced efficacy of FMT. Individuals not responding to FMT and/or patients treated for rCDI with antimicrobials alone retained overgrowth of *Candida.* In a mouse model of CDI, the presence of *C. albicans* was associated with reduced efficacy of FMT, while use of antifungal therapy helped restore efficacy (Zuo *et al.*, 2018).

While the perturbation of gut bacteriophage and fungal profiles observed in rCDI and their influence upon FMT outcomes are of interest, their significance as a potential contribution as a mechanism of efficacy of FMT remains unclear. For example, given the narrow range that bacteriophages possess, any anti-*C. difficile* lytic phages in the gut virome post-FMT would have had to have been originated from *C. difficile* in the donor virome; given that *C. difficile* carriage is a near universal exclusion criterion for inclusion as an FMT donor, this would likely be impossible in practice. Similarly, given the established relationship between antimicrobial treatment and overgrowth of *Candida* within the gut, any changes in gut mycobiota profiles may possibly only be proxies of gut bacterial alterations. As such, the specific contribution of bacteriophages and fungi to the efficacy of FMT remains undefined.

1.6.6.3. Bacteriocins and host defence molecules:

Bacteriocins are anti-microbial compounds which are produced by bacteria themselves, and may be bactericidal or bacteriostatic. Most typically, bacteriocin activity is primarily directed against bacteria of close phylogeny to the bacteria which produce them (Becattini, Taur & Pamer, 2016).

There are a number of bacteriocins which are well-established to have anti-*C. difficile* activity. One of the best characterised of these is thuricin CD, a bacteriocin produced by *Bacillus thuringiensis* DPC 6431, a bacterium cultured from a human stool sample (Rea *et al.*, 2010). Thuricin CD consists of two peptides, Trn-α and Trn-β, which have been demonstrated to act synergistically to kill a range of *C. difficile* ribotypes, including those associated with significant clinical disease, such as 027. Of particular interest, thuricin CD appears to be narrow-spectrum, with little activity against most bacterial genera other than *Clostridioides,* and no adverse effects against a wide-range of GI commensal bacteria (Rea *et al.*, 2010).

In addition to bacteriocins and related compounds produced by the microbiota, there are also a range of host defence molecules present within the lumen of the GI tract which have potent anti-microbial activity, including defensins, cathelicidins and lysozyme (Baktash *et al.*, 2018). Certain defensins and cathelicidins (including LL-37, derived from lysis of a precursor in epithelial cells and selected immune cells in humans) have been demonstrated as having the ability of kill a range of *C. difficile* strains, and to have a synergistic effect when co-administered with antibiotics (Nuding *et al.*, 2014).

At present, there are no published studies that have specifically investigated the possible association between successful FMT and changes in bacteriocin profiles. Of interest, in a study of 18 patients receiving FMT as treatment for rCDI, levels of LL-37 in plasma were significantly increased three months later in successfully-treated patients (Konturek *et al.*, 2016). Since cathelicidins have an apparent role in the host of minimising disruption of the gut barrier, these changes may have purely been a proxy of intestinal recovery after CDI (Konturek *et al.*, 2016), and further mechanistic studies are merited to explore further.

1.6.6.4. Gut microbiota-bile acid interactions:

Bile acids are cholesterol derivatives with a steroid ring component which are predominantly found within secreted bile (in comparison, *bile salts* specifically refers to sodium and potassium salts of bile acids). After their initial synthesis within the liver, bile acids are conjugated with the amino acids taurine or glycine, which increases solubility (Staley *et al.*, 2017b). After initial concentration with the gallbladder, bile acids pass within bile into the biliary tree and, via the common bile duct, into the duodenum. At the terminal ileum, approximately 95% of bile acids will be reabsorbed via the enterohepatic circulation, whilst the remaining 5% (~400-800mg per day) are not recovered, and will continue through the distal gut of the terminal ileum and into the colon (Hofmann, 1999). Within the colon, there is a small amount of further bile acid reabsorption, with <5% of bile acid lost through faeces. *Primary* bile acids are defined as those produced within the liver itself, whilst the terms *secondary* and *tertiary* bile acids are defined as those produced in the distal gut through microbially-mediated metabolism of primary bile acids (Mullish *et al.*, 2018b).

Until approximately two decades ago, it was generally considered that bile acids were simply surfactant molecules responsible for the emulsification and solubilisation of dietary lipids. However, since this point, there has been rapidly-growing recognition of the diverse roles of bile acids within the host, and in particular their role as endogenous ligands for host cell receptors. These include the nuclear receptor farnesoid X receptor (FXR), and the G protein-coupled plasma membrane bile acid receptor TGR5, which both exhibit varying affinities for different bile acids and their moieties (Mullish *et al.*, 2018b; Wahlström *et al.*, 2016). Via their actions as FXR and TGR5 agonists, bile acids contribute to a wealth of host physiological processes, including the modulation of lipid, glucose and energy homeostasis, as well as the regulation of bile acid synthesis, conjugation and transport (Mullish *et al.*, 2018b).

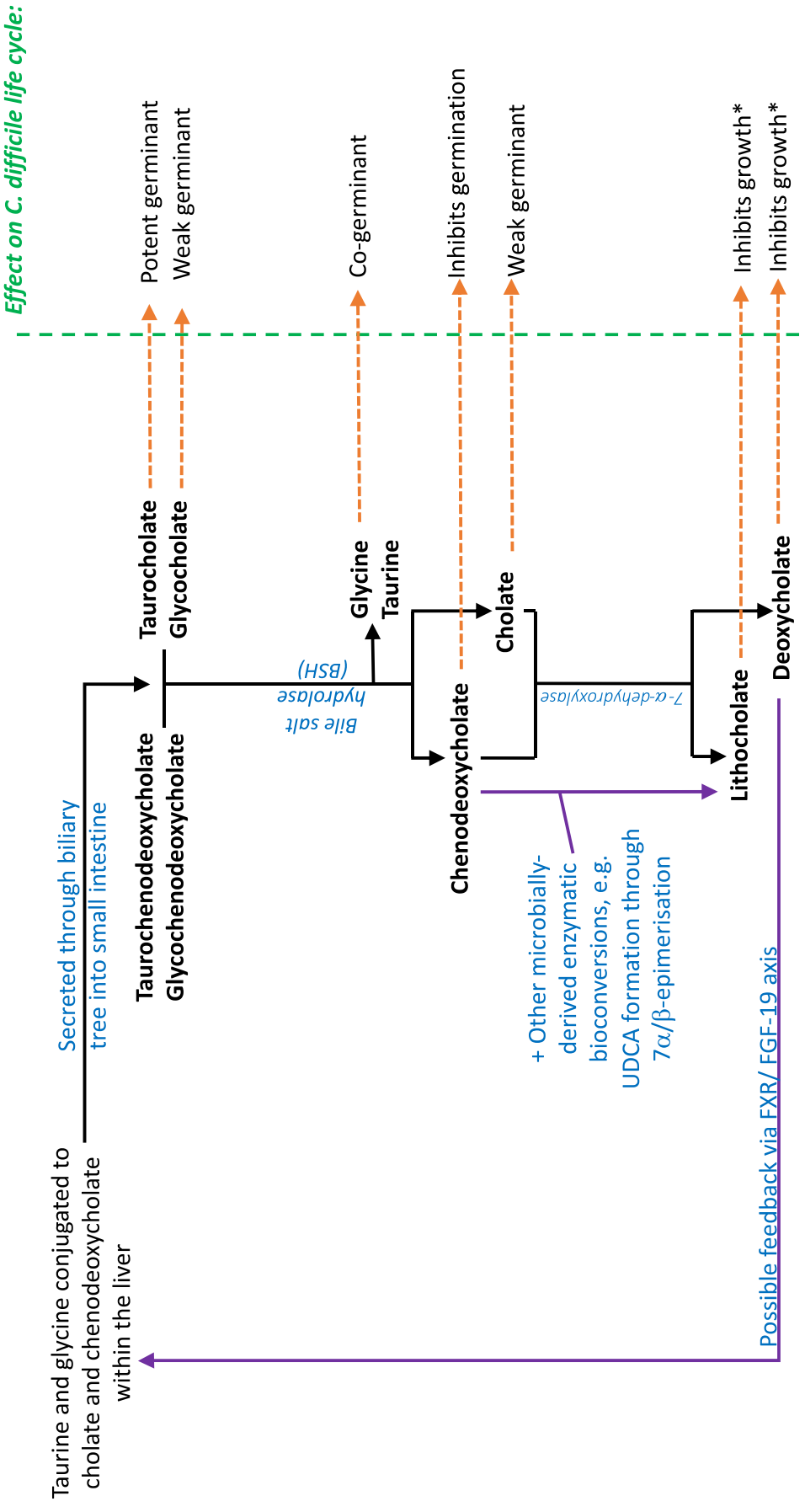
The interest in a possible relevance of bile acids to CDI stems from an initial observation from *in vitro* studies that a number of different bile acids have markedly different effects on the ability of *C. difficile* to undergo germination or vegetative growth (**Figure 1.2**). In particular, the conjugated primary bile acid taurocholate (TCA) is a very potent trigger to *C. difficile* germination, using glycine as co-germinant (Sorg & Sonenshein, 2008). Unconjugated primary bile acids have differential effects on *C. difficile*, with cholate (CA) being a mild trigger to germination (Sorg & Sonenshein, 2008), while chenodeoxycholate (CDCA) inhibits it (Sorg & Sonenshein, 2009). The effect of CDCA in inhibiting *C. difficile* germination is apparently more potent than CA in inducing it; however, CDCA is absorbed by the colonic epithelium ten times faster than CA, therefore likely reducing levels below that needed for significant inhibition *in vivo* (Mekhjian, Phillips & Hofmann, 1979). The *C. difficile* bile acid germinant receptor was only recently identified as CspC, a germination-specific protease (Francis *et al.*, 2013). The secondary bile acids – including deoxycholate (DCA) and lithocholate (LCA) – are able to potently inhibit the vegetative growth and toxin activity of *C. difficile* (Sorg & Sonenshein, 2008; Theriot *et al.*, 2014; Thanissery, Winston & Theriot, 2017). Similarly, DCA and LCA (at physiological concentrations) are able to inhibit TCA-mediated *C. difficile* germination (Theriot *et al.*, 2014; Thanissery, Winston & Theriot, 2017). Whilst most of these *in vitro* effects are maintained over a wide range of concentrations of the bile acid in question, DCA is exceptional in that high concentrations of it are conversely able to cause *C. difficile* germination (Sorg & Sonenshein, 2008).

The transition from conjugated primary bile acids to secondary bile acids *in vivo* principally involves two enzymatic steps, with both enzymes produced by microbes but not mammals (**Figure 1.2**). The first principle step is undertaken by **bile salt hydrolases** (**BSHs** (EC 3.5.1.24)**;** also referred to as choloylglycine hydrolase), which deconjugate the taurine and glycine groups from the sterol core of bile acids via a hydrolysis reaction, and consequently reform the unconjugated primary bile acids CA and CDCA (**Figure 1.3**). This process has been described as a 'gateway reaction', since it is the key initial reaction in microbially-mediated bile metabolism, without which subsequent steps are not able to occur (Jones *et al.*, 2008). The second principle enzymatic step is performed via **7-α-dehydroxylase,** whereby unconjugated primary bile acids are converted to secondary bile acids, including DCA and LCA. The distinction is made between *secondary* bile acids (those produced by microbial metabolism of primary bile acids by BSH and 7-α-dehydroxylase) and *tertiary* bile acids (those produced by other forms of microbial modification, e.g. dehydrogenation or sulfation, or the epimerisation of CDCA to synthesise ursodeoxycholic acid (UDCA)) (Wahlström *et al.*, 2016; Mullish *et al.*, 2018b).

A direct link between microbiota, bile acids and farnesoid X receptor (FXR) signalling has been demonstrated in rodents through the use of germ-free or antibiotic-treated animals (Wahlström *et al.*, 2016). The interplay between these factors in humans remains less clear, although successful FMT for rCDI is associated with increased circulating levels of fibroblast growth factor (FGF)-19 and reduction in FGF-21, consistent with increased FXR signalling post-FMT (Monaghan *et al.*, 2018) (discussed more extensively in **Section 4.4.1**).

Figure 1.2 (page 57): Schematic of gut microbiota-bile acid interactions in humans. With particular reference to potential relevance to CDI; see main text of **Section 1.6.6.4**. *: DCA and LCA inhibit TCA-mediated *C. difficile* germination at physiological concentrations, but DCA can trigger *C. difficile* germination at high concentrations. Reproduced from (Mullish *et al.*, 2019).

Figure 1.2:


Effect on C. difficile life cycle:
Potent germinant
Weak germinant
Co-germinant
Inhibits germination
Weak germinant
Inhibits growth*
Inhibits growth*
Taurocholate
Glycocholate
Glycine
Taurine
Cholate
Deoxycholate
Bile salt hydrolase (BSH)
7-α-dehydroxylase
Taurochenodeoxycholate
Glycochenodeoxycholate
Chenodeoxycholate
Lithocholate
Secreted through biliary tree into small intestine
+ Other microbially-derived enzymatic bioconversions, e.g. UDCA formation through 7α/β-epimerisation
Possible feedback via FXR/ FGF-19 axis
Taurine and glycine conjugated to cholate and chenodeoxycholate within the liver

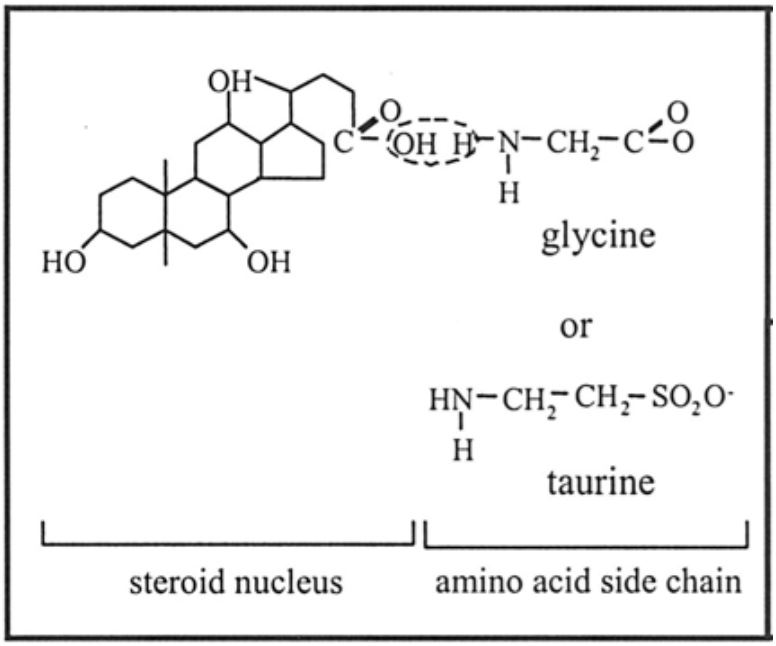


Figure 1.3: Chemistry of BSH. BSH cleaves the amide bond between the carboxyl group of a bile acid and the amino group of the amino acid (taurine or glycine), resulting in a deconjugated bile acid and free amino acid moiety. Reproduced with permission from (Begley, Hill & Gahan, 2006).

BSHs are found predominantly within the bacterial phyla *Bacteroidetes, Firmicutes,* and *Actinobacteria,* but are widely-distributed throughout most major bacterial divisions and archaea species of the gut microbiota (Jones *et al.*, 2008). Annotation for a *bsh* gene has been identified in 26.03% of all bacterial strains in the Human Microbiota Project microbiota reference genome (Song *et al.*, 2019). At least eight different *bsh* genes exist, with each resultant form of BSH enzyme having specific distinct properties relating to optimal pH, and specificity for taurine- or glycine-conjugated bile acids (Jones *et al.*, 2008) (**Appendix 1**). However, key aspects of protein secondary structure and the core active site (including the thiol group of cystine-1) appear conserved by all forms of BSH (Song *et al.*, 2019; Begley, Hill & Gahan, 2006; Dong & Lee, 2018). BSH are predominantly expressed within the cytoplasm of bacteria as homotetrameric proteins, but may also be identified extracellularly and/or in oligomeric forms (Bustos *et al.*, 2018). BSH-producing members of *Firmicutes* appear capable of degrading a range of conjugated bile acids, whereas those derived from *Bacteroidetes* are generally only active against tauro-conjugated bile acids (Jones *et al.*, 2008). BSHs appear to have less preference for the sterol core (Foley *et al.*, 2019).

7-α-dehydroxylase mediates a complex, multi-step process (involving eight different proteins) that converts primary to secondary bile acids via the removal of the hydroxyl group of C-7. One key element of this process is only performed by strictly anaerobic bacteria with the bile acid-inducible (*bai*) operon, which encodes a NAD(H)-dependent 3-dehydro-4-bile acid oxidoreductase (Ridlon, Kang & Hylemon, 2006). Based on current microbial genomic annotation, it is estimated that only a very small percentage of commensal gut microbiota members possess 7-α-dehydroxylation activity, with those organisms that do predominantly belonging to the genera *Clostridium* clusters XIVa and XI (Kitahara *et al.*, 2000; Ridlon, Kang & Hylemon, 2006). Generation of secondary bile acids creates a more hydrophobic bile acid pool, enabling the elimination of such bile acids within faeces.

Since bile acid metabolism *in vivo* is so strongly influenced by gut microbiota functionality – and given the marked effect of various bile acids upon different aspects of the *C. difficile* life cycle – the theory has arisen that this pathway may also be relevant to the pathogenesis of CDI and/or efficacy of FMT in humans (Mullish *et al.*, 2019). Specifically, it has been hypothesised that:

- A key contributory mechanism to CDI pathogenesis is disruption of the gut microbiota by antibiotics (and/or other insults), and particularly loss of gut microbial community members with BSH and/or 7-α-dehydroxylase functionality. The direct consequence of this is gut enrichment of TCA (promoting *C. difficile* germination) and loss of DCA (permitting vegetative growth of *C. difficile*).
- A key mechanism of efficacy of FMT in treating CDI is rapid, sustained recolonisation of the gut microbiota with bacteria that possess BSH and/or 7-α-dehydroxylase functionality. The consequence of this is restoration of the normal bile acid *milieu* of the gut, and removal of key triggers for *C. difficile* germination and/ or vegetative growth.

Several strands of evidence support the concept that gut microbiota-derived bile-metabolising enzymes have a key contribution to colonisation resistance, and that perturbation of this may contribute to CDI pathogenesis:

Bile salt hydrolase: Allegretti and colleagues compared the stool microbiota of patients with a first episode of CDI, patients with recurrent CDI, and healthy controls (Allegretti *et al.*, 2016). Using an inferential metagenomic tool (PICRUSt), researchers demonstrated that the predicted *bsh* gene abundance in the stool microbiota of rCDI patients was significantly lower than both patients with primary CDI and healthy controls. There was a trend observed towards reduced predicted stool *bsh* gene abundance in patients with primary CDI compared to controls, although this did not reach statistical significance. In the context of the hypothesis proposed above, it may have been expected that predicted stool *bsh* gene abundance would be significantly reduced in patients with primary CDI compared to controls; potential confounders within this study include possible underpowering, along with the limitation of assessing gut microbiota BSH functionality through an inferential software tool (given that reference databases may have incomplete or incorrect annotation of genes; see **Section 2.2.1.3**), rather than through the use of direct functional BSH assays.

7-α-dehydroxylase: Buffie and colleagues first used mathematical modelling to compare gut microbiota profiles from antibiotic-exposed mice with different levels of CDI vulnerability, as well as to compare profiles from patients with CDI with those who were *C. difficile* carriers (Buffie *et al.*, 2014). They identified the presence of an operational taxonomic unit (OTU) corresponding to *Clostridium scindens* as a key microbiota factor influencing colonisation resistance. In bacterial transfer experiments, they demonstrated that the degree of colonisation of the gut microbiota of antibiotic-treated mice with *C. scindens* correlated with degree of infection resistance. Given that *C. scindens* is one of the few commensal bacteria with 7-α-dehydroxylase activity, the researchers hypothesised that the ability to produce secondary bile acids was the link between *C. scindens* colonisation and infection resistance; they provided evidence for this by demonstrating that infection resistance was reversed after addition of the bile acid sequestrant cholestyramine (Buffie *et al.*, 2014). The same association between *C. scindens* and CDI resistance was observed after the organism was administered to germ-free mice (Studer *et al.*, 2016).

However, while it has been reported that FMT for rCDI is associated with changes in the stool bile acid profile (Weingarden *et al.*, 2014), there has been no investigation regarding the

impact of FMT upon the dynamics and activity of BSH and 7-α-dehydroxylase. As such, this is one of the major aims of this project (see **Aims and Objectives, Section 1.6.7**).

1.6.6.5. Short chain fatty acids:

These are fatty acid molecules with aliphatic tails of 1-6 carbons (as compared to those with 7-12 carbons (medium chain fatty acids; MCFAs)). Acetate (C2), propionate (C3) and butyrate (C4) together form ≥95% of all SCFAs (Cook & Sellin, 1998). The major source of these in the host is bacterial fermentation of partially and non-digestible carbohydrates (principally dietary, but also endogenous) within the colon. Alternatively, branched chain amino acids (including valine, leucine and isoleucine) may also be used for fermentation into SCFAs, with the result being production of branched chain SCFAs including isobutyrate and isovalerate (Yao, Muir & Gibson, 2016). Concentrations of SCFA are highest within the proximal colon (up to 140mM), but are still detectable at least 20mM in the distal colon and terminal ileum (Wong *et al.*, 2006). Approximately 80-90% of SCFAs produced are absorbed in the colon, with the rest excreted within faeces (García-Villalba *et al.*, 2012). The quantities and profile of SCFA produced in healthy individuals is influenced by a number of factors, including the colonic microbiota profile, the substrate sources available, and the GI tract transit time (Macfarlane & Macfarlane, 2003).

SCFAs were originally thought to principally act only as an energy source for colonocytes. However, over the past 20 years, there has been increasing recognition of the diverse range of complex host functions which SCFAs contribute to or directly mediate, including: inhibition of pathogen growth, resolution of inflammation (Maslowski *et al.*, 2009), and maintenance of gut barrier function (Chen *et al.*, 2017), among other functions (Nicholson *et al.*, 2012).

There are several lines of evidence suggesting an association between antibiotic use, gut microbiota-mediated SCFA synthetic ability and CDI. A mixture of SCFA at the concentration found in the healthy hamster caecum (or butyrate by itself) was able to inhibit growth of *C. difficile in vitro* (Rolfe, 1984). Mice treated with the broad-spectrum antibiotic cefoperazone for 10 days experienced a marked decline in stool levels of a range of SCFAs (including a 66-fold reduction in valerate and 28-fold reduction in isovalerate), and higher SCFA levels correlated with protection from *C. difficile* growth (Theriot *et al.*, 2014). In a mouse model of

CDI, a diet deficient in carbohydrate which may be fermented into SCFA perpetuated the length of time with CDI; in contrast, a diet rich in a complex mixture of such carbohydrates (or even administration of inulin alone) helped to minimise *C. difficile* burden in mice (Hryckowian *et al.*, 2018). On stool microbiota analysis, patients with CDI have been demonstrated to have reduced relative abundance of microorganisms with butyrate-producing capacity, including the bacterial families *Rumincoccaceae* and *Lachnospiraceae* (Song *et al.*, 2013).

Until recently, there had been no data investigating the impact of FMT for rCDI upon gut SCFA dynamics. However, recent experiments performed concurrently in the same laboratory as those in this thesis (Dr Julie McDonald and Prof Julian Marchesi, Imperial College) have explored this area, using a complex *in vitro* system (chemostat) to model the distal gut (McDonald *et al.*, 2018a). Chemostats systems allow the user to mimic certain spatial, temporal and environmental conditions found in the human gut (Macfarlane & Macfarlane, 2007). Bacterial communities established in chemostats are stable, reproducible and representative of the gut microbiota communities found *in vivo* (McDonald *et al.*, 2013), allowing longitudinal sampling to closely monitor the effect of an intervention. Recent chemostat experiments of the distal gut in CDI have demonstrated that while levels of the SCFAs acetate and butyrate spontaneously recover after cessation of antibiotics, the C5 SCFA valerate does not, but is restored by FMT. This raises the possibility that valerate restoration is a further key functionality restored through FMT, which may be of clinical relevance. However, at present, whether these same changes occur in patients with CDI receiving FMT is unknown. As such, this is one of the major aims of this project (see **Aims and Objectives, Section 1.6.7**).

1.6.7. Aims and Objectives:

1.6.7.1. Aims:

The central aim of this study was to explore the contribution of perturbed gut microbiota-host metabolism interactions to the pathogenesis of CDI, with a particular emphasis on gut microbiota-derived bile-metabolising enzymes.

As an extension of this, a further major aim of this study was to assess whether a key mechanism of efficacy of FMT in treating rCDI is through restoration of this interaction to a pre-morbid state.

1.6.7.2. Objectives:

The specific objectives of this study were as follows:

1. To investigate the hypothesis that loss of gut microbiota-derived bile-metabolising enzymes (particularly bile salt hydrolases (BSHs)) is a key contributory factor to the pathogenesis of CDI (and/or influencing whether recurrence occurs or not), via the resulting enrichment in the gut of bile acids able to trigger the germination of *C. difficile* (i.e. taurocholic acid), and loss of bile acids which inhibit the vegetative growth of *C. difficile* (i.e. deoxycholic acid).

2. To investigate the hypothesis that a major mechanism by which FMT acts as an efficacious treatment for rCDI is through restoration of gut microbiota members with bile-metabolising functionality (particularly BSH), via the subsequent degradation in the gut of bile acids able to trigger the germination of *C. difficile* (i.e. taurocholic acid), and restoration of bile acids which inhibit the vegetative growth of *C. difficile* (i.e. deoxycholic acid).

3. To investigate the hypothesis that other gut microbiota-host metabolism interactions are also contributory to the efficacy of FMT in treating rCDI. Specifically, to explore whether an additional key mechanism of action for FMT is restoration of gut microbial functionality

for the production of short chain fatty acids (SCFAs) (in particular, valerate, which inhibits the vegetative growth of *C. difficile*).

4. To investigate objectives 1-3 using the following workflow:

- Observational investigation – deep phenomic profiling of human samples collected from patients with rCDI pre- and post-FMT (along with donor samples), and serial human samples collected from patients with a first episode of CDI (both recurrers and non-recurrers).
- Mechanistic investigation – using *in vitro* and *in vivo* modelling of CDI to investigate the specific impact of bile-metabolising enzymes (and particularly BSH) upon the ability of *C. difficile* to undergo germination and/or vegetative growth.

Chapter 2. Methods:

2.1. Human samples:

2.1.1. Ethics:

Collection and analysis of human samples for the studies within received approval from the UK National Research Ethics Centres (13/LO/1867) and Research and Development/ Research Ethics Board approval from all collaborating institutions.

2.1.2. Recurrent *Clostridioides difficile* infection/ faecal microbiota transplant samples:

2.1.2.1. Introduction:

Analysis was performed on two different sample sets that were collected from patients from recurrent *Clostridioides difficile* infection pre- and post-FMT, together with healthy stool donors. These are hereafter referred to as the 'initial human dataset' and 'validation human dataset'.

2.1.2.2. Initial human dataset - collection of samples and FMT protocols:

For the initial human dataset, stool samples were collected from participants with rCDI (26 participants; samples collected pre- and at 8-10 weeks post-successful FMT) and their FMT donors (17 participants). Post-FMT were collected at 8-10 weeks post-successful FMT administration, since absence of diarrhoea at this point is acknowledged by most expert opinions as the definition of a successful FMT for rCDI (Mullish *et al.*, 2018d, 2018c; Kelly *et al.*, 2015b). Of note, samples within this dataset were all collected from patients successfully entering remission from CDI after a single FMT. While a small number of patients being treated by the FMT service from which the samples of this dataset were derived experienced failure to respond to an initial FMT, stool samples from such patients were *not* part of this dataset, and are the subject of analysis in future postgraduate student study at Imperial College London. rCDI for this dataset was diagnosed on a combination of clinical and laboratory criteria. Specifically, with regards to clinical status, participants with CDI had recurrence of diarrhoea (>3 unformed bowel movements every 24 hours) within eight weeks of completing a prior course of treatment (with resolution of diarrhoea with anti-CDI antibiotics for prior episodes), with no clear alternate explanation for diarrhoea. All patients had originally been diagnosed with CDI during the primary episode of disease with laboratory

testing via positive ELISA for toxins A/B, with confirmation via PCR. This testing had been repeated at the time of suspected recurrence, but positive stool tests were not obligatory for diagnosing rCDI; nevertheless, such laboratory testing had a role in helping to distinguish diarrhoea related to recurrent CDI from post-infectious irritable bowel syndrome, secondary lactose intolerance, and other disease mimics. For CDI participants, samples were collected shortly prior to FMT (whilst on suppressive doses of anti-CDI antibiotics), and again at 8-10 weeks post-successful FMT.

For preparation of FMT material, a fresh stool donation (>50 grams) was collected from a donor, and processed within the laboratory within 6 hours of defaecation. No 'pooling' of samples from different donors was performed. The stool donation was mixed with 200ml of phosphate-buffered saline and filtered using a stomacher bag to produce 180ml of faecal slurry. The slurry was mixed with 20ml of 100% glycerol (to give a final concentration of 10% glycerol (v/v)), and kept stored at -80^{o}C for up to six months. FMT was administered either by colonoscopy or via capsule, with 4 litres of polyethylene glycol bowel preparation administered to all patients on the day prior to FMT. Where FMT was performed via colonoscopy, frozen slurry was thawed at 4^{o}C, reconstituted with 160ml of phosphate-buffered saline, drawn into syringes, and administered via the endoscope. Capsule preparation and administration was as previously-outlined (Kao *et al.*, 2017). Specifically, faecal slurry (approximately 200ml) was mixed with 40ml of 100% glycerol, and centrifuged (400 x *g*) at room temperature for 20 minutes. After decanting the supernatant, it was centrifuged (at 10000 x *g*) for 30 minutes at 4^{o}C. The supernatant was discarded, and the remaining microbial pellet was mixed to incorporate residual liquid to allow pipetting into capsules. Gelatin capsules were then filled and further encapsulated, were flash frozen at -55^{o}C on dry ice, and were kept stored at -80^{o}C for up to six months. Forty capsules were manufactured from each initial stool donation. Donors were selected/ screened using conventional health questionnaire/ laboratory screening protocols (Mullish *et al.*, 2015, 2018d, 2018c; Kelly *et al.*, 2015b).

2.1.2.3. Validation human dataset - collection of samples and FMT protocols:

For validation of initial findings (together with further exploration of the dynamics of microbiome/ metabonome changes), stool samples (together with those of paired urine and

serum) were also analysed for rCDI patients from a Canadian randomised controlled trial investigating capsulised *vs* colonoscopic FMT as rCDI treatment (18 participants; samples collected pre- and at 1, 4 and 12 weeks post-FMT), together with donors (five participants) (Kao *et al.*, 2017). While the clinical aspect of this study had included 116 participants, full availability of serial stool samples was only available for 43 patients, and 18 of these were randomly chosen (9 receiving capsule FMT, 9 receiving colonoscopic FMT) for the analyses performed within this thesis. The protocols for FMT preparation and administration are the same as those described in **Section 2.1.2.2.** Crude stool and FMT slurry derived from the same donation (i.e. separate aliquots of the same stool sample both before and after processing into FMT) were collected from these donors where possible. This study was co-ordinated by Dr Dina Kao, a collaborating gastroenterologist at the University of Alberta, Canada. Of note, samples within this dataset were all collected from patients successfully entering remission from CDI after a single FMT. While a small number of patients being treated in the trial from which the samples of this dataset were derived experienced failure to respond to an initial FMT, samples collected from such patients were *not* part of this dataset, since such samples were unavailable for analysis.

2.1.2.4. Patients with primary *Clostridioides difficile* infection - collection of samples:

A prospective study was performed on patients with a first episode of uncomplicated CDI (29 participants) (Allegretti *et al.*, 2018). The study was co-ordinated by Dr Jessica Allegretti, a collaborating gastroenterologist at Brigham and Women's Hospital/ Harvard Medical School, USA. Stool samples were collected from all patients at the time of diagnosis (i.e. prior to antimicrobial therapy), and then once weekly up until six weeks; however, if recurrence occurred within six weeks, the last weekly stool sample collected prior to recurrence was the last sample collected. Patients were defined as non-recurrers if no recurrence of diarrhoeal disease had occurred at six weeks after diagnosis of primary CDI; recurrence was defined as diarrhoea with a positive *C. difficile* stool toxin.

2.2. Gut microbiota analysis:

2.2.1. Overview of the principles of gut microbiota analysis:

2.2.1.1. Introduction:

Until relatively recently, investigation of the gut microbiota was primarily focused on culture-based techniques. Such techniques have the drawbacks of being labour-intensive and time-demanding in light of the diverse and specific needs required to optimise culture for different bacterial species, including use of selective media, requirement for an anaerobic environment, etc. Despite such drawbacks, culture-based techniques are still a valuable tool for studying the gut microbiota. For example, Browne and colleagues used a workflow applying complex, broad-range bacteriological media to allow culture of 96% of bacterial abundance at the genus level and 90% at the species level (Browne *et al.*, 2016).

However, over the past two decades, there has been an explosion of use of next-generation microbial sequencing technologies as a high-throughput, comprehensive means of defining the composition – and giving insight into the function – of the gut microbiota. The most common sample type used for analysis in human gut microbiota studies is faecal samples, since they are non-invasive and can be collected at home by study participants relatively easily using commercial kits. Although it is recognised that there are significant differences in the microbiota composition of faecal and mucosal biopsy samples both in health and disease (Carstens *et al.*, 2018), faecal samples are the generally-favoured sample for gut microbiota analysis on the grounds of practicality. Rectal swab may be a relatively non-invasive and straightforward alternative (Jones *et al.*, 2018).

This study applies a microbial sequencing technique known as metataxonomic sequencing, which is summarised below (also discussed in (Mullish *et al.*, 2018a)).

2.2.1.2. Metataxonomic sequencing (16S rRNA gene sequencing):

The 16S rRNA gene is present in all prokaryotes, and consists of nine hypervariable regions of variable sequence and length, interspersed by highly-conserved regions (Baker, Smith & Cowan, 2003). The DNA sequences of these hypervariable regions are phylogenetically distinct for a given species, and therefore sequencing of these regions allows researchers to determine the bacterial composition of each sample.

Metataxonomic sequencing involves the use of PCR amplification and sequencing of one or more of these variable regions of the 16S rRNA gene from DNA extracted from the sample. PCR primers are designed to bind to conserved regions of the gene that flank one or more hypervariable regions (Van de Peer, Chapelle & De Wachter, 1996). Following the initial PCR of the 16S rRNA gene, the PCR product is amplified, purified, and used as a template in a second PCR reaction that adds a unique combination of barcoded indices to each sample (Illumina, n.d.). Barcoded indices permit many samples to be pooled and sequenced in parallel. Subsequently, amplicons are purified, mixed together at equimolar concentrations, denatured and sequenced. Whilst several sequencing instruments are available commercially, analysis of samples in this study used the Illumina MiSeq platform (at St Mary's Hospital Campus, Imperial College London), which is capable of sequencing 300-400 base pair paired-end reads (Illumina, n.d.). Initial next-generation sequencing platforms (such as 454 pyrosequencing) used to perform 16S rRNA gene sequencing have been superseded by those from Illumina, in light of lower rates of errors of gene and contig sequencing, particularly with regards to homopolymer-assocated single base errors (Luo *et al.*, 2012). Metataxonomic sequencing is recognised as reliably classifying bacterial taxa down to the genus levels in humans (and, in some cases, down to species level), but only down to family level in murine samples (Janda & Abbott, 2007).

More specific details about the methodology for DNA extraction and metataxonomic analysis techniques used in this thesis is provided in **Sections 2.2.2** and **2.2.3.**

2.2.1.3. Other techniques for microbiota analysis:

The initial molecular techniques that emerged for gut microbiota analysis included fingerprinting techniques (including denaturing gradient gel electrophoresis (DGGE) and terminal fragment length polymorphism (T-RFLP)), as well as fluorescent *in situ* hybridisation (FISH) (Zoetendal, Rajilic-Stojanovic & de Vos, 2008). However, such techniques lacked sufficient resolution for in depth analysis of a complex, dense ecosystem such as the gut, being able to define the most abundant taxa only, and are now used less commonly. There have also been techniques that avoid genomic sequencing altogether, with the principal example being microarrays. These arrays permit phylogenetic profiling of microbial

communities of interest through hybridisation of extracted DNA against array probes corresponding to either single-stranded full or partial 16S rRNA genes (Claesson *et al.*, 2010). However, drawbacks of microarrays include the fact that it is difficult to formulate probes able to differentiate closely-related taxa, and that the depth and breadth of profiling is limited by the number of probes that can be included in a single array.

Although 16S rRNA gene sequencing was applied in this study, there are other next-generation sequencing techniques available to analyse the microbiota, including *shotgun metagenomic sequencing* (the sequencing of the entire metagenome within a sample) and *metatranscriptomics* (sequencing of RNA from the sample, with the aim of assessing gene expression) (Claesson, Clooney & O'Toole, 2017). While these are powerful techniques for investigating microbiota composition and functionality, they have a number of drawbacks, including concerns related to the accuracy and completion of reference genome databases, the potential for bias related to the complex sequencing and PCR steps, the requirement for a large amount of nucleic acid to sequence, and their considerable cost.

These drawbacks have been a drive to the development of computational tools that infer metagenomic content from 16S rRNA gene amplicon sequencing counts; by comparing such counts to reference prokaryotic genomic databases, it is possible to obtain predicted copy number for a wide variety of genes. A number of these tools now exist, and many have been validated by demonstrating comparable results when shotgun metagenomics and metataxonomics/ use of the tool in question are performed on the same defined microbial community (Iwai *et al.*, 2016). While such tools are a helpful and broadly-accurate adjunct for exploration of microbiota function (particularly in light of their simplicity and speed of use), the accuracy of their output is clearly limited by the degree to which metagenomic databases are appropriately annotated/ completed, and confirmatory experiments (e.g. qPCR) are typically required (Mullish *et al.*, 2018b). In line with these principles, one such tool (named Piphillin) has been used in microbiota analysis in this work (Iwai *et al.*, 2016) (also see **Section 2.2.5**), with confirmatory PCR and enzyme assays experiments (see **Sections 2.4-2.5**).

2.2.2. DNA extraction and 16S rRNA gene sequencing:

All samples for microbiome profiling analysis were collected and kept frozen until processing for analysis consistent with best practice protocols (Mullish *et al.*, 2018b, 2018a). DNA was extracted from ~250 mg of stool using the PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, California, USA) using manufacturer's instructions, but with the addition of a bead-beating step at speed 8 for 3 minutes via use of a Bullet Blender Storm (Chemobio Ltd, St Albans, UK) (Mullish *et al.*, 2018b). Extracted DNA was quantified using a Qubit 2.0 Fluorometer (ThermoFischer Scientific, Hemel Hempstead, UK), aliquoted, and stored at -80^{o}C pending downstream assays.

Sample libraries were prepared following Illumina's 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, n.d.) with several modifications (Mullish *et al.*, 2018b). Firstly, the V1-V2 regions of the 16S rRNA gene were amplified using previously-reported primers (Mullish *et al.*, 2018b) (also **Table 2.1**).

Table 2.1: Primers used for 16S rRNA gene sequencing on the Illumina MiSeq.

Primer name	Primer sequence
28F-YM (forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAGTTTGATYMTGGCTCAG
28F-Borrellia (forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAGTTTGATCCTGGCTTAG
28F-Chloroflex (forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAATTTGATCTTGGTTCAG
28F-Bifido (forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGGTTCGATTCTGGCTCAG
388R (reverse primer)	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGCTGCCTCCCGTAGGAGT

The forward primer mix was composed of four different forward primers, mixed at a final ratio of 4: 1: 1: 1 (28F-YM: 28F-Borrellia: 28FChloroflex: 28F-Bifdo). Bases in bold are the MiSeq adapter sequences.

Secondly, index PCR reaction products were cleaned up and normalised using the SequalPrep Normalization Plate Kit (Life Technologies, Paisley, UK). Sample libraries were quantified using the NEBNext Library Quant Kit for Illumina (New England Biolabs, Hitchin, UK).

Sequencing was performed in house at Imperial College London on an Illumina MiSeq platform (Illumina Inc., Saffron Walden, UK) using the MiSeq Reagent Kit v3 (Illumina) and paired-end 300bp chemistry.

2.2.3. Metataxonomic analysis:

The output data from MiSeq was analysed using the Mothur package (v1.35.1) following the MiSeq standard operating protocol pipeline (Kozich *et al.*, 2013). Sequence alignments were performed using the Silva bacterial database (www.arb-silva.de/), and the RDP database reference sequence files were used for sequence classification using the Wang method (Wang *et al.*, 2007). Operational taxonomic unit (OTU) taxonomies (from phylum to genus level) were established using the RDP MultiClassifier Script. Data was resampled and normalised to the lowest read count in Mothur (11604 reads per sample), which resulted in >99.5% coverage within each sample. Where possible, species were identified from OTU data using a standard nucleotide BLAST of the 16S rRNA gene sequences (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes) with strict criteria (query cover 100% and ≥97% identity, with no other candidate species above ≥97% identity) (Stackebrandt & Goebel, 1994). Genus-level annotation was made where query cover was 100% and ≥94% identity. Non-metric multidimensional scaling (NMDS) plots and permutational multivariate ANOVA (PERMANOVA) p-values were generated using the UniFrac weighted distance matrix generated from Mothur, and analysed using the Vegan library within the R statistical package (Foundation, n.d.). Extended error bar plots were generated at different taxonomic levels using the Statistical Analysis of Metagenomic Profiles (STAMP) software package, applying White's non-parametric *t*-test with Benjamini-Hochberg false discovery rate (FDR) correction (Parks *et al.*, 2014). Ecological indices - including α-diversity (Shannon diversity index, H') and richness (total number of bacterial taxa observed, S_{obs}) - were calculated within Mothur and R using the Vegan package, and statistical tests were performed using GraphPad Prism v7.03. A *p*-value of 0.05 and a *q*-value of 0.05 was considered significant.

Changes in microbial composition were also assessed down to the OTU level. Differences in means of relative proportions >1% were measured between donor and pre-FMT samples, and between pre-FMT and post-FMT samples, using White's non-parametric test and Benjamini-

Hochberg FDR. From these data, OTUs were analysed that were enriched in donors in comparison to pre-FMT samples, and those enriched post-FMT in comparison to pre-FMT samples.

Sequencing data from this study (in fastq-format) are publicly available for download at the European Nucleotide Archive (ENA) database using study accession number PRJEB30298 (http://www.ebi.ac.uk/ena/data/view/PRJEB30298).

2.2.4. 16S rRNA gene qPCR:

For quantification of the prokaryotic biomass present in each sample, 16S rRNA gene qPCR was performed using DNA extracted from stool, applying a previously-published protocol (Liu *et al.*, 2012). A total of 20µl was made up for each qPCR reaction, consisting of the following: 1x Platinum Supermix with ROX (Life Technologies, Carlsbad, USA), 1.8 µM BactQUANT forward primer (5'-CCTACGGGAGGCAGCA-3'), 1.8 µM BactQUANT reverse primer (5'-GGACTACCGGGTATCTAATC-3'), 225nM probe ((6FAM) 5'-CAGCAGCCGCGGTA-3' (MGBNFQ)), PCR grade water (Roche, Penzberg, Germany), and 5µl DNA. Each plate also included an *E. coli* DNA (Sigma-Aldrich) standard curve, consisting of 3-300,000 copies per reaction in 10-fold serial dilutions, and a 'no template' negative control. All samples, standards, and controls were performed in triplicate. Extracted DNA samples were diluted to ensure they fell within the standard curve. The Applied Biosystems StepOnePlus Real-Time PCR System was used for amplification and real-time fluorescence detections using the following PCR cycling conditions: 50°C for 3 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A Mann-Whitney test was to compare 16S rRNA gene copy number between donor and FMT (both pre- and post-FMT) samples, and Wilcoxon signed-rank test to compare changes between pre- and post-FMT.

2.2.5. Prediction of bile-metabolising function of gut microbiota from metataxonomic data:

The inferential tool, Piphillin, was used to predict selected functionalities (including bile-metabolising functionality) of the stool microbiota as established from the metaxonomic data (Second Genome Therapeutics, n.d.; Iwai *et al.*, 2016). The algorithm of this tool uses direct nearest-neighbour matching between 16S rRNA gene sequencing datasets and microbial

genomic databases to infer the metagenomic content of samples. Piphillin was used with BioCyc version 21.0 as reference database, and applying 97% identity cut-off. Inference of gene abundance was assessed for a number of selected potentially-relevant genes, including 'choloylglycine hydrolase-RXN' (an alternative name for BSH) and for '7-alpha-hydroxysteroid-dehydrogenase-RXN'. Statistical testing between groups was performed using Mann-Whitney U for donor *vs* pre- or post-FMT, and Wilcoxon rank sum testing for pre- vs post-FMT .

2.3. Metabonomic analysis:

2.3.1. Overview of the principles of metabonomic analysis:

2.3.1.1. Introduction:

Metabonomics is defined as the, 'the quantitative measurement of the dynamic multi-parametric metabolic response of multicellular systems to pathophysiological stimuli' (Nicholson, Lindon & Holmes, 1999). Practically, it may be viewed as a technique that enables the detection, identification and quantification of metabolites responsible for mediating the phenotypic expression of altered metabolism resulting from a biological challenge (Mullish *et al.*, 2018a). In the context of gut microbiota analysis, identified metabolites may represent products of host metabolism, microbial metabolism, or host-microbiota co-metabolism. Metabonomics is performed through the analysis of biofluids or samples using spectroscopic techniques - in particular, nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry – with the subsequent use of advanced data analysis techniques (including multivariate statistical tools) to analysis the spectral data produced. Even when using a particular technique, a wide range of specific spectroscopic analytic protocols are available based upon biofluids/ samples available, nature of the metabolites of interest (molecular weight, polarity, etc).

This project involves the use of mass spectrometry for bile acid profiling, as well as for gas chromatography for SCFA identification and quantification. However, all forms of mass spectrometry apply similar principles, with the first step of the process being generation of gas phase ions of the compound within a vacuum. The molecular ion generated initially undergoes fragmentation, with the product ion undergoing fragmentation itself, etc. The mass spectrometer separates ions based on their mass-to-charge (*m/z*) ratio (through use of

an electric and/or magnetic field), which are then detected and recorded in proportion to their abundance. A mass spectrum is ultimately generated, plotting *m/z* ratio against ion abundance. Within the spectrum of a particular pure molecular metabolite, the molecular ion (if present) appears at the highest value of *m/z* (with ions containing heavier isotopes next following), enabling establishment of the compound's molecular mass (Przybylski, 2014).

2.3.1.2. Overview of mass spectrometry for bile acid profiling:

Mass spectrometry techniques have become well-established for bile acid analysis, due to their high sensitivity and specificity compared to other assays. In particular, high resolution time-of-flight spectrometry using a soft ionisation method (electrospray ionisation; ESI) coupled with ultra-performance liquid chromatography has become the analytical method of choice, since it can provide comprehensive coverage of bile acids from a range of complex biological samples with only relatively modest sample preparation (Sarafian *et al.*, 2015; Mullish *et al.*, 2018b; Przybylski, 2014). Use of an ultra-performance liquid chromatography system (as opposed to a conventional LC system) has a number of advantages, the main one being an approximate ten-fold reduction in experimental running time. More specific details on the methodology for bile acid profiling used in these experiments is given in **Section 2.3.2.**

2.3.1.3. Overview of mass spectrometry for short chain fatty acid identification and quantification:

While a range of methods have been explored, gas chromatography (GC) is well-established as the separation technique of choice for the identification of SCFA. Mass spectrometry (MS) appears to be the detector system with the highest sensitivity and specificity (Mills, Walker & Mughal, 1999), and GC-MS techniques have therefore become the reference standard. A derivatisation step is typically applied after the extraction step, as this aids in reducing the volatility of SCFAs (García-Villalba *et al.*, 2012). More specific details on the methodology for SCFA quantification used in these experiments is given in **Section 2.3.3.**

2.3.1.4. Introduction to multivariate statistical analysis of metabonomics data:

Multivariate statistical analysis in metabonomics utilises both unsupervised and supervised statistical techniques. The following section gives further insight into these techniques.

2.3.1.4.1. Unsupervised analysis:

Principal components analysis (PCA) is the most widely-used unsupervised approach. In the context of metabonomics, it uses as its input a data matrix where rows contain observations and columns contain variables, and applies a linear transformation technique (named orthogonal transformation) to convert the large set of variables in the original matrix to *principal components*, lower dimensionality data with minimal loss of information and preservation of variance (Worley & Powers, 2013). The first principal component is that with the largest possible variance (i.e. the component 'explaining' the largest part of the variance of the data table). The second component is computed under the constraint of being orthogonal to the first component and to have the largest possible variance, and so on for further components (Abdi & Williams, 2010).

This reduction in data complexity aids interpretability by allowing comparison of class differences and identification of outliers. Interpretation is typically performed on a two-dimensional space initially defined by the first and second principal components. In particular, data may be visualised on a scores plot, with data points representing all observations projected into two-dimensional space, and where points clustered together share similarity (Abdi & Williams, 2010).

There is also the requirement to scale data; this is particularly required for datasets where metabolites may have marked variability in their concentrations, to prevent dominance of variables with particularly large variance. A variety of scaling methods have been suggested, although pareto scaling is the preferred technique for UPLC-MS. Pareto (par) scaling requires each variable to be divided by the square root of its standard deviation, with the aim of reducing the relative importance of large values (Worley & Powers, 2013).

In these experiments, PCA has been applied on bile acid profiling metabonomic datasets for initial visualisation of spectral data based upon key variables, e.g. pre- *vs* post-FMT. Further specific details about methodology are provided in **Section 2.3.2.3.**

2.3.1.4.2. Supervised analysis:

A number of different supervised modelling techniques are available, with the best established being the partial least squares (PLS) technique. This is a multivariate linear regression modelling technique that, in the context of metabonomics, regresses one matrix containing metabonomic data against another with quantitative data of the sample (e.g. response to an intervention). Subsequent developments in this technique have included the introduction of an 'orthogonal filtration' step, whereby variance orthogonal to the variation of interest is removed (Fonville *et al.*, 2010).

A further advancement has been the development of projection to latent structures-discriminant analysis (PLS-DA), at its orthogonal equivalent, OPLS-DA. These apply the principles of PLS, but in the quantitative data in the second matrix, class membership is also indicated. The algorithm is instructed to maximally separate/ discriminate the observations in the matrix containing metabonomic data based upon the class membership specified in the second matrix (Worley & Powers, 2013). In all PLS-based analyses, the terminology is applied that the addition of class membership into the model (*Y* matrix) facilitates the selection of discriminating features (*X* matrix). In the analysis of such models, R2X and R2Y are terms used to indicate the proportion of explained variance attributable to the *X* and *Y* matrices respectively; the term Q2Y is used to denote the proportion of the predicted variance attributable to the model's *Y* matrix.

In these experiments, OPLS-DA has been applied on bile acid profiling metabonomic datasets to discriminate spectral data based on relevant classes such as pre- *vs* post-FMT (for FMT datasets), or recurrers *vs* non-recurrers (for the primary CDI dataset). Further specific details about methodology are provided in **Section 2.3.2.3.**

2.3.2. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) profiling of bile acids:

2.3.2.1. Preparation of faecal samples:

All samples for metabonomic analysis were collected and kept frozen until processing for analysis consistent with best practice protocols (Sarafian *et al.*, 2015; Gratton *et al.*, 2016; Mullish *et al.*, 2018b). Faecal samples were lyophilized for >24 hours using a VirTis Benchtop

BTP 8ZL freeze dryer (BPS, UK). The lyophilised samples were weighed, and bile acids were extracted using a 2:1:1 (vol) mixture of water, acetonitrile and 2-propanol in a Biospec bead beater with 1.0 mm Zirconia beads. After centrifugation (16000 x *g*, 20 minutes) the supernatant was filtered using 0.45 µm microcentrifuge filters with nylon membranes (Costar, Corning).

Samples were randomised, and bile acid analysis of faecal extracts was performed using ACQUITY UPLC (Waters Ltd, Elstree, UK) coupled to a Xevo G2 Q-ToF mass spectrometer equipped with an electrospray ionization source operating in negative ion mode (ESI-), using the method described by Sarafian and colleagues (Sarafian *et al.*, 2015).

Quality control (QC) samples were prepared using equal parts of the faecal filtrates. QC samples were used as a performance monitor for the assay (Sangster *et al.*, 2006), and as a proxy to remove features with high variation. QC samples were also spiked with mixtures of bile acid standards (55 bile acid standards including 36 non-conjugated, 12 conjugated with taurine, seven conjugated with glycine (Steraloids, Newport, RI, USA)) and were analysed along with the stool samples to determine the chromatographic retention times of bile acids and to aid in metabolite identification. Specifically, QCs were injected 10 times prior to initiating the run (to condition the column), once per every subsequent 10 injections, and again at the end of the run to assess MS stability and analytical reproducibility. Blank samples were also run to assess for constituents and impurities of the extraction solvents.

Waters raw data files were converted to NetCDF format and data were extracted using XCMS (v1.50) package with R (v3.1.1) software. XCMS is open-access software for the pre-processing of LC-MS software that allows the user to input preferred thresholds for a number of key variables (including peak width, peak intensity, etc) (Smith *et al.*, 2006). Probabilistic quotient normalisation (Veselkov *et al.*, 2011) was used to correct for dilution effects and chromatographic features with coefficient of variation higher than 30% in the QC samples were excluded from further analysis. Features were quantified as relative intensities, i.e. relative values of the integrated area under the curve for all identified chromatographic features. The relative intensities of the features were corrected to the dry weight of the faecal samples. For identification of bile acids from the final dataset, expected *m/z* ratios of

standards were established using appropriate metabonomic databases (particularly the Human Metabolome Database), and those of retention times were established using an in-house database derived from previous UPLC-MS analysis of bile acid standards.

2.3.2.2. Preparation of urine and serum samples:

As for faecal samples, all urine and serum samples for metabonomic analysis were collected and kept frozen until processing for analysis consistent with best practice protocols (Sarafian *et al.*, 2015; Gratton *et al.*, 2016; Mullish *et al.*, 2018b). Urine and serum samples were thawed and centrifuged (9500 x *g*, 20 minutes), and supernatant retained. Bile acids were extracted from 75 µl of urine and serum samples by adding 225 µl of cold methanol, followed by incubation at -20^{o}C for >2 hours. Tubes were centrifuged (9500 x *g*, 20 minutes) and 120 µl of supernatant loaded into vials. QCs were prepared as described for faecal samples. Sample processing and data analysis also followed the sample principles are outlined in **Section 2.3.2.1.**

2.3.2.3. Multivariate statistics analysis of UPLC-MS bile acid profiling data:

The data table obtained as output from XCMS was introduced to SIMCA 14.1 (MKS Umetrics AB), and data was pareto-scaled. Principal component analysis (PCA) was performed in order to visualise clustering of the samples, as well as in order to use the QC samples to assess the quality of the run. In addition, supervised analysis (using OPLS-DA) was undertaken in order to demonstrate the features responsible for the discrimination between different groups. OPLS-DA models were validated using CV-ANOVA, which provides a significance test of the null hypothesis of equal residuals between the model under validation, and a randomly-fitted model which uses the same data (Eriksson, Trygg & Wold, 2008). S-plots were used to visualise the highly-influential discriminatory features, and depict the covariance and the correlation structure between the X-variables and the predictive score t[1] of the model. Features at the far ends of the plot have a very high reliability whilst having a high model influence due to their high variance in the dataset (Wiklund *et al.*, 2008).

2.3.2.4. Integration of metataxonomic and bile acid profiling data:

Regularised Canonical Correlation Analysis (rCCA) was applied to correlate metataxonomic data with UPLC-MS bile acid profiling data from the same samples using the mixOmics library

within R (Lê Cao, González & Déjean, 2009; Gonzalez *et al.*, 2008). This technique maximises the correlation between the two data sets X and Y, using principles that overlap with that of PCA (see **Section 2.3.1.4.1.**). The shrinkage method was applied to determine regularisation parameters. Unit representation plots were generated using the plotIndiv function, where each sample is represented as a single point on the scatter plot, and samples were projected into two-dimensional/ XY-variate space. Correlation circle plots were generated using the plotVar function. On these plots, strong correlations between variables are plotted outside of the inner circle (i.e. correlations where $r>0.5$). Variables are represented through their projections onto the planes defined by their respective canonical variables. Strong positive correlations are present when variables are projected in the same direction from the origin, and strong negative correlations are present when variables are projected in opposite directions. Variables present at farther distances from the origin have stronger correlations.

2.3.3. Gas chromatography mass spectrometry for quantification of short chain fatty acids:

A targeted gas chromatography mass spectrometry protocol was used for the detection, identification and quantification of short chain fatty acids (SCFA) via adaptation of a protocol as previously-described (García-Villalba *et al.*, 2012). The protocol used ethyl acetate (Sigma) for the extraction of volatile compounds from stool, whilst tert-butyl methyl ether (MTBE) (Sigma) was used for extraction from serum and/or urine. In addition, derivatisation was performed using MTBSTF + 1% TBDMSCl (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchloro-silane) (Sigma). As for LCMS, QC samples were also prepared and run as a performance monitor for the assay. Calibration curves were obtained via the analysis of SCFA standards (Sigma) of known concentrations in full scan mode to allow specific quantification of each SCFA. MTBE with 100 parts per million (ppm) methyl stearate (Sigma) was used as internal standard.

Samples were randomised and analysed on an Agilent 7890B GC system coupled to an Agilent 5977A mass selective detector (Agilent, Santa Clara, California). Data analysis was performed using MassHunter software (Agilent), using retention times as stated in **Table 2.2**. Extracted ion chromatograms of the target ion selected for each SCFA were integrated, and the peak area was normalised to the internal standard to correct for variability in the instrument response. Whilst MassHunter performs automatic integration of spectral peaks, all peak

integration was also confirmed manually. SCFA concentrations were analysed through the use of univariate analysis, comparing groups (e.g. pre- *vs* post-FMT).

Table 2.2: Retention times used for the identification of spectral peaks of different SCFAs in GC-MS.

Metabolite	Retention time (minutes)
Short chain fatty acid:	
Acetate	2.50
Propionate	3.07
Isobutyrate	3.20
Butyrate	3.40
2-methylbutyrate	3.70
Isovalerate	3.80
Valerate	4.20
Caproate	4.90
Lactate	6.14
2-hydroxybutyrate	6.40
Internal standard:	
Methyl stearate	7.88

2.4. Bile salt hydrolase enzyme activity assay:

Faecal water was prepared and total faecal protein quantified using a similar method to that previously-described by Morris and co-workers (Morris & Marchesi, 2016), but with the addition of bacterial and mammalian protease inhibitor cocktails (G Biosciences, St Louis, MO, USA), as well as DTT to 1mM final concentration (Roche, Welwyn Garden City, UK) to minimise enzyme oxidation (Smith *et al.*, 2014).

The BSH assay itself was an adaptation of the conventional precipitation-based assay (Ling *et al.*, 1994; Tanaka *et al.*, 2000; Smith *et al.*, 2014). This assay exploits the observation that when unconjugated secondary bile acids are exposed to a pH < ~6.0, they will precipitate. As such, taurine- or glycine-conjugated secondary bile acids may be incubated with a BSH-containing solution, and the rate of precipitation formation reflects the rate of liberation of unconjugated secondary bile acid, providing a useful proxy of the activity of the BSH present (Mullish *et al.*, 2018b). Such BSH assays are typically performed in ambient air; of note, BSH-producing bacteria have relative insensitivity to oxygen exposure, whilst 7-α-dehydroxylase-

producing bacteria are all obligate anaerobes (Thomas *et al.*, 1997). As such, any secondary bile acid synthesised during the course of this assay represents the hydrolytic activity of BSH upon conjugated secondary bile acid, rather than 7-α-dehydroxylase-mediated conversion of primary to secondary bile acid.

The assay was performed in a clear flat-bottomed 96-well microtitre plate and incubated at 37°C at pH 5.8 for up to 8 hours. In a total volume of 200μl, 500μg of faecal protein was incubated with sodium phosphate buffer (pH 5.8, final concentration of 0.02mM), and taurodeoxycholic acid (TDCA) (Merck, Damstadt, Germany) (at final concentration 1mM). BSH has been demonstrated to have comparable activity for the hydrolysis of TDCA as for the hydrolysis of TCA (Tanaka *et al.*, 2000), making TDCA a suitable substrate for the assay. To prevent evaporation during incubation, wells were overlaid with 50μl of light paraffin oil (0.85g/ml; PanReac AppliChem, Barcelona, Spain) (Tanaka *et al.*, 2000). Samples were assayed in triplicate, with precipitation of insoluble deoxycholic acid monitored by absorbance measurement at 600nm (A_{600}) using a microplate reader (MultiSkan Go, Thermo Scientific, Dartford, UK). Faecal protein incubated with phosphate-buffered saline served as a negative control, and faecal protein incubated with varying concentrations of deoxycholic acid (Merck) was used to establish a standard curve to quantify precipitate formation.

2.5. Real-time PCR for the quantification of *bsh* and *baiCD* gene copy number:

2.5.1. Bacteria used as standards for real-time PCR:

The bacteria used as standards for the qPCR and for batch cultures experiments were obtained by the following route. *Bacteroides plebius*, *Bacteroides ovatus*, *Bacteroides vulgatus, Collinsella aerofaciens* and *Blautia obeum* had been previously isolated from the stool of a healthy male donor in his 20's (by Dr Julie A K McDonald, Imperial College London). *Bacteroides plebius* was isolated from fastidious anaerobe agar plates (Acumedia, USA) with 5% horse blood (VWR, USA). *Bacteroides ovatus* and *Bacteroides vulgatus* were isolated from nutrient agar plates (Sigma-Aldrich, USA). *Blautia obeum* was isolated from de Man, Rogosa and Sharpe agar plates (Sigma-Aldrich). *Collinesella aerofaciens* was isolated from tryptic soy agar plates (Sigma-Aldrich).

DNA extraction was performed on the isolates using the EZNA Bacterial DNA Kit (Omega, USA) applying the manufacturer's protocol, but with the addition of a bead beating using the Bullet Blender Storm (speed 8 for 3 minutes). A ~900 bp region of the 16S rRNA gene was amplified using previously published primers (Ben-Dov *et al.*, 2006), and DNA was sequenced at Macrogen Europe. Isolates were identified by performing a standard nucleotide BLAST of the 16S rRNA gene sequences (NCBI) (see **Section 2.2.3** for weblink).

2.5.2. Real-time PCR protocol:

qPCR was performed using extracted DNA as the template in order to quantify gene abundance/ copy number of relevant genes. Specifically, gene copy number was quantified for:

i) Specified groups of *bsh* (using degenerate primer sets previously-designed and optimised by our group; work performed by myself with Dr Julie McDonald, Dr Diya Kapila and Miss Grace Barker, Imperial College London) **(Table 2.3)** (Mullish *et al.*, 2018b)**.**

ii) *bai*CD*,* using primers well-established within the literature (Wells *et al.*, 2003).

Table 2.3: Primer sequences and PCR conditions for *bsh* and *baiCD* qPCR.

Group	Primer Sequence (5'-3')	F/R	Cycling Conditions	Expected Product Size (bp)
***bsh* 1A**	CACATATTGTGGCACGAACAATHGAR TGGGG	F	95°C for 10 min, (95°C for 15 sec, 55°C for 1 min) x 40 cycles	570
	CTGTGCCCGGATACAGATTAACRTAR TTRTT	R		
***bsh* 1B**	CGGCGTTCCGCATTTYTAYGARAA	F	95°C for 10 min, (95°C for 15 sec, 55°C for 1 min) x 40 cycles	318
	GTTCAATGCCAATCGGAATATCRAAR TTRTT	R		
***bsh* 3C/E**	TTTTGGCCGAACACTGGAYTAYGARTT	F	95°C for 5 min, (95°C for 15 sec, 54°C for 30 sec, 72 for 10 min) x 40 cycles	774
	TCAACGGAGCCCAGAATATGRAARA AYTG	R		
***bai*CD**	GGWTTCAGCCCRCAGATGTTCTTTG	F	94°C for 2 min, (94°C for 20 sec, 52°C for 30 sec, 69°C for 90 sec) x 35 cycles, 68°C for 10 min	745
	GAATTCCGGGTTCATGAACATTCTKCKAAG	R		

Each PCR reaction used a total reaction volume of 25 µl, consisting of 20 µl master mix and 5 µl diluted DNA (i.e. 12.5 ng total per reaction). All DNA was diluted in buffer EB (Qiagen, Hilden, Germany). A standard master mix consisting of 12.5 µl of 2x SYBR green master mix (ThermoFisher Scientific, Waltham, Massachusetts, USA), 5.5 µl PCR grade water (Roche, Penzberg, Germany), 1 µl of 10 µM forward primer (Eurofins Genomics, Wolverhampton, UK) and 1 µl of 10 µM reverse primer (Eurfins Genomics) was used. One bacterial strain from the relevant reference group was selected as a standard for each primer set (*bsh* group 1A – *Bacteroides plebius; bsh* group 1B – *Bacteroides ovatus; bsh* group 3C – *Blautia obeum; baiCD* – *Clostridium scindens* (DSMZ 5676, Braunschweig, Germany). Serial dilutions of each isolate were used to create a standard curve. Whilst *bsh* primers were degenerate, each primer set used was specific for an individual BSH group. Thermocycling conditions for each primer set are summarised in **Table 2.3.** A melt curve stage was performed post-cycling, to enable

confirmation of primer specificity. PCR products were also visualised using the 2200 Tapestation System (Aligent Technologies, Santa Clara, California, USA) in combination with D1000 reagents and D100 screentapes (Agilent Technologies), following the manufacturer's instructions.

Gene copy number was calculated from qPCR data using the following formula: gene abundance = (quantity (ng) x 6.022 x 10^{23} (gene copy number/ mol))/ (length of product x 1 x 10^{9} (ng/g) x 600 (g/mol)). Mean copy number was calculated for each set of triplicates, and was divided by the total DNA per reaction to establish average gene copy number per ng of DNA.

2.6. *Clostridioides difficile* batch cultures:

2.6.1. *C. difficile* spore preparation and enumeration:

C. difficile spores were prepared from strains via the application of previously-described methodology (Freeman, O 'Neill & Wilcox, 2003). Three different strains of *C. difficile* were used, namely DS1684 (ribotype 010), CD630 (ribotype 012) and R20291 (ribotype 027). *C. difficile* strain DS1684 (ribotype 010) is a non-toxigenic strain of *C. difficile,* whilst the two other strains used are toxin-producing. R20291 (ribotype 027) is a hypervirulent strain, whilst CD630 (ribotype 012) is a virulent multidrug resistant strain. Ribotype 027 is a well-established cause of hospital outbreaks of CDI, being previously responsible for a number of CDI epidemics in Europe and remaining prevalent in North America; (Wilcox *et al.*, 2012; Waslawski *et al.*, 2013; Davies *et al.*, 2014); ribotype 012 was formerly one of the more common *C. difficile* ribotypes found in mainland China (Huang *et al.*, 2009), although is now of decreasing prevalence within this region. These two toxigenic strains are genetically and phenotypically well-characterised (Dawson *et al.*, 2011), and so were thought to be appropriate to use as representative ribotypes.

For spore preparation, *C. difficile* ribotypes 010, 012 and 027 were grown anaerobically on fastidious anaerobe agar (FAA) plates supplemented with 5% defibrinated horse blood (VWR, Radnor, USA) and incubated at 37°C for 7 days. Using a sterile loop, the growth was removed from the plates and resuspended in 1ml of sterile water. Next, 1ml of 95% ethanol was mixed with the cell suspension and was incubated at room temperature for 1 hour. The cell

suspension was centrifuged at 3000 x *g* and the pellet resuspended in 1ml sterile water. Spores were stored at -80°C until required.

Spores were enumerated by preparing serial 10-fold dilutions in PBS (Sigma-Aldrich) and plating the dilutions on Braziers Cycloserine Cefoxitin Egg Yolk agar plates (containing Braziers Cycloserine Cefoxitin Egg Yolk agar base (Lab M), cycloserine 250 mg/L (VWR), cefoxitin 8 mg/L (Sigma-Aldrich), 8% egg yolk emulsion (SLS, Nottingham, United Kingdom), 2% lysed defibrinated horse blood (VWR), and lysozyme 5 mg/l (Sigma-Aldrich) (Crowther *et al.*, 2014). Plates were incubated anaerobically at 37°C for 48 hours and the colonies were enumerated.

2.6.2. Batch culture protocol:

The batch culture experiments were based upon the principle that TCA is a potent pro-germination trigger to *C. difficile* spores; as such, the addition of BSH-producing organisms to media containing TCA and *C. difficile* spores would therefore be expected to significantly reduce *C. difficile* germination (through the hydrolysis of TCA to taurine and cholic acid, with cholic acid being only a weak *C. difficile* pro-germinant (Sorg & Sonenshein, 2008)).

Batch cultures were performed via adaptation of a previously-described protocol (Sorg & Sonenshein, 2008). A range of different bacterial species established to produce BSH from different BSH groups (and *Clostridium scindens,* as a known 7-α-dehydroxylase-producer, but which is not annotated as possessing a *bsh* gene) were incubated in sBHI (supplemented Brain Heart Infusion broth (Sigma-Aldrich), with 5mg/ml yeast extract (Sigma-Aldrich), and 0.1% w/v L-cysteine (Sigma-Aldrich)), with or without 1% w/v TCA added. These bacterial species were partly selected based upon changes seen in the metataxonomic analysis between the stool microbiota pre- and post-FMT. Specifically, the microorganisms selected had all been shown to be reduced in mean proportion in the gut microbiota of pre-FMT patients in comparison to donors and/or post-FMT samples.

The bacterial species used in batch cultures also included wild-type *E. coli* MG1655 (which does not contain *bsh* genes within its genome), along with two forms of *E. coli* MG1655 into which *bsh* genes had been cloned using pBKminiTn*7*GM2 under the control of the P44 promotor (Joyce *et al.*, 2014) (gifted from Dr Cormac Gahan and Dr Susan Joyce, SFI Research

Centre APC Microbiome Ireland, University College Cork, Ireland). Each of these forms of MG1655 also had StrepR and RifR persistence, allowing selection of these organisms on plate culture through the use of streptomycin and/or rifampicin respectively. The two species that constitutively produced BSH are hereafter referred to as '*E. coli* BSH_{low}' (containing a gene for BSH with narrow substrate range against conjugated bile acids, derived from *Lactobacillus salivarius*); and '*E. coli* BSH_{high}' (containing a gene for BSH with high glycine and taurine-deconjugating activity, obtained from *Bifidobacterium adolescentis*) (**Figure 2.1**). These bacterial species were used as a means of directly identifying the specific contribution of BSH to TCA-mediated *C. difficile* germination.

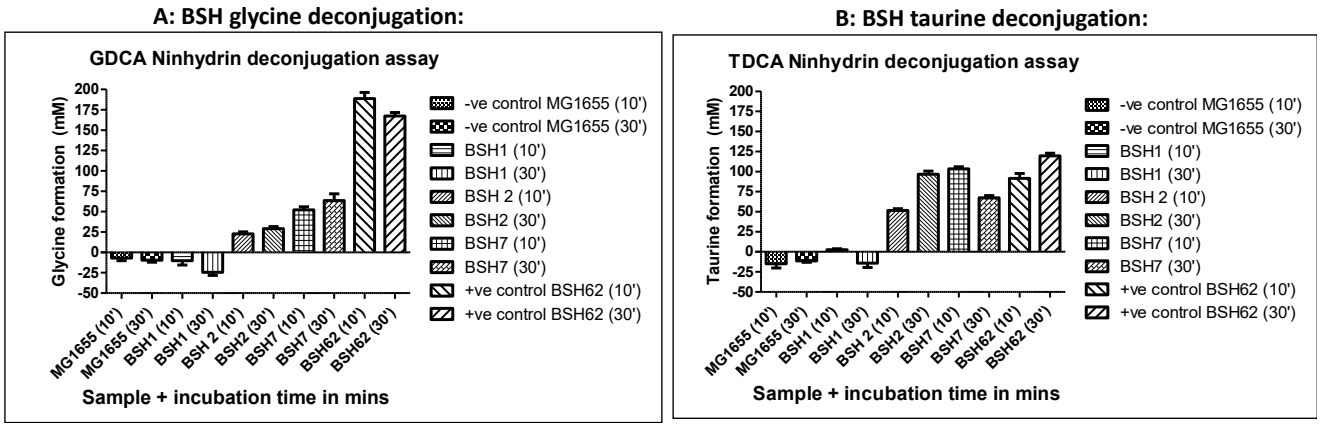


Figure 2.1: Demonstration of *in vitro* BSH activity of *E. coli* MG1655, both wild-type (BSH-negative) and strains engineered to express BSH. BSH 1, 2, 7 and 62: different forms of *E. coli* MG1655 that had been engineered to express BSH. 10' and 30': minutes after instigation of reaction that BSH activity was assayed. *In vitro* BSH activity was assessed for both A) glycine-conjugated bile acids (using glycodeoxycholic acid (GDCA) as substrate); and B) tauro-conjugated bile acids (using taurodeoxycholic acid (TDCA) as substrate). BSH1 = *E. coli* BSH_{low} (i.e. BSH with relatively low taurine-deconjugating ability only); BSH62 = *E. coli* BSH_{high} (i.e. BSH with relatively high glycine-and taurine-deconjugating activity). BSH activity was established via the ninhydrin assay (Tanaka *et al.*, 2000), i.e. measurement of the rate of liberation of taurine or glycine as a proxy of rate of BSH-mediated hydrolysis of a conjugated bile acid (described in more detail in **Section 2.8**). Data kindly provided by (and reproduced with permission of) Dr Susan Joyce and Dr Cormac Gahan, SFI Research Centre APC Microbiome Ireland, University College Cork, Ireland.

The basic protocol for the batch culture involved the incubation of *C. difficile* spores with spent (centrifuged and then filtered) supernatant from bacteria of interest that had been incubated with sBHI media either with or without TCA. Specifically, after overnight incubation within sBHI, cultures of bacteria of interest were centrifuged at 20000 x *g* for 10 minutes at

4°C; supernatant from the culture was diluted 1:3 with sBHI without added TCA, and again filter sterilised (0.2μm).

C. difficile spores from the three different ribotypes outlined above (010, 012 and 027) were resuspended in supernatant in triplicate, and an OD_{600} reading taken on a microplate reader at time zero (adjusted to OD_{600} of ~0.1 with supernatant/sBHI mix), and again after overnight incubation. An increased OD_{600} reading after overnight incubation was interpreted as indicating that spores had undergone germination and had grown as vegetative cells (Sorg & Sonenshein, 2008).

In addition, UPLC-MS was performed on batch culture supernatants to establish bile acid profiles, and BSH activity assays were performed on spent supernatant from selected batch cultures. For UPLC-MS bile acid profiling on batch culture supernatants, 75μl of supernatant was added to 225μl of cold methanol, followed by incubation at -30°C for at least 2 hours. Tubes were then centrifuged at 9500 x *g* at 4°C for 20 minutes, and 120μl of product loaded into vials. Subsequent UPLC-MS analysis was otherwise as already described.

Incubation for all batch cultures occurred at 37°C in an ElectroTek AW 400TG Anaerobic Workstation (ElectroTek, West Yorkshire, UK).

2.7. Recurrent *Clostridioides difficile* mouse model:

2.7.1. Ethics and animal welfare:

Mouse experiments were performed under the authority of the UK Home Office outlined in the Animals (Scientific Procedures) Act 1986 after ethical review by the Imperial College London Animal Welfare and Ethical Review Body (PPL 70/7969). Adherence was made to the standards articulated in the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Kilkenny *et al.*, 2010).

Wild-type C57BL/6 mice (8-10 weeks old; female) were purchased from Envigo (Huntingdon, UK) and acclimatised for one week before use. Mice were housed five per cage in individually ventilated cages with autoclaved food (RM1, Special Diet Services, Essex, United Kingdom),

bedding (Aspen chip 2 bedding, NEPCO, Warrensburg, New York) and water (provided *ad libitum*). Mice were subjected to a 12-hour light and 12-hour dark cycle at 20^{o}C-22^{o}C.

All handling of mice/ collection of samples was performed by Dr Thomas B Clarke and Ms Izabela Glegola-Madjeska, both MRC Centre for Molecular Bacteriology and Infection, Imperial College London. The design of all mouse experiments, preparation of antibiotics, preparation and quantification of *C. difficile* and *E. coli*, and all analysis of mouse samples was entirely my own work.

2.7.2. Mouse model:

The mouse model used in this work was an adaptation and extension of a previously-published mouse of rCDI and FMT (Seekatz *et al.*, 2015). In this model, 5- to 8-week-old male and female C57BL/6 mice received five days of oral cefoperazone to render them susceptible to *C. difficile,* and were two days later exposed to *C. difficile* spores (strain 630 (ATCC BAA-1382), a toxin-producing strain) via oral gavage. Between days 4 and 9 after infection, mice were treated with oral vancomycin. FMT was administered to mice via oral gavage either on day 11 post-infection, day 12 post-infection or two FMTs on both days. FMT was prepared via the pooling of faecal samples collected from 10 conventional, untreated, age-matched mice. The researchers validated this as a model of CDI relapse by demonstrating that by the time of completion of a course of vancomycin, mice had almost negligible stool *C. difficile* total viable counts (TVC) and toxin activity, along with minimal colitis; within a few days of completion of vancomycin, mice experienced a CDI relapse, as evidenced via stool *C. difficile* TVC and toxin activity at comparably high levels to that found pre-vancomycin, together with histological evidence of severe colitis (Seekatz *et al.*, 2015). The researchers further demonstrated that administration of FMT on two consecutive days after completion of vancomycin (i.e. FMT on protocol days 11 and 12) prevented CDI relapse (specifically, no increase in stool *C. difficile* TVC and toxin activity to levels higher than that found at the completion of vancomycin); furthermore, this remission from CDI was maintained for at least three weeks after FMT (Seekatz *et al.*, 2015).

In experiments performed for this thesis, mice were initially given cefoperazone 0.5mg/ml (Melford, Ipswich, UK) in their drinking water for 5 days (day -7 to day -2), followed by

challenge with ~10^3 *C. difficile* spores (DS1684 (ribotype 010)) by oral gavage on day 0 (spore quantification as described in **Section 2.6.1.**). Mice were treated with vancomycin 0.4mg/ml together with streptomycin 5mg/ml (both Melford, Ipswich, UK) in their drinking water for 3 days (days 4–7), followed by autoclaved antibiotic-free water for the remainder of the experiment. On both days 9 and 10, mice were fed by oral gavage either with ~10^9 colony-forming units (CFU) of wild-type *E. coli* (*n*=5) or *E. coli* BSH_{high} (*n*=5) (the rationale for administering streptomycin into drinking water prior to *E. coli* exposure was as a selection pressure to promote *E. coli* colonisation, given that all *E. coli* MG1655 used in these experiments expressed streptomycin-resistance genes within their genome). This particular protocol was used with the aim of recapitulating the dynamics of FMT administration to patients with rCDI. Mice were not fasted before oral gavages and all interventions were performed during the light cycle.

A summary of the protocol used in these studies for the mouse model for rCDI/ FMT is demonstrated in **Figure 5.6.**

2.7.3. Outcome measures from mouse model:

Serial faecal samples were collected from each mouse and *C. difficile* total viable counts (TVCs) quantified until *E. coli* colonisation began to decline. Mouse stool samples were collected directly into Carey-Blair medium and immediately homogenised by vortexing. *Clostridioides difficile* total viable counts (TVCs) were enumerated by performing serial 10-fold dilutions of faecal supernatant in PBS and plating onto Braziers Cycloserine Cefotixin Egg Yolk agar plates (as described earlier, with the addition of moxifloxacin 2mg/l (VWR)) in triplicate using the method of Miles and Misra (Miles, Misra & Irwin, 1938). Plates were incubated anaerobically at -37^{o}C for 2 days, and colonies were enumerated. *E. coli* CFUs were quantified by plating on MacConkey agar plates supplemented with rifampicin 50μg/ml (Melford, Ipswich, UK).

2.8. Purification of BSH enzyme:

It was recognised that purified BSH enzyme may have a useful role in future experiments (e.g. for use in future mouse works evaluating the role of BSH in preventing CDI), and therefore expression and purification of recombinant BSH enzyme was attempted.

The *bsh* gene from *Bifidobacterium adolescentis* was that selected for cloning, given that this had been demonstrated *in vitro* and *in vivo* to have high level BSH activity in the experiments using *E. coli* BSH_{high}. The *Bifidobacterium adolescentis bsh* gene was assembled from synthetic oligonucleotides and/or PCR products (960 bp), and inserted into the bacterial expression vector pTrcHis2a_A085 (Invitrogen, Thermo Fischer Scientific, UK). Plasmid DNA was purified from transformed bacteria, and concentration determined by UV spectroscopy, with the final construct verified by sequencing (Invitrogen, Thermo Fischer Scientific, UK). The map of the resulting plasmid is demonstrated is **Figure 2.2.**

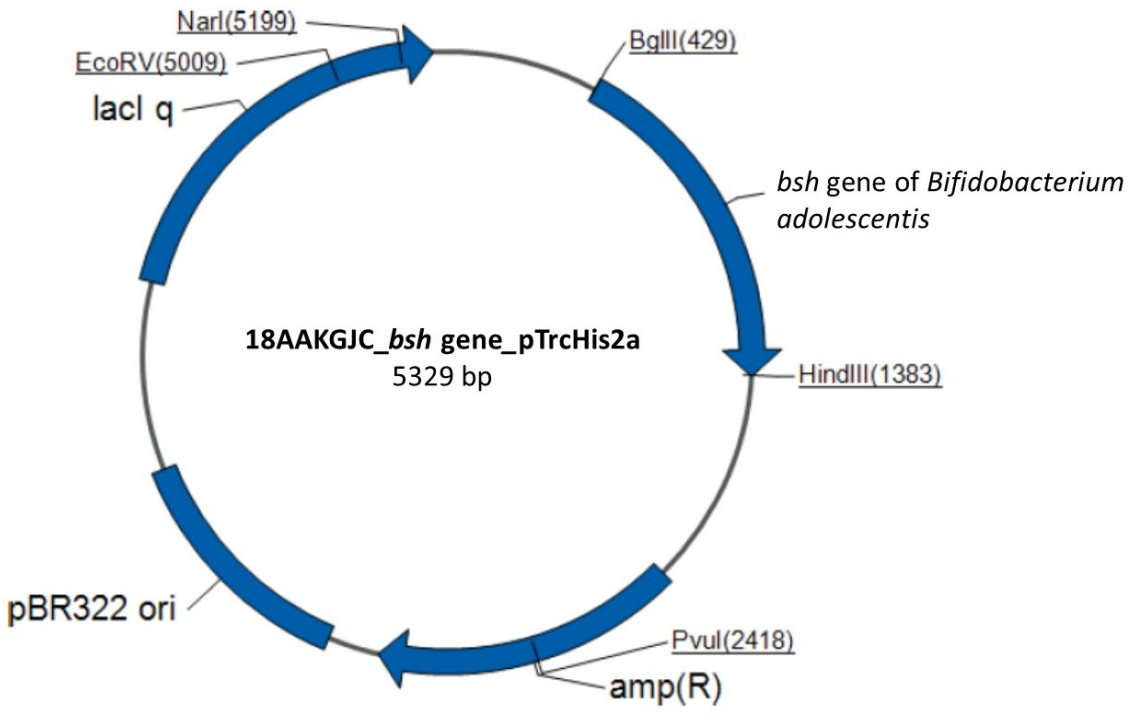


Figure 2.2: Plasmid map of pTrcHis2a_A085 vector containing *bsh* gene of *Bifidobacterium adolescentis.* As provided by Invitrogen, Thermo Fischer Scientific, UK.

Subsequent steps for expression and cloning were adapted from protocols as previously-described (Rani, Anandharaj & Ravindran, 2017; Kumar *et al.*, 2013). For expression of recombinant BSH (which was His-tagged (6x)), the plasmid was transformed into the expression host *E. coli* BL21-AI™ OneShot Chemically Competent cells (Invitrogen, Thermo Fischer Scientific, UK). Specifically, *E. coli* BL21-AI™ cells transformed with the plasmid were cultured overnight on LB agar plates with added ampicillin (concentration 100μg/ml)

(Melford, Ipswich, UK). Transformant cells were inoculated into lysogeny broth (LB) medium containing ampicillin from a starting OD_{600} of 0.01-0.1. Cultures were grown until reaching mid-log phase (OD_{600} ~0.4), at which point they were induced with L-arabinose (final concentration 0.2%) and isopropyl-β-d-thiogalactopyranoside (IPTG; final concentration 1mM) (both Invitrogen, Thermo Fischer Scientific, UK) for 3 hours to allow protein expression.

To purify protein, cells were centrifuged at 10000 x *g* for 10 minutes at 4°C. Cells were resuspended in lysis buffer containing 50mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM dithiothreitol (DTT), and 1x SIGMAFAST™ protease inhibitor cocktail (Sigma). Sonication was performed with resuspended cells on ice using 6 cycles of 20 seconds on/off with a 60% amplitude and 50% duty cycle on ice with a 3mm probe. Sonicated cells were centrifuged again at 10000 x *g* for 10 minutes at 4°C, and cell pellet and lysate were separated. BSH from the lysed cells was purified using His slurry/ Ni^{2+}-NTA agarose column using the manufacturer's protocol (Qiagen, Manchester, UK). Buffer containing imidazole (50-500mM) was used for elution, and protein concentration within eluted fractions was quantified using Bradford reagent (Bio-Rad, Watford UK). Protein purity was analysed using 12% (weight/volume) sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). No anti-BSH antibody was available to use on Western blot to confirm the presence of the purified protein; however, the plasmid vector had a C-terminal *c-myc* epitope fused to the inserted *bsh* gene. As such, the presence of purified BSH was further confirmed by Western blot using a mouse-derived primary antibody against c-myc (1:5000), and a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:10000).

BSH activity of the purified protein was assessed using the ninhydrin assay (Rani, Anandharaj & Ravindran, 2017; Tanaka *et al.*, 2000). As described above, this is an assay that allows measurement of the rate of liberation of taurine or glycine, which may be used as a proxy of rate of BSH-mediated hydrolysis of a conjugated bile acid. Specifically, an experimental reaction mixture of 200μl was prepared, containing 178μl of sodium phosphate buffer (0.1M, pH 6.0), 10μl of purified BSH protein, 2μl of 1M DTT (Roche, Welwyn Garden City, UK), and 10μl of 100mM conjugated bile acid (either taurocholic or glycocholic acid (Sigma-Aldrich)). The reaction mixture was incubated on a laboratory shaker at 37°C for 30 minutes. At this

point, the reaction was terminated by mixing 50µl of the reaction mixture with 50µl of 15% (w/v) trichloroacetic acid (Sigma-Aldrich), and precipitates were removed by centrifugation at 13000 x *g* at room temperature for 10 minutes. To enable estimation of concentrations of taurine or glycine that had been liberated by BSH's activity, 50µl of the supernatant from above was mixed with 950µl of ninhydrin reagent (Sigma-Aldrich) and 100µl of sodium-citrate buffer (0.5M, pH 5.5 (Sigma-Aldrich)). This mixture was placed in a laboratory shaker heated to 96^{o}C for 14 minutes, before cooling in an ice bath for 3 minutes. A standard curve (using either taurine or glycine, as relevant) was also constructed using the same protocol to enable specific quantification of the concentration of amino acid that had been liberated. Absorbance was measured on a spectrophotometer at 570nm, and – via combination of the results from the Bradford assay and use of the standard curve – enzyme activity was quantified as micromoles of taurine/ glycine released per minute per mg of BSH protein. All experiments were performed in triplicate.

2.9. Statistical analysis:

The analytical pathway for microbial sequencing data is summarised in **Section 2.2.3**, whilst multivariate UPLC-MS bile acid profiling data analysis is described in **Section 2.3.2.3.** As already described, correlation of metataxonomic and metabolomics data was undertaken via regularised Canonical Correlation Analysis (rCCA), using the mixOmics library within R (Le Cao, Kim-Anh, Rohart F, Gonzalez I, n.d.) (see **Section 2.3.2.4**).

Statistical analysis was performed using GraphPad Prism, v7.03. Mann-Whitney U test was used to compare the donor group with pre-FMT or post-FMT groups. Wilcoxon rank sum test, Friedman test or Kruskal-Wallis tests were used as appropriate to compare pre-FMT with post-FMT samples, and to compare different time points for collection of samples from patients with first CDI. Where multiple statistical testing was performed, Benjamini-Hochberg false discovery rate (FDR) correction was applied, unless otherwise stated. All statistics were two-tailed tests, unless otherwise stated.

Chapter 3: Initial exploration of the effect of faecal microbiota transplant for recurrent *Clostridioides difficile* infection upon gut microbiota bile-metabolising functionality:

3.1. Introduction:

The experiments in this chapter were an initial investigation of the hypothesis that recurrent CDI is associated with loss of gut microbiota members with bile-metabolising functionality, and that FMT is associated with restoration of this function. This investigation was performed using crude stool samples collected from patients with recurrent CDI prior to FMT, and again at 8-12 weeks after successful FMT. Crude stool samples were also analysed from the associated healthy stool donors.

3.2. Methods:

The same samples underwent a number of different analyses to explore the hypothesis. The initial analysis was performed to establish gut microbiota profile (using metataxonomics and associated analyses) and bile acid profiles. Based on the results of this, the activity and gene copy numbers of bile-metabolising enzymes, in particular BSH and 7-α-dehydroxylase, was also assayed.

3.3. Results:

3.3.1. Clinical details of subjects in this dataset:

Patient characteristics for the initial human dataset are summarised in **Table 3.1**; none of the included patients had inflammatory bowel disease. The average time to first CDI recurrence in these patients was 1.7 weeks (range: 1 – 6 weeks).

Table 3.1: Clinical characteristics of rCDI patients receiving FMT in the initial human dataset.

Characteristic	Value
Age (years) (mean +/- SD)	66 +/- 13
Sex	65% female (17/26)
BMI pre-FMT (kg/m^2)	25.01 +/- 7
Median recurrences	3 (range: 1 - 5)
Median prior CDI-related hospitalisations	1 (range: 1-3)
Reported antibiotic use prior to CDI	88% (23/26)
Reported previous failed vancomycin taper	58% (15/26)
Route of administration	31% capsulised (8/26), 69% colonoscopy (18/26)
PPI use	42% (11/26)
Statin use	12% (3/26)

Reproduced from (Mullish *et al.*, 2019).

A summary of the characteristics of the donors who provided samples for the initial human dataset is given in **Table 3.2**.

Table 3.2: Clinical characteristics of donors providing stool for FMT in the initial human dataset.

Characteristic	Value
Age (years) (mean +/- SD)	28 +/- 9
Sex	24% female (4/17)
BMI at initial recruitment (kg/m^2)	23.08 +/- 4
Declared time since last course of antibiotics (months) (mean +/- SD)	32.50 +/- 11

3.3.2. Gut microbiota analysis:

3.3.2.1. Introduction:

Stool microbiota analysis was performed on the samples from the initial human dataset, with the aim of evaluating whether successful FMT for rCDI was associated with an increased relative abundance of microbes with bile-metabolising capacity within the faecal microbiota. Similarly, a further aim was to compare the profile of bacteria producing bile-metabolising enzymes in the stool of healthy donors with that found in the stool of rCDI patients pre-FMT.

3.3.2.2. Real-time PCR of 16S rRNA gene:

Results from these PCRs are demonstrated in **Figure 3.1.** There was no significant difference in the stool bacterial load detectable between donors, pre-FMT and post-FMT, as evaluated by assaying the copy number of 16S rRNA genes in DNA extracted from stool (i.e. $p>0.05$ between all groups).

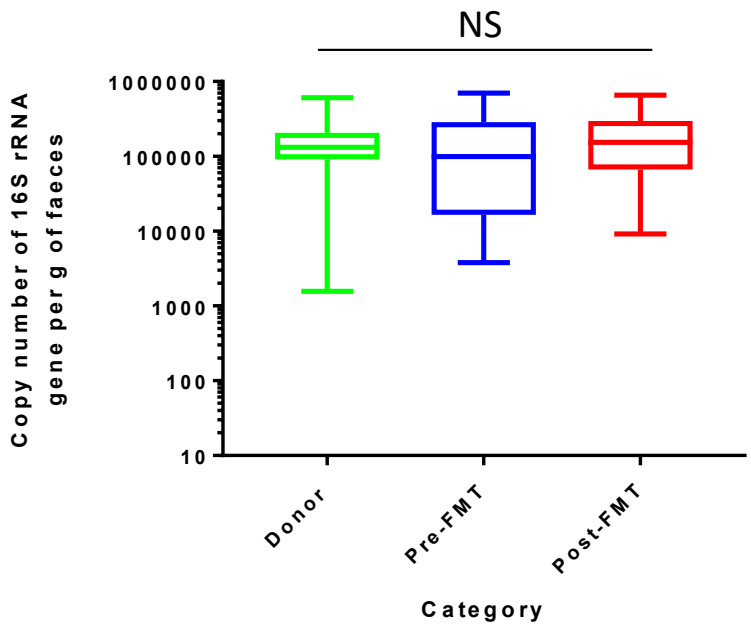


Figure 3.1: Effect of FMT for rCDI upon stool copy number of 16S rRNA gene. As established via qPCR. NS: non-significant (p>0.05). (Donors: n=17; rCDI patients pre- and post-FMT: n=26). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019)).

3.3.2.3. Ecological indices:

Results from these analyses are presented in **Figure 3.2.** Successful FMT for rCDI was associated with restoration of stool microbiota alpha diversity (as assessed by Shannon diversity index; **Figure 3.2A**) and richness (S_{obs}; **Figure 3.2B**) from the low levels found pre-FMT up to much higher levels comparable to healthy stool donors (p<0.0001 between pre- and post-FMT for both Shannon diversity index and richness, Wilcoxon rank sum test; p<0.0001 between donor and pre-FMT for both Shannon diversity index and richness, Mann Whitney test). Stool collected pre-FMT demonstrated profoundly altered microbial community structure (as measured by non-metric multidimensional scaling (NMDS); **Figure 3.2C**) as compared to healthy donor and post-FMT samples (p<0.001, PERMANOVA); conversely, post-FMT and donor samples demonstrated comparable microbial community structure.

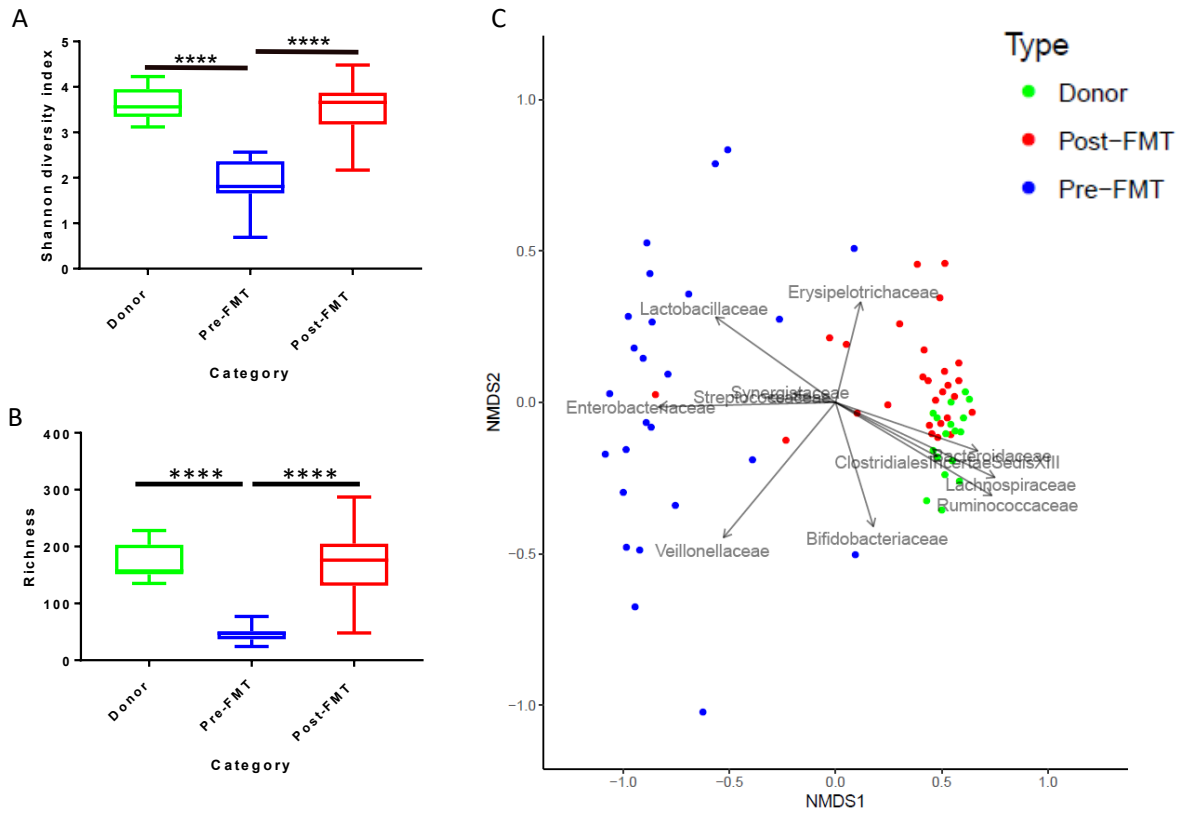


Figure 3.2: Effect of FMT for rCDI upon gut microbial diversity and community composition. A: Shannon diversity index for faecal samples (****, p<0.0001, Mann-Whitney test for donor *vs* pre-FMT, Wilcoxon rank sum test for pre-FMT vs post-FMT); B: Richness (S_{obs}, total number of bacterial taxa observed) (****, p<0.0001); C: Non-metric multidimensional scaling (NMDS) plot at family-level (p<0.001 across all three groups, PERMANOVA). (Donors: n=17; rCDI patients pre- and post-FMT: n=26). In A and B: centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Reproduced from (Mullish *et al.*, 2019)).

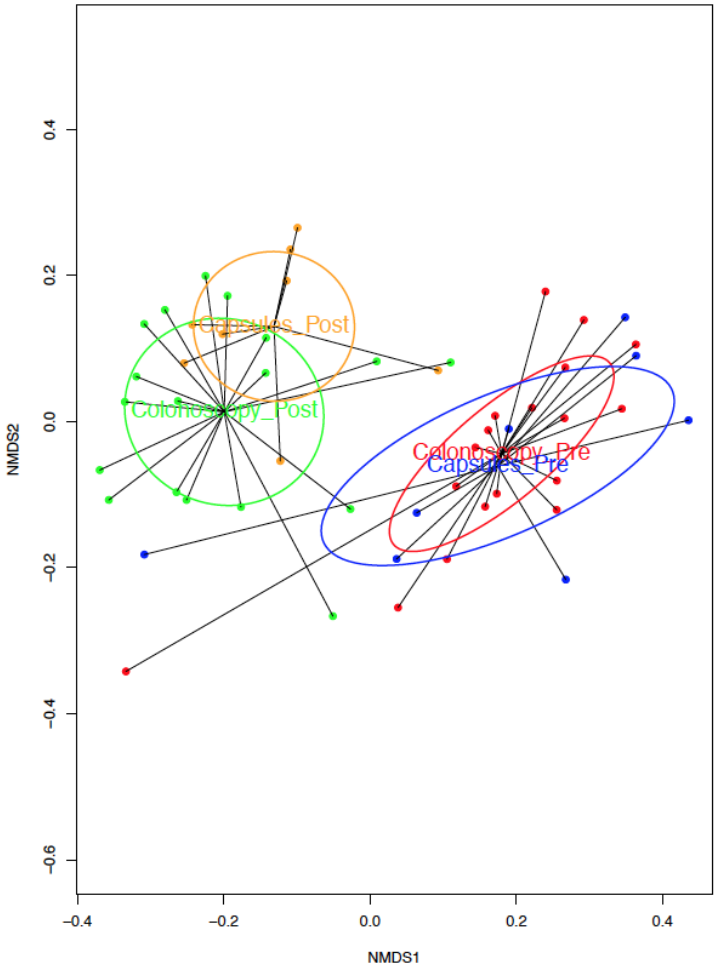


Figure 3.3: NMDS plot showing different routes of FMT administration (colonoscopy *vs* capsule administration) for pre- and post-FMT samples. There were no significant differences between stool microbial community structure for patients treated via capsule or colonoscopy pre-FMT (p=0.288, PERMANOVA), or post-FMT (p=0.288, PERMANOVA). Blue: capsule-treated patients pre-FMT; red: colonoscopy-treated patients, pre-FMT; yellow: capsule-treated patients, post-FMT; green: colonoscopy-treated patients, post-FMT. (Capsules: n=8; colonoscopy: n=18). (Capsulised FMT: n=8; colonoscopic FMT: n=18). Reproduced from (Mullish *et al.*, 2019).

All stool samples were inseparable by microbial community structure pre-FMT and subsequently post-FMT, regardless of whether colonoscopy or capsule administration was used (p = 0.288, PERMANOVA, **Figure 3.3**).

3.3.2.4. Comparison of stool metataxonomic profiles between donors, pre-FMT and post-FMT samples:

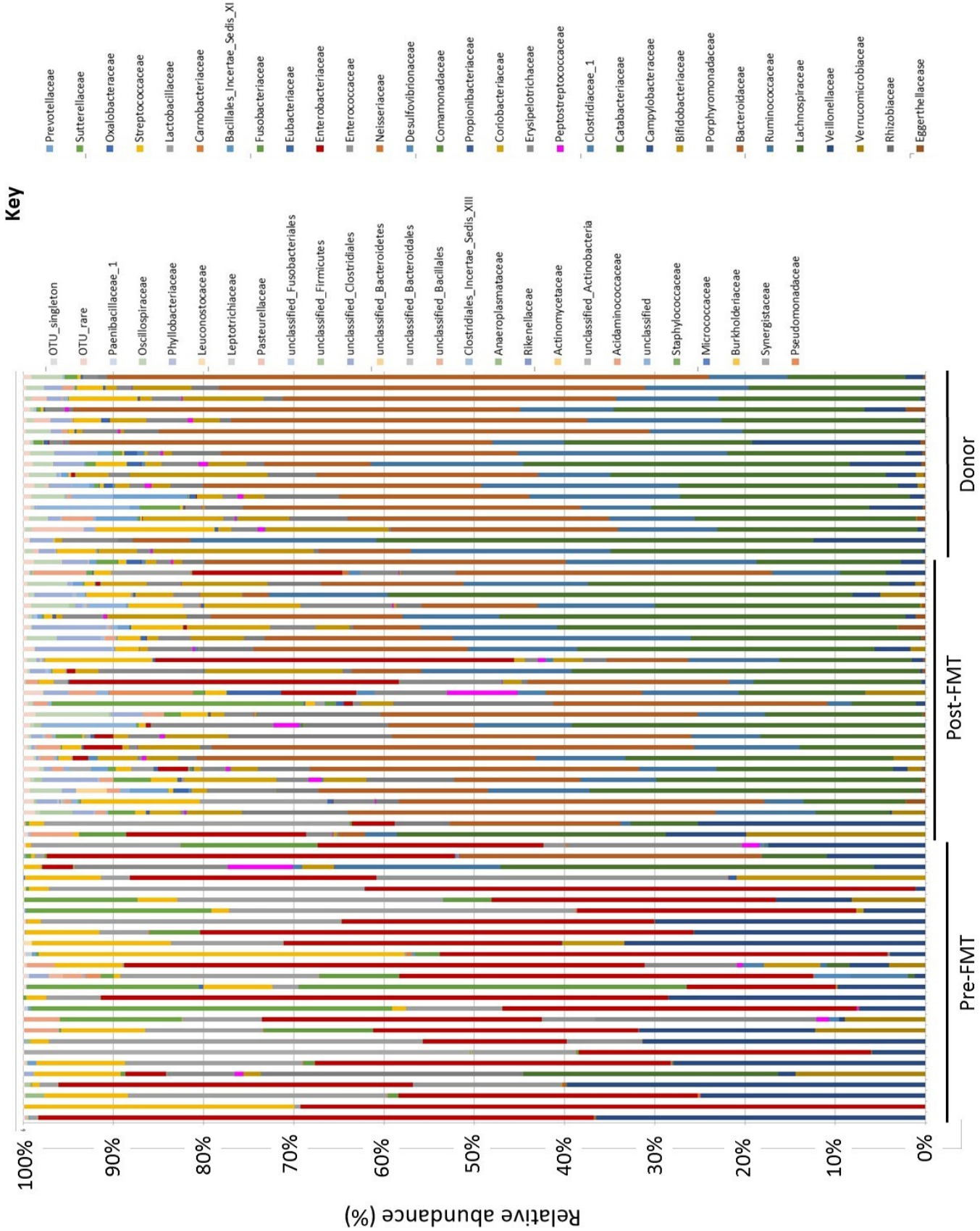
16S rRNA gene sequencing data (metataxonomics) derived from the initial human dataset was analysed at multiple taxonomic levels.

At the family level, rCDI patients pre-FMT had lower relative abundances of the bacterial families *Lachnospiraceae, Ruminococcaceae* and *Bacteroidaceae,* and higher relative abundances of *Enterobacteriaceae, Lactobacillaceae* and *Veillonellaceae* compared to healthy donors (p<0.01 in all cases, Whites' non-parametric *t*-test with Benjamini-Hochberg FDR correction; **Figure 3.3** and **Figure 3.4**). These families were all found at similar relative abundances in post-FMT and healthy donor samples.

Where possible (see **Section 2.2.3**), metataxonomic analysis included evaluation even at the species level. Results of metataxonomic analysis at the species level are displayed in **Figure 3.5 and 3.6.** Bacterial species enriched in the faecal microbiota of healthy donors compared to pre-FMT samples were notable for a range of BSH-producing organisms (see **Appendix 1**), including members of group 1 (*Bacteroides ovatus, q*=0.017; *Bacteroides uniformis, q*=0.007), group 2 (*Bifidobacterium dentium, q*=0.014; *Collinsella aerofaciens, q*=0.009; *Bifidobacterium longum, q*=0.011) and group 3 (*Bacteroides vulgatus, q*=0.003; *Faecalibacterium prausnitzii, q*=0.003; *Eubacterium rectale, q*=0.005; *Blautia obeum, q*=0.014) (**Figure 3.5**). Similarly, bacterial species enriched in the faecal microbiota post-FMT compared to those pre-FMT also included members of all BSH groups, including group 1 (*Bacteroides uniformis, q*=0.005; *Bacteroides ovatus, q*=0.009; *Parabacteroides distasonis, q*=0.003), group 2 (*Collinsella aerofaciens, q*=0.006; *Bifidobacterium dentium, q*=0.029) and group 3 (*Bacteroides vulgatus, q*=0.002; *Eubacterium rectale, q*=0.004; *Blautia obeum, q*=0.009; *Faecalibacterium prausnitzii, q*=0.003) (**Figure 3.6**). Even where species-level annotation was not possible, the higher level taxonomic categorisation was often for taxa which are known to contain multiple BSH-producing bacteria (i.e. for unclassified *Bacteroides, q*=0.003 for donor *vs* pre-FMT samples (**Figure 3.5**), and *q*=0.006 for pre- *vs* post-FMT samples (**Figure 3.6**)).

It was observed that two species of *Lactobacilli* which produce BSH (*Lactobacillus salvarius* and *Lactobacillus plantarum*) were overall enriched in pre-FMT samples compared to donor and/or post-FMT samples. On further analysis, this was only the case of the 4/26 patients taking probiotics prior to FMT (these *Lactobacilli* are included in many common probiotic supplements). These four patients were all taking regular probiotic supplements until shortly before FMT, and did not restart following their FMT.

Figure 3.4: Relative abundance plot of stool metataxonomic profiles at family level. Each column represents an individual patient/ donor.



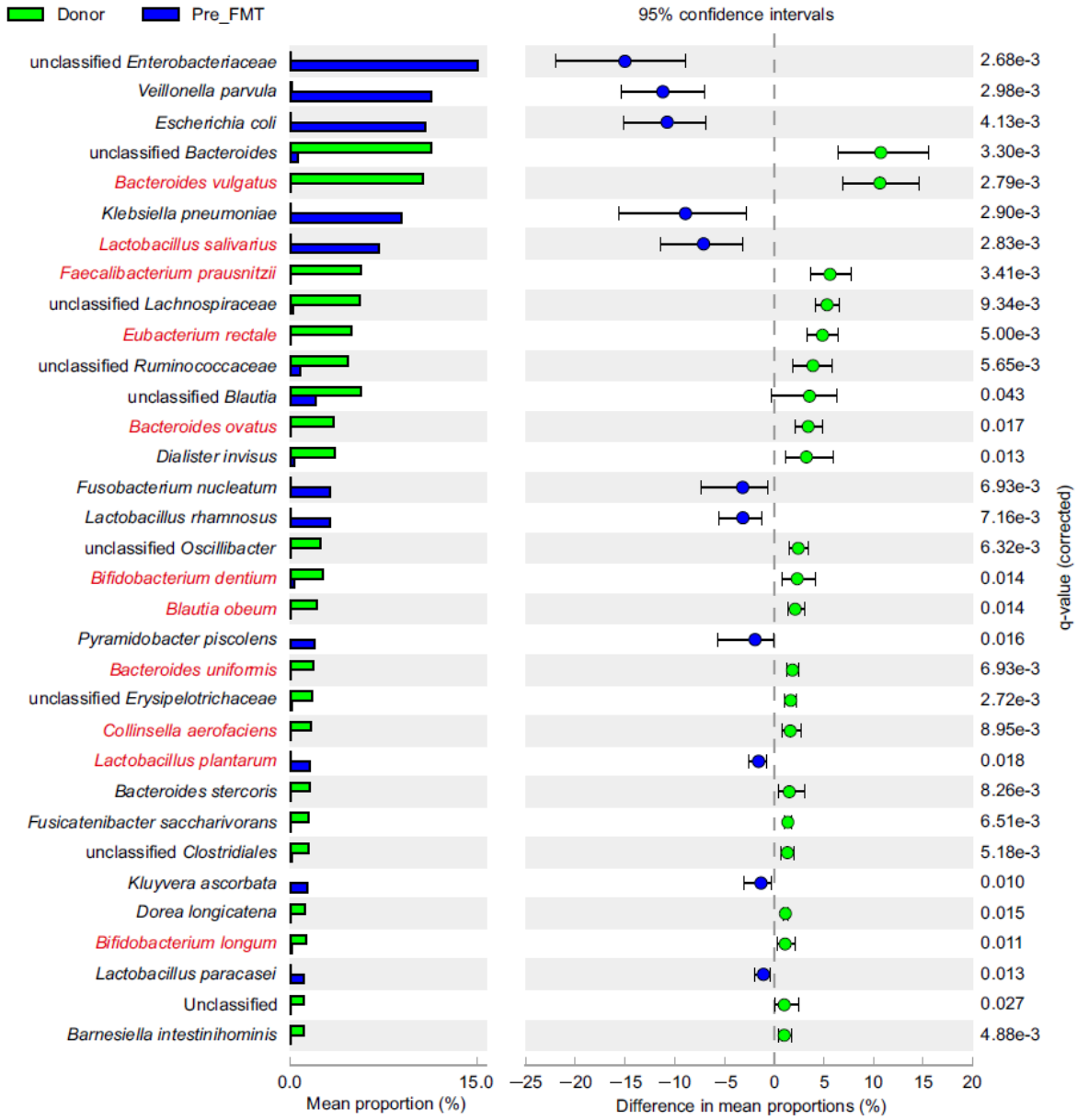


Figure 3.5: Species differences in 16S rRNA gene sequencing data in rCDI patients pre-FMT compared to donors. Extended error bar plots (mean percentage proportional change with 95% confidence intervals), with bacterial species changing significantly measured by White's non-parametric test with Benjamini-Hochberg correction. Names in red: known BSH-producing bacterial species (see **Appendix 1**). (Donors: *n*=17; rCDI patients pre-FMT: *n*=26). Reproduced from (Mullish *et al.*, 2019).

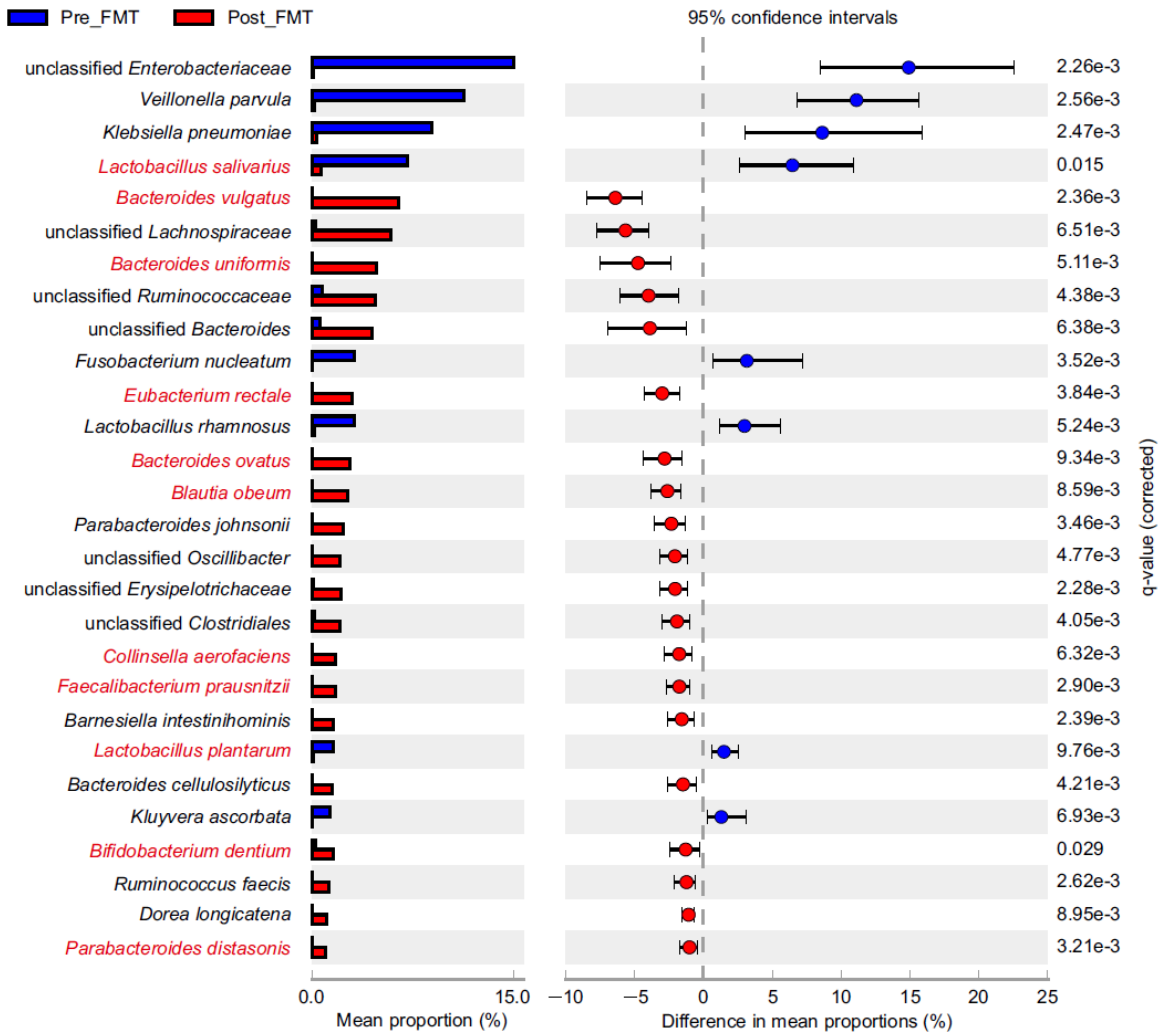


Figure 3.6: Species differences in 16S rRNA gene sequencing data in rCDI patients pre-FMT compared to post-FMT. Extended error bar plots (mean percentage proportional change with 95% confidence intervals), with bacterial species changing significantly measured by White's non-parametric test with Benjamini-Hochberg correction. Names in red: known BSH-producing bacterial species (see **Appendix 1**). (rCDI patients pre- and post-FMT: *n*=26). Reproduced from (Mullish *et al.*, 2019).

Analysis was also undertaken at the OTU level (**Figure 3.7** and **Table 3.3**). Once again, pre-FMT stool samples were characterised by a significantly reduced proportions of OTUs corresponding to BSH-producing organisms (across all BSH classes) in comparison to both post-FMT and healthy donor samples (**Figure 3.7** and **Table 3.3A and B**). The only OTU identified that could be labelled as *Clostridium scindens,* the archetypal 7-α-dehydroxylase-producing organism, was found in significantly reduced proportions between pre-FMT samples in comparison to donors (*q*=0.007, **Table 3.3C**), but with mean proportion difference of <1%. In contrast, this OTU was not identified as being significantly enriched post-FMT in comparison to pre-FMT samples (*q*=1.096, **Table 3.3C**). However, it was observed that there was a decreased relative abundance of the genus *Clostridium* cluster XIVa (i.e. the genus most strongly associated with 7-α-dehydroxylase-producing organisms (Ridlon, Kang & Hylemon, 2006)) in pre-FMT samples compared to donor samples (*q*=0.007), as well as in pre-FMT samples compared to post-FMT samples (*q*=0.005).

Figure 3.7 (page 104): OTU differences in 16S rRNA gene sequencing data in rCDI patients compared to donor or post-FMT. Extended error bar plots (mean percentage proportional change with 95% confidence intervals), with OTUs changing significantly measured by White's non-parametric test with Benjamini-Hochberg correction, using threshold of differences between mean proportions >1%. A: Donor *vs* pre-FMT; B: Pre-FMT *vs* post-FMT. Asterisks indicate OTUs changed in both comparisons. (Donors: *n*=17; rCDI patients pre- and post-FMT: *n*=26). Reproduced from (Mullish *et al.*, 2019).

Table 3.3 (page 105): Changes in mean proportions of OTUs after FMT for rCDI. A: OTUs enriched in donors compared to pre-FMT; B: OTUs enriched post-FMT compared to pre-FMT; C: Changes in the OTU corresponding to *Clostridium scindens,* the archetypal 7-α-dehydroxylase-producing bacterium. Annotation of species was performed after running OTU sequencing through Microbial BLAST. Where criteria were not met for annotation of genus or species, annotations as assigned by Mothur were used instead (these are the OTUs where no query cover or identity figure is quoted). OTUs highlighted in blue: microbes with *bsh* genes. (Donors: *n*=17; rCDI patients pre- and post-FMT: *n*=26). Reproduced from (Mullish *et al.*, 2019).

Figure 3.7:

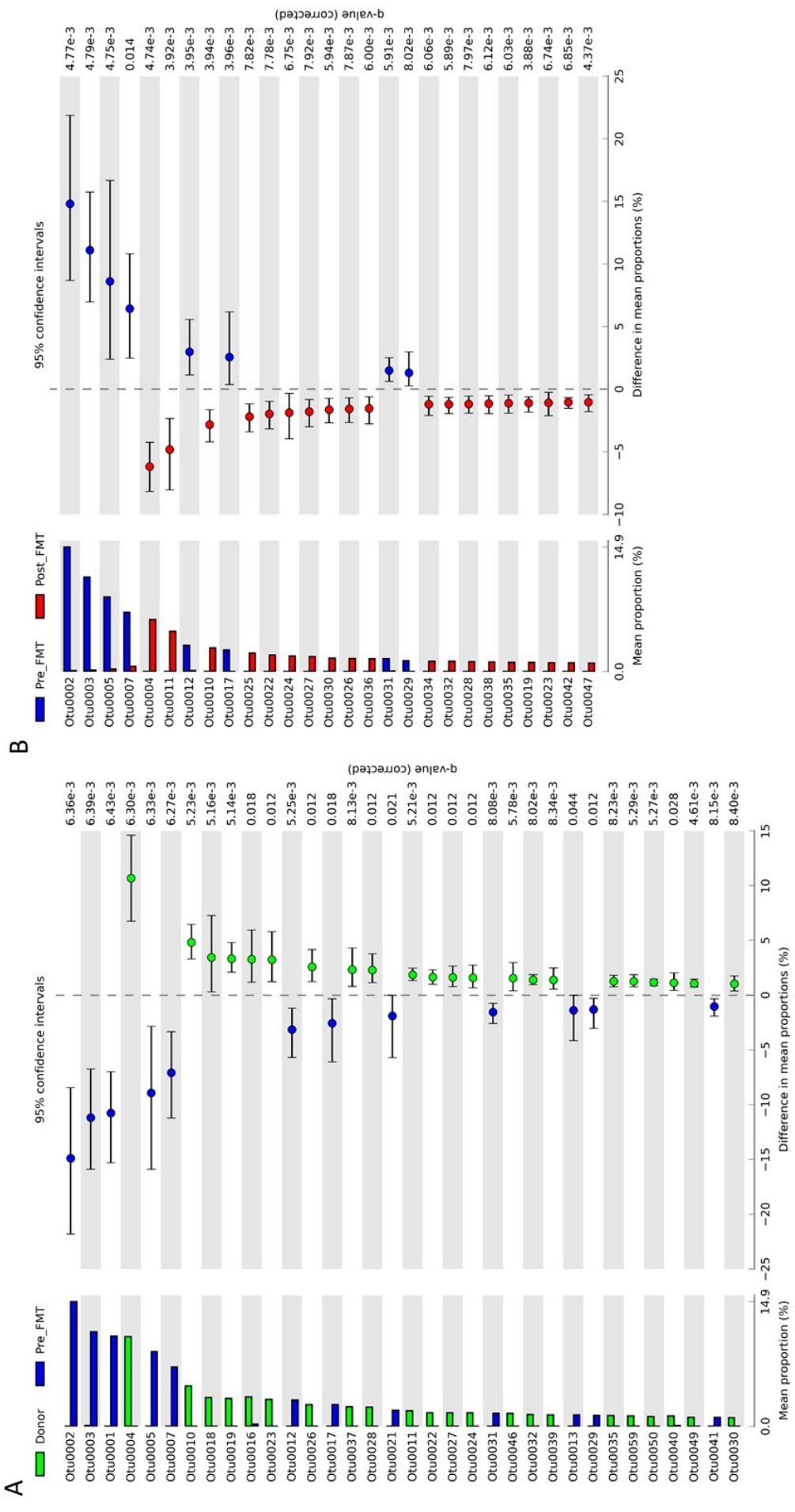

A
Donor
Pre_FMT
95% confidence intervals
Mean proportion (%)
Difference in mean proportions (%)
q-value (corrected)
B
Pre_FMT
Post_FMT
95% confidence intervals
Mean proportion (%)
Difference in mean proportions (%)
q-value (corrected)

Table 3.3:

A

OTUs enriched in donors vs pre-FMT:				
OTU	**Identity**	**Query cover (%)**	**Identity (%)**	**q value for difference in mean proportions (corrected)**
Otu0004	*Bacteroides vulgatus*	100	100	0.00630
Otu0010	*Eubacterium rectale*	100	100	0.00523
Otu0018	Unclassified *Bacteroides*	100	96	0.00516
Otu0019	*Faecalibacterium prausnitzii*	100	99	0.00514
Otu0016	*Dialister invisus*	100	100	0.018
Otu0023	Unclassified *Bacteroides*	-	-	0.012
Otu0026	*Bifidobacterium adolescentis*	100	100	0.012
	Bifidobacterium dentium	100	97	
Otu0037	*Bacteroides caccae*	100	99	0.00813
Otu0028	*Bacteroides ovatus*	100	99	0.012
Otu0011	*Bacteroides uniformis*	100	99	0.00521
Otu0022	*Bacterium LF-3*	100	100	0.012
Otu0027	*Collinsella aerofaciens*	100	99	0.012
Otu0024	Unclassified *Bacteroides*	100	94	0.012
Otu0046	*Bacteroides stercoris*	100	99	0.00578
Otu0032	*Anaerostipes hadrus*	100	100	0.00802
Otu0039	Unclassified *Ruminococcaceae*	-	-	0.00834
Otu0035	Unclassified *Oscillibacter*	-	-	0.00832
Otu0059	*Faecalibacterium prausnitzii*	100	98	0.00529
Otu0050	*Fusicatenibacter saccharivorans*	100	99	0.00527
Otu0040	*Bifidobacterium longum*	100	100	0.028
	Bifidobacterium sanguini	100	97	
Otu0049	Unclassified *Lachnospiraceae*	-	-	0.00461
Otu0030	*Barnesiella intestinihominis*	100	99	0.00840

B

OTUs enriched post-FMT compared to pre-FMT:				
OTU	**Identity**	**Query cover (%)**	**Identity (%)**	**q value for difference in mean proportions (corrected)**
Otu0004	*Bacteroides vulgatus*	100	100	0.00474
Otu0011	*Bacteroides uniformis*	100	99	0.00392
Otu0010	*Eubacterium rectale*	100	100	0.00394
Otu0025	*Parabacteroides johnsonii*	100	98	0.00782
Otu0022	*Bacterium LF-3*	100	100	0.00778
Otu0024	Unclassified *Bacteroides*	100	94	0.00675
Otu0027	*Collinsella aerofaciens*	100	99	0.00792
Otu0030	*Barnesiella intestinihominis*	100	99	0.00594
Otu0026	*Bifidobacterium adolescentis*	100	100	0.00787
	Bifidobacterium dentium	100	97	
Otu0036	*Bacteroides cellulosilyticus*	100	98	0.00600
Otu0034	*Blautia obeum*	100	97	0.00606
Otu0032	*Anaerostipes hadrus*	100	100	0.00589
Otu0028	*Bacteroides ovatus*	100	99	0.00797
Otu0038	*Bacteroides ovatus*	100	97	0.00612
Otu0035	Unclassified *Oscillibacter*	-	-	0.00603
Otu0019	*Faecalibacterium prausnitzii*	100	99	0.00388
Otu0023	Unclassified *Bacteroides*	-	-	0.00674
Otu0042	*Blautia obeum*	100	98	0.00685
Otu0047	*Parabacteroides distasonis*	100	98	0.00437

C

OTUs enriched in donors vs pre-FMT:				
OTU	**Identity**	**Query cover (%)**	**Identity (%)**	**q value for difference in mean proportions (corrected)**
Otu0212	*Clostridium scindens*	100	99	0.00663
OTUs enriched post-FMT compared to pre-FMT:				
OTU	**Identity**	**Query cover (%)**	**Identity (%)**	**q value for difference in mean proportions (corrected)**
Otu0212	*Clostridium scindens*	100	99	1.096

3.3.2.5. Inference of metagenomic content from stool metataxonomic data using Piphillin: Piphillin was used to infer metagenomic content using the 16S rRNA gene sequencing data (Iwai *et al.*, 2016; Mullish *et al.*, 2018b). Applying this, there were a significantly reduced proportion of sequences predicted to represent both BSH (**Figure 3.8A**) and 7-α-dehydroxylase (**Figure 3.8B**) in pre-FMT samples in comparison to donors (p<0.0001, Mann-Whitney test), but with a significant increase in predicted content of both in post-FMT samples as compared to pre-FMT (p<0.05, Wilcoxon rank sum test).

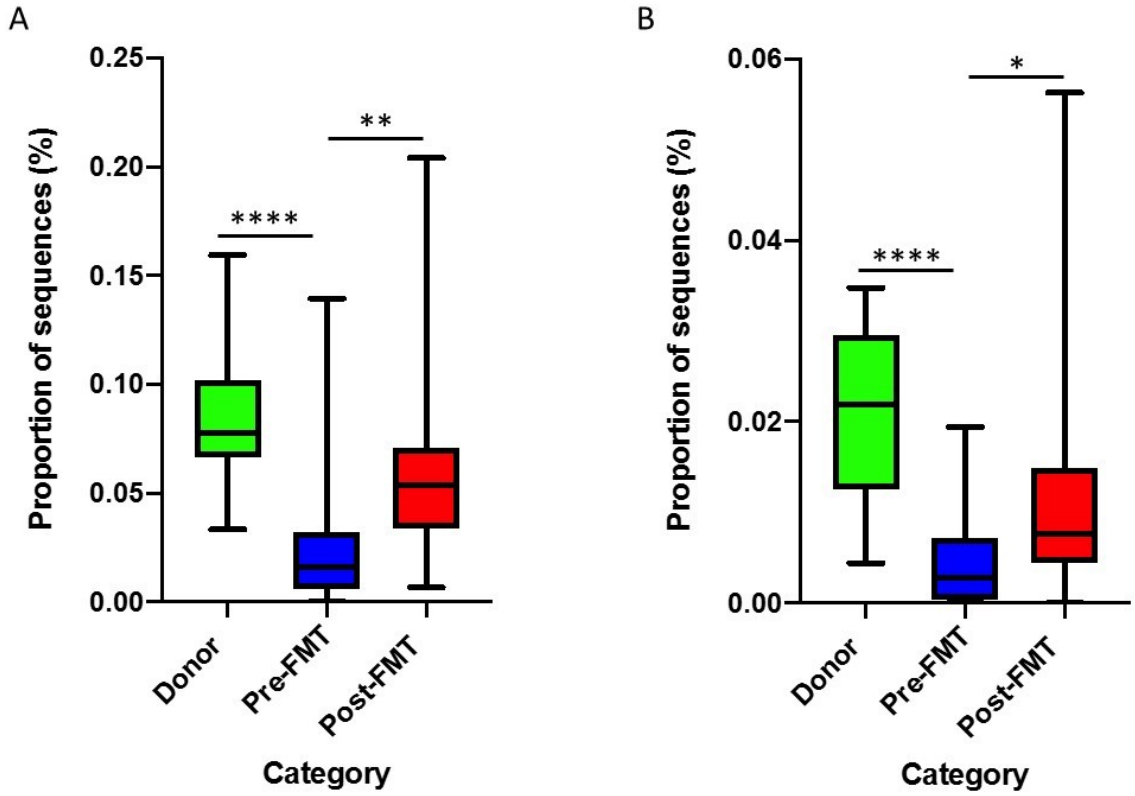


Figure 3.8: Effect of FMT for rCDI upon inferred faecal microbiota function. As established using Piphillin as inferentional tool and BioCyc database, with analysis performed in STAMP. A: Inferred 'choloylglycine-hydrolase-RXN' functionality; B: Inferred '7-alpha-hydroxysteroid-dehydrogenase-RXN' functionality (*, p<0.05; **, p<0.01; ****, p<0.0001; Mann-Whitney U for donor *vs* pre-FMT, Wilcoxon rank sum testing for pre- vs post-FMT). (Donors: n=17; rCDI patients pre- and post-FMT: n=26). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points.

3.3.3. Ultra-performance liquid chromatography-mass spectrometry for stool bile acid profiling:

3.3.3.1. Introduction:

UPLC-MS for stool bile acid profiling was also performed on stool samples from the initial dataset. The aim of these assays was to investigate the impact of FMT for rCDI upon the gut bile acid metabolism, and particularly to explore the changes in key bile acids known to impact the ability of *C. difficile* to undergo germination and/or vegetative growth.

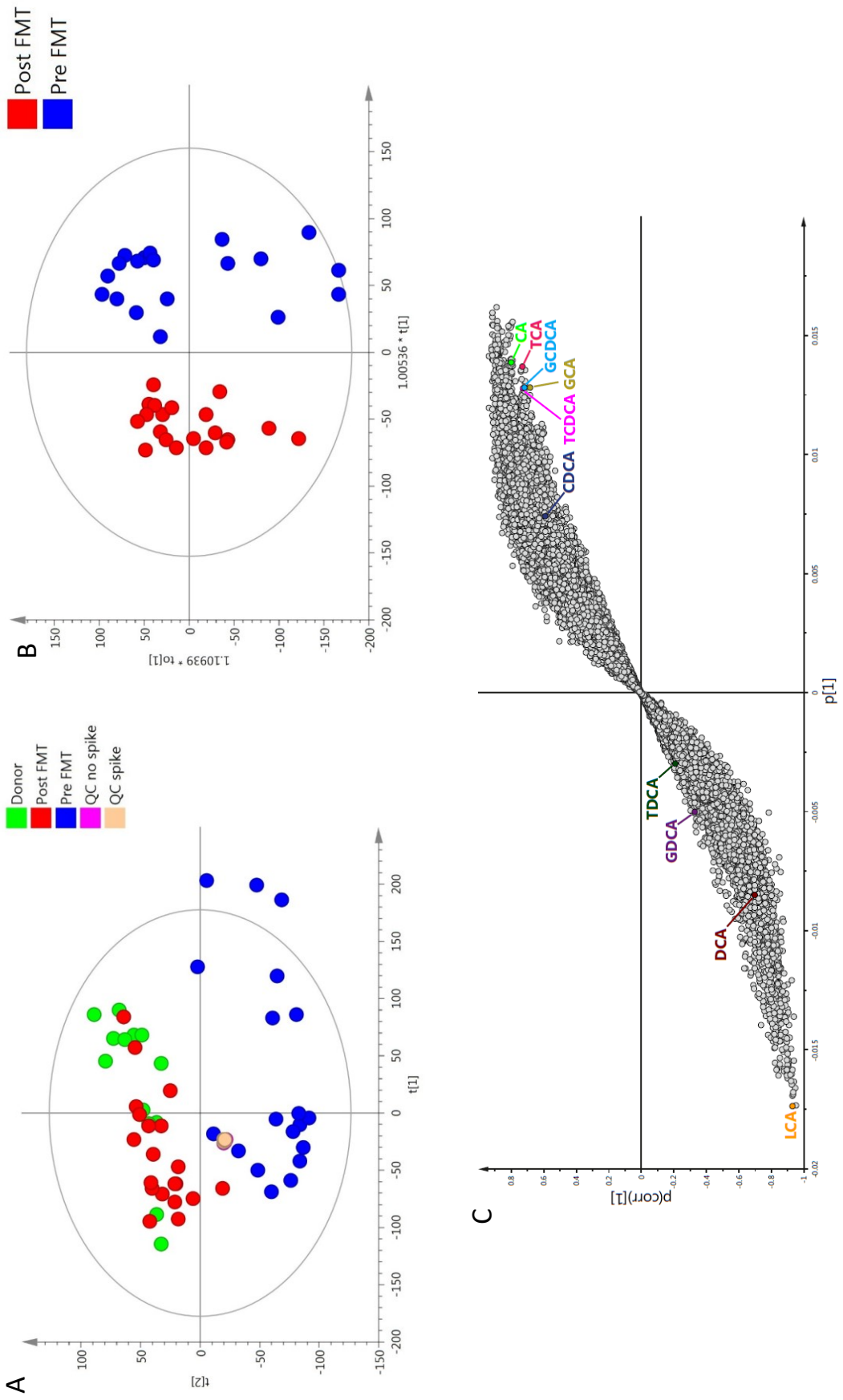
3.3.3.2. Multivariate analysis:

On multivariate analysis of UPLC-MS bile acid profiling data from the initial dataset, unsupervised principal component analysis (PCA) demonstrated close clustering of quality control (QC) samples, consistent with stability of the machine over the course of the run/ reproducibility of the assay (**Figure 3.9A**). On closer analysis, the PCA showed clustering of donor and post-FMT samples, but clear separation of both groups from pre-FMT samples (i.e. separation by the second principal component) (**Figure 3.9A**).

Supervised analysis was performed with orthogonal projections to latent structures discriminant analysis (OPLS-DA) to analyse the features responsible for discrimination between donor and pre-FMT groups (**Figure 3.10A**), and between pre-FMT and post-FMT groups (**Figure 3.9B**). Discriminatory feature identification was performed from OPLS-DA model data via S-plot, with pre-FMT samples showing an enrichment in primary bile acids (including both conjugated and unconjugated forms) and loss of secondary bile acids as compared to post-FMT and healthy donor samples (**Figure 3.9C, Figure 3.10B**). OPLS-DA model validation was performed using CV-ANOVA (**Table 3.4**).

Figure 3.9 (page 108): Effect of FMT for rCDI upon stool bile acid profiles. Assessed via multivariate analysis of UPLC-MS bile acid profiling data from the initial dataset. A: PCA scores plot; B: OPLS-DA scores plot, comparing pre-FMT and post-FMT samples; C: OPLS-DA S-plot of pre- vs post-FMT data. QC: quality controls. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; LCA: lithocholic acid; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid. (Donors: *n*=17; rCDI patients pre- and post-FMT: *n*=26). Reproduced from (Mullish *et al.*, 2019).

Figure 3.9:


A
Donor
Post FMT
Pre FMT
QC no spike
QC spike
t[1]
t[2]
B
Post FMT
Pre FMT
1.00536 * t[1]
1.10939 * to[1]
C
CA
TCA
GCDCA
CDCA TCDCA GCA
TDCA
GDCA
DCA
LCA
p[1]
p(corr)[1]

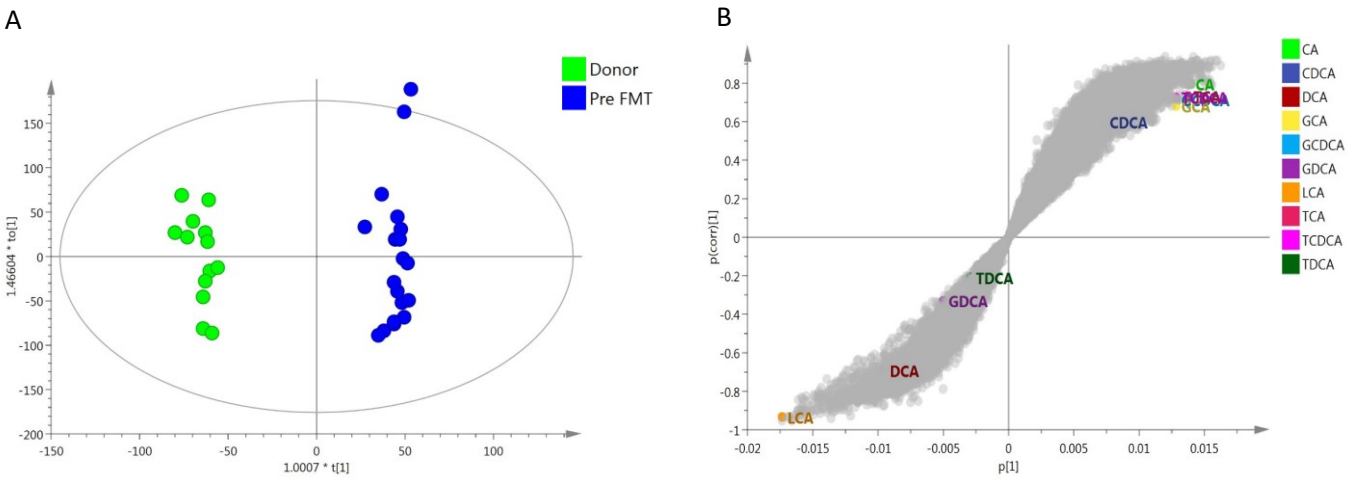


Figure 3.10: Further UPLC-MS bile acid analysis of the initial dataset. A: OPLS-DA scores plot, comparing donor and pre-FMT samples; B: OPLS-DA S-plot of donor vs pre-FMT data, as assessed via multivariate analysis of UPLC-MS bile acid profiling data. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; LCA: lithocholic acid; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid. (Donors: *n*=17; rCDI patients pre-FMT: *n*=26). Adapted from (Mullish *et al.*, 2019).

Table 3.4: Model characteristics for OPLS-DA models.

Multi-variate model	R^2X	Q^2	*p* value
Donor vs pre-FMT	0.533	0.933	1.85×10^{-13}
Pre- vs post-FMT	0.437	0.839	5.24×10^{-13}

Validation of the models is expressed with *p* values derived from CV-ANOVA. Reproduced from (Mullish *et al.*, 2019).

3.3.3.3. Univariate analysis:

Univariate analysis supported these findings (**Figure 3.11**). In particular, pre-FMT samples demonstrated enrichment in TCA (**Figure 3.11A**; the major *C. difficile* germination factor) and loss of DCA (**Figure 3.11H**; a potent inhibitor of the vegetative growth of *C. difficile*) compared to healthy donor samples ($p<0.01$, Mann-Whitney test), whilst post-FMT samples were

characterised by restoration of both bile acids back to levels comparable to donors (p<0.001, Wilcoxon rank sum test).

Figure 3.11:

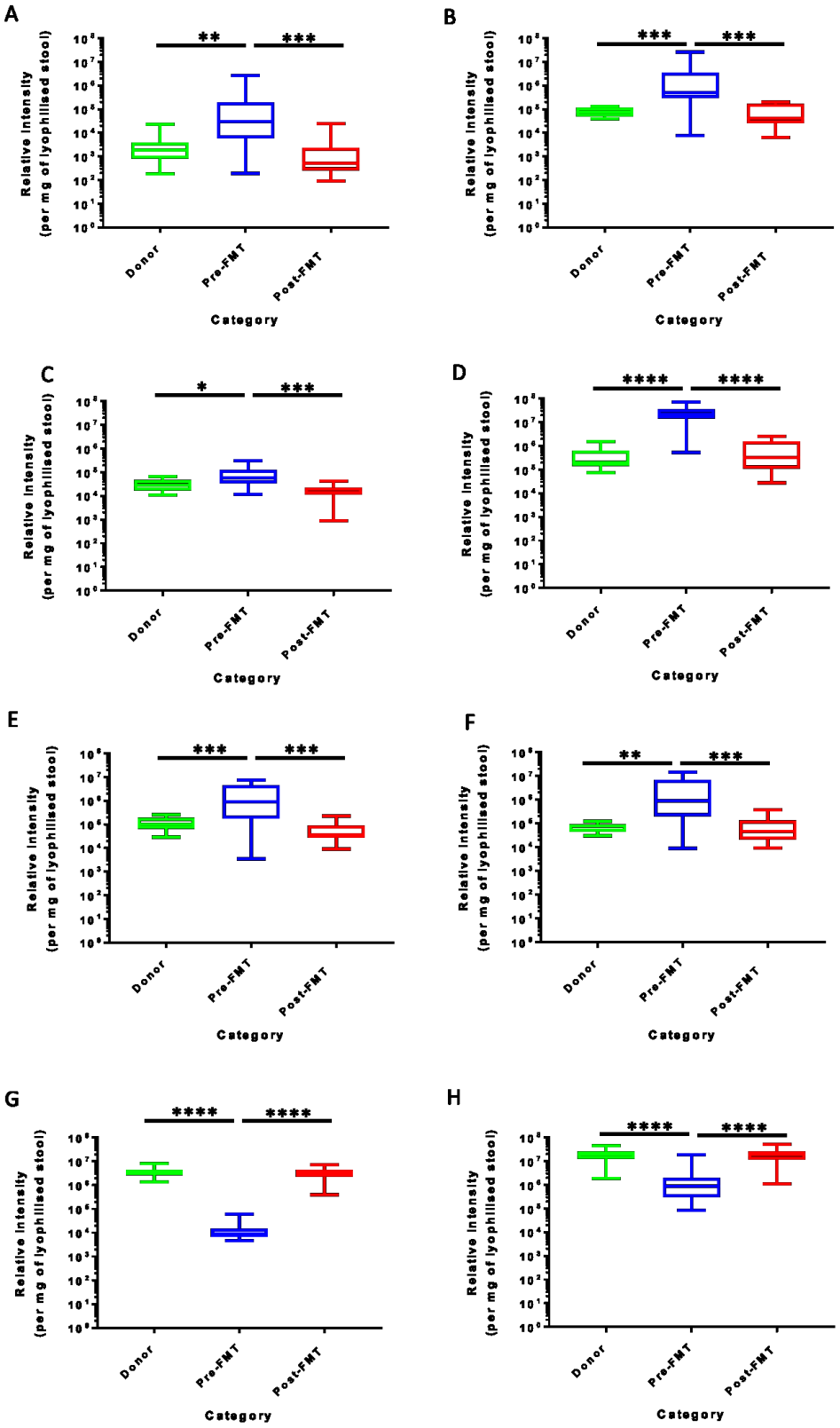


Figure 3.11 (page 110): Univariate analysis of the effect of FMT for rCDI upon faecal profiles of specific bile acids from the initial dataset, as assessed using UPLC-MS bile acid profiling data. A: Taurocholic acid; B: Glycocholic acid; C: Chenodeoxycholic acid; D: Cholic acid; E: Taurochenodeoxycholic acid; F: Glycochenodeoxycholic acid; G: Lithocholic acid; H: Deoxycholic acid (*, p<0.05; **, p<0.01; ***, p< 0.001; ****, p<0.0001; Mann-Whitney U for donor *vs* pre- or post-FMT, Wilcoxon rank sum testing for pre- vs post-FMT). (Donors: n=17; rCDI patients pre- and post-FMT: n=26). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019).

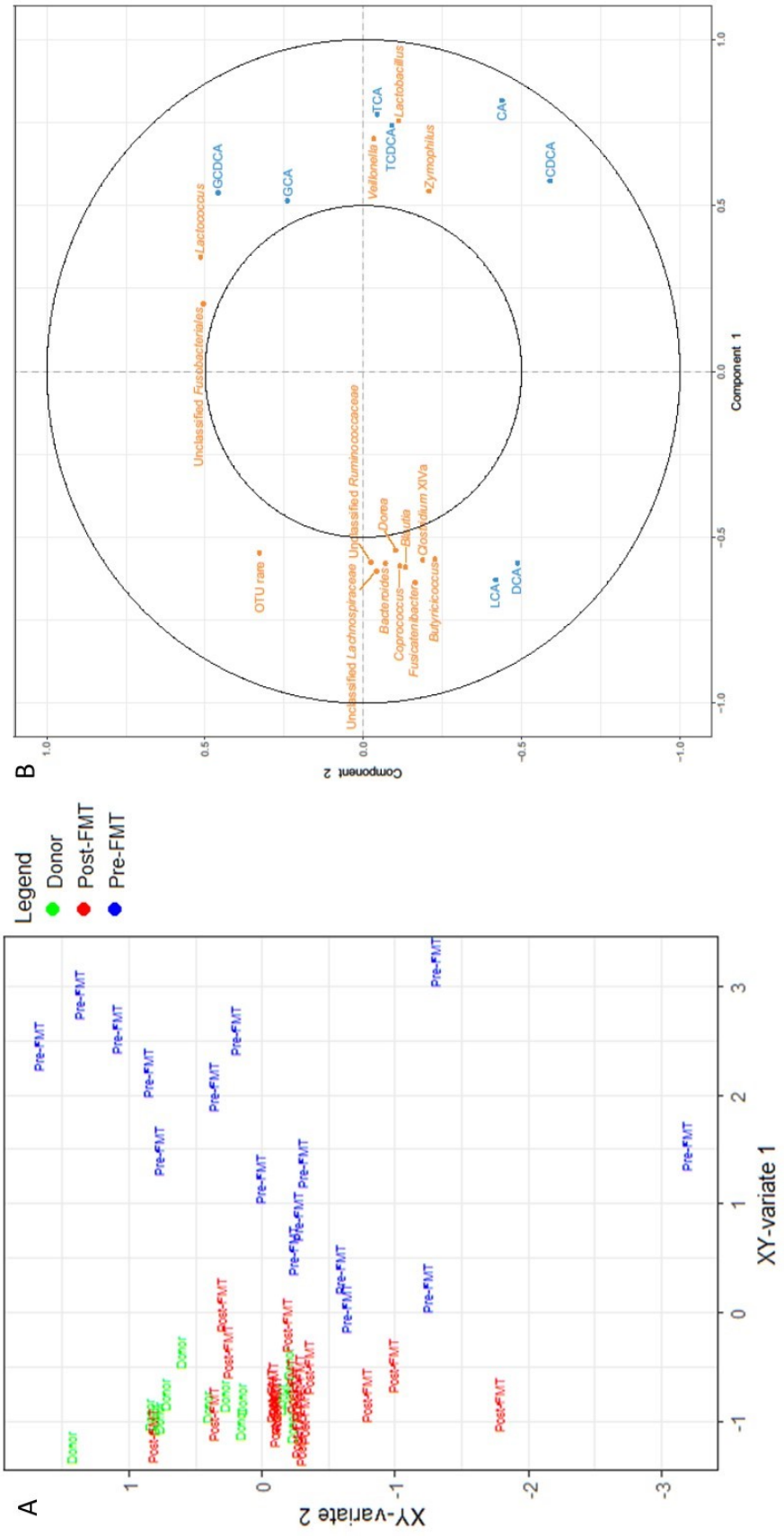
3.3.4. Integration of metataxonomic and bile acid profiling data:

rCCA modelling was applied to integrate metataxonomic and bile acid profiling data, with the aim of evaluating which specific bacterial taxa may be associated with particular bile acid changes. Results are demonstrated in **Figure 3.12.**

The unit representation plot (i.e. representation of integrated data in XY-space) demonstrated marked separation of pre- and post-FMT samples, but considerable overlap between donor and post-FMT samples (**Figure 3.12A**). A correlation circle plot demonstrated negative correlations between levels of TCA and the abundance of the bacterial genera *Bacteroides* and *Blautia,* both known to include representative BSH-producing organisms that were significantly increased after FMT in the metaxonomic data (**Figure 3.12B**). Furthermore, there was positive correlation between the genus *Clostridium* cluster XIVa (known to contain 7-α-dehydroxylase producing organisms (Kitahara *et al.*, 2000; Ridlon, Kang & Hylemon, 2006)) and the secondary bile acids DCA and lithocholic acid (LCA) (**Figure 3.12B**).

Figure 3.12 (page 112): Regularised Canonical Correlation Analysis (rCCA) model correlating 16S rRNA gene sequencing data (genus level) and bile acid data. A: Unit representation plot for the two canonical variables (metataxonomics and stool bile acids); B: Correlation circle plot between pre-FMT and post-FMT samples. Bile acids are shown in blue and bacterial genera are shown in orange. TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid. (Donors: n=17; rCDI patients pre- and post-FMT: n=26). Reproduced from (Mullish *et al.*, 2019).

Figure 3.12:


A
B
Legend
Donor
Post-FMT
Pre-FMT
XY-variate 1
XY-variate 2
Component 1
Component 2
Unclassified Fusobacteriales
Lactococcus
GCDCA
GCA
Veillonella
TCA
TCDCA
Lactobacillus
Zymophilus
CA
CDCA
OTU rare
Unclassified Lachnospiraceae
Unclassified Ruminococcaceae
Dorea
Blautia
Clostridium XlVa
Bacteroides
Coprococcus
Fusicatenibacter
Butyricicoccus
LCA
DCA

3.3.5. Gene copy number for bile-metabolising enzymes:

3.3.5.1. Introduction:

Using samples from the initial dataset, qPCR was performed for a range of *bsh* genes from different groups, together with the *bai*CD gene that is found in bacteria with 7-α-dehydroxylase activity. Given the association already established between restoration of BSH-producing organisms into the gut microbiota post-FMT and recovery or premorbid stool bile acid profiles, the aim here was to more directly interrogate whether successful FMT for rCDI was associated with restoration of gut microbiota bile-metabolising enzymes.

3.3.5.2. *bsh* gene copy number:

Results are shown in **Figure 3.13.** For *bsh* genes across all groups assayed (i.e. groups 1A, 1B and 3C (**Appendix 1**)), *bsh* gene copy number was significantly reduced in pre-FMT samples compared to that found post-FMT ($p<0.05$, Wilcoxon rank sum test) and in healthy donors ($p<0.001$, Mann-Whitney U test).

Figure 3.13:

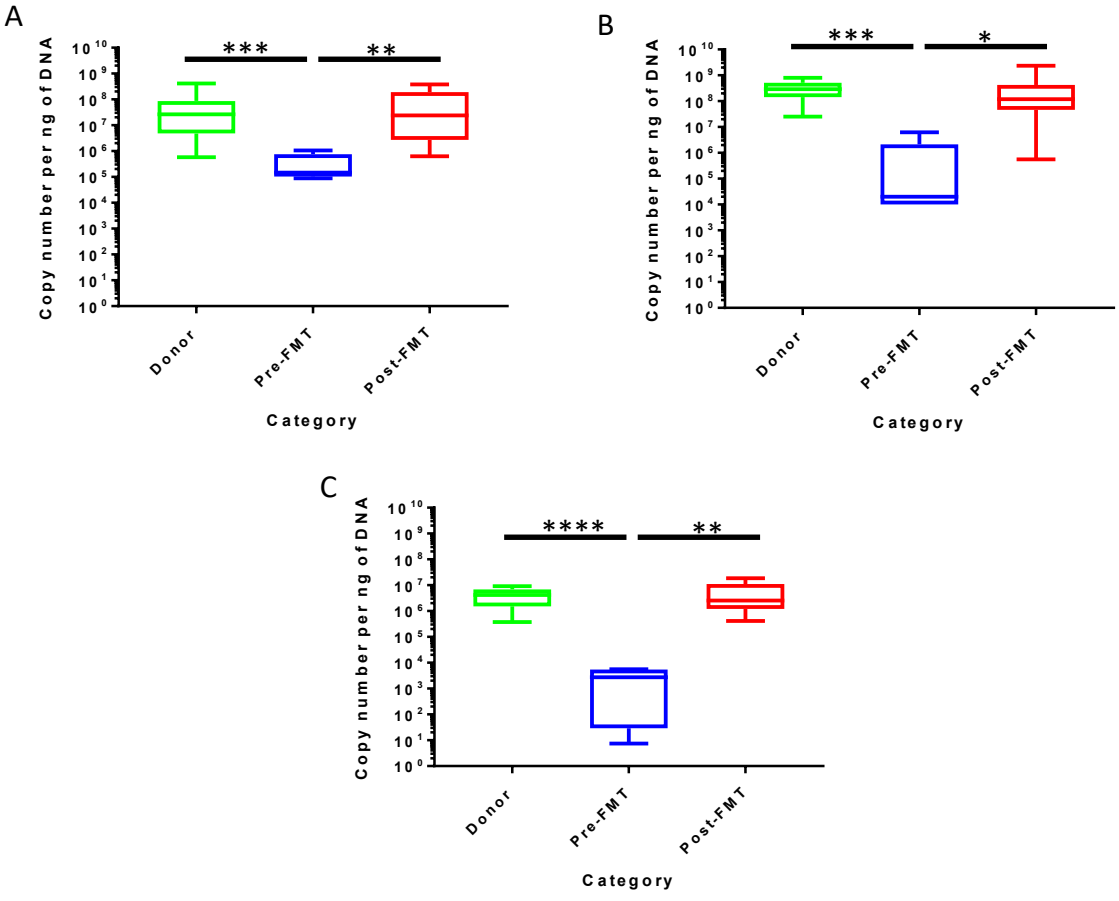


Figure 3.13 (page 113): Effect of FMT upon *bsh* gene copy number. A: *bsh* group 1A; B: *bsh* group 1B; C: *bsh* group 3C (*, p<0.05; **, p<0.01; ***; p<0.001; ****, p<0.0001; Mann-Whitney U for donors *vs* pre-FMT, Wilcoxon rank sum test for pre-FMT *vs* post-FMT). (Donors: n=17; rCDI patients pre- and post-FMT: n=26). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Reproduced from (Mullish *et al.*, 2019).

3.3.5.3. *bai*CD gene copy number:

Results are shown in **Figure 3.14.** Successful FMT for rCDI was again associated with a restoration of gene copy number back to levels similar to that of donors. Gene copy number for *bai*CD in donors and post-FMT was noted to be markedly lower than for each *bsh* gene assayed from the same study participants.

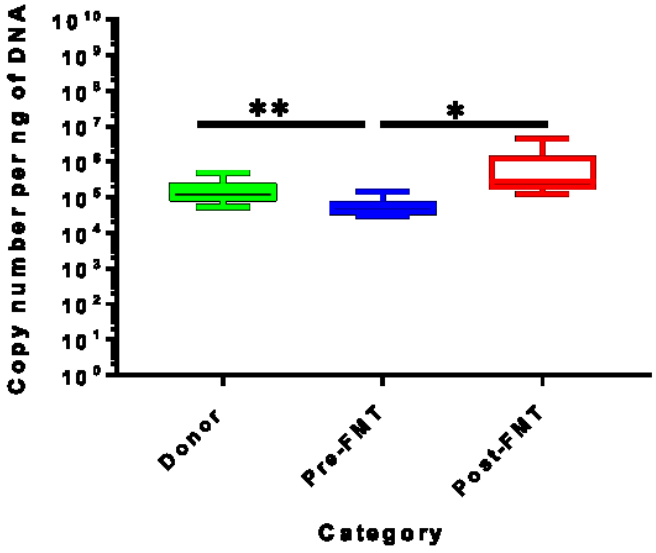


Figure 3.14: Effect of FMT upon gene copy number of the *bai*CD operon of 7-α-dehydroxylase. (*, p<0.05; **, p<0.01; Mann-Whitney U for donors *vs* pre-FMT, Wilcoxon rank sum test for pre-FMT *vs* post-FMT). (Donors: n=17; rCDI patients pre- and post-FMT: n=26). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019).

3.3.6. Bile salt hydrolase activity assay:

3.3.6.1. Introduction:

These assays were also performed on the stool samples in this dataset. As for the qPCR, the aim was further evaluation of the effect of FMT upon restoration of gut BSH functionality.

3.3.6.2. BSH activity:

Results for these assays are shown in **Figure 3.15.** The pattern seen in BSH activity was comparable to that seen with *bsh* gene copy number, i.e. stool BSH activity was found to be practically negligible pre-FMT, but was restored by FMT to levels similar to that of healthy donors (p<0.05 for pre- *vs* post-FMT, Wilcoxon rank sum test). It was observed that there was inter-individual variability in the pattern of BSH activity between different healthy donors (**Figure 3.16**), potentially implying that different individuals may have a distinct pattern of gut microbiota community members with BSH functionality.

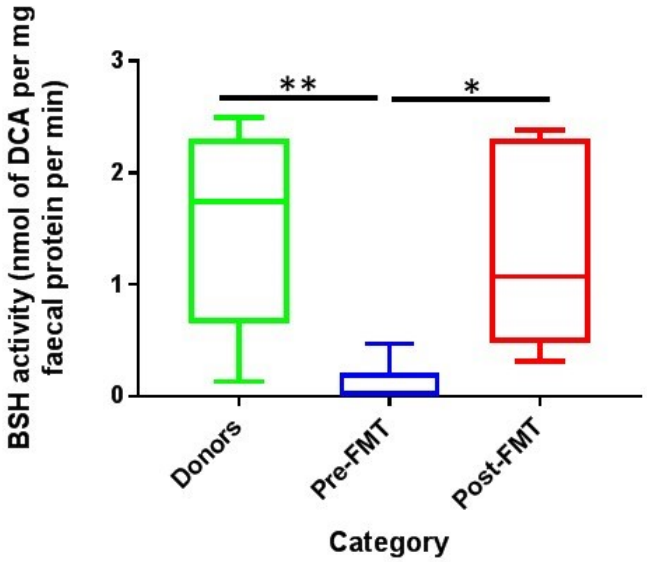


Figure 3.15: Effect of FMT upon BSH activity. As assessed through performance of the BSH precipitation assay upon faecal supernatant (*, p <0.05; **, p<0.01, Mann-Whitney U for donors *vs* pre-FMT, Wilcoxon rank sum for pre- *vs* post-FMT). (Donors: n=17; rCDI patients pre- and post-FMT: n=26). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019).

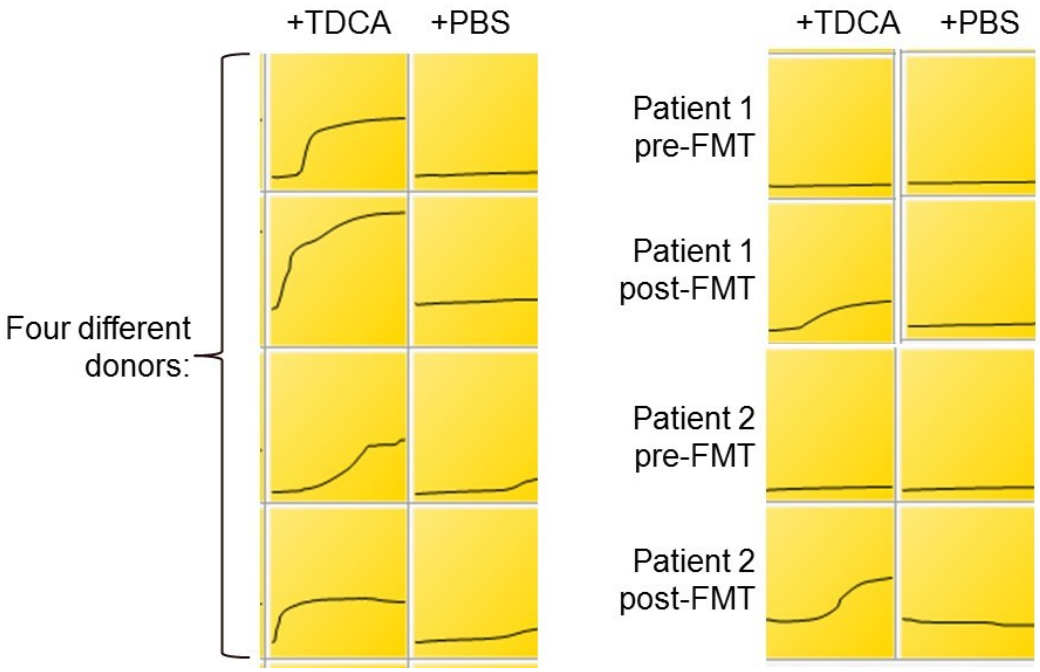


Figure 3.16: Representative spectrophotometer patterns obtained during the BSH activity assay. Horizontal axis is time, and vertical axis is OD_{600}. Whilst the level of BSH activity in post-FMT/ healthy donor samples was consistently greater than that of pre-FMT samples, it was also observed that the morphology of spectrophotometer patterns was markedly different for different donor stool samples. TDCA: taurodeoxycholic acid; PBS: phosphate-buffered saline.

3.4. Discussion and analysis:

3.4.1. Gut microbiota analysis:

Consistent with previous studies (van Nood *et al.*, 2013; Weingarden *et al.*, 2014; Kao *et al.*, 2017), successful FMT for rCDI was associated with restoration of measures of ecological diversity and richness of the microbiota back to a level comparable to that of healthy donors (**Section 3.3.2.3**). Based on these results, it may initially seem surprising that 16S rRNA gene qPCR results demonstrated comparable bacterial load between pre- and post-FMT samples, since it may intuitively be expected that this would be reduced in patients with rCDI who have experienced prolonged exposure to antimicrobials (**Section 3.3.2.2**). However, it has been demonstrated both in human subjects treated with a range of antibiotics (Panda *et al.*, 2014) and a chemostat model of CDI generated with clindamycin (McDonald *et al.*, 2018a) that following an initial decrease in bacterial load, levels quickly recover to levels comparable with that before antibiotics. While the use of antibiotics kills a number of bacterial taxa within the

gut microbiota, it also creates a selection pressure that favours overgrowth of those remaining microbiota members that are antibiotic-resistant; in the case of antibiotics that are most strongly associated with CDI risk, these are particularly Gram-negative bacteria (Panda *et al.*, 2014).

A number of studies have previously explored the effect of FMT for rCDI upon the gut microbiota using metataxonomics (Weingarden *et al.*, 2015, 2014; Hamilton *et al.*, 2013; Staley *et al.*, 2017a, 2018), shotgun metagenomics (Shankar *et al.*, 2014; Smillie *et al.*, 2018; Kao *et al.*, 2017), or even microarray analysis (Fuentes *et al.*, 2014; Shankar *et al.*, 2014; Jalanka *et al.*, 2016), and this study supports and builds upon the results from these previous studies. From these previous studies, a number of key principles about gut microbiota changes after FMT have become well-established. Recipients of FMT for rCDI show changes in gut microbiota towards a profile similar to that of healthy donors within a day of FMT; while there is a variable degree of divergence over the course of follow-up, recipient gut microbiota profiles remain broadly-comparable to that of healthy donors for at least six months post-FMT (Weingarden *et al.*, 2015) and even up to one year (Jalanka *et al.*, 2016). The effect of FMT for rCDI upon the stool microbiota is comparable to that seen in the mucosal microbiota (Jalanka *et al.*, 2016). While it has previously been demonstrated that gut microbiota changes in patients with rCDI treated with either fresh or frozen FMT are comparable (Hamilton *et al.*, 2013; Staley *et al.*, 2017a), a novel finding of these studies was that such changes are also the same regardless of whether an upper GI (capsule) or lower GI (colonoscopic) route of FMT administration was used.

In the metataxonomic analysis undertaken here, rCDI patients pre-FMT had lower relative abundances of the bacterial families *Lachnospiraceae, Ruminococcaceae* and *Bacteroidaceae,* and higher relative abundances of *Enterobacteriaceae, Lactobacillaceae* and *Veillonellaceae* compared to healthy donors and post-FMT patients (**Figure 3.4**), which is consistent with the finding of other studies (Weingarden *et al.*, 2015, 2014; Staley *et al.*, 2018). These other studies have tended to report their bacterial profiling data at the family taxonomic level, but generally not at genus or species level. Even when shotgun metagenomics has been performed for such studies in both the rCDI and non-CDI context (which theoretically should allow resolution of bacterial profiles down to strain level), limitations in current microbial

genomic annotation has meant that a significant proportion of reads remain unclassified, or classification is at no better resolution than at order level (Kao *et al.*, 2017; Hildebrand *et al.*, 2016), in essence giving no additive value compared to a metataxonomic study. However, while the studies within this chapter performed microbiota profiling with metataxonomics and qPCR of genes of interest (on the grounds of available expertise in data interpretation, cost of experimentation, etc), there are clear theoretical reasons why a well-performed metagenomic experiment may potentially yield additional information. For instance, metagenomic analysis may enable strain-level tracking of bacteria, allowing direct assessment of whether bacteria in the post-FMT stool metagenome were derived from their associated donor. Furthermore, such analysis may enable novel 'hypothesis generation' regarding potential additional mechanisms of FMT's efficacy in treating rCDI, by identifying the full spectrum of metagenomic material that is enriched in post-FMT stool compared to that pre-FMT.

The few studies that have reported microbiota profiles at the genus and species level of resolution have observed comparable taxonomic changes post-FMT to those seen here, including a marked and sustained enrichment in the genera *Bacteroides* and *Clostridium* clusters XIVa (Fuentes *et al.*, 2014; Jalanka *et al.*, 2016), as well as in a number of similar bacterial species (including *Bacteroides ovatus, Faecalibacterium prausnitzii* and *Blautia obeum* (Shankar *et al.*, 2014)). However, this latter study is limited in that it only included three rCDI/ FMT recipients, and only measured species abundance through the use of a microarray technique, drawbacks of which have already been discussed (**Section 2.2.1.3**). The microarray used in the study of Shankar and colleagues only allowed differentiation of 65 distinct bacterial species, when it is estimated that there may be >1000 separate bacterial species in datasets such as this (Smillie *et al.*, 2018). Being able to define microbiota changes in an experiment such as this at a high level of resolution (ideally species, or even strain) is clearly particularly of importance for researchers interested in exploiting FMT studies to develop therapeutics compromised of 'defined microbial communities', as any higher taxonomic level would give too vague an insight as to what bacteria should be potentially considered for inclusion. In the metataxonomic analysis performed here, it was possible to obtain species (or at least genus level) annotation in many cases, even despite the strict criteria applied for doing so (Stackebrandt & Goebel, 1994). Key reasons that it was possible

to get this level of resolution here but not in older studies include the development in reference databases for 16S rRNA gene sequences, and increased experience of using next-generation sequencing platforms for studies such as these (i.e. optimisation of primers, hypervariable region of choice, etc) (Watts *et al.*, 2017). It might be argued that using comparison of culture results of stool collected before and after FMT may also allow high-level resolution evaluation of the effect of FMT upon microbial communities (Browne *et al.*, 2016); however, as discussed in **Section 2.2.1.1.**, such experiments would be very time consuming and laborious in order to ensure coverage of all the preferred culturing conditions for different bacteria.

Many previous comparable sequencing studies have explored pre- and post-FMT microbiota composition without an *a priori* hypothesis about which specific taxa are expected to change and/or their associated functions. One further area of novelty within this present study was that, since *bsh* (Jones *et al.*, 2008) and 7-α-dehydroxylase (Ridlon, Kang & Hylemon, 2006) gene annotation has already been defined for the human gut metagenome, it was possible to compare taxa with presumed bile-metabolising functionality between groups, via use of both metataxonomics and metagenome inference data. While the key finding was the reduced abundance of taxa with this functionality in rCDI patients pre-FMT compared to healthy donors and post-FMT, this clearly requires more direct evaluation of microbiota functionality before any conclusions about the hypothesis may be drawn. It is particularly interesting to note that *C. scindens* abundance in the gut microbiota was reduced pre-FMT in comparison with donors (albeit with <1% change in mean abundance), but there was no increase in abundance of this species post-FMT, suggesting that the increase in *bai*CD post-FMT was by yet unidentified bacteria with 7-α-dehydroxylation activity.

In microbial sequencing studies such as that performed here, it is often assumed that any bacteria taxa which are at low relative abundance/ absent in the pre-FMT gut microbiota profile but present post-FMT – and which are present in the donor gut microbiota profile – must have been transferred from donor to recipient. However, this clearly cannot be assumed from observational data such as these. For example, one explanation for the increased relative abundance of a number of members of the genus *Bacteroides* observed post-FMT here is transfer from the donor and rapid colonisation of the recipient. However,

an alternative explanation is that these *Bacteroides* members are still present at low levels in the pre-FMT gut microbiota but have been suppressed in the context of the dominant microbiota-metabonome *milieu* of CDI. By extension, it may be that such bacteria can only freely grow and become restored to their pre-morbid relative abundance after a factor related to the FMT kills the *Enterobacteriaceae* members which dominate the pre-FMT microbial ecosystem. As such, based on this metataxonomic data alone, it cannot specifically be concluded that successful FMT for rCDI is associated with the transfer of bacteria with bile-metabolising capacity from donor to recipient, and that was the drive for the further analyses in these and subsequent chapters. An extension of this analysis that may have been of relevance to addressing this would be to compare changes in metataxonomic profiles after successful FMT compared to that in patients with FMT failures. However, no FMT failure samples were available to analyse – this is largely a reflection of the very high efficacy of the treatment.

Recognising this issue, a variety of approaches have been attempted to help differentiate transfer of taxa from donors into recipient *vs* reconstitution within the recipient from the host microbiota. One such approach is the use of the tool SourceTracker, which provides a Bayesian approach to attempt to estimate the proportion of bacteria in a 'sink' microbial community (such as the post-FMT microbiota) which are derived from a source population (i.e. a donor) (Knights *et al.*, 2011). When SourceTracker has been applied to rCDI datasets, it has been demonstrated that patients cured by FMT showed significantly greater donor similarity within one week of FMT than patients who had further recurrence (Staley *et al.*, 2017a). Further analysis demonstrated that ~50% of the post-FMT gut microbiota of FMT responders were derived from donor microbiota, with an additional 20-30% of the community structure similar to (but not derived from) that of healthy donors (Staley *et al.*, 2018). As such, the current consensus is that both transfer and colonisation of donor microbiota and re-establishment of suppressed host microbiota are contributors to the restored gut microbiota profile observed after FMT for rCDI. One clear limitation of SourceTracker is that it does not specifically define the identity of the transferred donor taxa. A newer tool, Strain Finder, has been very recently described, which assesses frequency of single nucleotide polymorphisms within strains (as established from shotgun metagenomic

data) to infer genotypes and track them over time, although this requires further validation before it can be more utilised more widely (Smillie *et al.*, 2018).

3.4.2. Bile acid analysis:

Although the metataxonomic analysis demonstrated an association between successful FMT and reconstitution of gut microbiota members with bile-metabolising functionality, the next logical question was whether that amounted to changes in bile acid metabolism in practice. As such, stool bile acid profiling was performed as a next step.

It is important to recognise that there are certain drawbacks with the use of stool samples as a tool to study gut bile acid metabolism. Both quantities and profile of bile acids within biofluids are influenced by whether a subject is fasting or post-prandial (Sarafian *et al.*, 2015); clearly, however, this cannot be controlled for in the collection of stool samples (as opposed to serum and urine) for analysis, given the unpredictability on when a patient will be able to provide a stool sample. Furthermore, it has been demonstrated that germ-free mice have increased expression of the gene for the ileal bile acid transporter (a key mediator of the enterohepatic circulation), and absorb more TCA than colonised mice (Sayin *et al.*, 2013). Given the marked shifts in microbiota that characterise FMT for rCDI in humans, it is clearly feasible that this finding has applicability to results in this study too. This is relevant to studies such as this one, as the implication from these findings is that stool bile acid profiles may not completely represent those of the small intestine. Nevertheless, stool is clearly the most practical biofluid/ material that may be routinely collected to explore gut bile acid metabolism, and has become the typical sample analysed in such studies.

One particular novelty of the bile acid analysis performed here was the use of multivariate analysis. The use of spiked standards allowed annotation of bile acids of interest, but many of the integrated spectral peaks identified were not annotated. At least a proportion of these represent chemical variants of these bile acids, e.g. sodium salts, ionised forms, sulfate conjugated forms, dimers, isotopes (e.g. carbon-13 variant), etc. However, a further proportion of these likely represent metabolites with similar physiochemical properties to bile acids that are also extracted in the sample preparation protocol, such as lipids (Sarafian *et al.*, 2015). Further work that would be of interest with this dataset would be to identify

integrated spectral peaks that differentiate groups in multivariate analysis, and attempt putative identification of them (based on *m/z* ratio and retention time) using reference databases and/or algorithms that perform automated annotation, e.g. ChemDistiller (Laponogov *et al.*, 2018). It is important to recognise that such putative identification would still at this stage be speculative, since no spiked standards other than bile acids were used in these studies – further research would be required using standards of these metabolites to explore further. Nonetheless, such a process could still be a useful 'hypothesis generation' exercise for the initial identification of other metabolites with potential relevance to CDI pathogenesis and/or the response to FMT.

The central findings from the bile acid analysis were that pre-FMT, there was enrichment in stool of primary conjugated bile acids (TCA, GCA, TCDCA and GCDCA), as well as unconjugated primary bile acids (CA and CDCA). It was also observed that successful FMT was associated with decline in levels of all these primary bile acids, and marked enrichment in secondary bile acids, including DCA and LCA (**Section 3.3.3.3**). Diet is recognised to affect bile acid homeostasis, and patients in this cohort are likely to have markedly different diets at the times that their pre- and post-FMT samples were collected in light of their different health status at both these points. As such, it is therefore reasonable to question whether change in diet played a significant contribution to the bile acid profiling results observed. In this cohort, rCDI patients post-successful FMT are likely to be eating a more diverse, energy-rich Western diet than prior to FMT; such a diet has been previously associated with reduced secondary bile acid production (rather than increased, as observed here) (Dermadi *et al.*, 2017), implying that dietary changes may not be a major contributory factor to these results.

These bile acid profiling findings are consistent with a number of previous studies that have explored stool bile acid profiles, including both in germ-free (Swann *et al.*, 2011) and antibiotic-treated mice susceptible to CDI (Theriot *et al.*, 2014), as well as humans with rCDI and/or after FMT (Weingarden *et al.*, 2014; Staley *et al.*, 2017a; Allegretti *et al.*, 2016; Brown *et al.*, 2018; Farowski *et al.*, 2019). It has even recently been demonstrated that stool LCA may have utility as a predictor of FMT response, with sensitivity and specificity of >90% (Farowski *et al.*, 2019). However, the findings in this thesis expand upon that of previous

studies, since these investigations have tended to identify a more limited range of bile acids than investigated here.

Primary conjugated bile acids are the substrates for BSH, while unconjugated primary bile acids are the substrates for 7-α-dehydroxylase; secondary bile acids are the ultimate product of this metabolic pathway. The pattern of changes of enriched TCA and diminished DCA in rCDI is particularly of interest, given that this together results in a potent trigger for germination and no inhibitory signal for the vegetative growth of *C. difficile.* As such, one feasible explanation is that patients with rCDI have an altered bile acid *milieu* in response to deficiencies in bile-metabolising microbiota, and this process is reversed through FMT. The rCCA data (demonstrating close negative correlation between TCA levels and relative abundance of bacterial genera with annotated BSH functionality, and positive correlation between that of 7-α-dehydroxylase-producing organisms and DCA levels) provides further support for this hypothesis (**Section 3.3.4**). However, for further evaluation of this, qPCR of bile-metabolising genes and BSH activity assays were performed.

3.4.3. qPCR of *bsh* genes and BSH enzyme activity assays:

The qPCR primers sets used for a range of *bsh* groups had been designed to be degenerate (Mullish *et al.*, 2018b), and therefore each set provides an insight into gene copy number for a range of different bacteria, as described in **Appendix 1**. qPCR was also performed for the *bai*CD operon; while this operon is not present in all bacteria with 7-α-dehydroxylating ability, it is present within the two bacterial species with particularly high activity of this enzyme, *Clostridium scindens* and *Clostridium hiranonis,* and most strains of these species will be amplified by this PCR (Wells *et al.*, 2003). Furthermore, *C. scindens* is particularly of interest within this context, because of its previous association with colonisation resistance from CDI (Buffie *et al.*, 2014). Even though this qPCR will not amplify certain bacteria with low activity for the bioconversion of primary to secondary bile acids (including *Clostridium leptum* and *Clostridium sordeii*), close correlation has been observed between *bai*CD gene copy number and 7-α-dehydroxylase *in vitro,* demonstrating the utility of this assay (Wells *et al.*, 2003).

The BSH activity assay used here is an adaptation of a conventional precipitation-based assay (Mullish *et al.*, 2018b). Practically, establishment of enzyme assays for BSH activity are reasonably straightforward, since the enzyme is relatively oxygen and temperature insensitive, and, and deconjugates at near to maximal efficacy at pH 5-6, as was used here (Thomas *et al.*, 1997, 2001). A new fluorescence-based probe has been described for high-throughput assessment of BSH activity (Brandvold *et al.*, 2019); however, while this probe has been developed to have high sensitivity for the detection of BSH enzymatic activity, significant deconjugation could only be detected in a human stool sample when spiked with pure BSH. Whilst 7-α-dehydroxylase enzyme activity assays would clearly be of interest, these are difficult to perform on stool samples because the enzyme is exquisitely sensitive to oxygen (Thomas *et al.*, 1997), and therefore such assays have only been successfully described using freshly-collected colonic contents taken at colonoscopy (Thomas *et al.*, 2001). Furthermore, 7-α-dehydroxylation demonstrates peak efficacy at a pH of 7-9 and substantially lower activity outside of this range, so would not be suitable for assessment by a precipitation assay anyway.

One interesting observation from the qPCR and enzyme assay data was the apparent variability in gut microbiota BSH gene copy number and activity even within a cohort of healthy donors (**Section 3.3.6.2**). This variability is consistent with a previous comparative metagenomic analysis of BSH, which also saw marked variation in overall abundance and origins of BSH proteins between different human gut metagenomes (Jones *et al.*, 2008). Age, gender, BMI and sex do not appear to affect BSH distribution, although geographical origin exerts a marked influence (Song *et al.*, 2019); the effect of diet has not been assessed. As such, even though the reasons for this inter-individual variability in BSH remains unclear, bile deconjugation shows marked functional redundancy within the gut microbiota. Many donors had only given FMT for one or two patients, and (as discussed above) there were no samples from patients with FMT failures. Therefore, it was not possible to gauge if, for example, a donor microbiota more enriched in particular BSH-producing taxa and/or one particular profile of *bsh* genes rather than another may influence outcome from FMT, but this would evidently be of interest.

The major finding from these qPCR and enzyme activity experiments was that successful FMT is associated with restoration of gut microbiota bile-metabolising enzyme number and function. Gene copy number for *bai*CD in donors and post-FMT was noted to be markedly lower than for each *bsh* gene assayed from the same study participants (**Section 3.3.5**), although the functional implications of this are unclear. However, the samples analysed within this chapter included pre-FMT samples, and samples post-FMT collected at 8-12 weeks. If a key mechanism of efficacy of FMT is restoration of BSH-producing microbiota which degrades TCA, then this functionality must act quickly so that *C. difficile* germination may be prevented. As such, assessment at 8-12 weeks post-FMT is by itself insufficient to make any strong inference about whether restoration of this functionality is of biological significance.

3.4.4. Summary:

The analyses performed in this chapter – microbiota, bile acid profiling, qPCR and enzymology – are all consistent with the central hypothesis. Specifically, patients with rCDI appear to be deficient in gut microbiota members with bile-metabolising capacity, with consequent enrichment in bile acids which promote *C. difficile* germination (i.e. TCA), and diminishment of bile acids which inhibit the vegetative growth of *C. difficile* (i.e. DCA). Furthermore, these abnormalities are reversed in patients receiving successful FMT, with bile-metabolising functionality restored to a pattern comparable to that of healthy donors. However, while these observational data support the main hypothesis, they have a number of limitations, particularly related to aspects of the methodology used, as well as timescales at which samples were collected.

To respond to these limitations, the following chapter reports analyses on further human samples using comparable techniques, but from additional datasets:

1. Samples collected pre- and post-FMT for rCDI, but with serial sampling in the post-FMT period; a ‘validation’ cohort.
2. Samples collected from patients with primary CDI, with serial samples collected on follow-up, and comparison of recurrers *vs* non-recurrers.

Chapter 4. Validation of the effect of faecal microbiota transplant for recurrent *Clostridioides difficile* infection upon gut microbiota bile-metabolising functionality, and microbiota-metabonome interactions in primary CDI:

4.1. Introduction:

The results from the initial dataset used a number of analyses of both the composition and function of the gut microbiota to demonstrate that microbiota-mediated bile metabolising functionality is reduced in patients with rCDI compared to patients successfully treated post-FMT, and/or their associated donors. This is also associated with the change of the bile acid *milieu* from one that predominantly signals to prevent the vegetative growth of *C. difficile,* to one that promotes germination. However, given that this was a relatively small dataset with samples from only one post-FMT time point, it was decided to repeat selected analyses upon an independent 'validation' dataset, with additional post-FMT time points, and where samples were available of the actual FMT slurry itself.

As discussed in the introduction, there is also interest in the apparent association between loss of microbiota-mediated SCFA production capacity and increased vulnerability to CDI (**Section 1.6.6.5**). Conversely, there is also interest as to whether restoration of colonic SCFA metabolism may be a mechanism underpinning the efficacy of FMT, and particularly with regards to the C5 SCFA, valerate (McDonald *et al.*, 2018a). As such, SCFA concentrations in different body compartments (urine, serum and stool) were also assayed for the validation cohort. Given that there is a growing interest as to whether different forms of FMT preparation may have comparable efficacy in treating rCDI (including the use of lyophilised and/ or filtered faecal slurry (Ott *et al.*, 2017)), SCFA concentrations were also compared between FMT material prepared using different protocols, using stool derived from the same specific donation.

Finally, while there is a growing understanding of microbiota-metabonome interactions in recurrent CDI, there is very limited knowledge as to these interactions in people with a primary episode of CDI. There are growing rates of recurrence after primary CDI, with recent data indicating this risk as 15-25% after a first episode of CDI, and as high as 40-65% for patients experiencing further recurrences (Cornely *et al.*, 2012a; Johnson *et al.*, 2014). Given

that gut microbiota bile-metabolising functionality appears to contribute to the pathogenesis of rCDI, it may also be hypothesised that this may contribute to whether a patient experiences recurrence or not after treatment for a primary episode of CDI. As such, serial stool samples were collected from patients with primary CDI from diagnosis until the time of recurrence, or until the time of presumed remission.

4.2. Methods:

For the validation dataset, no microbial sequencing was performed by myself, since these samples had already undergone shotgun metagenomic sequencing and analysis by associates of Dr Dina Kao at the University of Alberta, Canada (Kao *et al.*, 2017; Monaghan *et al.*, 2018); however, these results were re-reviewed and interpreted in the context of the hypothesis of this work. Serial stool samples from patients with rCDI receiving FMT were analysed via UPLC-MS for bile acid profiling, and BSH activity assays. Serial stool, urine and serum samples from these patients were also analysed by GC-MS for SCFA profiling; furthermore, FMT slurries derived from the same specific stool sample (but prepared using varied protocols) were also assessed for SCFA quantification.

For the primary CDI dataset, no microbial sequencing was performed by myself, since this was undertaken by colleagues of Dr Jessica Allegretti at Brigham and Women's Hospital/ Harvard University, USA (Allegretti *et al.*, 2018). Serial stool samples from primary CDI patients were analysed by UPLC-MS for bile acid profiling, and via BSH activity assays.

4.3. Results:

4.3.1. Validation human dataset:

4.3.1.1. Clinical details of the included patients:

Demographics of the patients in the validation human dataset are as previously-described (Kao *et al.*, 2017). The major finding from this study had been the demonstration of the non-inferiority of capsulised FMT to colonoscopic in the treatment of rCDI. Importantly, this study had used the same faecal slurry from a single stool donation for the preparation of administered colonoscopic FMT as for the preparation of FMT capsules.

4.3.1.2. Shotgun metagenomic sequencing:

As stated above, this was not performed by myself, but existing data was re-reviewed in light of the hypothesis of this study. A summary of data is given in **Appendix 2**. Successful FMT for rCDI was associated with an increased abundance of genera in the stool metagenome including *Bacteroides, Faecalibacterium, Ruminococcus* and *Blautia,* as well as unclassified members of the family *Lachnospiraceae* ($q \leq 0.003$, White's non-parametric test with Benjamini-Hochberg FDR correction). These same taxa were enriched in the stool metagenome at both week 4 and week 12 post-successful FMT. Such metagenomic changes are very similar to the metataxonomic changes seen in the initial human dataset (**Section 3.3.2.4**). All of these genera are recognised as containing bacterial species with annotated *bsh* genes (**Appendix 1**), whilst the family *Lachnospiraceae* contains the genus *Clostridium* cluster XIVa, the major source of 7-α-dehydroxylase-producing bacteria.

4.3.1.3. Ultra-performance liquid chromatography-mass spectrometry for stool bile acid profiling:

4.3.1.3.1. Multivariate analysis:

Results from this analysis are displayed in **Table 4.1** and **Figure 4.1.** All bile acid profiling in this analysis was performed on crude stool samples, both in the case of donors and recipients. On PCA, as for the initial human dataset, pre-FMT stool bile acid profiles once again clustered with marked separation from those of post-FMT and donor samples (**Figure 4.1A**). Furthermore, donor and post-FMT sample profiles overlapped in their clustering at all times points assayed (i.e. week 1, week 4 and week 12) post-successful FMT for rCDI (**Figure 4.1A**).

Supervised analysis with OPLS-DA enabled the construction of valid models separating pre-FMT samples from those post-FMT at week 1 (**Figure 4.1B; Table 4.1**), week 4 (**Figure 4.1C; Table 4.1**) and week 12 (**Figure 4.1D; Table 4.1**). However, it was not possible to construct valid OPLS-DA models to separate post-FMT samples by time point, demonstrating that post-FMT bile acid changes are rapid in onset and maintained. Comparable to the results with the microbiota analysis (**Section 3.1.1.3**), it was not possible to construct valid OPLS-DA models to separate the stool bile acid profiles of rCDI patients based on route of administration (i.e. colonoscopic *vs* capsule), either pre- or post-FMT administration.

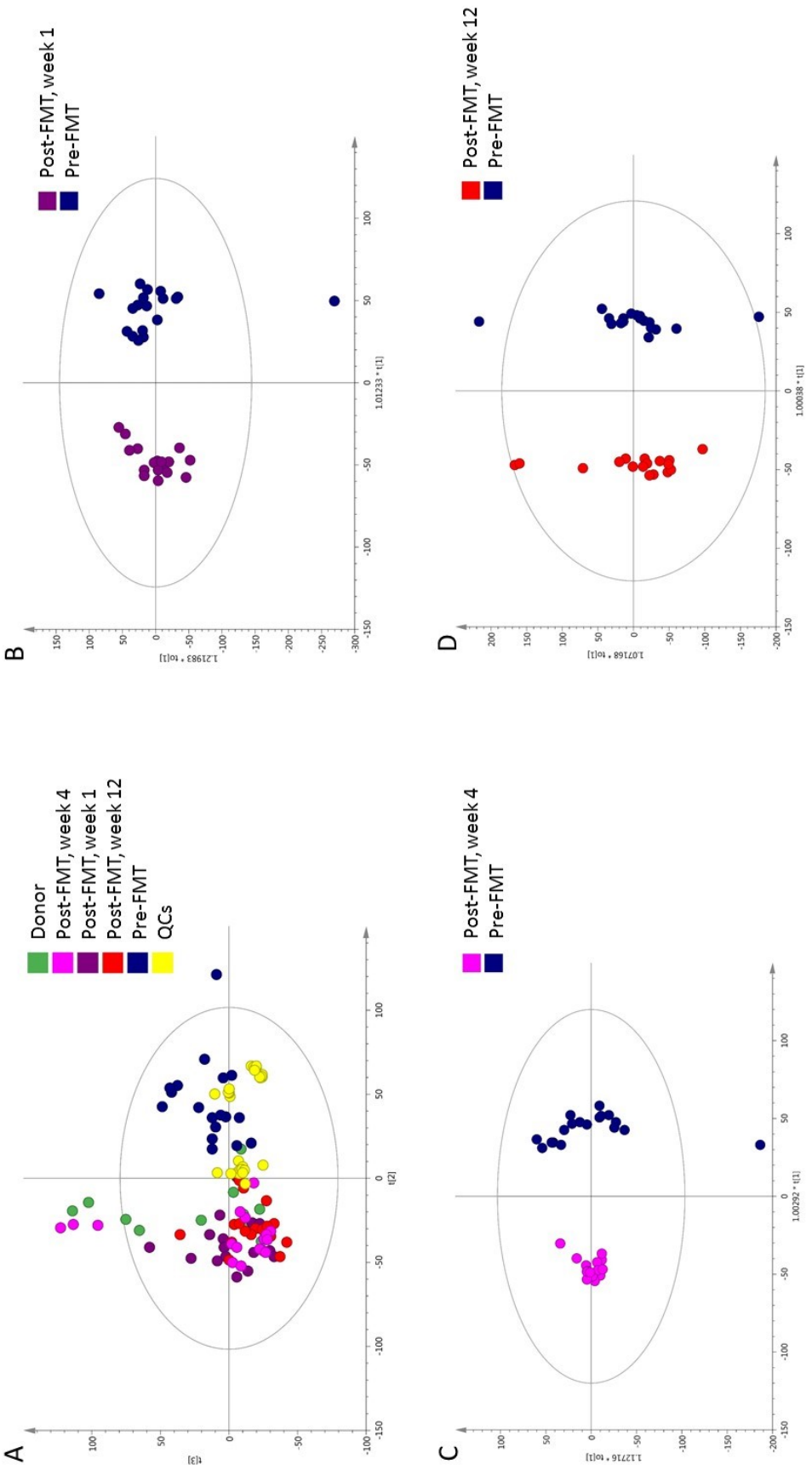
Table 4.1: Model characteristics for OPLS-DA models derived from the validation human dataset.

Multivariate model	R^2X	Q^2	*p* value
OPLS-DA modelling by time point:			
Donor vs pre-FMT	0.615	0.942	1.01×10^{-10}
Pre- vs post-FMT, week 1	0.568	0.909	1.20×10^{-11}
Pre- vs post-FMT, week 4	0.612	0.924	5.06×10^{-11}
Pre- vs post-FMT, week 12	0.593	0.931	3.53×10^{-13}
Post-FMT, week 1 vs post-FMT, week 4	No valid model constructed		
Post-FMT, week 1 vs post-FMT, week 12	No valid model constructed		
Post-FMT, week 4 vs post-FMT, week 12	No valid model constructed		
OPLS-DA modelling by route of administration:			
All pre-FMT samples, capsule vs colonoscopic administration	No valid model constructed		
All post-FMT samples, capsule vs colonoscopic administration	No valid model constructed		

Validation of the models is expressed with *p* values derived from CV-ANOVA.

Figure 4.1 (page 130): Effect of FMT for rCDI upon stool bile acid profiles. Assessed via multivariate analysis of UPLC-MS bile acid profiling data from the validation human dataset. A: PCA scores plot; B: OPLS-DA scores plot, comparing pre-FMT and post-FMT, week 1 samples; C: OPLS-DA scores plot, comparing pre-FMT and post-FMT, week 4 samples; D: OPLS-DA scores plot, comparing pre-FMT and post-FMT, week 12 samples. QC: quality controls. (Donors: *n*=8 (5 crude stool samples, 3 FMT slurry samples); rCDI patients pre- and post-FMT: *n*=18).

Figure 4.1:

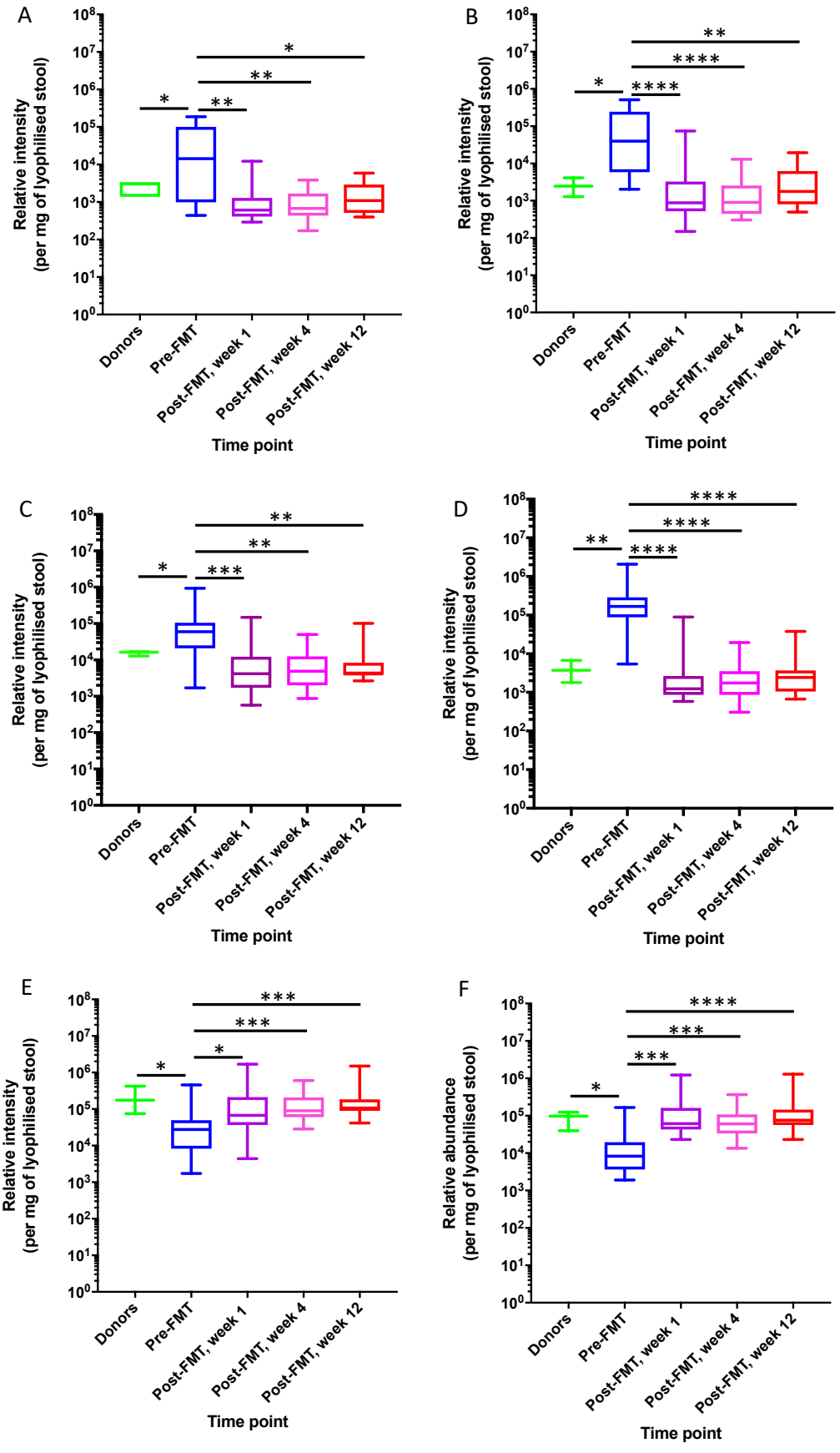

A
Donor
Post-FMT, week 4
Post-FMT, week 1
Post-FMT, week 12
Pre-FMT
QCs
B
Post-FMT, week 1
Pre-FMT
C
Post-FMT, week 4
Pre-FMT
D
Post-FMT, week 12
Pre-FMT

4.3.1.3.2. Univariate analysis:

Univariate analysis was performed on stool bile acid profiles for the specific bile acids most closely recognised as affecting *C. difficile* germination and vegetative growth; results of this are displayed in **Figure 4.2.** Consistent with the initial human dataset, successful FMT for rCDI was found to be associated with rapid and sustained loss of all stool primary bile acids in comparison to pre-FMT levels (p<0.05, Friedman's test with Benjamini-Hochberg FDR), including TCA (**Figure 4.2A**), GCA (**Figure 4.2B**), CDCA (**Figure 4.2C**), and CA (**Figure 4.2D**). Conversely, successful FMT was again noted to be characterised by a maintained restoration of secondary bile acids (p<0.05, Friedman's test with Benjamini-Hochberg FDR), including DCA (**Figure 4.2E**) and LCA (**Figure 4.2F**).

Figure 4.2 (page 132): Univariate analysis of the effect of FMT for rCDI upon faecal profiles of specific bile acids from the validation dataset, as assessed using UPLC-MS bile acid profiling data. A: Taurocholic acid; B: Glycocholic acid; C: Chenodeoxycholic acid; D: Cholic acid; E: Deoxycholic acid; F: Lithocholic acid (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; Mann-Whitney U for donor *vs* pre- or post-FMT, Friedman test with Benjamini-Hochberg FDR for pre- *vs* post-FMT). (Donors: n=5; rCDI patients pre- and post-FMT: n=18). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019; Monaghan *et al.*, 2018).

Figure 4.2:


A
B
C
D
E
F
Relative intensity (per mg of lyophilised stool)
Relative abundance (per mg of lyophilised stool)
Donors
Pre-FMT
Post-FMT, week 1
Post-FMT, week 4
Post-FMT, week 12
Time point

4.3.1.4. Bile salt hydrolase activity assay:

To further explore the timescale of gut BSH changes post-FMT, the BSH activity assay was also performed on the validation human dataset (**Figure 4.3**). This demonstrated that BSH activity was restored to levels comparable to donors within one week of successful FMT, and was maintained at these levels at four and 12 weeks post-FMT (p<0.05, Friedman test with Benjamini-Hochberg FDR). Similarly, BSH activity was significantly lower in pre-FMT samples compared to that found in either donor crude stool or FMT slurry (p<0.01, Mann-Whitney test; **Figure 4.3**).

Figure 4.3:

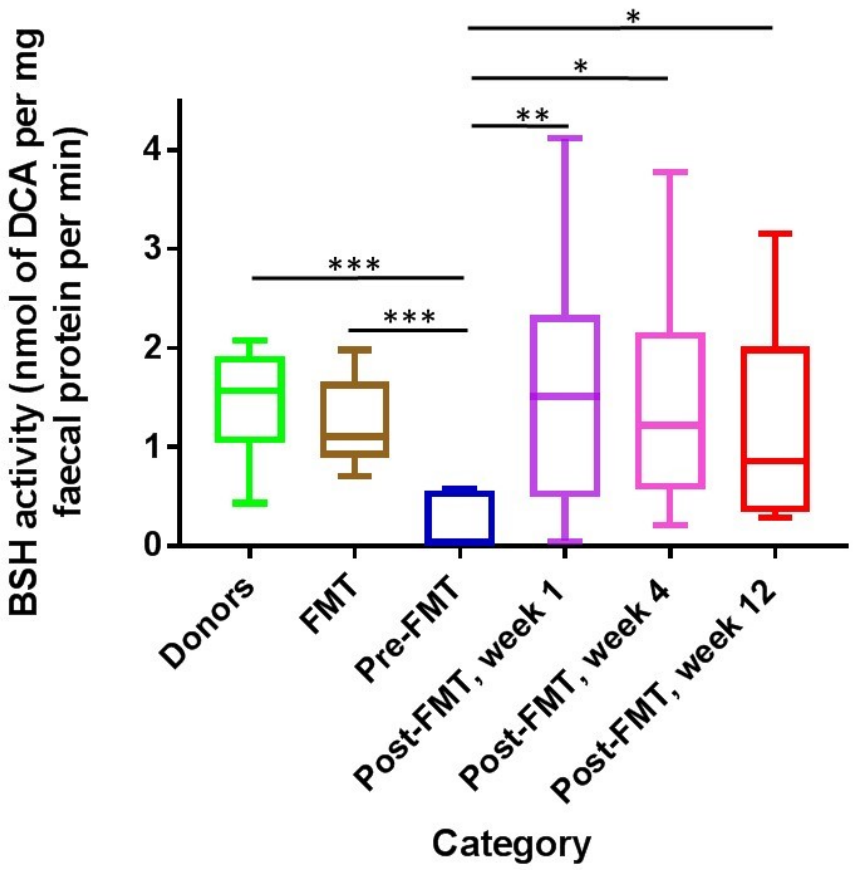


Figure 4.3 (page 133): Dynamics of changes in BSH activity after FMT for rCDI. Assessed via analysis of BSH activity in stool and FMT slurry via precipitation assay. Samples were collected from patients (and their matched donors) in a randomised trial of colonoscopy vs capsule FMT as treatment for rCDI (Kao *et al.*, 2017). Regarding donor samples, crude stool ('donors') and FMT slurry ('FMT') derived from the same donation (i.e. separate aliquots of the same stool sample both before and after processing into FMT) were collected from these donors where possible (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; Mann-Whitney U for donors/ FMT slurry vs pre-FMT, Friedman test with Benjamini-Hochberg FDR for pre-FMT vs post-FMT). (Donor crude stools ('donors'): $n=5$; donor FMT slurry ('FMT'): $n=3$; rCDI patients pre- and post-FMT: $n=18$). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019).

4.3.1.5. Gas chromatography-mass spectrometry for short chain fatty acid quantification:

4.3.1.5.1. Introduction:

GC-MS was performed on matched urine, serum and stool samples collected from patients in the validation dataset for the detection, identification and quantification of SCFAs. As described above, the aim was to investigate the impact of successful FMT for rCDI upon gut SCFA levels; particularly of interest was the levels of valerate, given its apparent influence on the ability of *C. difficile* to undergo vegetative growth (McDonald *et al.*, 2018a).

4.3.1.5.2. Urinary SCFA:

Results for the analysis of the effect of FMT for rCDI upon urinary levels of SCFAs are demonstrated in **Figure 4.4**. FMT appeared to have almost no impact on the urinary levels of almost all SCFA. However, it was noted that urinary levels of both valerate and caproate were lower in pre-FMT samples than they were in donor urine ($p<0.05$, Mann-Whitney U test). In addition, urinary levels of 2-methylbutyrate were higher at week 1 post-FMT compared to pre-FMT samples ($p<0.01$, Friedman test with Benjamini-Hochberg FDR), but not at later time points.

Figure 4.4 (page 135): Analysis of the effect of FMT for rCDI upon urinary profiles of SCFAs, as assessed using GC-MS. A: Acetate; B: Propionate; C: Isobutyrate; D: Butyrate; E: 2-methylbutyrate; F: Isovalerate; G: Valerate; H: Caproate; I: 2-hydroxybutyrate (*, $p<0.05$; **, $p<0.01$; Mann-Whitney U for donor *vs* pre- or post-FMT, Friedman test with Benjamini-Hochberg FDR for pre- *vs* post-FMT). (Donors: $n=5$; rCDI patients pre- and post-FMT: $n=18$). Data presented as mean with standard deviation.

Figure 4.4:

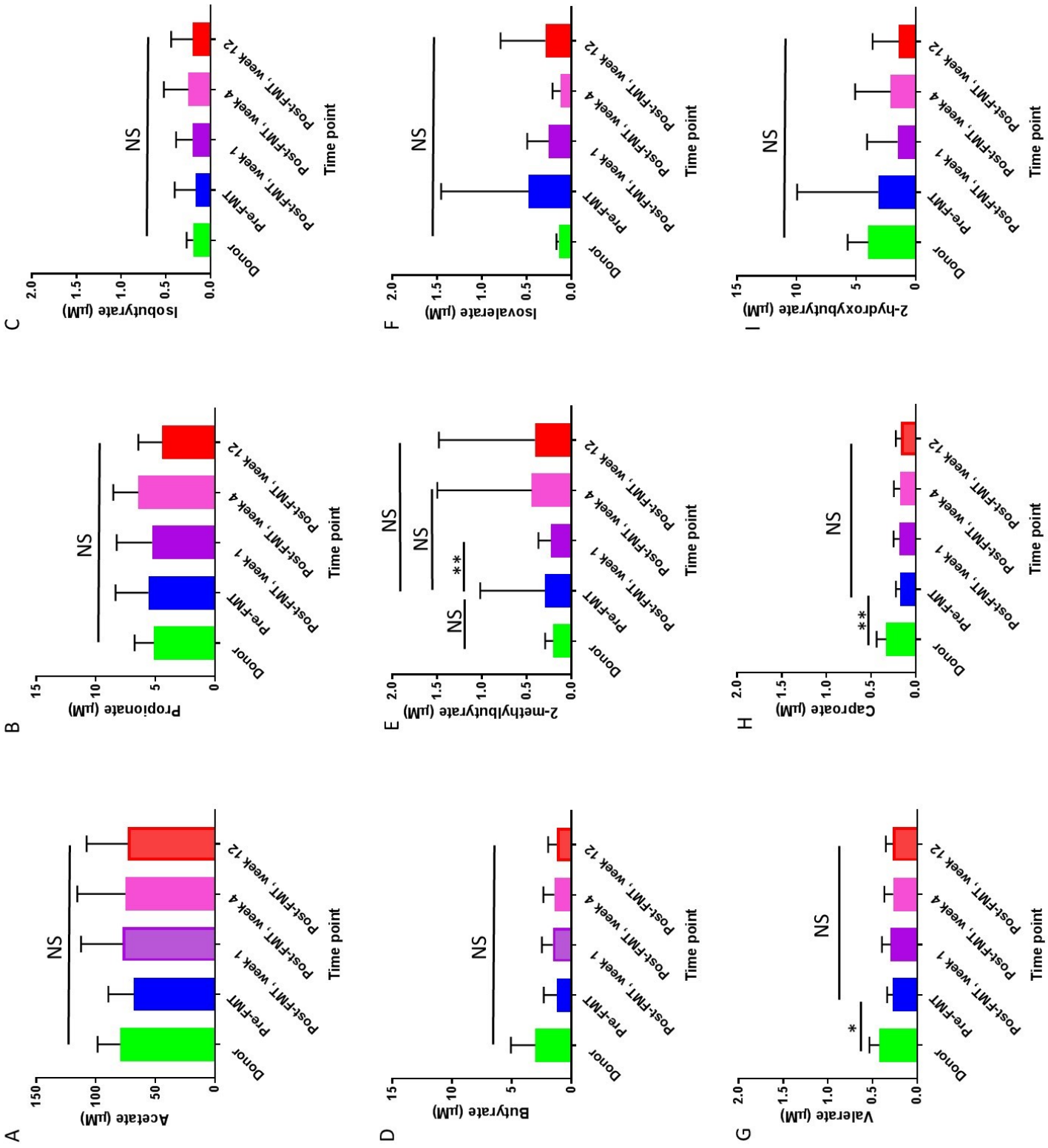

A
Acetate (μM)
B
Propionate (μM)
C
Isobutyrate (μM)
D
Butyrate (μM)
E
2-methylbutyrate (μM)
F
Isovalerate (μM)
G
Valerate (μM)
H
Caproate (μM)
I
2-hydroxybutyrate (μM)
NS
*
**
Donor
Pre-FMT
Post-FMT, week 1
Post-FMT, week 4
Post-FMT, week 12
Time point

Figure 4.5:

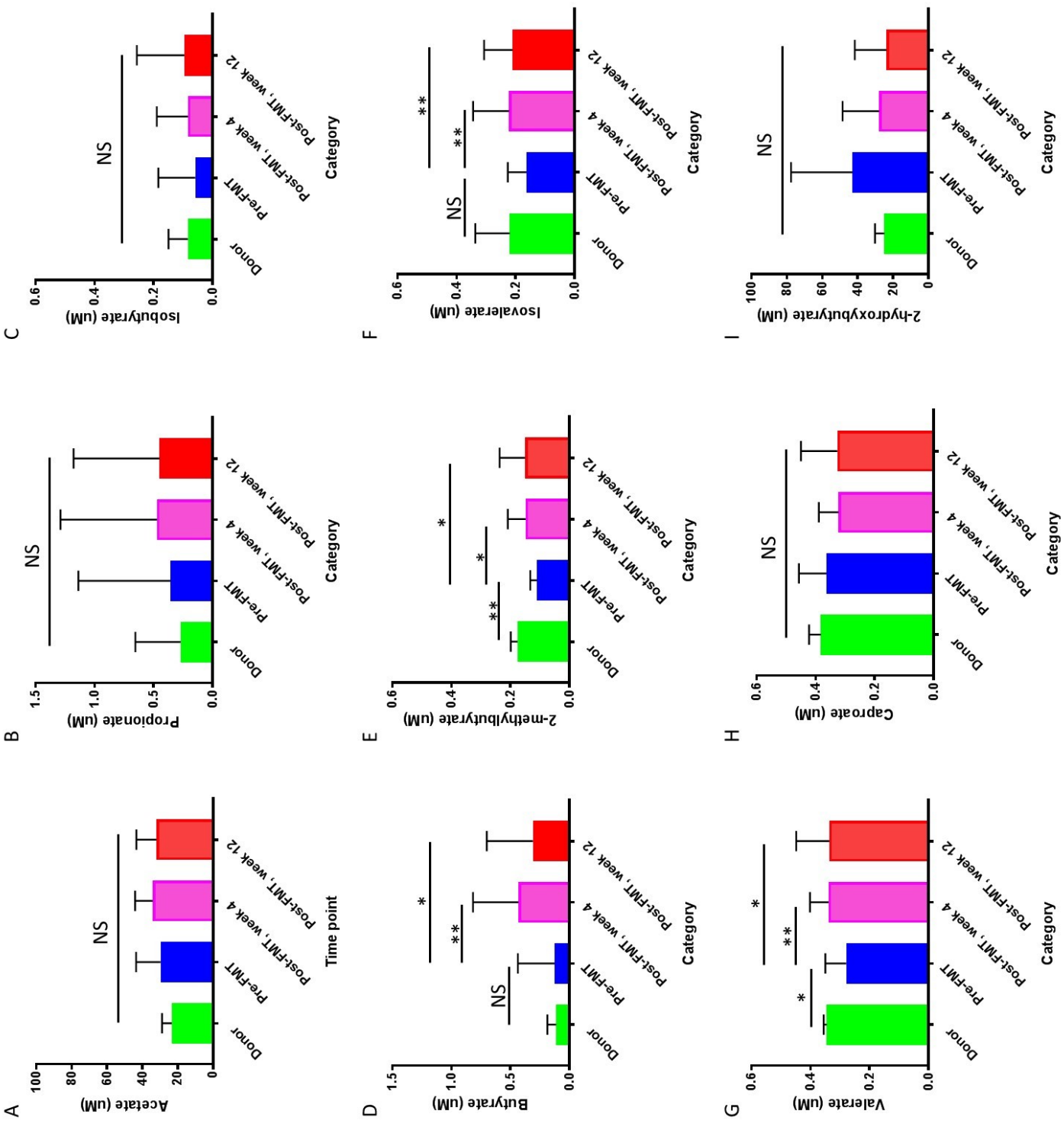

A
Acetate (uM)
NS
Donor
Pre-FMT
Post-FMT, week 4
Post-FMT, week 12
Time point
B
Propionate (uM)
NS
Category
C
Isobutyrate (uM)
NS
D
Butyrate (uM)
NS
*
**
E
2-methylbutyrate (uM)
F
Isovalerate (uM)
G
Valerate (uM)
H
Caproate (uM)
I
2-hydroxybutyrate (uM)

Figure 4.5 (page 136): Analysis of the effect of FMT for rCDI upon serum profiles of SCFAs, as assessed using GC-MS. A: Acetate; B: Propionate; C: Isobutyrate; D: Butyrate; E: 2-methylbutyrate; F: Isovalerate; G: Valerate; H: Caproate; I: 2-hydroxybutyrate (*, p<0.05; **, p<0.01; Mann-Whitney U for donor *vs* pre- or post-FMT, Friedman test with Benjamini-Hochberg FDR for pre- *vs* post-FMT). (Donors: n=5; rCDI patients pre- and post-FMT: n=18). Data presented as mean with standard deviation.

4.3.1.5.3. Serum SCFA:

Results for the analysis of changes in serum levels of SCFAs after FMT for rCDI are presented in **Figure 4.5** (N.B. no serum samples were collected at week 1 post-FMT). Successful FMT was associated with sustained increases in serum levels of butyrate, 2-methylbutyrate, isovalerate and valerate (**Figure 4.5D-G;** p <0.05, Friedman test with Benjamini-Hochberg FDR). Whilst there was a trend towards reduced serum levels of a number of SCFAs in pre-FMT samples compared to healthy donors, this only reached statistical significance for 2-methylbutyrate (**Figure 4.5E**; p <0.01, Mann-Whitney U test) and valerate (**Figure 4.5G**; p <0.01, Mann-Whitney U test).

4.3.1.5.4. Faecal SCFA:

Results for the analysis of the effect of FMT for rCDI upon stool levels of SCFAs are displayed in **Figure 4.6**. Faecal levels of all assayed SCFAs were significantly lower in patients with rCDI pre-FMT than in the stool of healthy donors (p<0.01, Mann-Whitney U). Successful FMT for rCDI was associated with rapid and sustained restoration of SCFAs to levels comparable to that of donors (p<0.01, Friedman test with Benjamini-Hochberg FDR), including the restoration of gut valerate levels (**Figure 4.6D**).

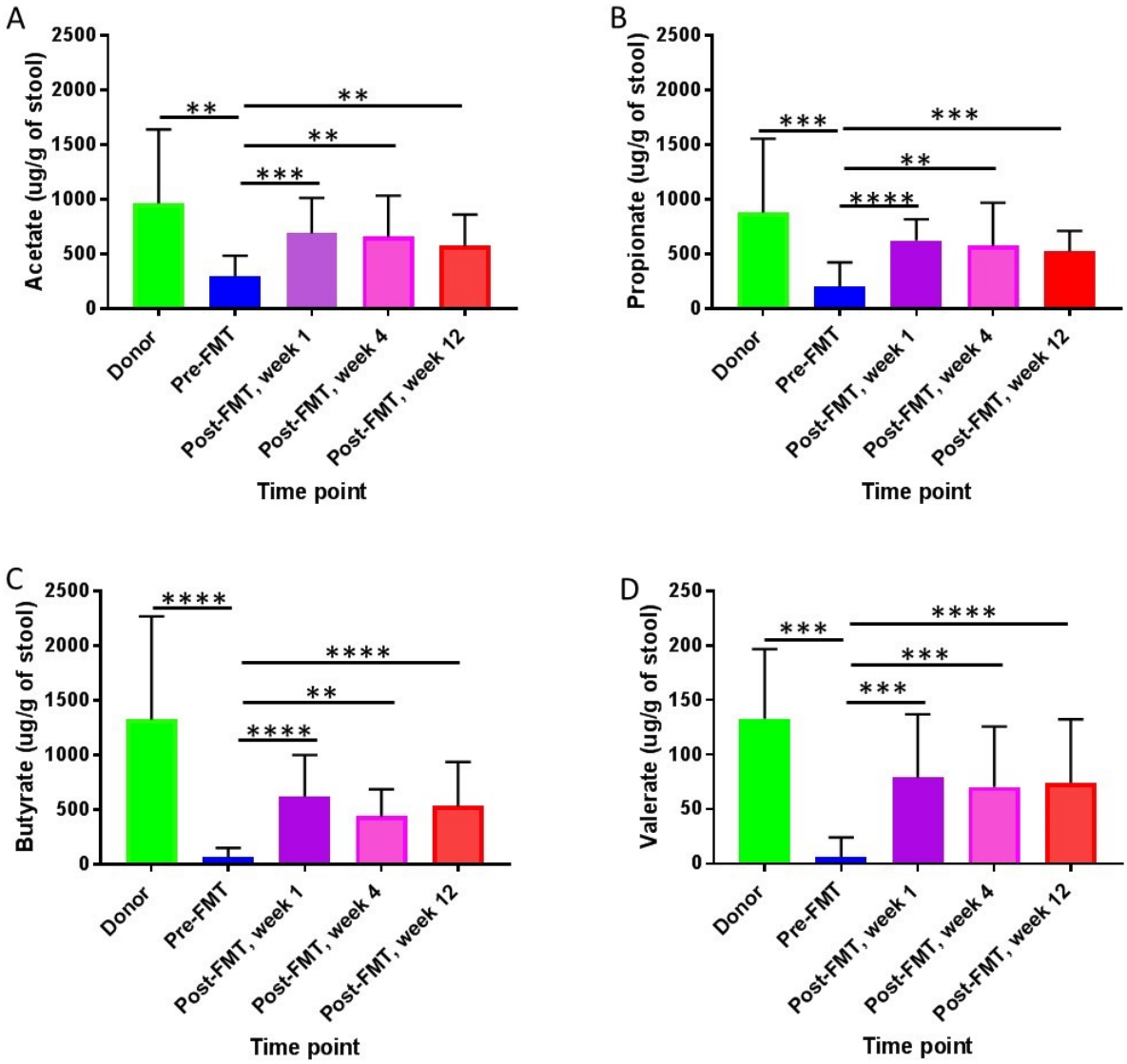


Figure 4.6: Analysis of the effect of FMT for rCDI upon faecal profiles of SCFAs, as assessed using GC-MS. A: Acetate; B: Propionate; C: Butyrate; D: Valerate (**, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; Mann-Whitney U for donor *vs* pre- or post-FMT, Friedman test with Benjamini-Hochberg FDR for pre- *vs* post-FMT) (Donors: $n=5$; rCDI patients pre- and post-FMT: $n=18$). Data presented as mean with standard deviation. Adapted from (McDonald *et al.*, 2018a).

The changes observed in stool valerate levels after FMT were the same regardless of route of administration (**Figure 4.7A**) or donor used (**Figure 4.7B**).

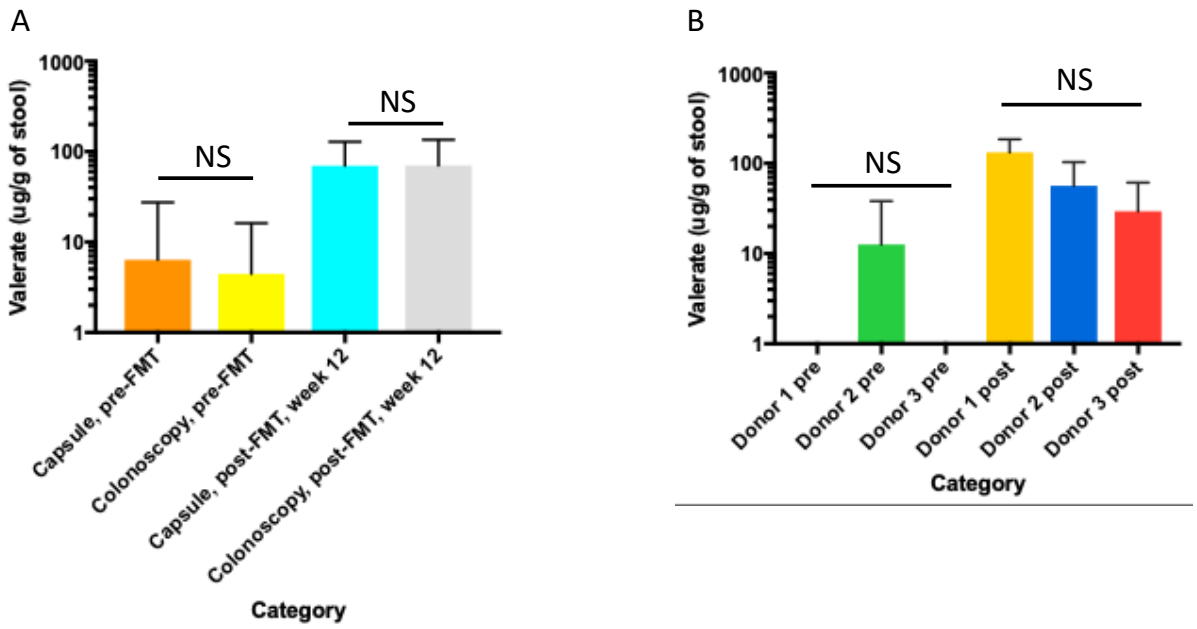


Figure 4.7: Factors potentially influencing post-FMT stool valerate changes. As assessed through comparison of pre-FMT stool valerate levels with post-FMT, week 12 levels. A: Impact of modality of administration (capsule *vs* colonoscopy) (rCDI patients receiving capsule FMT: *n*=9; rCDI patients receiving colonoscopic FMT: *n*=9); B: Impact of donor used (using samples from three different donors who had given at least three stool donations for FMT/ analysis) (Kruskal-Wallis with Benjamini-Hochberg FDR). Data presented as mean with standard deviation.

4.3.1.5.5. Influence of FMT preparation protocol upon SCFA concentrations:

Investigation was also performed as to the influence of the modality of FMT preparation (i.e. whether lyophilisation or filtration steps were used) upon the concentration of SCFA within it; results of this are shown in **Figure 4.8**. It was noted that conventional FMT preparation protocols (Kao *et al.*, 2017) and/or generation of sterile faecal filtrate (Ott *et al.*, 2017) resulted in small but non-significant reductions in the concentrations of all SCFA assayed within the transplant material. In contrast, both forms of lyophilisation assessed (lyophilised FMT and lyophilised faecal filtrate) resulted in significantly increased concentrations of all SCFA assayed, with a particularly marked increase observed for lyophilised faecal filtrate (p<0.0001, lyophilised faecal filtrate *vs* crude stool, 2-way ANOVA with Benjamini-Hochberg FDR).

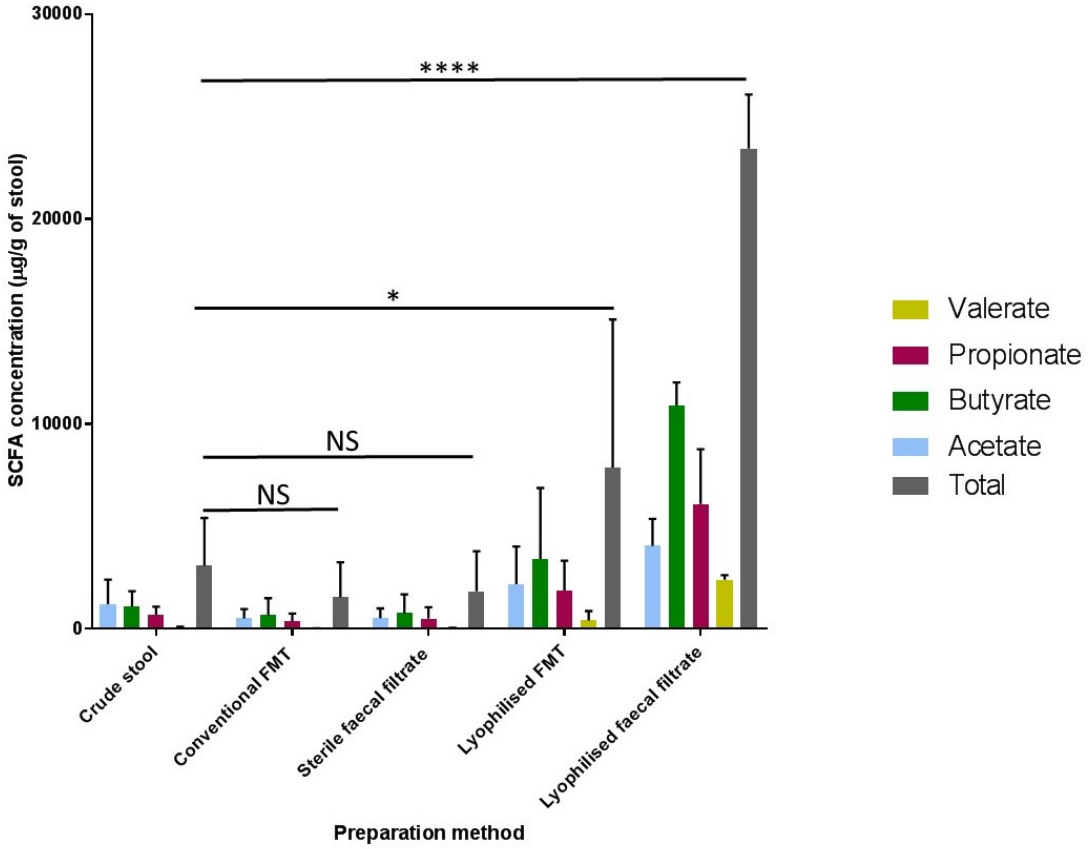


Figure 4.8: Influence of modality of FMT preparation used upon SCFA concentrations within it. Aliquots of a single homogenised stool sample provided by a donor were prepared as conventional FMT, sterile faecal filtrate, lyophilised FMT and a lyophilised faecal filtrate. Data was pooled from three separate donors who had given at least three stool donations for FMT/ analysis. Analysis of SCFA was performed via GC-MS (*, p<0.05; ****, p<0.0001; 2-way ANOVA with Benjamini-Hochberg correction). Data presented as mean with standard deviation.

4.3.2. Primary episode of *Clostridioides difficile* infection:

4.3.2.1. Clinical details:

This analysis was performed on serial stool samples collected from a prospective study of 29 patients with a first episode of uncomplicated CDI, all of whom were treated with vancomycin (Allegretti *et al.*, 2018). Mean age was 59.2 +/- 16.8 years; 20 patients were female. Patients were defined as non-recurrers if no recurrence of diarrhoeal disease had occurred at six weeks after diagnosis of primary CDI; recurrence was defined as a return of diarrhoea with a positive *C. difficile* stool toxin within six weeks of a diagnosis of primary CDI. Over the course of the study, 10 of these patients experienced recurrent disease (average time to recurrence: 1.9 weeks).

4.3.2.2. Metataxonomic profiling:

This was performed on the samples in this cohort by Dr Jessica Allegretti and her team, but results were not available for reference to in this thesis.

4.3.2.3. Ultra-performance liquid chromatography-mass spectrometry for stool bile acid profiling:

4.3.2.3.1. Introduction:

These experiments were performed to investigate whether there were differences in stool bile acid profiles between patients with first CDI developing recurrence compared to those remaining in remission with antimicrobial therapy. The particular focus was on those bile acids established to affect the ability of *C. difficile* to undergo germination and/or vegetative growth, and especially TCA and DCA. If such differences were seen, this may support the hypothesis that re-establishment of gut microbial communities with bile-metabolising functionality after treatment for CDI influence is a key factor in influencing whether remission is maintained or not.

4.3.2.3.2. Multivariate analysis:

Multivariate analysis was performed on all stool samples collected within this dataset. No valid OPLS-DA model could be constructed for the comparison of baseline samples from patients who went on to recur *vs* patients without future recurrence. In contrast, there were significant differences between these groups on supervised analysis of stool bile acid profiles obtained at the final time point, as indicated by a valid OPLS-DA model (**Table 4.2** and **Figure 4.9A**). Discriminatory feature identification was performed from OPLS-DA model data via S-plot, with stool samples at the final time point from those with recurrence demonstrating enrichment in primary bile acids (including TCA) and loss of secondary bile acids as compared to those without recurrence (including DCA) (**Figure 4.9B**).

Table 4.2: Model characteristics for OPLS-DA models derived from the primary CDI human dataset.

OPLS-DA multivariate model	R^2X	Q^2	*p* value
Recurrers, baseline *vs* non-recurrers, baseline	No valid model constructed		
Recurrers, final time point *vs* non-recurrers, final time point	0.372	0.552	0.0075

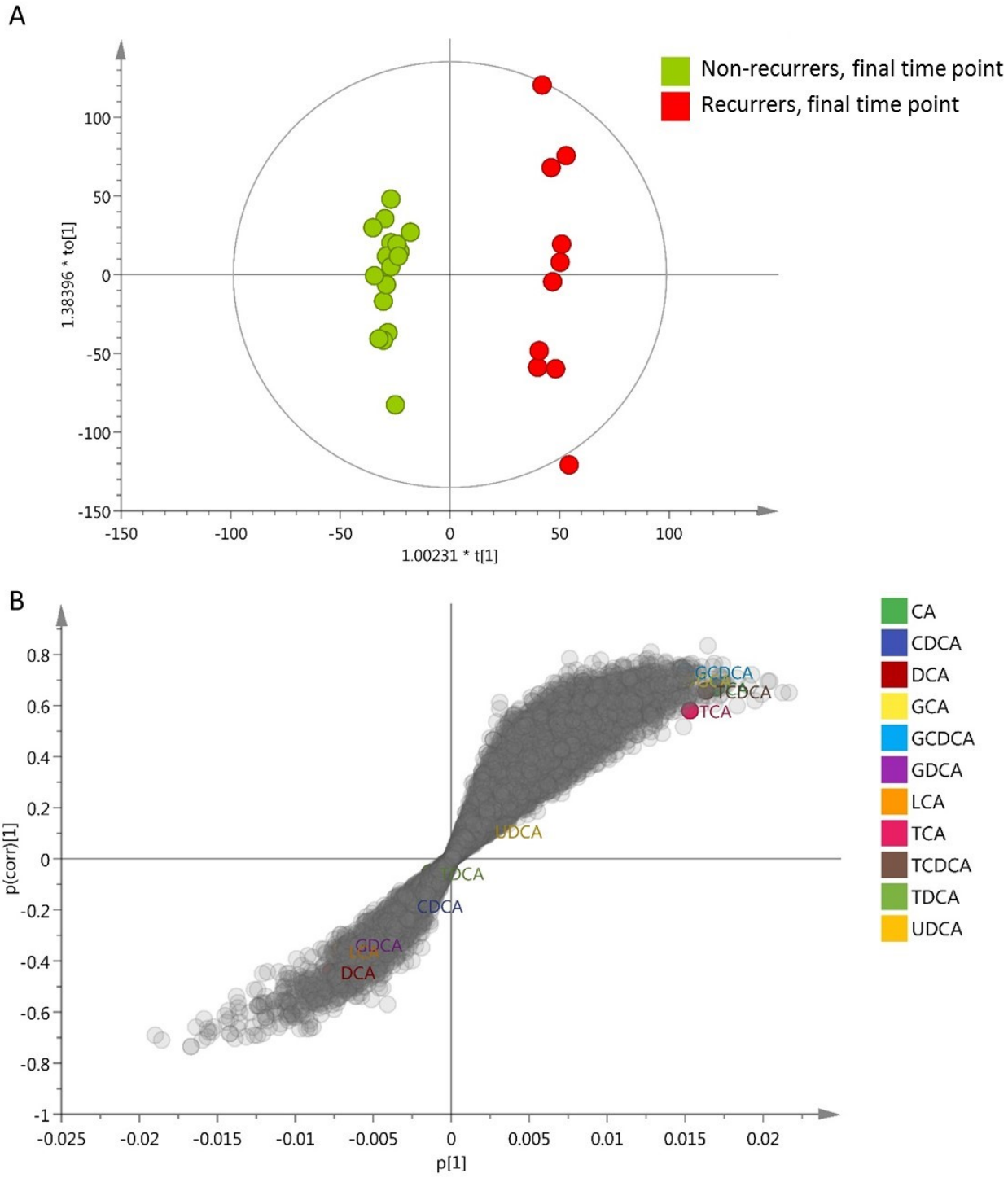


Figure 4.9: Multivariate analysis of the primary CDI dataset as assessed via UPLC-MS bile acid analysis. A: OPLS-DA scores plot, final time point of stool collection from recurrers *vs* that from non-recurrers (i.e. week 6 after diagnosis for the latter); B: OPLS-DA S-plot of recurrers *vs* non-recurrer data, as assessed via multivariate analysis of UPLC-MS bile acid profiling data. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; LCA: lithocholic acid; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; UDCA: ursodeoxycholic acid. (Recurrers: *n*=10; non-recurrers: *n*=19).

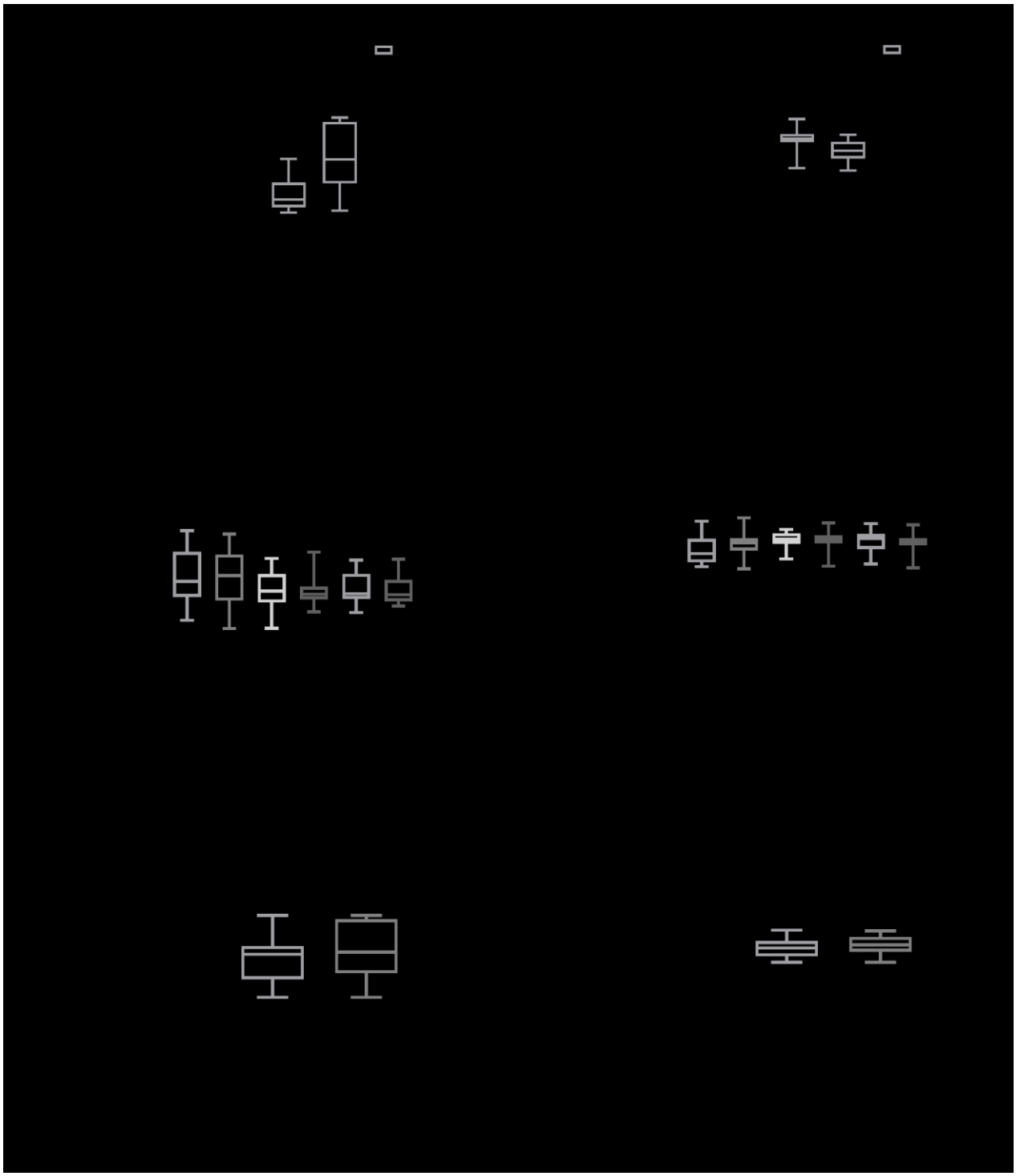
4.3.2.3.3. Univariate analysis:

In light of the results from the multivariate analysis, the univariate analysis was performed with a focus on those bile acids known to directly affect the ability of *C. difficile* to germinate and/or undergo vegetative growth, specifically TCA and DCA; results are summarised in **Figure 4.10**.

There were no significant differences in stool TCA and DCA levels in baseline samples between future recurrers *vs* non-recurrers (**Figure 4.10A and B**). However, by the final time point assayed, there was significantly higher stool TCA and lower stool DCA in patients with recurrence compared to those without (p<0.05, Mann-Whitney U). In non-recurring patients, there were significant reductions in stool TCA over time (p<0.01 for diagnosis *vs* week 6, Kruskal-Wallis with Benjamini-Hochberg FDR; **Figure 4.10C**), but no notable changes in stool DCA levels (**Figure 4.10D**). In contrast, patients with recurrence demonstrated no change in stool TCA levels during serial sampling (**Figure 4.10E**), but did show reduction in stool DCA levels over time (p<0.05 for diagnosis *vs* at relapse; **Figure 4.10F**).

Figure 4.10 (page 144): Comparative dynamics of stool bile acids changes in patients with first CDI who recur *vs* non-recurrers, as assessed using UPLC-MS bile acid profiling data. A: Comparison of stool TCA levels at baseline and at the final time point sampled; B: Comparison of stool DCA levels at baseline and at the final time point sampled; C: Time course of stool TCA, non-recurrers; D: Time course of stool DCA, non-recurrers; E: Time course of stool TCA, recurrers; F: Time course of stool DCA, recurrers. The final time point at which stool was assayed for non-recurring patients was week 6 after diagnosis (patients were defined as non-recurrers if no recurrence of diarrhoeal disease had occurred at six weeks after diagnosis of primary CDI) (*, p<0.05; **, p<0.01; Mann-Whitney U for recurrers *vs* non-recurrers, Kruskal-Wallis test with Benjamini-Hochberg FDR for comparison of time points). (Recurrers: n=10; non-recurrers: n=19). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Allegretti *et al.*, 2018).

Figure 4.10:



4.3.2.4. Bile salt hydrolase activity assay:

Following on from the stool bile acid profiling studies, BSH activity assays were performed on these samples with the aim of evaluating whether restoration of gut BSH functionality may be a factor that explains the above stool TCA results, and influences whether a patient experiences recurrence of CDI or not.

Results from these studies are given in **Figure 4.11.** No significant difference was found in BSH activity at baseline amongst patients who went on to relapse *vs* those who did not (p>0.05, Mann-Whitney U), but activity was significantly higher at the final time point assessed in non-recurrers in comparison to recurrers (p<0.05; **Figure 4.11A**). Whilst there was a trend towards recovery in stool BSH activity amongst patients who did not relapse over time, this did not reach statistical significance (p>0.05, Kruskal-Wallis with Benjamini-Hochberg FDR correction; **Figure 4.11B**). Amongst patients who experienced recurrence, no recovery of gut BSH activity was seen between diagnosis and the time of relapse (p>0.05; **Figure 4.11C**).

Figure 4.11:

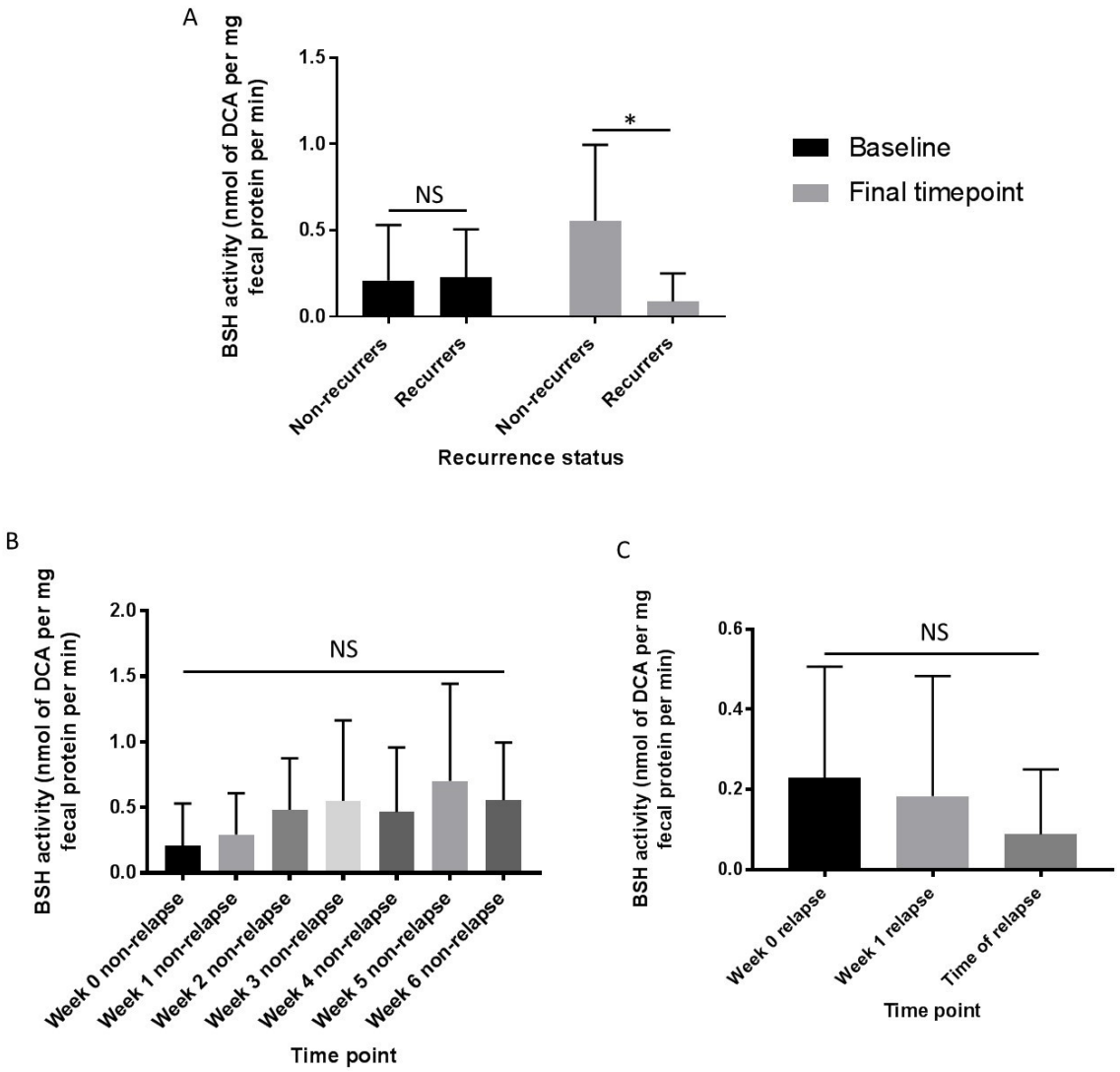


Figure 4.11 (page 145): Comparative dynamics of stool BSH activity changes in patients with first CDI who recur *vs* non-recurrers. As assessed using precipitation assay. A: Comparison of stool BSH activity at baseline and at the final time point sampled; B: Time course of stool BSH activity, non-recurrers; C: Time course of stool BSH activity, recurrers. NS: non-significant. Final time point assayed for non-recurring patients was week 6 after diagnosis (*, $p<0.05$; Mann-Whitney U for recurrers *vs* non-recurrers, Kruskal-Wallis test with Benjamini-Hochberg FDR for comparison of time points). (Recurrers: $n=10$; non-recurrers: $n=19$). Data presented as mean with standard deviation. Adapted from (Allegretti *et al.*, 2018).

4.4. Discussion and analysis:

4.4.1. Gut microbiota-bile acid interactions in recurrent *Clostridioides difficile* infection, and the impact of FMT:

As for the initial dataset, the main finding of this analysis was that successful FMT for rCDI is associated with reconstitution of microbiota community members with presumed bile-metabolising functionality, restoration of a normal gut bile acid *milieu*, and recovery of gut bile-salt hydrolase activity. These changes are observed within a week of FMT, and maintained at up to 12 weeks, which is the typical point in time at which a patient would be defined as being fully in remission from CDI after successful treatment (Mullish *et al.*, 2018d).

Although such changes in bile acid profiles are interesting in themselves, results obtained using an observational dataset such as this does not directly answer whether these changes are of sufficient magnitude to be of biological significance, or purely an epiphenomenon with no direct physiological consequences. However, data from previous studies from humans and mice have provided supportive evidence that the metabolic/ bile acid *milieu* found in CDI *in vivo* is sufficient to directly influence *C. difficile*'s life cycle. In particular, exposure of *C. difficile* spores to the bile acid *milieu* found in antibiotic-treated mouse caecum (Theriot *et al.*, 2014) or human stool pre-FMT (Weingarden *et al.*, 2016b) was sufficient to cause spore germination, whilst that of the non-antibiotic-treated mouse caecum (Theriot *et al.*, 2014) or human stool post-FMT (Weingarden *et al.*, 2016b) prevented germination and vegetative growth of *C. difficile*.

In addition, results from a collaborative study performed during the course of the experiments in this thesis give further support for physiological consequences for the host related to the gut bile acid *milieu* in CDI. A proteomic array was performed upon serum

collected from patients in the validation cohort with the primary aim of investigating the effect of FMT for rCDI upon serum inflammatory markers (Monaghan *et al.*, 2018). Of the 73 proteomic markers compared, significant differences between pre- and post-levels were only observed for two markers, fibroblast growth factor (FGF)-19 and -21. In particular, compared to pre-FMT, there was a statistically significant increase in serum FGF-19 levels post-FMT (at both week 4 and week 12), and a significant decrease in serum FGF-21 levels (**Figure 4.12**).

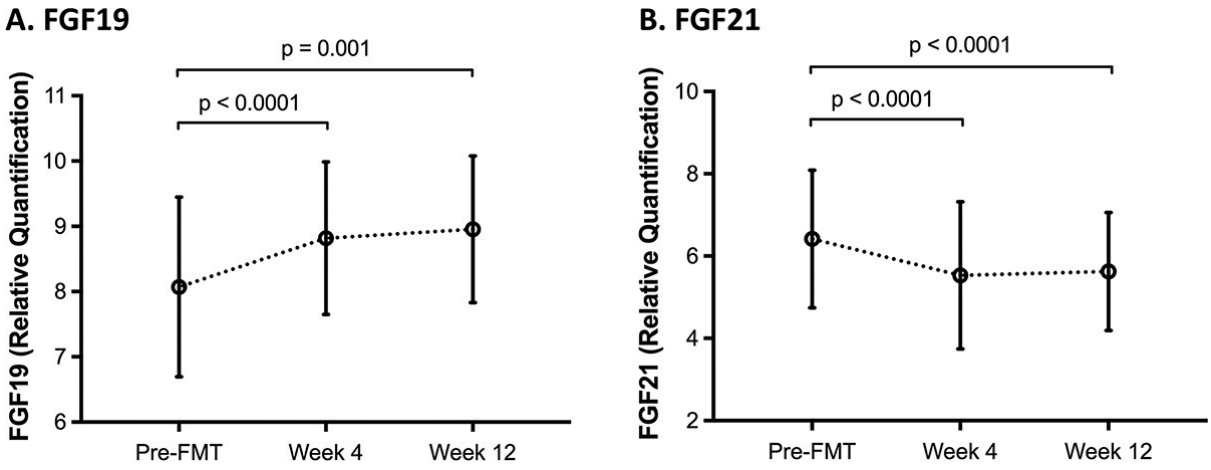


Figure 4.12: Effect of FMT for rCDI upon relative protein expression values of fibroblast growth factors within serum. Protein expression expressed as Normalised Protein eXpression (NPX) values. A: FGF-19; B: FGF-21. As assessed by repeated measures ANOVA with Bonferroni FDR correction. (FMT recipients: *n*=43). Data presented as mean with standard deviation. Reproduced from (Monaghan *et al.*, 2018).

This is of interest and relevance, since both FGFs are secreted into the portal circulation in response to activation of the nuclear receptor farnesoid X receptor (FXR) within the ileum; FXR is a protein which is abundantly expressed in the liver and ileum, and is recognised as a key endogenous regulator of host bile acid metabolism (Lefebvre *et al.*, 2009). In humans, the most potent endogenous ligand for FXR is CDCA; the secondary bile acids DCA and LCA are moderate FXR agonists, whilst CA also has modest agonist activity (Wahlström *et al.*, 2016). After ileal FXR stimulation and consequent secretion of FGFs into blood, FGFs bind to the FGFR4/β Klotho receptor complex on hepatocytes. The main outcome of this interaction is negative feedback control on hepatic bile acid synthesis and transport out of hepatocytes, but other effects include modulation of key metabolic pathways involved in glucose, lipid and energy metabolism (Benoit *et al.*, 2017). FGF-19 and FGF-21 contribute to the same

physiological processes but have an inverse relationship, with FGF-19 being secreted during feeding, and FGF-21 produced during fasting (Zhang *et al.*, 2015).

Prior to this study, a link between gut microbiota, intestinal bile acid metabolism and FGF signalling had been observed, but only in mouse models. Specifically, germ-free and antibiotic-treated mice have markedly reduced ileal *Fgf15* gene expression (FGF-15 being the murine orthologue of human FGF-19 (Sayin *et al.*, 2013). Given the absence of BSH in these mice, they experience accumulation of tauro-β-muricholic acid within the gut; this bile acid is an FXR antagonist, and therefore hypothesised to be a key factor contributing to the association between the gut microbiota and FXR signalling seen in mice (Sayin *et al.*, 2013). However, the extent to which this mechanism may be extrapolated to humans is unclear because of differences in key aspects of relevant physiology between mice and humans, such as the differences in FGF orthologues present in both, and the fact that tauro-β-muricholic acid is only present at relatively modest levels in the human gut compared to other conjugated bile acids (Monaghan *et al.*, 2018). Nevertheless, on further examination of the data from the validation human dataset, there is evidence that successful FMT for rCDI is associated with a rapid and sustained reduction in the amount of tauro-β-muricholic acid in stool to a comparable level to that of healthy donors (**Figure 4.13**), and this merits further evaluation within the human setting. An alternative hypothesis to explain the association between microbiota, bile acid and FXR signalling changes in humans is that post-FMT for rCDI, the reduced level of a potent FXR agonist (CDCA) is offset by increased levels of two moderate FXR agonists (DCA and LCA), with a net upregulation of the ileal FXR-FGF pathway (Parks *et al.*, 1999; Monaghan *et al.*, 2018). The dietary differences of rCDI patients between pre- and post-FMT are unlikely to be the explanation for the FXR changes seen, given that these are more likely to result in FXR suppression rather than activation (as also discussed in **Section 3.4.2**).

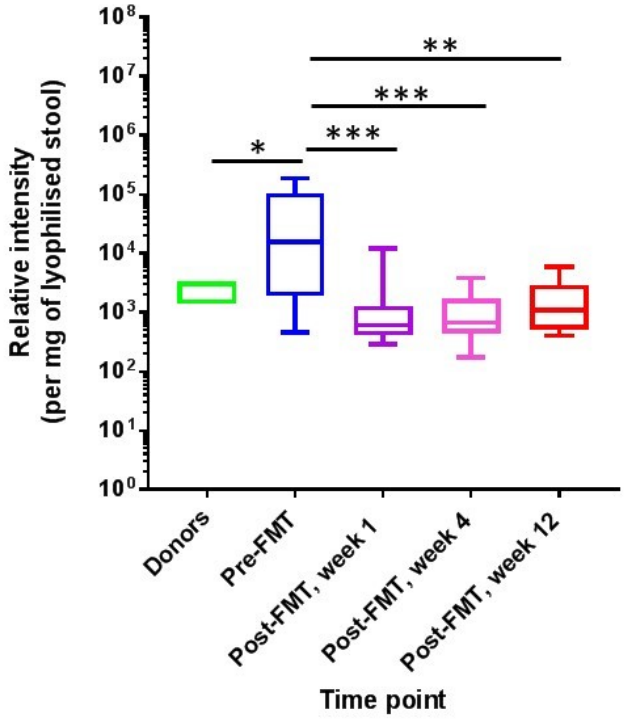


Figure 4.13: The effect of FMT for rCDI upon faecal tauro-β-muricholic acid levels. As assessed within the validation dataset, using UPLC-MS bile acid profiling data (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; Mann-Whitney U for donor *vs* pre- or post-FMT, Friedman test with Benjamini-Hochberg FDR for pre- *vs* post-FMT). (Donors: $n=5$; rCDI patients pre- and post-FMT: $n=18$). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points.

This FXR data is also a salient reminder that not only does the gut microbiota influence bile acid metabolism, but bile acids directly influence the survival and growth of members of the gut microbiota. Conjugated bile acids have been recognised as having more potent antimicrobial effects than unconjugated bile acids, and during colonisation of the human GI tract, bacteria are exposed to inhibitory levels of conjugated bile acids (Begley, Gahan & Hill, 2005; Jones *et al.*, 2008). As such, BSH-producing organisms are sometimes described as 'bile tolerant', since their hydrolytic action facilitates GI tract colonisation and development of the gut microbiota. This has been demonstrated experimentally too – for instance, wild-type *Listeria innocua* (which does not have a *bsh* gene) does not colonise mice as well as *L. innocua* engineered to express *bsh* genes (Jones *et al.*, 2008). Further evidence has been described recently that FXR-mediated changes in bile metabolism directly impact the gut microbiota. In a study where healthy volunteers or mice were administered obeticholic acid (OCA) (a bile acid analogue and farnesoid X receptor agonist), endogenous bile acid synthesis was

suppressed, and a reversible induction of Gram-positive bacteria (particularly a relative enrichment in *Firmicutes* was observed) (Friedman *et al.*, 2018). Interestingly, researchers also noted that the single bacterial species that was most enriched post-OCA, *Streptococcus thermophilus,* did not have an annotated *bsh* gene.

Collectively, these experiments on bile tolerance support the concept that the more conjugated bile acids entering the gut, the more selection there is for colonisation with BSH-producing organisms which can degrade potentially toxic conjugated bile acids (and vice-versa). Within the framework of the hypothesis of this project, this concept of bile tolerance – and the apparent contribution of FXR signalling - suggests a more complex integrated model than originally proposed as the potential mechanism of efficacy of FMT for rCDI:

1. The BSH-deficient, conjugated bile acid-rich environment of the rCDI gut promotes germination of *C. difficile,* and provides a hostile environment for colonisation for many bacteria.
2. The absence of secondary bile acids in the CDI gut ensures there is no inhibitory signals to *C. difficile* vegetative growth, and may directly influence the apparent low levels of ileal FXR signalling. Low levels of FXR signalling promotes further hepatic bile acid synthesis – including the generation of TCA – which further drives *C. difficile* germination.
3. After FMT, the presence of BSH-producing bacteria facilitates colonisation through degradation of toxic conjugated bile acids; in the process, TCA is degraded, and there is consequently less signal for *C. difficile* germination.
4. BSH is the gateway step to subsequent bile acid metabolism, and secondary bile acids are formed. DCA inhibits *C. difficile* growth, and may directly contribute to increased FXR signalling. FXR-mediated reduction in hepatic bile acid synthesis minimises further TCA entering the small intestine; this reduces any drive to *C. difficile* germination, and may limit intestinal overgrowth by BSH-producing bacteria.

Whilst this suggests BSH as the central driver to the efficacy of FMT (but with a feasible additive contribution of 7-α-dehydroxylase), the specific contribution of these enzymes to initial vulnerability to CDI (and its potential contribution to recurrence) remains less clear. As such, this was explored via the primary CDI study.

4.4.2. Gut microbiota-bile acid interactions in primary *Clostridioides difficile* infection, and their potential influence upon recurrence risk:

In this study, serial stool bile acid profiles and BSH activity were measured for patients with primary CDI, with the aim of evaluating whether different profiles were observed in patients who went on to experience recurrence of CDI compared to patients who did not. One central finding was that in patients without recurrence, there was a gradual but continuous reduction in gut TCA levels over time (but, in contrast, no statistically significant change in gut DCA levels), and significantly higher BSH levels at the last time point sampled compared to that in recurrers. In patients who went on to develop recurrence, there was no change in gut TCA levels, but there was a decline in gut DCA.

One further factor that requires consideration when starting to interpret these data is that patients with primary CDI were treated with vancomycin for 10-14 days, and this medication has its own effects on gut microbiota-bile acid interactions. In particular, vancomycin has been demonstrated to particularly suppress members of the *Firmicutes* phylum (and particularly *Clostridium* clusters IV and XIVa) to a considerably greater extent than the *Bacteroidetes* (in which BSH-producing organisms are predominantly found (**Appendix 1**)) (Reijnders *et al.*, 2016; Vrieze *et al.*, 2014). These microbiota changes have been accompanied by loss of faecal secondary bile acids and enrichment in primary unconjugated bile acids (Vrieze *et al.*, 2014; Reijnders *et al.*, 2016). This is clearly consistent with vancomycin resulting in predominantly reduced gut microbiota 7-α-dehydroxylation rather than BSH activity. There are at least two implications from such data for this project.

1. Firstly – one limitation to the interpretation of the human data from the two rCDI/FMT cohorts described here was that all patients were taking vancomycin almost right up to the point of the FMT, and so it is difficult to differentiate what microbiota changes seen between pre- and post-FMT were directly FMT-related, and which reflected microbiota recovery after cessation of vancomycin. This suggests that any microbiota enrichment in *Bacteroidetes* post-FMT (and, by implication, most restored BSH activity, since *Bacteroidetes* is the major source) is unlikely to solely reflect gut microbiota recovery without the selection pressure of vancomycin.
2. This widens the interpretation of the DCA data for primary CDI. For example, in recurring patients, the reduction in stool DCA levels observed between diagnosis and week 1/ time

of recurrence may reflect a transient loss of 7-α-dehydroxylase in response to vancomycin therapy, or may imply a more significant permanent loss of this functionality. The suggestion that the latter may be the case is implied through the observation that non-recurring patients did not show any significant change in stool DCA over serial follow-up.

There is relatively limited literature on gut microbiota-mediated bile acid metabolism in primary CDI (as opposed to recurrent CDI) in humans. Allegretti and colleagues compared gut microbiota and bile acid profiles from patients with rCDI to patients with primary CDI and healthy controls. Using an inferential metagenomic tool (PICRUSt), researchers observed that the predicted *bsh* gene abundance was significantly lower in rCDI than in primary CDI and/or healthy controls. In contrast, however, no significant difference in predicted abundance was seen between healthy controls and those with primary CDI (Allegretti *et al.*, 2016). Solbach and colleagues analysed *bai*CD gene copy number in stool samples taken from *C. difficile* negative stool, patients with CDI, and patients either colonised with toxigenic or non-toxigenic *C. difficile* but without actual CDI. They reported a significantly reduced prevalence of samples containing any detectable *bai*CD amongst CDI stool compared to stool from all other groups, but no actual significant difference in overall *bai*CD copy number between groups (Solbach *et al.*, 2018). The conclusion made by these researchers was that the presence or absence of *bai*CD within the gut microbiota may influence colonisation resistance and risk of CDI, but not the actual copy number present itself.

In a distal gut chemostat model (which more closely resembled primary than recurrent CDI), enrichment in primary and loss of secondary bile acids was observed during clindamycin treatment, but levels of all bile acids rapidly reversed back to original levels after cessation of antibiotics (McDonald *et al.*, 2018a). *C. difficile* total viable counts remained high despite restoration of secondary bile acid levels comparable to those observed before administration of antibiotics. One conclusion from these experiments is, given that the general pattern of changes in gut bile acid profiles in primary CDI is similar in a chemostat to *in vivo,* that these observations primarily reflect changes in the dynamics of bile-metabolising enzymes (rather than, for instance, changes in FXR signalling). A further conclusion is that restoration of DCA to pre-morbid levels may not be sufficient to limit vegetative growth of *C. difficile,* despite the effects seen by DCA upon *C. difficile in vitro.*

Collectively, these studies suggest a model whereby patients treated successfully for primary CDI are most likely to retain a state of non-recurrence where they experience recovery of gut microbiota members with BSH functionality, and consequently loss of the pro-germination trigger, TCA. The central risk factor for recurrence is absence of recolonisation of the gut microbiota with BSH producing members. However, an additional factor may be non-recovery (or further loss of) 7-α-dehydroxylase-producing bacteria from the gut, which may itself be influenced by the use of vancomycin as therapy.

Several further experiments would help to expand upon the results of these pilot data from patients with primary CDI. The number of included patients was small (particularly with regards to recurrence), and repetition with larger numbers – and associated increased power to detect between-group differences – would evidently be desirable. Comparison of metabonome data to microbial sequencing data would also clearly provide insightful corroborative data. A further direction of interest would be *ex vivo* experiments, i.e. incubation of *C. difficile* spores or vegetative cells in faecal supernatant (and/or an artificial bile acid solution), containing bile acids at relative concentrations to those identified in primary CDI *in vivo*). This would allow more direct identification of the net effect of varying bile acid changes upon *C. difficile*'s life cycle directly.

4.4.3. Gut microbiota-short chain fatty acid interactions in recurrent *Clostridioides difficile* infection, and the impact of FMT:

4.4.3.1. Short chain fatty acids in recurrent *Clostridioides difficile* infection:

SCFA concentrations were measured within urine, serum and stool. Evaluation of metabolites of interest in different body compartments is often insightful as to the whole body availability of those metabolites. No significant differences were noted in the urinary SCFA concentrations between donors, pre-FMT and post-FMT, on analysis of spot urine samples (**Section 4.3.1.5.2**); however, concentrations were overall very low, which is consistent with the recent observation that only 0.05% of SCFAs are excreted within urine (with most excretion ultimately via the respiratory route, after oxidation to carbon dioxide) (Boets *et al.*, 2017). This same study also observed that systemic availability of colonic SCFA was very variable (between 36% for acetate and 2% for butyrate), as well as bioconversions from one SCFA to another mediated by the gut microbiota (Boets *et al.*, 2017), which all add to the

complexity of interpretation of results here. One further complexity in the interpretation of urinary metabonomic data results is that urine can display marked variability of osmolality (based upon hydration status, use of diuretics, degree of kidney impairment, etc), which in turn may affect the apparent concentration of metabolites quantified within the sample (Vogl *et al.*, 2016). As such, best practice for metabonomic analysis of spot urine samples (such as those used here) is to normalise data, such as through the use of creatinine content or osmolality (Vogl *et al.*, 2016).

Successful FMT was found to be associated with recovery of serum butyrate, 2-methylbutyrate, valerate and isovalerate to levels similar to that found pre-FMT (**Section 4.3.1.5.3**). The recovery of the branched chain fatty acids (BCFAs), isovalerate and 2-methylbutyrate, is particularly of interest. There are different routes to synthesis of BCFAs, but a key pathway is gut microbiota fermentation from branched chain amino acids (isovalerate from valine, 2-methylbutyrate from leucine) via a specialised branched-chain-keto acid dehydrogenase complex (Portune *et al.*, 2016). The effect of FMT for rCDI upon BCFA levels has not previously been well-explored, although one recent study observed a trend towards (but not statistically significant) increases in their levels within stool after successful FMT (Kellingray *et al.*, 2018). The impact of BCFA upon host health is relatively poorly-explored, although there is recent evidence that BCFA can affect adipocyte lipid and glucose metabolism, with the net result being improved insulin sensitivity (Heimann *et al.*, 2016). As discussed earlier, systemic effects of SCFA have also been increasingly-recognised, including effects upon host metabolism and satiety (Flint *et al.*, 2015). As such, rather than FMT for rCDI purely exerting local effects within the gut, there may be broader implications for host physiology as well. This is discussed further within the **Discussion, Section 6.2**.

Analysis in this present study also demonstrated that successful FMT was associated with restoration of stool acetate, butyrate, propionate and valerate (**Section 4.3.1.5.4**; assessment was not possible for BCFA within stool). Whilst prior human studies have also demonstrated recovery of acetate, butyrate and propionate after FMT (Brown *et al.*, 2018; Seekatz *et al.*, 2018), none have investigated the effect of FMT for rCDI upon valerate metabolism. Many bacteria which were found to be at increased relative abundance in the stool microbiota post-FMT in the metataxonomic analysis within the past chapter are also recognised as producers

of SCFA (Flint *et al.*, 2015), including acetate-producers (*Blautia obeum, Bacteroides vulgatus, Collinsella aerofaciens*), butyrate-producers (*Eubacterium rectale, Anaerostipes hadrus, Faecalibacterium prausnitzii*) and propionate-producers (*Bacteroides vulgatus*). Much less is understood about which bacteria are responsible for the production of valerate, although there appears to be several separate microbially-mediated pathways, which have been most closely-linked with *Clostridium* species. Specifically, some *Clostridium* species can ferment ethanol and propionate to valerate (Bornstein & Barker, 1948); in another pathway, proline is first reduced to 5-aminovalerate by *Clostridium* species in a Stickland-type fermentation, before further gut bacteria ferment 5-aminovalerate to valerate (Barker, D'Ari & Kahn, 1987).

However, one key factor that makes it difficult to interpret any potential changes in SCFA concentrations between pre- and post-FMT for rCDI is that it would clearly be expected that patients have markedly different diets after FMT. Unfortunately, detailed dietary diaries were not kept for patients who provided samples for this study. Patients often transition from a situation pre-FMT where they are severely debilitated by chronic diarrhoea, to a situation where their gut inflammation has resolved, and any associated cachexia and anorexia is no longer present. Unsurprisingly, weight gain post-FMT is well-documented (Monaghan *et al.*, 2018). However, given that colonic SCFA production is a function both of the gut microbiota and of the availability of dietary substrates, it may be difficult to differentiate whether the change of one of these factors (or both in concert) explains any observed alterations in SCFA concentrations post-FMT.

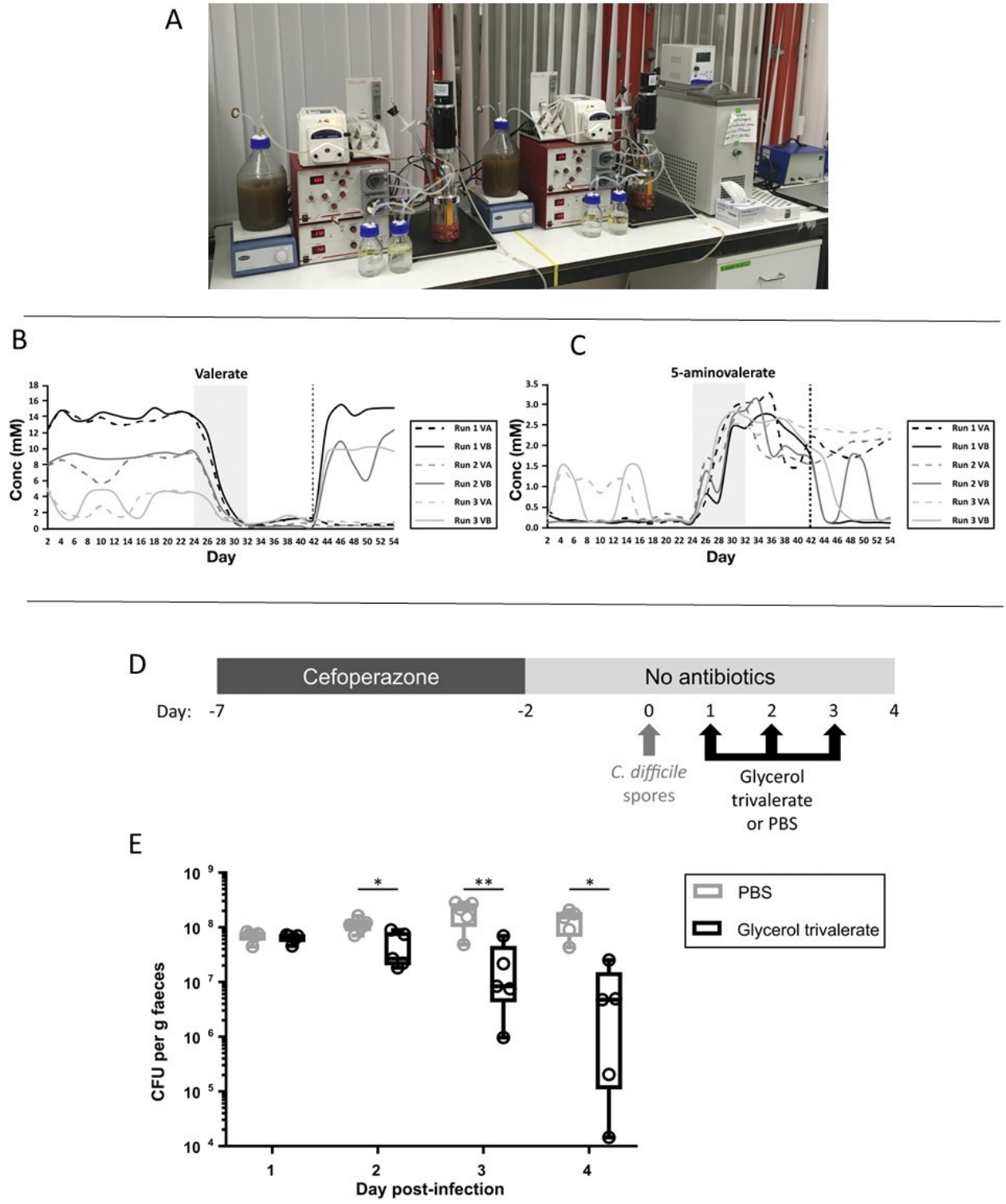
One way to control for this is to model CDI *in vitro,* ensuring that the level of fermentable polysaccharides and polypeptides available is constant throughout. One such model that may be used to assess this that has already been alluded to is a chemostat model. In the laboratory where the experiments of this thesis were undertaken, experiments using a chemostat model of CDI and FMT have also recently been performed (McDonald *et al.*, 2018a). In these experiments, two identical chemostat models of distal gut CDI were established; one vessel was treated with FMT (derived from a healthy screened donor), whilst the other was treated with saline (**Figure 4.14A**). Three separate twin chemostat experiments were performed. While there was a significant decline in levels of all measurable SCFA during clindamycin dosing of the chemostat, there was a spontaneous recovery of acetate and butyrate after

cessation of antibiotics. However, of particular interest was the observation that the C5 SCFA valerate did not recover spontaneously after completion of antibiotics, but did recover when vessels were treated with FMT (**Figure 4.14B**). Conversely, valerate's precursor, 5-aminovalerate, was enriched after clindamycin administration but only restored to baseline by FMT (**Figure 4.14C**). Batch culture experiments demonstrated an inverse relationship between valerate concentration and growth of a range of *C. difficile* strains; in contrast, changes in valerate concentration did not appear to affect the growth of a range of gut commensal bacteria. In a mouse model of CDI (**Figure 4.14D**), mice administered glycerol trivalerate (GTV) had a significant reduction in faecal *C. difficile* total viable counts compared to mice administered saline (**Figure 4.14E**). More specifically, 200µl of 15mM GTV was administered to mice as three administrations on consecutive days via oral gavage (this dose was chosen in light of the results of *C. difficile*/ valerate batch cultures). Glycerol is well-established as a bacterial cryopreservative, with frozen FMT typically stored in 10-20% glycerol, without any marked adverse effect upon commensal bacteria (Costello *et al.*, 2015); as such, it is much more feasible that the reduced *C. difficile* total viable count represented the action of valerate rather than glycerol.

Collectively, these data support the concept that valerate is profoundly depleted in the gut of those with CDI, that it is restored through FMT, and that it has direct inhibitory effects on the vegetative growth of *C. difficile,* but without adverse effects upon gut commensal bacteria.

Figure 4.14 (page 157): Investigation of the role of the impact of FMT for CDI upon SCFA dynamics. A: twin-vessel single-stage chemostat models (courtesy of Dr Julie A K McDonald, Imperial College London). Three separate simultaneous runs were made of vessels A and B, each using a different stool donor; B: effect of FMT for CDI upon chemostat valerate concentrations (dashed line: saline-treated cultures; solid line: FMT-treated cultures; shaded grey box: clindamycin-dosing period; vertical dotted line: day of FMT or saline dosing); C: effect of FMT for CDI upon concentrations of the valerate precursor, 5-aminovalerate; D: experimental protocol to investigate the impact of valerate on a mouse model of CDI; E: changes in faecal *C. difficile* colony-forming units after administration of glycerol trivalerate or PBS to mice (mice receiving PBS: n=5; mice receiving glycerol trivalerate: n=5). (*, p<0.05; **, p<0.01) (centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points). PBS: phosphate-buffered saline; VA: vessel A; VA: vessel B. Adapted from (McDonald *et al.*, 2018a).

Figure 4.14:


A
B
Valerate
Conc (mM)
Day
Run 1 VA
Run 1 VB
Run 2 VA
Run 2 VB
Run 3 VA
Run 3 VB
C
5-aminovalerate
Conc (mM)
Day
D
Cefoperazone
No antibiotics
Day: -7
-2
0
1
2
3
4
C. difficile spores
Glycerol trivalerate or PBS
E
CFU per g faeces
Day post-infection
PBS
Glycerol trivalerate

4.4.3.2. Short chain fatty acid concentrations within faecal microbiota transplant material:

The protocol used for the generation of FMT appears to have very marked effects on the SCFA concentration (including valerate) within the final preparation (**Section 4.4.3.2**). Lyophilisation was associated with a particularly marked increase in SCFA concentrations. Consistent with a recent study (Seekatz *et al.*, 2018), concentrations of all assayed SCFAs in this study were reduced in conventional FMT slurry and sterile faecal filtrate as compared to crude stool.

Further recent work has also explored the impact of FMT preparation protocol upon gut microbiota composition and functionality within FMT. Using propidium monoazide (PMA), researchers demonstrated that ~50% of the bacteria within stool processed rapidly within strictly anaerobic conditions into fresh FMT were non-viable, and this figure rose to ~80% after a freeze-thaw cycle (Papanicolas *et al.*, 2019). Stool processing within ambient air resulted in up to 12-fold reductions in the abundance of a number of commensal bacteria, including *Faecalibacterium prausnitzii*; *F. prausnitzii* is particularly of interest since it is both a BSH- and SCFA-producer, and was identified as being enriched in the stool microbiota post-FMT in this study (**Section 3.3.2.4**). Papanicolas and colleagues also observed that faecal slurries produced in ambient air demonstrated reduced butyrate and acetate levels compared to those prepared anaerobically. However, it is important to note that - at least in the context of CDI – there is currently no evidence that anaerobic preparation of FMT results in an improved efficacy compared to preparation in anaerobic air (Mullish *et al.*, 2018d, 2018c). Furthermore, it is also feasible that spore-forming bacteria within donor stool (which will survive faecal manipulation in ambient air, and only germinate in the appropriate environmental *milieu* found within the GI tract) are potential contributors to the efficacy of FMT (Quraishi, McNally & van Schaik, 2019).

A conclusion from these results is that FMT preparation protocols should attempt stool manipulation in as physiological environment as is possible, with consideration of temperature, exposure to oxygen, and other variables that are recognised to impact SCFA metabolism. However, in clinical practice, there may be a 'trade-off' between what would be the ideal situation regarding a preparation process theoretically, and what is practically

achievable. For instance, even if there were a strong scientific case for anaerobic preparation of FMT, the very nature of stool collection from donors and FMT administration means that it is difficult to eliminate any exposure of faecal material to oxygen prior to patient administration. Regarding specific protocols for FMT preparation, lyophilisation appears of interest because of its association with increased SCFA concentrations, and further research should focus upon the impact of lyophilisation upon other microbiota components of interest (including other classes of metabolites, bile-metabolising enzymes, etc).

4.4.4. Summary:

Experiments within this chapter demonstrate that production of SCFA (and likely BCFA) is also restored within the colon by successful FMT. Most of interest is the restoration of valerate, given that this appears to have profound inhibitory effects against the vegetative growth of *C. difficile* but no adverse actions against other gut commensal bacteria. Particular SCFA and BCFA are also restored in serum by FMT; this is of noteworthy because of the growing recognition of the systemic effects that these fatty acids may exert.

The experiments described within this chapter also provide further supportive evidence that loss of bile-metabolising functionality of the gut microbiota is a key contributory factor to the establishment of CDI, influences whether recurrence occurs or not after treatment of primary CDI, and is reversed in rCDI through successful FMT. Perturbations of gut microbiota-bile acid metabolism interactions in rCDI are also associated with changes in FXR signalling, which may further exacerbate the condition (e.g. through promoting increased hepatic synthesis of TCA). However, the data presented within this chapter are observational, and do not evaluate the specific direct interaction between BSH and/or 7-α-dehydroxylase, bile acids, and *C. difficile*. As such, the next chapter provides further *in vitro* and *in vivo* experiments to explore this in greater detail.

5. Mechanistic studies into the impact of bile salt hydrolase activity upon the germination of *Clostridioides difficile in vitro* and *in vivo:*

5.1. Introduction:

The analysis of human samples had collectively demonstrated data that gut microbiota bile-metabolising functionality is markedly reduced in patients with CDI, and is restored to and maintained at pre-morbid levels/ levels comparable to healthy donors after FMT. However, these are observational data, and no samples were analysed from patients with FMT failure. As such, these results do not intrinsically distinguish restoration of gut microbiota bile-metabolising functionality as being a direct mechanism of the efficacy of FMT from being an incidental change associated with it. For this reason, mechanistic/ CDI modelling studies were performed both *in vitro* and *in vivo*, to directly explore the interaction between bile-metabolising enzymes (particularly BSH) and *C. difficile.*

5.2. Methods:

A general description of the methodology used for batch cultures, the mouse model and BSH purification is described within the **Methods, Section 2.6-2.8**. Specific details of the methodology for each of these experiments is described in the relevant section below.

5.3. Results:

5.3.1. *Clostridioides difficile* batch cultures:

5.3.1.1. Introduction:

These experiments involved preparation of spent culture supernatants by first incubating bacteria of interest in broth with 1% w/v TCA; these were different BSH-producing bacteria that had been seen to enrich in relative abundance within the stool microbiota post-FMT compared to pre-FMT in the initial dataset (**Figure 3.6**), and collectively represent all major BSH groups. After overnight incubation, cultures were centrifuged and filter-sterilised. *C. difficile* spores were incubated in sBHI broth supplemented with the spent culture supernatant. Using this setup, it was expected that *C. difficile* spores incubated with spent culture supernatants without BSH activity would have TCA available to stimulate germination and therefore grow, while *C. difficile* spores incubated with spent culture supernatants with BSH activity would not have TCA available to stimulate germination and therefore would not

grow. In addition, consideration was given to batch cultures including bacteria with 7-α-dehydroxylase activity, since the ability of such bacteria to convert primary bile acids to secondary bile acids (including DCA and LCA) would be expected to reduce the vegetative growth of *C. difficile.*

The primary endpoint of these experiments was spectrophotometric measurement of each batch culture within a microplate reader, to establish OD_{600}. This is an endpoint well-established from comparable studies; an increased OD_{600} reading after overnight incubation was interpreted as indicating that *C. difficile* spores had undergone germination and had grown as vegetative cells (Sorg & Sonenshein, 2008).

C. difficile spores in sBHI supplemented with 1% TCA was used as positive control in all cases; all statistical testing of results was performed relative to this sample for the particular ribotype under assessment. 1% TCA was chosen, since this has been the concentration used as a positive control in other comparable batch culture studies (Sorg & Sonenshein, 2008).

As described in the **Methods** chapter (**Section 2.6.2**), all batch cultures were repeated for three different strains of *C. difficile,* i.e. DS1684 (ribotype 010), CD630 (ribotype 012) and R20291 (ribotype 027). *C. difficile* strain DS1684 (ribotype 010) is a non-toxigenic strain of *C. difficile,* whilst the two other strains used are toxin-producing. In particular, ribotype 027 is a recognised cause of hospital outbreaks of CDI, being previously responsible for a number of CDI epidemics in Europe and remaining prevalent in North America; (Wilcox *et al.*, 2012; Waslawski *et al.*, 2013; Davies *et al.*, 2014) (**Section 1.5**); ribotype 012 was formerly one of the more common *C. difficile* ribotypes found in mainland China (Huang *et al.*, 2009), although is now of decreasing prevalence within this region. It was considered that demonstration of comparable results for all three of these ribotypes would suggest at least a degree of generalisability of results. Furthermore, given that two of these ribotypes are well-established as being able to cause severe CDI disease, they are of clear clinical relevance to include in experiments.

5.3.1.2. Batch cultures using naturally BSH-producing organisms:

For initial experiments, *C. difficile* spores were incubated with spent supernatant from BSH-expressing microorganisms that had been incubated with TCA. The microorganisms selected were those which had been shown to be reduced in mean proportion in the gut microbiota of pre-FMT patients in comparison to donors and/or post-FMT samples in the analysis of the initial human dataset (**Section 3.3.2.4**), and which collectively represented most BSH groups (**Appendix 1**). Specifically, these bacteria were:

Bacteroides ovatus – from BSH group 1B.
Collinsella aerofaciens – from BSH group 2.
Bacteroides vulgatus – from BSH group 3C.
Blautia obeum – from BSH group 3C.

Two microorganisms were picked from BSH group 3C, given both that this is a large group, and also given that a marked proportion of the bacteria significantly reduced in mean abundance in pre-FMT samples in comparison to post-FMT/ donors were from this BSH group.

Results for these batch culture experiments are displayed in **Figure 5.1**. For all *C. difficile* ribotypes assayed, supernatant from the broth of each of the BSH-producing microbes assayed significantly reduced *C. difficile* germination ($p<0.0001$, ANOVA with multiple group comparison, Benjamini-Hochberg FDR correction; **Figure 5.1**).

As an additional control for these experiments, spent supernatant was used from TCA-supplemented broth in which vegetative *C. difficile* had been cultured (strain DS1864); this was used to assess whether metabolites produced by *C. difficile* in culture may limit its own germination and/or vegetative growth. Results from this batch culture were that *C. difficile* failed to affect its own germination (**Figure 5.1**).

A potential alternative route for assessing whether it was the BSH of these bacteria specifically that was responsible for the suppressed germination observed (as opposed to, for instance, other bacterially-derived metabolites or anti-microbial peptides) was to attempt to use a specific BSH inhibitor. A number of BSH inhibitors have been derived from experiments

using pure strains/ purified enzyme from a range BSH-producing bacteria, including agents known to disrupt thiol group stability (including copper sulphate and copper chloride), antibiotics (including oxytetracycline and doxycycline) and a range of other agents (including riboflavin and caffeic acid phenethyl ester) (Smith *et al.*, 2014; Wang *et al.*, 2012; Tanaka *et al.*, 2000; Dong & Lee, 2018). Preliminary batch culture experiments were performed using a range of the most potent BSH inhibitors, but a number of issues made them unsuitable for further use, including toxicity against bacteria within the batch cultures (copper salts) and poor solubility in media (riboflavin). As such, an alternative approach was applied to assess the specific action of BSH within the batch cultures.

Figure 5.1:

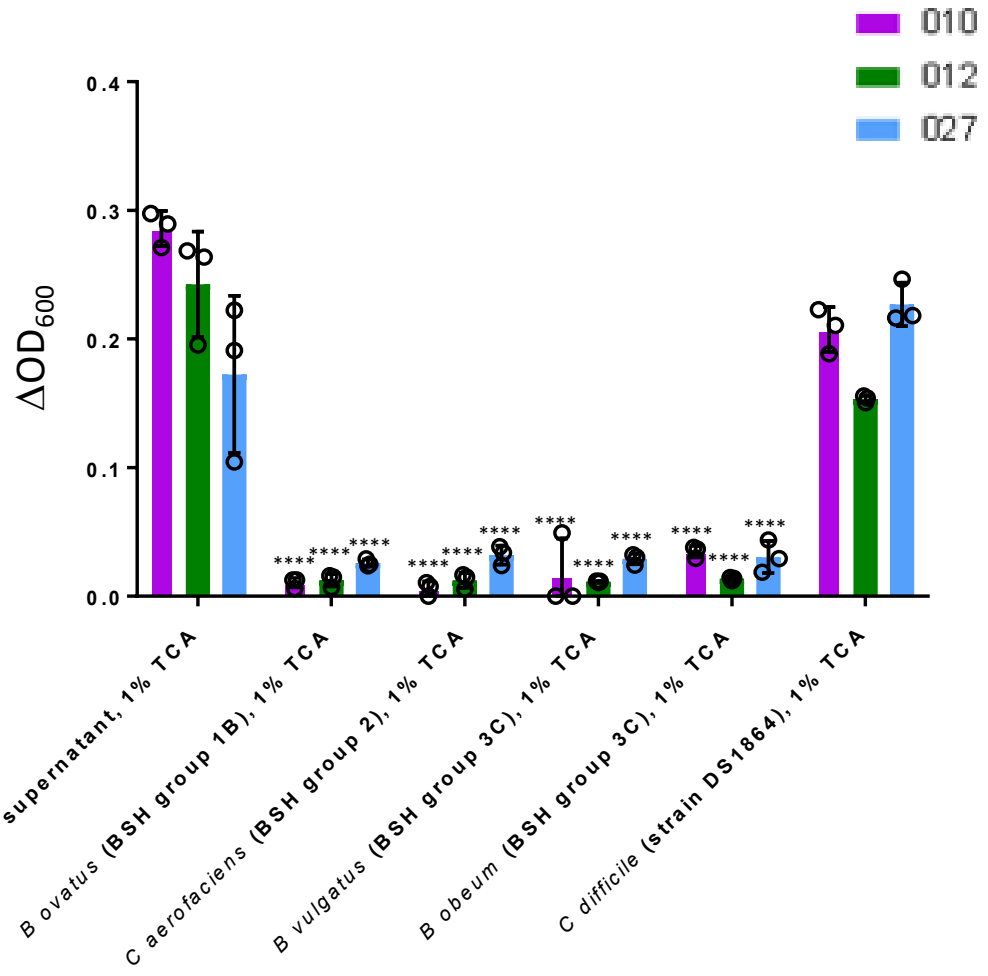


Figure 5.1 (page 163): *Clostridioides difficile* batch cultures – naturally BSH-producing organisms. Changes in spectrophotometer reading (ΔOD_{600}) after overnight incubation of *C. difficile* spores (three ribotypes assayed: 010, 012, 027; each experiment run in triplicate) in sBHI +/- TCA in which bacterial species of interest had been cultured for 24 hours. *C. difficile* spores in sBHI supplemented with 1% TCA ('No supernatant, 1% TCA') was used as positive control in all cases; statistical testing shown was performed relative to this sample for the particular ribotype under assessment. Results are shown for batch cultures of naturally BSH-producing microbial species found to be affected by FMT in metataxonomic analysis, and vegetative *C. difficile* as control (****, p<0.0001; ANOVA with multiple group comparisons, Benjamini-Hochberg FDR correction). Key: 010 is a non-toxigenic *C. difficile* ribotype, whilst 012 and 027 are both toxigenic ribotypes. Each experiment was performed in triplicate, and for each of the three different *C. difficile* ribotypes. Data presented as mean with standard deviation. Adapted from (Mullish *et al.*, 2019).

5.3.1.3. Batch cultures using engineered *Escherichia coli (+/- bsh)*:

It was next assessed whether BSH alone was sufficient to mediate inhibition of *C. difficile* germination, or if if this reflected an alternative aspect of bacterial metabolism within the cultures. For the second set of batch culture experiments, the bacterial strains of interest included wild-type *E. coli* (which lacks a *bsh* gene), as well as two forms of *E. coli* into which *bsh* genes had been cloned to be constitutively expressed (i.e. '*E. coli* BSH_{low}', containing a *bsh* gene with narrow substrate range/ moderate activity against conjugated bile acids; and '*E. coli* BSH_{high}', containing a *bsh* gene with high glycine and taurine-deconjugating activity) (Joyce *et al.*, 2014) (see **Figure 2.1**). Given that these bacterial species differed only in their *bsh* gene, results from these batch cultures could be used to directly assess the impact of BSH-mediated hydrolysis of TCA upon *C. difficile.* For these batch cultures, *C. difficile* spores in sBHI with no added TCA was used as a negative control.

Results from these batch cultures are summarised in **Figure 5.2**. While spent supernatant from the culture of wild-type/ BSH-negative *E. coli* did not affect the ability of *C. difficile* to undergo germination, supernatant from both forms of *bsh* gene-expressing *E. coli* significantly reduced *C. difficile* germination across all ribotypes tested (p<0.0001, ANOVA with multiple group comparison, Benjamini-Hochberg FDR correction) (**Figure 5.2**). *C. difficile* germination was significantly lower for all three ribotypes when incubated in TCA-supplemented supernatant from an *E. coli* BSH_{high} batch culture as compared to *E. coli* BSH_{low} (p<0.05; **Figure 5.2**). These results imply that the higher the level of BSH activity present within a batch

culture, the greater the degree of TCA hydrolysis, and the lower the trigger for germination of *C. difficile.*

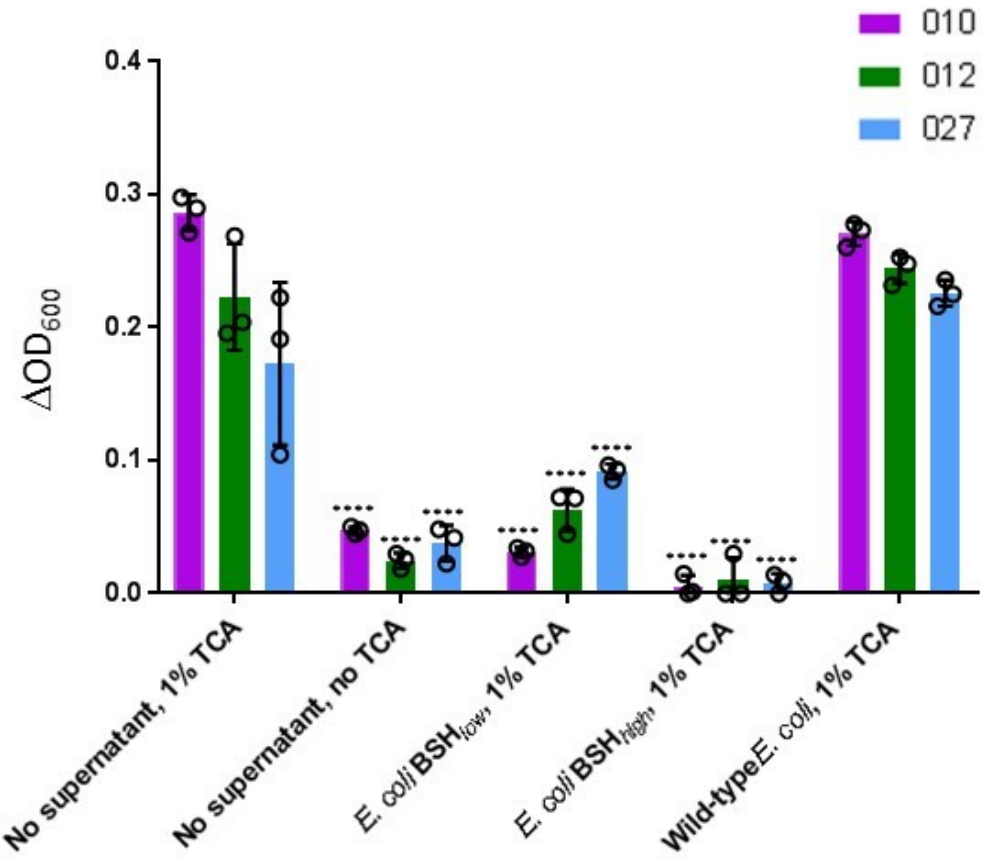


Figure 5.2: *Clostridioides difficile* batch cultures – *E. coli* +/- *bsh* genes. Changes in spectrophotometer reading (ΔOD_{600}) after overnight incubation of *C. difficile* spores (three ribotypes assayed: 010, 012, 027; each experiment run in triplicate) in sBHI +/- TCA in which bacterial species of interest had been cultured for 24 hours. *C. difficile* spores in sBHI supplemented with 1% TCA ('No supernatant, 1% TCA') was used as positive control in all cases; statistical testing shown was performed relative to this sample for the particular ribotype under assessment. Results shown here are from batch cultures of native *E. coli* (which lacks a *bsh* gene), and two forms of *E. coli* into which *bsh* genes had been cloned *(E. coli* BSH_{low} *= E. coli* expressing BSH with low deconjugation ability; *E. coli* BSH_{high} *= E. coli* expressing BSH with high deconjugation activity). (****, $p<0.0001$; ANOVA with multiple group comparisons, Benjamini-Hochberg FDR correction). Key: 010 is a non-toxigenic *C. difficile* ribotype, whilst 012 and 027 are both toxigenic ribotypes. Each experiment was performed in triplicate, and for each of the three different *C. difficile* ribotypes. Data presented as mean with standard deviation. Adapted from (Mullish *et al.*, 2019).

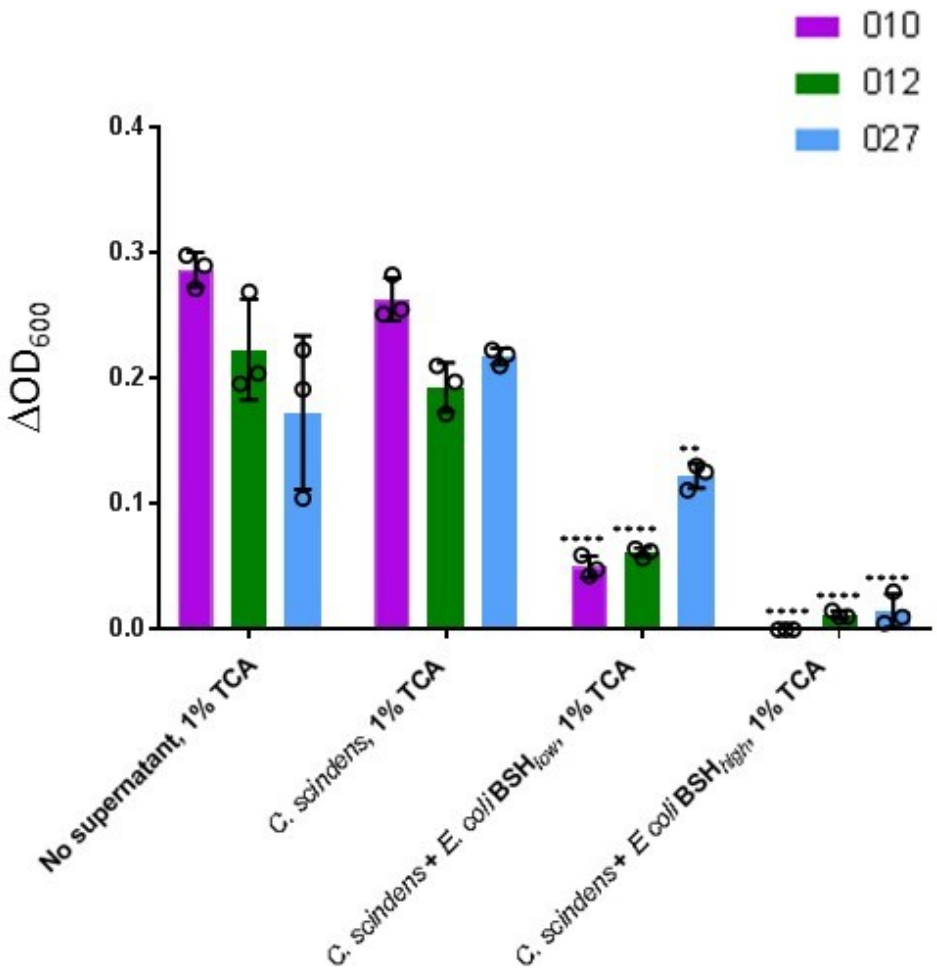
5.3.1.4. Batch cultures using 7-α-dehydroxylase +/- BSH:

None of the bacterial species of interest used in the first two sets of batch cultures were annotated as having the ability to convert primary to secondary bile acids (i.e. no annotated 7-α-dehydroxylase gene). However, it was also clearly important to directly assess the impact of 7-α-dehydroxylase functionality (either with or without the co-presence of BSH) in a batch culture setting, given the marked effect of secondary bile acids (and particularly DCA) in the inhibition of the vegetative growth of *C. difficile.* As such, a further set of batch cultures were performed to investigate this.

C. difficile spores were cultured in spent supernatant from *Clostridium scindens* (i.e. the archetypal 7-α-dehydroxylase-producing bacterium); *C. scindens* had been incubated with TCA, either by itself or also in co-culture with BSH-expressing *E. coli.* Results from these batch cultures are shown in **Figure 5.3**. *C. scindens* spent supernatant did not affect *C. difficile* germination ability by itself, but germination was significantly reduced when *C. scindens* was co-incubated with BSH-expressing *E. coli* (p<0.01, ANOVA with multiple group comparisons, Benjamini-Hochberg FDR correction).

Figure 5.3 (page 167): *Clostridioides difficile* batch cultures – *C. scindens* +/- engineered *E. coli*. Changes in spectrophotometer reading (ΔOD_{600}) after overnight incubation of *C. difficile* spores (three ribotypes assayed: 010, 012, 027; each experiment run in triplicate) in sBHI +/- TCA in which bacterial species of interest had been cultured for 24 hours. *C. difficile* spores in sBHI supplemented with 1% TCA ('No supernatant, 1% TCA') was used as positive control in all cases; statistical testing shown was performed relative to this sample for the particular ribotype under assessment. Results shown here are from batch cultures of *C. scindens* +/- BSH-expressing *E. coli* (*E. coli* BSH_{low} = *E. coli* expressing BSH with low deconjugation ability; *E. coli* BSH_{high} = *E. coli* expressing BSH with high deconjugation activity). (**, p<0.01; ****, p<0.0001; ANOVA with multiple group comparisons, Benjamini-Hochberg FDR correction). Key: 010 is a non-toxigenic *C. difficile* ribotype, whilst 012 and 027 are both toxigenic ribotypes. Each experiment was performed in triplicate, and for each of the three different *C. difficile* ribotypes. Data presented as mean with standard deviation. Adapted from (Mullish *et al.*, 2019).

Figure 5.3:



5.3.1.5. Further analysis of batch cultures:

For the first two sets of batch culture experiments (i.e. those using naturally BSH-producing bacteria, and those using engineered BSH-expressing *E. coli*), it was presumed that BSH had been active during overnight incubation and degraded TCA within the media into cholic acid. To seek a more direct demonstration of this, two different further sets of experiments were performed.

Firstly, UPLC-MS for bile acid profiling was performed on leftover media from the end of selected batch culture experiments. Taurocholic acid: cholic acid (TCA: CA) ratios were measured, with high ratios interpreted as low/ absent BSH activity. Results from these experiments are displayed in **Figure 5.4.** As predicted, all batch cultures including naturally BSH-producing bacteria and those using BSH-producing *E. coli* demonstrated low TCA: CA

ratios (p<0.0001 for all these batch cultures *vs* negative control, ANOVA with multiple groups comparison, Benjamini-Hochberg FDR correction). All batch culture experiments in which *C. difficile* germination was suppressed were characterised by undetectable levels of DCA within supernatant (other than batch cultures containing *C. scindens* and *bsh*-expressing *E. coli,* where DCA was detectable). As such, these experiments provide further supportive evidence that the direct hydrolysis of TCA (rather than the formation of secondary bile acids) was the key mechanism underlying suppression of *C. difficile* germination in these batch cultures.

Figure 5.4:

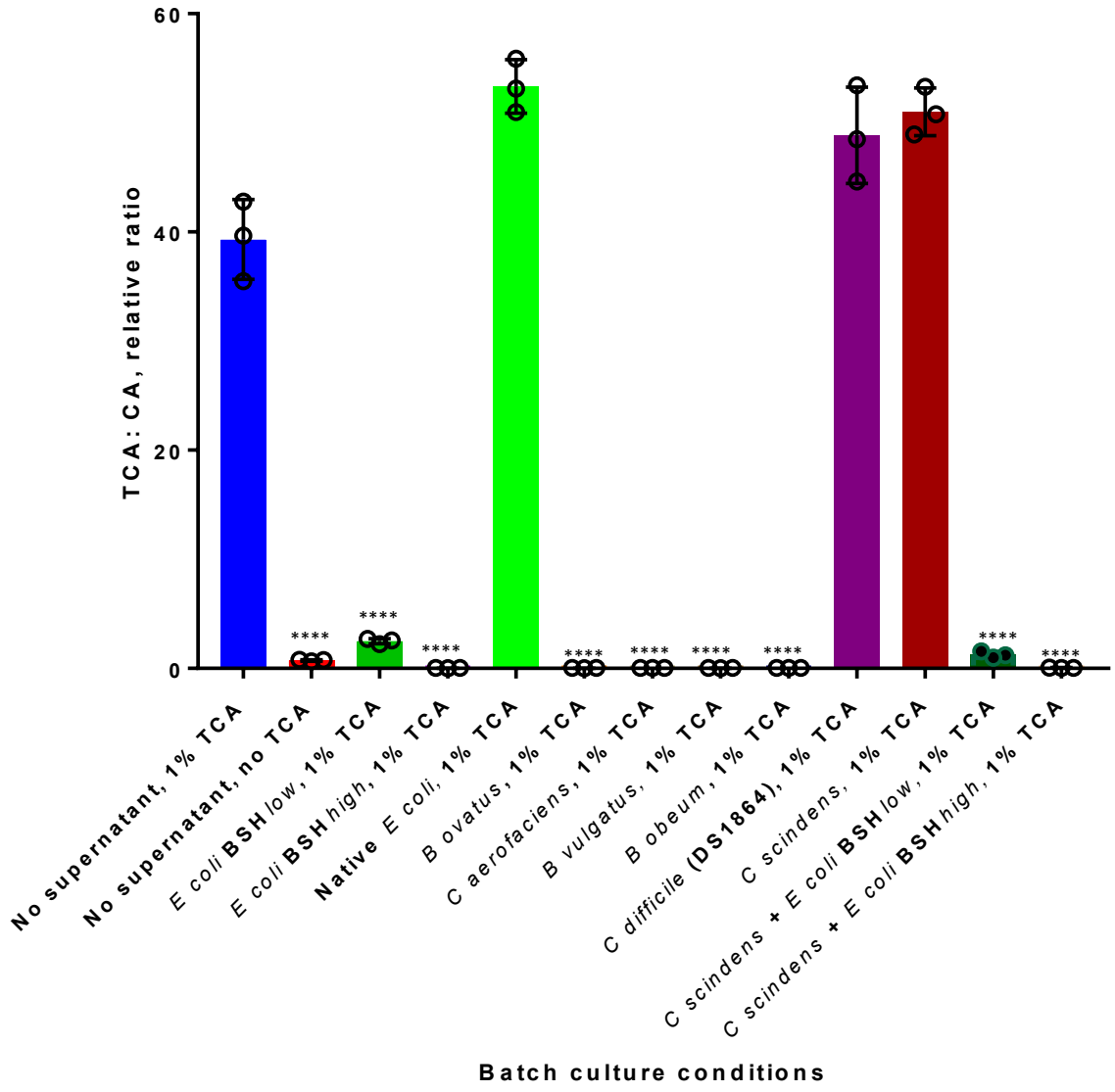


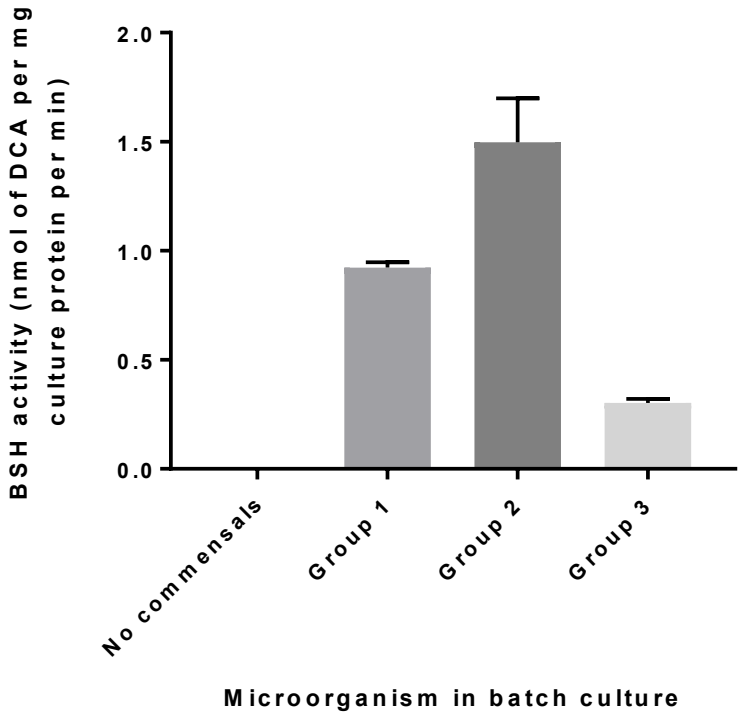
Figure 5.4 (page 168): Bile acid profiles within media at the end of *C. difficile* batch culture experiments. As established via UPLC-MS of media. Taurocholic acid: cholic acid (TCA: CA) ratios were calculated at the end of batch culture experiments (each experiment run in triplicate); high ratios are consistent with low/ absent BSH activity, and low ratios are consistent with high BSH activity. *C. difficile* spores in sBHI supplemented with 1% TCA ('No supernatant, 1% TCA') was used as positive control in all cases. Statistical testing shown was performed in all cases relative to 'No supernatant, 1% TCA' (****, p<0.0001, ANOVA with multiple groups comparison, Benjamini-Hochberg FDR correction). Each experiment was performed in triplicate. Data presented as mean with standard deviation. Adapted from (Mullish *et al.*, 2019).

Secondly, investigation of the actual BSH activity within the filtered spent supernatant was performed for selected batch cultures. Whilst centrifugation followed by filtering of the batch culture would be expected to remove vegetative cells, it was hypothesised that there would still be significant BSH activity present (with the BSH present arising from bacterial secretion and/or lysis of bacteria). Results of these experiments are potentially of clinical relevance, since sterile faecal filtrate has been demonstrated to have comparable efficacy to conventional FMT in the treatment of rCDI, suggestive of a soluble factor mediating the efficacy of FMT (Ott *et al.*, 2017). As such, given that BSH is a potential candidate for being one such mediator, the effect of filtration upon BSH activity within solution is evidently of key interest.

Results from these experiments are demonstrated in **Figure 5.5**. BSH activity was measured on batch cultures involving selected naturally-BSH producing bacteria from group 1 *(Bacteroides ovatus*), group 2 (*Collinsella aerofaciens*) and group 3 (*Bacteroides vulgatus*). In all cases, marked BSH activity was detected, at levels comparable to that found in healthy/ post-FMT human stool samples.

Figure 5.5 (page 170): BSH activity in *C. difficile* batch cultures. As assessed by performance of BSH activity assay on sterile filtered spent supernatant from batch cultures containing microorganisms from different BSH groups (each experiment run in triplicate). Group 1: batch culture from *Bacteroides ovatus;* Group 2: batch culture from *Collinsella aerofaciens;* Group 3: batch culture from *Bacteroides vulgatus.* Each experiment was performed in triplicate. Data presented as mean with standard deviation. Adapted from (Mullish *et al.*, 2019).

Figure 5.5:



In summary, cultures containing bacteria commonly found in the stool of healthy people/ post-FMT – and with BSH activity of comparable levels to these patients - were able to fully-suppress *C. difficile* germination through the rapid hydrolysis of TCA. The close comparability of the BSH activity found in the stool of healthy/ post-FMT patients and that of batch cultures here would support the assertion that this mechanism may also be important for the suppression of germination of *C. difficile in vivo.*

5.3.2. Mouse model of recurrent *Clostridioides difficile* infection:

5.3.2.1. Introduction and protocol:

Whilst these batch culture results demonstrate that BSH-mediated hydrolysis of TCA is sufficient to fully-suppress *C. difficile* germination *in vitro*, they are evidently a much-simplified model of CDI. For a more representative model with greater comparability to human CDI disease, an established mouse model of rCDI/ FMT was adapted (Seekatz *et al.*, 2015). Specifically - after completion of a course of vancomycin, instead of being administered conventional FMT, mice were either orally administered wild-type/ BSH-negative *E. coli* or *E. coli* BSH_{high}. Since the two arms of mice within this study were treated identically in all regards apart from whether or not they had been administered BSH, this

experiment aided demonstration of the specific interaction between BSH action and *C. difficile in vivo.*

A summary of the protocol used in these studies for the mouse model for rCDI/ FMT is demonstrated in **Figure 5.6.**

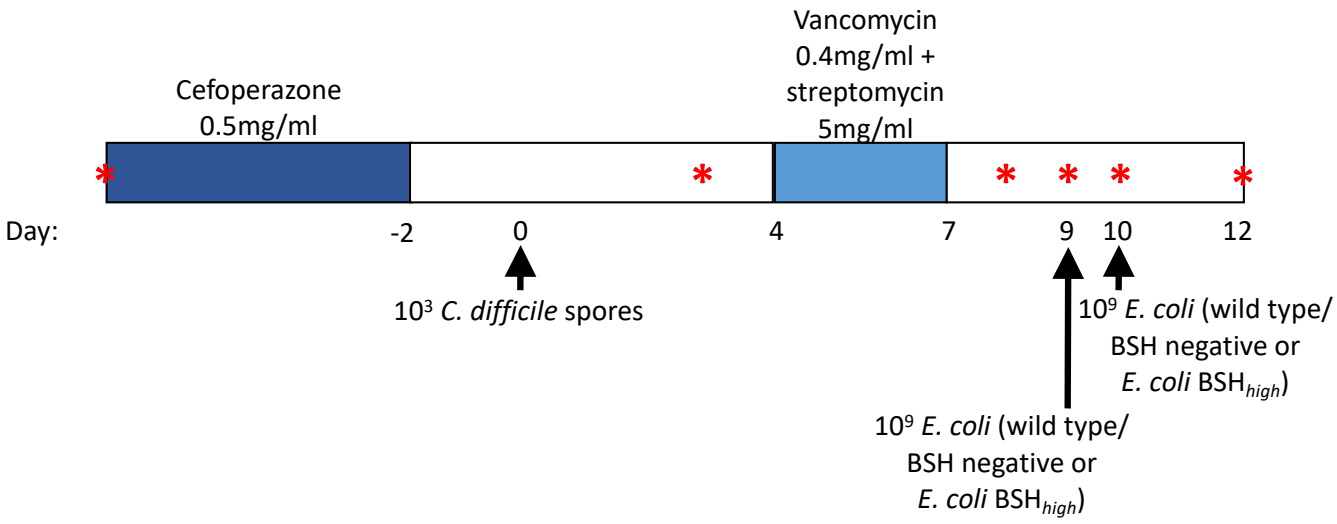


Figure 5.6: Protocol/ timeline used for the mouse model of recurrent *Clostridioides difficile* infection. This protocol was adapted from a previously-described model (see main text) (Seekatz *et al.*, 2015). *: Time points at which stool samples were collected for *C. difficile* +/- *E. coli* counts. As reported in (Mullish *et al.*, 2019).

5.3.2.2. Mouse model results:

5.3.2.2.1. Confirmation of validity of model:

C. difficile was not detectable in the stool of any of the mice in either arm at the start of the experiment. However, colonisation of all mice with *C. difficile* was confirmed at three days post-*C. difficile* spore administration (~10^3 spores per mouse via oral gavage) via analysis of plate counts for total viable counts (TVCs) in stool (**Figure 5.7**). At this time point, no significant difference in stool *C. difficile* CFU/g count was detectable between both arms of mice (p>0.05 on day 3, Mann-Whitney U test, **Figure 5.7B**). By the time of completion of vancomycin, *C. difficile* was at almost negligible CFU/g in the stool of all mice (p>0.05 on day 8, Mann-Whitney U test, **Figure 5.7B).**

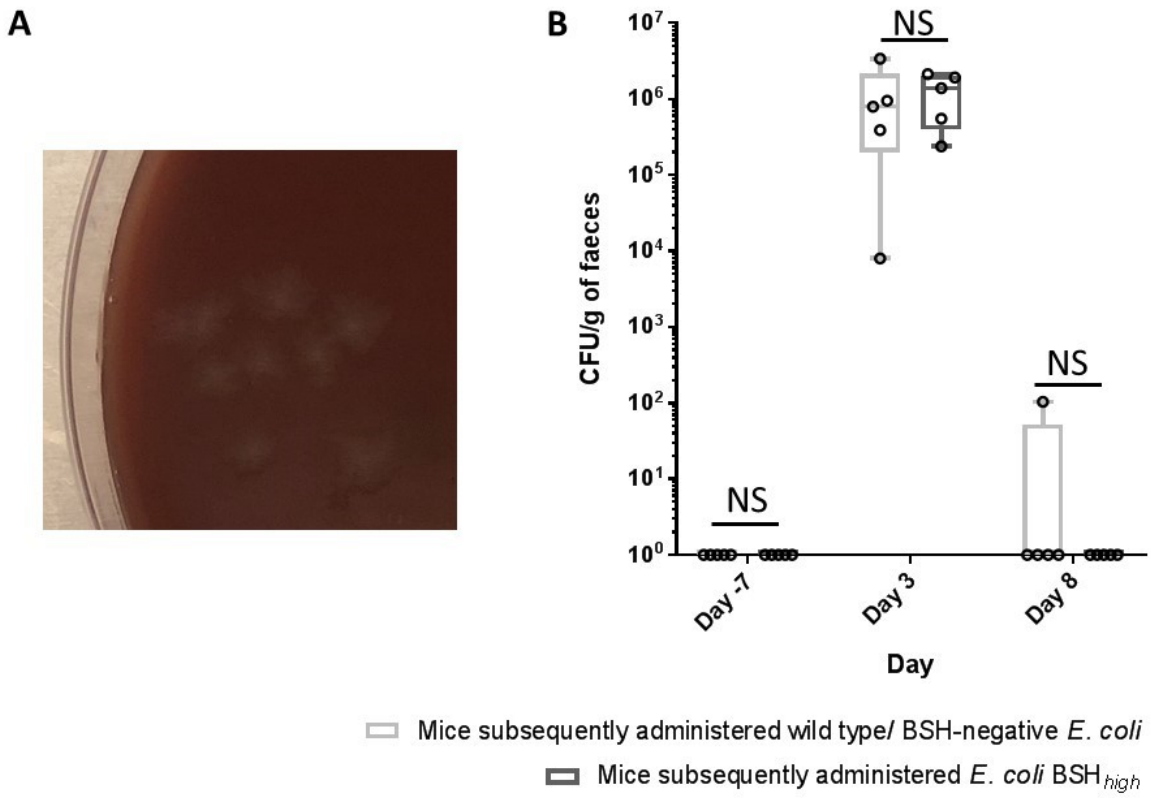


Figure 5.7: Stool *C. difficile* counts during mouse modelling experiments. A: Typical morphology for *C. difficile* CFUs seen on mCCEY (moxifloxacin-Braziers Cycloserine Cefotixin Egg Yolk) agar plates; B: Stool *C. difficile* titres (expressed as CFU/g of faeces) at three and eight days following exposure to *C. difficile* spores, as established from serial dilutions of faecal supernatant and plate counts ($p > 0.05$, Mann-Whitney U test). (Mice subsequently administered wild-type/ BSH-negative *E. coli*: $n=5$; mice subsequently administered BSH-positive *E. coli*: $n=5$). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019)

Quantification of administered *E. coli* was performed via plate counts on selective media (MacConkey agar plates supplemented with rifampicin 50μg/ml; the *E. coli* administered to mice in both arms possessed a rifampicin resistance gene (Joyce *et al.*, 2014)) (**Figure 5.8A**). Rifampicin-resistant *E. coli* was not detected in the stool of any mice prior to commencement of the experiment. Plate dilutions were performed on residual suspended *E. coli* inoculum used for oral gavage to mice for confirmation of the titre of *E. coli* administered to them; this verified that the *E. coli* titres administered to both arms of mice were comparable for both rounds of oral gavage ($p > 0.05$, Mann-Whitney U test**; Figure 5.8B**).

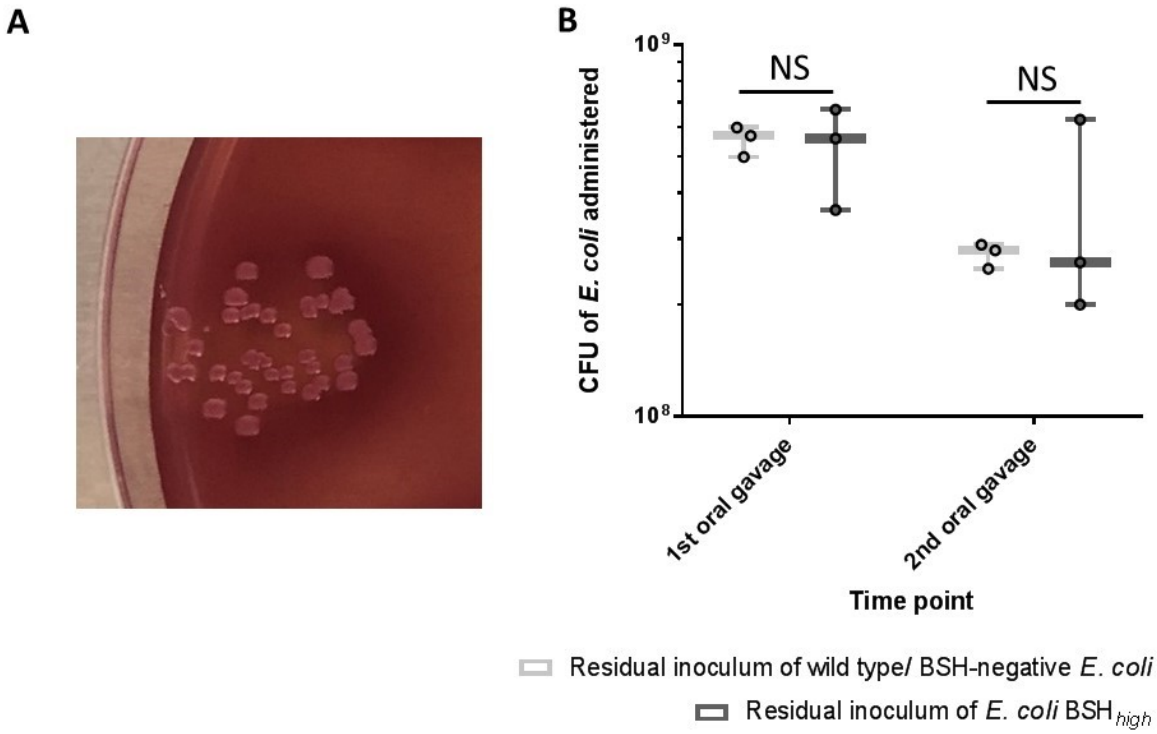


Figure 5.8: Counting administered *E. coli* during mouse modelling experiments. A: Typical morphology for *E. coli* CFUs seen on MacConkey agar plates supplemented with rifampicin 50μg/ml; B: *E. coli* titres (expressed as CFU) gavaged to mice at both time point, as established from plate counts of residual suspended *E. coli* inoculum used for oral gavage to mice (p> 0.05, Mann-Whitney U test). All plating performed in triplicate. (Mice administered wild-type/ BSH-negative *E. coli*: n=5; mice administered BSH-positive *E. coli*: n=5). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019).

5.3.2.2.2. Effect of BSH upon rCDI mouse model:

On day 12 of the protocol (i.e. three days after initial *E. coli* administration), *E. coli* colonisation was at comparable, high levels in both groups of mice (mean CFU per gram of faeces of 1.49 x 10^9 in mice administered *E. coli* BSH_{high} *vs* 1.18 x 10^9 in mice administered wild-type/ BSH-negative *E. coli*; p>0.05, Mann-Whitney U) (**Figure 5.9A**). As such, it was interpreted that this was a valid time point for the comparison of *C. difficile* TVCs between both arms of mice.

On day 12, total viable counts of *C. difficile* were significantly lower in the stool of mice who had been administered *E. coli* BSH_{high} *vs* mice administered wild-type/ BSH-negative *E. coli* (mean CFU per gram of faeces of 6.92 x 10^7 *vs* 2.70 x 10^8 respectively; p <0.05, Mann-Whitney U). This equated to an ~70% reduction in stool *C. difficile* total vegetative cell counts (**Figure 5.9B**).

Given that the treatment of mice in both arms was identical apart from whether they received exposure to *bsh* genes or not, these data were evidence that BSH alone is able to limit the recurrence of CDI in the mammalian host.

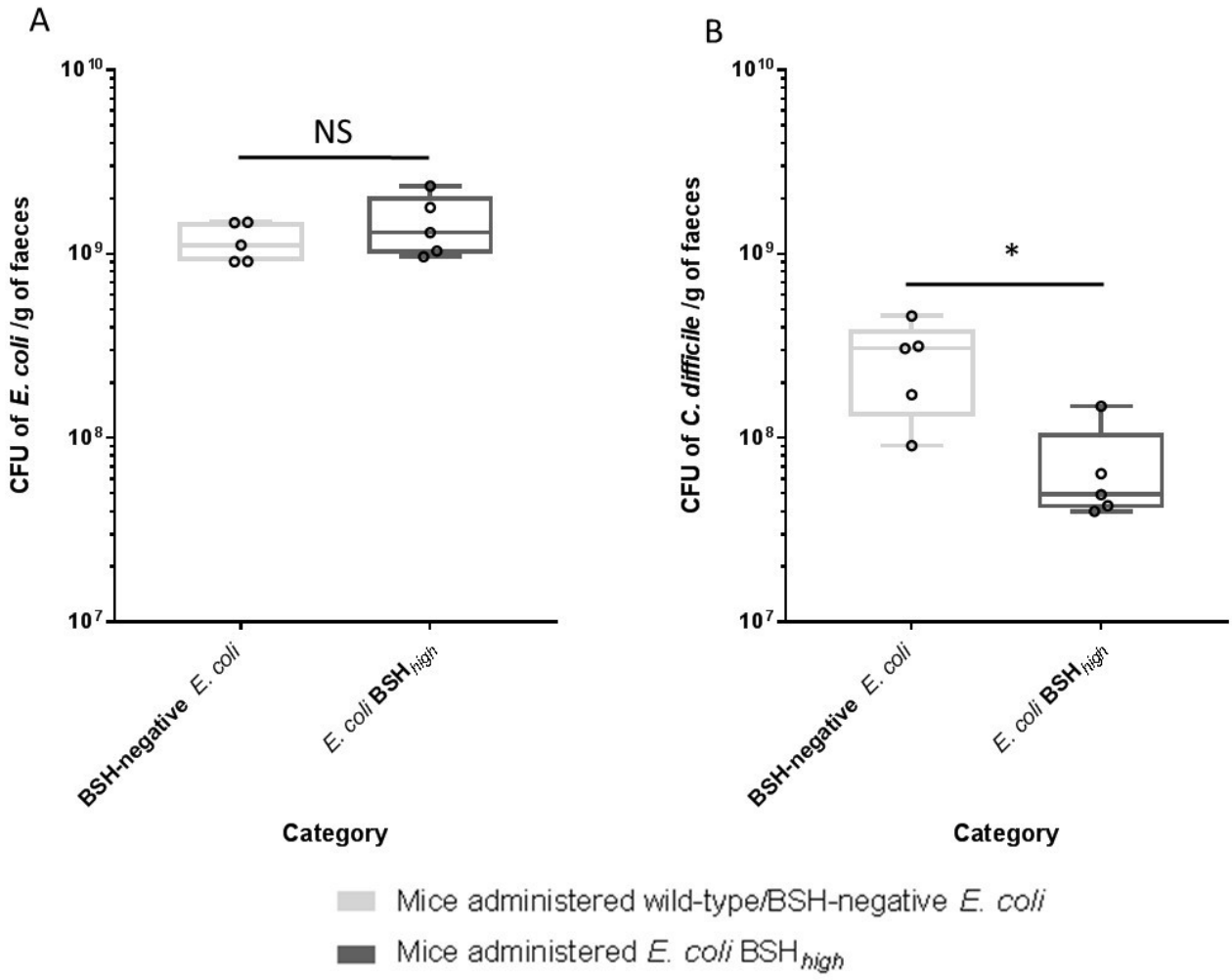


Figure 5.9: Impact of BSH upon a mouse model of rCDI. Assessed using a mouse model of rCDI/ FMT was used that was adapted from a previously-described model (Seekatz *et al.*, 2015). A: Counts of administered *E. coli*; B: total vegetative counts of *C. difficile* on day 12. CFU counts as established from plate counts of serial dilutions of faecal supernatant (*, p<0.05, Mann-Whitney U test). (Mice administered wild-type/ BSH-negative *E. coli*: n=5; mice administered BSH-positive *E. coli*: n=5). Centre of box: median; box hinges: 25 – 75% percentile; whiskers: minimum to maximum data points. Adapted from (Mullish *et al.*, 2019).

5.3.3. Purification of bile salt hydrolase:

5.3.3.1. Introduction:

From the above experiments, the potential utility of purified BSH enzyme with high activity was recognised. Firstly, it was recognised that purified BSH may be useful for a number of *in vitro* experiments that may not be possible with live bacteria (e.g. investigation of BSH inhibitors that are toxic to vegetative bacterial cells). Secondly, purified BSH would clearly lend itself well to use in further chemostat experiments and mouse experimentation (e.g. potential use of BSH as a primary preventative therapy against the development of CDI), particularly since it would enable the possibility of 'dose finding' experiments in a more robust way than using BSH-producing bacteria.

5.3.3.2. Expression and purification of BSH:

For expression and purification of BSH, the highly-active *bsh* gene from *Bifidobacterium adolescentis* that had been cloned into *E. coli* BSH_{high} was used. As described in the **Methods** chapter (**Section 2.8**), a pTrcHis2a_A085 plasmid containing the *bsh* gene of interest was used to transform *E. coli* BL21-AI™ OneShot Chemically Competent cells (Invitrogen, Thermo Fischer Scientific, UK). Induction of transformed cells was performed with L-arabinose and isopropyl-β-d-thiogalactopyranoside for 3 hours to allow protein expression. Purification of protein was performed from lysed cells using His slurry/ Ni^{2+}-NTA agarose column, before elution using buffer containing imidazole, with the aim of eluting the protein in native condition.

Aliquots of sample from all stages of the purification process were run on SDS-PAGE (**Figure 5.10A**). In the elution steps, the single clearest band (particularly noticeable in the final elutate) was at ~37kDa, which is the approximate molecular weight of a bile salt hydrolase monomer (the molecular weight of BSH monomers has been described as ranging from 28-50 kDa (Geng & Lin, 2016)). BSH *in vivo* is a tetramer (Rossocha *et al.*, 2005), and other notable bands were in regions consistent with the dimer, trimer and tetramer of the protein (**Figure 5.10A**). As described in **Section 2.8**, no anti-BSH antibody was available to use on Western blot to confirm the presence of the purified protein; however, the plasmid vector had a C-terminal *c-myc* epitope fused to the inserted *bsh* gene. As such, the presence of purified BSH was confirmed by Western blot using a mouse-derived primary antibody against c-myc, and

a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody. Results from the Western blot are demonstrated below (**Figure 5.10B**); bands were seen in all four elutates in the region of ~100kDa (i.e. the approximate molecular mass of c-myc), but were particularly prominent in elutates 2 and 3.

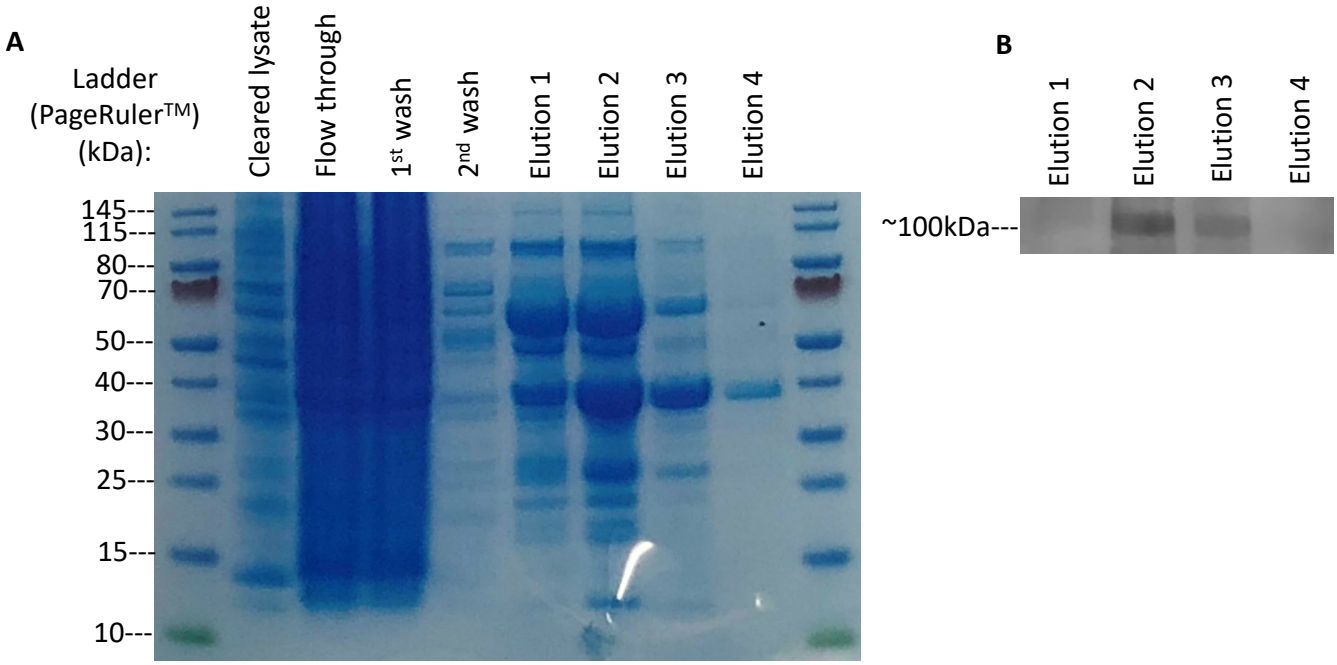


Figure 5.10: Expression and purification of BSH. A: SDS-PAGE analysis, with aliquots from all steps of protein purification. B: Western blot of purified BSH using mouse-derived anti-c-myc primary antibody, and HRP-conjugated anti-mouse secondary antibody.

5.3.3.3. Enzyme activity of purified BSH:

To assess for BSH functionality of the purified protein, a ninhydrin assay was performed (see **Section 2.8**). A ninhydrin assay was chosen rather than the previously-used precipitation-based BSH assay, since the elution buffer/ imidazole chosen for the protein elution had pH ~8.0, making it difficult to use in an experimental protocol where the pH by necessity must be <6.0 (Mullish *et al.*, 2018b). Enzyme activity of different elutates (as assessed by ninhydrin assay) are summarised in **Table 5.1.**

Table 5.1: Enzyme activity of purified BSH.

Category	Enzyme activity (μmol of taurine/ minute/ mg protein) (mean ± SD)
Elutate 1	0.56 ± 0.029
Elutate 2	1.62 ± 0.049
Elutate 3	7.122 ± 0.064
Elutate 4	13.35 ± 0.021

As assessed via ninhydrin assay.

It was noted that progressive elution steps were associated with fewer residual protein purification products, but higher BSH activity. This was suggestive that protein products seen in earlier elutates may be contaminants that inhibit the activity of BSH, but had been removed through successive purification cycles.

5.4. Discussion and analysis:

5.4.1. Overview:

Even though the data from the human datasets described in the last two chapters were compelling for the central significance of gut bile-metabolising functionality to CDI, there were evidently limits to their interpretability given their observational nature and the techniques used. For example, one obvious drawback with interpreting the rCDI/ FMT human sample data is (as is standard with most human studies of FMT and rCDI), the included patients were taking vancomycin at the time of collection of pre-FMT samples, meaning that it cannot definitively be concluded that the restored gut bile-metabolising functionality that was observed post-FMT represents transfer of BSH/ 7-α-dehydroxylase-producing organisms from the donor, rather than the recovery of species in the gut microbiota of recipients that were being suppressed by vancomycin. It was for this reason that batch cultures and mouse experiments were performed to allow assessment of the direct impact of these enzymes (and particularly BSH activity) upon *C. difficile* in the rCDI setting.

5.4.2. The role of primary bile acids and bile salt hydrolase in *Clostridioides difficile* infection:

The focus of the studies described in this chapter was regarding the potential role of BSH (rather than that of 7-α-dehydroxylase) in CDI. The human data already presented had shown

that restored BSH activity/ loss of TCA after FMT appeared to be rapid in onset and sustained for at least several months, which would be consistent with the hypothesis that this is a contributory mechanism to the efficacy of FMT. Recovery of gut BSH activity appeared to be a factor which prevented patients with primary CDI from recurring, while the potential contribution of 7-α-dehydroxylase was less clearly demonstrated (**Section 4.3.2**). Furthermore, BSH is the key 'gateway' step in metabolism of bile acids within the distal gut, without which other steps of microbiota-mediated bile acid metabolism cannot proceed.

As discussed in the previous chapter (**Sections 4.4.1-4.4.2**), it is also hypothesised that BSH-producing organisms aid gut microbiota colonisation, through their ability to degrade conjugated bile acids that are associated with bile toxicity to many bacterial taxa. Of note, there are likely also other advantages associated with the presence of BSH, including the ability to liberate amino acids which may be used as a nutrient source (Tanaka *et al.*, 2000) and/or the property of inducing changes in the structure of the bacterial lipid cell membrane to improve survival (Taranto *et al.*, 2003). There are also a number of different strands of evidence in the literature that suggest that BSH-producing bacteria may colonise the gut easily after FMT for rCDI, whilst 7-α-dehydroxylase-producing bacteria may not. For example, the bacterial families *Lachnospiraceae, Bacteroidaceae* and *Ruminococcaceae* have been shown to be those most likely to engraft and which are most closely associated with rCDI patients entering remission after FMT (Staley *et al.*, 2018); however, 7-α-dehydroxylase-producing organisms are predominantly in the *Lachnospiraceae* family only. Similarly, metagenomic mapping identified that *Clostridiales* appeared to be poor colonisers post-FMT, and that colonisation success was negatively correlated with the number of genes in the genome of transplanted bacteria related to sporulation (Lee *et al.*, 2017).

In patients with and animal models of CDI and rCDI, few vegetative cells of *C. difficile* remain in the gut after completion of vancomycin (Seekatz *et al.*, 2015). Intuitively, for further recurrence to occur, TCA-mediated germination of *C. difficile* is likely to be a key contributory factor, with germination occurring from residual spores residing within the distal gut (Seekatz *et al.*, 2015). As such, the key focus of experiments in this chapter was to assess the extent to which BSH-mediated hydrolysis of TCA limited the germination of *C. difficile.*

On performing batch cultures in TCA-rich media with a range of naturally BSH-producing bacterial species from across the range of BSH groups (all of which were of increased relative abundance within the gut microbiota after FMT in the metataxonomic data obtained here), *C. difficile* germination was almost completely inhibited. It was not possible from these experiments alone to deduce that BSH activity *per se* was responsible for the limited *C. difficile* germination observed (as opposed to, for instance, another metabolite produced by these bacteria, or production of an antimicrobial peptide). However, this was confirmed after batch cultures were repeated with different forms of engineered *E. coli* that differed only in the presence or absence of a *bsh* gene. Germination was not inhibited in the presence of *C. scindens* alone, but was when BSH-producing bacteria and *C. scindens* were added to the culture together. BSH was still highly active even in filtered spent supernatant. Batch culture results were very similar for three different *C. difficile* ribotypes (one non-toxigenic, two toxigenic), suggesting clinical relevance and applicability. However, there are some obvious limitations of batch cultures. Firstly, batch cultures only had TCA added, rather than the complex mixture of multiple bile acids and other metabolites observed in the distal gut *in vivo* (although previous similar studies *ex vivo* have shown comparable results (Theriot *et al.*, 2014; Weingarden *et al.*, 2016b)). Secondly, batch cultures do not include an aspect of FXR signalling, immune responses, or other host factors that may clearly be contributory to the life cycle of *C. difficile in vivo.*

As such, this was the impetus for going on to perform a rCDI mouse model. This mouse model was an adaptation of one which has previously been validated for recreating the dynamics of changes in *C. difficile* total viable counts as seen in humans with CDI treated with vancomycin who go on to experience recurrence of disease and are then treated with FMT (Seekatz *et al.*, 2015). Instead of administering mice conventional FMT, mice were administered either wild-type/ BSH-negative *E. coli* or BSH-expressing *E. coli* at titres comparable to that typically found in stool (Costello *et al.*, 2015), with the aim of directly evaluating the impact of BSH itself upon CDI. It was possible to demonstrate a significant reduction in stool *C. difficile* total viable count in mice treated with BSH-positive *E. coli* compared to those treated with BSH-negative *E. coli* (~70% reduction). However, the mouse experiments also had some limitations. Most notably, because of the ethics that had been granted for this project, a non-toxigenic form of *C. difficile* was used; as such, it was not possible to perform measures such as toxin titres,

histological assessment of distal gut, etc, to more robustly assess whether a reduced *C. difficile* total viable count also translated into true improvement in CDI-related colitis. Hamster models of CDI have certain advantages over mouse models (including lower resistance to infection with *C. difficile,* and a phenotype more representative of human disease in CDI) (Hutton *et al.*, 2014), so may be the preferred choice of model for potential further animal work involving toxigenic strains of the bacterium. A further limitation of this animal study was that this was a short-term mouse experiment only, designed to specifically assess the effect of BSH upon *C. difficile* germination *in vivo*; given the discussion above about BSH and bacterial fitness burden, it is also relevant to consider the longer-term effect of BSH supplementation upon gut microbiota composition, microbiota-host interactions etc, but that was not assessed here. In addition, since no measurement was made of the specific quantity or activity of BSH administered to mice, it is unclear whether this experiment resulted in restoration of BSH to pre-morbid levels, or if it reached supraphysiological levels. A possible way to more physiologically explore the contribution of BSH *in vivo* would be to administer CDI animals either a native BSH-producing bacterium, or the same bacterium but with a BSH knockout.

No prior mouse model experiments have been reported that have been designed to assess the role of BSH in the treatment of CDI. In contrast, there have been rodent studies that have demonstrated that 7-α-dehydroxylase-producing organisms (in particular, *Clostridium scindens*) partly protect the host against the development of CDI (Buffie *et al.*, 2014; Studer *et al.*, 2016). However, on closer evaluation, such studies also provide some insight as to a possible contribution of BSH as well. For instance, in the study by Buffie and colleagues, antibiotic-treated mice who were administered *C. scindens* before exposure to *C. difficile* spores experienced a small but significant reduction in *C. difficile* total viable counts compared to mice administered PBS instead of *C. scindens* (Buffie *et al.*, 2014). In contrast, mice administered a cocktail of four bacteria prior to exposure to *C. difficile* spores experienced a very marked reduction in *C. difficile* TVCs compared to control mice; this four-bacteria cocktail included *Barnesiella intestinihominis,* which is a known BSH-producing bacterium (**Appendix 1**). In the study by Studer and colleagues, germ-free mice were stably-colonised with a twelve-bacteria cocktail which included bacteria with annotated *bsh* genes (although none were common commensal human gut bacteria), and such mice had minimal

CDI resistance; however, further addition of *C. scindens* resulted in transient CDI resistance (Studer *et al.*, 2016). However, researchers also presented ileal bile acid profiles, which demonstrated that mice stably-colonised with the twelve-bacteria cocktail had significantly higher ileal TCA levels than those found even in antibiotic-treated wild-type mice, implying that there was only modest BSH functionality in the gut of the stably-colonised mice, and potentially explaining their vulnerability to CDI. Together, these studies infer that 7-α-dehydroxylation has an important contributory role to colonisation resistance, but that colonisation resistance is promoted further in the presence of gut BSH functionality.

Collectively, these experiments provide strong supportive evidence for restoration of gut BSH functionality – with associated hydrolysis of conjugated bile acids, including TCA - being a mediator of the efficacy of FMT. There are other intuitive reasons that would support restoration of BSH-producing organisms to be a key contributor to FMT's success in the treatment of rCDI. Firstly, as already alluded to, BSH has a relatively high prevalence in healthy stool, relative insensitivity to oxygen, and robust activity over a wide pH range (Thomas *et al.*, 1997; Begley, Hill & Gahan, 2006); in contrast, 7-α-dehydroxylase functionality exists only in very few bacteria, and the enzyme itself is highly oxygen-sensitive (Thomas *et al.*, 1997)). However unrefined the preparation process of the slurry is for FMT is (i.e. regardless of ambient air, diluent or route of administration used, etc), FMT remains highly-effective at treating rCDI. Of particular note, even sterile faecal filtrate - prepared in a commercial blender, and not in anaerobic conditions – appears to be of comparable efficacy to conventional FMT at treating rCDI (Ott *et al.*, 2017). It is more feasible that BSH (rather than 7-α-dehydroxylase) is able to reach the distal gut functionally intact after FMT (particularly in the scenario of sterile faecal filtrate, where no spores are present in the administered material).

5.4.3. The role of secondary bile acids and 7-α-dehydroxylase in *Clostridioides difficile* infection:

Batch culture data demonstrated that *C. scindens* supplementation alone was unable to limit *C. difficile* growth, but a combination of BSH-producing bacteria together with *C. scindens* was able to do this. No mouse model was performed here evaluating the potential efficacy of 7-

α-dehydroxylase in treating CDI, and no experiment administering *C. scindens* alone to mice with CDI has been described in the literature to date. Of note, a mouse model of CDI was successfully treated with a mixture of six bacteria, none of which are recognised to contain 7-α-dehydroxylase activity (Lawley *et al.*, 2012). Furthermore, *bai*CD gene abundance is not different in the stool of CDI and *C. difficile*-negative patients (after correction for total bacterial load), and the *bai*CD gene is not consistently detectable in stool after successful FMT for rCDI (Solbach *et al.*, 2018). A further recent study using microbial sequencing and culture demonstrated comparable results (Amrane *et al.*, 2018).

However, it is feasible that as well as degradation of TCA, an additional explanation for the association between the recovery of gut microbiota-bile acid interactions post-FMT and CDI eradication may be through the restitution of secondary bile acids. Specifically, DCA and LCA both recover post-FMT to levels commensurate with donors, which has an 'anti-*C. difficile*' effect principally through inhibition of the organism's vegetative growth (Sorg & Sonenshein, 2008; Thanissery, Winston & Theriot, 2017). There are at least two explanations for how FMT may contribute to this finding. One such explanation is that the restitution of gut BSH functionality post-FMT creates a larger pool of deconjugated primary bile acids, the substrate for further gut bacterial enzyme degradation and conversion of primary into secondary bile acids within the colon. An additional explanation is that FMT may also be associated with the restitution of microorganisms with 7-α-dehydroxylase activity. The human data described earlier identified the enrichment in stool of unconjugated primary bile acids (CDCA and CA) and reduction in *bai*CD operon copy number pre-FMT in comparison to healthy donors, and that *bai*CD copy number/ predicted 7-α-dehydroxylase functionality was restored by FMT.

If FMT is associated with the restitution of microorganisms with 7-α-dehydroxylase activity, one possible explanation is that these organisms (or their spores) are transferred within the FMT itself; alternatively, the altered gut metabolic *milieu* post-FMT (after restoration of BSH-producing microbes) may facilitate recovery and growth of 7-α-dehydroxylase-producing bacteria from the host gut microbiota that were suppressed pre-FMT. The restoration of BSH by FMT is accompanied by degradation of TCA into CA, and there are now several strands of evidence that increased gut CA levels facilitate the growth of 7-α-dehydroxylating bacteria.

CA feeding to rats resulted in a marked increase in the relative abundance of *Firmicutes* within the gut microbiota and particularly in *Clostridium* cluster XIVa (Islam *et al.*, 2011), with further experiments in mice demonstrating that this included a 1000-fold increase in 7-α-dehydroxylating bacteria from within this genus specifically (Ridlon *et al.*, 2013). Other data also support the concept that 7-α-dehydroxylating bacteria are very sensitive to the metabolites in their immediate environment; for instance, gene expression profiling after incubation of *C. scindens* with bile acids resulted in complex and differential responses to CA and DCA, including the expression of potentially-novel bile acid-inducible genes which are contributory to CA metabolism (Devendran *et al.*, 2019). It is recognised that 7-α-dehydroxylating bacteria can use primary bile acids as an electron acceptor, facilitating increased ATP formation and growth (Ridlon, Kang & Hylemon, 2006).

Interesting, a further potential role for 7-α-dehydroxylating bacteria in inhibiting the growth of *C. difficile* (in addition to secondary bile acid production) has also recently been identified. Specifically, *in vitro, Clostridium scindens* was identified as producing the tryptophan-derived antibiotic 1-acetyl-β-carboline, which inhibited the growth of *C. difficile* (as well as other human gut bacteria). This antibiotic is produced particularly in the presence of CA, and acts most potently against *C. difficile* in the presence of DCA or LCA (Kang *et al.*, 2019).

5.4.4. Summary:

BSH-mediated hydrolysis of conjugated bile acids (particularly TCA) is sufficient *in vitro* and *in vivo* to significantly reduce *C. difficile* germination. Given that this functionality is absent pre-FMT and restored and maintained post-FMT, this mechanism appears to be a key contributory factor to the efficacy of FMT. The restoration of secondary bile acid production post-FMT has a potent effect of inhibiting *C. difficile* growth. Although it appears that restoration of 7-α-dehydroxylase functionality alone appears insufficient to treat CDI, joint restitution of BSH and 7-α-dehydroxylase functionality has a potent anti-*C. difficile* effect.

6. Discussion:

6.1. Overview:

In these studies, it has been demonstrated that gut microbiota-mediated bile-metabolising enzyme functionality influences vulnerability to *C. difficile* infection, and restoration of bile salt hydrolase functionality is a key mediator of the efficacy of faecal microbiota transplant. Furthermore, it has also been demonstrated that successful FMT is associated with the restoration of short chain fatty acid metabolism, consistent with recent data demonstrating that valerate has potent effects in inhibiting the vegetative growth of *C. difficile* (McDonald *et al.*, 2018a).

In this Discussion, an overview is given of other key relevant areas that have not already been discussed.

6.2. Wider implications of microbiota-metabonome changes after FMT:

6.2.1. Overview:

Up until this point, the implication of changes in the gut microbiota and bile, SCFA and BCFA metabolism after FMT have principally been assessed in the context of their direct impact upon the *C. difficile* life cycle. While some potential additional relevant effects have been considered (e.g. changes in FXR signalling), there are several more that have not been discussed before now - including anti-bacterial, anti-inflammatory and immune modulatory effects – that are discussed here.

6.2.2. Bile acid-mediated effects:

One interesting indication of gut microbiota-bile acid interactions having a physiological influence on the whole host level has been the demonstration of profound changes to the bile acid profiles in multiple body compartments of germ-free and antibiotic-treated animals as compared to wild-type animals. In particular, germ-free/ antibiotic-treated rats demonstrated marked enrichment in the proportion of taurine-conjugated bile acids that were detected in body compartments as diverse as the liver, kidney and heart (Swann *et al.*, 2011). Furthermore, germ-free mice that were monocolonised with *Bacteroides thetaiotaomicron* with a *bsh* gene (as opposed to those monocolonised with the same

bacterium but without a *bsh* gene) demonstrated a reduction in weight gain, in addition to transcriptional changes in metabolic, circadian rhythm and immunological pathways in both the gut and the liver (Yao *et al.*, 2018).

Similarly, FXR expression has also been identified in a wide variety of tissue and organ systems (including the brain of mice and humans (Huang *et al.*, 2016)), suggesting the much wider physiological roles of bile acid-FXR interactions than in purely glucose and lipid metabolism, as originally believed. With particular regards to intestinal disease, recent data have implicated ileal FXR signalling as having further roles in preventing/ resolving colitis, maintaining intestinal barrier integrity, and in limiting malignant transformations within cancer stem cells that might promote adenoma to adenocarcinoma transitions (Fu *et al.*, 2019). In particular, FXR activation has been demonstrated to inhibit bacterial overgrowth and block mucosal injury in mouse ileum (Inagaki *et al.*, 2006), and is associated with reduced expression of NF-κB target genes (including TNF-α and IL-1β) that regulate the host innate immune response (Vavassori *et al.*, 2009). In mice with chemically-induced colitis (via dextran sodium sulfate (DSS) or trinitrobenzenesulfonic acid (TNBS)), administration of an FXR agonist resulted in significantly reduced colonic inflammation and a more intact intestinal barrier. Such benefits were no longer observed when the FXR agonist was administered in Fxr-null DSS-colitis animals, confirming that the mechanism was FXR-mediated (Gadaleta *et al.*, 2011). Such data infer that the restoration of bile acid homeostasis after FMT for rCDI may be beneficial not only through the direct effects upon *C. difficile,* but also via FXR-mediated maintenance of a structurally and functionally intact intestinal mucosa.

It has also only recently been recognised that bile acids are endogenous ligands for a wide range of host receptors in addition to FXR and TGR-5, including other nuclear receptors (i.e. pregnane X receptor (Staudinger *et al.*, 2001) and vitamin D receptor (Makishima *et al.*, 2002)), as well as cell membrane receptors (i.e. muscarinic receptors (Sheikh Abdul Kadir *et al.*, 2010) and sphingosine-1-phosphate receptor 2 (Studer *et al.*, 2012)). These various receptor systems are activated to different extents by different forms of bile acids; specifically, while FXR and TGR-5 are most strongly activated by CDCA and secondary bile acids, both of the cell membrane receptors listed here are particularly activated by conjugated bile acids. Given that gut microbiota perturbations clearly are associated with

such profound alterations of the dominant bile acid moieties in different organ systems, it might be inferred that the gut microbiota may significantly influence the degree to which various bile acid-host receptor pathways are activated or inhibited in the host.

6.2.3. Short chain fatty acid-mediated effects:

SCFAs have similarly been demonstrated as having a wide range of physiological roles. As already alluded to (**Sections 1.6.6.5** and **4.4.3**), many of these effects are distant from the colon, including roles in energy metabolism and satiety regulation (principally mediated by propionate and acetate) (Cani *et al.*, 2019), as well as anti-tumorigenic effects (Tan *et al.*, 2014).

Other important actions have been defined within the intestinal epithelium. Butyrate is a key energy source of colonocytes, and also mediates a number of local functions that are protective against infection. For instance, butyrate promotes the integrity of the intestinal barrier via several different mechanisms, including modulation of the expression of tight junction proteins (Kelly *et al.*, 2015a). It has also been demonstrated that butyrate contributes to the inflammasome activation within colonic intraepithelial cells, and – partly via the production of IL-18 - to increase the expression of mucins and antimicrobial peptides (Levy *et al.*, 2015). In addition, butyrate is able to inhibit NF-κB activation and improve rodent TNBS-induced colitis (Segain *et al.*, 2000).

SCFAs have also been demonstrated to have a more global impact on different aspects of the immune system via a number of alternative mechanisms, including effects upon immune cell differentiation and recruitment, reduction in pro-inflammatory mediators, and an increase in anti-inflammatory mediators (Gonçalves, Araújo & Di Santo, 2018). While some of these experiments have been *in vitro* or *ex vivo,* others have confirmed the impact of SCFAs experimentally; for instance, in a study involving germ- and pathogen-free mice, SCFAs were demonstrated to regulate the size and function of the colonic regulatory T cell population, which was directly shown to be a protective mechanism against colitis in mice (Smith *et al.*, 2013).

One particular SCFA-mediated action of recent interest is their impact upon histone deacetylases (HDAC). The acetylation of lysine residues within histones induces gene activation by facilitating transcription factors gaining access to promoter regions. HDACs cleave acetyl groups from histones, and therefore reduce gene transcription. SCFAs are recognised as being HDAC inhibitors (with butyrate as the most potent inhibitor), and as such have marked effects on increasing transcription of a wide-variety of genes. SCFA-mediated HDAC inhibition results in an anti-inflammatory immune phenotype via actions upon on a number of immune cells (Tan *et al.*, 2014). The reason that this particular mechanism of SCFA activity is of interest here is that it has recently been demonstrated *in vitro* that gut commensal bacteria derived from healthy human stool are directly able to inhibit HDAC via the production of valerate (Yuille *et al.*, 2018); this is relevant since administration of exogenous valerate has already been shown to ameliorate chemically-induced colitis in a rodent model via HDAC inhibition (Glauben *et al.*, 2006). As such, this provides another potential mechanistic link between restoration of gut valerate production after FMT and resolution of *C. difficile* colitis.

6.3. Other potential mechanisms of efficacy of faecal microbiota transplant:

6.3.1. Overview:

This project has focused upon the contributory role of gut microbiota-metabolite interactions to the host vulnerability to CDI and the efficacy of FMT. Major alternative theories regarding mechanisms of efficacy of FMT have already been discussed (**Section 1.6.6**). However, there are a number of other potential avenues of interest that are currently relatively poorly-explored, and are summarised here.

6.3.2. Microbiota-immune system interactions:

The interaction between the mucosal microbiota and the host immune system is complex and bidirectional, with evidence that this interaction plays an important role in maintaining the integrity of gut barrier function and protecting against enteropathogens, including *C. difficile* (Hooper, Littman & Macpherson, 2012). Perturbation of this interaction may directly contribute to the pathogenesis of GI infection; for instance, in mice with CDI, it has been demonstrated that translocation of commensal bacteria across the intestinal epithelial barrier

is critical for neutrophil recruitment through the induction of an IL-1β-mediated positive feedback loop (Hasegawa *et al.*, 2012).

Only recently has the effect of FMT upon the innate and adaptive immune response been investigated, through the administration of FMT in a mouse DSS-model of colitis (Burrello *et al.*, 2018). FMT derived from healthy wild-type mice was delivered into colitic mice, and was observed to produce a rapid and sustained improvement in colonic inflammation, as well as marked modulation of the immunological response. Specifically, FMT from healthy mice was associated with IL-10 production by T helper cells, invariant natural killer T (iNKT) cells and antigen-presenting cells, as well as the reduced ability of dendritic cells, monocytes and macrophages to present major histocompatibility class II-dependent bacterial antigens to colonic T cells. FMT derived from the stool of mice treated with DSS and/or broad-spectrum antibiotics failed to produce similar improvements in colonic inflammation, or the observed immunological responses (Burrello *et al.*, 2018).

As such, it may be hypothesised that further benefits of FMT for rCDI may include modulation of the host innate and adaptive immune response that may contribute to colonisation resistance. However, there are several lines of evidence that question the degree to which host immunological response may be contributory to the efficacy of FMT for rCDI. Firstly, FMT appears of similar efficacy even in patients who are highly immunocompromised compared to patients who are immunocompetent (Patel *et al.*, 2013; Kelly *et al.*, 2014), and has been demonstrated to have similar effects on *C. difficile* dynamics in a chemostat system to those observed *in vivo* (McDonald *et al.*, 2018a). Secondly, RAG1-null mice (which lack B and T cells) are able to recover from CDI to a similar degree as wild-type mice, and reconstitution of IgG antitoxin antibody was found to be insufficient to clear *C. difficile* (Leslie *et al.*, 2019). Collectively, these data suggest that the changes exerted by FMT on host immune response are not sufficient by themselves to explain its efficacy, but may be at least partly contributory to the resolution of colitis, perhaps even independently of the effects of FMT against *C. difficile* directly.

6.3.3. Scavenging of carbohydrate and amino acid sources by *Clostridioides difficile:*

C. difficile has the almost unique ability to exploit changes in the availability of/ competition for nutrients in the antibiotic-treated gut to its own survival advantage, by scavenging a number of sources of carbohydrates and amino acids.

For example, the outer mucus layer found within the mammalian intestine includes carbohydrate chains capped with an acetylated version of the 9-carbon monosaccharide sialic acid, an adaptation which protects the heavily-glycosylated components of mucus from the actions of bacterial endo- and exoglycosidases. However, certain bacteria have evolved sialidases and esterases to liberate sialic acid; furthermore, bacteria that lack these enzymes (such as *C. difficile*) may still have the ability to scavenge this carbohydrate from bacteria that do, and take up and metabolise sialic acid themselves (Robinson, Lewis & Lewis, 2017). In the antibiotic-treated gut, the loss of competitors that scavenge sialic acid is used to the advantage of *C. difficile,* since it has almost unopposed access to any liberated sialic acid, and can use this to rapidly expand (Ng *et al.*, 2013). However, it has been established from an *in vitro* study that other gut microbiota members can outcompete *C. difficile* for sialic acid, as well as other carbohydrate sources (including glucose, N-acetylglucosamine, N-acetylneuraminic acid) (Wilson & Perini, 1988). As such, an additional function of FMT may be restoration of gut microbiota that provide increased competition for gut carbohydrates such as sialic acid, depriving *C. difficile* of an energy source that it may otherwise exploit to facilitate its growth.

C. difficile also has the ability to scavenge amino acids to its competitive advantage. Antibiotic-treated mice demonstrated reduced expression of genes within their gut microbiota related to amino acid uptake and metabolism (and an increased concentration of 12 gut amino acids, particularly proline) in comparison to untreated mice. *C. difficile* is capable of using proline as a sole energy source via Stickland fermentation, and uses proline particularly effectively to promote its own growth and gut colonisation in an environment with low levels of DCA, as is found in the gut in CDI (Battaglioli *et al.*, 2018). FMT in mice with CDI was noted to remove free proline availability; a small number of other bacteria within the gut microbiota of wild-type mice were observed to have the ability to utilise proline as an energy source, including *Dorea longicatena* and a member of *Lachnospiraceae* (Battaglioli *et*

al., 2018). It is interesting to note that in the metataxonomic studies performed here, *Dorea longicatena* was noted to be at lower relative abundance in the stool microbiota in rCDI pre-FMT samples than found in donors and post-FMT (**Section 2.2.1.2**). This is therefore suggestive that while *C. difficile* can exploit proline metabolism to facilitate its colonisation and growth in an antibiotic-exposed gut, FMT may minimise this ability by restoring other bacteria which may outcompete *C. difficile* for metabolism of proline (and/or other amino acids).

6.3.4. Contribution of other organic acids:

6.3.4.1. Succinate:

While SCFAs are the most widely-studied end products of primary fermentation within the distal gut in mammals, other common end products of fermentation include the organic acids succinate and lactate, which are metabolised by other gut bacteria (Ferreyra *et al.*, 2014). While gut succinate levels are low in healthy conditions, levels can markedly increase after antibiotic treatment, due to loss of bacterial taxa that take up and utilise it as a nutritional source (McDonald *et al.*, 2018a). However, *C. difficile* has the ability to metabolise succinate to butyrate, and to use this as an energy source to rapidly expand. It performs this function particularly avidly in a gut treated with antibiotics/chemically-induced diarrhoea (where succinate levels are high and alternative sources of SCFA are low) (Ferreyra *et al.*, 2014).

In the healthy gut, succinate levels normal remain low due to the presence of members of *Bacteroidetes* and the *Negativicutes* class of *Firmicutes*, which can degrade succinate to propionate (Louis & Flint, 2017). As such, it may be hypothesised that by restoring microbiota members with the ability to cross-feed succinate, FMT deprives *C. difficile* of a nutrient source that it could otherwise exploit for expansion.

6.3.4.2. Medium chain fatty acids:

Similar to the impact of SCFAs, it has also been observed that medium chain fatty acids (i.e. those fatty acids with 7-12 carbon tails) have antimicrobial activity against a range of human bacterial pathogens, including *C. difficile.* In humans, these fatty acids are normally derived from plant oils (e.g. virgin coconut oil) and/or dairy products. In particular, the C12 MCFA lauric acid inhibited the growth of multiple isolates of *C. difficile in vitro* through the damage

and disruption of both its cell membrane and cytoplasm, via the production of reactive oxygen species (Shilling *et al.*, 2013; Yang *et al.*, 2018). Furthermore, administration of lauric acid limited the extent of disease in a CDI mouse model, both via its direct effects on *C. difficile* and via limitation of host proinflammatory cytokine production (Yang *et al.*, 2018).

It may be expected that healthy donors (with a rich and diverse diet) may have higher levels of MCFAs within the metabolic *milieu* of their distal gut than those with rCDI (who are often elderly, frail and debilitated). Therefore, it is possible that transfer of MCFAs from donor to recipient may be a contributory factor to the efficacy of FMT. However, this would clearly represent a transient delivery of MCFAs immediately following the FMT, rather than a sustained exposure. As such, even though MCFA administration is of interest as a potential novel, targeted approach for the treatment of CDI, it is at most only likely to form a minor contributory factor to the efficacy of FMT.

6.3.5. Abiotic factors – the potential role of zinc:

While the emphasis of this work has been on biological factors influencing the vulnerability to CDI and efficacy of FMT, there are also data implicating the relevance of abiotic factors as well.

One such factor of specific interest has been dietary metal intake, and particularly that of zinc. Mice fed a high zinc diet developed marked shifts in their gut microbiota composition (with reduced microbial diversity), and developed CDI after exposure to a lower threshold of antibiotics than mice on a conventional diet (Zackular *et al.*, 2016). Mice fed a high zinc diet exposed to antibiotics and *C. difficile* displayed increased *C. difficile* toxin titres, altered immune responses and more marked caecal inflammation than control animals. Researchers also demonstrated that the ability of calprotectin to combat CDI were partly mediated by the ability of this protein to bind and limit exposure to zinc (Zackular *et al.*, 2016).

As previously discussed, there has been a recent increase in human cases of CDI (particularly within the community) where patients have had no obvious prior exposure to antibiotics (**Section 1.2**). One proposed explanation is that zinc supplementation in livestock feed may have resulted in higher rates of *C. difficile* colonisation within livestock animals, and

consequently higher rates of transfer of zinc and *C. difficile* into the human food chain (Zackular & Skaar, 2018). It may also be proposed that a potential mechanism of efficacy of FMT could be related to the transfer from donor to recipient of faecal slurry containing low levels of zinc, and/or high levels of zinc-binding proteins such as calprotectin. However, there are no supportive data for these theories at present.

6.4. Clinical implications of this project:

As summarised earlier, there are a number of important drawbacks that currently exist with FMT clinically, including its unpalatability, complex regulation, the potential need for invasive administration, the possible risk of transmission of infection, and/or the theoretical transfer of a gut microbiota associated with increased risk of disease (Mullish *et al.*, 2018e; Mullish & Williams, 2015). Consequently, there is a widely-acknowledged need for more refined, targeted therapies for CDI.

A proposal that may arise from this work is that the administration of BSH- or valerate-producing gut microbiota members (or the administration of purified BSH enzyme and/or valerate alone) – merits further evaluation as an alternative CDI treatment strategy. Administration of a microbial community containing BSH-producing bacteria (such as those identified in this study) might be expected – like FMT - to require a single administration only, given the apparent ability of these organisms to easily colonise the gut; in contrast, to attain comparable efficacy, delivery of purified BSH enzyme is likely to require a more prolonged administration. However, prior to considering whether purified BSH enzyme may have viability as a clinical therapy, further studies would be required to establish whether pH changes throughout the GI tract and/or host digestive enzymes (i.e. derived from the stomach or pancreas) may influence enzyme activity. The latter may be particularly relevant; in the development of the BSH enzyme activity used in these experiments, it was noted that addition of broad-range mammalian and bacterial protease inhibitor cocktails to the experimental mixture improved the activity detected from the assay (see **Section 2.4**), suggesting that proteases within the gut may degrade BSH *in vivo.* In the mouse model of CDI where the impact of valerate replacement was assessed, it was administered in the form of glycerol trivalerate (GTV) (McDonald *et al.*, 2018a). Administration of oral valerate alone would likely be inadequate as it would be absorbed within the upper gastrointestinal tract;

however, the glycerol head of GTV is cleaved by pancreatic lipases, liberating free valerate into the distal gut, making this a preferable method of administration. However, while proof of concept of 'BSH/valerate therapy' has been demonstrated in a batch culture and mouse model setting, this would evidently merit evaluation within a clinical trial setting before it could be considered further as treatment for human patients with rCDI. It is also important to acknowledge that FMT may provide additional mechanisms of efficacy in treating rCDI (**Section 1.6.6**), and such benefits may be missed through the use of BSH/valerate supplementation alone. However, given that BSH and valerate act upon two different targets within the *C. difficile* life cycle – germination and vegetative growth respectively – then it may be that their administration in combination results in a particularly potent anti-*C. difficile* effect.

The concept of prevention of *C. difficile* germination as a treatment strategy for CDI has previously been partly explored, but principally based around preventing TCA interacting with the *C. difficile* germination receptor. Specifically, mutagenesis of the *C. difficile* germination receptor, CspC, limits TCA-related *C. difficile* germination and reduces mortality from CDI in a Syrian hamster model (Francis *et al.*, 2013), while bile acid analogues that disrupt the interaction of TCA with this receptor have also shown promise (Stoltz *et al.*, 2017; Howerton, Patra & Abel-Santos, 2013). However, the clear potential advantage of BSH as a therapeutic strategy (as opposed to purely interrupting TCA-CspC interaction) is that it actually degrades TCA and restores gut bile acid homeostasis, rather than purely minimises the effect of TCA.
There have also been certain other previous attempts at manipulating bile acid metabolism as a novel means of treating CDI, including:

- *Anion-binding resins*: These were first utilised in CDI with the primary aim of binding toxin, but such resins also act as bile acid sequestrants, so could potentially have a role in binding TCA. In practice, however, such resins have demonstrated poor efficacy in the treatment of CDI, as discussed in **Section 1.5.2.3**; this may reflect the non-specific nature of their bile acid binding properties, including the ability to sequester secondary bile acids as well as TCA (Buffie *et al.*, 2014).
- *Deoxycholic acid:* Direct administration of DCA is an alternative treatment strategy that has been proposed for CDI; however, this would require very careful consideration of

pharmacokinetics and pharmacodynamics, since excess intestinal DCA is well-recognised as a direct cause of colorectal tumorigenesis (Dong *et al.*, 2018).

- *Bile acid analogues:* The tertiary bile acid ursodeoxycholic acid (UDCA) is structurally similar to lithocholic acid, and has been demonstrated to modestly reduce vegetative growth/ TCA-mediated germination of *C. difficile in vitro* (Weingarden *et al.*, 2016a; Palmieri *et al.*, 2018). Mortality was the same in a CDI hamster model for animals treated with UDCA *vs* those who were not (Palmieri *et al.*, 2018); however, a case report and a small case series have demonstrated low rates of CDI recurrence in humans patients treated with UDCA in addition to antimicrobial therapy (Weingarden *et al.*, 2016a; Webb *et al.*, 2019). Similarly, as well as being a potent FXR agonist, obeticholic acid also modestly reduces *C. difficile* germination and vegetative growth, and has been demonstrated as having efficacy at treating CDI in a rodent model (Tessier *et al.*, 2015).

However, once again, the clear drawbacks of these approaches is that they have anti-*C. difficile* effects without exerting any sustained recovery of colonisation resistance by fully restoring bile acid homeostasis.

Furthermore, the experiments presented here have focused on the use of BSH and valerate in the setting of *treatment* of CDI, rather than as *prevention*; given that CDI originates from the faeco-oral transmission of *C. difficile* spores, there is certainly at least a strong case for exploration of the use of BSH in disease prevention. More specifically, BSH and valerate supplementation could have utility as a potential novel strategy for prevention of CDI in those at high risk, e.g. patients likely to require prolonged antibiotic courses. In addition, FMT has a small but appreciable failure rate, and there is currently no rational targeted biological means by which donors are selected. Donor screening is expensive and demanding (McSweeney *et al.*, 2018). Assays of stool from potential donors for BSH- and valerate-producing organisms (and/or levels of BSH and valerate, or BSH activity) may be one such means to achieving this aim.

6.5. Final summary:

Clostridioides difficile is a very well-adapted scavenger, being able to exploit any of a number of different perturbations of the composition or function of the gut microbiota of the antibiotic-treated host to promote its germination, colonisation and vegetative growth within

the distal gut. Specifically, these include germination in response to increased gut taurocholic acid levels, unrestrained vegetative growth in the absence of secondary bile acids and short chain fatty acids (including valerate), and unopposed scavenging of carbohydrate, amino acid and organic acid sources to allow rapid expansion. Loss of gut commensal bacteria also results in disruption of other key host physiological processes (including loss of ileal FXR signalling, breakdown in intestinal barrier function, perturbation of immune response, etc), which may act in concert with *C. difficile* toxin production to result in marked colitis.

While *Clostridioides difficile* infection was conventionally easy to treat with antimicrobials, *C. difficile* has once again adapted. Increasing rates of metronidazole failure means that this antimicrobial is no longer first-line therapy, while the emergence of hypervirulent strains (itself potentially arising from the ability of *C. difficile* to exploit a new dietary carbohydrate, trehalose) has been responsible for rising morbidity and mortality associated with *C. difficile* globally. As such, recurrence rates for CDI remain high. The research here has inferred that after primary CDI, the ability of the host to repopulate the gut ecosystem with bacteria with bile-metabolising functionality – with the associated restoration of the pre-morbid gut bile acid homeostasis – may be an important contributory factor as to whether recurrence occurs or not.

Conventional antimicrobial therapy kills *C. difficile,* but has little direct impact on restoring colonisation resistance. However, salvation may come from an unlikely source: faecal material. Faecal microbiota transplant is a highly-effective therapy for recurrent CDI, but its mechanisms of efficacy have previously remained elusive. The apparent comparable efficacy of sterile faecal filtrate to conventional FMT suggests that soluble mediators – rather than intact microorganisms – may be important mediators of FMT. One key mechanism of efficacy demonstrated in this work is the rapid and sustained restoration of gut microbial gut bile salt hydrolase function, a gateway function upon which all further microbial bile acid metabolism depends. BSH-producing organisms are well-adapted to colonising the gut, as degradation of conjugated bile acids removes a major source of bile toxicity for many bacteria. The BSH-mediated degradation of taurocholic acid – a major pro-germinant trigger to *C. difficile* – minimises further germination, and therefore further recurrence of disease.

Taurocholic acid is degraded by BSH into cholic acid. Cholic acid promotes the growth of 7-α-dehydroxylase-producing bacteria within the gut, which convert primary into secondary bile acids, including deoxycholic acid. Deoxycholic acid has potent effects against *C. difficile* that minimise vegetative growth. 7-α-dehydroxylase-producing organisms also produce tryptophan-derived antimicrobials, including 1-acetyl-β-carboline, which also limits *C. difficile* growth. Such bile acid changes post-FMT are also associated with increased ileal FXR signalling; this may be the source of a number of beneficial anti-*C. difficile* effects, including further limitation of TCA production, upregulated innate immunity, and intestinal barrier repair.

This thesis (and associated work) also demonstrate the role for FMT-mediated changes in nutritional sources for *C. difficile* influencing its ability to undergo vegetative growth. More specifically, successful FMT is associated with restoration of gut short- and branched-chain fatty acid production. In particular, the 5-carbon SCFA, valerate, is able to potently inhibit the growth of *C. difficile,* while having minimal adverse effects on other gut commensal bacteria. Restored SCFAs are likely to have other beneficial effects contributing to the resolution of *C. difficile* colitis, including those related to maintenance of the gut barrier, and anti-inflammatory effects within the colonic mucosa. Gut community members restored by FMT may also outcompete *C. difficile* in scavenging carbohydrate and amino acids sources of nutrition.

Furthermore, FMT may also have additional mechanisms of efficacy, including those related to anti-microbial peptides, and/or transfer of bacteriophages and fungi. Collectively, this increased understanding of the mechanisms of efficacy of FMT for rCDI raises the prospect of formulation of refined, targeted therapies (e.g. purified BSH enzyme with glycerol trivalerate) that avoid the drawbacks associated with the use of conventional FMT. This preparation may also have a role in prevention of CDI in high-risk patients.

In addition, microbiota-metabonome interactions appear not just to produce effects within the gut lumen or intestinal mucosa, but also have the apparent potential to generate more marked host physiological changes both within the intestine and systemically. Effects upon the immune response and diverse metabolic signalling pathways are two key such areas of

note. This final point is of interest in the non-CDI setting too, as clinicians and scientists begin to move on from solely defining the microbiota changes that appear to characterise a wide range of clinical conditions, but also start to explore whether manipulation of the gut microbiota (by FMT or otherwise) may be a successful novel approach for therapeutic intervention.

Funding Supporting this Thesis:

This includes funding for preliminary work occurring prior to the formal start of the experiments in this thesis.

- NIHR Academic Clinical Fellowship in Gastroenterology and Hepatology (October 2012 – September 2015).

- Imperial Healthcare Charity research fellowship (September 2015 – September 2017).

- Medical Research Council (MRC) Clinical Research Training Fellowship (RCUK/ UKRI Innovation Fellowship (September 2017 – present).

- NIHR Imperial Biomedical Research Centre (October 2012 – present).

Abstracts and Publications Arising from this Thesis:

This includes abstracts and publications arising from preliminary work during my NIHR Academic Clinical Fellowship before formally commencing this work.

1. Abstracts:

Allegretti J.R., Hurtado J., Carrellas M., Marcus J., Phelps E., Wong W.F., Marchesi J.R., Mullish B.H., McDonald J.A.K., Pechlivanis A., Barker G.F., Miguens Blanco J., Sagi S., Bohm M., Kelly C.R., Kassam Z., Grinspan A., Fischer M. The Icon study: inflammatory bowel disease and recurrent *Clostridium difficile* infection: outcomes after fecal microbiota transplantation. *Gastroenterology* (2019); 156(6): S2-S3. Oral presentation at DDW 2019, San Diego, USA.

McDonald J.A.K., Perez J.L., Mullish B.H., Marchesi J.R. Growth inhibition of *Clostridioides difficile* by short and medium chain fatty acids. *Gastroenterology* (2019); 156(6): S898. Poster of distinction at DDW 2019, San Diego, USA.

Allegretti J.R., Kassam Z., Chiang A.L., Mullish B.H., Carrellas M., Hurtado J., Marchesi J.R., McDonald J.A.K., Pechlivanis A., Barker G.F., Miguens Blanco J., Wong W.F., Geradin Y., Silverstein M., Kennedy K., Thompson C.C. Fecal microbiota transplantation for the treatment of obesity: a randomized, placebo-controlled pilot trial. *Gastroenterology* (2019); 156(6): S129. Plenary presentation at DDW 2019, San Diego, USA.

Churchward M.A., Michaud E.R., Miguens Blanco J., Garcia Perez I., Mullish B.H., Marchesi J.R, Xu H., Kao D.H., Todd K. Effect of short chain fatty acids on gut-brain axis using a microglial cell model. *Gastroenterology* (2019); 156(6); S455. Poster of distinction at DDW 2019, San Diego, USA.

Ghani R., Gan C., Mullish B., Ferizoli V., Davies F., Thursz M., Marchesi J., Dasgupta R., Minhas S. 291 – Prevalence of recurrent extended-spectrum beta-lactamase (ESBL) urinary tract infections (UTIs) in patients within a urology service. Introducing the concept of faecal

microbiota transplantation (FMT) as a treatment modality. *Eur Urol Suppl* (2019); 18(1): e375-e376. Oral presentation at EUA 2019, Barcelona, Spain.

Allegretti J.A., Mullish B.H., Kassam Z., Carrellas M., Marchesi J.R., Smith M., Gerardin Y., Timberlake S., Pratt D.S., Korzenik J.R. Bile acid profiles are not altered by fecal microbiota transplantation for the treatment of primary sclerosing cholangitis. *Am J Gastroenterol* (2018); 113: S574-575. Poster presentation at ACG 2018, Philadelphia, USA: category award (liver); presidential poster award.

Ghani R., Mookherjee S., Mullish B., Thursz M., Marchesi J., Pavlu J., Davies F. Impact on mortality, length of stay, and antibiotic use in allogenic and autologous stem cell transplant patients colonized with carbapenemase-producing Enterobacteriaceae. *Open Forum Infectious Diseases* (2018); 5: S353-S354. Poster presentation at ID Week 2018, San Francisco, USA.

McSweeney B., Allegretti J.R., Fischer M., Monaghan T., Mullish B.H., Petrof E.O., Phelps E.L, Wong K., Xu H., Chis R., Kao D. Potential motivators and deterrents for stool donors: a multicentre study. *Gastroenterology* (2018); 154: S1051. Poster presentation at DDW 2018, Washington DC, USA.

Allegretti J.R., Mullish B.H., Bogart E., Shu E., Dong X., McDonald J.A.K, Pechlivanis A., Marchesi J.R., Gerber G., Bry L. Microbiome and metabolic markers of *Clostridium difficile* recurrence. *Gastroenterology* (2018); 154: S8-S9. Oral presentation at DDW 2018, Washington DC, USA.

McDonald J.A.K., Mullish B.H., Pechlivanis A., Li J.V., Nicholson J.L, Holmes E., Thursz M.R., Marchesi J.R. A novel route to controlling *Clostridioides difficile* growth via short chain fatty acid and bile acid modulation. *Gastroenterology* (2018); 154: S8. Oral presentation at DDW 2018, Washington DC, USA.

Mullish B.H., McDonald J.A.K., Kao D., Allegretti J.R., Petrof E.O., Pechlivanis A., Barker G.F., Atkinson S.R, Williams H.R.T., Thursz M.R., Marchesi J.R. Gut microbiota-host bile acid

metabolism interactions in *Clostridium difficile* infection: the explanation for the efficacy of faecal microbiota transplantation? *Gut* (2017); 66: A33-A34. Oral presentation at BSG 2017, Manchester, UK.

Mullish B.H., McDonald J.A.K., Kao D.H., Allegretti J.R., Petrof E.O., Pechlivanis A., Atkinson S.R, Williams H.R.T., Thursz M.R., Marchesi J.R. Understanding the mechanisms of efficacy of fecal microbiota transplantation in the treatment of *Clostridium difficile* infection: the potential role of bile-metabolising enzymes. *Gastroenterology* (2017); 152: S47. Oral presentation at DDW 2017, Chicago, USA; young investigator award to B. H. Mullish.

Mullish B.H., McDonald J.A.K., Pechlivanis A., Rees D.N., Williams H.R.T., Thursz M.R., Marchesi J.R. Understanding the efficacy of faecal microbiota transplantation in *Clostridium difficile* infection: re-establishment of gut microbiota with the ability to degrade bile? *Gut* (2016); 65: A184. Poster presentation at BSG 2016, Liverpool, UK.

Quraishi M.N., Segal J.P., Mullish B.H., McCune V.L., Hawkey P.M., Colville A., Williams H.R.T., Hart A.L, Iqbal T.H. National survey of practice of faecal microbiota transplantation for *Clostridium difficile* infection in the United Kingdom. *Gut* (2016); 65: A23-A24. Oral presentation at BSG 2016, Liverpool, UK.

2. Publications: (*: joint first author)

Allegretti J.R., Kassam Z., Mullish B.H., Chiang A., Carrellas M., Hurtado J., Marchesi J.R., McDonald J.A.K., Pechlivanis A., Barker G.F., Miguens Blanco J., Garcia-Perez I., Wong W.F., Gerardin Y., Silverstein M., Kennedy K., Thompson C. Effects of fecal microbiota transplantation with oral capsules in obese patients. *Clin Gastroenterol Hepatol* (2019); doi: 10.1016/j.cgh.2019.07.006

Allegretti J.R., Mullish B.H., Fischer M., Kelly C. The evolution of the use of faecal microbiota transplantation and emerging clinical indications. *Lancet* (2019); accepted.

McSweeney B, Allegretti J.R., Fischer M., Xu H., Goodman K.J., Monaghan T., McLeod C., Mullish B.H., Petrof E.O., Phelps E.L., Chis R., Edmison A., Juby A., Ennis-Davis R., Roach B., Wong K., Kao D. In search of stool donors: a multicentre study of prior knowledge, perceptions, motivators, and deterrents among potential donors for fecal microbiota transplantation. *Gut Microbes* (2019); doi: 10.1080/19490976.2019.1611153

Mullish B.H., McDonald J.A.K., Pechlivanis A., Allegretti J.R., Kao D., Barker G.B., Kapila D., Petrof D., Joyce S.A., Gahan G.M., Glegola-Madesjska I., Williams H.R.T., Holmes E., Clarke T.B., Thursz M.R, Marchesi J.R. Microbial bile salt hydrolases mediate the efficacy of faecal microbiota transplant in the treatment of recurrent *Clostridioides difficile* infection. *Gut* (2019); doi: 10.1136/gutjnl-2018-317842

Mullish B.H., Ghani R., McDonald J.A.K., Marchesi J.R. Faecal microbiota transplant for eradication of multidrug resistant Enterobacteriaceae: a lesson in applying best practice? *Clin Microbiol Infect* (2019); doi: 10.1016/j.cmi.2019.01.010

Segal J.P.*, Mullish B.H.*, Quraishi M.N.*, Acharjee A., Williams H.R.T, Iqbal T., Hart A.L., Marchesi J.R. The application of omics techniques to understand the role of the gut microbiota in inflammatory bowel disease. *Therap Adv Gastroenterol* (2019);12:1756284818822250.

Allegretti J.A., Kassam Z., Carrellas M., Mullish B.H., Marchesi J.R., Pechlivanis A., Smith M., Gerardin Y., Timberlake S., Pratt D.S., Korzenik J.R. Fecal microbiota transplantation in patients with primary sclerosing cholangitis: a pilot clinical trial. *Am J Gastroenterol* (2019): doi: 10.14309/ajg0000000000000115

McIlroy J., Segal J.P., Mullish B.H., Quraishi M.N., Gasbarrini A., Cammarota G., Ianiro G. Current and future targets for faecal microbiota transplantation. *Human Microbiome Journal* (2018); doi: 10.1016/j.humic.2018.08.004

Mullish B.H.*, Quraishi M.N.*, Segal J.P.*, Williams H.R.T., Goldenberg S.D. Introduction to the joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) faecal microbiota transplant guidelines. *J Hosp Infect* (2018); 100(2): 130-132.

Monaghan T.*, Mullish B.H.*, Patterson J., Wong G.K., Marchesi J.R., Xu H., Jilani T., Kao D. Effective fecal microbiota transplantation for recurrent *Clostridioides difficile* infection in humans is associated with increased signalling in the bile acid-farnesoid X receptor-fibroblast growth factor pathway. *Gut Microbes* (2018); Sep 5:1-7, doi: 10.1080/19490976.2018.1506667

Mullish B.H.*, Quraishi M.N.*, Segal J.P.*, McCune V.L., Baxter M., Marsden G.L., Moore D., Colville A., Bhala N., Iqbal T.H., Settle C., Kontkowski G., Hart A.L, Hawkey P.M., Williams H.R.T., Goldenberg S.D. The use of faecal microbiota transplant as treatment for recurrent or refractory *Clostridium difficile* infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *J Hosp Infect* (2018); Suppl 1: S1-S31.

Mullish B.H.*, Quraishi M.N.*, Segal J.P.*, McCune V.L., Baxter M., Marsden G.L., Moore D., Colville A., Bhala N., Iqbal T.H., Settle C., Kontkowski G., Hart A.L, Hawkey P.M., Williams H.R.T., Goldenberg S.D. The use of faecal microbiota transplant as treatment for recurrent or refractory *Clostridium difficile* infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut* (2018); 67(11): 1920-1941.

McDonald J.A.K., Mullish B.H., Pechlivanis A., Liu Z., Brignardello J., Kao D., Holmes E., Li J.V., Clarke T.B., Thursz M.R., Marchesi J.R. Inhibiting growth of *Clostridioides difficile* by restoring valerate, produced by the intestinal microbiota. *Gastroenterology* (2018); 155(5):1495-1507.e15

Mullish B.H., Osborne L.S., Marchesi J.R., McDonald J.A.K. The implementation of omics technologies in cancer microbiome research. *Ecancermedicalscience* (2018); 12: 864, doi: 10.3332/ecancer.2018.864

Mullish B.H. Letter: improvements in mental health after faecal microbiota transplantation – an underexplored treatment-related benefit? *Aliment Pharmacol Ther* (2018); 47(11): 1562-1563.

Mullish B.H., Williams H.R.T. *Clostridium difficile* infection and antibiotic-associated diarrhoea. *Clin Med (Lond)* (2018); 18(3): 237-241.

Mullish B.H., McDonald J.A.K., Thursz M.R., Marchesi J.R. Antibiotic-associated disruption of microbiota composition and function in cirrhosis is restored by fecal transplant. *Hepatology* (2018); 68(3): 1205.

Mullish B.H., Pechlivanis A., Barker G.F., Thursz M.R., Marchesi J.R., McDonald J.A.K. Functional microbiomics: evaluation of gut microbiota-bile acid metabolism interactions in health and disease. *Methods* (2018); 149: 49-58.

King A., Mullish B.H., Pechlivanis A., Williams H.R.T., Aylin P. Comparative epidemiology of *Clostridium difficile* infection: England and the USA. *Int J Qual Health Care* (2018); 29(6): 785-791.

Mullish B.H., McDonald J.A.K., Thursz M.R., Marchesi J.R. Fecal microbiota transplant from a rational stool donor improves hepatic encephalopathy: a randomized clinical trial. *Hepatology* (2017); 66(4): 1354-1355.

Innes A.J., Mullish B.H., Fernando F., Adams G., Marchesi J.R., Apperley J.F., Brannigan E., Davies F., Pavlu J. Faecal microbiota transplant: a novel biological approach to extensively drug-resistant organism-related non-relapse mortality. *Bone Marrow Transplant* (2017); 52(10): 1452-1454.

Ding N.S., Mullish B.H., McLaughlin J., Hart A., Marchesi J.R. Meeting update: faecal microbiota transplantation – bench, bedside, courtroom? *Frontline Gastroenterol* (2018); 9(1): 45-48.

Quraishi M.N., Segal J.P., Mullish B.H., McCune V.L., Hawkey P.M., Colville A., Williams H.R.T., Hart A.L, Iqbal T.H. National survey of practice of faecal microbiota transplantation for *Clostridium difficile* infection in the UK. *J Hosp Infect* (2017); 95(4): 444-445.

Gratton J., Phetcharaburanin J., Mullish B.H., Williams H.R.T, Thursz M., Nicholson J.K., Holmes E., Marchesi J.R., Li J.V. Optimized sample handling strategy for metabolic profiling of human feces. *Anal Chem* (2016); 88(9): 4661-4668.

Mullish B.H., Williams H.R.T. Obstacles to establishing an NHS faecal transplant programme. *BMJ* (2015); 351: h6043.

Mullish B.H. Case 25-2014: A man with ulcerative colitis and bloody diarrhea. *N Engl J Med* (2014); 371(19): 1848.

Mullish B.H., Marchesi J.R., Thursz M.R., Williams H.R.T. Microbiome manipulation with faecal microbiome manipulation as a therapeutic strategy in *Clostridium difficile* infection. *QJM* (2015); 108(5): 355-359.

References:

Abdi, H. & Williams, L.J. (2010) Principal component analysis. *Wiley Interdisciplinary Reviews: Computational Statistics*. [Online] 2 (4), 433–459. Available from: doi:10.1002/wics.101 [Accessed: 26 February 2019].

Allegretti, J.R., Kassam, Z., Carrellas, M., Mullish, B.H., et al. (2019a) Fecal Microbiota Transplantation in Patients With Primary Sclerosing Cholangitis. *The American Journal of Gastroenterology*. [Online] 1. Available from: doi:10.14309/ajg.0000000000000115 [Accessed: 9 February 2019].

Allegretti, J.R., Kassam, Z., Mullish, B.H., Chiang, A., et al. (2019b) Effects of Fecal Microbiota Transplantation With Oral Capsules in Obese Patients. *Clinical Gastroenterology and Hepatology*. [Online] Available from: doi:10.1016/j.cgh.2019.07.006 [Accessed: 13 July 2019].

Allegretti, J.R., Kearney, S., Li, N., Bogart, E., et al. (2016) Recurrent Clostridium difficile infection associates with distinct bile acid and microbiome profiles. *Alimentary Pharmacology and Therapeutics*. [Online] 43 (11), 1142–1153. Available from: doi:10.1111/apt.13616 [Accessed: 7 October 2017].

Allegretti, J.R., Mullish, B.H., Bogart, E., Shu, E., et al. (2018) 25 - Microbiome and Metabolic Markers of Clostridium Difficile Recurrance. *Gastroenterology*. [Online] 154 (6), S-8-S-9. Available from: doi:10.1016/S0016-5085(18)30514-6 [Accessed: 15 February 2019].

Allen, S.J., Wareham, K., Wang, D., Bradley, C., et al. (2013) Lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea and Clostridium difficile diarrhoea in older inpatients (PLACIDE): a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet*. [Online] 382 (9900), 1249–1257. Available from: doi:10.1016/S0140-6736(13)61218-0 [Accessed: 23 February 2019].

Amrane, S., Bachar, D., Lagier, J.C. & Raoult, D. (2018) Clostridium scindens Is Present in the Gut Microbiota during Clostridium difficile Infection: a Metagenomic and Culturomic Analysis. *Journal of Clinical Microbiology*. [Online] 56 (5), e01663-17. Available from: doi:10.1128/jcm.01663-17 [Accessed: 8 December 2018].

Anand, A. & Glatt, A.E. (1993) Clostridium difficile infection associated with antineoplastic chemotherapy: a review. *Clinical Infectious Diseases*. [Online] 17 (1), 109–113. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8353229 [Accessed: 22

February 2019].

Archibald, L.K., Banerjee, S.N. & Jarvis, W.R. (2004) Secular trends in hospital-acquired Clostridium difficile disease in the United States, 1987-2001. *Journal of Infectious Diseases*. [Online] 189 (9), 1585–1589. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15116293 [Accessed: 23 February 2019].

Bajaj, J.S., Kassam, Z., Fagan, A., Gavis, E.A., et al. (2017) Fecal Microbiota Transplant from a Rational Stool Donor Improves Hepatic Encephalopathy: A Randomized Clinical Trial. *Hepatology*. [Online] 66 (6), 1727–1738. Available from: doi:10.1002/hep.29306 [Accessed: 9 June 2017].

Baker, G.C., Smith, J.J. & Cowan, D.A. (2003) Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods*. [Online] 55 (3), 541–555. Available from: doi:10.1016/J.MIMET.2003.08.009 [Accessed: 1 May 2018].

Baktash, A., Terveer, E.M., Zwittink, R.D., Hornung, B.V.H., et al. (2018) Mechanistic Insights in the Success of Fecal Microbiota Transplants for the Treatment of Clostridium difficile Infections. *Frontiers in microbiology*. [Online] 9, 1242. Available from: doi:10.3389/fmicb.2018.01242 [Accessed: 20 February 2019].

Banerjee, P., Merkel, G.J. & Bhunia, A.K. (2009) Lactobacillus delbrueckii ssp. bulgaricus B-30892 can inhibit cytotoxic effects and adhesion of pathogenic Clostridium difficile to Caco-2 cells. *Gut Pathogens*. [Online] 1 (1), 8. Available from: doi:10.1186/1757-4749-1-8 [Accessed: 23 February 2019].

Barker, H.A., D'Ari, L. & Kahn, J. (1987) Enzymatic reactions in the degradation of 5-aminovalerate by Clostridium aminovalericum. *Journal of Biological Chemistry*. [Online] 262 (19), 8994–9003. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3597403 [Accessed: 4 March 2019].

Barr, J.J., Auro, R., Furlan, M., Whiteson, K.L., et al. (2013) Bacteriophage adhering to mucus provide a non-host-derived immunity. *Proceedings of the National Academy of Sciences*. [Online] 110 (26), 10771–10776. Available from: doi:10.1073/pnas.1305923110 [Accessed: 20 February 2019].

Bartlett, J.G. (2002) Clinical practice. Antibiotic-associated diarrhea. *New England Journal of Medicine*. [Online] 346 (5), 334–339. Available from: doi:10.1056/NEJMcp011603 [Accessed: 23 February 2019].

Bartsch, S.M., Umscheid, C.A., Fishman, N. & Lee, B.Y. (2013) Is Fidaxomicin Worth the Cost?

An Economic Analysis. *Clinical Infectious Diseases*. [Online] 57 (4), 555–561. Available from: doi:10.1093/cid/cit346 [Accessed: 23 February 2019].

Battaglioli, E.J., Hale, V.L., Chen, J., Jeraldo, P., et al. (2018) *Clostridioides difficile* uses amino acids associated with gut microbial dysbiosis in a subset of patients with diarrhea. *Science Translational Medicine*. [Online] 10 (464), eaam7019. Available from: doi:10.1126/scitranslmed.aam7019 [Accessed: 10 March 2019].

Baxter, M., Ahmad, T., Colville, A. & Sheridan, R. (2015) Fatal Aspiration Pneumonia as a Complication of Fecal Microbiota Transplant. *Clinical Infectious Diseases*. [Online] 61 (1), 136–137. Available from: doi:10.1093/cid/civ247 [Accessed: 22 October 2017].

Baxter, M. & Colville, A. (2016) Adverse events in faecal microbiota transplant: a review of the literature. *Journal of Hospital Infection*. [Online] 92 (2), 117–127. Available from: doi:10.1016/j.jhin.2015.10.024 [Accessed: 20 March 2017].

Becattini, S., Taur, Y. & Pamer, E.G. (2016) Antibiotic-Induced Changes in the Intestinal Microbiota and Disease. *Trends in Molecular Medicine*. [Online] 22 (6), 458–478. Available from: doi:10.1016/j.molmed.2016.04.003.

Begley, M., Gahan, C.G.M. & Hill, C. (2005) The interaction between bacteria and bile. *FEMS Microbiology Reviews*. [Online] 29 (4), 625–651. Available from: doi:10.1016/J.FEMSRE.2004.09.003 [Accessed: 1 March 2019].

Begley, M., Hill, C. & Gahan, C.G.M. (2006) Bile salt hydrolase activity in probiotics. *Applied and environmental microbiology*. [Online] 72 (3), 1729–1738. Available from: doi:10.1128/AEM.72.3.1729-1738.2006 [Accessed: 18 January 2018].

Ben-Dov, E., Shapiro, O.H., Siboni, N. & Kushmaro, A. (2006) Advantage of using inosine at the 3′ termini of 16S rRNA gene universal primers for the study of microbial diversity. *Applied and Environmental Microbiology*. [Online] 72 (11), 6902–6906. Available from: doi:10.1128/AEM.00849-06 [Accessed: 1 December 2017].

Benoit, B., Meugnier, E., Castelli, M., Chanon, S., et al. (2017) Fibroblast growth factor 19 regulates skeletal muscle mass and ameliorates muscle wasting in mice. *Nature Medicine*. [Online] 23 (8), 990–996. Available from: doi:10.1038/nm.4363 [Accessed: 16 January 2018].

Bliss, D.Z., Johnson, S., Savik, K., Clabots, C.R., et al. (1998) Acquisition of Clostridium difficile and Clostridium difficile-associated diarrhea in hospitalized patients receiving tube feeding. *Annals of Internal Medicine*. [Online] 129 (12), 1012–1019. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/9867755 [Accessed: 22 February 2019].

Boets, E., Gomand, S. V., Deroover, L., Preston, T., et al. (2017) Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study. *The Journal of Physiology*. [Online] 595 (2), 541–555. Available from: doi:10.1113/JP272613 [Accessed: 4 March 2019].

Boix, V., Fedorak, R.N., Mullane, K.M., Pesant, Y., et al. (2017) Primary Outcomes From a Phase 3, Randomized, Double-Blind, Active-Controlled Trial of Surotomycin in Subjects With Clostridium difficile Infection. *Open Forum Infectious Diseases*. [Online] 4 (1), ofw275. Available from: doi:10.1093/ofid/ofw275 [Accessed: 23 February 2019].

Bonhoff, M., Drake, B.L. & Miller, C.P. (1954) Effect of streptomycin on susceptibility of intestinal tract to experimental Salmonella infection. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*. [Online] 86 (1), 132–137. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13177610 [Accessed: 1 April 2019].

Bornstein, B.T. & Barker, H.A. (1948) The energy metabolism of Clostridium kluyveri and the synthesis of fatty acids. *Journal of Biological Chemistry*. [Online] 172 (2), 659–669. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18901185 [Accessed: 4 March 2019].

Bourgault, A.-M., Lamothe, F., Toye, B., Oughton, M., et al. (2011) Host and Pathogen Factors for Clostridium difficile Infection and Colonization. *New England Journal of Medicine*. [Online] 365 (18), 1693–1703. Available from: doi:10.1056/nejmoa1012413 [Accessed: 22 February 2019].

Boyle, R.J., Robins-Browne, R.M. & Tang, M.L.K. (2006) Probiotic use in clinical practice: What are the risks? *American Journal of Clinical Nutrition*. [Online]. 83 (6) pp.1256–1264. Available from: doi:10.1093/ajcn/83.6.1256 [Accessed: 23 February 2019].

Brandvold, K.R., Weaver, J.M., Whidbey, C. & Wright, A.T. (2019) A continuous fluorescence assay for simple quantification of bile salt hydrolase activity in the gut microbiome. *Scientific Reports*. [Online] 9 (1), 1359. Available from: doi:10.1038/s41598-018-37656-7 [Accessed: 28 February 2019].

Bristol, A., Hubert, S., Hofmann, F. & Baer, H. (2017) Formulation development of SYN-004 (ribaxamase) oral solid dosage form, a β-lactamase to prevent intravenous antibiotic-associated dysbiosis of the colon. *International Journal of Pharmaceutics*. [Online] 534

(1–2), 25–34. Available from: doi:10.1016/j.ijpharm.2017.10.001 [Accessed: 23 February 2019].

Brito, G.A.C., Guerrant, R.L., Carper, H.T., Mandell, G.L., et al. (2002) Clostridium difficile Toxin A Alters In Vitro–Adherent Neutrophil Morphology and Function. *The Journal of Infectious Diseases*. [Online] 185 (9), 1297–1306. Available from: doi:10.1086/340236 [Accessed: 22 February 2019].

Brouwer, M.S.M., Roberts, A.P., Hussain, H., Williams, R.J., et al. (2013) Horizontal gene transfer converts non-toxigenic Clostridium difficile strains into toxin producers. *Nature Communications*. [Online] 4 (1), 2601. Available from: doi:10.1038/ncomms3601 [Accessed: 23 February 2019].

Brown, J.R.-M., Flemer, B., Joyce, S.A., Zulquernain, A., et al. (2018) Changes in microbiota composition, bile and fatty acid metabolism, in successful faecal microbiota transplantation for Clostridioides difficile infection. *BMC Gastroenterology*. [Online] 18 (1), 131. Available from: doi:10.1186/s12876-018-0860-5 [Accessed: 31 August 2018].

Browne, H.P., Forster, S.C., Anonye, B.O., Kumar, N., et al. (2016) Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature*. [Online] 533 (7604), 543–546. Available from: doi:10.1038/nature17645 [Accessed: 25 February 2019].

Bryson, V. & Vogel, H.J. (1965) *Evolving genes and proteins : a symposium held at the Institute of Microbiology of Rutgers, the State University, with support from the National Science Foundation*. [Online]. Academic Press. Available from: https://www.sciencedirect.com/science/book/9781483227344#ancpart3 [Accessed: 28 February 2018].

Buffie, C.G., Bucci, V., Stein, R.R., McKenney, P.T., et al. (2014) Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature*. [Online] 517 (7533), 205–208. Available from: doi:10.1038/nature13828 [Accessed: 7 October 2017].

Burrello, C., Garavaglia, F., Cribiù, F.M., Ercoli, G., et al. (2018) Therapeutic faecal microbiota transplantation controls intestinal inflammation through IL10 secretion by immune cells. *Nature Communications*. [Online] 9 (1), 5184. Available from: doi:10.1038/s41467-018-07359-8 [Accessed: 23 February 2019].

Bustos, A.Y., Font de Valdez, G., Fadda, S. & Taranto, M.P. (2018) New insights into bacterial

bile resistance mechanisms: the role of bile salt hydrolase and its impact on human health. *Food Research International*. [Online] 112, 250–262. Available from: doi:10.1016/j.foodres.2018.06.035 [Accessed: 8 March 2019].

Camacho-Ortiz, A., Gutiérrez-Delgado, E.M., Garcia-Mazcorro, J.F., Mendoza-Olazarán, S., et al. (2017) Randomized clinical trial to evaluate the effect of fecal microbiota transplant for initial Clostridium difficile infection in intestinal microbiome John Green (ed.). *PLOS ONE*. [Online] 12 (12), e0189768. Available from: doi:10.1371/journal.pone.0189768 [Accessed: 22 January 2018].

Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanović, M., et al. (2017) European consensus conference on faecal microbiota transplantation in clinical practice. *Gut*. [Online] 66 (4), 569–580. Available from: doi:10.1136/gutjnl-2016-313017 [Accessed: 20 March 2017].

Cammarota, G., Masucci, L., Ianiro, G., Bibbò, S., et al. (2015) Randomised clinical trial: faecal microbiota transplantation by colonoscopy vs. vancomycin for the treatment of recurrent Clostridium difficile infection. *Alimentary Pharmacology and Therapeutics*. [Online] 41 (9), 835–843. Available from: doi:10.1111/apt.13144 [Accessed: 20 March 2017].

Cani, P.D., Van Hul, M., Lefort, C., Depommier, C., et al. (2019) Microbial regulation of organismal energy homeostasis. *Nature Metabolism*. [Online] 1 (1), 34–46. Available from: doi:10.1038/s42255-018-0017-4 [Accessed: 7 March 2019].

Le Cao, Kim-Anh, Rohart F, Gonzalez I, D.S. (n.d.) *CRAN - Package mixOmics*. [Online]. Available from: https://cran.r-project.org/web/packages/mixOmics/index.html [Accessed: 3 January 2018].

Carstens, A., Roos, A., Andreasson, A., Magnuson, A., et al. (2018) Differential clustering of fecal and mucosa-associated microbiota in 'healthy' individuals. *Journal of Digestive Diseases*. [Online] 19 (12), 745–752. Available from: doi:10.1111/1751-2980.12688 [Accessed: 25 February 2019].

Castagliuolo, I., Riegler, M.F., Valenick, L., LaMont, J.T., et al. (1999) Saccharomyces boulardii protease inhibits the effects of Clostridium difficile toxins A and B in human colonic mucosa. *Infection and Immunity*. [Online] 67 (1), 302–307. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9864230 [Accessed: 22 February 2019].

Chang, T.W., Bartlett, J.G., Gorbach, S.L. & Onderdonk, A.B. (1978) Clindamycin-induced

enterocolitis in hamsters as a model of pseudomembranous colitis in patients. *Infection and immunity*. [Online] 20 (2), 526–529. Available from: http://www.ncbi.nlm.nih.gov/pubmed/669810 [Accessed: 21 February 2019].

Chen, T., Kim, C.Y., Kaur, A., Lamothe, L., et al. (2017) Dietary fibre-based SCFA mixtures promote both protection and repair of intestinal epithelial barrier function in a Caco-2 cell model. *Food and Function*. [Online] 8 (3), 1166–1173. Available from: doi:10.1039/c6fo01532h [Accessed: 24 February 2019].

Cheng, Y.-W., Phelps, E., Ganapini, V., Khan, N., et al. (2019) Fecal microbiota transplantation for the treatment of recurrent and severe *Clostridium difficile* infection in solid organ transplant recipients: A multicenter experience. *American Journal of Transplantation*. [Online] 19 (2), 501–511. Available from: doi:10.1111/ajt.15058 [Accessed: 17 February 2019].

Claesson, M.J., Clooney, A.G. & O'Toole, P.W. (2017) A clinician's guide to microbiome analysis. *Nature Reviews Gastroenterology & Hepatology*. [Online] 14 (10), 585–595. Available from: doi:10.1038/nrgastro.2017.97 [Accessed: 1 May 2018].

Claesson, M.J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., et al. (2010) Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Research*. [Online] 38 (22), e200. Available from: doi:10.1093/nar/gkq873 [Accessed: 1 May 2018].

Clements, A.C., Magalhães, R.J.S., Tatem, A.J., Paterson, D.L., et al. (2010) Clostridium difficile PCR ribotype 027: assessing the risks of further worldwide spread. *The Lancet Infectious Diseases*. [Online] 10 (6), 395–404. Available from: doi:10.1016/S1473-3099(10)70080-3 [Accessed: 22 February 2019].

Collins, J., Robinson, C., Danhof, H., Knetsch, C.W., et al. (2018) Dietary trehalose enhances virulence of epidemic Clostridium difficile. *Nature*. [Online] 553 (7688), 291–294. Available from: doi:10.1038/nature25178 [Accessed: 10 March 2019].

Cook & Sellin (1998) Review article: short chain fatty acids in health and disease. *Alimentary Pharmacology and Therapeutics*. [Online] 12 (6), 499–507. Available from: doi:10.1046/j.1365-2036.1998.00337.x [Accessed: 24 February 2019].

Cornely, O.A., Crook, D.W., Esposito, R., Poirier, A., et al. (2012a) Fidaxomicin versus vancomycin for infection with Clostridium difficile in Europe, Canada, and the USA: a

double-blind, non-inferiority, randomised controlled trial. *The Lancet Infectious Diseases*. [Online] 12 (4), 281–289. Available from: doi:10.1016/S1473-3099(11)70374-7 [Accessed: 9 February 2018].

Cornely, O.A., Miller, M.A., Louie, T.J., Crook, D.W., et al. (2012b) Treatment of First Recurrence of Clostridium difficile Infection: Fidaxomicin Versus Vancomycin. *Clinical Infectious Diseases*. [Online] 55 (suppl 2), S154–S161. Available from: doi:10.1093/cid/cis462 [Accessed: 23 February 2019].

Costello, S.P., Conlon, M.A., Vuaran, M.S., Roberts-Thomson, I.C., et al. (2015) Faecal microbiota transplant for recurrent Clostridium difficile infection using long-term frozen stool is effective: Clinical efficacy and bacterial viability data. *Alimentary Pharmacology and Therapeutics*. [Online] 42 (8), 1011–1018. Available from: doi:10.1111/apt.13366.

Costello, S.P., Hughes, P.A., Waters, O., Bryant, R. V., et al. (2019) Effect of Fecal Microbiota Transplantation on 8-Week Remission in Patients With Ulcerative Colitis. *JAMA*. [Online] 321 (2), 156. Available from: doi:10.1001/jama.2018.20046 [Accessed: 20 February 2019].

Costello, S.P., Soo, W., Bryant, R. V., Jairath, V., et al. (2017) Systematic review with meta-analysis: faecal microbiota transplantation for the induction of remission for active ulcerative colitis. *Alimentary Pharmacology & Therapeutics*. [Online] 46 (3), 213–224. Available from: doi:10.1111/apt.14173 [Accessed: 7 October 2017].

Crobach, M.J.T., Planche, T., Eckert, C., Barbut, F., et al. (2016) European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for Clostridium difficile infection. *Clinical Microbiology and Infection*. [Online] 22 Suppl 4, S63-81. Available from: doi:10.1016/j.cmi.2016.03.010 [Accessed: 22 October 2017].

Crowther, G.S., Chilton, C.H., Todhunter, S.L., Nicholson, S., et al. (2014) Comparison of planktonic and biofilm-associated communities of Clostridium difficile and indigenous gut microbiota in a triple-stage chemostat gut model. *Journal of Antimicrobial Chemotherapy*. [Online] 69 (8), 2137–2147. Available from: doi:10.1093/jac/dku116 [Accessed: 9 October 2017].

D'Agostino, R.B., Collins, S.H., Pencina, K.M., Kean, Y., et al. (2014) Risk estimation for recurrent clostridium difficile infection based on clinical factors. *Clinical Infectious Diseases*. [Online] 58 (10), 1386–1393. Available from: doi:10.1093/cid/ciu107

[Accessed: 22 February 2019].

Daley, P., Louie, T., Lutz, J.E., Khanna, S., et al. (2017) Surotomycin versus vancomycin in adults with Clostridium difficile infection: primary clinical outcomes from the second pivotal, randomized, double-blind, Phase 3 trial. *Journal of Antimicrobial Chemotherapy*. [Online] 72 (12), 3462–3470. Available from: doi:10.1093/jac/dkx299 [Accessed: 23 February 2019].

Dancer, S.J., Kirkpatrick, P., Corcoran, D.S., Christison, F., et al. (2013) Approaching zero: temporal effects of a restrictive antibiotic policy on hospital-acquired Clostridium difficile, extended-spectrum β-lactamase-producing coliforms and meticillin-resistant Staphylococcus aureus. *International Journal of Antimicrobial Agents*. [Online] 41 (2), 137–142. Available from: doi:10.1016/j.ijantimicag.2012.10.013 [Accessed: 22 February 2019].

Davies, K.A., Longshaw, C.M., Davis, G.L., Bouza, E., et al. (2014) Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID). *The Lancet Infectious Diseases*. [Online] 14 (12), 1208–1219. Available from: doi:10.1016/S1473-3099(14)70991-0 [Accessed: 6 February 2019].

Dawson, L.F., Valiente, E., Donahue, E.H., Birchenough, G., et al. (2011) Hypervirulent Clostridium difficile PCR-Ribotypes Exhibit Resistance to Widely Used Disinfectants Adam Driks (ed.). *PLoS ONE*. [Online] 6 (10), e25754. Available from: doi:10.1371/journal.pone.0025754 [Accessed: 6 February 2019].

Debast, S.B., Bauer, M.P., Kuijper, E.J., Allerberger, F., et al. (2014) European society of clinical microbiology and infectious diseases: Update of the treatment guidance document for Clostridium difficile infection. *Clinical Microbiology and Infection*. [Online] 20 (S2), 1–26. Available from: doi:10.1111/1469-0691.12418 [Accessed: 2 October 2017].

Department of Health (2012) *Clostridium difficile: updated guidance on diagnosis and reporting - GOV.UK*. [Online]. 2012. Available from: https://www.gov.uk/government/publications/updated-guidance-on-the-diagnosis-and-reporting-of-clostridium-difficile [Accessed: 23 February 2019].

Dermadi, D., Valo, S., Ollila, S., Soliymani, R., et al. (2017) Western diet deregulates bile acid homeostasis, cell proliferation, and tumorigenesis in colon. *Cancer Research*. [Online]

77 (12), 3352–3363. Available from: doi:10.1158/0008-5472.CAN-16-2860.

Devendran, S., Shrestha, R., Alves, J.M.P., Wolf, P.G., et al. (2019) *Clostridium scindens* ATCC 35704: integration of nutritional requirements, the complete genome sequence, and global transcriptional responses to bile acids. *Applied and Environmental Microbiology*. [Online] Available from: doi:10.1128/AEM.00052-19 [Accessed: 5 March 2019].

Dong, W., Liu, L., Dou, Y., Xu, M., et al. (2018) Deoxycholic acid activates epidermal growth factor receptor and promotes intestinal carcinogenesis by ADAM 17-dependent ligand release. *Journal of Cellular and Molecular Medicine*. [Online] 22 (9), 4263–4273. Available from: doi:10.1111/jcmm.13709 [Accessed: 6 March 2019].

Dong, Z. & Lee, B.H. (2018) Bile salt hydrolases: Structure and function, substrate preference, and inhibitor development. *Protein Science*. [Online] 27 (10), 1742–1754. Available from: doi:10.1002/pro.3484 [Accessed: 8 March 2019].

Draper, L.A., Ryan, F.J., Smith, M.K., Jalanka, J., et al. (2018) Long-term colonisation with donor bacteriophages following successful faecal microbial transplantation. *Microbiome*. [Online] 6 (1), 220. Available from: doi:10.1186/s40168-018-0598-x [Accessed: 21 December 2018].

Dubberke, E.R., Lee, C.H., Orenstein, R., Khanna, S., et al. (2018) Results From a Randomized, Placebo-Controlled Clinical Trial of a RBX2660—A Microbiota-Based Drug for the Prevention of Recurrent Clostridium difficile Infection. *Clinical Infectious Diseases*. [Online] 67 (8), 1198–1204. Available from: doi:10.1093/cid/ciy259 [Accessed: 17 February 2019].

Eiseman, B., Silen, W., Bascom, G.S. & Kauvar, A.J. (1958) Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. *Surgery*. [Online] 44 (5), 854–859. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13592638 [Accessed: 17 February 2019].

Enache-Angoulvant, A. & Hennequin, C. (2005) Invasive Saccharomyces Infection: A Comprehensive Review. *Clinical Infectious Diseases*. [Online] 41 (11), 1559–1568. Available from: doi:10.1086/497832 [Accessed: 22 February 2019].

Eriksson, L., Trygg, J. & Wold, S. (2008) CV-ANOVA for significance testing of PLS and OPLS® models. *Journal of Chemometrics*. [Online] 22 (11–12), 594–600. Available from: doi:10.1002/cem.1187 [Accessed: 1 February 2018].

Farowski, F., Solbach, P., Tsakmaklis, A., Brodesser, S., et al. (2019) Potential biomarkers to

predict outcome of faecal microbiota transfer for recurrent Clostridioides difficile infection. *Digestive and Liver Disease*. [Online] Available from: doi:10.1016/j.dld.2019.01.012 [Accessed: 28 February 2019].

Farrow, M.A., Chumbler, N.M., Lapierre, L.A., Franklin, J.L., et al. (2013) Clostridium difficile toxin B-induced necrosis is mediated by the host epithelial cell NADPH oxidase complex. *Proceedings of the National Academy of Sciences*. [Online] 110 (46), 18674–18679. Available from: doi:10.1073/pnas.1313658110 [Accessed: 8 March 2019].

Feil, W., Cosentini, E., Riegler, M., Bischof, G., et al. (2008) Clostridium difficile toxin B is more potent than toxin A in damaging human colonic epithelium in vitro. *Journal of Clinical Investigation*. [Online] 95 (5), 2004–2011. Available from: doi:10.1172/jci117885 [Accessed: 22 February 2019].

Fekety, R., Silva, J., Kauffman, C., Buggy, B., et al. (1989) Treatment of antibiotic-associated Clostridium difficile colitis with oral vancomycin: comparison of two dosage regimens. *American Journal of Medicine*. [Online] 86 (1), 15–19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2910090 [Accessed: 23 February 2019].

Ferreyra, J.A., Wu, K.J., Hryckowian, A.J., Bouley, D.M., et al. (2014) Gut microbiota-produced succinate promotes C. Difficile infection after antibiotic treatment or motility disturbance. *Cell Host and Microbe*. [Online] 16 (6), 770–777. Available from: doi:10.1016/j.chom.2014.11.003 [Accessed: 10 March 2019].

Feuerstadt, P., Das, R. & Brandt, L.J. (2014) The Evolution of Urban C. difficile Infection (CDI): CDI in 2009–2011 Is Less Severe and has Better Outcomes Than CDI in 2006–2008. *The American Journal of Gastroenterology*. [Online] 109 (8), 1265–1276. Available from: doi:10.1038/ajg.2014.167 [Accessed: 21 February 2019].

Fischer, M., Sipe, B.W., Rogers, N.A., Cook, G.K., et al. (2015) Faecal microbiota transplantation plus selected use of vancomycin for severe-complicated *Clostridium difficile* infection: description of a protocol with high success rate. *Alimentary Pharmacology & Therapeutics*. [Online] 42 (4), 470–476. Available from: doi:10.1111/apt.13290 [Accessed: 6 October 2017].

Flint, H.J., Duncan, S.H., Scott, K.P. & Louis, P. (2015) Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society*. [Online] 74 (01), 13–22. Available from: doi:10.1017/S0029665114001463 [Accessed: 4 March 2019].

Foley, M.H., O'Flaherty, S., Barrangou, R. & Theriot, C.M. (2019) Bile salt hydrolases:

Gatekeepers of bile acid metabolism and host-microbiome crosstalk in the gastrointestinal tract Laura J. Knoll (ed.). *PLOS Pathogens*. [Online] 15 (3), e1007581. Available from: doi:10.1371/journal.ppat.1007581 [Accessed: 8 March 2019].

Fonville, J.M., Richards, S.E., Barton, R.H., Boulange, C.L., et al. (2010) The evolution of partial least squares models and related chemometric approaches in metabonomics and metabolic phenotyping. *Journal of Chemometrics*. [Online] 24 (11–12), 636–649. Available from: doi:10.1002/cem.1359 [Accessed: 26 February 2019].

Foundation, R. (n.d.) *R: The R Project for Statistical Computing*. [Online]. Available from: https://www.r-project.org/ [Accessed: 27 November 2017].

Francis, M.B., Allen, C.A., Shrestha, R. & Sorg, J.A. (2013) Bile Acid Recognition by the Clostridium difficile Germinant Receptor, CspC, Is Important for Establishing Infection Michael R. Wessels (ed.). *PLoS Pathogens*. [Online] 9 (5), e1003356. Available from: doi:10.1371/journal.ppat.1003356 [Accessed: 18 January 2018].

Freedberg, D.E., Salmasian, H., Cohen, B., Abrams, J.A., et al. (2016) Receipt of antibiotics in hospitalized patients and risk for clostridium difficile infection in subsequent patients who occupy the same bed. *JAMA Internal Medicine*. [Online] 176 (12), 1801–1808. Available from: doi:10.1001/jamainternmed.2016.6193 [Accessed: 22 February 2019].

Freeman, J., O 'Neill, F.J. & Wilcox, M.H. (2003) Effects of cefotaxime and desacetylcefotaxime upon Clostridium difficile proliferation and toxin production in a triple-stage chemostat model of the human gut. *Journal of Antimicrobial Chemotherapy*. [Online] 52, 96–102. Available from: doi:10.1093/jac/dkg267 [Accessed: 3 January 2018].

Friedman, E.S., Li, Y., Shen, T.-C.D., Jiang, J., et al. (2018) FXR-Dependent Modulation of the Human Small Intestinal Microbiome by the Bile Acid Derivative Obeticholic Acid. *Gastroenterology*. [Online] Available from: doi:10.1053/J.GASTRO.2018.08.022 [Accessed: 8 October 2018].

Fu, T., Coulter, S., Yoshihara, E., Oh, T.G., et al. (2019) FXR Regulates Intestinal Cancer Stem Cell Proliferation. *Cell*. [Online] 176 (5), 1098-1112.e18. Available from: doi:10.1016/J.CELL.2019.01.036 [Accessed: 8 March 2019].

Fuentes, S., van Nood, E., Tims, S., Heikamp-de Jong, I., et al. (2014) Reset of a critically disturbed microbial ecosystem: faecal transplant in recurrent Clostridium difficile infection. *The ISME Journal*. [Online] 8 (8), 1621–1633. Available from:

doi:10.1038/ismej.2014.13 [Accessed: 7 December 2018].

Gadaleta, R.M., van Erpecum, K.J., Oldenburg, B., Willemsen, E.C.L., et al. (2011) Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. *Gut*. [Online] 60 (4), 463–472. Available from: doi:10.1136/gut.2010.212159 [Accessed: 7 March 2019].

García-Villalba, R., Giménez-Bastida, J.A., García-Conesa, M.T., Tomás-Barberán, F.A., et al. (2012) Alternative method for gas chromatography-mass spectrometry analysis of short-chain fatty acids in faecal samples. *Journal of Separation Science*. [Online] 35 (15), 1906–1913. Available from: doi:10.1002/jssc.201101121 [Accessed: 23 February 2018].

Geng, W. & Lin, J. (2016) Bacterial bile salt hydrolase: An intestinal microbiome target for enhanced animal health. *Animal Health Research Reviews*. [Online]. 17 (2) pp.148–158. Available from: doi:10.1017/S1466252316000153 [Accessed: 24 February 2019].

Gerber, M. & Ackermann, G. (2008) OPT-80, a macrocyclic antimicrobial agent for the treatment of Clostridium difficile infections: a review. *Expert Opinion on Investigational Drugs*. [Online] 17 (4), 547–553. Available from: doi:10.1517/13543784.17.4.547 [Accessed: 23 February 2019].

Gerding, D.N., Cornely, O.A., Grill, S., Kracker, H., et al. (2019) Cadazolid for the treatment of Clostridium difficile infection: results of two double-blind, placebo-controlled, non-inferiority, randomised phase 3 trials. *The Lancet Infectious Diseases*. [Online] Available from: doi:10.1016/S1473-3099(18)30614-5 [Accessed: 23 February 2019].

Gerding, D.N., Johnson, S., Peterson, L.R., Mulligan, M.E., et al. (1995) Clostridium difficile-associated diarrhea and colitis. *Infection Control & Hospital Epidemiology*. [Online] 16 (8), 459–477. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7594392 [Accessed: 21 February 2019].

Gerding, D.N., Meyer, T., Lee, C., Cohen, S.H., et al. (2015) Administration of Spores of Nontoxigenic *Clostridium difficile* Strain M3 for Prevention of Recurrent *C difficile* Infection. *JAMA*. [Online] 313 (17), 1719. Available from: doi:10.1001/jama.2015.3725 [Accessed: 6 October 2017].

Geric, B., Rupnik, M., Gerding, D.N., Grabnar, M., et al. (2004) Distribution of Clostridium difficile variant toxinotypes and strains with binary toxin genes among clinical isolates in an American hospital. *Journal of Medical Microbiology*. [Online] 53 (9), 887–894.

Available from: doi:10.1099/jmm.0.45610-0 [Accessed: 22 February 2019].

Gill, H.S. (2003) Probiotics to enhance anti-infective defences in the gastrointestinal tract. *Bailliere's Best Practice and Research in Clinical Gastroenterology*. [Online]. 17 (5) pp.755–773. Available from: doi:10.1016/S1521-6918(03)00074-X [Accessed: 23 February 2019].

Gill, H.S., Rutherfurd, K.J. & Cross, M.L. (2001) Dietary probiotic supplementation enhances natural killer cell activity in the elderly: An investigation of age-related immunological changes. *Journal of Clinical Immunology*. [Online] 21 (4), 264–271. Available from: doi:10.1023/A:1010979225018 [Accessed: 23 February 2019].

Glauben, R., Batra, A., Fedke, I., Zeitz, M., et al. (2006) Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *Journal of Immunology*. [Online] 176 (8), 5015–5022. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16585598 [Accessed: 14 March 2019].

Goldenberg, J.Z., Yap, C., Lytvyn, L., Lo, C.K.F., et al. (2017) Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. *Cochrane Database of Systematic Reviews*. [Online]. 2017 (12) p.CD006095. Available from: doi:10.1002/14651858.CD006095.pub4 [Accessed: 23 February 2019].

Gonçalves, P., Araújo, J.R. & Di Santo, J.P. (2018) A Cross-Talk Between Microbiota-Derived Short-Chain Fatty Acids and the Host Mucosal Immune System Regulates Intestinal Homeostasis and Inflammatory Bowel Disease. *Inflammatory Bowel Diseases*. [Online] 24 (3), 558–572. Available from: doi:10.1093/ibd/izx029 [Accessed: 7 March 2019].

Gonzalez, I., Déjean, S., Martin, P. & Baccini, A. (2008) **CCA** : An *R* Package to Extend Canonical Correlation Analysis. *Journal of Statistical Software*. [Online] 23 (12), 1–14. Available from: doi:10.18637/jss.v023.i12 [Accessed: 27 November 2017].

Gould, L.H. & Limbago, B. (2010) Clostridium difficile in food and domestic animals: a new foodborne pathogen? *Clinical Infectious Diseases*. [Online] 51 (5), 577–582. Available from: doi:10.1086/655692 [Accessed: 22 February 2019].

Goy, G., Widmer, A.F., Rudin, S., Frei, R., et al. (2007) Rapid and Reliable Diagnostic Algorithm for Detection of Clostridium difficile. *Journal of Clinical Microbiology*. [Online] 46 (1), 328–330. Available from: doi:10.1128/jcm.01503-07 [Accessed: 23 February 2019].

Gratton, J., Phetcharaburanin, J., Mullish, B.H., Williams, H.R.T., et al. (2016) Optimized

Sample Handling Strategy for Metabolic Profiling of Human Feces. *Analytical Chemistry*. [Online] 88 (9). Available from: doi:10.1021/acs.analchem.5b04159.

de Groot, P., Scheithauer, T., Bakker, G.J., Prodan, A., et al. (2019) Donor metabolic characteristics drive effects of faecal microbiota transplantation on recipient insulin sensitivity, energy expenditure and intestinal transit time. *Gut*. [Online] Available from: doi:10.1136/gutjnl-2019-318320.

de Groot, P.F., Keller, J.J., van Beurden, Y.H., Nieuwdorp, M., et al. (2016) Complications, effectiveness, and long term follow-up of fecal microbiota transfer by nasoduodenal tube for treatment of recurrent Clostridium difficile infection. *United European Gastroenterology Journal*. [Online] 5 (6), 868–879. Available from: doi:10.1177/2050640616678099 [Accessed: 19 January 2018].

Guery, B., Menichetti, F., Anttila, V.J., Adomakoh, N., et al. (2017) Extended-pulsed fidaxomicin versus vancomycin for Clostridium difficile infection in patients 60 years and older (EXTEND): A randomised, controlled, open-label, phase 3b/4 trial. *The Lancet Infectious Diseases*. [Online] 18 (3), 296–307. Available from: doi:10.1016/S1473-3099(17)30751-X [Accessed: 31 January 2018].

de Gunzburg, J., Ghozlane, A., Ducher, A., Le Chatelier, E., et al. (2018) Protection of the Human Gut Microbiome From Antibiotics. *The Journal of Infectious Diseases*. [Online] 217 (4), 628–636. Available from: doi:10.1093/infdis/jix604 [Accessed: 23 February 2019].

Halkjær, S.I., Christensen, A.H., Lo, B.Z.S., Browne, P.D., et al. (2018) Faecal microbiota transplantation alters gut microbiota in patients with irritable bowel syndrome: results from a randomised, double-blind placebo-controlled study. *Gut*. [Online] 67 (12), 2107–2115. Available from: doi:10.1136/gutjnl-2018-316434 [Accessed: 22 February 2019].

Hall, I.C. & O'Toole, E. (1935) Intestinal flora in new-born infants. *American Journal of Diseases of Children*. [Online] 49 (2), 390. Available from: doi:10.1001/archpedi.1935.01970020105010 [Accessed: 21 February 2019].

Hamilton, M.J., Weingarden, A.R., Sadowsky, M.J. & Khoruts, A. (2012) Standardized Frozen Preparation for Transplantation of Fecal Microbiota for Recurrent Clostridium difficile Infection. *The American Journal of Gastroenterology*. [Online] 107 (5), 761–767. Available from: doi:10.1038/ajg.2011.482 [Accessed: 20 March 2017].

Hamilton, M.J., Weingarden, A.R., Unno, T., Khoruts, A., et al. (2013) High-throughput DNA

sequence analysis reveals stable engraftment of gut microbiota following transplantation of previously frozen fecal bacteria. *Gut Microbes*. [Online] 4 (2), 125–135. Available from: doi:10.4161/gmic.23571 [Accessed: 27 February 2019].

Hargreaves, K.R. & Clokie, M.R.J. (2015) A taxonomic review of Clostridium difficile phages and proposal of a novel genus, 'Phimmp04likevirus'. *Viruses*. [Online] 7 (5), 2534–2541. Available from: doi:10.3390/v7052534 [Accessed: 20 February 2019].

Hasegawa, M., Kamada, N., Jiao, Y., Liu, M.Z., et al. (2012) Protective Role of Commensals against Clostridium difficile Infection via an IL-1 -Mediated Positive-Feedback Loop. *The Journal of Immunology*. [Online] 189 (6), 3085–3091. Available from: doi:10.4049/jimmunol.1200821 [Accessed: 8 March 2019].

He, M., Miyajima, F., Roberts, P., Ellison, L., et al. (2013) Emergence and global spread of epidemic healthcare-associated Clostridium difficile. *Nature Genetics*. [Online] 45 (1), 109–113. Available from: doi:10.1038/ng.2478 [Accessed: 22 February 2019].

Hecht, G., Koutsouris, A., Pothoulakis, C., LaMont, J.T., et al. (1992) Clostridium difficile toxin B disrupts the barrier function of T84 monolayers. *Gastroenterology*. [Online] 102 (2), 416–423. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1732112 [Accessed: 22 February 2019].

Hecker, M.T., Obrenovich, M.E., Cadnum, J.L., Jencson, A.L., et al. (2016) Fecal Microbiota Transplantation by Freeze-Dried Oral Capsules for Recurrent Clostridium difficile Infection. *Open forum infectious diseases*. [Online] 3 (2), ofw091. Available from: doi:10.1093/ofid/ofw091 [Accessed: 31 January 2018].

Heimann, E., Nyman, M., Pålbrink, A.-K., Lindkvist-Petersson, K., et al. (2016) Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes. *Adipocyte*. [Online] 5 (4), 359–368. Available from: doi:10.1080/21623945.2016.1252011 [Accessed: 4 March 2019].

Hickson, M., D'Souza, A.L., Muthu, N., Rogers, T.R., et al. (2007) Use of probiotic Lactobacillus preparation to prevent diarrhoea associated with antibiotics: Randomised double blind placebo controlled trial. *British Medical Journal*. [Online] 335 (7610), 80–83. Available from: doi:10.1136/bmj.39231.599815.55 [Accessed: 14 July 2019].

Hildebrand, F., Li, S.S., Benes, V., Salojärvi, J., et al. (2016) Durable coexistence of donor and recipient strains after fecal microbiota transplantation. *Science*. [Online] 352 (6285), 586–589. Available from: doi:10.1126/science.aad8852 [Accessed: 23 March 2019].

Hofmann, A.F. (1999) The Continuing Importance of Bile Acids in Liver and Intestinal Disease. *Archives of Internal Medicine*. [Online] 159 (22), 2647. Available from: doi:10.1001/archinte.159.22.2647 [Accessed: 23 November 2017].

Hooper, L. V., Littman, D.R. & Macpherson, A.J. (2012) Interactions Between the Microbiota and the Immune System. *Science*. [Online] 336 (6086), 1268–1273. Available from: doi:10.1126/science.1223490 [Accessed: 7 March 2019].

Hota, S.S., Sales, V., Tomlinson, G., Salpeter, M.J., et al. (2017) Oral Vancomycin Followed by Fecal Transplantation Versus Tapering Oral Vancomycin Treatment for Recurrent *Clostridium difficile* Infection: An Open-Label, Randomized Controlled Trial. *Clinical Infectious Diseases*. [Online] 64 (3), 265–271. Available from: doi:10.1093/cid/ciw731 [Accessed: 10 October 2017].

Howell, M.D., Novack, V., Grgurich, P., Soulliard, D., et al. (2010) Iatrogenic gastric acid suppression and the risk of nosocomial Clostridium difficile infection. *Archives of Internal Medicine*. [Online] 170 (9), 784–790. Available from: doi:10.1001/archinternmed.2010.89 [Accessed: 22 February 2019].

Howerton, A., Patra, M. & Abel-Santos, E. (2013) A New Strategy for the Prevention of Clostridium difficile Infection. *The Journal of Infectious Diseases*. [Online] 207 (10), 1498–1504. Available from: doi:10.1093/infdis/jit068 [Accessed: 25 January 2018].

Hryckowian, A.J., Van Treuren, W., Smits, S.A., Davis, N.M., et al. (2018) Microbiota-accessible carbohydrates suppress Clostridium difficile infection in a murine model. *Nature Microbiology*. [Online] 3 (6), 662–669. Available from: doi:10.1038/s41564-018-0150-6 [Accessed: 25 February 2019].

Hu, M.Y., Maroo, S., Kyne, L., Cloud, J., et al. (2008) A Prospective Study of Risk Factors and Historical Trends in Metronidazole Failure for Clostridium difficile Infection. *Clinical Gastroenterology and Hepatology*. [Online] 6 (12), 1354–1360. Available from: doi:10.1016/j.cgh.2008.06.024 [Accessed: 23 February 2019].

Huang, C., Wang, J., Hu, W., Wang, C., et al. (2016) Identification of functional farnesoid X receptors in brain neurons. *FEBS Letters*. [Online] 590 (18), 3233–3242. Available from: doi:10.1002/1873-3468.12373 [Accessed: 7 March 2019].

Huang, H., Fang, H., Weintraub, A. & Nord, C.E. (2009) Distinct ribotypes and rates of antimicrobial drug resistance in Clostridium difficile from Shanghai and Stockholm. *Clinical Microbiology and Infection*. [Online] 15 (12), 1170–1173. Available from:

doi:10.1111/J.1469-0691.2009.02992.X [Accessed: 6 February 2019].

Huttner, B.D., de Lastours, V., Wassenberg, M., Maharshak, N., et al. (2019) A five-day course of oral antibiotics followed by faecal transplantation to eradicate carriage of multidrug-resistant Enterobacteriaceae: A Randomized Clinical Trial. *Clinical Microbiology and Infection*. [Online] Available from: doi:10.1016/j.cmi.2018.12.009 [Accessed: 12 January 2019].

Hutton, M.L., Mackin, K.E., Chakravorty, A. & Lyras, D. (2014) Small animal models for the study of Clostridium difficile disease pathogenesis. *FEMS Microbiology Letters*. [Online]. 352 (2) pp.140–149. Available from: doi:10.1111/1574-6968.12367 [Accessed: 16 July 2019].

Hvas, C.L., Jørgensen, S.M.D., Jørgensen, S.P., Storgaard, M., et al. (2019) Fecal Microbiota Transplantation is Superior to Fidaxomicin for Treatment of Recurrent Clostridium difficile Infection. *Gastroenterology*. [Online] 0 (0). Available from: doi:S0016508518354349 [Accessed: 31 January 2019].

Ianiro, G., Masucci, L., Quaranta, G., Simonelli, C., et al. (2018) Randomised clinical trial: faecal microbiota transplantation by colonoscopy plus vancomycin for the treatment of severe refractory *Clostridium difficile* infection-single versus multiple infusions. *Alimentary Pharmacology & Therapeutics*. [Online] 48 (2), 152–159. Available from: doi:10.1111/apt.14816 [Accessed: 17 February 2019].

Illumina (n.d.) *16S Metagenomic Sequencing Library Preparation*. [Online]. Available from: https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html [Accessed: 27 November 2017a].

Illumina (n.d.) *MiSeq Specifications*. [Online]. Available from: https://emea.illumina.com/systems/sequencing-platforms/miseq/specifications.html [Accessed: 1 May 2018b].

Imhann, F., Bonder, M.J., Vich Vila, A., Fu, J., et al. (2016) Proton pump inhibitors affect the gut microbiome. *Gut*. [Online] 65 (5), 740–748. Available from: doi:10.1136/gutjnl-2015-310376.

Inagaki, T., Moschetta, A., Lee, Y.-K., Peng, L., et al. (2006) Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proceedings of the National Academy of Sciences*. [Online] 103 (10), 3920–3925. Available from: doi:10.1073/pnas.0509592103 [Accessed: 26 June 2018].

Islam, K.B.M.S., Fukiya, S., Hagio, M., Fujii, N., et al. (2011) Bile Acid Is a Host Factor That Regulates the Composition of the Cecal Microbiota in Rats. *Gastroenterology*. [Online] 141 (5), 1773–1781. Available from: doi:10.1053/J.GASTRO.2011.07.046 [Accessed: 8 March 2019].

Iwai, S., Weinmaier, T., Schmidt, B.L., Albertson, D.G., et al. (2016) Piphillin: Improved Prediction of Metagenomic Content by Direct Inference from Human Microbiomes Zhili He (ed.). *PLOS ONE*. [Online] 11 (11), e0166104. Available from: doi:10.1371/journal.pone.0166104 [Accessed: 8 October 2017].

Jalanka, J., Hillamaa, A., Satokari, R., Mattila, E., et al. (2018) The long-term effects of faecal microbiota transplantation for gastrointestinal symptoms and general health in patients with recurrent *Clostridium difficile* infection. *Alimentary Pharmacology & Therapeutics*. [Online] 47 (3), 371–379. Available from: doi:10.1111/apt.14443 [Accessed: 26 February 2018].

Jalanka, J., Mattila, E., Jouhten, H., Hartman, J., et al. (2016) Long-term effects on luminal and mucosal microbiota and commonly acquired taxa in faecal microbiota transplantation for recurrent Clostridium difficile infection. *BMC Medicine*. [Online] 14 (1), 155. Available from: doi:10.1186/s12916-016-0698-z [Accessed: 7 December 2018].

Janda, J.M. & Abbott, S.L. (2007) 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *Journal of Clinical Microbiology*. [Online] 45 (9), 2761–2764. Available from: doi:10.1128/JCM.01228-07 [Accessed: 25 February 2019].

Jiang, Z.D., Ajami, N.J., Petrosino, J.F., Jun, G., et al. (2017) Randomised clinical trial: faecal microbiota transplantation for recurrent Clostridum difficile infection – fresh, or frozen, or lyophilised microbiota from a small pool of healthy donors delivered by colonoscopy. *Alimentary Pharmacology and Therapeutics*. [Online] 45 (7), 899–908. Available from: doi:10.1111/apt.13969 [Accessed: 6 October 2017].

Jiang, Z.D., Garey, K.W., Price, M., Graham, G., et al. (2007) Association of Interleukin-8 Polymorphism and Immunoglobulin G Anti-Toxin A in Patients With Clostridium difficile-Associated Diarrhea. *Clinical Gastroenterology and Hepatology*. [Online] 5 (8), 964–968. Available from: doi:10.1016/j.cgh.2007.04.018 [Accessed: 22 February 2019].

Johnsen, P.H., Hilpüsch, F., Cavanagh, J.P., Leikanger, I.S., et al. (2018) Faecal microbiota transplantation versus placebo for moderate-to-severe irritable bowel syndrome: a

double-blind, randomised, placebo-controlled, parallel-group, single-centre trial. *The Lancet Gastroenterology and Hepatology*. [Online] 3 (1), 17–24. Available from: doi:10.1016/S2468-1253(17)30338-2 [Accessed: 31 January 2018].

Johnson, S., Louie, T.J., Gerding, D.N., Cornely, O.A., et al. (2014) Vancomycin, Metronidazole, or Tolevamer for Clostridium difficile Infection: Results From Two Multinational, Randomized, Controlled Trials. *Clinical Infectious Diseases*. [Online] 59 (3), 345–354. Available from: doi:10.1093/cid/ciu313 [Accessed: 21 February 2019].

Joint FAO/WHO Working Group Report (2002) *Food and Agriculture Organization of the United Nations World Health Organization*. [Online]. Available from: http://www.fao.org/es/ESN/Probio/probio.htm [Accessed: 23 February 2019].

Jones, B. V, Begley, M., Hill, C., Gahan, C.G.M., et al. (2008) Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America*. [Online] 105 (36), 13580–13585. Available from: doi:10.1073/pnas.0804437105 [Accessed: 7 October 2017].

Jones, R.B., Zhu, X., Moan, E., Murff, H.J., et al. (2018) Inter-niche and inter-individual variation in gut microbial community assessment using stool, rectal swab, and mucosal samples. *Scientific Reports*. [Online] 8 (1), 4139. Available from: doi:10.1038/s41598-018-22408-4 [Accessed: 25 February 2019].

Joyce, S.A., MacSharry, J., Casey, P.G., Kinsella, M., et al. (2014) Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. *Proceedings of the National Academy of Sciences of the United States of America*. [Online] 111 (20), 7421–7426. Available from: doi:10.1073/pnas.1323599111 [Accessed: 4 January 2018].

Juul, F.E., Garborg, K., Bretthauer, M., Skudal, H., et al. (2018) Fecal Microbiota Transplantation for Primary *Clostridium difficile* Infection. *New England Journal of Medicine*. [Online] 378 (26), 2535–2536. Available from: doi:10.1056/NEJMc1803103 [Accessed: 17 February 2019].

Kang, D.-W., Adams, J.B., Gregory, A.C., Borody, T., et al. (2017) Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. *Microbiome*. [Online] 5 (1), 10. Available from: doi:10.1186/s40168-016-0225-7 [Accessed: 22 February 2019].

Kang, J.D., Myers, C.J., Harris, S.C., Kakiyama, G., et al. (2019) Bile Acid 7α-Dehydroxylating

Gut Bacteria Secrete Antibiotics that Inhibit Clostridium difficile: Role of Secondary Bile Acids. *Cell Chemical Biology*. [Online] 26 (1), 27-34.e4. Available from: doi:10.1016/J.CHEMBIOL.2018.10.003 [Accessed: 9 March 2019].

Kao, D., Roach, B., Silva, M., Beck, P., et al. (2017) Effect of oral capsule– vs colonoscopy-delivered fecal microbiota transplantation on recurrent Clostridium difficile infection: A randomized clinical trial. *JAMA - Journal of the American Medical Association*. [Online] 318 (20), 1985–1993. Available from: doi:10.1001/jama.2017.17077 [Accessed: 12 December 2017].

Kedderis, G.L., Argenbright, L.S. & Miwa, G.T. (1989) Covalent Interaction of 5-Nitroimidazoles with DNA and Protein in Vitro: Mechanism of Reductive Activation. *Chemical Research in Toxicology*. [Online] 2 (3), 146–149. Available from: doi:10.1021/tx00009a004 [Accessed: 21 February 2019].

Kelesidis, T. & Pothoulakis, C. (2012) Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. *Therapeutic Advances in Gastroenterology*. [Online] 5 (2), 111–125. Available from: doi:10.1177/1756283X11428502 [Accessed: 22 February 2019].

Kellingray, L., Gall, G. Le, Defernez, M., Beales, I.L.P., et al. (2018) Microbial taxonomic and metabolic alterations during faecal microbiota transplantation to treat Clostridium difficile infection. *Journal of Infection*. [Online] 77 (2), 107–118. Available from: doi:10.1016/j.jinf.2018.04.012 [Accessed: 4 March 2019].

Kelly, C.J., Zheng, L., Campbell, E.L., Saeedi, B., et al. (2015a) Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell Host & Microbe*. [Online] 17 (5), 662–671. Available from: doi:10.1016/j.chom.2015.03.005 [Accessed: 7 March 2019].

Kelly, C.P., Aboudola, S., Michetti, P., Warny, M., et al. (2002) Saccharomyces boulardii Stimulates Intestinal Immunoglobulin A Immune Response to Clostridium difficile Toxin A in Mice. *Infection and Immunity*. [Online] 69 (4), 2762–2765. Available from: doi:10.1128/iai.69.4.2762-2765.2001 [Accessed: 22 February 2019].

Kelly, C.P., Chong Nguyen, C., Palmieri, L.J., Pallav, K., et al. (2019) Saccharomyces boulardii CNCM I-745 Modulates the Fecal Bile Acids Metabolism During Antimicrobial Therapy in Healthy Volunteers. *Frontiers in Microbiology*. [Online] 10, 336. Available from: doi:10.3389/fmicb.2019.00336 [Accessed: 15 March 2019].

Kelly, C.P. & LaMont, J.T. (2008) *Clostridium difficile* — More Difficult Than Ever. *New England Journal of Medicine*. [Online] 359 (18), 1932–1940. Available from: doi:10.1056/NEJMra0707500 [Accessed: 20 March 2017].

Kelly, C.P., Pothoulakis, C. & LaMont, J.T. (1994) Clostridium difficile Colitis. *New England Journal of Medicine*. [Online] 330 (4), 257–262. Available from: doi:10.1056/NEJM199401273300406 [Accessed: 22 February 2019].

Kelly, C.R., Ihunnah, C., Fischer, M., Khoruts, A., et al. (2014) Fecal microbiota transplant for treatment of Clostridium difficile infection in immunocompromised patients. *Am J Gastroenterol*. [Online] 109 (7), 1065–1071. Available from: doi:10.1038/ajg.2014.133 [Accessed: 20 March 2017].

Kelly, C.R., Kahn, S., Kashyap, P., Laine, L., et al. (2015b) Update on Fecal Microbiota Transplantation 2015: Indications, Methodologies, Mechanisms, and Outlook. *Gastroenterology*. [Online] 149 (1), 223–237. Available from: doi:10.1053/j.gastro.2015.05.008 [Accessed: 3 January 2018].

Kelly, C.R., Khoruts, A., Staley, C., Sadowsky, M.J., et al. (2016) Effect of Fecal Microbiota Transplantation on Recurrence in Multiply Recurrent Clostridium difficile Infection. *Annals of Internal Medicine*. [Online] 165 (9), 609–616. Available from: doi:10.7326/M16-0271 [Accessed: 6 October 2017].

Kelly, C.R., Kim, A.M., Laine, L. & Wu, G.D. (2017) The AGA's Fecal Microbiota Transplantation National Registry: An Important Step Toward Understanding Risks and Benefits of Microbiota Therapeutics. *Gastroenterology*. [Online] 152 (4), 681–684. Available from: doi:10.1053/j.gastro.2017.01.028 [Accessed: 9 October 2017].

Khanna, S., Pardi, D.S., Aronson, S.L., Kammer, P.P., et al. (2012) The epidemiology of community-acquired clostridium difficile infection: A population-based study. *American Journal of Gastroenterology*. [Online] 107 (1), 89–95. Available from: doi:10.1038/ajg.2011.398 [Accessed: 22 February 2019].

Khanna, S., Pardi, D.S., Kelly, C.R., Kraft, C.S., et al. (2016) A Novel Microbiome Therapeutic Increases Gut Microbial Diversity and Prevents Recurrent *Clostridium difficile* Infection. *Journal of Infectious Diseases*. [Online] 214 (2), 173–181. Available from: doi:10.1093/infdis/jiv766 [Accessed: 3 October 2017].

Khoruts, A., Dicksved, J., Jansson, J.K. & Sadowsky, M.J. (2009) Changes in the Composition of the Human Fecal Microbiome After Bacteriotherapy for Recurrent Clostridium

Difficile-associated Diarrhea. *Journal of Clinical Gastroenterology*. [Online] 44 (5), 1. Available from: doi:10.1097/MCG.0b013e3181c87e02 [Accessed: 24 February 2019].

Khoruts, A. & Sadowsky, M.J. (2016) Understanding the mechanisms of faecal microbiota transplantation. *Nature Reviews Gastroenterology & Hepatology*. [Online] 13 (9), 508–516. Available from: doi:10.1038/nrgastro.2016.98 [Accessed: 3 October 2017].

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., et al. (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biology*. [Online] 8 (6), e1000412. Available from: doi:10.1371/journal.pbio.1000412 [Accessed: 6 February 2019].

King, A., Mullish, B.H., Williams, H.R.T. & Aylin, P. (2017) Comparative epidemiology of Clostridium difficile infection: England and the USA. *International Journal for Quality in Health Care*. [Online] 29 (6), 785–791. Available from: doi:10.1093/intqhc/mzx120 [Accessed: 6 October 2017].

Kitahara, M., Takamine, F., Imamura, T. & Benno, Y. (2000) Assignment of Eubacterium sp. VPI 12708 and related strains with high bile acid 7alpha-dehydroxylating activity to Clostridium scindens and proposal of Clostridium hylemonae sp. nov., isolated from human faeces. *International Journal of Systemic and Evoluntionary Microbiology*. [Online] 50 (3), 971–978. Available from: doi:10.1099/00207713-50-3-971 [Accessed: 29 November 2017].

Knights, D., Kuczynski, J., Charlson, E.S., Zaneveld, J., et al. (2011) Bayesian community-wide culture-independent microbial source tracking. *Nature Methods*. [Online] 8 (9), 761–763. Available from: doi:10.1038/nmeth.1650 [Accessed: 27 February 2019].

Kokai-Kun, J.F., Roberts, T., Coughlin, O., Le, C., et al. (2019) Use of ribaxamase (SYN-004), a β-lactamase, to prevent Clostridium difficile infection in β-lactam-treated patients: a double-blind, phase 2b, randomised placebo-controlled trial. *The Lancet Infectious Diseases*. [Online] 0 (0). Available from: doi:10.1016/S1473-3099(18)30731-X [Accessed: 17 March 2019].

Konijeti, G.G., Sauk, J., Shrime, M.G., Gupta, M., et al. (2014) Cost-effectiveness of Competing Strategies for Management of Recurrent Clostridium difficile Infection: A Decision Analysis. *Clinical Infectious Diseases*. [Online] 58 (11), 1507–1514. Available from: doi:10.1093/cid/ciu128 [Accessed: 17 February 2019].

Konturek, P.C., Koziel, J., Dieterich, W., Haziri, D., et al. (2016) Successful therapy of

Clostridium difficile infection with fecal microbiota transplantation. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society*. [Online] 67 (6), 859–866. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28195066 [Accessed: 15 March 2019].

Koon, H.W., Ho, S., Hing, T.C., Cheng, M., et al. (2014) Fidaxomicin inhibits Clostridium difficile toxin a-mediated enteritis in the mouse ileum. *Antimicrobial Agents and Chemotherapy*. [Online] 58 (8), 4642–4650. Available from: doi:10.1128/AAC.02783-14 [Accessed: 23 February 2019].

Kootte, R.S., Levin, E., Salojärvi, J., Smits, L.P., et al. (2017) Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition. *Cell Metabolism*. [Online] 26 (4), 611-619.e6. Available from: doi:10.1016/j.cmet.2017.09.008 [Accessed: 11 June 2018].

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., et al. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Applied and Environmental Microbiology*. [Online] 79 (17), 5112–5120. Available from: doi:10.1128/AEM.01043-13 [Accessed: 27 November 2017].

Kreutzer, E.W. & Milligan, F.D. (1978) Treatment of antibiotic-associated pseudomembranous colitis with cholestyramine resin. *The Johns Hopkins Medical Journal*. [Online] 143 (3), 67–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/691920 [Accessed: 23 February 2019].

Kumar, R., Rajkumar, H., Kumar, M., Varikuti, S.R., et al. (2013) Molecular cloning, characterization and heterologous expression of bile salt hydrolase (Bsh) from Lactobacillus fermentum NCDO394. *Molecular Biology Reports*. [Online] 40 (8), 5057–5066. Available from: doi:10.1007/s11033-013-2607-2 [Accessed: 8 February 2019].

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*. [Online] 33 (7), 1870–1874. Available from: doi:10.1093/molbev/msw054 [Accessed: 28 February 2018].

Kyne, L., Warny, M., Qamar, A. & Kelly, C.P. (2001a) Association between antibody response to toxin A and protection against recurrent Clostridium difficile diarrhoea. *Lancet*. [Online] 357 (9251), 189–193. Available from: doi:10.1016/S0140-6736(00)03592-3

[Accessed: 22 February 2019].

Kyne, L., Warny, M., Qamar, A. & Kelly, C.P. (2001b) Association between antibody response to toxin A and protection against recurrent Clostridium difficile diarrhoea. *Lancet*. [Online] Available from: doi:10.1016/S0140-6736(00)03592-3.

Kyne, L., Warny, M., Qamar, A. & Kelly, C.P. (2002) Asymptomatic Carriage of Clostridium difficile and Serum Levels of IgG Antibody against Toxin A. *New England Journal of Medicine*. [Online] 342 (6), 390–397. Available from: doi:10.1056/nejm200002103420604 [Accessed: 22 February 2019].

Lagier, J.-C., Delord, M., Million, M., Parola, P., et al. (2015) Dramatic reduction in Clostridium difficile ribotype 027-associated mortality with early fecal transplantation by the nasogastric route: a preliminary report. *European Journal of Clinical Microbiology & Infectious Diseases*. [Online] 34 (8), 1597–1601. Available from: doi:10.1007/s10096-015-2394-x.

Laponogov, I., Sadawi, N., Galea, D., Mirnezami, R., et al. (2018) ChemDistiller: an engine for metabolite annotation in mass spectrometry Jonathan Wren (ed.). *Bioinformatics*. [Online] 34 (12), 2096–2102. Available from: doi:10.1093/bioinformatics/bty080 [Accessed: 1 February 2019].

Larson, H.E., Parry, J. V, Price, A.B., Dolby, J., et al. (1977) Undescribed toxin in pseudomembranous colitis. *British Medical Journal*. [Online] 1 (6071), 1246–1248. Available from: doi:10.1136/bmj.1.6071.1246 [Accessed: 22 February 2019].

Lawley, T.D., Clare, S., Walker, A.W., Stares, M.D., et al. (2012) Targeted Restoration of the Intestinal Microbiota with a Simple, Defined Bacteriotherapy Resolves Relapsing Clostridium difficile Disease in Mice Michael S. Gilmore (ed.). *PLoS Pathogens*. [Online] 8 (10), e1002995. Available from: doi:10.1371/journal.ppat.1002995 [Accessed: 2 May 2018].

Lawley, T.D. & Walker, A.W. (2013) Intestinal colonization resistance. *Immunology*. [Online] 138 (1), 1–11. Available from: doi:10.1111/j.1365-2567.2012.03616.x [Accessed: 21 February 2019].

Lawson, P.A., Citron, D.M., Tyrrell, K.L. & Finegold, S.M. (2016) Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. *Anaerobe*. [Online] 40, 95–99. Available from: doi:10.1016/j.anaerobe.2016.06.008 [Accessed: 7 October 2017].

Lê Cao, K.A., González, I. & Déjean, S. (2009) IntegrOmics: An R package to unravel relationships between two omics datasets. *Bioinformatics*. [Online] 25 (21), 2855–2856. Available from: doi:10.1093/bioinformatics/btp515 [Accessed: 27 November 2017].

Leav, B.A., Blair, B., Leney, M., Knauber, M., et al. (2010) Serum anti-toxin B antibody correlates with protection from recurrent Clostridium difficile infection (CDI). *Vaccine*. [Online] Available from: doi:10.1016/j.vaccine.2009.10.144.

Lee, C.H., Steiner, T., Petrof, E.O., Smieja, M., et al. (2016) Frozen vs fresh fecal microbiota transplantation and clinical resolution of diarrhea in patients with recurrent clostridium difficile infection a randomized clinical trial. *JAMA - Journal of the American Medical Association*. [Online] 315 (2), 142–149. Available from: doi:10.1001/jama.2015.18098 [Accessed: 6 October 2017].

Lee, S.T.M., Kahn, S.A., Delmont, T.O., Shaiber, A., et al. (2017) Tracking microbial colonization in fecal microbiota transplantation experiments via genome-resolved metagenomics. *Microbiome*. [Online] 5 (1), 50. Available from: doi:10.1186/s40168-017-0270-x [Accessed: 9 October 2018].

Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., et al. (2009) Role of Bile Acids and Bile Acid Receptors in Metabolic Regulation. *Physiological Reviews*. [Online] 89 (1), 147–191. Available from: doi:10.1152/physrev.00010.2008 [Accessed: 16 January 2018].

Leffler, D.A. & Lamont, J.T. (2015) *Clostridium difficile* Infection Dan L. Longo (ed.). *New England Journal of Medicine*. [Online] 372 (16), 1539–1548. Available from: doi:10.1056/NEJMra1403772 [Accessed: 13 September 2018].

Leslie, J.L., Vendrov, K.C., Jenior, M.L. & Young, V.B. (2019) The Gut Microbiota Is Associated with Clearance of Clostridium difficile Infection Independent of Adaptive Immunity. *mSphere*. [Online] 4 (1), e00698-18. Available from: doi:10.1128/mSphereDirect.00698-18 [Accessed: 8 March 2019].

Lessa, F.C., Mu, Y., Bamberg, W.M., Beldavs, Z.G., et al. (2015) Burden of *Clostridium difficile* Infection in the United States. *New England Journal of Medicine*. [Online] 372 (9), 825–834. Available from: doi:10.1056/NEJMoa1408913 [Accessed: 13 September 2018].

Levy, M., Thaiss, C.A., Zeevi, D., Dohnalová, L., et al. (2015) Microbiota-Modulated Metabolites Shape the Intestinal Microenvironment by Regulating NLRP6 Inflammasome Signaling. *Cell*. [Online] 163 (6), 1428–1443. Available from:

doi:10.1016/j.cell.2015.10.048 [Accessed: 7 March 2019].

Limon, J.J., Skalski, J.H. & Underhill, D.M. (2017) Commensal Fungi in Health and Disease. *Cell Host and Microbe*. [Online]. 22 (2) pp.156–165. Available from: doi:10.1016/j.chom.2017.07.002 [Accessed: 22 February 2019].

Ling, W.H., Korpela, R., Mykkänen, H., Salminen, S., et al. (1994) Lactobacillus strain GG supplementation decreases colonic hydrolytic and reductive enzyme activities in healthy female adults. *The Journal of Nutrition*. [Online] 124 (1), 18–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8283290 [Accessed: 19 March 2017].

Liu, C.M., Aziz, M., Kachur, S., Hsueh, P.-R., et al. (2012) BactQuant: An enhanced broad-coverage bacterial quantitative real-time PCR assay. *BMC Microbiology*. [Online] 12 (1), 56. Available from: doi:10.1186/1471-2180-12-56 [Accessed: 3 January 2018].

Liu, H.-C., Wu, Y.-H., Lin, H.-J., Wu, T.-C., et al. (2013) Risk Factors of Fecal Toxigenic or Non-Toxigenic Clostridium difficile Colonization: Impact of Toll-Like Receptor Polymorphisms and Prior Antibiotic Exposure Markus M. Heimesaat (ed.). *PLoS ONE*. [Online] 8 (7), e69577. Available from: doi:10.1371/journal.pone.0069577 [Accessed: 22 February 2019].

Louie, T.J., Miller, M.A., Mullane, K.M., Weiss, K., et al. (2011) Fidaxomicin versus Vancomycin for Clostridium difficile Infection. *New England Journal of Medicine*. [Online] 364 (5), 422–431. Available from: doi:10.1056/NEJMoa0910812 [Accessed: 20 March 2017].

Louis, P. & Flint, H.J. (2017) Formation of propionate and butyrate by the human colonic microbiota. *Environmental Microbiology*. [Online] 19 (1), 29–41. Available from: doi:10.1111/1462-2920.13589 [Accessed: 10 March 2019].

Luo, C., Tsementzi, D., Kyrpides, N., Read, T., et al. (2012) Direct Comparisons of Illumina vs. Roche 454 Sequencing Technologies on the Same Microbial Community DNA Sample Francisco Rodriguez-Valera (ed.). *PLoS ONE*. [Online] 7 (2), e30087. Available from: doi:10.1371/journal.pone.0030087 [Accessed: 27 February 2019].

Lyerly, D.M., Ball, D.W., Toth, J. & Wilkins, T.D. (1988) Characterization of cross-reactive proteins detected by Culturette Brand Rapid Latex Test for Clostridium difficile. *Journal of Clinical Microbiology*. [Online] 26 (3), 397–400. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3128574 [Accessed: 13 July 2019].

Lyras, D., O'Connor, J.R., Howarth, P.M., Sambol, S.P., et al. (2009) Toxin B is essential for

virulence of Clostridium difficile. *Nature*. [Online] 458 (7242), 1176–1179. Available from: doi:10.1038/nature07822 [Accessed: 22 February 2019].

Macfarlane, G.T. & Macfarlane, S. (2007) Models for intestinal fermentation: association between food components, delivery systems, bioavailability and functional interactions in the gut. *Current Opinion in Biotechnology*. [Online] 18 (2), 156–162. Available from: doi:10.1016/j.copbio.2007.01.011 [Accessed: 25 February 2019].

Macfarlane, S. & Macfarlane, G.T. (2003) Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society*. [Online] 62 (01), 67–72. Available from: doi:10.1079/PNS2002207 [Accessed: 25 February 2019].

Magill, S.S., Edwards, J.R., Bamberg, W., Beldavs, Z.G., et al. (2014) Multistate Point-Prevalence Survey of Health Care–Associated Infections. *New England Journal of Medicine*. [Online] 370 (13), 1198–1208. Available from: doi:10.1056/NEJMoa1306801 [Accessed: 21 February 2019].

Makishima, M., Lu, T.T., Xie, W., Whitfield, G.K., et al. (2002) Vitamin D Receptor As an Intestinal Bile Acid Sensor. *Science*. [Online] 296 (5571), 1313–1316. Available from: doi:10.1126/science.1070477 [Accessed: 14 March 2019].

Marchesi, J.R., Adams, D.H., Fava, F., Hermes, G.D.A., et al. (2016) The gut microbiota and host health: a new clinical frontier. *Gut*. [Online] 65 (2), 330–339. Available from: doi:10.1136/gutjnl-2015-309990 [Accessed: 20 March 2017].

Marchesi, J.R. & Ravel, J. (2015) The vocabulary of microbiome research: a proposal. *Microbiome*. [Online] 3 (1), 31. Available from: doi:10.1186/s40168-015-0094-5 [Accessed: 2 May 2018].

Martin, J.S.H., Monaghan, T.M. & Wilcox, M.H. (2016) Clostridium difficile infection: Epidemiology, diagnosis and understanding transmission. *Nature Reviews Gastroenterology and Hepatology*. [Online]. 13 (4) pp.206–216. Available from: doi:10.1038/nrgastro.2016.25 [Accessed: 20 March 2017].

Maslowski, K.M., Vieira, A.T., Ng, A., Kranich, J., et al. (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature*. [Online] 461 (7268), 1282–1286. Available from: doi:10.1038/nature08530 [Accessed: 24 February 2019].

McDonald, J.A.K., Mullish, B.H., Pechlivanis, A., Liu, Z., et al. (2018a) Inhibiting Growth of Clostridioides difficile by Restoring Valerate, Produced by the Intestinal Microbiota.

Gastroenterology. [Online] 155 (5), 1495-1507.e15. Available from: doi:10.1053/j.gastro.2018.07.014 [Accessed: 27 July 2018].

McDonald, J.A.K., Schroeter, K., Fuentes, S., Heikamp-deJong, I., et al. (2013) Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model. *Journal of Microbiological Methods*. [Online] 95 (2), 167–174. Available from: doi:10.1016/j.mimet.2013.08.008 [Accessed: 25 February 2019].

McDonald, L.C., Gerding, D.N., Johnson, S., Bakken, J.S., et al. (2018b) Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clinical Infectious Diseases*. [Online] 31 (5), 431–455. Available from: doi:10.1093/cid/cix1085 [Accessed: 22 February 2018].

McDonald, L.C., Killgore, G.E., Thompson, A., Owens, R.C., et al. (2005) An Epidemic, Toxin Gene–Variant Strain of *Clostridium difficile*. *New England Journal of Medicine*. [Online] 353 (23), 2433–2441. Available from: doi:10.1056/NEJMoa051590 [Accessed: 12 December 2017].

McSweeney, B., Allegretti, J.R., Fischer, M., Monaghan, T., et al. (2018) Tu1894 - Potential Motivators and Deterents for Stool Donors: A Multicenter Study. *Gastroenterology*. [Online] 154 (6), S-1051. Available from: doi:10.1016/S0016-5085(18)33520-0 [Accessed: 24 January 2019].

Mekhjian, H.S., Phillips, S.F. & Hofmann, A.F. (1979) Colonic absorption of unconjugated bile acids - Perfusion studies in man. *Digestive Diseases and Sciences*. [Online] 24 (7), 545–550. Available from: doi:10.1007/BF01489324 [Accessed: 8 March 2019].

Merrick, M.J. & Edwards, R.A. (1995) Nitrogen control in bacteria. *Microbiological Reviews*. [Online] 59 (4), 604–622. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8531888%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC239390 [Accessed: 13 July 2019].

Miles, A.A., Misra, S.S. & Irwin, J.O. (1938) The estimation of the bactericidal power of the blood. *The Journal of Hygiene*. [Online] 38 (6), 732–749. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20475467 [Accessed: 5 October 2018].

Mills, G.A., Walker, V. & Mughal, H. (1999) Headspace solid-phase microextraction with 1-pyrenyldiazomethane in-fibre derivatisation for analysis of faecal short-chain fatty acids. *Journal of Chromatography B: Biomedical Sciences and Applications*. [Online] 730

(1), 113–122. Available from: doi:10.1016/S0378-4347(99)00211-X [Accessed: 25 February 2019].

Mirzaei, M.K. & Maurice, C.F. (2017) Ménage à trois in the human gut: interactions between host, bacteria and phages. *Nature Reviews Microbiology*. [Online] 15 (7), 397–408. Available from: doi:10.1038/nrmicro.2017.30 [Accessed: 20 February 2019].

Moayyedi, P., Surette, M.G., Kim, P.T., Libertucci, J., et al. (2015) Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology*. [Online] 149 (1), 102-109.e6. Available from: doi:10.1053/j.gastro.2015.04.001.

Mogg, G.A., Arabi, Y., Youngs, D., Johnson, M., et al. (1980) Therapeutic trials of antibiotic associated colitis. *Scandinavian Journal of Infectious Diseases*. [Online] (Suppl 22), 41–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7010533 [Accessed: 23 February 2019].

Mograbi, J., Mahamid, M., Bishara, J., Nseir, W., et al. (2013) Obesity as a Risk Factor for Clostridium difficile Infection. *Clinical Infectious Diseases*. [Online] 57 (4), 489–493. Available from: doi:10.1093/cid/cit280 [Accessed: 22 February 2019].

Monaghan, T., Mullish, B.H., Patterson, J., Wong, G.K.S.K., et al. (2018) Effective fecal microbiota transplantation for recurrent Clostridioides difficile infection in humans is associated with increased signalling in the bile acid-farnesoid X receptor-fibroblast growth factor pathway. *Gut Microbes*. [Online] 1–7. Available from: doi:10.1080/19490976.2018.1506667 [Accessed: 9 September 2018].

Morris, L.S. & Marchesi, J.R. (2016) Assessing the impact of long term frozen storage of faecal samples on protein concentration and protease activity. *Journal of Microbiological Methods*. [Online] 123, 31–38. Available from: doi:10.1016/j.mimet.2016.02.001 [Accessed: 19 March 2017].

Mullish, B.H., Marchesi, J.R., Thursz, M.R. & Williams, H.R.T. (2015) Microbiome manipulation with faecal microbiome transplantation as a therapeutic strategy in Clostridium difficile infection. *QJM*. [Online] 108 (5), 355–359. Available from: doi:10.1093/qjmed/hcu182 [Accessed: 20 March 2017].

Mullish, B.H., McDonald, J.A.K., Pechlivanis, A., Allegretti, J.R., et al. (2019) Microbial bile salt hydrolases mediate the efficacy of faecal microbiota transplant in the treatment of recurrent Clostridioides difficile infection. *Gut*. [Online] gutjnl-2018-317842. Available

from: doi:10.1136/gutjnl-2018-317842 [Accessed: 11 February 2019].

Mullish, B.H., Osborne, L.S., Marchesi, J.R. & McDonald, J.A.A.K. (2018a) The implementation of omics technologies in cancer microbiome research. *ecancermedicalscience*. [Online] 12. Available from: doi:10.3332/ecancer.2018.864 [Accessed: 5 September 2018].

Mullish, B.H., Pechlivanis, A., Barker, G.F., Thursz, M.R., et al. (2018b) Functional microbiomics: Evaluation of gut microbiota-bile acid metabolism interactions in health and disease. *Methods*. [Online] 149, 26 April, 49–58. Available from: doi:10.1016/j.ymeth.2018.04.028 [Accessed: 2 May 2018].

Mullish, B.H., Quraishi, M.N., Segal, J.P., McCune, V.L., et al. (2018c) The use of faecal microbiota transplant as treatment for recurrent or refractory Clostridium difficile infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Journal of Hospital Infection*. [Online] 100, S1–S31. Available from: doi:10.1016/j.jhin.2018.07.037 [Accessed: 5 September 2018].

Mullish, B.H., Quraishi, M.N., Segal, J.P., McCune, V.L., et al. (2018d) The use of faecal microbiota transplant as treatment for recurrent or refractory Clostridium difficile infection and other potential indications: Joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut*. [Online] 67 (11), 1920–1941. Available from: doi:10.1136/gutjnl-2018-316818 [Accessed: 5 September 2018].

Mullish, B.H., Quraishi, M.N., Segal, J.P., Williams, H.R.T., et al. (2018e) Introduction to the joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) faecal microbiota transplant guidelines. *Journal of Hospital Infection*. [Online] 100 (2), 130–132. Available from: doi:10.1016/j.jhin.2018.07.028 [Accessed: 19 September 2018].

Mullish, B.H. & Williams, H.R.T. (2018) Clostridium difficile infection and antibiotic-associated diarrhoea. *Clinical Medicine, Journal of the Royal College of Physicians of London*. [Online] 18 (3), 237–241. Available from: doi:10.7861/clinmedicine.18-3-237.

Mullish, B.H. & Williams, H.R.T. (2015) Obstacles to establishing an NHS faecal transplant programme. *British Medical Journal*. [Online] 351 (nov19 5), h6043–h6043. Available from: doi:10.1136/bmj.h6043 [Accessed: 20 March 2017].

Nale, J., Redgwell, T., Millard, A. & Clokie, M. (2018) Efficacy of an Optimised Bacteriophage

Cocktail to Clear Clostridium difficile in a Batch Fermentation Model. *Antibiotics*. [Online] 7 (1), 13. Available from: doi:10.3390/antibiotics7010013 [Accessed: 20 February 2019].

Nale, J.Y., Chutia, M., Carr, P., Hickenbotham, P.T., et al. (2016a) 'Get in Early'; Biofilm and Wax Moth (Galleria mellonella) Models Reveal New Insights into the Therapeutic Potential of Clostridium difficile Bacteriophages. *Frontiers in Microbiology*. [Online] 7, 1383. Available from: doi:10.3389/fmicb.2016.01383 [Accessed: 20 February 2019].

Nale, J.Y., Spencer, J., Hargreaves, K.R., Trzepiński, P., et al. (2016b) Bacteriophage combinations significantly reduce Clostridium difficile growth in vitro and proliferation in vivo. *Antimicrobial Agents and Chemotherapy*. [Online] 60 (2), 968–981. Available from: doi:10.1128/AAC.01774-15 [Accessed: 20 February 2019].

National Institute for Health and Care Excellence (NICE) (2014) *Faecal microbiota transplant for recurrent Clostridium difficile infection | Guidance and guidelines | NICE*. [Online]. 2014. NICE. Available from: https://www.nice.org.uk/guidance/ipg485 [Accessed: 2 October 2017].

Neal, M.D., Alverdy, J.C., Hall, D.E., Simmons, R.L., et al. (2011) Diverting Loop Ileostomy and Colonic Lavage. *Annals of Surgery*. [Online] 254 (3), 423–429. Available from: doi:10.1097/SLA.0b013e31822ade48 [Accessed: 23 February 2019].

Nelson, R.L., Suda, K.J. & Evans, C.T. (2017) Antibiotic treatment for Clostridium difficile-associated diarrhoea in adults. *Cochrane Database of Systematic Reviews*. [Online]. 2017 (3) p.CD004610. Available from: doi:10.1002/14651858.CD004610.pub5 [Accessed: 23 February 2019].

Ng, K.M., Ferreyra, J.A., Higginbottom, S.K., Lynch, J.B., et al. (2013) Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature*. [Online] 502 (7469), 96–99. Available from: doi:10.1038/nature12503 [Accessed: 10 March 2019].

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., et al. (2012) Host-Gut Microbiota Metabolic Interactions. *Science*. [Online] 336 (6086), 1262–1267. Available from: doi:10.1126/science.1223813 [Accessed: 24 February 2019].

Nicholson, J.K., Lindon, J.C. & Holmes, E. (1999) 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica*. [Online] 29 (11),

1181–1189. Available from: doi:10.1080/004982599238047 [Accessed: 5 February 2018].

van Nood, E., Vrieze, A., Nieuwdorp, M., Fuentes, S., et al. (2013) Duodenal Infusion of Donor Feces for Recurrent Clostridium difficile. *New England Journal of Medicine*. [Online] 368 (5), 407–415. Available from: doi:10.1056/NEJMoa1205037 [Accessed: 20 March 2017].

Nuding, S., Frasch, T., Schaller, M., Stange, E.F., et al. (2014) Synergistic effects of antimicrobial peptides and antibiotics against clostridium difficile. *Antimicrobial Agents and Chemotherapy*. [Online] 58 (10), 5719–5725. Available from: doi:10.1128/AAC.02542-14 [Accessed: 20 February 2019].

Ofir, G. & Sorek, R. (2018) Contemporary Phage Biology: From Classic Models to New Insights. *Cell*. [Online] 172 (6), 1260–1270. Available from: doi:10.1016/j.cell.2017.10.045 [Accessed: 20 February 2019].

OpenBiome (2019) *OpenBiome*. [Online]. 2019. Available from: https://www.openbiome.org/ [Accessed: 1 February 2018].

Ott, S.J., Waetzig, G.H., Rehman, A., Moltzau-Anderson, J., et al. (2017) Efficacy of Sterile Fecal Filtrate Transfer for Treating Patients With Clostridium difficile Infection. *Gastroenterology*. [Online] 152 (4), 799-811.e7. Available from: doi:10.1053/j.gastro.2016.11.010 [Accessed: 9 June 2017].

Padua, D. & Pothoulakis, C. (2016) Novel approaches to treating Clostridium difficile-associated colitis. *Expert Review of Gastroenterology & Hepatology*. [Online] 10 (2), 193–204. Available from: doi:10.1586/17474124.2016.1109444 [Accessed: 13 September 2018].

Palmieri, L.-J., Rainteau, D., Sokol, H., Beaugerie, L., et al. (2018) Inhibitory Effect of Ursodeoxycholic Acid on Clostridium difficile Germination Is Insufficient to Prevent Colitis: A Study in Hamsters and Humans. *Frontiers in microbiology*. [Online] 9, 2849. Available from: doi:10.3389/fmicb.2018.02849 [Accessed: 12 March 2019].

Panda, S., El khader, I., Casellas, F., López Vivancos, J., et al. (2014) Short-Term Effect of Antibiotics on Human Gut Microbiota Paul D. Cotter (ed.). *PLoS ONE*. [Online] 9 (4), e95476. Available from: doi:10.1371/journal.pone.0095476 [Accessed: 26 February 2019].

Papanicolas, L.E., Choo, J.M., Wang, Y., Leong, L.E.X., et al. (2019) Bacterial viability in faecal

transplants: Which bacteria survive? *EBioMedicine*. [Online] Available from: doi:10.1016/J.EBIOM.2019.02.023 [Accessed: 21 February 2019].

Paquet-Bolduc, B., Beaulieu, C., Trottier, S., Loungnarath, V., et al. (2012) Impact of the Type of Diagnostic Assay on Clostridium difficile Infection and Complication Rates in a Mandatory Reporting Program. *Clinical Infectious Diseases*. [Online] 56 (1), 67–73. Available from: doi:10.1093/cid/cis840 [Accessed: 23 February 2019].

Paramsothy, S., Kamm, M.A., Kaakoush, N.O., Walsh, A.J., et al. (2017) Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet (London, England)*. [Online] 389 (10075), 1218–1228. Available from: doi:10.1016/S0140-6736(17)30182-4.

Paramsothy, S., Nielsen, S., Kamm, M.A., Deshpande, N.P., et al. (2018) Specific Bacteria and Metabolites Associated with Response to Fecal Microbiota Transplantation in Patients with Ulcerative Colitis. *Gastroenterology*. [Online] Available from: doi:10.1053/j.gastro.2018.12.001 [Accessed: 24 February 2019].

Parks, D.H., Tyson, G.W., Hugenholtz, P. & Beiko, R.G. (2014) STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics*. [Online] 30 (21), 3123–3124. Available from: doi:10.1093/bioinformatics/btu494 [Accessed: 27 November 2017].

Parks, D.J., Blanchard, S.G., Bledsoe, R.K., Chandra, G., et al. (1999) Bile acids: Natural ligands for an orphan nuclear receptor. *Science*. [Online] 284 (5418), 1365–1368. Available from: doi:10.1126/science.284.5418.1365 [Accessed: 17 January 2018].

Patel, N.C., Griesbach, C.L., DiBaise, J.K. & Orenstein, R. (2013) Fecal Microbiota Transplant for Recurrent Clostridium difficile Infection: Mayo Clinic in Arizona Experience. *Mayo Clinic Proceedings*. [Online] 88 (8), 799–805. Available from: doi:10.1016/j.mayocp.2013.04.022 [Accessed: 9 October 2017].

Van de Peer, Y., Chapelle, S. & De Wachter, R. (1996) A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Research*. [Online] 24 (17), 3381–3391. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8811093 [Accessed: 25 February 2019].

Pepin, J., Valiquette, L. & Cossette, B. (2005) Mortality attributable to nosocomial Clostridium difficile-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *Canadian Medical Association Journal*. [Online] 173 (9), 1037–1042. Available from: doi:10.1503/cmaj.050978 [Accessed: 21 February 2019].

Peri, R., Aguilar, R.C., Tüffers, K., Erhardt, A., et al. (2019) The impact of technical and clinical factors on fecal microbiota transfer outcomes for the treatment of recurrent *Clostridioides difficile* infections in Germany. *United European Gastroenterology Journal*. [Online] 205064061983991. Available from: doi:10.1177/2050640619839918 [Accessed: 23 March 2019].

Petrof, E.O., Gloor, G.B., Vanner, S.J., Weese, S.J., et al. (2013) Stool substitute transplant therapy for the eradication of Clostridium difficile infection: 'RePOOPulating' the gut. *Microbiome*. [Online] 1 (1), 3. Available from: doi:10.1186/2049-2618-1-3 [Accessed: 3 October 2017].

Pfizer (2019) *NCT03090191 | Clostridium Difficile Infection Clinical Trial | Pfizer*. [Online]. 2019. Available from: https://www.pfizer.com/science/find-a-trial/nct03090191 [Accessed: 22 February 2019].

Philips, C.A., Pande, A., Shasthry, S.M., Jamwal, K.D., et al. (2017) Healthy Donor Fecal Microbiota Transplantation in Steroid-Ineligible Severe Alcoholic Hepatitis: A Pilot Study. *Clinical Gastroenterology and Hepatology*. [Online]. 15 (4) pp.600–602. Available from: doi:10.1016/j.cgh.2016.10.029 [Accessed: 11 March 2019].

Pillai, A. & Nelson, R. (2008) Probiotics for treatment of Clostridium difficile-associated colitis in adults. *Cochrane Database of Systematic Reviews*. [Online]. (1) p.CD004611. Available from: doi:10.1002/14651858.CD004611.pub2 [Accessed: 23 February 2019].

Portune, K.J., Beaumont, M., Davila, A.-M., Tomé, D., et al. (2016) Gut microbiota role in dietary protein metabolism and health-related outcomes: The two sides of the coin. *Trends in Food Science & Technology*. [Online] 57 (B, SI), 213–232. Available from: doi:10.1016/j.tifs.2016.08.011 [Accessed: 4 March 2019].

Pothoulakis, C., Sullivan, R., Melnick, D.A., Triadafilopoulos, G., et al. (1988) Clostridium difficile toxin A stimulates intracellular calcium release and chemotactic response in human granulocytes. *Journal of Clinical Investigation*. [Online] 81 (6), 1741–1745. Available from: doi:10.1172/JCI113514 [Accessed: 22 February 2019].

Przybylski, M. (2014) Mass Spectrometry. In: *Handbook of Spectroscopy*. [Online]. Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA. pp. 355–406. Available from: doi:10.1002/9783527654703.ch11 [Accessed: 25 February 2019].

Public Health England (2013) *Updated guidance on the management and treatment of Clostridium difficile infection*. [Online]. 2013. Public Health England. Available from:

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/321891/Clostridium_difficile_management_and_treatment.pdf [Accessed: 20 March 2017].

Qazi, T., Amaratunga, T., Barnes, E.L., Fischer, M., et al. (2017) The risk of inflammatory bowel disease flares after fecal microbiota transplantation: Systematic review and meta-analysis. *Gut Microbes*. [Online] 8 (6), 574–588. Available from: doi:10.1080/19490976.2017.1353848 [Accessed: 16 October 2017].

Quraishi, M.N., McNally, A. & van Schaik, W. (2019) Do we really understand how faecal microbiota transplantation works? *EBioMedicine*. [Online] Available from: doi:10.1016/j.ebiom.2019.03.024.

Quraishi, M.N., Segal, J., Mullish, B., McCune, V.L., et al. (2017) National survey of practice of faecal microbiota transplantation for Clostridium difficile infection in the UK. *Journal of Hospital Infection*. [Online]. 95 (4) pp.444–445. Available from: doi:10.1016/j.jhin.2016.10.023 [Accessed: 7 October 2017].

Rackaityte, E. & Lynch, S. V. (2018) Rules of engagement in the gut microbiome. *Nature Medicine*. [Online] 24 (11), 1642–1644. Available from: doi:10.1038/s41591-018-0242-0 [Accessed: 23 February 2019].

Rani, R.P., Anandharaj, M. & Ravindran, A.D. (2017) Characterization of Bile Salt Hydrolase from Lactobacillus gasseri FR4 and Demonstration of Its Substrate Specificity and Inhibitory Mechanism Using Molecular Docking Analysis. *Frontiers in Microbiology*. [Online] 8, 1004. Available from: doi:10.3389/fmicb.2017.01004 [Accessed: 8 February 2019].

Rea, M.C., Sit, C.S., Clayton, E., O'Connor, P.M., et al. (2010) Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proceedings of the National Academy of Sciences of the United States of America*. [Online] 107 (20), 9352–9357. Available from: doi:10.1073/pnas.0913554107 [Accessed: 20 February 2019].

Redelings, M.D., Sorvillo, F. & Mascola, L. (2007) Increase in *Clostridium difficile* –related Mortality Rates, United States, 1999 **–** 2004. *Emerging Infectious Diseases*. [Online] 13 (9), 1417–1419. Available from: doi:10.3201/eid1309.061116 [Accessed: 21 February 2019].

Reigadas, E., Alcalá, L., Gómez, J., Marín, M., et al. (2018) Breakthrough Clostridium difficile

Infection in Cirrhotic Patients Receiving Rifaximin. *Clinical Infectious Diseases*. [Online] 66 (7), 1086–1091. Available from: doi:10.1093/cid/cix918 [Accessed: 22 February 2019].

Reijnders, D., Goossens, G.H., Hermes, G.D.A., Zoetendal, E.G., et al. (2016) Clinical and Translational Report Effects of Gut Microbiota Manipulation by Antibiotics on Host Metabolism in Obese Humans: A Randomized Double-Blind Placebo-Controlled Trial Cell Metabolism Clinical and Translational Report Effects of Gut Microbiota Manipulation by Antibiotics on Host Metabolism in Obese Humans: A Randomized Double-Blind Placebo-Controlled Trial. *Cell Metabolism*. [Online] 24, 63–74. Available from: doi:10.1016/j.cmet.2016.06.016 [Accessed: 7 October 2018].

Reynolds, P.E. (2005) Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology & Infectious Diseases*. [Online] 8 (11), 943–950. Available from: doi:10.1007/bf01967563 [Accessed: 21 February 2019].

Ridlon, J.M., Alves, J.M., Hylemon, P.B. & Bajaj, J.S. (2013) Cirrhosis, bile acids and gut microbiota. *Gut Microbes*. [Online] 4 (5), 382–387. Available from: doi:10.4161/gmic.25723 [Accessed: 9 March 2019].

Ridlon, J.M., Kang, D.-J. & Hylemon, P.B. (2006) Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research*. [Online] 47 (2), 241–259. Available from: doi:10.1194/jlr.R500013-JLR200 [Accessed: 7 October 2017].

Robinson, L.S., Lewis, W.G. & Lewis, A.L. (2017) The sialate *O* -acetylesterase EstA from gut *Bacteroidetes* species enables sialidase-mediated cross-species foraging of 9- *O* -acetylated sialoglycans. *Journal of Biological Chemistry*. [Online] 292 (28), 11861–11872. Available from: doi:10.1074/jbc.M116.769232 [Accessed: 10 March 2019].

Rolfe, R.D. (1984) Role of volatile fatty acids in colonization resistance to Clostridium difficile. *Infection and immunity*. [Online] 45 (1), 185–191. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6735467 [Accessed: 9 October 2017].

Rossen, N.G., Fuentes, S., van der Spek, M.J., Tijssen, J.G., et al. (2015) Findings From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative Colitis. *Gastroenterology*. [Online] 149 (1), 110-118.e4. Available from: doi:10.1053/j.gastro.2015.03.045.

Rossocha, M., Schultz-Heienbrok, R., Von Moeller, H., Coleman, J.P., et al. (2005) Conjugated bile acid hydrolase is a tetrameric N-terminal thiol hydrolase with specific recognition

of its cholyl but not of its tauryl product. *Biochemistry*. [Online] 44 (15), 5739–5748. Available from: doi:10.1021/bi0473206 [Accessed: 17 February 2019].

Sangster, T., Major, H., Plumb, R., Wilson, A.J., et al. (2006) A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. *The Analyst*. [Online] 131 (10), 1075. Available from: doi:10.1039/b604498k [Accessed: 29 November 2017].

Sanofi (2017) *Sanofi - Sanofi ends development of Clostridium difficile vaccine - Sanofi*. [Online]. 2017. Available from: https://mediaroom.sanofi.com/en/press-releases/2017/sanofi-ends-development-of-clostridium-difficile-vaccine/ [Accessed: 21 February 2019].

Sarafian, M.H., Lewis, M.R., Pechlivanis, A., Ralphs, S., et al. (2015) Bile Acid Profiling and Quantification in Biofluids Using Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry. *Analytical Chemistry*. [Online] 87 (19), 9662–9670. Available from: doi:10.1021/acs.analchem.5b01556 [Accessed: 8 October 2017].

Satokari, R., Mattila, E., Kainulainen, V. & Arkkila, P.E.T. (2015) Simple faecal preparation and efficacy of frozen inoculum in faecal microbiota transplantation for recurrent *Clostridium difficile* infection - an observational cohort study. *Alimentary Pharmacology & Therapeutics*. [Online] 41 (1), 46–53. Available from: doi:10.1111/apt.13009 [Accessed: 9 October 2017].

Sayin, S.I., Wahlström, A., Felin, J., Jäntti, S., et al. (2013) Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metabolism*. [Online] 17 (2), 225–235. Available from: doi:10.1016/j.cmet.2013.01.003 [Accessed: 16 January 2018].

Scanlan, P.D. & Marchesi, J.R. (2008) Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and -independent analysis of faeces. *The ISME Journal*. [Online] 2 (12), 1183–1193. Available from: doi:10.1038/ismej.2008.76 [Accessed: 11 March 2019].

Second Genome Therapeutics (n.d.) *Piphillin*. [Online]. Available from: http://secondgenome.com/solutions/resources/data-analysis-tools/piphillin/ [Accessed: 14 February 2018].

Seekatz, A.M., Theriot, C.M., Molloy, C.T., Wozniak, K.L., et al. (2015) Fecal Microbiota Transplantation Eliminates Clostridium difficile in a Murine Model of Relapsing Disease.

Infection and Immunity. [Online] 83 (10), 3838–3846. Available from: doi:10.1128/IAI.00459-15 [Accessed: 5 October 2018].

Seekatz, A.M., Theriot, C.M., Rao, K., Chang, Y.-M., et al. (2018) Restoration of short chain fatty acid and bile acid metabolism following fecal microbiota transplantation in patients with recurrent Clostridium difficile infection. *Anaerobe*. [Online] 53, 64–73. Available from: doi:10.1016/J.ANAEROBE.2018.04.001 [Accessed: 4 March 2019].

Segain, J.P., Raingeard de la Blétière, D., Bourreille, A., Leray, V., et al. (2000) Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut*. [Online] 47 (3), 397–403. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10940278 [Accessed: 7 March 2019].

Seres Therapeutics (n.d.) *Seres Therapeutics Press Release*. [Online]. Available from: http://ir.serestherapeutics.com/phoenix.zhtml?c=254006&p=irol-newsArticle&ID=2240833 [Accessed: 17 February 2019].

Shankar, V., Hamilton, M.J., Khoruts, A., Kilburn, A., et al. (2014) Species and genus level resolution analysis of gut microbiota in Clostridium difficile patients following fecal microbiota transplantation. *Microbiome*. [Online] 2 (1), 13. Available from: doi:10.1186/2049-2618-2-13 [Accessed: 27 February 2019].

Sheikh Abdul Kadir, S.H., Miragoli, M., Abu-Hayyeh, S., Moshkov, A. V., et al. (2010) Bile Acid-Induced Arrhythmia Is Mediated by Muscarinic M2 Receptors in Neonatal Rat Cardiomyocytes Alicia J. Kowaltowski (ed.). *PLoS ONE*. [Online] 5 (3), e9689. Available from: doi:10.1371/journal.pone.0009689 [Accessed: 14 March 2019].

Shen, N.T., Maw, A., Tmanova, L.L., Pino, A., et al. (2017) Timely Use of Probiotics in Hospitalized Adults Prevents Clostridium difficile Infection: A Systematic Review With Meta-Regression Analysis. *Gastroenterology*. [Online] 152 (8), 1889-1900.e9. Available from: doi:10.1053/j.gastro.2017.02.003 [Accessed: 23 February 2019].

Shilling, M., Matt, L., Rubin, E., Visitacion, M.P., et al. (2013) Antimicrobial Effects of Virgin Coconut Oil and Its Medium-Chain Fatty Acids on *Clostridium difficile*. *Journal of Medicinal Food*. [Online] 16 (12), 1079–1085. Available from: doi:10.1089/jmf.2012.0303 [Accessed: 9 March 2019].

Shim, J.K., Johnson, S., Samore, M.H., Bliss, D.Z., et al. (1998) Primary symptomless colonisation by Clostridium difficile and decreased risk of subsequent diarrhoea. *Lancet*. [Online] 351 (9103), 633–636. Available from: doi:10.1016/S0140-

6736(97)08062-8 [Accessed: 23 February 2019].

Shin, J.H., High, K.P. & Warren, C.A. (2016) Older is not wiser, immunologically speaking: Effect of aging on host response to Clostridium difficile infections. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*. [Online]. 71 (7) pp.916–922. Available from: doi:10.1093/gerona/glv229 [Accessed: 8 March 2019].

Singh, H., Nugent, Z., Yu, B.N., Lix, L.M., et al. (2017) Higher Incidence of Clostridium difficile Infection Among Individuals With Inflammatory Bowel Disease. *Gastroenterology*. [Online] 153 (2), 430-438.e2. Available from: doi:10.1053/j.gastro.2017.04.044 [Accessed: 22 February 2019].

Smillie, C.S., Sauk, J., Gevers, D., Friedman, J., et al. (2018) Strain Tracking Reveals the Determinants of Bacterial Engraftment in the Human Gut Following Fecal Microbiota Transplantation. *Cell Host & Microbe*. [Online] 23 (2), 229-240.e5. Available from: doi:10.1016/J.CHOM.2018.01.003 [Accessed: 27 February 2019].

Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R., et al. (2006) XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification. *Analytical Chemistry*. [Online] 78 (3), 779–787. Available from: doi:10.1021/ac051437y [Accessed: 26 February 2019].

Smith, K., Zeng, X., Lin, J., Chincholkar, S., et al. (2014) Discovery of Bile Salt Hydrolase Inhibitors Using an Efficient High-Throughput Screening System Huaijun Zhou (ed.). *PLoS ONE*. [Online] 9 (1), e85344. Available from: doi:10.1371/journal.pone.0085344 [Accessed: 19 March 2017].

Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., et al. (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Tregcell homeostasis. *Science*. [Online] 341 (6145), 569–573. Available from: doi:10.1126/science.1241165 [Accessed: 6 March 2019].

Solbach, P., Chhatwal, P., Woltemate, S., Tacconelli, E., et al. (2018) BaiCD gene cluster abundance is negatively correlated with Clostridium difficile infection Daniel Paredes-Sabja (ed.). *PLOS ONE*. [Online] 13 (5), e0196977. Available from: doi:10.1371/journal.pone.0196977 [Accessed: 20 August 2018].

Song, Y., Garg, S., Girotra, M., Maddox, C., et al. (2013) Microbiota Dynamics in Patients Treated with Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection Gabriele Berg (ed.). *PLoS ONE*. [Online] 8 (11), e81330. Available from:

doi:10.1371/journal.pone.0081330 [Accessed: 24 February 2019].

Song, Z., Cai, Y., Lao, X., Wang, X., et al. (2019) Taxonomic profiling and populational patterns of bacterial bile salt hydrolase (BSH) genes based on worldwide human gut microbiome. *Microbiome*. [Online] 7 (1), 9. Available from: doi:10.1186/s40168-019-0628-3 [Accessed: 24 February 2019].

Sorg, J.A. & Sonenshein, A.L. (2008) Bile Salts and Glycine as Cogerminants for Clostridium difficile Spores. *Journal of Bacteriology*. [Online] 190 (7), 2505–2512. Available from: doi:10.1128/JB.01765-07 [Accessed: 7 October 2017].

Sorg, J.A. & Sonenshein, A.L. (2009) Chenodeoxycholate Is an Inhibitor of *Clostridium difficile* Spore Germination. *Journal of Bacteriology*. [Online] 191 (3), 1115–1117. Available from: doi:10.1128/JB.01260-08 [Accessed: 18 January 2018].

Souza, M.H., Melo-Filho, A.A., Rocha, M.F., Lyerly, D.M., et al. (1997) The involvement of macrophage-derived tumour necrosis factor and lipoxygenase products on the neutrophil recruitment induced by Clostridium difficile toxin B. *Immunology*. [Online] 91 (2), 281–288. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9227329 [Accessed: 22 February 2019].

Stackebrandt, E. & Goebel, B.M. (1994) Taxonomic Note: A Place for DNA-DNA Reassociation and 16s rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic Bacteriology*. [Online]. 44 (4). Available from: www.microbiologyresearch.org [Accessed: 28 February 2019].

Staley, C., Kaiser, T., Vaugh, B.P., Graiziger, C.T., et al. (2018) Predicting recurrence of Clostridium difficile infection following encapsulated fecal microbiota transplantation. *Microbiome*. [Online] submitted (1), 166. Available from: doi:10.1186/s40168-018-0549-6 [Accessed: 6 October 2018].

Staley, C., Vaughn, B.P., Graiziger, C.T., Singroy, S., et al. (2017a) Community dynamics drive punctuated engraftment of the fecal microbiome following transplantation using freeze-dried, encapsulated fecal microbiota. *Gut Microbes*. [Online] 8 (3), 276–288. Available from: doi:10.1080/19490976.2017.1299310 [Accessed: 27 February 2019].

Staley, C., Weingarden, A.R., Khoruts, A. & Sadowsky, M.J. (2017b) Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Applied Microbiology and Biotechnology*. [Online] 101 (1), 47–64. Available from: doi:10.1007/s00253-016-8006-6 [Accessed: 23 November 2017].

Staudinger, J.L., Goodwin, B., Jones, S.A., Hawkins-Brown, D., et al. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proceedings of the National Academy of Sciences*. [Online] 98 (6), 3369–3374. Available from: doi:10.1073/pnas.051551698 [Accessed: 14 March 2019].

Stoltz, K.L., Erickson, R., Staley, C., Weingarden, A.R., et al. (2017) Synthesis and Biological Evaluation of Bile Acid Analogues Inhibitory to *Clostridium difficile* Spore Germination. *Journal of Medicinal Chemistry*. [Online] 60 (8), 3451–3471. Available from: doi:10.1021/acs.jmedchem.7b00295 [Accessed: 7 October 2017].

Studer, E., Zhou, X., Zhao, R., Wang, Y., et al. (2012) Conjugated bile acids activate the sphingosine-1-phosphate receptor 2 in primary rodent hepatocytes. *Hepatology*. [Online] 55 (1), 267–276. Available from: doi:10.1002/hep.24681 [Accessed: 14 March 2019].

Studer, N., Desharnais, L., Beutler, M., Brugiroux, S., et al. (2016) Functional Intestinal Bile Acid 7α-Dehydroxylation by Clostridium scindens Associated with Protection from Clostridium difficile Infection in a Gnotobiotic Mouse Model. *Frontiers in Cellular and Infection Microbiology*. [Online] 6, 191. Available from: doi:10.3389/fcimb.2016.00191 [Accessed: 2 May 2018].

Suez, J., Zmora, N., Zilberman-Schapira, G., Mor, U., et al. (2018) Post-Antibiotic Gut Mucosal Microbiome Reconstitution Is Impaired by Probiotics and Improved by Autologous FMT. *Cell*. [Online] 174 (6), 1406-1423.e16. Available from: doi:10.1016/j.cell.2018.08.047 [Accessed: 23 February 2019].

Surawicz, C.M., Brandt, L.J., Binion, D.G., Ananthakrishnan, A.N., et al. (2013a) Guidelines for Diagnosis, Treatment, and Prevention of Clostridium difficile Infections. *The American Journal of Gastroenterology*. [Online] 108 (4), 478–498. Available from: doi:10.1038/ajg.2013.4.

Surawicz, C.M., Brandt, L.J., Binion, D.G., Ananthakrishnan, A.N., et al. (2013b) Guidelines for Diagnosis, Treatment and Prevention of Clostridium difficile Infections. *The American Journal of Gastroenterology*. [Online] 108 (4), 478–498. Available from: doi:10.1038/ajg.2013.4 [Accessed: 21 February 2019].

Swann, J.R., Want, E.J., Geier, F.M., Spagou, K., et al. (2011) Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *Proceedings of the National Academy of Sciences*. [Online] 108 (Supplement_1), 4523–4530. Available

from: doi:10.1073/pnas.1006734107 [Accessed: 7 October 2017].

Swindells, J., Brenwald, N., Reading, N. & Oppenheim, B. (2010) Evaluation of diagnostic tests for Clostridium difficile infection. *Journal of Clinical Microbiology*. [Online] 48 (2), 606–608. Available from: doi:10.1128/JCM.01579-09 [Accessed: 23 February 2019].

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., et al. (2014) The Role of Short-Chain Fatty Acids in Health and Disease. *Advances in Immunology*. [Online] 121, 91–119. Available from: doi:10.1016/B978-0-12-800100-4.00003-9 [Accessed: 7 March 2019].

Tanaka, H., Hashiba, H., Kok, J. & Mierau, I. (2000) Bile salt hydrolase of Bifidobacterium longum-biochemical and genetic characterization. *Applied and Environmental Microbiology*. [Online] 66 (6), 2502–2512. Available from: doi:10.1128/AEM.66.6.2502-2512.2000 [Accessed: 19 March 2017].

Taranto, M.P., Fernandez Murga, M.L., Lorca, G. & De Valdez, G.F. (2003) Bile salts and cholesterol induce changes in the lipid cell membrane of Lactobacillus reuteri. *Journal of Applied Microbiology*. [Online] 95 (1), 86–91. Available from: doi:10.1046/j.1365-2672.2003.01962.x [Accessed: 8 March 2019].

Tessier, M.E.M., Andersson, H., Ross, C., Peniche-trujillo, A., et al. (2015) Mo1850 Obeticholic Acid (INT-747) Confers Disease Protection Against Clostridium difficile Infection. *Gastroenterology*. [Online] 148 (4), S-726. Available from: doi:10.1016/S0016-5085(15)32479-3 [Accessed: 26 June 2018].

Thanissery, R., Winston, J.A. & Theriot, C.M. (2017) Inhibition of spore germination, growth, and toxin activity of clinically relevant C. difficile strains by gut microbiota derived secondary bile acids. *Anaerobe*. [Online] 45, 86–100. Available from: doi:10.1016/j.anaerobe.2017.03.004 [Accessed: 17 January 2018].

Theriot, C.M., Koenigsknecht, M.J., Carlson, P.E., Hatton, G.E., et al. (2014) Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. *Nature Communications*. [Online] 5, 3114. Available from: doi:10.1038/ncomms4114 [Accessed: 7 October 2017].

Thomas, C., Stevenson, M. & Riley, T. V (2003) Antibiotics and hospital-acquired Clostridium difficile-associated diarrhoea: A systematic review. *Journal of Antimicrobial Chemotherapy*. [Online]. 51 (6) pp.1339–1350. Available from: doi:10.1093/jac/dkg254 [Accessed: 22 February 2019].

Thomas, L.A., King, A., French, G.L., Murphy, G.M., et al. (1997) Cholylglycine hydrolase and

7α-dehydroxylase optimum assay conditions in vitro and caecal enzyme activities ex vivo. *Clinica Chimica Acta*. [Online] 268 (1–2), 61–72. Available from: doi:10.1016/S0009-8981(97)00169-1 [Accessed: 30 November 2017].

Thomas, L.A., Veysey, M.J., French, G., Hylemon, P.B., et al. (2001) Bile acid metabolism by fresh human colonic contents: a comparison of caecal versus faecal samples. *Gut*. [Online] 49 (6), 835–842. Available from: doi:10.1136/GUT.49.6.835 [Accessed: 19 February 2018].

Valiquette, L., Fulop, K., Ruel, J., Alary, M.-E., et al. (2005) Increasing Risk of Relapse after Treatment of Clostridium difficile Colitis in Quebec, Canada. *Clinical Infectious Diseases*. [Online] 40 (11), 1591–1597. Available from: doi:10.1086/430315 [Accessed: 23 February 2019].

Valneva (2019) *Clostridium difficile – Valneva R&D*. [Online]. 2019. Available from: https://www.valneva.com/en/rd/vla84 [Accessed: 22 February 2019].

Vavassori, P., Mencarelli, A., Renga, B., Distrutti, E., et al. (2009) The Bile Acid Receptor FXR Is a Modulator of Intestinal Innate Immunity. *The Journal of Immunology*. [Online] 183 (10), 6251–6261. Available from: doi:10.4049/jimmunol.0803978 [Accessed: 28 June 2018].

Veselkov, K.A., Vingara, L.K., Masson, P., Robinette, S.L., et al. (2011) Optimized Preprocessing of Ultra-Performance Liquid Chromatography/Mass Spectrometry Urinary Metabolic Profiles for Improved Information Recovery. *Analytical Chemistry*. [Online] 83 (15), 5864–5872. Available from: doi:10.1021/ac201065j [Accessed: 29 November 2017].

Vickers, R.J., Tillotson, G.S., Nathan, R., Hazan, S., et al. (2017) Efficacy and safety of ridinilazole compared with vancomycin for the treatment of Clostridium difficile infection: a phase 2, randomised, double-blind, active-controlled, non-inferiority study. *The Lancet Infectious Diseases*. [Online] 17 (7), 735–744. Available from: doi:10.1016/S1473-3099(17)30235-9 [Accessed: 23 February 2019].

Vogl, F.C., Mehrl, S., Heizinger, L., Schlecht, I., et al. (2016) Evaluation of dilution and normalization strategies to correct for urinary output in HPLC-HRTOFMS metabolomics. *Analytical and bioanalytical chemistry*. [Online] 408 (29), 8483–8493. Available from: doi:10.1007/s00216-016-9974-1 [Accessed: 11 July 2019].

Vrieze, A., Van Nood, E., Holleman, F., Salojärvi, J., et al. (2012) Transfer of Intestinal

Microbiota From Lean Donors Increases Insulin Sensitivity in Individuals With Metabolic Syndrome. *Gastroenterology*. [Online] 143 (4), 913-916.e7. Available from: doi:10.1053/j.gastro.2012.06.031 [Accessed: 15 October 2017].

Vrieze, A., Out, C., Fuentes, S., Jonker, L., et al. (2014) Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. *Journal of Hepatology*. [Online] 60 (4), 824–831. Available from: doi:10.1016/J.JHEP.2013.11.034 [Accessed: 7 October 2018].

Wahlström, A., Sayin, S.I., Marschall, H.U. & Bäckhed, F. (2016) Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metabolism*. [Online]. 24 (1) pp.41–50. Available from: doi:10.1016/j.cmet.2016.05.005 [Accessed: 29 November 2017].

Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R. (2007) Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology*. [Online] 73 (16), 5261–5267. Available from: doi:10.1128/AEM.00062-07 [Accessed: 27 November 2017].

Wang, Y., Wiesnoski, D.H., Helmink, B.A., Gopalakrishnan, V., et al. (2018) Fecal microbiota transplantation for refractory immune checkpoint inhibitor-associated colitis. *Nature Medicine*. [Online] 24 (12), 1804–1808. Available from: doi:10.1038/s41591-018-0238-9 [Accessed: 22 February 2019].

Wang, Z., Zeng, X., Mo, Y., Smith, K., et al. (2012) Identification and characterization of a bile salt hydrolase from Lactobacillus salivarius for development of novel alternatives to antibiotic growth promoters. *Applied and Environmental Microbiology*. [Online] 78 (24), 8795–8802. Available from: doi:10.1128/AEM.02519-12 [Accessed: 30 November 2017].

Warny, M., Pepin, J., Fang, A., Killgore, G., et al. (2005) Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. *Lancet*. [Online] 366 (9491), 1079–1084. Available from: doi:10.1016/S0140-6736(05)67420-X [Accessed: 22 February 2019].

Waslawski, S., Lo, E.S., Ewing, S.A., Young, V.B., et al. (2013) Clostridium difficile ribotype diversity at six health care institutions in the United States. *Journal of Clinical Microbiology*. [Online] 51 (6), 1938–1941. Available from: doi:10.1128/JCM.00056-13 [Accessed: 6 February 2019].

Watts, G.S., Youens-Clark, K., Slepian, M.J., Wolk, D.M., et al. (2017) 16S rRNA gene sequencing on a benchtop sequencer: accuracy for identification of clinically important bacteria. *Journal of Applied Microbiology*. [Online] 123 (6), 1584–1596. Available from: doi:10.1111/jam.13590 [Accessed: 28 February 2019].

Webb, B.J., Brunner, A., Lewis, J., Ford, C.D., et al. (2019) Repurposing an Old Drug for a New Epidemic: Ursodeoxycholic Acid to Prevent Recurrent *Clostridioides difficile* Infection. *Clinical Infectious Diseases*. [Online] 68 (3), 498–500. Available from: doi:10.1093/cid/ciy568 [Accessed: 6 March 2019].

Weingarden, A., González, A., Vázquez-Baeza, Y., Weiss, S., et al. (2015) Dynamic changes in short- and long-term bacterial composition following fecal microbiota transplantation for recurrent Clostridium difficile infection. *Microbiome*. [Online] 3 (1), 10. Available from: doi:10.1186/s40168-015-0070-0 [Accessed: 27 February 2019].

Weingarden, A.R., Chen, C., Bobr, A., Yao, D., et al. (2014) Microbiota transplantation restores normal fecal bile acid composition in recurrent Clostridium difficile infection. *AJP: Gastrointestinal and Liver Physiology*. [Online] 306 (4), G310–G319. Available from: doi:10.1152/ajpgi.00282.2013 [Accessed: 7 October 2017].

Weingarden, A.R., Chen, C., Zhang, N., Graiziger, C.T., et al. (2016a) Ursodeoxycholic Acid Inhibits Clostridium difficile Spore Germination and Vegetative Growth, and Prevents the Recurrence of Ileal Pouchitis Associated With the Infection. *Journal of Clinical Gastroenterology*. [Online] 50 (8), 624–630. Available from: doi:10.1097/MCG.0000000000000427 [Accessed: 6 March 2019].

Weingarden, A.R., Dosa, P.I., DeWinter, E., Steer, C.J., et al. (2016b) Changes in Colonic Bile Acid Composition following Fecal Microbiota Transplantation Are Sufficient to Control Clostridium difficile Germination and Growth Gayatri Vedantam (ed.). *PLOS ONE*. [Online] 11 (1), e0147210. Available from: doi:10.1371/journal.pone.0147210 [Accessed: 7 October 2017].

Wells, J.E., Williams, K.B., Whitehead, T.R., Heuman, D.M., et al. (2003) Development and application of a polymerase chain reaction assay for the detection and enumeration of bile acid 7alpha-dehydroxylating bacteria in human feces. *Clinica Chimica Acta*. [Online] 331 (1–2), 127–134. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12691873 [Accessed: 8 October 2017].

Wiklund, S., Johansson, E., Sjöström, L., Mellerowicz, E.J., et al. (2008) Visualization of

GC/TOF-MS-Based Metabolomics Data for Identification of Biochemically Interesting Compounds Using OPLS Class Models. *Analytical Chemistry*. [Online] 80 (1), 115–122. Available from: doi:10.1021/ac0713510 [Accessed: 1 February 2018].

Wilcox, M.H., Gerding, D.N., Poxton, I.R., Kelly, C., et al. (2017) Bezlotoxumab for Prevention of Recurrent *Clostridium difficile* Infection. *New England Journal of Medicine*. [Online] 376 (4), 305–317. Available from: doi:10.1056/NEJMoa1602615 [Accessed: 20 March 2017].

Wilcox, M.H., Shetty, N., Fawley, W.N., Shemko, M., et al. (2012) Changing Epidemiology of Clostridium difficile Infection Following the Introduction of a National Ribotyping-Based Surveillance Scheme in England. *Clinical Infectious Diseases*. [Online] 55 (8), 1056–1063. Available from: doi:10.1093/cid/cis614 [Accessed: 6 February 2019].

Wilson, K.H. & Perini, F. (1988) Role of competition for nutrients in suppression of Clostridium difficile by the colonic microflora. *Infection and immunity*. [Online] 56 (10), 2610–2614. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3417352 [Accessed: 10 March 2019].

Wiström, J., Norrby, S.R., Myhre, E.B., Eriksson, S., et al. (2001) Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. *Journal of Antimicrobial Chemotherapy*. [Online] 47 (1), 43–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11152430 [Accessed: 22 February 2019].

Wong, J.M.W., de Souza, R., Kendall, C.W.C., Emam, A., et al. (2006) Colonic health: fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*. [Online] 40 (3), 235–243. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16633129 [Accessed: 24 February 2019].

Worley, B. & Powers, R. (2013) Multivariate Analysis in Metabolomics. *Current Metabolomics*. [Online] 1 (1), 92–107. Available from: doi:10.2174/2213235X11301010092 [Accessed: 26 February 2019].

Wullt, M., Johansson Hagslätt, M.L., Odenholt, I. & Berggren, A. (2007) Lactobacillus plantarum 299v enhances the concentrations of fecal short-chain fatty acids in patients with recurrent Clostridium difficile-associated diarrhea. *Digestive Diseases and Sciences*. [Online] 52 (9), 2082–2086. Available from: doi:10.1007/s10620-006-9123-3 [Accessed: 23 February 2019].

Yang, H.-T., Chen, J.-W., Rathod, J., Jiang, Y.-Z., et al. (2018) Lauric Acid Is an Inhibitor of

Clostridium difficile Growth in Vitro and Reduces Inflammation in a Mouse Infection Model. *Frontiers in Microbiology*. [Online] 8, 2635. Available from: doi:10.3389/fmicb.2017.02635 [Accessed: 9 March 2019].

Yao, C.K., Muir, J.G. & Gibson, P.R. (2016) Review article: insights into colonic protein fermentation, its modulation and potential health implications. *Alimentary Pharmacology & Therapeutics*. [Online] 43 (2), 181–196. Available from: doi:10.1111/apt.13456 [Accessed: 24 February 2019].

Yao, L., Seaton, S.C., Ndousse-Fetter, S., Adhikari, A.A., et al. (2018) A selective gut bacterial bile salt hydrolase alters host metabolism. *eLife*. [Online] 7. Available from: doi:10.7554/eLife.37182 [Accessed: 23 March 2019].

Youngster, I., Mahabamunuge, J., Systrom, H.K., Sauk, J., et al. (2016) Oral, frozen fecal microbiota transplant (FMT) capsules for recurrent Clostridium difficile infection. *BMC Medicine*. [Online] 14 (1), 134. Available from: doi:10.1186/s12916-016-0680-9.

Youngster, I., Russell, G.H., Pindar, C., Ziv-Baran, T., et al. (2014a) Oral, Capsulized, Frozen Fecal Microbiota Transplantation for Relapsing Clostridium difficile Infection. *JAMA - Journal of the American Medical Association*. [Online] 312 (17), 1772–1778. Available from: doi:10.1001/jama.2014.13875 [Accessed: 6 October 2017].

Youngster, I., Sauk, J., Pindar, C., Wilson, R.G., et al. (2014b) Fecal Microbiota Transplant for Relapsing Clostridium difficile Infection Using a Frozen Inoculum From Unrelated Donors: A Randomized, Open-Label, Controlled Pilot Study. *Clinical Infectious Diseases*. [Online] 58 (11), 1515–1522. Available from: doi:10.1093/cid/ciu135 [Accessed: 6 October 2017].

Yuille, S., Reichardt, N., Panda, S., Dunbar, H., et al. (2018) Human gut bacteria as potent class I histone deacetylase inhibitors in vitro through production of butyric acid and valeric acid Daotai Nie (ed.). *PLoS ONE*. [Online] 13 (7), e0201073. Available from: doi:10.1371/journal.pone.0201073 [Accessed: 7 March 2019].

Zackular, J.P., Moore, J.L., Jordan, A.T., Juttukonda, L.J., et al. (2016) Dietary zinc alters the microbiota and decreases resistance to Clostridium difficile infection. *Nature Medicine*. [Online] 22 (11), 1330–1334. Available from: doi:10.1038/nm.4174 [Accessed: 25 March 2019].

Zackular, J.P. & Skaar, E.P. (2018) The role of zinc and nutritional immunity in *Clostridium difficile* infection. *Gut Microbes*. [Online] 00–00. Available from:

doi:10.1080/19490976.2018.1448354 [Accessed: 25 March 2019].

Zhang, F., Yu, L., Lin, X., Cheng, P., et al. (2015) Minireview: Roles of Fibroblast Growth Factors 19 and 21 in Metabolic Regulation and Chronic Diseases. *Molecular Endocrinology*. [Online] 29 (10), 1400–1413. Available from: doi:10.1210/me.2015-1155 [Accessed: 17 January 2018].

Zoetendal, E.G., Rajilic-Stojanovic, M. & de Vos, W.M. (2008) High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut*. [Online] 57 (11), 1605–1615. Available from: doi:10.1136/gut.2007.133603 [Accessed: 27 February 2019].

Zuckerlandl, E. & Pauling, L. (1965) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*. 4, 406–425.

Zuo, T., Wong, S.H., Cheung, C.P., Lam, K., et al. (2018) Gut fungal dysbiosis correlates with reduced efficacy of fecal microbiota transplantation in Clostridium difficile infection. *Nature Communications*. [Online] 9 (1), 3663. Available from: doi:10.1038/s41467-018-06103-6 [Accessed: 22 February 2019].

Zuo, T., Wong, S.H., Lam, K., Lui, R., et al. (2017) Bacteriophage transfer during faecal microbiota transplantation in Clostridium difficile infection is associated with treatment outcome. *Gut*. [Online] gutjnl-2017-313952. Available from: doi:10.1136/gutjnl-2017-313952 [Accessed: 7 October 2017].

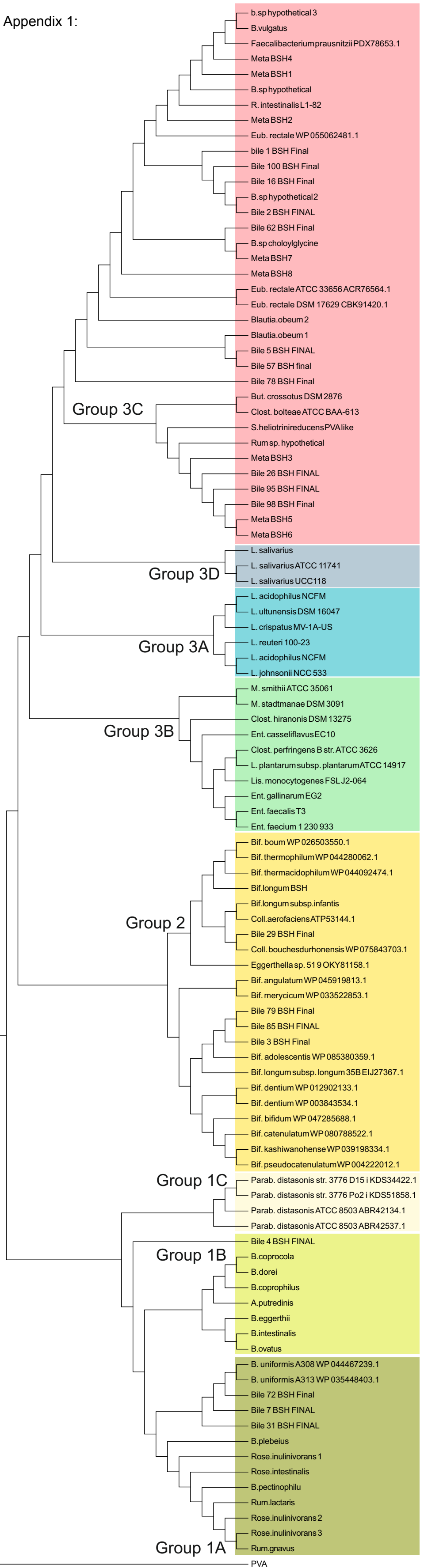
Appendices:

Appendix 1: Evolutionary relationships of BSH genes with their taxonomic hosts shown. The evolutionary history was inferred using the Neighbor-Joining method (Zuckerlandl & Pauling, 1965). The optimal tree with the sum of branch length = 11.24114695 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Bryson & Vogel, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 102 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 145 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher & Tamura, 2016). Reproduced from (Mullish *et al.*, 2019).

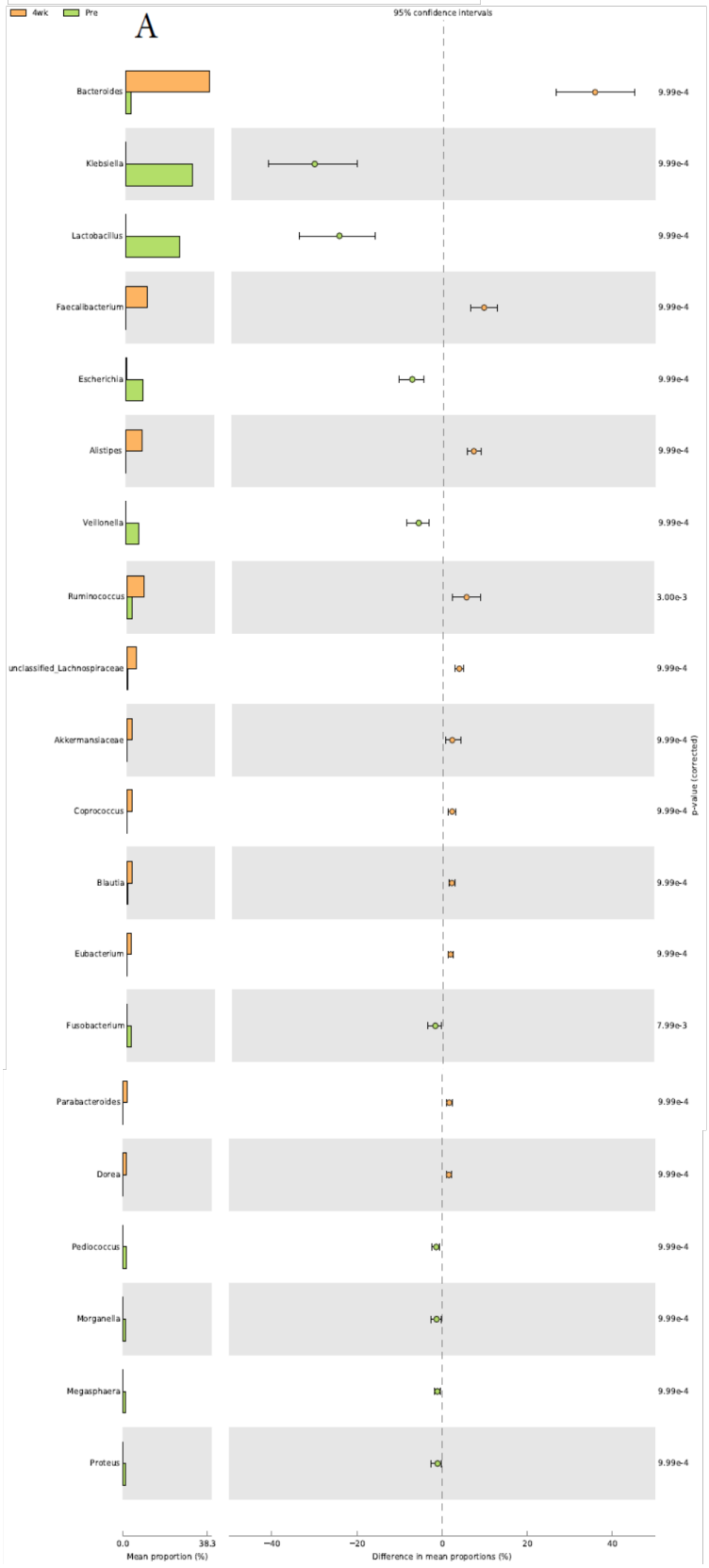
Appendix 2: Effect of FMT for rCDI upon genus-level gut bacterial profiles. As derived from the validation human dataset. Extended error bar plots, with genera changing significantly measured by White's non-parametric test with Benjamini-Hochberg correction, using threshold between mean proportions of >1%. A: pre-FMT vs 4 weeks post-FMT; B: pre-FMT vs 12 weeks post-FMT. (Donors: *n*=5; rCDI patients pre- and post-FMT: *n*=18). Reproduced from (Monaghan *et al.*, 2018).

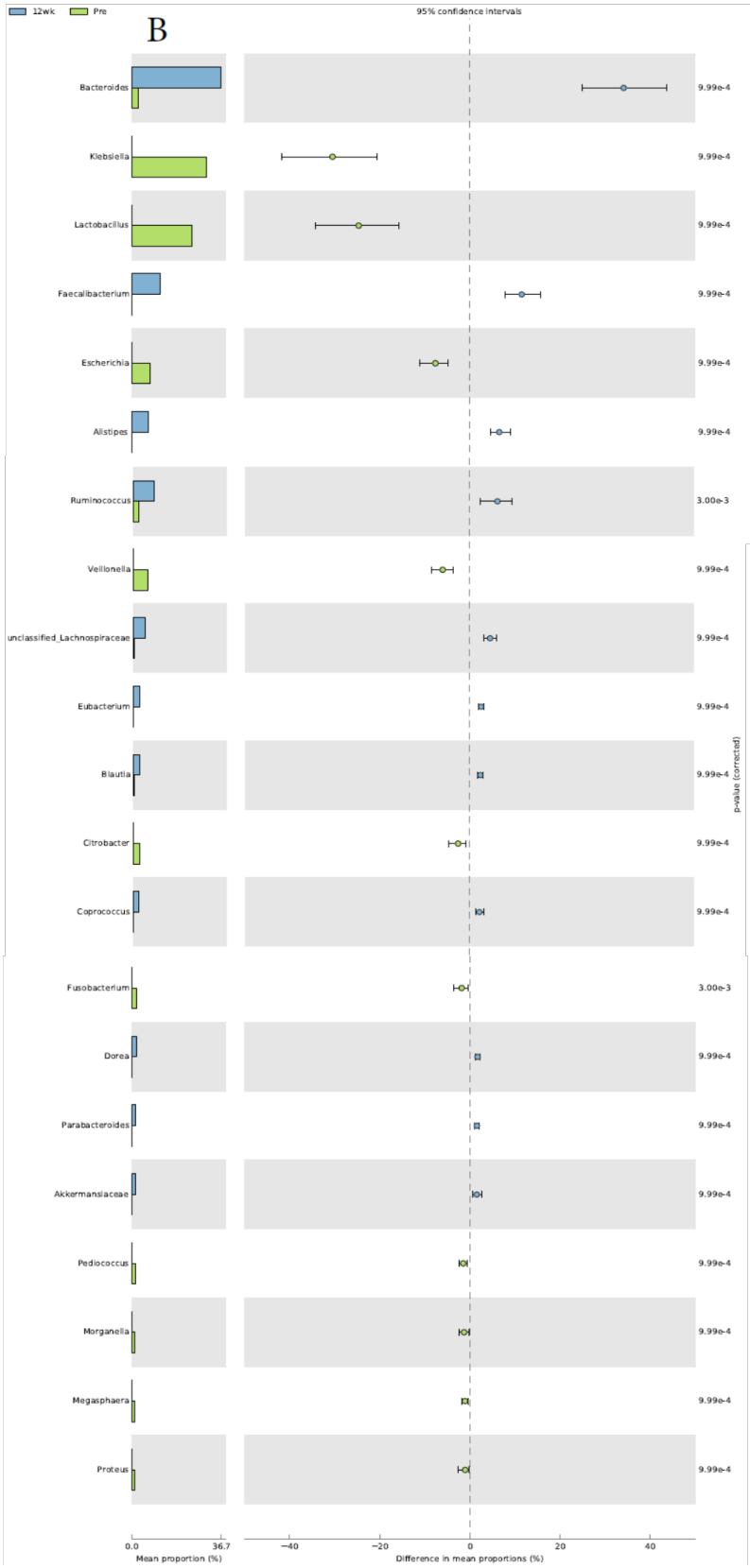
Appendix 3: Permissions for reproduction of material from published journal articles/ abstracts.

Appendix 1:


b.sp hypothetical 3
B.vulgatus
Faecalibacterium prausnitzii PDX78653.1
Meta BSH4
Meta BSH1
B.sp hypothetical
R. intestinalis L1-82
Meta BSH2
Eub. rectale WP 055062481.1
bile 1 BSH Final
Bile 100 BSH Final
Bile 16 BSH Final
B.sp hypothetical2
Bile 2 BSH FINAL
Bile 62 BSH Final
B.sp choloylglycine
Meta BSH7
Meta BSH8
Eub. rectale ATCC 33656 ACR76564.1
Eub. rectale DSM 17629 CBK91420.1
Blautia.obeum 2
Blautia.obeum 1
Bile 5 BSH FINAL
Bile 57 BSH final
Bile 78 BSH Final
But. crossotus DSM 2876
Clost. bolteae ATCC BAA-613
S.heliotrinireducens PVA like
Rum sp. hypothetical
Meta BSH3
Bile 26 BSH FINAL
Bile 95 BSH FINAL
Bile 98 BSH Final
Meta BSH5
Meta BSH6
Group 3C
L. salivarius
L. salivarius ATCC 11741
L. salivarius UCC118
Group 3D
L. acidophilus NCFM
L. ultunensis DSM 16047
L. crispatus MV-1A-US
L. reuteri 100-23
L. acidophilus NCFM
L. johnsonii NCC 533
Group 3A
M. smithii ATCC 35061
M. stadtmanae DSM 3091
Clost. hiranonis DSM 13275
Ent. casseliflavus EC10
Clost. perfringens B str. ATCC 3626
L. plantarum subsp. plantarum ATCC 14917
Lis. monocytogenes FSL J2-064
Ent. gallinarum EG2
Ent. faecalis T3
Ent. faecium 1 230 933
Group 3B
Bif. boum WP 026503550.1
Bif. thermophilum WP 044280062.1
Bif. thermacidophilum WP 044092474.1
Bif.longum BSH
Bif.longum subsp.infantis
Coll.aerofaciens ATP53144.1
Bile 29 BSH Final
Coll. bouchesdurhonensis WP 075843703.1
Eggerthella sp. 51 9 OKY81158.1
Group 2
Bif. angulatum WP 045919813.1
Bif. merycicum WP 033522853.1
Bile 79 BSH Final
Bile 85 BSH FINAL
Bile 3 BSH Final
Bif. adolescentis WP 085380359.1
Bif. longum subsp. longum 35B EIJ27367.1
Bif. dentium WP 012902133.1
Bif. dentium WP 003843534.1
Bif. bifidum WP 047285688.1
Bif. catenulatum WP 080788522.1
Bif. kashiwanohense WP 039198334.1
Bif. pseudocatenulatum WP 004222012.1
Group 1C
Parab. distasonis str. 3776 D15 i KDS34422.1
Parab. distasonis str. 3776 Po2 i KDS51858.1
Parab. distasonis ATCC 8503 ABR42134.1
Parab. distasonis ATCC 8503 ABR42537.1
Bile 4 BSH FINAL
B.coprocola
B.dorei
B.coprophilus
A.putredinis
B.eggerthii
B.intestinalis
B.ovatus
Group 1B
B. uniformis A308 WP 044467239.1
B. uniformis A313 WP 035448403.1
Bile 72 BSH Final
Bile 7 BSH FINAL
Bile 31 BSH FINAL
B.plebeius
Rose.inulinivorans 1
Rose.intestinalis
B.pectinophilu
Rum.lactaris
Rose.inulinivorans 2
Rose.inulinivorans 3
Rum.gnavus
Group 1A
PVA

Appendix 2


4wk
Pre
A
95% confidence intervals
Bacteroides
Klebsiella
Lactobacillus
Faecalibacterium
Escherichia
Alistipes
Veillonella
Ruminococcus
unclassified_Lachnospiraceae
Akkermansiaceae
Coprococcus
Blautia
Eubacterium
Fusobacterium
Parabacteroides
Dorea
Pediococcus
Morganella
Megasphaera
Proteus
9.99e-4
3.00e-3
7.99e-3
p-value (corrected)
0.0
38.3
Mean proportion (%)
−40
−20
0
20
40
Difference in mean proportions (%)


12wk
Pre
B
95% confidence intervals
Bacteroides
Klebsiella
Lactobacillus
Faecalibacterium
Escherichia
Alistipes
Ruminococcus
Veillonella
unclassified_Lachnospiraceae
Eubacterium
Blautia
Citrobacter
Coprococcus
Fusobacterium
Dorea
Parabacteroides
Akkermansiaceae
Pediococcus
Morganella
Megasphaera
Proteus
9.99e-4
3.00e-3
p-value (corrected)
0.0
36.7
Mean proportion (%)
−40
−20
0
20
40
Difference in mean proportions (%)

AMERICAN SOCIETY FOR MICROBIOLOGY

Title: Bile Salt Hydrolase Activity in Probiotics

Author: Máire Begley,Colin Hill,Cormac G. M. Gahan

Publication: Applied and Environmental Microbiology

Publisher: American Society for Microbiology

Date: Mar 3, 2006

Permissions Request

ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation. If your thesis, or dissertation, is to be published commercially, then you must reapply for permission.

BACK CLOSE WINDOW

Title: Microbial bile salt hydrolases mediate the efficacy of faecal microbiota transplant in the treatment of recurrent *Clostridioides difficile* infection

Author: Benjamin H Mullish,Julie A K McDonald,Alexandros Pechlivanis,Jessica R Allegretti,Dina Kao,Grace F Barker,Diya Kapila,Elaine O Petrof,Susan A Joyce,Cormac G M Gahan,Izabela Glegola-Madejska,Horace R T Williams,Elaine Holmes,Thomas B Clarke,Mark R Thursz,Julian R Marchesi

Publication: Gut

Publisher: BMJ Publishing Group Ltd.

Date: Feb 11, 2019

Welcome to Rightslink

Important notice - please read.
This article is Open Access, published under the terms of a Creative Commons licence. Please refer back to the article to check the applicable licence (details are in the article Footnotes).
Articles published under CC-BY-NC permit non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited. Permission only needs to be obtained for commercial use and can be done via the RightsLink system below.
Articles published under the CC-BY permit unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

BMJ Publishing Group Ltd. has partnered with Copyright Clearance Center's Rightslink service to offer a variety of options for reusing BMJ Publishing Group content. Select the "I would like to ..." drop-down menu to view the many reuse options available to you.

I would like to... make a selection

Hinari initiative

Title: Inhibiting Growth of Clostridioides difficile by Restoring Valerate, Produced by the Intestinal Microbiota

Author: Julie A.K. McDonald,Benjamin H. Mullish,Alexandros Pechlivanis,Zhigang Liu,Jerusa Brignardello,Dina Kao,Elaine Holmes,Jia V. Li,Thomas B. Clarke,Mark R. Thursz,Julian R. Marchesi

Publication: Gastroenterology

Publisher: Elsevier

Date: November 2018

Journal

Gut Microbes ›

Latest Articles

Brief Report

Effective fecal microbiota transplantation for recurrent *Clostridioides difficile* infection in humans is associated with increased signalling in the bile acid-farnesoid X receptor-fibroblast growth factor pathway

Tanya Monaghan, Benjamin H Mullish, Jordan Patterson, Gane KS Wong, Julian R Marchesi, Huiping Xu, ...show all

Received 24 Apr 2018, Accepted 25 Jul 2018, Published online: 05 Sep 2018

Download citation https://doi.org/10.1080/19490976.2018.1506667 Check for updates

Full Article Figures & data References Supplemental Citations

Metrics © Licensing PDF

People also read

Article commentary

Regulating stool for microbiota transplantation ›

Pilar N. Ossorio et al.

Gut Microbes
Latest Articles

Published online: 13 Sep 2018

Editorial

Non-alcoholic fatty liver disease and cardiovascular risk: an update ›

Benjamin H. Mullish et al.

Expert Review of Gastroenterology & Hepatology
Volume 12, 2018 - Issue 12

Published online: 16 Oct 2018

Consequences of bile salt biotransformations by intestinal bacteria ›

OXFORD UNIVERSITY PRESS LICENSE TERMS AND CONDITIONS

Mar 13, 2019

This Agreement between Imperial College London -- Benjamin Mullish ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4546950185988
License date	Mar 13, 2019
Licensed content publisher	Oxford University Press
Licensed content publication	International Journal for Quality in Health Care
Licensed content title	Comparative epidemiology of *Clostridium difficile* infection: England and the USA
Licensed content author	King, Alice; Mullish, Benjamin H
Licensed content date	Sep 11, 2017
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	Dr Benjamin Mullish
Publisher of your work	Imperial College London
Expected publication date	Mar 2019
Permissions cost	0.00 GBP
Value added tax	0.00 GBP
Total	0.00 GBP
Title	Dr Benjamin Mullish
Institution name	Imperial College London
Expected presentation date	Mar 2019
Portions	Figure 1 to be used with identifiers
Requestor Location	Imperial College London Division of Integrative Systems Medicine and Digestive Disease 10th Floor, QEQM Wing, St Mary's Hospita South Wharf Road, Paddington London, other W2 1NY United Kingdom Attn: Imperial College London
Publisher Tax ID	GB125506730
Billing Type	Invoice
Billing Address	Imperial College London Division of Integrative Systems Medicine and Digestive Disease 10th Floor, QEQM Wing, St Mary's Hospita South Wharf Road, Paddington London, United Kingdom W2 1NY Attn: Benjamin H Mullish
Total	0.00 GBP
Terms and Conditions	

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.

2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.
3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.
4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.
5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.
6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com
7. If the credit line or acknowledgement in our publication indicates that any of the figures, images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.
8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.
9. This license is personal to you and may not be sublicensed, assigned or transferred by you to any other person without Oxford University Press's written permission.
10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
12. Other Terms and Conditions:
v1.4

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Title: 25 - Microbiome and Metabolic Markers of Clostridium Difficile Recurrance

Author: Jessica R. Allegretti,Benjamin H. Mullish,Eli Bogart,Edina Shu,Xiaoxi Dong,Julie A. McDonald,Alexandros Pechlivanis,Julian Marchesi,Georg Gerber,Lynn Bry

Publication: Gastroenterology

Publisher: Elsevier

Date: May 2018