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

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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
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Acknowledgements and Dedication:

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

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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
<table>
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<tr>
<td>GCDCA</td>
<td>glycochenodeoxycholic acid</td>
</tr>
<tr>
<td>GDCA</td>
<td>glycodeoxycholic acid</td>
</tr>
<tr>
<td>GDH</td>
<td>glutamate dehydrogenase</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTV</td>
<td>glycerol trivalerate</td>
</tr>
<tr>
<td>HCAI</td>
<td>healthcare-associated infection</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase(s)</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNKT</td>
<td>invariant natural killer T (cells)</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-d-thiogalactopyranoside</td>
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<tr>
<td>LCA</td>
<td>lithocholic acid</td>
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<tr>
<td>mCCEY</td>
<td>moxifloxacin-Braziers Cycloserine Cefotixin Egg Yolk</td>
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<tr>
<td>MCFA</td>
<td>medium-chain fatty acid(s)</td>
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<tr>
<td>MRSA</td>
<td>methicillin-resistant <em>Staphylococcus aureus</em></td>
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<td>NAAT</td>
<td>nucleic acid amplification test</td>
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<tr>
<td>NAP1</td>
<td>North American PFGE type 1</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer (cell)</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPX</td>
<td>Normalised Protein eXpression</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OCA</td>
<td>obeticholic acid</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>orthogonal projections to latent structures discriminant analysis</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PERMANOVA</td>
<td>permutational multivariate analysis of variance</td>
</tr>
<tr>
<td>PLS</td>
<td>partial least squares</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PMA</td>
<td>propidium monoazide</td>
</tr>
<tr>
<td>PPI</td>
<td>proton pump inhibitor(s)</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PSC</td>
<td>primary sclerosing cholangitis</td>
</tr>
<tr>
<td>QC</td>
<td>quality control(s)</td>
</tr>
<tr>
<td>(q)PCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>rCCA</td>
<td>regularised canonical correlation analysis</td>
</tr>
<tr>
<td>rCDI</td>
<td>recurrent <em>Clostridioides difficile</em> infection</td>
</tr>
<tr>
<td>RCT</td>
<td>randomised controlled trial</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>sBHI</td>
<td>supplemented brain heart infusion</td>
</tr>
<tr>
<td>SCFA</td>
<td>short chain fatty acids</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SOP</td>
<td>standard operating procedure</td>
</tr>
<tr>
<td>STAMP</td>
<td>statistical analysis of metagenomic profiles</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>terminal restriction fragment length polymorphism</td>
</tr>
<tr>
<td>TCA</td>
<td>taurocholic acid</td>
</tr>
<tr>
<td>Tcd</td>
<td>toxin of <em>C. difficile</em></td>
</tr>
<tr>
<td>TCDCA</td>
<td>taurochenodeoxycholic acid</td>
</tr>
<tr>
<td>TDCA</td>
<td>taurodeoxycholic acid</td>
</tr>
<tr>
<td>TGR5</td>
<td>Takeda G-protein-coupled receptor 5</td>
</tr>
<tr>
<td>TNBS</td>
<td>trinitrobenzenesulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TVC</td>
<td>total viable count(s)</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>UDCA</td>
<td>ursodeoxycholic acid</td>
</tr>
<tr>
<td>UPLC-MS</td>
<td>ultra-performance liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>VRE</td>
<td>vancomycin-resistant <em>Enterococci</em></td>
</tr>
</tbody>
</table>
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 asymptotically 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 10000 patients in 1996 up to 84 cases per 10000 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>
<thead>
<tr>
<th>Risk factor</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Rates of CDI in patients &gt;65 years old are approximately 10-fold higher than in younger patients (Lessa <em>et al</em>., 2015; Pepin, Valiquette &amp; Cossette, 2005).</td>
</tr>
<tr>
<td>Recent antibiotic exposure</td>
<td>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 &amp; Riley, 2003).</td>
</tr>
<tr>
<td>Hospitalisation/institutionalisation</td>
<td>Recent hospitalisation, prolonged hospitalisation (&gt;7 days) and/or prolonged antibiotic courses are all associated with an increased risk of CDI (Padua &amp; Pothoulakis, 2016).</td>
</tr>
<tr>
<td></td>
<td>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 <em>et al</em>., 2016).</td>
</tr>
<tr>
<td></td>
<td>Having a normal place of residence in a long-term care facility (e.g. nursing home) is also a CDI risk factor.</td>
</tr>
<tr>
<td>Acid-suppressing medications</td>
<td>Both PPI and H2-receptor antagonist use increase risk, but particularly PPIs (Bourgault <em>et al</em>., 2011; Howell <em>et al</em>., 2010).</td>
</tr>
<tr>
<td></td>
<td>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 <em>et al</em>., 2016).</td>
</tr>
<tr>
<td>Chronic metabolic, GI and/or liver disease</td>
<td>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 <em>et al</em>., 2017).</td>
</tr>
<tr>
<td></td>
<td>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 <em>et al</em>., 2018).</td>
</tr>
<tr>
<td></td>
<td>Obesity is also associated with an increased CDI risk (Mograbi <em>et al</em>., 2013).</td>
</tr>
</tbody>
</table>
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...
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 (Lyra 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.

<table>
<thead>
<tr>
<th>Test</th>
<th>Details</th>
</tr>
</thead>
</table>
| 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 ribaxamase-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 bezlotoxumab 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
35

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, Rutherford & 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 heterogeneous 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°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:

<table>
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<tr>
<th>Pathogen screening:</th>
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<tbody>
<tr>
<td>• Hepatitis A IgM</td>
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<tr>
<td>• Hepatitis B (HBsAg and HBeAb)</td>
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<tr>
<td>• Hepatitis C antibody</td>
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<tr>
<td>• Hepatitis E IgM</td>
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<tr>
<td>• HIV -1 and -2 antibodies</td>
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<tr>
<td>• HTLV-1 and -2 antibodies</td>
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<tr>
<td>• Treponema pallidum antibodies (TPHA, VDRL)</td>
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<td>• Epstein-Barr virus IgM and IgG*</td>
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<td>• Cytomegalovirus IgM and IgG*</td>
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<td>• Strongyloides stercoralis IgG</td>
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<td>• Entamoeba histolytica serology</td>
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<th>General/metabolic screening:</th>
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<td>• Full blood count with differential.</td>
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<td>• Creatinine and electrolytes</td>
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<td>• Liver enzymes (including albumin, bilirubin, aminotransferases, gamma-glutamyltransferase and alkaline phosphatase).</td>
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<td>• C-reactive protein</td>
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<tr>
<td>• Clostridium difficile PCR</td>
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<tr>
<td>• Campylobacter, Salmonella, and Shigella by standard stool culture and/ or PCR</td>
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<tr>
<td>• Shiga toxin-producing Escherichia coli by PCR.</td>
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<tr>
<td>• Multi-drug resistant bacteria, at least carbapenemase-producing Enterobacteriaceae (CPE) and extended-spectrum beta-lactamases (ESBL)*.</td>
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<tr>
<td>• Stool ova, cysts and parasite analysis, including for Microsporidia.</td>
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<td>• Faecal antigen for Cryptosporidium and Giardia.</td>
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<tr>
<td>• Acid fast stain for Cyclospora and Isospora.</td>
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<td>• Helicobacter pylori faecal antigen.</td>
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<td>• Norovirus, Rotavirus PCR.</td>
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Table 1.3: 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*

Taurine and glycine conjugated to cholate and chenodeoxycholate within the liver are secreted through biliary tree into small intestine.

- Taurochenodeoxycholate
- Glycochenodeoxycholate

Bile salt hydrolase (BSH) converts:

- Taurocholate
- Glycocholate

+ Other microbially-derived enzymatic bioconversions, e.g., UDCA formation through 7α/β-epimerisation.

Possible feedback via FXR/FGF-19 axis.
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 *Ruminococcaceae* 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 diarrhea with anti-CDI antibiotics for prior episodes), with no clear alternate explanation for diarrhea. 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°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°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°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°C on dry ice, and were kept stored at -80°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/ metabolome changes), stool samples (together with those of paired urine and
serum) were also analysed for rCDI patients from a Canadian randomised controlled trial investigating capsule 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-recurers if no recurrence of diarrheal 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-consuming, 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-associated 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 metatranscriptomics/ 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°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.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>28F-YM (forward primer)</td>
<td>TCGTCGGCACGTCAGATGTGTAAGAGCACAGGAGTTTGTATGCTCAG</td>
</tr>
<tr>
<td>28F-Borrellia (forward primer)</td>
<td>TCGTCGGCACGTCAGATGTGTAAGAGCACAGGAGTTTGTACCTGGCTT</td>
</tr>
<tr>
<td>28F-Chloroflex (forward primer)</td>
<td>TCGTCGGCACGTCAGATGTGTAAGAGCACAGGAATTTCATCTGGTCAG</td>
</tr>
<tr>
<td>28F-Bifido (forward primer)</td>
<td>TCGTCGGCACGTCAGATGTGTAAGAGACAGGGGTTAGCTGGCTCAG</td>
</tr>
<tr>
<td>388R (reverse primer)</td>
<td>GTCTCGTGGGCTCGAGATGTAAGAGACAGTGCTGCTCCCGTAGGAGT</td>
</tr>
</tbody>
</table>

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: 28F-Chloroflex: 28F-Bifido). 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/Blastcgi?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, Sobs) - 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’- CCTACGGAGGGCAGCA-3’), 1.8 µM BactQUANT reverse primer (5’- GGACTACGGGTATCTATCAAC-3’), 225nM probe ((6FAM) 5’-CAGCAGCCGCGGA-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 metataxonomic 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°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% TBDMSCI (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.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short chain fatty acid:</strong></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>2.50</td>
</tr>
<tr>
<td>Propionate</td>
<td>3.07</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>3.20</td>
</tr>
<tr>
<td>Butyrate</td>
<td>3.40</td>
</tr>
<tr>
<td>2-methylbutyrate</td>
<td>3.70</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>3.80</td>
</tr>
<tr>
<td>Valerate</td>
<td>4.20</td>
</tr>
<tr>
<td>Caproate</td>
<td>4.90</td>
</tr>
<tr>
<td>Lactate</td>
<td>6.14</td>
</tr>
<tr>
<td>2-hydroxybutyrate</td>
<td>6.40</td>
</tr>
<tr>
<td><strong>Internal standard:</strong></td>
<td></td>
</tr>
<tr>
<td>Methyl stearate</td>
<td>7.88</td>
</tr>
</tbody>
</table>

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 (A₆₀₀) 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). Collinsella 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) baiCD, using primers well-established within the literature (Wells et al., 2003).
Table 2.3: Primer sequences and PCR conditions for bsh and baiCD qPCR.

<table>
<thead>
<tr>
<th>Group</th>
<th>Primer Sequence (5'-3')</th>
<th>F/R</th>
<th>Cycling Conditions</th>
<th>Expected Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bsh 1A</td>
<td>CACATATTGTGGCAGAAAACTG</td>
<td>F</td>
<td>95°C for 10 min, (95°C for 15 sec, 55°C for 1 min) x 40 cycles</td>
<td>570</td>
</tr>
<tr>
<td></td>
<td>CTGTGCCGGATACAGATTTAAT</td>
<td>R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bsh 1B</td>
<td>CGCGTTCCGCATTTYTAGGAA</td>
<td>F</td>
<td>95°C for 10 min, (95°C for 15 sec, 55°C for 1 min) x 40 cycles</td>
<td>318</td>
</tr>
<tr>
<td></td>
<td>GTTCAATGCAAATCGGAATATCRA</td>
<td>R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bsh 3C/E</td>
<td>TTTTGCCGAAACACTGGAYGARTT</td>
<td>F</td>
<td>95°C for 5 min, (95°C for 15 sec, 54°C for 30 sec, 72 for 10 min) x 40 cycles</td>
<td>774</td>
</tr>
<tr>
<td></td>
<td>TCAACGGAGCCCAATATGGRAAR AYG</td>
<td>R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>baiCD</td>
<td>GGWTTCAGCCRCAGATTTTCTTTG</td>
<td>F</td>
<td>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</td>
<td>745</td>
</tr>
<tr>
<td></td>
<td>GAATCCCGGTTCATGAAACTTCKKAAG</td>
<td>R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 (Eurofins 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 (Agilent 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 pBKminiTn7GM2 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\textsubscript{low}’ (containing a gene for BSH with narrow substrate range against conjugated bile acids, derived from \textit{Lactobacillus salivarius}); and ‘E. coli BSH\textsubscript{high}’ (containing a gene for BSH with high glycine and taurine-deconjugating activity, obtained from \textit{Bifidobacterium adolescentis}) (Figure 2.1). These bacterial species were used as a means of directly identifying the specific contribution of BSH to TCA-mediated \textit{C. difficile} germination.

The basic protocol for the batch culture involved the incubation of \textit{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...
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°C-22°C.

All handling of mice/ collection of samples was performed by Dr Thomas B Clarke and Ms Izabela Glegola-Gledjeska, 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 \( \sim 10^3 \) 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 \( \sim 10^9 \) colony-forming units (CFU) of wild-type E. coli \((n=5)\) or E. coli BSH\textsubscript{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°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 \textit{bsh} gene from \textit{Bifidobacterium adolescentis} was that selected for cloning, given that this had been demonstrated \textit{in vitro} and \textit{in vivo} to have high level BSH activity in the experiments using \textit{E. coli} BSH\textsubscript{high}. The \textit{Bifidobacterium adolescentis bsh} gene was assembled from synthetic oligonucleotides and/or PCR products (960 bp), and inserted into the bacterial expression vector pTrcHis2a\textunderscore 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.

![Plasmid map of pTrcHis2a\textunderscore A085 vector containing \textit{bsh} gene of \textit{Bifidobacterium adolescentis}. As provided by Invitrogen, Thermo Fischer Scientific, UK.](image)

Subsequent steps for expression and cloning were adapted from protocols as previously-described (Rani, Anandharaj & Ravindran, 2017; Kumar \textit{et al.}, 2013). For expression of recombinant BSH (which was His-tagged (6x)), the plasmid was transformed into the expression host \textit{E. coli} BL21\textsuperscript{Al}\textsuperscript{™} OneShot Chemically Competent cells (Invitrogen, Thermo Fischer Scientific, UK). Specifically, \textit{E. coli} BL21\textsuperscript{Al}\textsuperscript{™} cells transformed with the plasmid were cultured overnight on LB agar plates with added ampicillin (concentration 100\textmu g/ml)
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 50\(\mu\)l of the reaction mixture with 50\(\mu\)l 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, 50\(\mu\)l of the supernatant from above was mixed with 950\(\mu\)l of ninhydrin reagent (Sigma-Aldrich) and 100\(\mu\)l of sodium-citrate buffer (0.5M, pH 5.5 (Sigma-Aldrich)). This mixture was placed in a laboratory shaker heated to 96\(^\circ\)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>
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<td>Age (years) (mean +/- SD)</td>
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</tr>
<tr>
<td>Sex</td>
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</tr>
<tr>
<td>BMI pre-FMT (kg/m²)</td>
<td>25.01 +/- 7</td>
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<tr>
<td>Median recurrences</td>
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<tr>
<td>Median prior CDI-related hospitalisations</td>
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<tr>
<td>Reported antibiotic use prior to CDI</td>
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<tr>
<td>Reported previous failed vancomycin taper</td>
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<tr>
<td>Route of administration</td>
<td>31% capsulised (8/26), 69% colonoscopy (18/26)</td>
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<tr>
<td>PPI use</td>
<td>42% (11/26)</td>
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<tr>
<td>Statin use</td>
<td>12% (3/26)</td>
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</table>

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.

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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).
### Table 3.3:

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<th>q value for difference in mean proportions (corrected)</th>
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### OTU enriched post-FMT compared to pre-FMT:

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### OTU enrichment data vs pre-FMT:

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### OTU enrichment post-FMT compared to pre-FMT:

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### OTU enrichment data vs pre-FMT:

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<th>Query cover (%)</th>
<th>Identity (%)</th>
<th>q value for difference in mean proportions (corrected)</th>
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<td>coliforms</td>
<td>100</td>
<td>100</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
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](image-url)  

**Figure 3.8:** Effect of FMT for rCDI upon inferred faecal microbiota function. As established using Piphillin as inferential 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 \textit{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 (\textbf{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) (\textbf{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 (\textbf{Figure 3.10A}), and between pre-FMT and post-FMT groups (\textbf{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 (\textbf{Figure 3.9C, Figure 3.10B}). OPLS-DA model validation was performed using CV-ANOVA (\textbf{Table 3.4}).

\textbf{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: \textit{n}=17; rCDI patients pre- and post-FMT: \textit{n}=26). Reproduced from (Mullish \textit{et al.}, 2019).
Figure 3.9:
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.

<table>
<thead>
<tr>
<th>Multi-variate model</th>
<th>$R^2_X$</th>
<th>$Q^2$</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor vs pre-FMT</td>
<td>0.533</td>
<td>0.933</td>
<td>$1.85 \times 10^{-13}$</td>
</tr>
<tr>
<td>Pre- vs post-FMT</td>
<td>0.437</td>
<td>0.839</td>
<td>$5.24 \times 10^{-13}$</td>
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</tbody>
</table>

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 metatonomic 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:
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 baiCD 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:
3.3.5.3. *baiCD* 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 *baiCD* 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 *baiCD* 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).](image)

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 baiCD 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 \textit{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 \textit{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 \textit{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 \textit{et al.}, 2017a). Further analysis demonstrated that \textasciitilde50\% 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 \textit{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 \textit{baiCD} in donors and post-FMT was noted to be markedly lower than for each \textit{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 \textit{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 \textit{C. difficile} germination (i.e. TCA), and diminishment of bile acids which inhibit the vegetative growth of \textit{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 time 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.

<table>
<thead>
<tr>
<th>Multivariate model</th>
<th>R²X</th>
<th>Q²</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OPLS-DA modelling by time point:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor vs pre-FMT</td>
<td>0.615</td>
<td>0.942</td>
<td>1.01 x 10⁻¹⁰</td>
</tr>
<tr>
<td>Pre- vs post-FMT, week 1</td>
<td>0.568</td>
<td>0.909</td>
<td>1.20 x 10⁻¹¹</td>
</tr>
<tr>
<td>Pre- vs post-FMT, week 4</td>
<td>0.612</td>
<td>0.924</td>
<td>5.06 x 10⁻¹¹</td>
</tr>
<tr>
<td>Pre- vs post-FMT, week 12</td>
<td>0.593</td>
<td>0.931</td>
<td>3.53 x 10⁻¹³</td>
</tr>
<tr>
<td>Post-FMT, week 1 vs post-FMT, week 4</td>
<td>No valid model constructed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-FMT, week 1 vs post-FMT, week 12</td>
<td>No valid model constructed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-FMT, week 4 vs post-FMT, week 12</td>
<td>No valid model constructed</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OPLS-DA modelling by route of administration:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All pre-FMT samples, capsule vs colonoscopic administration</td>
<td>No valid model constructed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All post-FMT samples, capsule vs colonoscopic administration</td>
<td>No valid model constructed</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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:
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. Relative intensity (per mg of lyophilised stool)

B. Relative intensity (per mg of lyophilised stool)

C. Relative intensity (per mg of lyophilised stool)

D. Relative intensity (per mg of lyophilised stool)

E. Relative intensity (per mg of lyophilised stool)

F. Relative abundance (per mg of lyophilised stool)
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 4.5:
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).
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-recurrences 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>
<thead>
<tr>
<th>OPLS-DA multivariate model</th>
<th>R²X</th>
<th>Q²</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrers, baseline vs non-recurers, baseline</td>
<td>No valid model constructed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrers, final time point vs non-recurers, final time point</td>
<td>0.372</td>
<td>0.552</td>
<td>0.0075</td>
</tr>
</tbody>
</table>
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-recurrences 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).
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-recurers 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 (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 baiCD 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 baiCD amongst CDI stool compared to stool from all other groups, but no actual significant difference in overall baiCD copy number between groups (Solbach et al., 2018). The conclusion made by these researchers was that the presence or absence of baiCD 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 iso-valerate to levels similar to that found pre-FMT (Section 4.3.1.5.3). The recovery of the branched chain fatty acids (BCFAs), iso-valerate 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 (iso-valerate 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 debilitating 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 CS 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; VB: vessel B. Adapted from (McDonald *et al.*, 2018a).
Figure 4.14:

A

B

C

D

E

C. difficile spores

Glycerol trivlarate or PBS

PBS

Glycerol trivlarate

CFU per g feces

Day post-infection

10^4

10^5

10^6

10^7

10^8

10^9

1

2

3

4
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 \( \text{OD}_{600} \). This is an endpoint well-established from comparable studies; an increased \( \text{OD}_{600} \) 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:**

![Graph showing ΔOD600 for different samples](image-url)
Figure 5.1 (page 163): *Clostridioides difficile* batch cultures – naturally BSH-producing organisms. Changes in spectrophotometer reading (ΔOD600) 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 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\textsubscript{low},’ containing a *bsh* gene with narrow substrate range/ moderate activity against conjugated bile acids; and ‘*E. coli* BSH\textsubscript{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\textsubscript{high} batch culture as compared to *E. coli* BSH\textsubscript{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 (ΔOD₆₀₀) 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 BSHₙₙₒₙ = E. coli expressing BSH with low deconjugation ability; *E. coli BSHₕᵢᵍₑₑ = 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).
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).
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).](image)

### 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 (~10³ 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 50$\mu$g/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 50 μg/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. 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.

![Box plots showing CFU counts of administered E. coli and C. difficile](image)

**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-Al™ 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 elutrate) 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.](image)

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.

<table>
<thead>
<tr>
<th>Category</th>
<th>Enzyme activity (μmol of taurine/ minute/ mg protein) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elutate 1</td>
<td>0.56 ± 0.029</td>
</tr>
<tr>
<td>Elutate 2</td>
<td>1.62 ± 0.049</td>
</tr>
<tr>
<td>Elutate 3</td>
<td>7.122 ± 0.064</td>
</tr>
<tr>
<td>Elutate 4</td>
<td>13.35 ± 0.021</td>
</tr>
</tbody>
</table>

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 metataxonomically 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, *baiCD* gene abundance is not different in the stool of CDI and *C. difficile*-negative patients (after correction for total bacterial load), and the *baiCD* 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 & Sonenschein, 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 *baiCD* operon copy number pre-FMT in comparison to healthy donors, and that *baiCD* 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 \textit{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 \textit{C. difficile} (McDonald \textit{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 \textit{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 \textit{et al.}, 2011). Furthermore, germ-free mice that were monocolonised with \textit{Bacteroides thetaiotaomicron} with a \textit{bsh} gene (as opposed to those monocolonised with the same
bacterium but without a \textit{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 \textit{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 \textit{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 \textit{et al.}, 2019). In particular, FXR activation has been demonstrated to inhibit bacterial overgrowth and block mucosal injury in mouse ileum (Inagaki \textit{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 \textit{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 \textit{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 \textit{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 \textit{et al.}, 2001) and vitamin D receptor (Makishima \textit{et al.}, 2002)), as well as cell membrane receptors (i.e. muscarinic receptors (Sheikh Abdul Kadir \textit{et al.}, 2010) and sphingosine-1-phosphate receptor 2 (Studer \textit{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 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
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 *Clostridiodiles 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.
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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:


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


2. Publications:  (*: joint first author)


References:


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


predict outcome of faecal microbiota transfer for recurrent Clostridioides difficile infection. *Digestive and Liver Disease*. [Online] Available from:


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


Kang, J.D., Myers, C.J., Harris, S.C., Kakiyama, G., et al. (2019) Bile Acid 7α-Dehydroxylating


Konturek, P.C., Koziel, J., Dieterich, W., Haziri, D., et al. (2016) Successful therapy of


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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).

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**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

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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

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