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# An investigation of the factors influencing *Clostridium difficile* germination, colonisation and persistence

A thesis submitted to the University of Glasgow for the degree of

Doctor of Philosophy

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Submitted September 2016

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# Author's Declaration

Unless explicitly stated otherwise, all of the experimental work presented in this thesis was performed by me, either independently or in conjunction with others. No part of this research or any part of this document has or will be presented for the fulfilment of any other degree or qualification.

Caitlin Jukes
September 2016

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# Abstract

*Clostridium difficile* is the leading cause of antibiotic associated diarrhoea worldwide. Infection typically follows treatment with broad spectrum antibiotics which disrupt the protective microbiota. The mechanisms of infection are being slowly unravelled and it is now known that the microbiota plays an important role in mediating colonisation resistance to *C. difficile*. *C. difficile* is spread by spores which once ingested germinate, a process stimulated by primary bile acids (BAs), particularly taurocholate (TC). The aim of the work presented was to further extend our understanding of the functional role BAs play in germination, colonisation resistance and disease severity. A final aim was to examine how the production of *para*-cresol, a bacteriostatic compound produced by *C. difficile*, impacts upon acute and relapsing infections.

To examine the role of the microbiota in *C. difficile* pathogenesis, three mouse models of infection were used, in which disease severity is dependent on the combination of antibiotics administered prior to *C. difficile* challenge. In the first model, administration of an antibiotic cocktail (ABC) followed by clindamycin induced more severe disease. In contrast, mice treated with clindamycin only became colonised, whilst those given the ABC alone were only transiently infected. Colonisation resistance was found to be associated with the absence of bacteria involved in bile metabolism. This is because unmodified bile acids (BAs) act as germinants for *C. difficile*, stimulation germination and bacterial outgrowth. Loss of bile salt hydrolase (BSH) producing bacteria did not correlate with increased severity of disease. Further analysis of the microbiome data suggests that severity may be linked to loss of bacteria associated with mucus layer integrity.

BAs are toxic molecules that can damage both bacterial membranes and their DNA. Limiting exposure to these compounds is essential for *C. difficile* to survive *in vivo*. Investigation of the genetic response to the TC post germination was investigated using RNA seq. This resulted in the identification of several genes, including efflux pumps that may be important in mediating *C. difficile* bile resistance. It is likely genes involved in bile resistance are important in enabling successful colonisation of *C. difficile*.

Finally the production of *para*-cresol in acute and relapsing infection was examined. Strains that were unable to generate *para*-cresol caused greater severity in the mouse model of disease. Extensive *in vitro* and *in vivo* experiments showed slightly increased toxin production in the non *para*-cresol producing R20291_*hpdA*::CT strain, providing one potential explanation for this unusual phenotype.

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# Abbreviations

| | |
|---|---|
| % | Percent |
| Δ | Deletion |
| °C | Degrees Celsius |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometre |
| AAD | Antibiotic associated diarrhoea |
| AHT | Anhydrotetracycline |
| BA(s) | Bile acid(s) |
| BAI | Bile acid inducible operon |
| BHI | Brain hear infusion |
| bp | Base pairs |
| BSH | Bile salt hydrolase |
| BSH+ve | *L. murinus* isolate with BSH activity |
| BSH-ve | *L. murinus* isolate lacking BSH activity |
| CA | Cholic acid |
| CBA | Conjugated bile acid |
| CBM | Columbia based media |
| CDCA | Chenodeoxycholic acid |
| CDT | *Clostridium difficile* binary toxin |
| CFU | Colony forming units |
| CI | Caecal index |
| Cm | Centimetres |
| CODEHOP | Consensus-degenerate hybrid oligonucleotide |
| CPD | Cysteine protease domain (CPD) |

| | |
|---|---|
| CROP | Combined repetitive oligo peptide repeat |
| CU | Cardiff University |
| CV | Crystal violet |
| DCA | Deoxycholic acid |
| DNA | Deoxyribonucleic acid |
| DMSO | Dimethyl Sulfoxide |
| DPA | Dipicolinic acid |
| DPI | Days post infection |
| DW | Drinking water |
| EHC | Enterohepatic circulation |
| FACs | Fluorescent Automated Cell sorter |
| FAM | Fastidious anaerobic media |
| FDA | Food and Drugs administration |
| FMT | Faecal microbial transplant |
| FS | Forespore |
| FXR | Farnesoid X receptor |
| g | Grams |
| GF | Germ free |
| GI | Gastrointestinal |
| GFP | Green fluorescence protein |
| GPF | Glasgow Polyomics facility |
| GTD | Glucosyl transferase domains (GTDs) |
| H + E | Haematoxylin and eosin |
| ICL | Imperial College London |
| IP | Intraperitoneal |
| Kb | Kilobase |

| | |
|---|---|
| KEGG | Kyoto Encyclopaedia of Genes and Genomes |
| Kg | Kilograms |
| LCA | Lithochoilc acid |
| LCGR | Liverpool centre for genomic research |
| La | Lumen associated |
| LB | Luria Bertani |
| LOV | Light, oxygen or voltage sensing |
| LSHTM | London School of Hygiene and Tropical Medicine |
| mg | Milligrams |
| mL | Millilitre |
| mM | Milli molar |
| M | Molar |
| MA | Muricholic acids |
| MC | Mother cell |
| mRNA | Messenger Ribonucleic acid |
| MRS | Man Rogosa Sharpe |
| MT | Mariner transposon |
| MVLA | Multilocus Variant number tandem repeat analysis |
| ng | Nanograms |
| OD | Optical density |
| OTUs | Operational taxonomic units |
| PaLoc | Pathogenicity locus |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| phiLOV | Photostable LOV |
| *p*-HPA | *Para*-hydroxyphenylacetate |

| | |
|---|---|
| PMC | Pseudomembranous colitis |
| PXF | Pregnane X receptor |
| RFC | Random Forest Classifier |
| RNA | Ribonucleic acid |
| RNAP | RNA polymerase |
| Rpm | Rotations per minute |
| R20291_*hpd*A::CT | *C. difficile* with a ClosTron insertion in the *hpd*A gene |
| R20291_*1754*::CT | *C. difficile* with a ClosTron insertion in CDR202911754 |
| SAM | S-adenosyl-L-methionine |
| SBA | Secondary bile acids |
| SCFAs | Short chain fatty acids |
| SDW | Sterile deionised water |
| SEM | Standard error of the mean |
| Ta | Tissue associated |
| tBSH | Truncated bile salt hydrolase |
| TcdA | Toxin A |
| TcdB | Toxin B |
| TC | Taurocholic acid |
| TMC | Toxin mega colon |
| TOF-MS | Time of flight mass spectrometry |
| TraDIS | Transposon Directed Insertion Sequencing |
| TSB | Tryptone soya broth |
| TY | Tryptone yeast |
| UK | United Kingdom |
| USA | United States of America |
| UoS | University of Sheffield |

| | |
|---|---|
| V | Volts |
| VDR | Vitamin D receptor |
| WCA | Wilkins-Chalgren anaerobic |
| WGS | Whole genome sequencing |
| WT | Wild type |

# 1 Introduction

## 1.1 *Clostridium difficile*

*C. difficile* is a Gram positive, obligate anaerobe which is capable of producing endospores. First isolated from a healthy infant's stool in 1935 (Hall & O'Toole, 1935) it is a disease largely of the modern antibiotic era, first being identified as the cause of clindamycin associated pseudomembranous colitis (PMC) in the early 1970's (Bartlett *et al.,* 1977). *C. difficile* is now recognised as the leading cause of antibiotic associated diarrhoea (AAD) worldwide causing an estimated 10-15% of cases of AAD and 95% of PMC (Bartlett, 1994). *C. difficile* disease is typically associated with the elderly (those over 65) (Rea *et al.,* 2012) and immunocompromised individuals. It is frequently seen in patients who have been treated with a number of predisposing antibiotics, which, include clindamycin, the cephalosporins and penicillin (Bartlett, 2002). Infection with *C. difficile* is associated with a wide range of symptoms from mild, self-resolving diarrhoea, to severe relapsing infection that can proceed to PMC, toxic mega colon (TMC) and sometimes death. It is thought that between 3-7% of the healthy adult population carry *C. difficile* as part of their normal flora (Pires *et al.,* 2016; Kato *et al.,* 2001) and in infants carriage rates are much higher (up to 70%) although this carriage is typically asymptomatic (Jangi & Lamont, 2010).

*C. difficile* is a complicated disease and can often cause relapsing and difficult to treat infections. This is estimated to occur in between 30-60% of patients (Viswanathan *et al.,* 2010) with the likelihood of relapse increasing with each successive episode (Khoruts & Sadowsky, 2011) which most likely reflects a persistent and ongoing disruption of the microbiota (Chang *et al.,* 2008). These patients are difficult to treat leading to poor quality of life. It is for these patients that new treatments for this infection need to be sought, as for many, traditional antibiotic therapy is no longer effective. This has resulted in a move towards bacterial therapies such as faecal microbial transplantation (FMT) and defined bacterial therapies, which will be discussed in detail in sections 1.5.1 and 1.5.2. The financial cost associated with *C. difficile* infection is also enormous with the USA estimated to have spent in

excess of $4.8 billion on treatment in 2008 alone (Dubberke & Olsen, 2012), whilst costs in Europe have been calculated to be approximately £8000 per patient (Wiegand *et al.,* 2012). Although improvements in antibiotic stewardship have resulted in the number of cases in the UK falling steadily since 2009 (Carter, 2013), cases are still challenging to treat. For this reason, greater understanding of the disease itself and improvement of treatment is essential. Although, many recent advances have been linked to increased knowledge of the role of the microbiota, a clearer understanding of the bacterial functions that influence *C. difficile* germination, colonisation and persistence are required.

### 1.1.1 Emergence of a hyper-virulent ribotype

In the early 2000s, the epidemiology of *C. difficile* underwent a dramatic change with a sudden increase in the rates of infection reported in the USA, Canada and the UK. The outbreaks were found to all be associated with the spread of a newly emergent clone referred to as NAP1/REA/027, which will be referred to as the 027 type for the remainder of this thesis (Warny *et al.,* 2005). The spread of this hyper-virulent epidemic clone was associated with higher levels of mortality and increased incidence of PMC and TMC. During this time, several severe outbreaks occurred culminating in the death of 25 patients at The Stoke Mandeville hospital in the UK (Katikireddi, 2005). Another outbreak across Quebec in Canada during 2003-2005 was estimated to have killed between 1440-2000 people (Pepin *et al.,* 2004; Eggertson 2005; Eggertson 2006). In addition to the high death rates, surviving patients infected with this strain suffered higher rates of antibiotic failure and relapse of disease (Marsh *et al.,* 2012).

### 1.1.2 The role of toxin production in *C. difficile* disease

#### 1.1.2.1 The pathogenicity locus and regulation of toxin production

Most of the clinical symptoms of *C. difficile* disease are associated with production of two toxins that result in epithelial damage and inflammation. These toxins, known as toxin A (TcdA) and toxin B (TcdB) are encoded on a 19.6kb pathogenicity locus (PaLoc), which is only found in pathogenic strains

of *C. difficile*. This locus consists of 5 genes that control toxin production, regulation and release (Figure 1.1).

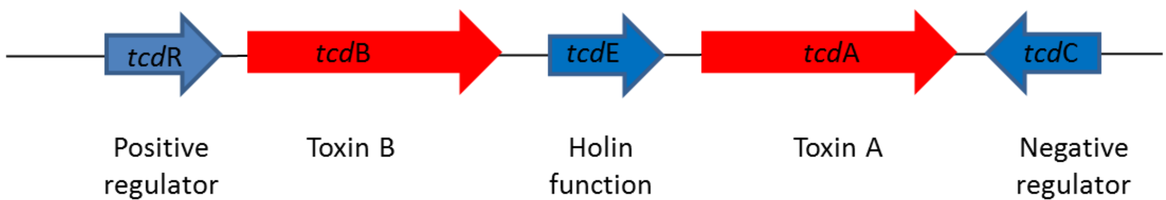



**Figure 1.1 The structure of the PaLoc in *C. difficile.* Adapted from Voth & Ballard (2005)**
The 19.6kb pathogenicity locus from *C. difficile* contains 5 genes. The *tcd*B and *tcd*A genes encode for toxins. The PaLoc contains two regulators with *tcd*R acting as a positive regulator and *tcd*C acting as a negative regulator. Toxin release is thought to be mediated by *tcd*E a putative holin.

Toxins are most highly expressed during the stationary phase of growth (Dupuy & Sonenshein 1998), with a transcriptional study showing high levels of expression of *tcd*R, *tcd*A, *tcd*B and *tcd*E during this phase. In contrast only low levels of *tcd*C were expressed during this phase with *tcd*C expressed predominantly during exponential phase. This suggests a role of TcdC as a negative regulator of toxin production (Hundsberger *et al.,* 1997). Investigation of the structure of TcdC suggests this repression may be achieved through its action as an anti-sigma factor enabling it to interfere with positive regulation that is mediated by tcdR. TcdR is a σ factor of RNA polymerase (RNAP) that enables the expression of both toxins genes by directing the RNAP to the toxin promoter regions (Mani & Dupuy, 2001). Repression of toxin production by TcdC is thought to be achieved through its ability to prevent the TcdR containing RNAP from recognising the toxin gene promoters, preventing binding (Matamouros *et al.,* 2007).

Early analysis of the hyper-virulent 027 strains reported that mutations in *tcd*C were associated with the production of higher levels of toxin *in vivo* (Dupuy *et al.,* 2008). This was supported by a study that created mutants in the *tcd*C gene and found that this resulted in increased toxin production and virulence in the hamster model of infection (Carter *et al.,* 2011). However, subsequent studies have reported no difference in toxin production. A landmark study by Cartman *et al.,* (2012) replicated the *tcd*C mutations associated with hyper virulence in R20291 in a non-hyper virulent 630 strain. They also reversed the Δ117 frameshift in R20291 that is thought to be

associated with increased toxin production. It was found that these changes had no impact on toxin production in either strain suggesting that these mutations may not be responsible for increased toxin production.

#### 1.1.2.2 The influence of CodY on toxin production

Wider control of toxin production is complicated. It is in part influenced by CodY, a global gene regulator that is involved in several important processes including toxin production and sporulation (Nawrocki *el al.,* 2016). A study that constructed a *cod*Y null strain found that lack of CodY activity resulted in the >4-fold overexpression of 146 genes and the under expression of a further 19. This included genes involved sporulation, toxin production, amino acid biosynthesis, fermentation pathways and surface proteins (Dineen *et al.,* 2010). Another study by Dineen *et al.,* (2007) found that inactivation of CodY resulted in increased expression of 5 genes within the pathogenicity locus including *tcd*R, *tcd*B, *tcd*E, *tcd*A and *tcd*C. It was also demonstrated that there was strong interaction between CodY and tcdR. This suggests that the majority of the CodY repression of toxin production is mediated through this interaction. It has also been shown that this repression is increased in the presence of branched chain amino acids and GTP, potentially highlighting a link between toxin production and nutrient limitation. This fits with earlier observations that toxins are predominately expressed in the stationary phase (1.1.2.1).

#### 1.1.2.3 Toxin action and receptor mediated endocytosis

For toxins to become active they must first be internalised within the cell. Both tcdA and tcdB contain 3 distinct domains that are involved in different processes. These domains are a glucosyl transferase domain (GDT), a cysteine protease domain (CPD) and a combined repetitive oligo-peptide repeat (CROP) (Figure 1.2). Each domain has a distinct role in toxin internalisation, activation and action. The CROP region is responsible for the binding of the toxin to the colonic epithelial cells with TcdA binding to carbohydrates and TcdB to poliovirus receptor-like 3. Receptor mediated endocytosis then occurs during which the toxin is internalised into the cell in acidified endosomes. The environment within the endosome allows the CROP domain to embed into the

endosomes membrane. After the toxin has embedded the CPD and GTD domains are transferred into the cytosol. Once within the cytosol inositol hexakisphosphate (IP6) triggers the cleavage of the CPD releasing the GTD (Figure 1.2). The GTD can then inactivate both Rho and Rac GTPases. Inactivation of these molecules leads to the disruption of tight junctions, actin rearrangement and apoptosis. Cell damage and death results in an influx of immune cells to the site and if inflammation is not controlled it can lead to severe inflammatory bowel disease typified by PMC. Inflammation is mediated by the release of inflammatory promoting cytokines such as IL-1, TNF-α and IL-8 which result neutrophil influx and destruction of the epithelial cell lining (Shen 2012; Warny *et al.,* 2000; Savidge *et al.,* 2003).

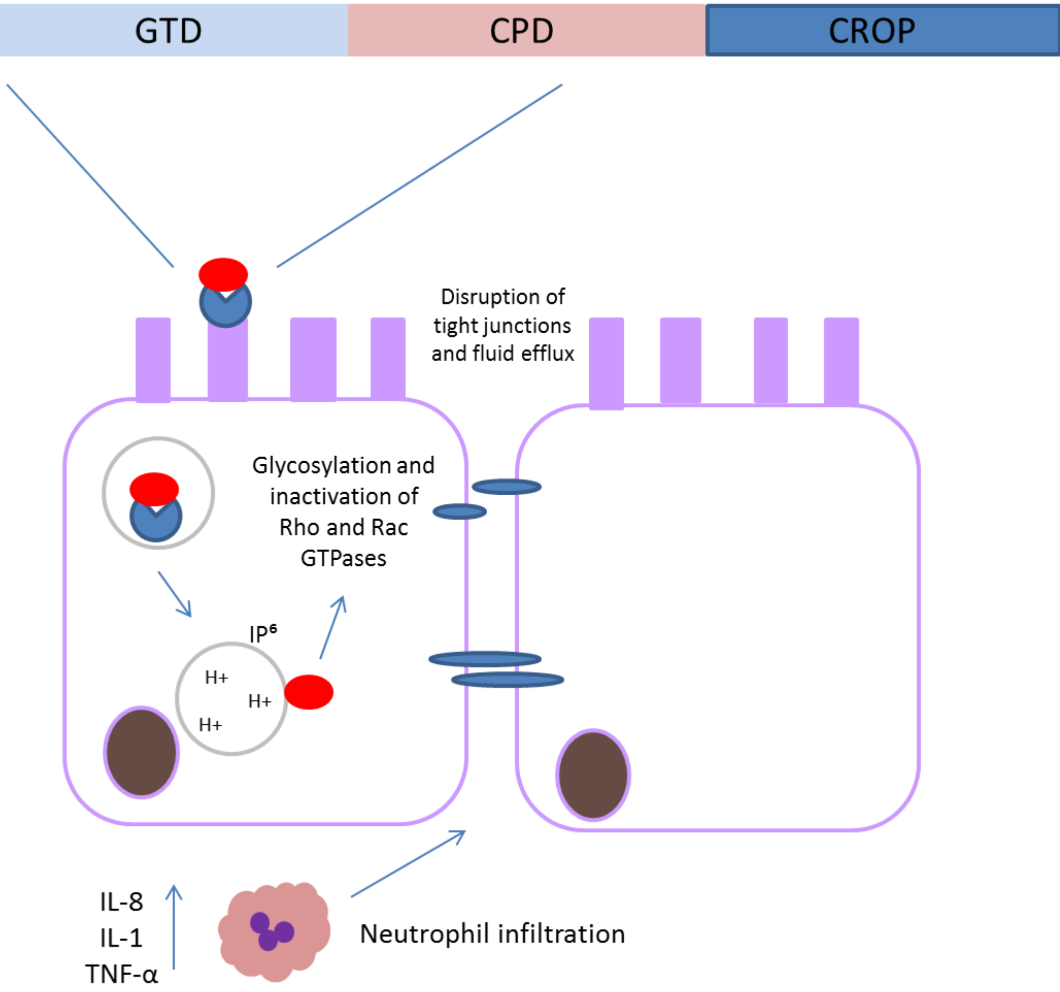



**Figure 1.2 Toxin structure, internalisation and action. Adapted from** (Abt, McKenney, & Pamer, 2016)
After binding to epithelial cells toxins are internalised into acidified endosomes. The CROP domain is embedded into the membrane of the endosome allowing the CPD and GTD to enter the cytosol where the toxin is then activated thought the cleavage of the CPD domain. The GTD then inactivates Rho and Rac GTPases resulting in the loss of tight junctions resulting in fluid efflux and cell death. Additionally, increases in the inflammatory cytokines IL-1, IL-8 and TNF-α result in an influx of immune cells to the site which can lead to inflammation.

Initial studies using purified toxins indicated that TcdA was solely responsible for the majority of disease symptoms, with hamsters and mice succumbing to disease when given purified TcdA alone (Lyerly *et al.,* 1985). However subsequent experiments using strains in which the individual toxin genes have been rendered inactive have now shown that typically both toxins are required to cause disease. Initially, work carried out by Lyras *et al.,* (2009), in which mutants in each toxin gene were used to infect hamsters, showed that TcdB not TcdA was essential for virulence. However a later study that also created mutants in each toxin gene found that mutation of either toxin was not sufficient to render the strain avirulent. However hamsters infected with

a strain producing TcdA only took longer to develop symptoms of disease than those only producing TcdB (Kuehne *et al.,* 2010). An additional study by the same group confirmed these findings and again found that although both toxins cause disease TcdB is required for severe disease (Kuehne *et al.,* 2014). Interestingly clinical isolates are frequently identified that are TcdA negative but TcdB positive, (Samra *et al.,* 2002; Pituch *et al.,* 2001; Alfa *et al.,* 2000; Limaye *et al.,* 2000) whereas TcdA only strains have never been isolated from humans (Di Bella *et al.,* 2016). This lack of TcdA only producing strains suggests that TcdB is essential for disease in humans.

In addition to TcdA and TcdB some isolates can also produce *C. difficile* binary toxin (CDT). Production of CDT has been associated with an increased risk of mortality within 30 days of infection (Gerding *et al.,* 2014). However, the role of CDT in disease is unclear with CDT production alone not sufficient to cause disease in hamsters (Geric *et al.,* 2006). A recent study found that CDT may increase virulence by inducing host inflammation via a Toll like receptor mediated pathway which in turn results in the suppression of the immune response (Cowardin *et al.,* 2016). The recent isolation of a strain expressing CDT alone from a patient suffering from severe diarrhoea (who tested negative for other intestinal pathogens), suggests that these strain may be able to cause disease in humans (Androga *et al.,* 2015). Further work to fully understand the role of CDT is required and its relative importance in disease is still not well understood.

## 1.2 *C. difficile* spore germination and outgrowth

The etiological agent of *C. difficile* is the spore. These are formed under conditions of stress, such as oxygen exposure and nutrient limitation. Spores are highly resistant to environmental insults such as heat, desiccation and antibiotic exposure, allowing *C. difficile* to persist in the environment, facilitating transfer to new hosts. To cause disease spores must germinate to form vegetative cells that subsequently produce toxins. In *C. difficile*, spore germination is triggered by a combination of BAs and glycine. This has been confirmed experimentally *in vitro* (Sorg & Sonenshein, 2008) and indirectly *in vivo* with caecal extracts from antibiotic treated mice less able to induce

spore germination after incubation with cholestyramine, a BA binding resin (Giel *et al.,* 2010). A recent study sought to determine the sites of spore germination and outgrowth *in vivo*. To do this they took *ex-vivo* extracts from the small intestine, caecum and colon of mice before and after antibiotic treatment. They found that the mouse small intestine continually promotes *C. difficile* spore germination, even without antibiotic treatment due to the presence of primary BAs. However, they found that outgrowth in the large intestine was only possible after antibiotic treatment and that this was due to decreases in the abundance of secondary BAs (Theriot *et al.,* 2015). It is therefore likely that germination is triggered early in the GI tract and that outgrowth in the caecum and colon only occurs when the level of secondary BAs decreases.

## 1.2.1 *C. difficile* spore formation

### 1.2.1.1 Genetic regulation of sporulation

*C. difficile* forms metabolically dormant spores under conditions of stress and nutrient limitation, although the precise signals are unknown. Spore formation is essential to aid transmission of *C. difficile* and to enable it to persist in the environment. The structure of the spore enables them to be highly resistance to a range of environmental insults including heating, desiccation, radiation and chemical stresses. As with toxin production the global regulator CodY is involved in sporulation with activation of sporulation genes associated with nutrient limitation (Nawrocki *et al.,* 2016). Genetic control of sporulation is controlled by the master regulator Spo0A, with inactivation of this gene completely removing the ability of *C. difficile* to form spores.

Activation of Spo0A is achieved through phosphorylation by sensor histidine kinases. After phosphorylation Spo0A induces the expression of $\sigma^H$, which then forms a positive feedback loop with Spo0A. Downstream from Spo0A are 4 compartment specific alternative sigma factors, σF, σE, σG and σK In the forespore (FS) σF activates SpoIIR, a signalling protein that can then activate σE in the mother cell (MC) and σG in the FS. After activation σE can then activate σK in the MC. These transcription factors then initiate the production

of specific genes involved in formation of the spore (Fimlaid *et al.,* 2013; Paredes-Sabja *et al.,* 2014; Pereira *et al.,* 2013).

#### 1.2.1.2 *Clostridium difficile* spore structure

The structure of the spore is what enables *C. difficile* to exhibit high levels of resistance to multiple stresses. The formation of the spore begins with the formation of a septum in the MC resulting in the formation of two unequal sized compartments. The FS is then engulfed by the MC and the several layers of the spore-coat assembled. The spore is a complex structure with several layers that help to confer resistance to environmental stresses and to protect the DNA, RNA and enzymes held within the spore core. Around this core is an inner membrane and a germ cell wall. These layers have low permeability thus preventing water and other small molecules from entering the spore core. This maintains the low water content of the spore is and protects from DNA damaging molecules. A thick peptidoglycan layer is then surrounded by an outer membrane which in turn is covered with the spore-coat. This consists of many unique proteins that help to mediate *C. difficile* spore resistance properties. The final layer is the exosporium which is also involved in aiding resistance of the spore. After formation of these layers the MC lyses and the spore is released into the environment where it can persist and disseminate. Under exposure to the right stimulus *C. difficile* can undergo germination to form a vegetative cell (Paredes-Sabja *et al.,* 2014).

### 1.2.2 Bile acids and glycine trigger spore germination

To cause active disease *C. difficile* spores must germinate to form vegetative cells that can then produce toxin. In *C. difficile* the most potent inducer of spore germination is the conjugated BA taurocholate (TC), which is only found in the GI tract of mammals and other vertebrates. Exposure of *C. difficile* spores to TC and glycine results in a rapid and irreversible induction of germination pathways (Figure 1.3).

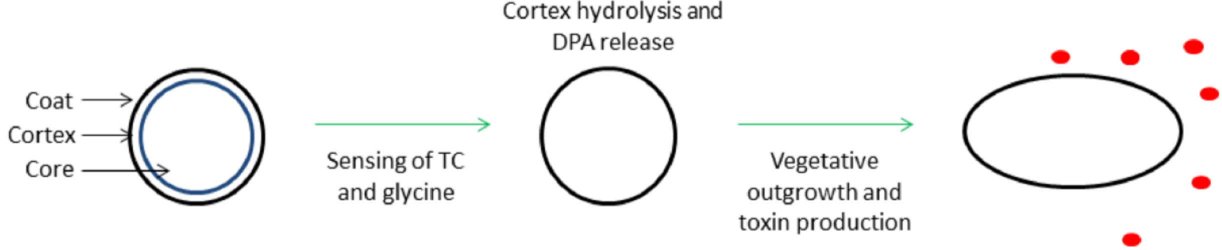



**Figure 1.3 Simplified schematic of *C. difficile* spore germination, vegetative outgrowth and toxin production**
The sensing of TC and glycine by the spore results in a rapid induction of germination and progression to vegetative outgrowth and toxin production (red circles). This is a two stage process with dipicolinic acid (DPA) release followed by cortex degradation.

Induction of spore germination is followed by rapid efflux of calcium and DPA (ca-DPA) followed by cortex hydrolysis. Vegetative outgrowth is followed by toxin production. Understanding the complex process of germination is essential to improve understanding of disease progression and may help to identify therapeutic targets of this disease.

#### 1.2.2.1 Genetic regulation of germination

To cause disease spores must first germinate to form vegetative cells. However, in comparison to *Bacillus subtilis* in which the different steps in germination are well defined, knowledge of the *C. difficile* germination pathway remains undeveloped. Initial progress was hampered by the failure to find any homologs to known germinant receptors (encoded by the ger operon) that regulate initiation of germination in *Bacillus* species. Knowledge of *C. difficile* spore germination is improving as a consequence of the increased availability of genetic tools that allow manipulation of the organism, including mutagenesis of individual genes (Heap *et al.*, 2010), and the creation of transposon mutant libraries (Dembek *et al.*, 2015; Cartman & Minton 2010). Additionally, the availability of multiple genome sequences has enabled easier study of this and other biological processes in *C. difficile*.

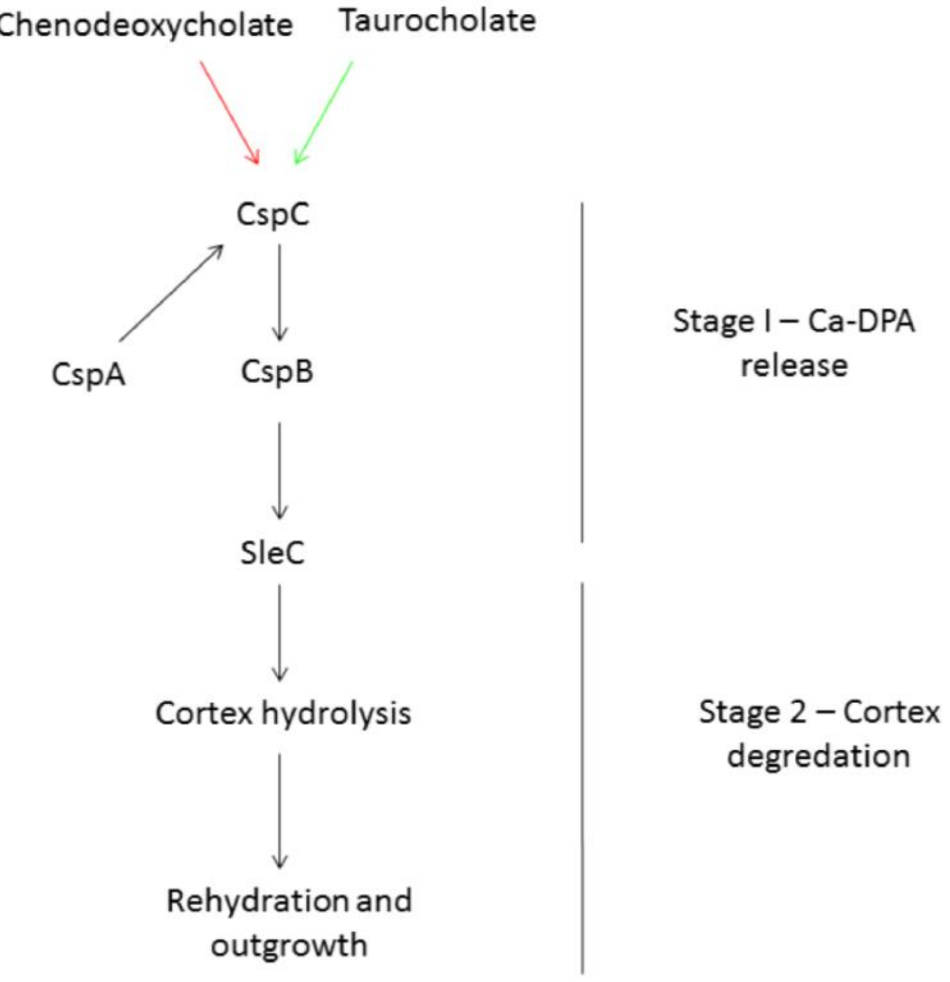



**Figure 1.4 Schematic of current understanding of *C. difficile* spore germination adapted from Olguín-Araneda *et al.,* (2015)**
The sensing of TC by CspC results in the rapid induction of spore germination. The protease CspB activates SleC which then activates spore cortex hydrolysis. The bacterium can then rehydrate and outgrow.

Spore germination in *C. difficile* is a two-step process, the first step, release of DPA triggers the second step of cortex break down (Figure 1.4). Germination is initiated through activation of the germinant receptor *csp*C, by TC. Mutants in *csp*C that are unable to respond to TC have severely attenuated germination rates *in vitro*. This mutant can still cause fatal infection in hamsters, although only 50% of animals succumb to infection compared to 100% of those infected with the WT. This suggests that either *C. difficile* is able to germinate spontaneously *in vivo* or that other germinants exist that can be sensed using an alternative pathway (Francis *et al.*, 2013). Activation of CspC leads to the loss of calcium and DPA, initiating the second

stage of germination, cortex hydrolysis. Hydrolysis is controlled by a single cortex hydrolase, SleC, which is essential for the transition to vegetative growth (Burns *et al.,* 2010). To be functional SleC must first be activated by the Csp family of proteases. In *C. difficile* this has been associated with the production of CspBA, a fusion of the active protease CspB to the inactive CspA (Francis *et al.,* 2015) Interestingly, although mutation of CspB results in decreased germination efficiency, it does not eradicate this process. This suggests that SleC may be activated by as yet undefined proteases (Adams *et al.,* 2013). Additionally, it would appear that CspA has a role in controlling the levels of CspC in the mature spore (Kevorkian *et al.,* 2016) with a nonsense mutation in the *csp*A region of *csp*BA gene resulting in severely decreased levels of germination (Francis *et al.,* 2013).

#### 1.2.2.2 Diverse responses to TC as a germinant

Although TC is known to induce spore germination, numerous studies have revealed that responses to TC vary considerably between isolates of *C. difficile* (Heeg *et al.,* 2012; Carlson *et al.,* 2015; Moore *et al.,* 2013). The implication of this is unclear, with higher rates of germination linked to both increased (Moore *et al.,* 2013) and decreased disease severity (Carlson *et al.,* 2015). Such variation in germination response has also been reported for other Clostridia including *Clostridium perfringens,* in which similar disparity between germination rates and disease and non-disease causing strains has been described (Akhtar *et al.,* 2009). Whilst the mechanisms by which TC induces germination have been well studied, little is known about the role of glycine in the process. Spores cannot geminate in the presence of TC alone, and glycine has been identified as an essential co-germinant (Sorg & Sonenshein, 2008). Kinetic analysis of germination suggests that glycine binding follows TC recognition, through an as yet unidentified receptor (Ramirez *et al.,* 2010).

#### 1.2.2.3 Other factors affecting germination

##### 1.2.2.3.1 Bile acids

Although TC and glycine are the most potent combination of germinants, several other compounds have been identified that influence *C. difficile* spore germination. These include other cholate derived BAs, such as cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA). The role of these compounds is complicated. For instance, CA is often reported as a germinant of *C. difficile*, and yet it has been frequently reported as unable to induce germination even after 6 hours of exposure (Ramirez *et al.,* 2010; Wheeldon *et al.,* 2008; Sorg & Sonenshein 2008). However CA has been shown to enhance colony formation on an agar plates (Sorg & Sonenshein, 2008). In contrast, CDCA has been reported as a competitive inhibitor of TC mediated germination with a higher affinity for the germinant receptor than TC (Sorg & Sonenshein, 2009). This has led to the development of potential treatments based around this compound, which will be discussed in section 1.5.3.1. Similarly DCA has been shown to inhibit vegetative growth of *C. difficile* (Sorg & Sonenshein, 2008). Interestingly, a subset of BAs only produced by mice, muricholic acids (MA), inhibit spore germination and growth of *C. difficile* (Francis *et al.,* 2013). The production of these BAs may provide an explanation as to why infections of mice with *C. difficile* have been classically difficult to achieve.

##### 1.2.2.3.2 Amino acids

In addition to glycine, several other amino acids have been shown to act as co-germinants of *C. difficile*. These include histidine, aspartic acid, arginine, serine and valine. Although these amino acids in isolation were unable to induce efficient germination, if used in combination with TC and glycine, these amino acids resulted in increased germination efficiency. This suggests that nutrient sensing may play a role in this process (Wheeldon *et al.,* 2011).

Whilst efficient *C. difficile* spore germination has been linked to TC and glycine, they are not essential, as *C. difficile* is able to germinate in rich media alone (Heeg *et al.,* 2012). This suggests that control of germination is

somewhat 'leaky' with initiation of germination possible in the absence of specific germinants. However, it is likely that *in vivo*, where competition is greater and nutrients are scarce, that this pathway is more tightly controlled.

## 1.3 BA production and function in the host

Although BAs play a role in triggering *C. difficile* spore germination, their main role in the host is to facilitate lipid absorption in the colon. BAs are cholesterol derivatives produced by liver hepatocytes. Their main function is to enable easy absorption of lipids and lipid soluble vitamins through the formation of micelles. BAs have also been found to interact with a number of host nuclear receptors including the farnesoid X receptor (FXR), pregnane X receptor (PXF) and the vitamin D receptor (VDR) (Zhou & Hylemon 2014; De Aguiar Vallim *et al.,* 2013) but these interactions fall outside of the remit of this thesis. Bile itself contains approximately 0.8% BAs with the rest consisting of water (97%), cholesterol, lipids, bilirubin and phosphatidylcholine.

There are two main BAs produced in the human body, CA and CDCA. Both CA and CDCA can be produced by the neutral pathway whereas the acidic pathway only produces CDCA. In addition to CA and DCA, rodents produce an additional group of murine specific MA (Botham & Boyd, 1983). The majority of BAs are produced by the neutral pathway. In this pathway cholesterol is converted to 7α-hydroxycholesterol by the enzyme cholesterol 7α-hydroxylase (CYP7A1). Conversion of 7α-hydroxycholesterol to CA is dependent on hydroxylation by sterol 12-alpha-hydroxylase (CYP8B1) followed by oxidation of cholesterol side chain by sterol 27-hydroxylase (CYP27A1). Oxidation alone of 7α-hydroxycholesterol by CYP27A1 results in the formation of CDCA. In the acidic pathway CYP27A1 converts cholesterol to 27-hydroxycholesterol which can then be converted into CDCA through hydroxylation by oxysterol 7α-hydroxylase (CYP7B1) (Figure 1.5) (De Aguiar Vallim *et al.,* 2013).

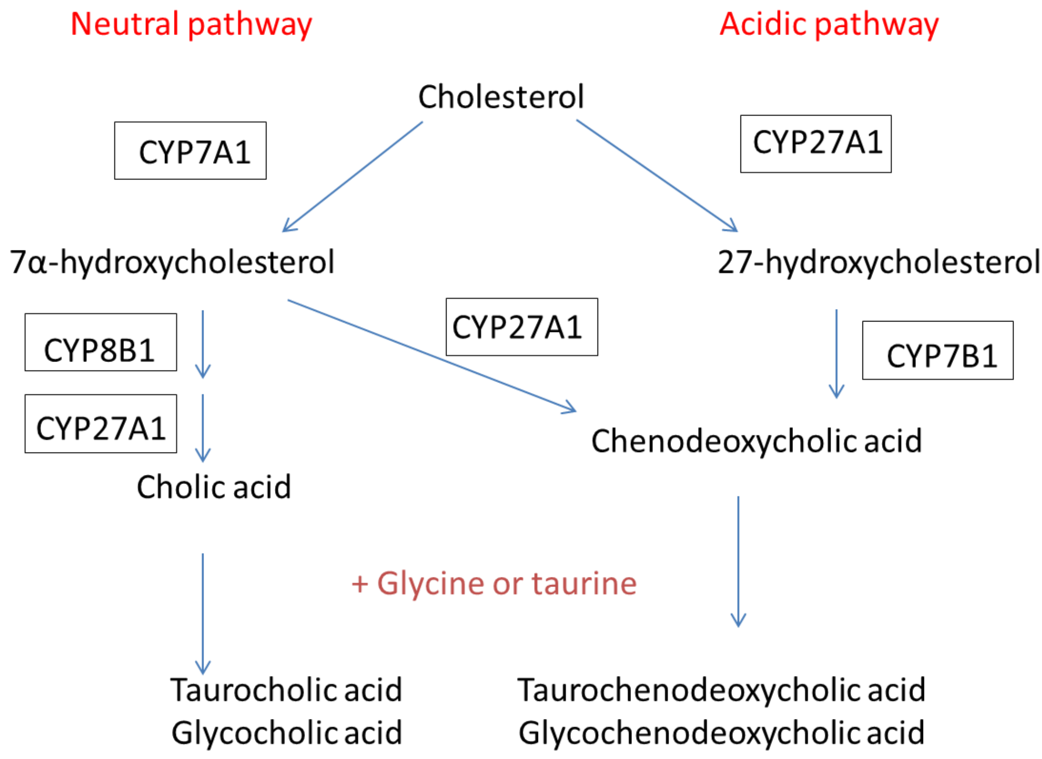



**Figure 1.5 Simplified schematic of production of conjugated primary BA from cholesterol** BAs are produced from cholesterol by the acidic and neutral pathways. The neutral pathway can result in the formation of both CA and CDCA whereas the acidic pathway only forms CDCA. Both CA and CDCA are then conjugated to either glycine or taurine before they are secreted into the gall bladder.

Approximately 95% of BAs are conjugated to either glycine or taurine. Conjugated bile acids are transported from the liver to the gall bladder where they are stored before their release into the duodenum after food consumption (Ridlon *et al.*, 2006; Ridlon *et al.*, 2016). Diet can impact upon the proportion of glycine to taurine conjugates, with people consuming a western diet having bile with a higher ratio of taurine to glycine conjugates (Hardison 1978). Such increases in taurine conjugation have been linked to elevated inflammation and expansion of pathobionts in mice (Devkota *et al.*, 2012). The type of conjugation can also be affected by other factors, including antibiotic treatment and the absence/disruption of the microbiota, which can increase the proportion of taurine conjugates and decrease BA diversity (Swann *et al.*, 2011).

BAs act as detergents due to their amphipathic nature, harbouring both hydrophobic and hydrophilic regions. This structure enables the formation of micelles around lipids, allowing uptake of lipids and lipid soluble vitamins

(Figure 1.6). Modification of BAs by intestinal bacteria is important as it alters their amphipathic structure decreasing their ability to form micelles and influences absorption.

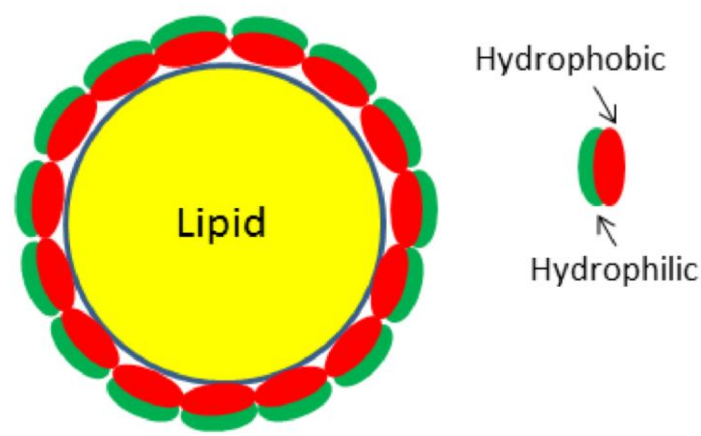



**Figure 1.6 Micelle formation around lipids and lipid soluble vitamins by BAs**
The amphipathic nature of BAs allows for the formation of micelles around lipids, facilitating their absorption.

To facilitate the efficient absorption of lipids there are high concentrations of BAs in the duodenum, jejunum and proximal ileum. The majority of BAs are reabsorbed in the colon and recycled through the body via enterohepatic circulation (EHC), an efficient process which happens multiple times throughout the day (Figure 1.7).

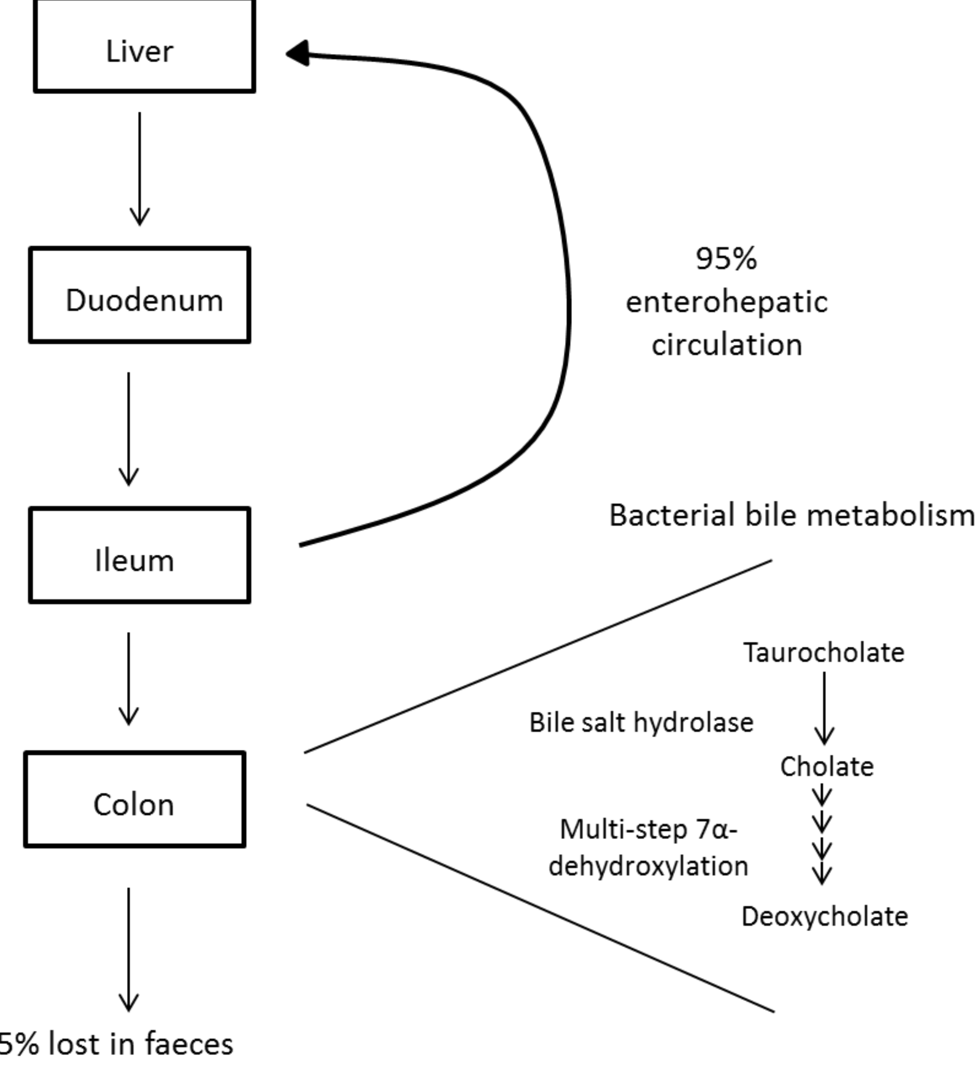



**Figure 1.7 Enterohepatic circulation of BAs through the gastrointestinal (GI) tract**
Enterohepatic circulation of BAs is the process by which BAs are reabsorbed allowing them to be recycled multiple times. As BAs move through the GI tract they are modified by members of the microbiota to form secondary BAs. The majority of BAs are reabsorbed and returned to the liver with only 5% are lost in faeces.

Deconjugated BAs and glycine CBAs can be passively reabsorbed, although there is also some active transport of BAs in the distal ileum. This process is very efficient with only small quantities (approx. 800mg) of BAs entering the colon each day (Ridlon *et al.,* 2006). These BAs are open to modification by members of the microbiota, the process of which will be discussed in section 1.3.2.

## 1.3.1 Bile acids impact the structure of the microbiota

As described in the previous sections, BAs play an important role in a number of important physiological processes. These include the capacity to alter the structure of the microbiota due to their toxicity. High levels of bile in the small intestine help to supress bacterial overgrowth, mostly through non-specific damage to the bacterial membrane (Merritt & Donaldson, 2009). In addition, some direct modification of BAs by resident bacteria increases their bactericidal activity, resulting in the inhibition of growth of a wide range of bacteria (Floch *et al.,* 1972). In a study by Islam *et al.,* (2011) it was found that the feeding of CA to mice led to changes in the structure of the caecal microbiota, including dominance by several Clostridial species. A similar study by Ridlon *et al.,* (2013) also observed that, mice fed 1% CA as part of their diet, showed an increase in the number of DCA producing bacteria. These studies suggest that BAs are important for shaping the environment in the GI tract by creating selective pressures that hinder growth of different bacteria species. As the host produces no equivalent enzymes to those generated by the microbiota, it is clear that bacteria are playing a key role in the diversity and population structure of each individual's microbiota.

## 1.3.2 Modification of Bile acids by intestinal bacteria

Many of the bacteria present within the GI tract possess enzymes that are involved in the modification of BAs. The conversion of conjugated BAs to secondary BAs is a complex, multistep enzymatic process (Figure 1.8). The gateway reaction involves the removal of the conjugated amino acid. This reaction is facilitated by the production of bile salt hydrolases (BSH) enzymes, which cleave the terminal taurine or glycine.

### 1.3.2.1 Bile salt hydrolases

BSH are part of the N-terminal nucleophilic hydrolase superfamily of enzymes and are produced by many bacterial species in the gut. The majority of BSH producers belonging to Gram positive species such as *Clostridium, Bacteroides, Lactobacillus* and *Enterococcus* (Begley *et al.,* 2005). BSH are typically intracellular enzymes, the expression of which linked to bacterial

growth (De Smet *et al.,* 1995). The exact purpose of BSH activity to individual bacteria is not well understood with multiple theories explored in the literature. Three main ideas remain in circulation. The first suggests that these bacteria are able utilise the amino acid, cleaved by the action of the BSH, as a carbon source giving them an advantage over other bacteria within the gut. Some bacterial species have been shown to utilise the amino acid that is freed by deconjugation (Begley et al., 2005). Both glycine and taurine can be further modified to ammonia and carbon dioxide, which may then be used in the formation of other metabolites (Patel *et al.,* 2010). A study by Van Eldere *et al.,* (1996) showed that some strains can utilise the free taurine as an electron acceptor.

It has also been proposed that BSH activity increases tolerance to BAs and therefore aids *in vivo* survival. A large number of bacteria have been found to have increased bile resistance due to BSH production (Gueimonde *et al.,* 2009; De Smet *et al.,* 1995; Grill *et al.,* 2000; Bi *et al.,* 2016). However the link between BSH activity and bile tolerance is disputed, with multiple studies finding no link between BSH activity and bile tolerance (Moser & Savage, 2001; Usman & Hosono, 1999). It could be that BSH do play some role in bile tolerance, but that in some bacteria this is part of a complex response to BAs meaning that BSH function is not the primary determinant of bile resistance (Fang *et al.,* 2009). However expression of a BSH is an attractive trait for probiotic bacteria (Patel *et al.,* 2010; Kumar *et al.,* 2012). In the pathogen *Listeria monocytogenes,* BSH production is considered a virulence factor as it is enables the bacteria to effectively colonise the GI tract, with mutation of this gene reducing colonisation of this pathogen (Dussurget *et al.,* 2002). A final potential role of BSH production is the incorporation of the modified BA into the membrane of the bacteria which may help the bacteria to adapt to environmental stressors and aid *in vivo* survival (Taranto *et al.,* 2003).

#### 1.3.2.2 7α-dehydroxylation of deconjugated BAs

Following deconjugation of the amino acid side chain, the BA is susceptible to further modifications. In the context of *C. difficile* infection the most important is 7α-dehydroxylation of the BA, which results in the formation of

DCA, which inhibits *C. difficile* outgrowth. The advantage of 7α-dehydroxylation to the bacteria that facilitate this process is unknown, although it may provide additional electron acceptors for anaerobic respiration (Mallonee & Hylemon, 1996). The production of DCA may also help to limit competition from other members of the microbiota including *C. difficile*. 7α-dehydroxylation completes the conversion from primary to secondary BA and is carried out as part of a multi-step reaction controlled by the BA inducible operon (BAI). Currently, only members of the Clostridia and Eubacteriaceae have been identified as able to carry out this process. This includes *C. scindens, Clostridium hylemonae, Clostridium sordellii, Clostridium hiranonis* and various unclassified Eubacteria (Ridlon et al., 2016). A simplified pathway of bacterial bile metabolism is shown in Figure 1.8.

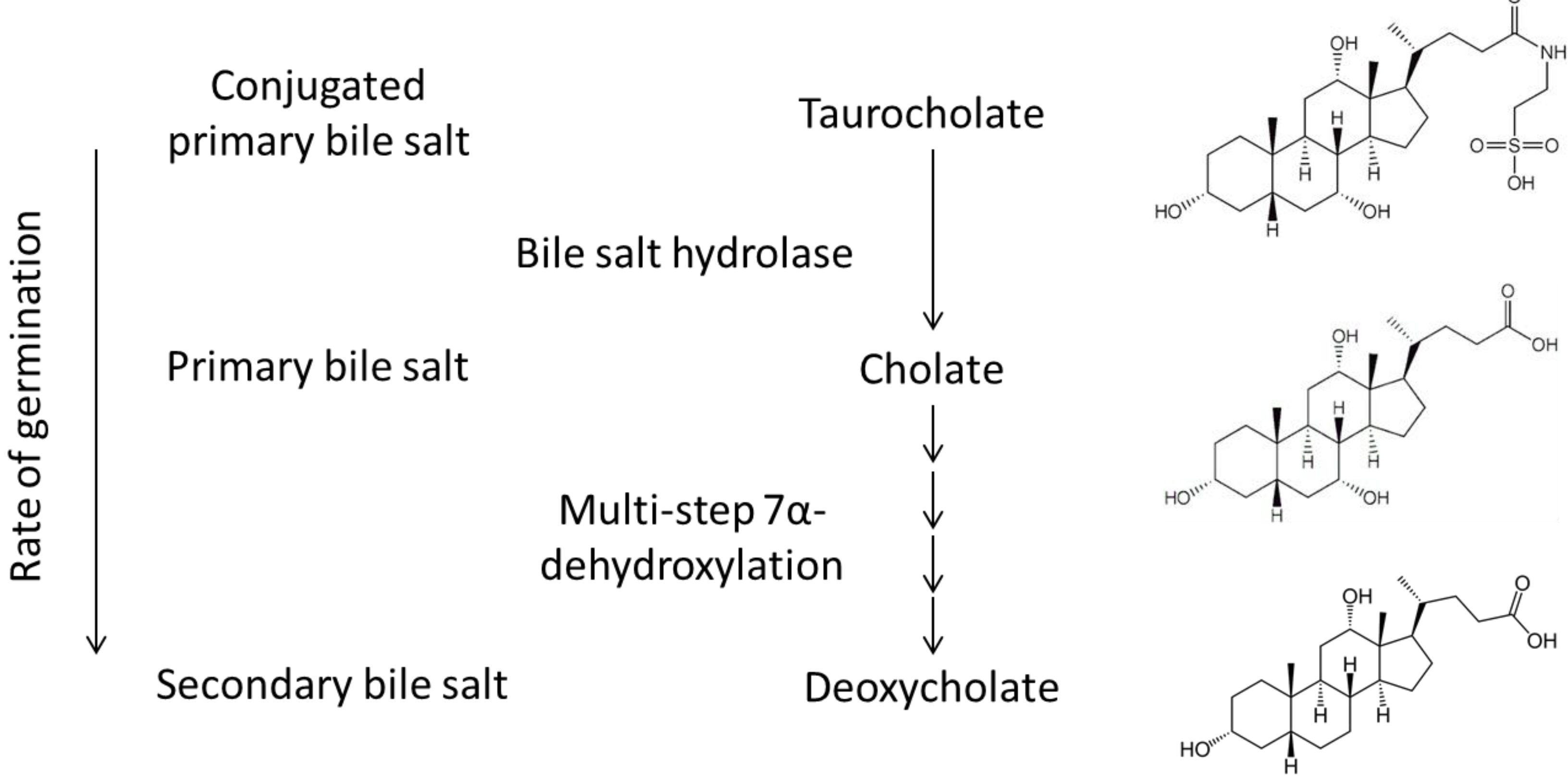



**Figure 1.8 A simplified diagram of bacterial enzymes involved in bile modification and the impact of this on the efficiency of *C. difficile* spore germination** Conjugated BAs are first modified by BSH which cleave the amino acid from the BA opening it up to further modification. In the context of *C. difficile* infection the most important secondary modification is 7α-dehydroxylation resulting in the formation of secondary BAs. As BAs are modified from conjugated to secondary their ability to induce *C. difficile* spore germination decreases.

7α-dehydroxylation of BAs is incredibly complex, requiring up to 10 different proteins individually encoded on the BAI. Whilst several bacteria encode this operon there is significant diversity observed between individual species (Ridlon *et al.,* 2016). The BAI from *C. scindens* is shown in Figure 1.9. The operon itself, encodes genes involved in its own regulation, BA uptake and modification including genes involved in the formation of either DCA or lithochoilc acid (LCA). In addition, the specialised BA transporter only binds CA or CDCA and not CBAs, meaning deconjugation by BSH is an essential prerequisite of 7α-dehydroxylation (Batta *et al.,* 1990; Van Eldere *et al.,* 1996).

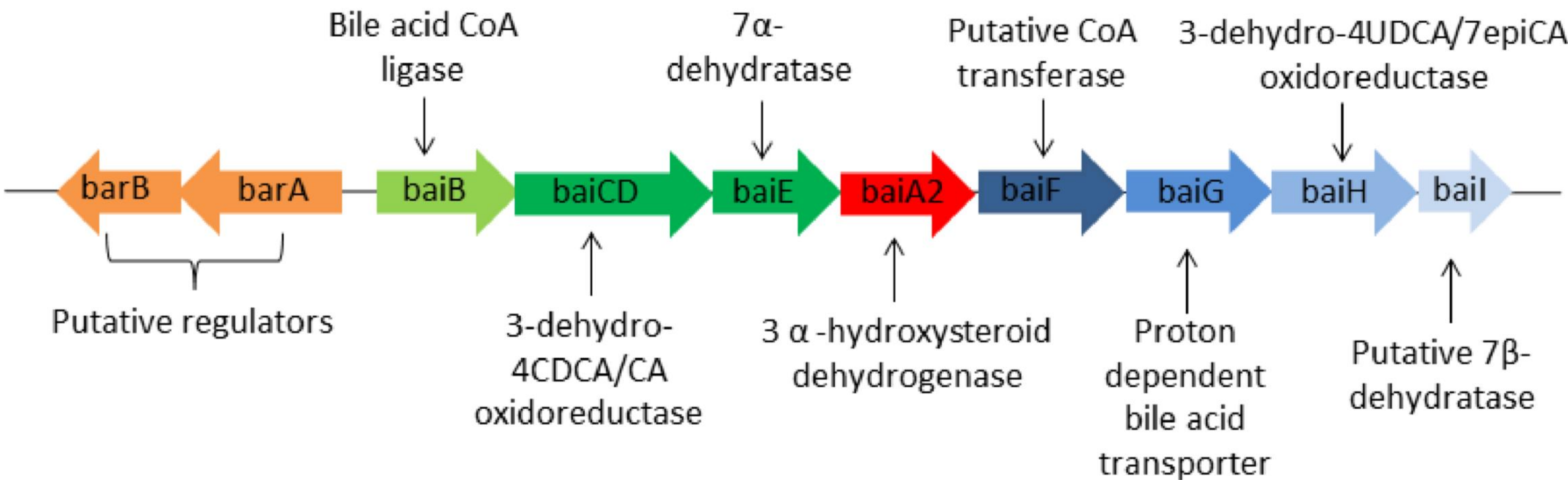



**Figure 1.9 The BAI operon from *C. scindens***
Figure showing the structure and function of the genes within the BAI operon in *C. scindens.*

This operon encoding this specialised function is possessed by a very small subset of bacteria (Stellwag & Hylemon 1978; Ferrari & Beretta 1977; Wells *et al.*, 2000). This process however, is highly efficient with the majority of BAs present in the healthy human colon being converted to their secondary form. Ultimately, the presence of these bacteria and activity of the enzymes determine the levels of secondary BAs present within the BA pool. This is important as these BAs are more toxic to cells and have been associated with increased incidence of colon cancer (Barrasa *et al.,* 2013; McGarr *et al.,* 2003). As western diets are known to influence the prevalence of bacteria associated with bile breakdown, this may help to explain the apparent recent increase in the frequency of this disease.

## 1.3.3 Bacterial bile acid resistance and virulence

### 1.3.3.1 BA induce resistance mechanisms in enteric bacteria and pathogens

All bacterial species that inhabit the GI tract come into contact with conjugated and free BAs. Damage associated with exposure is frequently linked to modification of membranes although BAs can also damage DNA, RNA and proteins. For this reason bacteria have evolved multiple mechanisms in order to deal with this toxicity and to utilise these molecules. Mechanisms for this include production of enzymes involved in the modification of CBAs and BAs (1.3.2), upregulation of efflux pumps, induction of DNA repair mechanism and modifications to the outer membrane (Begley *et al.,* 2005).

One of the best-characterised mechanisms for resistance is the increased expression of efflux pumps, which allow rapid efflux of BA before damage can occur. Modification of efflux pump regulation and production following bile exposure has been demonstrated in a huge range of commensal and pathogenic bacteria including *Salmonella typhimurium, Escherichia coli, Campylobacter jejuni, Pseudomonas aeruginosa* and *Vibrio cholerae* (Prouty *et al.,* 2004; Okoli *et al.,* 2007; Gunn 2000). The most studied example in this context are the *acr*AB genes expressed by *E. coli*. Homologs of these genes are found in a large range of enteric bacteria including *S. typhimurium* and *Bifidobacterium longum* (Baucheron et al., 2014; Gueimonde *et al.,* 2009). Upregulation of efflux pumps has been shown to increase resistance to antibiotics and other stressors such as solvents and detergents (Prouty *et al.,* 2004). Efflux pumps involved in bile

resistance have now been identified in a multitude of Gram positive and Gram negative bacteria and seem to be incredibly important factor influencing bile resistance (Gunn, 2000). In addition to increased efflux of BAs, *Salmonella enterica* has been shown to remodel its peptidoglycan layer in response to exposure to sub lethal concentrations of DCA (Hernández *et al.,* 2015) and to alter lipopolysaccharide transport which also helps to increase bile resistance (Hernández *et al.,* 2012)

#### 1.3.3.2 Modulation of virulence by BA

A number of bacterial species have been found to modify expression of genes as a consequence of exposure to a variety of BAs. This includes genes involved in BA resistance as described in section 1.3.3.1, but also genes involved in regulating virulence. Amongst these are genes associated with a number of virulence-associated traits including motility, biofilm formation and epithelial cell invasion. The regulation of virulence genes in response to bile has been shown in an array of pathogens including, *E. coli* (Hamner *et al.,* 2013), *Salmonella typhimurium* (Prouty *et al.,* 2004), *Shigella flexneri* (Pope *et al.,* 1995), *Vibrio cholera* (Krukonis & DiRita, 2003) and *L. monocytogenes* (Dussurget *et al.,* 2002) Interestingly responses to bile vary between different bacterial species, mostly likely due to differences in pathogenesis.

As the concentration and ratio of particular BAs varies considerably along the length of the GI tract it is possible that bacteria have adapted regulation and expression of virulence traits to ensure expression at the most appropriate location within the gut. This seems to be the case for *S. typhimurium* and *E. coli* which both downregulate several virulence genes in the presence of BAs. In *E. coli* O157:H7, incubation with BAs (an undefined mixture consisting mostly of glycocholate and TC) results in the significant downregulation of 41 genes from the locus of enterocyte effacement (LEE), which includes genes involved in attachment to epithelial cells, and upregulation of genes for iron scavenging and metabolism (Hamner *et al.,* 2013). In *S. typhimurium* the presence of bile has been shown in multiple studies to result in reduced expression of both SPI-1 and SPI-2 genes resulting in decreased invasion of epithelial cells (Prouty, Brodsky, Manos, *et al.,* 2004; Prouty & Gunn 2000; Hernández *et al.,* 2012). As concentrations of BAs are high in the lumen, but decrease with proximity to the

epithelial surface, regulation of these genes by BAs could halt expression of genes for invasion until they are required. Interestingly *S. flexneri* shows the opposite control of adherence and invasion to *S. typhimurium* with increased invasion in the presence of DCA (Pope *et al.,* 1995). A later study by Olive *et al.,* (2007) found that DCA directly interacts with IpaD protein at the tip of the needle complex of the type III secretion system. This interaction causes a conformational change that facilitates binding of IpaB resulting in increased capability to invade epithelial cells.

In addition to changes associated with invasion, BAs have also been shown to influence genes involved in motility and biofilm formation. Exposure to BAs results increased levels of swarming motility and decreased toxin production in *V. cholerae* (Gupta & Chowdhury, 1997). This same increase in motility was also found by Krukonis & DiRita (2003) and was thought to be due to increased flagella formation. Increased motility enabled the bacteria to penetrate the mucus layer more effectively and access the underlying epithelial cells. It has also been found that different BAs have different effects on biofilm formation in *V. cholera* with a crude bile mix found to increase biofilm formation through increased production of polysaccharides (Hung *et al.,* 2006). Interestingly exposure to TC was also found to stimulate biofilm dispersal in *V. cholera* suggesting that different BAs have different impacts upon the same organism (Hay & Zhu, 2015). DCA has also been found to increase biofilm formation in *C. jejuni,* by stimulating production of flagella resulting in increased adherence (Svensson *et al.,* 2014).

Considering that *C. difficile* also encounters this environment during infection and the fact that BAs, particularly TC, play such a pivotal role in germination and outgrowth (Sorg & Sonenshein, 2008, 2009), it is not surprising that this area has been subject to recent significant scientific interest. It is likely that further attention will be paid to this area as currently nothing is known about how BAs influence genes involved in bile resistance in *C. difficile* (Sistrunk *et al.,* 2016). This interest also stems from our increased understanding of the composition and functional activity of the gut microbiome which is known to be influential in *C. difficile* infection.

# 1.4 The human gut microbiota

## 1.4.1 The structure and function of the human gut microbiota

The human gut is colonised following birth by a huge number of bacteria, viruses and fungi. Bacteria are the most studied of these symbionts and in the GI tract there are 10 times as many bacterial cells as there are human cells (Luckey, 1972). Bacteria in this location encode an astonishing 150 times more unique genes than the human genome and provide many important functions that support human health (Qin *et al.*, 2010). The gut is thought to harbour 500-1000 species with large variation between individuals. Interestingly, the metabolic potential of the microbiome shows greater conservation, probably reflecting the high level of functional redundancy between bacterial species (Figure 1.10).

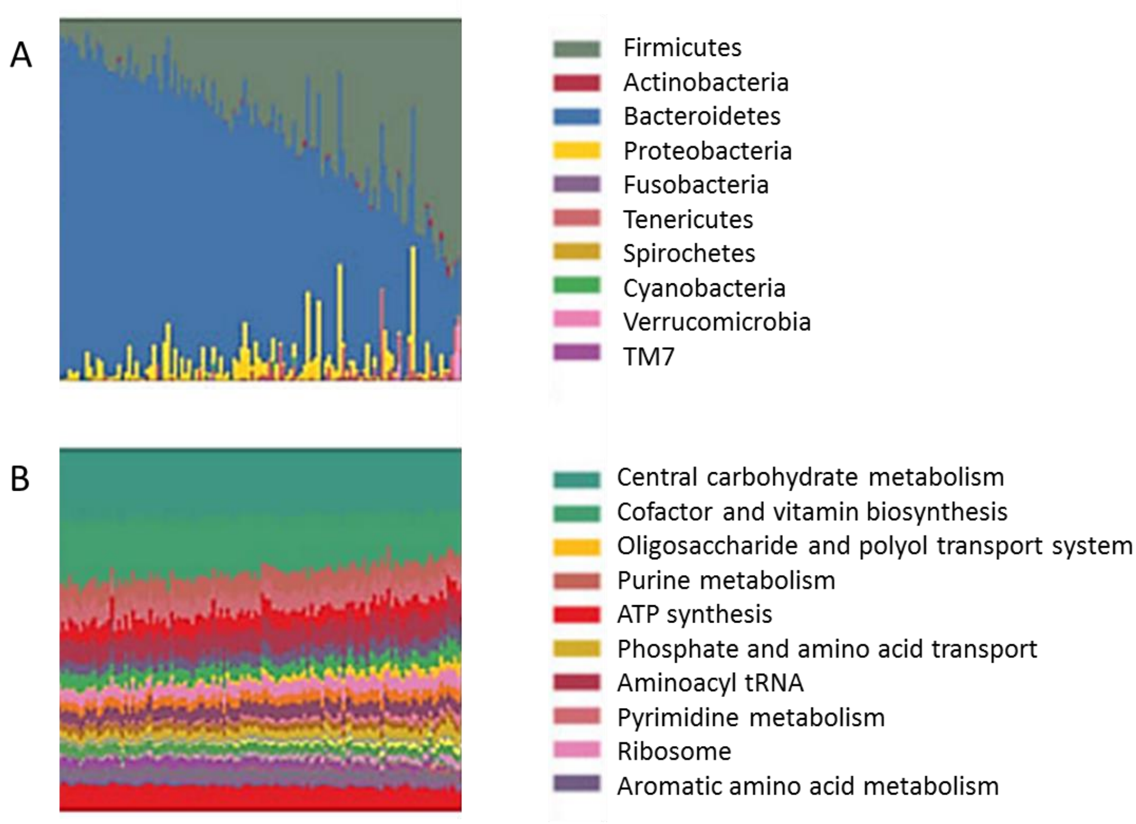



**Figure 1.10 Structure and function of the Human gut microbiome from Huttenhower *et al.*, (2013)**
A) The bacterial phyla present in stool samples from healthy human subjects. There is large variation between subjects with different proportions of bacterial phyla present in each sample. B) Investigation of metabolic pathways present within human stool samples. The metabolic activity of the microbiota is much more highly conserved compared to the species present.

The abundance of bacteria and the species present within specific body sites varies along the length of the GI tract. The density of bacteria increases throughout the GI tract, with the colon being the most densely populated site with an estimated $10^{11}$ CFU/ml. The typical bacteria associated with the different sites in the GI tract are shown in Figure 1.11.

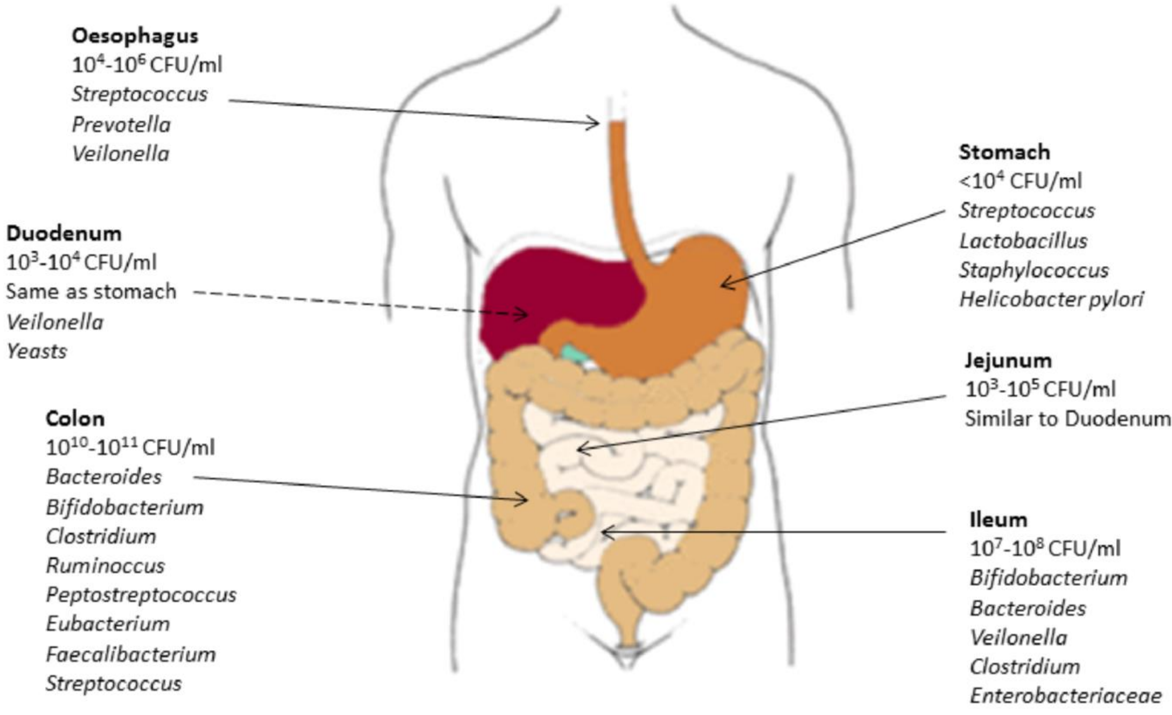



**Figure 1.11 The distribution and identification of microorganisms along the GI tract. Adapted from *Tiihonen et al., 2010***
This figure highlights the abundance and distribution of the bacterial species in several locations along the GI tract.

There is additional variation even within a specific location, with the luminal bacteria differing from those found intimately associated with the mucosa (Ouwehand *et al.,* 2004; Swidsinski *et al.,* 2002; Zoetendal *et al.,* 2002).

The microbes in our gut play a number of important roles in health, development of local immune responses and disease. Much information has been gleaned from studies performed on germ free (GF) animals. In these animals' the structural integrity and linked immune system remain undeveloped (Bauer *et al.,* 2006). Additionally, such animals require higher calorie intake and suffer from vitamin deficiencies, however they also live longer and are more resistant to developing kidney stones (Wilks, 2007), thus demonstrating the complex relationship that exists between the host and their commensal bacteria.

A major area of interest is the potential role of the microbiota in influencing autoimmune diseases and infection, with disruption being increasingly linked to irritable bowel syndrome (IBS) (Dupont, 2014), Crohn's disease (Dicksved *et al.*, 2008) and obesity (Ley *et al.*, 2005; DiBaise *et al.*, 2012). For infectious disease, one of the most important functions of the microbiota is its role in colonisation resistance and protection from colonisation with pathogenic bacteria. This concept was first described in 1962 when it was defined as 'antibiotic associated susceptibility' and was linked to the increased vulnerability of mice to *Salmonella* infection following streptomycin treatment (Bohnhoff & Miller, 1962). Antibiotic use remains one of the major factors influencing microbial diversity within the microbiome and it is their use that can be linked, in the majority of cases, to disruption of colonisation resistance.

### 1.4.2 The microbiome and the immune system

The proximity of many of these bacteria and their metabolic by-products to the epithelium of the GI tract means that the immune system must be perfectly balanced to ensure elimination of potential pathogens whilst limiting the response to commensals. Homeostasis of this system is crucial, with over-activation of host defences and/or mucosal defects linked with a range of inflammatory diseases. One mechanism by which this balance is achieved is the physical separation achieved through the continual production and secretion of mucus by goblet cells. Cross linking of mucin results in the formation of a two layered structure. A tightly cross-linked and adherent inner mucus layer and a more loosely linked outer layer. Bacteria are unable to penetrate the inner layer, decreasing contact between luminal bacteria and epithelial cells. The outer mucus layer is colonised by commensal bacteria, with the thickness of this layer increasing with progress through the GI tract, as the bacterial load increases (Johansson *et al.*, 2011). Mucus production is stimulated by several bacterial factors including peptidoglycan and lipopolysaccharides, which have been shown to ameliorate reduced mucus production in GF mice (Petersson *et al.*, 2011). The mucus layer is not just a physical barrier but contains several immune molecules that help to prevent contact of luminal bacteria with the epithelia. This includes secretory IgA, Reg-IIIγ and antimicrobial peptides (AMPs) which help to keep the inner mucus layer free of bacteria (Maynard *et al.*, 2012). Homeostasis of this system is important with dysregulation of mucin

production linked to inflammatory disorders like colitis (Van der Sluis *et al.*, 2006; Heazlewood *et al.*, 2008). Furthermore, development of host immunity appears to be triggered by species specific microbes, with GF mice only able to develop normal immune responses if transplanted with organisms from the mature mouse gut (Chung *et al.*, 2012).

### 1.4.3 The aging microbiota

One of the main risk factors associated with *C. difficile* susceptibility is ageing, with the majority of cases seen in patients aged 65 or above (Rea *et al.*, 2012). It is now recognised that the composition of the microbiota changes as people age with significant alterations seen in individuals living in healthcare facilities (Claesson *et al.*, 2012; McDonald *et al.*, 2016). Whilst most of these studies showed a large amount of variation in the changes associated with aging, the overall impression is a decrease in stability and diversity within the microbiome of the elderly (Tiihonen *et al.*, 2010; Claesson *et al.*, 2012; Claesson *et al.*, 2011). These changes may reflect changes to the complexity of diet, which can be restricted by poor dental health and problems with digestion. Elderly people are also likely to be taking prescription medications with 75% of over 65s taking at least one drug (Chrischilles *et al.*, 1992). Several of these medications, including proton pump inhibitors (PPIs), antibiotics (as discussed in section 1.4.4) and laxatives have been found to influence the microbiota (Tiihonen *et al.*, 2010). Large scale changes in the composition of the microbiota can influence the production and concentration of short chain fatty acids (SCFA) with decreased levels of butyrate and increased concentrations of phenol and branched fatty acids observed. This change in profile represents a shift to putrefaction rather than saccharolytic fermentation (Woodmansey 2007; Woodmansey *et al.*, 2004). Alteration of the SCFA profile can also favour decreases in barrier function (Suzuki *et al.*, 2007) and modulation of immune response (Meijer *et al.*, 2010). As a consequence such changes may contribute to the increased rates of *C. difficile* infection observed in this age group.

### 1.4.4 Impact of antibiotic treatment on the microbiota

The number of prescriptions written for antibiotics each year in England is estimated to be in the region of 34 million with antibiotic exposure identified as

the biggest risk factor for developing *C. difficile* infection (Furuya-Kanamori *et al.,* 2015). Antibiotics, particularly those with broad spectrum activity, have a huge impact on the structure and function of the bacteria in the microbiota and there is increasing evidence that taking antibiotics in the early years of life can result in profound and long lasting changes to the structure of the microbiota (Yassour *et al.,* 2016; Jernberg *et al.,* 2007). In particular their use alters the diversity of the microbiota known as dysbiosis. Persistent dysbiosis has been shown to be intrinsically linked to both acute and relapsing *C. difficile* infection and therapies that restore this lost diversity have been found to be highly effective.

### 1.4.5 The microbiota in *C. difficile* infection

The structure of the microbiota associated with *C. difficile* infection has now been studied extensively in both mouse and man. Infection is typically associated with disproportional blooms of *Enterobacteriaceae, Enterococcus* and *Proteobacteria* such as *E. coli* (Reeves *et al.,* 2011; Fuentes *et al.,* 2014; Schubert *et al.,* 2015). Such large changes in population structure have been linked to significant changes in the gut metabolome. A study by Antunes *et al.,* (2011) found 87% of the metabolites within the gut were altered following antibiotic treatment. Key changes included reduction in a number of short chain fatty acids (SCFAs) including butyrate, altered BA modifications (1.4.5.2) and presence of certain sugar alcohols and amino acids (Theriot *et al.,* 2014). These changes, favour *C. difficile* colonisation, germination and outgrowth particularly through the production of primary BAs and succinate (Ferreyra *et al.,* 2014; Curtis *et al.,* 2014). Such changes can persist for many weeks after antibiotic treatment potentially leaving the host susceptible to *C. difficile* infection over long periods of time (Theriot *et al.*, 2014).

#### 1.4.5.1 Influence of gut dysbiosis in chronic relapsing infection

As discussed, large proportion of patients who contract *C. difficile* suffer from repeated, relapsing infection, either as a consequence of reinfection by a new strain, or, relapse of existing disease. Relapse as a consequence of recurrence accounts for between 64-87% of such cases, making it more common than reinfection (Figueroa *et al.,* 2012; Johnson *et al.,* 1989; Alonso *et al.,* 2001; Dé

*et al.,* 2000). The reason for repeated relapse is unclear, however a study by Chang *et al.,* (2008) found that patients who were suffering from relapsing disease had limited diversity within the structure of the microbiota. Additionally it has been found that patients suffering from long term dysbiosis have significantly increased numbers of *Parabacteroides*, Enterobacteriaceae, *Veillonella* and *Streptococcus* with these patients found to be at higher risk of infection (Khanna *et al.,* 2016). This would suggest that *C. difficile* is able to persist in environments in which the diversity is limited.

At present it is unclear in what form *C. difficile* persists within the gut or whether the organism is able to manipulate the local environment to maintain the modified niche to perpetuate its persistence. However it has been hypothesised that production of *para*-cresol, a bacteriostatic compound, may assist the capacity to persist (Dawson *et al.,* 2011; Dawson *et al.,* 2008; Selmer & Andrei, 2001). *C. difficile* is among the few bacterial species that is able to produce *para-cresol* (Elsden *et al.,* 1976; Hafiz & Oakley, 1976) with strains of *C. difficile* associated with high rates of relapse able to produce and tolerate higher quantities of this compound (Dawson *et al.,* 2008; *Dawson et al.,* 2011). The capacity of bacteria to produce compounds that limit the growth of other species is well documented. Typical examples include bacteriocin production, nutrient limitation and production of toxic compounds.

In *S. typhimurium* production of a bacteriocin called colicin Ib (ColIb) allows the bacteria to outcompete closely related *Enterobacteriaceae* but only in an inflamed gut (Petkova Nedialkova *et al.,* 2014). Similarly bacteriocin production by *Enterococcus faecalis* enables it to outcompete other non-bacteriocin producing *E. faecalis* strains in a mouse model (Kommineni *et al.,* 2015). More general mechanisms of niche maintenance involve the production of inhibitory molecules and metabolites. These often involve the production of a compound to which the producer is highly tolerant to but which can damage other bacteria. An example of this is the production of DCA by *C. scindens* as described in section 1.3.2.2. DCA is toxic to many other bacterial species including *C. difficile* so production of this compound may help *C. scindens* to maintain its niche within the gut by creating an environment in which it can flourish. It may

be that *para*-cresol production by *C. difficile* represents a similar mechanism of niche maintenance but this requires further investigation.

#### 1.4.5.2 Antibiotic impact on bacterial bile metabolism

Multiple antibiotics have been associated with changes in BA metabolism including streptomycin (Antunes *et al.,* 2011), cefoperazone (Theriot *et al.,* 2014; Theriot *et al.,* 2015), clindamycin (Andréasson *et al.,* 1988; Theriot *et al.,* 2015) and vancomycin (Theriot & Young 2014; Theriot *et al.,* 2015). Clindamycin is commonly used to render animals susceptible to *C. difficile* before infection (Sun *et al.,* 2011; Chen *et al.,* 2008; Reeves *et al.,* 2011; Bartlett *et al.,* 1977; Larson & Borriello 1990). As clindamycin is circulated within bile and is present in high concentrations in bile (Leigh, 1981) it is likely that high levels of the antibiotic will be present in areas populated by bacteria involved in bile metabolism. The loss of members of the microbiome that are able to modify taurine conjugated BAs results in the formation of an environment in which *C. difficile* is able to germinate, grow and produce toxins resulting in disease (Figure 1.12).

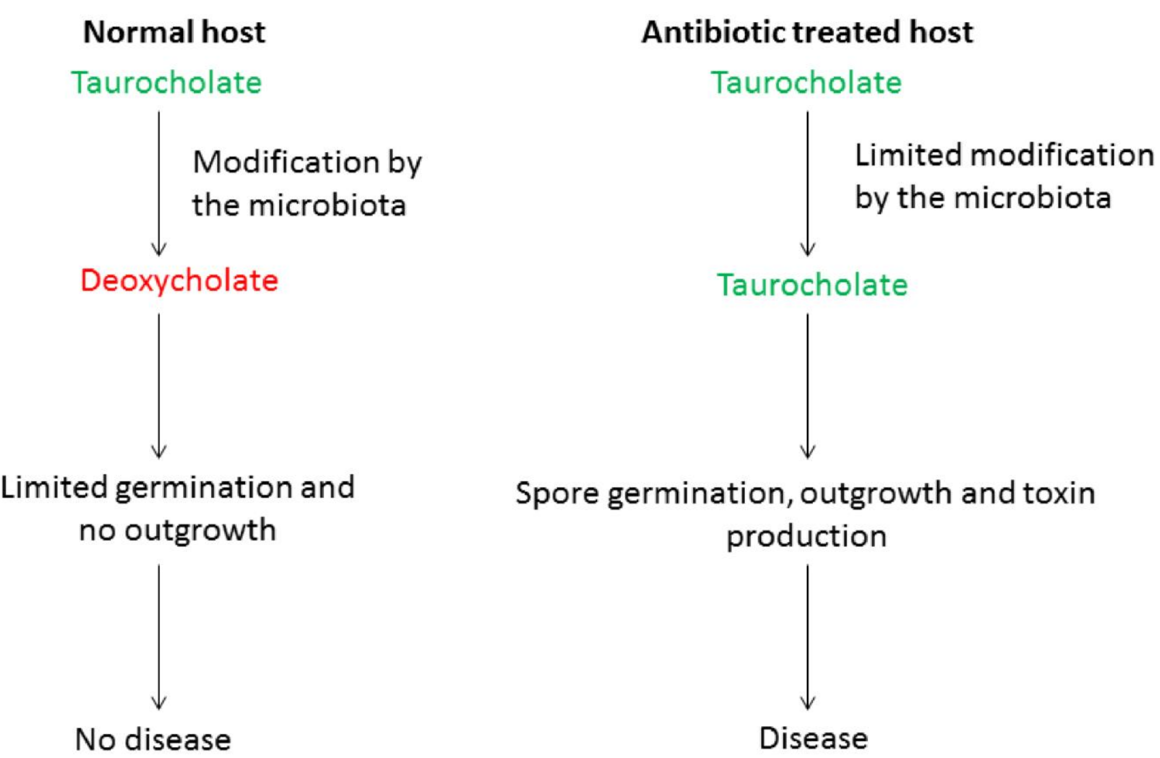



**Figure 1.12 The impact of antibiotic exposure on the microbiota, bile metabolism and *C. difficile* infection**
Antibiotic exposure can disrupt the intestinal microflora, resulting in a decrease in species able to modify primary bile salts (taurocholate) to secondary bile salts (deoxycholate) which creates an environment that promotes *C. difficile* germination and outgrowth.

## 1.5 Current and developing treatments

Due to the complicated nature of *C. difficile* infection, treatment can prove challenging. The significant rates of relapse and recurrence suggest that current treatment of acute disease is not effective and exploration of alternatives is required (1.1). Currently the recommended treatment is with vancomycin or metronidazole, however, failure rates are high and with reports of high rates of relapse (Vardakas *et al.*, 2012) and reduced susceptibility to these treatments (Baines *et al.*, 2008). It has also been found that exposure to these antibiotics increases susceptibility to a range of hospital pathogens including *C. difficile* (Lewis *et al.*, 2015; Wafa *et al.*, 2008) As decreased microbial diversity has been linked to infection, restoration and protection of this niche has now become the focus of a huge number of emerging therapeutics. This includes development of narrow spectrum antibiotics such as fidaxomicin which has been shown to be as effective as vancomycin (Johnson & Wilcox, 2012) but with lower rates of relapse (Drekonja *et al.*, 2012). There have also been efforts to utilise *C.*

*difficile* specific bacteriophage therapy (Nale et al., 2016) and the use and delivery of BA analogues (1.5.3.1). All these approaches aim to target *C. difficile* whilst protecting the microbiota from further damage. An alternative approach is the direct replacement of the microbiota either through FMT or targeted bacteriotherapy. At present, FMT is still reserved for use in only the most difficult to treat cases, but with the increasing rate of treatment failure the need for change is apparent.

### 1.5.1 Faecal microbial transplantation

FMT was first performed on patients with PMC in 1958 at the University of Colorado, Veterans Administration Hospital. In this small study, four patients were treated with faecal retention enemas. This treatment was found to be highly effective and the treating physicians observed that if a more precise preparation of microorganisms could be identified and delivered in capsules, that this would be a “more aesthetic and more effective” treatment. Since this there have been intermittent reports of this non-standard type of treatment for *C. difficile*. Today, the effectiveness of this approach has been widely reported in the literature with hundreds of patients being treated. The first official clinical trial conducted to compare the efficacy of FMT to traditional antibiotic therapy reported a 90% cure rate after two FMT infusions compared to 31% for traditional treatment with vancomycin. The trial was so successful that it was ended prematurely to allow those being treated with vancomycin to be given the more effective FMT (van Nood *et al.,* 2013). Meta-analysis of multiple studies report success rates for FMT of between 89.7-91.2% and recurrence rates of 5.5% (Li *et al.,* 2015; Kassam *et al.,* 2013). These rates suggest that FMT is much more effective than traditional antibiotic therapy, with the use of fresh versus frozen stool seemingly having no impact on efficacy (Hamilton *et al.,* 2013; Youngster *et al.,* 2014). Efforts to understand how this treatment works have been helped by our increasing knowledge of the composition of the ‘healthy’ human gut microbiota as discussed in section 1.4. Interestingly, assessment of microbial population structure of patients before and after FMT, have shown that the composition of the recipients gut microbiota shifts to one that resembles that of the donor (Weingarden *et al.,* 2014). In some studies, this change persists for years (Broecker *et al.,* 2016) with significant increases in bacterial diversity. Overall these changes include significant increases in Bacteroidetes and

Firmicutes with concurrent decreases in the proportion of Proteobacteria (van Nood *et al.,* 2013; Khoruts *et al.,* 2010; Hamilton *et al.,* 2013; Shankar *et al.,* 2014; Weingarden *et al.,* 2015; Seekatz *et al.,* 2014)

Unsurprisingly, given the importance of BAs in *C. difficile* germination and outgrowth, it has been found that changes in BA composition are associated with infection in both mice (Theriot *et al.,* 2014) and humans (Allegretti *et al.,* 2016). It has now been shown that FMT is able to restore normal BA metabolism in these patients providing one explanation to the mechanism of *C. difficile* control (Weingarden *et al.,* 2014; Weingarden *et al.,* 2016).

However, several issues around the widespread implementation of FMT exist. These include the possibility of transmitting pathogens and obvious problems with the aesthetics of this approach. For this reason there has been a move towards the creation of treatments that are easier to administer and tolerate, such as delivery within an oral capsule (Youngster *et al.,* 2014). Additionally the long-term consequences of such large scale modifications to the human microbiome remain unknown. Short term side effects such as bloating and diarrhoea have been reported but it is too soon to know if there are any long term implications (Li *et al.,* 2015). Many of the FMT procedures that take place in the USA utilise pre-screened faecal matter provided by not for profit stool banks such as Openbiome and AdvancingBio. It is likely in the future that there will be a shift towards the use of more defined bacteriotherapy increasing the need for a good understanding of how these therapies work. For this reason, much work has focused on producing a defined, safe and evidence based bacteriotherapy that can be embraced by both patients and regulatory agencies.

### 1.5.2 Defined bacteriotherapy for treatment of *C. difficile*

The development of an effective treatment based on a defined combination of bacteria has been on-going for many years. One benefit of these synthetic mixtures is the potential to validate the safety and effectiveness of each formulation. This is more difficult for FMT as, although each donation is screened for potential pathogens, it is difficult to ensure that all risk is removed. Amongst the first reported successes was the use of a human derived strain *Lactobacillus* GG that was used to successfully treat 6 patients (Gorbach

*et al.,* 1987). Two years later a combination of 10 bacteria that suppressed the growth of *C. difficile in vitro,* was successfully used to treat a further 6 patients with recovery associated with restoration of *Bacteroides* to the gut (Tvede & Rask-Madsen, 1989). More recently a combination of 6 bacteria, isolated from a healthy mouse, was able to suppress infection as effectively as an infusion of unmodified donor faeces. Interestingly, other combinations of bacteria were unable to suppress infection, suggesting it is the functional capacity of the combination that is important for protection. However, it is important to note that many of the species used were species that were specific to the murine gut, potentially limiting direct application to humans subjects (Lawley *et al.,* 2012). Another synthetic mix developed by Petrof *et al.,* (2013) graphically termed 'RePOOPulate' consists of 33 bacterial species isolated from a human faecal sample. These can be delivered at known abundances, are pathogen free and contain bacteria that have been screened for the presence of antibiotic resistance genes. RePOOPulate has been used to successfully treat 2 patients who had previously failed to respond to antibiotic therapy. A recent study from the same group suggests that the combination is effective as it both restores diversity and protects against inflammation caused by TcdA (Martz *et al.,* 2016). Clinical studies using such defined mixtures of bacteria are currently underway to treat *C. difficile* infection (Khanna *et al.,* 2016; Dubberke *et al.,* 2014).

### 1.5.3 Targeting bile metabolism to treat *C. difficile*

As BAs play an important role in modulating spore germination and vegetative outgrowth of *C. difficile*, there are numerous studies that attempt to utilise BAs as treatment for *C. difficile*.

#### 1.5.3.1 Chenodeoxycholate and bile acid analogues as competitive inhibitor of germination

CDCA is a competitive inhibitor of *C. difficile* germination and has been proposed as a potential therapeutic agent (Sorg & Sonenshein, 2009). Analogues of this compound have been found to act as even more efficient inhibitors of spore germination and were also shown to resist modification by 7α-dehydroxylation (Sorg & Sonenshein, 2010). Although able to inhibit *C. difficile* germination *in vitro,* these compounds have not been tested *in vivo*. An alternative treatment using a TC analogue, CamSA (cholate meta-sulfonic

benzene derivative) (Howerton *et al.,* 2011), was shown to be able to protect mice in a dose dependent manner by inhibiting spore germination (Howerton *et al.,* 2013). Although this proved successful in pre-clinical studies, there have been no subsequent reports of its use in the clinical setting.

#### 1.5.3.2 A move towards functionality

A recent and ground breaking study by Buffie *et al.,* (2014) found that the use of a single species of bacteria, *C. scindens,* was able to increase resistance to *C. difficile* infection in mice. They proposed that this was achieved through the production of DCA, which can inhibit the outgrowth of *C. difficile*. Mice that were treated with *C. scindens* had a lower mortality rate and lower toxin titres in their faeces. Full protection was only achieved when *C. scindens* was administered in conjunction with 3 other bacterial species. This suggests that although the production of secondary BAs is important there must also be other factors that impact colonisation and outgrowth.

## 1.6 Animal models of *C. difficile* infection

Much of our knowledge of *C. difficile* infection has been gleaned from the use of animal models. Multiple animal models of infection have been described in the literature to study various aspects of infection such as colonisation, transmission and virulence. These include the Syrian Golden hamster, several inbred species of mice, gnotobiotic piglets, rats, rabbits, guinea pigs, prairie dogs, rhesus monkeys, quails and zebrafish embryos (Hutton *et al.,* 2014; Best *et al.,* 2015). The most commonly used of these models are the hamster and mouse models of infection and these will be discussed in detail in this section.

### 1.6.1 Syrian Golden hamster model for acute infection

The Syrian Golden hamster has been considered the 'gold standard' model of this infection as animals develop many symptoms that parallel those seen in clinical disease. Animals are administered clindamycin 5 days prior to infection to disrupt the microflora and to render them susceptible to infection (Buckley *et al.,* 2011). After infection animals are monitored for signs of disease and culled when they become moribund (Figure 1.13).

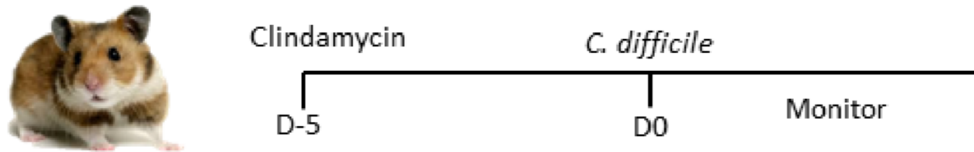



**Figure 1.13 Typical infection timeline for hamster experiments**
Animals are given a single dose of clindamycin before infection with *C. difficile*. After infection animals are monitored closely for signs of disease and culled when they become moribund as determined by a temperature drop to below 35℃.

Following infection with toxigenic strains of *C. difficile* animals rapidly develop symptoms including diarrhoea (wet tail), lethargy, hunching and quickly become moribund. The histology of the caecal and colonic tissues mirrors inflammation and tissue haemorrhage seen in human disease. As a consequence this model has been used to shed light on several aspects of the infection including virulence, colonisation, transmission and the role of toxins in disease. In addition, this model has been used to determine the efficacy of several prototypical vaccines and therapies (O'Brien *et al.,* 2005; Kink & Williams 1998; Kokkotou *et al.,* 2008). However, the rapidly fatal infection *C. difficile* causes in hamsters does not reflect the infection outcome of *C. difficile* in the majority of human cases. For this reason the development of models that display a wider range of disease outcomes that more closely reflect those seen in humans were required. An additional problem is a lack of suitable immunological tools that allow evaluation of the host response to this infection. The recent development of several mouse models of infection is helping to expand our overall knowledge of *C. difficile* host pathogen interactions.

## 1.6.2 Mouse models of infection

The use of mouse models to investigate *C. difficile* pathogenesis is increasing with several different mouse models being developed in recent years. These models utilise either conventional mice or GF mice with a humanised microbiota (Collins *et al.,* 2015) with conventional mice used most commonly. Untreated mice are resistant to infection and, as with humans, susceptibility to infection must be induced through disruption of the microbiota. In recent years a huge

number of mouse models have been developed in which different antibiotic pre-treatments lead to different disease susceptibilities. These models typically use one antibiotic or a combination of antibiotics to disrupt colonisation resistance. This includes models that utilise a single dose of clindamycin (Buffie *et al.*, 2012), delivery of cefoperazone in the drinking water (Theriot *et al.*, 2011) and use of a cocktail of antibiotics followed by a single dose of clindamycin (Chen *et al.*, 2008). Use of these models has allowed the study of longer term infection, relapsing infection and more severe disease, much of which would not have been possible in the hamster model of infection (Figure 1.14).

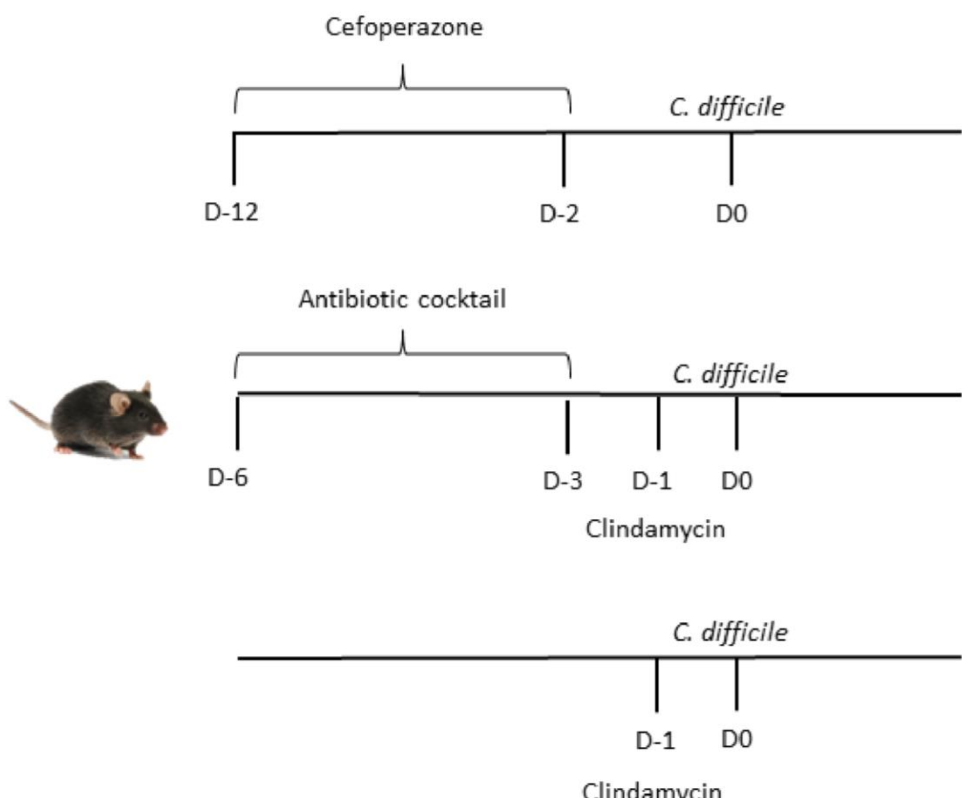



**Figure 1.14 The most commonly used mouse models of infection**
The 3 most common mouse models of infection are shown in the diagram above. One model uses delivery of cefoperazone in the drinking water for 10 days to sensitise mice to infection. Another uses a single dose of clindamycin. A variation of this model uses an antibiotic cocktail prior to clindamycin treatment and which results in increased disease severity.

All of these treatments render mice susceptible to infection but interestingly they result in differing infection outcomes. One model that has been used frequently is one that uses an antibiotic cocktail (ABC) of 5 antibiotics in conjugation with clindamycin treatment to induce susceptibility to more severe disease (Chen *et al.*, 2008). Mice treated with this regime display symptoms that more closely resembles infection in man as animals do not uniformly succumb to

infection but follow a range of disease outcomes from diarrhoea to significant weight loss (Collins *et al.,* 2015; Reeves *et al.,* 2011). In contrast use of clindamycin alone results in colonisation of animals but with minimal weight loss and severity. Use of these different antibiotic regimes allows for investigation of different disease states, increasing the usefulness of these models even further. One of the main advantages of the mouse model is that the animals do not appear as sensitive to the action of TcdA and TcdB. This means that studies of temporal changes in the composition of the microbiota during antibiotic treatment, infection and relapse can be determined.

## 1.7 Aims of this study

As the structure of the microbiota and BAs plays an essential role in modulating *C. difficile* infection the initial aim of this work was to;

1. Understand the population structure of the antibiotic treated gut and its influence on susceptibility to *C. difficile* colonisation and disease severity. This was achieved using several mouse models in which the extent of antibiotic pre-treatment and disease are linked.

2. To determine specific role of BAs and BA modifying bacteria on *C. difficile* germination and colonisation resistance.

3. To establish how increased concentrations of TC can affect both germination and subsequent growth of *C. difficile*. This was achieved through the construction of a transposon mutant library and RNA-seq to identify those genes whose expression was significantly altered in the presence of TC.

4. To investigate the influence of the bacteriostatic compound *para*-cresol produced by *C. difficile* in persistence and relapsing infection.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Enzymes

All restriction enzymes and Phusion high fidelity DNA polymerase were purchased from New England BioLabs Hitchin UK. Thermo Start Reddy Mix® was purchased from Thermo Fisher.

### 2.1.2 Antibiotics

#### 2.1.2.1 For use in culture media

Antibiotics were dissolved, filter sterilised using a 0.2 µm Ministart® syringe filter and stored at -20 °C before use. Antibiotics were added to sterilised liquid media immediately before use. For inclusion in agar, the media was allowed to cool before the addition of the antibiotics. Concentrations of routinely used antibiotics are shown in Table 2-1.

**Table 2-1 Antibiotics for *in vitro* work**

| Antibiotic | Stock concentration | Working concentration | Diluent |
|---|---|---|---|
| Chloramphenicol | 12.5 mg/ml | 12.5 µg/ml | Ethanol |
| Anhydrotetracycline | 500 µg/ml | 50-100 ng/ml | Ethanol |
| Lincomycin | 20 mg/ml | 20 µg/ml | Ethanol |
| Erythromycin | 5 mg/ml | 5 µg/ml | Ethanol |
| Clindamycin | 20 mg/ml | 20 µg/ml | Sterile deionised water (SDW) |
| Thiamphenicol | 1.5 mg/ml | 15 µg/ml | Dissolve in acetic acid and make up volume with SDW |

#### 2.1.2.2 For *in vivo* use

For *in vivo* use, antibiotics were dissolved and sterilised using a 0.2 µm Ministart® syringe filter. Antibiotics that were delivered in the drinking water

were prepared fresh on the day of use. Clindamycin was stored at -20 °C prior to use.

**Table 2-2 Antibiotics for *in vivo* use**

| Antibiotic | Concentration | Method of delivery | Diluent |
|---|---|---|---|
| Kanamycin | 400 mg/L | *Ab libitum* in the drinking water as a cocktail | SDW |
| Gentamycin | 35 mg/L | | SDW |
| Colistin | 850000 (U/L) | | SDW |
| Vancomycin | 45 mg/L | | SDW |
| Metronidazole | 215 mg/L | | Dissolve in acetic acid and make up volume with SDW |
| Clindamycin | 250 mg/L | | SDW |
| Clindamycin | 15 mg/ml | 200 µl Oral gavage | SDW |

## 2.2 Bacterial strains and vectors

### 2.2.1 Storage of bacterial strains

#### 2.2.1.1 Preparation of bacterial glycerol stocks

Non-sporulating bacterial strains were stored at -80 °C as glycerol stocks. These were prepared from single colonies inoculated and grown to mid-log phase in BHI. This culture was then aliquoted into 800 µl volumes of culture which were mixed with 20 % glycerol, before storage at -20 °C. Stocks of *E.coli* were prepared in an identical manner except colonies were grown to mid log phase in LB broth.

#### 2.2.1.2 Preparation of spore stocks of *Clostridium difficile* for *in vivo* infections

*C. difficile* strains were stored at -80 °C as spores. Spores were subject to the following protocol to limit clumping as this is important to ensure accuracy of the infectious dose during *in vivo* experiments. Briefly, 100 ml of BHI broth was inoculated with a single colony and grown anaerobically for 7 days at 37 °C after which cells were harvested by centrifugation for 10 minutes at 2950 g. The

resultant pellet was resuspened and left for 1h in 75 % ethanol and vortexed every 10 minutes. The pellet was again collected by centrifugation before being treated, at 37 °C, with 10ml of phosphate buffered saline (PBS) containing 1 % sarkosyl for 1 hour. Spores were again harvested by centrifugation before resuspension in 10 ml PBS containing 0.125 M Tris buffer (pH 8) and 10 mg/ml lysozyme before incubation at 37 °C overnight. The following day, samples were layered onto 10 ml of 50 % sucrose and centrifuged at 2950 g for 20 minutes. The resultant pellet was then resuspened in 10 ml PBS containing 200 mM EDTA, 300 ng/ml proteinase K and 1 % sarkosyl and incubated at 37 °C for at least 3 hours. Samples were layered onto 10 ml of 50 % sucrose and centrifuged as described previously. Finally the pellet was washed twice in 10 ml PBS before being resuspened in 1-2 ml PBS. The purified spores were then aliquoted into 200 µl volumes and stored at -80 °C.

### 2.2.2 Isolation of bacteria from mouse faecal samples

Bacteria were recovered from faecal pellets collected from each animal, for aerobic bacteria pellets were placed immediately in Brain Heart Infusion (BHI) broth. The samples were homogenised then serially diluted in BHI and plated on to BHI, Man Rogosa Sharpe (MRS), Fastidious anaerobic media (FAM), Columbia base media (CBM) supplemented with horse blood (5%), Wilkins-Chalgren anaerobic (WCA) media and Luria Bertani (LB). To enable recovery of anaerobic organisms, pellets were collected in preconditioned BHI and transported in anaerobic conditions to ensure maximum recovery of organisms. Bacterial growth was recovered from this range of agars following incubation at 37 °C for between 24-72 hours.

### 2.2.3 Bacterial strains used in this work

The bacterial strains utilised as part of this work are detailed in Table 2-3. Bacteria were stored at -80 °C in 20 % glycerol or as spores. For long term storage, plasmids were transformed into *E. coli* DH5α.

**Table 2-3; Bacterial species and strains used in this work**

| Species | Origin | Antibiotic resistance |
|---|---|---|
| *C. difficile* BI-7 | Trevor Lawley | Clindamycin |
| *C. difficile* R20291 | Lisa Dawson | Erythromycin |
| *C. difficile* R20291_*hpdA*::CT | Lisa Dawson | Lincomycin |
| *C. difficile* R20291_*1754*::CT | Lisa Dawson | Lincomycin |
| *C. difficile* R20291 harbouring pRPF215 (transposon) | This work | Thiamphenicol |
| *E. coli* CA434 harbouring pRPF215 (transposon) | Rob Fagan | Chloramphenicol |
| *Dermococcus* | This work | - |
| *Citrobacter* | This work | - |
| *Escherichia fergusonii Shigella sonnei or E.coli* | This work | - |
| *E.fergusonii S. sonnei or E.coli* | This work | - |
| *Enterococcus faecium* | This work | - |
| *Enterococcus casseliflavus* | This work | - |
| *Enterococcus* | This work | - |
| *Enterococcus* | This work | - |
| *Lactobacillus apodemi* | This work | - |
| *L. apodemi* | This work | - |
| *Kocuria rhizophila* | This work | - |
| *K. rhizophila* | This work | - |
| *Micrococcus luteus* | This work | - |
| *Enhydrobacter or Moraxella* | This work | - |
| *Staphylococcus lentus* | This work | - |
| *Staphylococcus warneri* | This work | - |
| *S. warneri* | This work | - |
| *S. warneri* | This work | - |
| *E.coli* DH5α | Thermo Fisher | Plasmid dependent |
| *E.coli* BL21 | Thermo Fisher | Plasmid dependent |

# 2.3 Molecular methods

## 2.3.1 Oligonucleotides

All oligonucleotides used in this study are listed in Table 2-4. All oligonucleotides were purchased from MWG Eurofins. Primers were diluted in DNAse and RNAse free water (Thermo Fisher) to a working concentration of 10 mM unless otherwise stated.

**Table 2-4 Oligonucleotides used in this study**

| Name | Description | Sequence |
|---|---|---|
| BSH G1 (F) | Degenerate primers for amplification of BSH from group 1<br><br>Primers provided by Prof Julian Marchesi | CGTCCAGGCCCGGACNATHGARTGGG |
| BSH G1 (R) | | GGTGGCGGAGGTCCAYTGNGT |
| 16s rRNA V4 (F) | Primers for amplification of the V4 region of the 16s rRNA gene. The forward primer is universal. The reverse primer contains a unique 12bp Golay barcode (red) to allow multiplexing of samples for sequencing<br><br>Primers provided by Dr Chris Quince | AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGT GTGNCAGCMGCCGCGGTAA |
| 16s rRNA V4 (R) | | CAAGCAGAAGACGGCATACGAGAT**GTCGAATTTGCG**AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT |
| S12tBSH | Production of both the tBSH from S12 and the full length BSH from S15 | GGGAGAAATTTTGACTATGAG |
| S15BSH | | ATGTGTACTGCAGTATCATTTAAAAC |
| BSH R | | TTAGTTAGCGTAATTGAAATG |
| S12CLO F | Primers for cloning of tBSH and BSH. Forward primers contain Sph1 restriction. Reverse primer contains a Xba1 site for cloning into pnz44. Additional S12 contains an ATG site to allow expression of S12 BSH | ATTGCATGCATGTTGGGGAGAAATTTTGACTATG |
| S15CLO F | | ATTGCATGCATGTGTACTGCAGTATCATTTAAAAC |
| BSHCLO R | | TAAGCATCTAGATTAGTTAGCGTAATTGAAATGTTG |
| hpdA F | Check for mutant in hpdA gene<br><br>Primers provided by Dr Lisa | TGTATGAATTTTTGTGCAGTGGATC |
| hpdA R | | TCGAAATTAGAAACTTGC |

| | | |
|---|---|---|
| | Dawson | GTTCAGTAAAC |
| MVLA_1 F | Primers directed to 7 VNTR regions in *C. difficile*. Pattern of PCR products is used for typing. | ATTAATCATATCCTACAGAACACGA |
| MVLA_1 R | | TAAAACAAATGATATAAACTGAAAAG |
| MVLA_2 F | | GTAGAAGGGGCAAATAATGAG |
| MVLA_2 R | | CCTTCTGGCTTCCTTGTAATA |
| MVLA_3 F | | AATTTTAAGTTAACGTTTTTCTACAT |
| MVLA_3 R | | AGCCATTTTTATCAATCCTTTCTAT |
| MVLA_4 F | | TCTGGGATGTAACTAGCGACTTGT |
| MVLA_4 R | | TCTTAGGGAATTTATTGGAGGAA |
| MVLA_5 F | | AGGAGCTTTATATGGACATTCAGGTAG |
| MVLA_5 R | | AATCTCTTTCAAACTCTTCAATCTCAAT |
| MVLA_6 F | | AACATATTTAGGCATTTTAGTC |
| MVLA_6 R | | GAGTATTATTTATCATTTGTGGGTATTA |
| MVLA_7 F | | GGTGCACATGCTGGTCCTG |
| MVLA_7 R | | AACGCATTAAATTTCACTCCTCATAC |

## 2.3.2 General PCR protocol

PCR was performed using Biometra Professional Thermocycler. Reactions were carried out in 0.2 ml PCR tubes (Sarstedt). A general PCR protocol and PCR reaction mixture used in this study are shown in Table 2-5 and Table 2-6. Annealing temperature and cycle number were varied for each primer set and this information is detailed in the corresponding sections. For general PCR reactions Thermo Start Reddy Mix was used, when PCR products were to be used for cloning or for V4 16s sequencing Phusion® High fidelity master mix was included to reduce PCR induced errors.

**Table 2-5 Reaction mixture for PCR reactions**

| Component | Quantity in 25µl reaction |
|---|---|
| 1X Master mix | 22.5 µl |
| Forward primer (10 mM) | 1 µl |
| Reverse primer (10 mM) | 1 µl |
| DNA template | Variable |
| Nuclease free water | Up to 25 µl |
| $MgCl_2$ (50 mM) | Dependent on reaction |

**Table 2-6 A general PCR protocol used in this study**

| Reaction step | Temperature (°C) | Time |
|---|---|---|
| Initial denaturing (x1) | 95 | 2 minutes |
| Denaturing (x30) | 95 | 30 seconds |
| Annealing (x30) | Variable | 30 seconds |
| Amplification (x30) | 72 | 1 minute per Kb |
| Amplification (x1) | 72 | 10 minutes |

### 2.3.3 Agarose gel electrophoresis

PCR products, plasmids and digests were all run on 1% agarose gels in 1xTAE buffer containing 10 µl GelRed (10000X) (Cambridge Biosciences) per 100 ml of gel. Gels were run at 140 V for 45 minutes. 1 Kb Plus DNA ladder (Thermo Fisher) was used to determine the size of DNA fragments.

### 2.3.4 Gel extraction and PCR purification

For gel extraction the band was excised from the gel using a clean scalpel. Extraction was carried out using the QIAquick gel extraction kit (Qiagen) as per the manufactures instructions. PCR purification was carried out using the QIAquick PCR purification kit (Qiagen) as per the manufactures instructions.

## 2.4 DNA extraction, 16s amplification and genome sequencing of bacteria

#### 2.4.1.1 Purification of genomic DNA from *C. difficile* and bacteria isolated from faeces

A single colony was inoculated into 20 ml of BHI or tryptone soy broth (TSB) and grown statically at 37 °C, aerobically or anaerobically, depending on the origin of the isolate. Cultures were incubated for 24 hours. DNA was extracted using a DNEasy Blood and Tissue kit (Qiagen) as per the manufactures instructions. DNA was stored at -80 °C until required.

#### 2.4.1.2 Amplification of 16s rRNA from bacteria

To determine the identity of bacteria isolated from the faecal samples PCR was used to amplify the V4 region of the 16s rRNA gene. The annealing temperature of the reaction was at 44 °C. The expected product size was 350 bp.

## 2.5 DNA extraction from faecal samples for microbiome analysis

Faecal samples were collected and stored at 5 °C for a maximum of 12 hours before DNA extraction. Extraction was carried out as described in the QIAgen soil kit with the addition of a homogenisation step using the Fastprep® machine (MpBio) at speed 6 m/s for 40 seconds. DNA was stored at -80 °C until required.

### 2.5.1 Amplification of the 16s rRNA gene from faecal DNA

To amplify the 16s rRNA gene the concentrations of faecal DNA were quantified using Nanodrop (Thermo Fisher) and diluted to 2 ng/µl before use. Each 25 µl reaction contained 12.5 µl of Phusion high fidelity master mix, 0.87 µl of forward primer, 0.87 µl of reverse primer (containing a 12 bp Golay barcode to allow multiplexing), 1.25 µl of DMSO, 6.5 µl of nuclease free water and 3 µl of DNA (2 ng/µl). The PCR reaction was limited to only 20 reaction cycles in order to reduce PCR induced error. PCRs were performed in triplicate and pooled. Pooled samples were run on a 1 % agarose gel at 125 V for 1 hour and the correct band (378 bp) excised and DNA recovered from the gel. Library preparation and

sequencing of 16s rRNA gene amplicons was carried out at the Liverpool Centre for Genomic Research (LCGR).

## 2.5.2 Microbiome analysis

All code used to carry out microbiome analysis is open source and is available at http://userweb.eng.gla.ac.uk/umer.ijaz/.

### 2.5.2.1 Processing of 16s amplicons

After gel extraction the 16s rRNA samples were sent to LCGR where the library was assembled and sequenced. After sequencing, the adaptor sequences were removed and the samples were demultiplexed using the unique Golay sequences associated with each sample (Table 2-4). This was carried out by the LCGR. All further analysis was carried out in conjunction with Dr Umer Ijaz.

As sequencing often produces reads that drop in quality towards the 3 prime end it necessary to trim sequences before further analysis. To do this we utilised Sickle, a program that examines the quality scores along a read and trims the read if the quality drops below the desired level. It also filters reads below a certain length. For this analysis sequences were trimmed when the phred score dropped below 20. In addition, sequences of 10 bp or less were removed before further processing. After quality trimming, paired end reads must be overlapped and mismatches and uncalled bases corrected. This was achieved using Pandaseq (Masella *et al.,* 2012). Reads required a minimum overlap of 20bp to be used. For removal of chimera sequences (a fusion of 2 or more sequences) and taxonomic assignment to OTUs Usearch was used (Edgar, 2010). OTUs were determined at a 97 % similarity level and assignments were determined using RDP classifier (Wang *et al.,* 2007), which gives identity at Phylum, Class, Order, Family and Genus level.

To further analyse the composition of the microbiome beyond merely OTU presence and absence it is necessary to construct maximum likelihood phylogenetic trees. These allow comparisons of abundance and the relatedness of OTUs to be undertaken and are required for the analysis of both alpha and beta diversity measures. To do this multiple sequence alignment was carried out in MAFFT (Katoh *et al.,* 2002). These alignments were then used to generate

maximum likelihood phylogenetic trees using FastTree (Price *et al.,* 2010). Data was then imported into R where additional analysis was carried out including creation of taxa plots, assessment of alpha and beta diversity and changes in OTU abundance.

#### 2.5.2.2 Metabolic prediction using Tax4fun

Analysis of the OTUs within microbiota samples is incredibly useful. However its main limitation is the lack of functional information. Bioinformatics packages that allow for the inference of metabolic potential from this data are now in existence. One such package is Tax4Fun. Tax4fun is an open source R package that uses the SILVA database and reference genomes to infer the likely metabolic potential of OTUs within a 16s dataset (Aßhauer *et al.,* 2015). To use Tax4fun it is necessary to create a biom file in QIIME. This was achieved by annotating the OTUs using the SILVA database which is compatible with the Tax4fun package. The biom file can then be used to extract KEGG information for the OTUs identified in the microbiome samples. Further analysis allows the changes in abundance of specific enzymes and KEGG pathways to be determined. In addition to this it was also possible to determine the importance of specific functions in classifying samples into treatment groups using a Random Forest Classifiers.

## 2.6 Determining the impact of BAs on *C. difficile* germination and outgrowth

### 2.6.1 Detection of BSH activity using a plate based assay

To test bacterial isolates for BSH activity bacteria were plated onto BHI media containing 0.5 % TC and incubated for up to 72 hours. BSH activity was determined by the presence of a halo of precipitate or by the formation of white granular colonies.

### 2.6.2 Amplification of BSH genes from microbiome samples

Primers for amplification of BSH were a kind gift from Professor Julian Marchesi (Cardiff University). These were designed using consensus-degenerate hybrid oligonucleotide (CODEHOP), which aligns the amino acid sequences of the

homologous enzymes from different bacteria and creates degenerate primers that are able to amplify all of the variations (Rose et al., 1998). Using this method numerous bacterial BSH can be amplified using a single set of primers. The PCR reaction was as described in 2.3.2. Reactions contained 1 µl of template DNA at 50 ng/µl. The annealing temperature of the reaction was 60 °C with an expected product size of 858 bp.

### 2.6.3 Amplification of *Lactobacillus murinus* BSH

Primers were designed to the BSH sequences recovered from the *L. murinus* genome sequences. Due to sequence homology a universal reverse primer was used for both *L. murinus* strains. In contrast forward primers were specifically designed to the individual strains. The annealing temperature of the reaction was 50 °C with an expected product size of 978 bp for the BSH+ve strain and 939 bp for the BSH-ve strain.

### 2.6.4 Attempts to clone and express the BSH from *Lactobacillus murinus*

#### 2.6.4.1 Plasmid purification

Plasmids were purified from 5 ml of LB culture using a QIAprep spin miniprep kit (Qiagen), using the manufacturers protocols.

#### 2.6.4.2 Amplification of *Lactobacillus murinus* BSH for use in cloning

Primers were designed containing restriction sites that were also found in the pnz44 plasmid. Forward primers contained the Sph1 restriction site and reverse primer Xba1 to allow ligation into pnz44 vector. The annealing temperature of the reaction was 50 °C.

#### 2.6.4.3 Restriction digest of pnz44 vector

Restriction digests were carried out in 50 µl volume reactions containing 5 µl of cutsmart buffer, max of 1 µg of template DNA, 10 units of Sph1 and of 20 units of Xba1. DNAse and RNase free water was added to a final volume of 50 µl. Restriction digests were carried out in a water bath at 37 °C for 3 hours followed by heat inactivation of the restriction enzymes.

#### 2.6.4.4 Ligation of *L. murinus* BSH into pnz44

Similarly digested vector and PCR fragment were ligated at room temperature for 15 minutes. A 20ul ligation reaction contained 400 units of T4 DNA ligase, 2 µl 10 X reaction buffer and vector and insert at a 3:1 molar ration. The reaction mix was made up to 20 µl with nuclease free water.

#### 2.6.4.5 Transformation of BSH containing pnz44

5 µl of ligation mixture was added to 50 µl of chemically competent DH5α *E. coli* and incubated on ice for 30 minutes. Cells were heat shocked at 42 °C for 45 seconds and then incubated on ice for 5 minutes. 300 µl of LB media was added and samples incubated at 37 °C with shaking before plating onto LB with the appropriate selection antibiotic.

### 2.6.5 Germination assays

#### 2.6.5.1 Impact of strains isolated from mouse faeces on *C. difficile* spore germination

To determine if strains recovered from the faeces were able to functionally modify BA *in vitro* 10 ml BHI broth was inoculated with a single colony of each isolate before incubation for 24 hours or until the culture reached an OD of approximately 1. Assays were carried out in triplicate with a minimum of 3 biological replicates for each sample. BHI was used as a negative control, BHI with 0.1 % TC as a positive control of maximum germination and BHI with 0.1 % CA to determine the ability of CA to induce germination. To determine if the strains were able to deconjugate TC to CA and impact germination 30 µl of each overnight culture was added to 3 ml of BHI with 0.1 % TC. The plate was incubated for 24 hours either aerobically or anaerobically depending on the origins of isolation. Cultures were sterilised using a 0.2 µm Ministart® syringe filter (Sartorius) to remove bacteria from the media and adjusted to pH7. The 12 well plates were incubated anaerobically for a minimum of 2 hours to condition the media. A minimum of 1 X $10^4$ spores were added and incubated for a further 2 hours to allow for germination of spores. Cultures were serially diluted to $10^{-3}$ and plated onto BHI agar containing 0.1 % TC giving total bacteria numbers. The remaining sample was heated at 65 °C for 20 minutes in order to kill and vegetative cells leaving only non-germinated spores. These spores were also

enumerated and from this the percentage of germination was calculated using the equation below.

$$Percentage\ germination = (Vegetative\ cells \div Total\ number\ cells) \times 100$$

#### 2.6.5.2 Impact of BSH expressing *E. coli* on *C. difficile* spore germination

To clarify the role of BSH activity in inhibition of *C. difficile* germination *E. coli* clones expressing BSH from the human gut microbiota were utilised (Jones *et al.,* 2008). The *E. coli* harbour BSH genes cloned into the constitutive pnz44 plasmid. This plasmid is a derivative of pnz8048 where the PnisA promoter has been replaced with the constitutive p44 promoter from *Lactococcus lactis*. Carriage of pnz44 confers chloramphenicol resistance (Mcgrath *et al.,* 2001). *E. coli* were grown overnight in LB broth containing chloramphenicol (12.5 ug/mL). 300 µl of this culture were used to inoculate 30 ml of BHI broth with 0.1 % TC and incubated for 24 hours. Cultures were centrifuged at 2950 g for 10 minutes and the supernatant filter sterilised using 0.2 µm Ministart® syringe filter. The rest of the assay was performed as in section 2.6.5.1.

### 2.6.6 The impact of BAs on bacterial growth

#### 2.6.6.1 Germination and outgrowth of *C. difficile* spores in the presence of different BAs

To investigate how different BAs influence germination and subsequent outgrowth of *C. difficile*, growth curves of the organism in the presence of different BAs were carried out. 100 µl of *C. difficile* spores were used to inoculate flasks containing 10 ml BHI, BHI with 0.1 % TC, BHI with 0.1 % CA or BHI with 0.1 % DCA. Cultures were incubated anaerobically at 37 °C. $OD_{600}$ measurements were taken every 3 hours for the first 12 hours with a final sample taken at 24 hours.

#### 2.6.6.2 Growth of *L. murinus* in the presence of TC

To determine the impact of TC on growth of *L. murinus* growth curves were carried out in MRS broth and MRS broth containing 0.1 % TC. Growth curves were set up by inoculating 100 µl of an overnight culture of *L. murinus* into 10 ml of MRS broth or MRS broth containing 0.1 % TC. Cultures were incubated

anaerobically at 37 °C. Samples were taken every 3 hours for 9 hours. Samples were serially diluted onto MRS agar and incubated for 24 hours before bacteria were enumerated.

### 2.6.7 Biofilm assays to determine the impact of TC on biofilm abundance and adherence

#### 2.6.7.1 Crystal violet assay to measure biofilm abundance in the presence and absence of TC

An overnight culture of *C. difficile* in BHIS-C media was used to seed a 6 well plates. Overnight cultures were grown in BHIS-C alone or with the addition of 0.1 % TC. 200 µl of an overnight culture was inoculated into 1.8 ml of pre-reduced BHIS-C media with or without 0.1 % TC, giving a total of 4 different growth combinations. Each biofilm was set up in triplicate with wells left uninoculated to serve as blanks. Plates were incubated anaerobically at 37 °C for 24 and 48 hours. After this time plates were removed from the cabinet and the media carefully removed using a vacuum pump. Biofilms were washed once with 1ml PBS before staining with 500 µl of 1 % crystal violet (CV). Plates were stained for 30 minutes and then washed twice with PBS to remove residual CV. CV was extracted from biofilms with the addition of 500 µl of methanol. Dilutions were performed to ensure accuracy of reading the sample at OD 544 nm. To allow effective absorbance measurements, 200 µl of the sample was transferred to a 96 well plate and the OD was determined at 544 nm using the FLOUstar OPTIMA plate reader (BMG labtech). The OD of the negative control well was subtracted from the samples to give an accurate reflection of biofilm formation.

#### 2.6.7.2 Assays to determine biofilm adherence to an abiotic surface in the presence and absence of TC

As with the biofilm method described in section (2.6.7.1) overnights were set up in BHIS-C alone or with the addition of 0.1 % TC. 1ml of overnight culture was used to seed 25 $cm^3$ vented tissue culture flasks containing 9 mls BHIS-C or BHIS-C 0.1 % TC, again giving a total of 4 growth conditions. Flasks were incubated statically and incubated anaerobically for 24 and 48 hours. After this time flasks were removed from the cabinet. Flasks were then mechanically disturbed to determine if the biofilm was adhered to the base of the flask (Dawson *et al.*,

2012). Photos were taken using a Panasonic lumix dms-t60 camera with samples placed on a light box.

## 2.7 Generation of a transposon mutant library

#### 2.7.1.1 Creation of a random transposon mutant library of *C. difficile*

The vector containing the mariner transposon pRPF215 was stored in *E. coli* CA434 and was a kind gift from Dr Rob Fagan (University of Sheffield). Maintenance of the plasmid was mediated by thiamphenicol resistance (Figure 2.1).

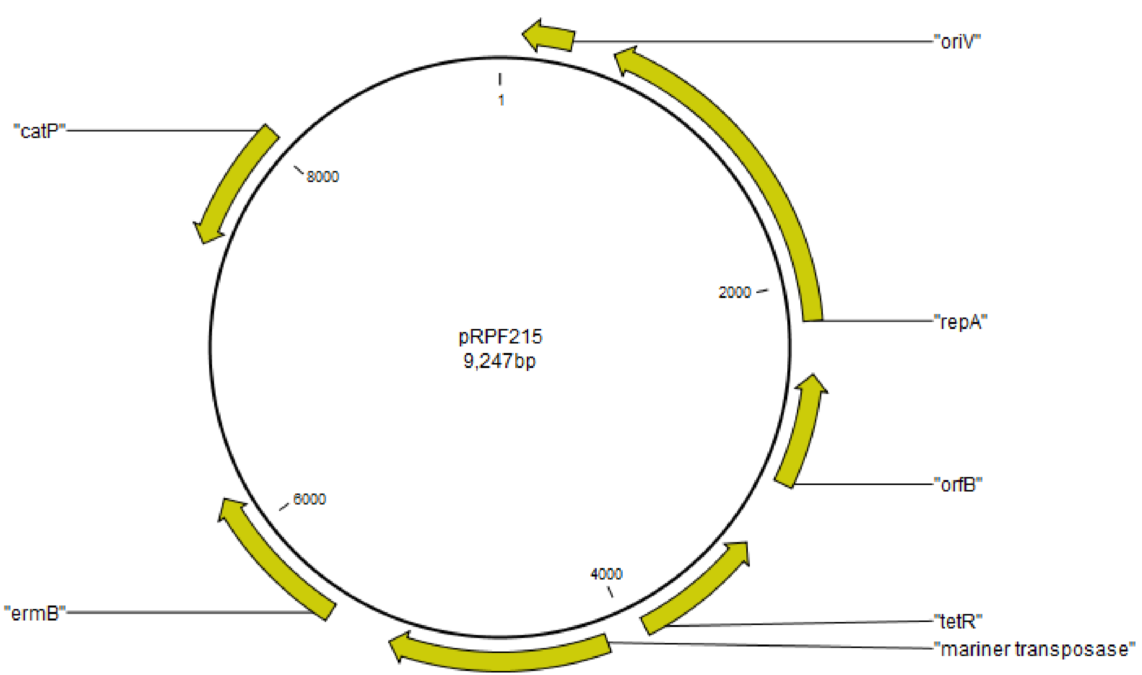



**Figure 2.1 Vector map of pRPF215**
The pRPF215 plasmid contains the mariner transposon which contains an ermB cassette to allow for selection of bacteria in which the transposons has integrated into the genome. Expression of the mariner transposon is under control of a tetracycline inducible promoter.

Regulation of expression of the mariner transposon is controlled by the addition of anhydrotetracycline (AHT). Following expression, this transposon is able to insert into any TA site within the chromosome, resulting in the generation a library of mutants in which the transposon is inserted randomly. The insertion of the transposon containing the *erm*B gene into the genome renders strains lincomycin resistance allowing for easy selection of transposon mutants. A

variation of pRPF215 in which a fluorescent LOV domain was linked to the C terminal end of the transposon (pYAA023) was also utilised in this project.

#### 2.7.1.2 Conjugation of pRPF215 and pYAA023 from *E. coli* CA434 into *C. difficile* R20291

Donor *E. coli* CA434 harbouring the pRPF215 or pYAA023 plasmid was streaked onto LB agar containing chloramphenicol (12.5 µg/ml). The recipient *C. difficile* strain was inoculated onto Braziers CCEY agar containing erythromycin (5 µg/ml). Overnight cultures of both the donor *E. coli* and recipient *C. difficile* strain were grown in antibiotic containing broth, the *E. coli* in LB and the *C. difficile* in BHI. 1.5 ml of the *E. coli* was harvested and centrifuged at 2950 g for 10 minutes. The pellet was washed gently in PBS and carefully resuspened in 200 µl of *C. difficile*. The conjugation mixture was spotted onto a non-selective BHI plate and incubated anaerobically at 37 °C. After 8 hours the conjugation mixture was scrapped from the plate and resuspened in 500 µl of PBS. 100 µl of this mixture was spread onto BHI containing cycloserine (250 µg/ml), cefoxitin (8 µg/ml) and thiamphenicol (15 µg/ml) to facilitate for selection of *C. difficile* containing the plasmid. A well isolated colony was subbed onto fresh selective agar. This step was repeated to purify *C. difficile* containing the transposon containing plasmid until no *E. coli* contamination remained.

#### 2.7.1.3 Generation of a transposon mutant library in *C. difficile*

To generate a transposon mutant library, *C. difficile* harbouring either pRPF215 or pYAA023 was streaked onto Braziers with thiamphenicol (15 µg/ml) and used to inoculate an overnight culture in BHI. The culture was then used the following day to inoculate (1:100) tryptone yeast broth with glucose (TY+G) and grown to an OD of 0.4. The culture was diluted to 1:5, 1:25 and 1:125 and plated onto Braziers CCEY agar containing AHT to induce the expression of the transposon. Plates also contained lincomycin (20 µg/ml) to select for transposon mutants. Colonies were scraped from the plate and stored as a pool in BHI with 12.5 % glycerol at -80 °C or used for the production of spores.

#### 2.7.1.4 Selection for *C. difficile* mutants unable to germinate in the presence of TC

Spores that were produced from the mutant library were harvested (2.2.1.2) and then incubated in BHI containing 10 % TC for 12 hours to induce germination of those spores able to respond to TC as a germinant. The sample was subsequently heat shocked at 65 °C for 30 minutes to kill any vegetative cells leaving only non-germinated spores. The remaining spores were then artificially germinated using thioglycollate and lysozyme. Spores were incubated in 2 mM of thioglycollate (pH 10) for 50 minutes and plated onto Braziers containing lincomycin (20 µg/ml) and 10 mg/ml lysozyme (Kamiya *et al.,* 1989). Colonies formed on these plates were considered to be germination mutants.

#### 2.7.1.5 Genome assembly and annotation

The code used for this analysis is open source and available at
http://userweb.eng.gla.ac.uk/umer.ijaz/

To identify the location of transposon insertion within the germination deficient mutants genome sequencing of strains was carried out. DNA was extracted and sequence carried out at GPF on the MiSeq platform. Read 1 and read 2 files were downloaded and the following steps carried out in order to assemble the genomes. Before genome assembly was carried out the quality scores of the reads was checked. Quality of reads was determined by examining the phred score associated with each read. Phred scores give a probability that a base pair is incorrect. For example, a phred score or 10 gives the probability that 1 in 10 nucleotides have been misidentified. A phred score of 20 gives a 1 in 100 chance and so on. For genome assembly any reads with a phred quality score lower than 35 were removed. Assembly was carried out using the Spades assembly tool. Briefly, this takes the read 1 and read 2 files and assembles them into contigs (Bankevich *et al.,* 2012). Contigs less than 1 kb in length were removed before annotation. Remaining contigs were annotated using PROKKA (Seemann, 2014). This creates a variety of files including a genbank file which can then be mined for genes of interest or analysed further. To identify the location of the transposon within the genome of the germination mutants the annotated contigs were imported into CLC genomics workbench and an internal blast search directed to the *erm*B gene carried out. Once this gene was identified with the

contigs the gene that is was present in was determined by carrying out a Blast search.

### 2.7.2 RNA collection, extraction and sequencing from *C. difficile*

To investigate the impact of TC on gene expression RNA-seq was carried out. *C. difficile* R20291 was grown shaking at 37 °C until an optical density (OD) of 0.4 was reached. To one sample, BHI containing TC was added to give a final concentration of 0.1 % TC. To the second sample an equal volume of BHI alone was added. This ensured that both samples were treated equivalently. In total, 3 biological replicates for each growth condition were prepared. Following the addition of BHI, or BHI containing TC samples were incubated with shaking at 37 °C for 1 hour after which 5 mls of culture was removed and added to 9 mls of pre-reduced RNA*later* (Thermo Fisher) and incubated for 15 minutes. To harvest the bacterial cells, samples were centrifuged for 15 minutes at 2950 g at 4 °C and the supernatant discarded. 20 units of rRNasin (Promega) was added and pellets were stored at -80 °C until RNA extraction. RNA extraction was completed using the FastRNA Pro blue kit (MP bio) following the manufacturer's instructions. Total RNA was DNAse treated using Turbo DNA-free kit (Ambion) rigorous treatment, with complete removal of DNA was confirmed by failure to amplify the 16s rRNA gene from this sample (2.4.1.2). Depletion of ribosomal RNA was carried out using Ribo-zero rRNA removal kit (Illumina). Testing of RNA quality, cDNA library preparation and sequencing of the cDNA library was carried out at the Glasgow Polyomics Facility (GPF).

### 2.7.3 RNA seq analysis to identify differentially expressed genes after TC exposure

Analysis of RNA-seq data was carried out in CLC Genomics Workbench version 7. A reference genome of *C. difficile* R20291 was downloaded and tracks created to allow alignment of reads to the genome. Differential gene expression was evaluated using Deseq2 package (Love *et al.*, 2014). This package investigates differential count data, in this case the number of cDNA reads aligned to genes in different growth conditions and then compares these. Analysis was carried out to the specifications given in the CLC RNA seq analysis tutorials.

## 2.8 *In vivo* models of infection

All *in vivo* experiments were carried out in line with the UK Animals (Scientific Procedures Act) 1986. Food and water was provided *ad libitum* and animals were kept at a constant room temperature of 20-22 °C with a 12 hour light/dark cycle. All mice used in this study were female C57bl/6 mice aged 6-8 weeks. After infection mice were monitored daily for weight loss and changes in behaviour. At the end of the experiments mice were humanely sacrificed by the schedule one method of cervical dislocation.

### 2.8.1 Different antibiotic treatments modulate disease outcome

Multiple mouse models were used to determine the impact of different antibiotic treatments on the microbiota. The first used a single dose of clindamycin (150 mg/kg) by oral gavage (200 µl) to induce susceptibility to infection. The second used treatment with an antibiotic cocktail (ABC) containing a daily dose of kanamycin (40 mg/kg), gentamicin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg), and vancomycin (4.5 mg/kg) (Sigma-Aldrich) *ad libitum* in the drinking water for 3 days. This is followed by a single dose of clindamycin (150 mg/Kg) given by oral gavage 2 days after the cessation of the antibiotic cocktail. Use of this model resulted in more severe disease. Multiple regimes based around these treatments were used and are shown in Figure 2.2.

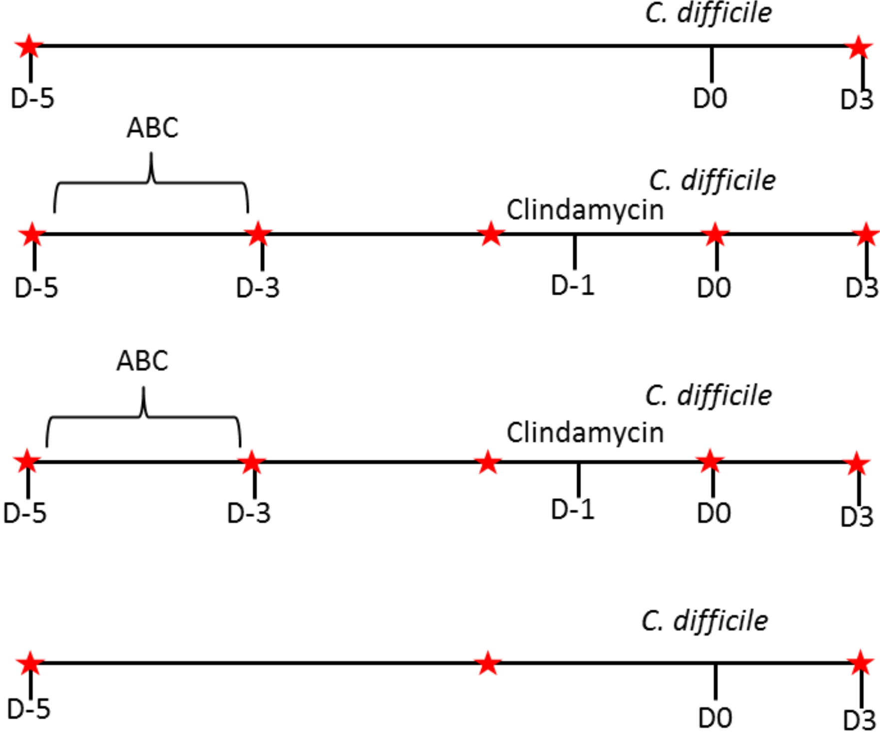



**Figure 2.2 Timeline of infection**
The figure above details the mouse models used to determine the impact of different antibiotic treatments on colonisation and disease severity. The ABC was administered for 3 days (D-5 to D-3). Clindamycin was given on D-1 and $10^5$ *C. difficile* spores on D0. Animals were culled at 4DPI. Stars indicate the timing of sampling for microbiome analysis.

During antibiotic treatment mice were transferred daily into clean sterile. On D0, $10^5$ *C. difficile* spores were administered by oral gavage to each animal. Faeces were collected prior to and post infection with *C. difficile* to monitor relative levels of infection (2.9.1). Following cull, toxin levels (2.9.4) and *C. difficile* numbers in the caecum and colon were enumerated (2.9.2).

#### 2.8.1.1 Bile salt extraction for mouse caecal samples

To prepare samples for Time of Flight mass spectrometer analysis (TOF-Mass spec) of BAs, 1 ml of caecum content, resuspened in PBS, was added to 3 mls of methanol to allow for extraction of BAs. This was mixed and centrifuged at 3000 g for 10 minutes. The supernatant was retained for analysis of BA composition.

#### 2.8.1.2 TOF-MS analysis of bile salts in mouse caecal samples

To determine the levels of TC and DCA in the caecum of mice, TOF-Mass spectrometry was used. This was carried by Dr Jia Li at Imperial Collage London using 6538 UHD Accurate Mass Q-TOF; Agilent Technologies. Parameters and methods are described in (Zhang *et al.*, 2016).

### 2.8.2 A mouse model to examine the role of *para-cresol* production in relapsing infection

The factors effecting relapsing *C. difficile* infection are not known. In order to investigate these factors further a relapsing model of *C. difficile* infection has been developed. This makes use of the observation that treatment of mice with clindamycin alone results in persistent colonisation, which although below the limit of detection can be induced by further use of clindamycin. This model was used specifically to determine the impact of *para*-cresol production in relapsing and acute infection. Mice were given clindamycin *ad libitum* in the drinking water (250 mg/L) for 3 days and then switched to normal drinking water. 2 days after cessation of clindamycin mice were administered $10^5$ spores of either *C. difficile* R20291 or R20291_*hpdA*::CT, which is unable to produce *para*-cresol. Faeces were taken daily to determine the level of colonisation with *C. difficile*. After cessation of shedding, relapse was induced with a single dose of clindamycin (150 mg/kg) by oral gavage on D35. Again, mice were monitored and faeces collected to determine levels of *C. difficile* in the faeces (Figure 2.3).

**Figure 2.3 Timeline of relapsing infection**
Clindamycin was given in the DW for 3 days (D-5 to D-3). Mice were switched to sterile DW for 2 days and then administered $10^5$ *C. difficile* spores at D0.

A variation of this model was also used to examine acute infection in which animals were culled at 2DPI. After cull, toxin levels and *C. difficile* numbers in the caecum and colon were enumerated.

#### 2.8.2.1 Colony PCR for detection of R20291_*hpdA*::CT from bacterial colonies

In these experiments all of the strains used are derived from the same parental R20291 strain making it not possible to differentiate between these strains with MVLA. Therefore it was necessary to ensure that each mouse was infected with

the correct strain of *C. difficile*. After isolation of *C. difficile* from faeces, colonies from each infection group were recovered used directly for amplification. Recovered colonies were added to 20 µl of SDW, heated at 95 °C for 20 minutes before incubation at -20 °C for 5 minutes to aid bacterial lysis. Samples were briefly centrifuged to sediment bacterial debris and 3 µl of the supernatant was used as the PCR template. Primers were directed to a region of the *hpd*A gene and a region within the ClosTron intron sequence. R20291_*hpdA*::CT will give a product of 408 bp whereas R20291 will not form a product due to the absence of ClosTron mutation. The annealing temperature of the reaction was 50 °C. This was used in conjunction with MVLA analysis to ensure that animals were infected with the correct strain of bacteria (2.9.3).

#### 2.8.2.2 *In vitro* growth of *C. difficile* with induction of para-cresol production in *C. difficile*

To determine the relative growth rates of R20291 and R20291_*hpdA*::CT comparative analysis was performed. In brief, strains were grown in BHI with the addition of 480 mg/L *para*-hydroxyphenylacetic acid (*p*-HPA), a precursor of *para-cresol* to induce *para*-cresol production. This was added to determine if production of *para*-cresol has a negative impact on the growth of R20291. Samples were taken every 3 hours for 9 hours and at 24 hours, 48 hours and 72 hours. At 24, 48 and 72 hours the proportion of spores was examined. To assess toxin production 1 ml of supernatant was taken and centrifuged at 18.8 g for 10 minutes and filter sterilised using a 0.2 µm Ministart® syringe filter. Samples were stored at -20 °C before used in toxin assays as described in section 2.9.4.

### 2.8.3 Prophylactic treatment with BSH-ve and BSH+ve *L. murinus*

Experiments were carried out to investigate the potential protective effect of BSH producing *L. murinus in vivo*. To induce susceptibility to infection, mice were given clindamycin (250 mg/L) in the drinking water for 3 days, as described in section 2.8.2. To prepare *L. murinus* for use *in vivo* MRS broth was inoculated and incubated for 9 hours. After 9 hours cultures were pelleted and washed twice with PBS. Samples were adjusted to a concentration of approximately $5x10^9$ CFU/ml. Each mouse was given 200 µl of *L. murinus* at 24 hours and 6 hours before infection mice equating to a dose of $10^9$ CFU of *L. murinus*. At D0 mice were administered $10^5$ *C. difficile* BI-7 spores (Figure 2.4).

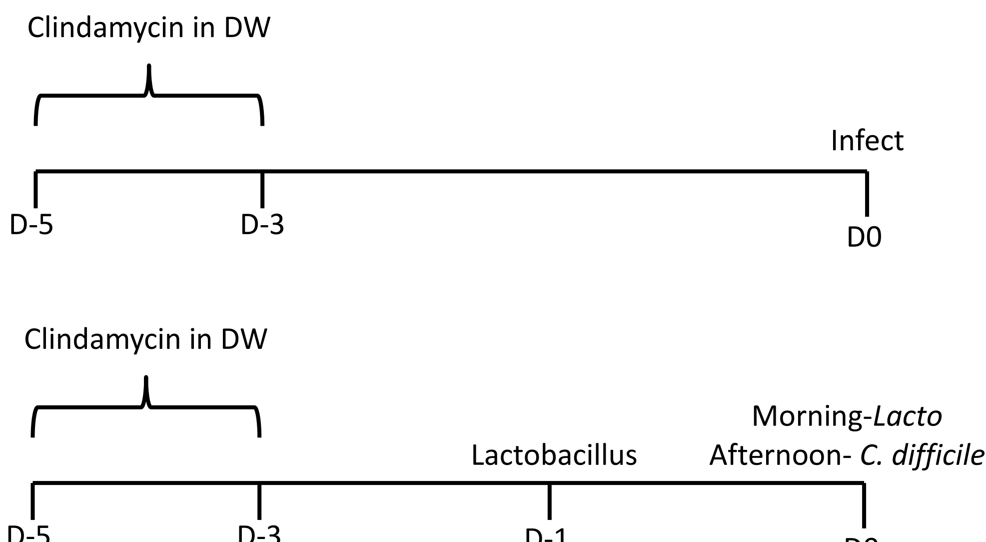



**Figure 2.4 Timelines of experiments to test efficacy of BSH *in vivo***
For all animals, clindamycin was delivered in the drinking water for 3 days. This was then replaced with sterile water for the remainder of the experiment. Approximately $10^9$ CFU of *L. murinus* was administered to each mouse on 24 h and 6 h prior to infection with $10^5$ spores of *C. difficile* BI-7.

Following *C. difficile* infection animals were monitored daily for changes in behaviour and weight loss.

## 2.9 General techniques for the measurement of *in vivo* colonisation and toxin production

A multitude of different mouse models were used in this work. The specifics of these are described in section 2.7. This section details the general techniques that are common to all *in vivo* experiments.

### 2.9.1 Detection of *C. difficile* in faeces as a measurement of colonisation

Total numbers of *C. difficile* in the faeces of mice were monitored daily post infection. Fresh faecal samples were collected, weighed and diluted tenfold in PBS. Dilutions were plated onto Braziers CCEY agar containing the appropriate antibiotic for selection and incubated at 37 °C for 48 hours before enumeration.

### 2.9.2 Culturing of *Clostridium difficile* from the caecum and colon

Upon *post-mortem*, the caecum and colon were harvested and cut longitudinally. *C. difficile* numbers in the caecum and colon were enumerated for lumen associated (LA) and tissue associated (TA) bacteria. Organ contents

were washed in 5 mls of PBS and tissue in 2 mls. Organ washes represent the LA bacteria. TA bacteria were recovered by homogenising the tissue. Organs were homogenised in 2 mls of PBS in the Stomacher Lab Blender 80 (Seaward Medical) and serial dilutions were plated onto Braziers CCEY agar containing the correct selection antibiotic for the strain used. Plates were incubated anaerobically for 48 hours. For spores the same protocol was used but with the addition of a heating step of 30 minutes at 65 °C to kill any vegetative cells. The number of vegetative cells was calculated by subtracting the number of spores from the total *C. difficile* counts.

### 2.9.3 Confirmation of identity of recovered bacteria using Multilocus Variant Number Tandem Repeat Analysis

After clindamycin treatment animals become highly susceptible to *C. difficile* infection. Although the significant efforts are made to limit inadvertent infection with environmental *C. difficile* it is necessary to identify any *C. difficile* recovered. This is determined using Multilocus Variant Number Tandem Repeat Analysis (MVLA). MVLA analysis is a method for typing bacterial strains including *C. difficile* (Marsh et al., 2006). It utilises 7 primer sets that amplify variable repeat regions from within the *C. difficile* genome resulting in the formation of a banding pattern that allows for discrimination of strains from one another. MVLA was used to confirm that *C. difficile* recovered from the faeces of mice was the same as the infecting strain in all experiments performed. In long term experiments numerous samples were taken for analysis each week. Primers used for MVLA are shown in Table 2-4. The annealing temperature of this PCR reaction was 50 °C. The expected banding pattern is different for each *C. difficile* strain.

### 2.9.4 Cellular assays for detection of *C. difficile* toxins

To semi-quantify the levels of TcdA and TcdB produced in the samples a cellular assay was used. HT29 cells which are sensitive to TcdA and Vero cells which are sensitive to TcdB were used to determine toxin levels in the caecum and colon. Cells were grown in Eagles Minimal essential medium (EMEM) supplemented with 10 % heat inactivated foetal calf serum (Thermo Fisher), 2 mM L-glutamine, 1 % Fungizone Amphotericin B 250 ug/ml (Life Technologies) and 1% Penicillin-

Streptomycin 10000 U/ml (Thermo Fisher). 96 wells plates were seeded with cells and grown at 37 °C with 5 % $CO_2$ until a confluent mono-layer was formed. The media was removed and the cells were washed with PBS before fresh EMEM was added. Before use in the assays samples were filter sterilised. For the caecum samples, 10 fold dilutions were performed, and for the colon samples 5 fold dilutions. To determine toxin levels in culture media, 2 or 5 fold dilutions were carried out using culture supernatant. In all cases, plates were incubated overnight at 37 °C with 5 % $CO_2$ before being washed twice with PBS and then fixed in 1 % formalin. Cells were fixed for 15 minutes and washed twice more with PBS and stained with Giemsa for a minimum of 1 hour. Giemsa stain was gently removed with running water and the plates examined by eye. High toxin level causes cell rounding and disassociation of cells from the plate which results in a lack of staining. Toxin level was determined as the highest dilution in which no cells and therefore no staining is present.

## 2.10 Statistical analysis

All analysis of 16s microbiome data was carried out in R version 3.1.3. For all other statistical analysis GraphPad Prism was used. The tests and parameters used are detailed in the figure legends throughout. Tests used included Students T-test, T-test with Welch correction, ANOVA with Tukey's correction and Kruskal-Wallis with Dunn's multiple comparison.

# 3 The impact of the microbiota on *C. difficile* colonisation and disease severity

## 3.1 Introduction

*C. difficile* is a disease intrinsically linked to disruption of the microbiome, with antibiotic treatment associated with reduction in microbial diversity, known to play a key role in *C. difficile* infection and disease severity (1.4.4 and 1.6.2). However, the precise mechanisms by which individual or combinations of microorganisms are able to suppress *C. difficile* colonisation and toxin release are unclear. Evidence of the importance of microbiome diversity is provided by the successful use of FMT to treat this disease (van Nood *et al.*,2013). Although clearly efficacious its clinical application has been restricted to patients suffering multiple relapses of disease. This reflects both issues with screening of faecal donations to limit indirect spread of pathogens to vulnerable patients, and aesthetic issues associated with the direct instillation of filtered faecal material into patients. For these reasons, identification and generation of a synthetic mix of microbes with the metabolic capacity to limit *C. difficile* colonisation is attractive. To date, several studies in both mouse and man have indicated that such an approach is feasible, with bacteriotherapy shown to suppress infection and reduce carriage of *C. difficile* in mice and humans (1.5.2). However, whilst much attention has been focussed on restoring microbial diversity, little consideration has been given to the functional metabolic capacity of these microbes. Additionally, not all combinations of bacteria are able to suppress infection (Lawley et al., 2012), further supporting the hypothesis that important functional aspects expressed by the microbiota are required for the restoration of colonisation resistance.

The aim of this study was to determine how different antibiotic combinations impact on the microbiota and how these changes modulate *C. difficile* disease severity. This required a combination of culture based and molecular techniques to investigate the diversity of the microbiome at different time points following antibiotic treatment and pre and post infection. Using these data, we hoped to identify those bacteria and their metabolic functions that influence disease outcome.

The TOF-mass spec data presented in this chapter was carried out by Dr Jia Li at Imperial College London. Bioinformatics analysis was carried out with the assistance of Dr Umer Ijaz.

## 3.2 Results

### 3.2.1 Optimisation of mouse models

Animal models of infection have been widely used for many years to study infection dynamics of *C. difficile in vivo*. Until recent times the most commonly used model was the hamster, which is easily colonised by *C. difficile* after antibiotic exposure. However, this model reflects only acute infection with animals often rapidly succumbing to infection (1.6.1). The development of mouse models that more closely reflect the spectrum of symptoms seen in human disease has allowed the role of the commensal microbiota in infection to be unravelled. This chapter utilises multiple mouse models of infection in order to determine if specific microbes or microbial functions are responsible for changes in disease severity and outcome. In these models the outcome of infection is influenced by the spectrum of antibiotics administered before infection (1.6.2). Multiple methods of antibiotic delivery were tested including use of oral gavage, *ad libitum* delivery in water and by intraperitoneal (IP) injection. It was found that administering the ABC in the water followed with clindamycin by gavage was the best method of delivery as it most closely replicated what has been seen in the literature. It also required minimal handling of animals, which reduces the possibility of cross infection with other strains of *C. difficile* that may contaminate the environment.

#### 3.2.1.1 Mouse body weights during infection

An important part of the licensure for these experiments is minimisation of animal suffering. As a consequence animals placed on procedure are continually monitored and any changes in behaviour or appearance monitored so that animals can be culled at a previously established humane endpoint. Weight loss in the context of these experiments is a good indicator of disease severity. Consequently, all mice within these experiments were weighed daily and animals suffering losses of greater than 15% of their weight after infection were culled.

In these initial experiments mice were treated with different antibiotic combinations before infection with *C. difficile* BI-7 and weight loss measured (2.8.1). The average weight loss of these animals is shown in Figure 3.1. To note only one mouse from the group treated with ABC plus clindamycin group reached this point and was subsequently culled 3DPI.

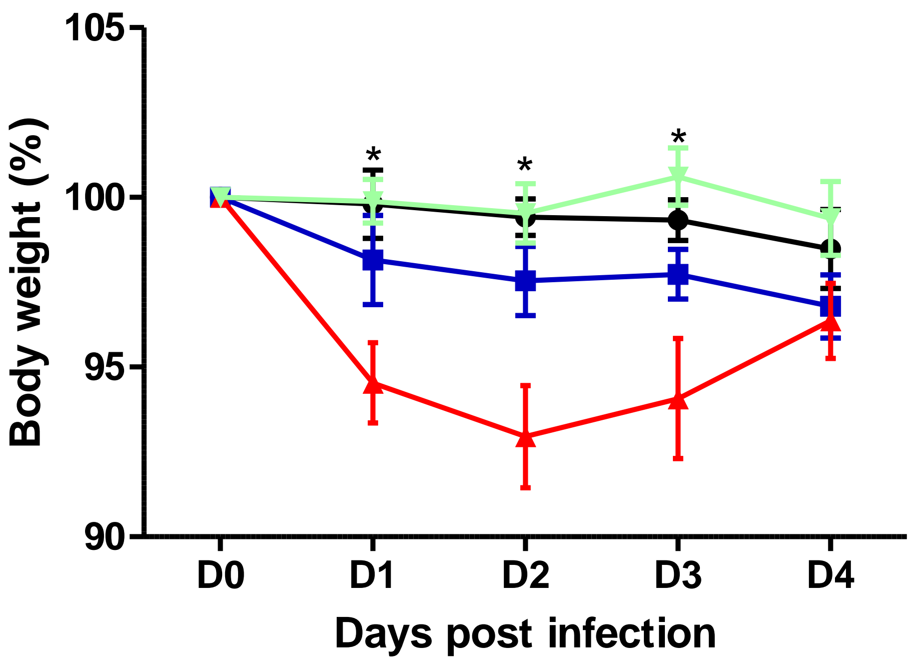



**Figure 3.1; Impact of different antibiotic pre-treatments on weight loss following infection with *C. difficile***
Mice were infected with $1x10^5$ spores of *C. difficile* BI-7 at D0. After infection mice were weighed daily during the course of the experiment and this was used as an indirect correlate of disease severity. Untreated mice (black), clindamycin treated (blue) and those treated with ABC (green) alone did not appear to lose any weight, with the average weights remaining consistent during this period. In contrast mice treated with both ABC and clindamycin (red) suffered from weight loss although some recovery was seen by day 3DPI. Error bars represent +/- SEM. (untreated n=5, ABC n=5, clindamycin n=10, ABC and clindamycin n=10). Significance was determined using a One way Anova with Tukey's correction. The untreated, ABC and clindamycin only treated groups were not significantly different from each other at any point. At 1DPI the ABC and clindamycin treated lost significantly more weight than the untreated and ABC groups (* = $p < 0.05$). At 2DPI the ABC and clindamycin treated group lost significantly more weight than all other groups (* = $p < 0.05$). At 3DPI the ABC and clindamycin group had lost significantly more weight than the ABC treated group (* = $p < 0.05$).

The weight of untreated and ABC only treated mice remained stable throughout infection with no weight loss observed. Clindamycin treated mice lost a small amount of weight but this was not significant when compared with untreated and ABC treated mice at any time point. In contrast mice treated with the ABC plus clindamycin with significantly higher weight loss seen at 1DPI (compared to

ABC and untreated), 2DPI (all other groups) and 3DPI (ABC), with recovery at 3DPI.

### 3.2.1.2 Shedding of Clostridium difficile in the faeces

To determine the extent of infection, shedding of *C. difficile* in the faeces was measured for 3 days post infection (DPI) (Figure 3.2).

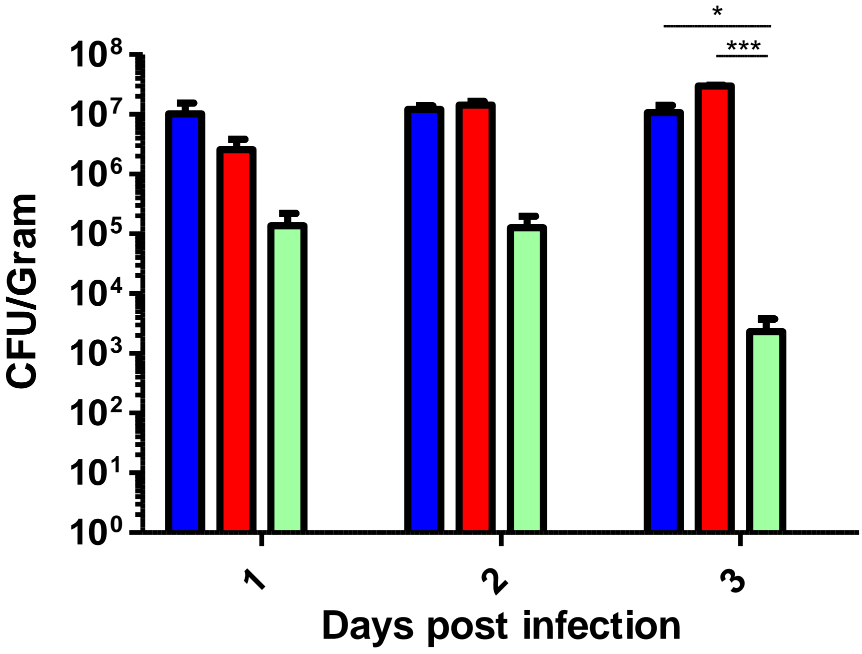



**Figure 3.2 Shedding of *C. difficile* in the faeces of mice in the first 3 days of infection**
Recovery of colony forming units (CFU) of *C. difficile* per gram of faeces from clindamycin treated (blue n=10) ABC plus clindamycin (red n=10) ABC only (green n=5) and untreated (white n=5). Mice treated with clindamycin alone or in conjunction with the ABC lost significantly more weight than those treated with the ABC alone. No organisms were recovered from the untreated mice. Error bars show the standard error of the mean (SEM). Significance determined using Welch's T-test (* indicates p = 0.0107 and ** indicates p= 0.0074).

These data reveal that in contrast to untreated mice, in which no bacteria were recovered from the faeces, samples from those treated with clindamycin or ABC plus clindamycin were highly colonised with *C. difficile*. These levels were significantly higher than those detected in faeces from animals treated with ABC alone at 3DPI. In addition, unlike the other groups, *C. difficile* colonisation in ABC only treated mice appeared to be initially lower (two logs) and was more rapidly cleared than the other two groups with very low levels ($10^3$CFU/g) of bacteria recovered.

#### 3.2.1.3 Colonisation of the caecum and colon

To determine the extent of colonisation of the gut tissue directly, mice were culled at 4DPI and the caecum and colon tissue harvested. The organisms were enumerated from either the lumen or from more intimately associated organisms recovered by homogenates of the tissue. The proportion of vegetative cells and spores were also determined as described in section 2.6.5.1 (Figure 3.3).

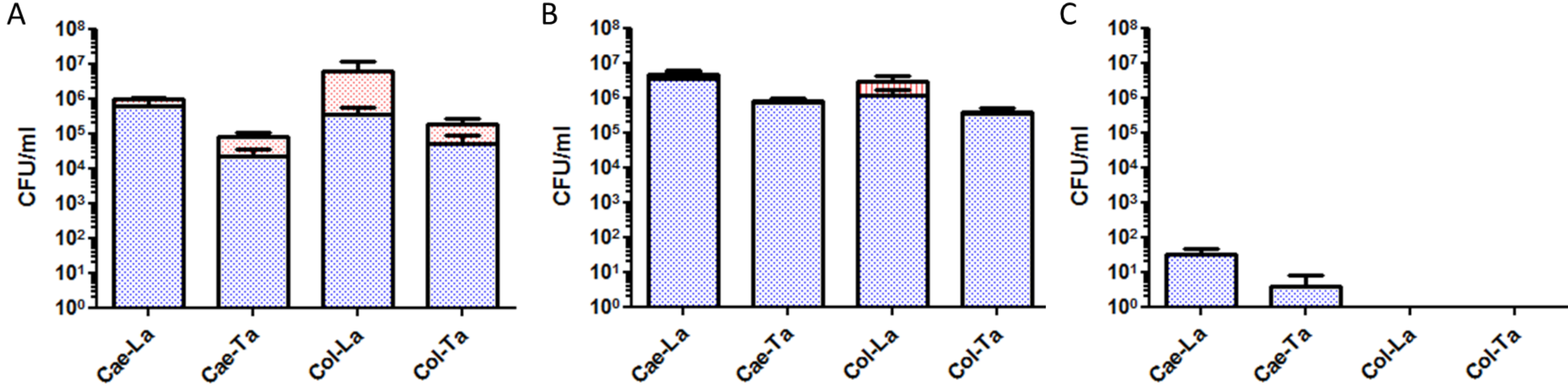



**Figure 3.3 Enumeration of *C. difficile* in the caecum and colon of infected animals**

CFU per ml of vegetative cells (blue) and spores (red) present in the caecum and colon of A) mice treated with clindamycin only (n=10) B) Antibiotic cocktail plus clindamycin (n=10) and C) ABC alone (n=5). Error bars represent +/- the SEM. No bacteria were recovered from the untreated mice. Statistical analysis was performed using Kruskal Wallis test with Dunn's comparison. This found that there were significantly more vegetative cells in the associated with the tissue in the caecum of the mice treated with the ABC and clindamycin (p= <0.5 although the impact of this is unclear.

This showed that mice treated with clindamycin alone, or, with ABC and clindamycin were highly colonised with *C. difficile* in both the caecum and colon. There was with no difference in the colonisation between these treatment groups. In contrast, no bacteria were recovered from either lumen or the tissue from untreated mice at 4DPI. Very low numbers of vegetative bacteria were recovered from the caecum of animals treated with ABC alone and no organisms recovered from the colon.

## 3.2.2 Effect of different treatment groups on mouse body weight and disease severity

### 3.2.2.1 Haematoxylin and eosin stained colon sections

To assess disease severity, tissue from the caecum and colon from mice from all treatment groups was additionally fixed, sectioned and stained with haematoxylin and eosin (H and E) to allow assessment of tissue damage. Representative images from sections of colon tissue are shown in Figure 3.4.

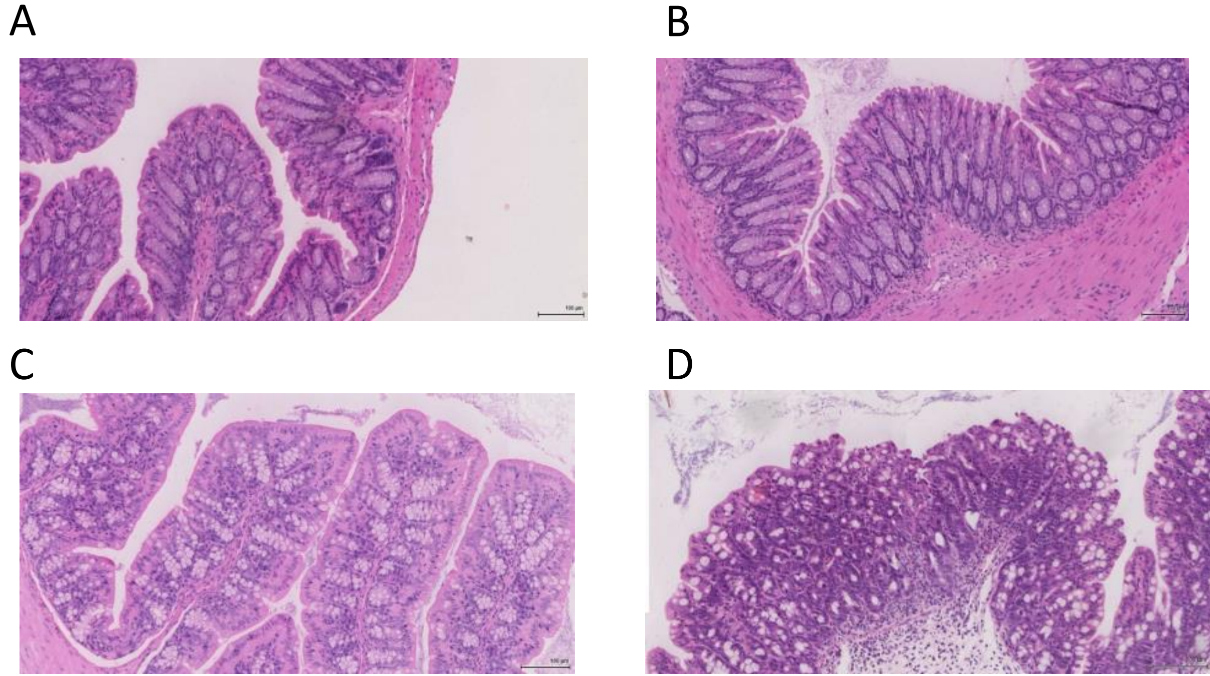



**Figure 3.4 Hemotoxylin and eosin stained colon sections from animals pre-treated with different antibiotic combinations.**

Tissue samples were taken 4DPI and immediately fixed in formalin. Sections were cut and H and E stained. Images represent typical histology in A) untreated but infected animals, those treated with clindamycin alone (B) those given ABC alone (C) and those given a combination of ABC and clindamycin. (D). Sections at 10X magnification. Scale bar in bottom right corner shows 100μm

These images reveal that whilst minimal damage to the tissue was observed in mice treated with clindamycin or ABC alone, disruption was apparent in the colon from those treated with the ABC/clindamycin combination. This includes damage to the epithelial surface and blood release into the epithelial tissue.

An alternative but effective method of assessing inflammation and tissue thickness can be provided by calculation of caecal index (CI). This is determined by assessing the proportion of the total body weight that can be attributed to the caeca. This was calculated for all animals using washed tissue recovered from animals 4DPI.

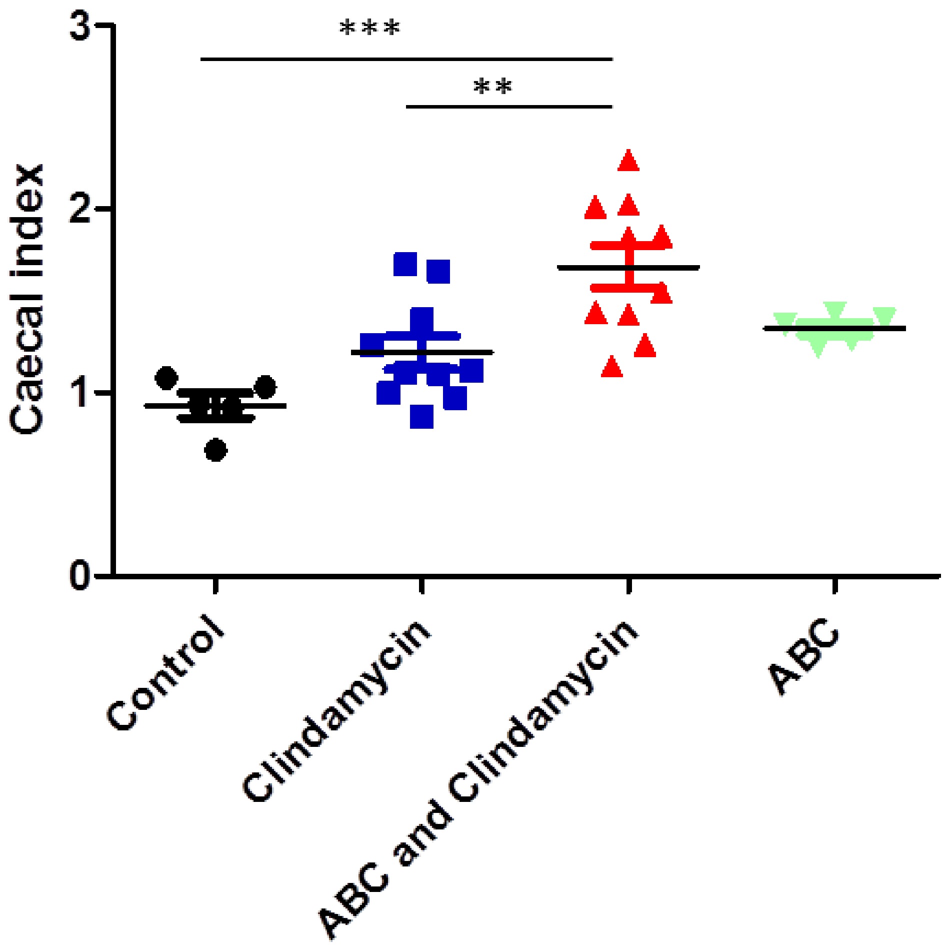



**Figure 3.5 Caecal index of mice from different treatment groups at 4DPI**
The caecal index for each animal was calculated by determining the relative proportion of the total body weight that can be attributed to the washed caecum from each animal. The CI for each animal is represented for untreated (black n=5), clindamycin alone (blue n=10), ABC/clindamycin combination (red n=10) and ABC alone (green n=5). The horizontal bar represents the mean CI for 10 animals per group. Statistical significance was determined using a one way ANOVA with Tukey's correction. Mice treated with the ABC and clindamycin had significantly higher caecal index than mice treated with clindamycin (** indicates p = < 00.1) or those that were untreated (*** indicates p < 0.0001).

These data revealed that mice that received ABC and clindamycin showed significantly higher CI than untreated animals or those given clindamycin alone. Although there are some slight differences between the other treatment groups none of these were significant. Mice treated with the ABC do have slightly elevated CI which suggests that there may be a role for the antibiotics alone in increased caecum weight. Interestingly, there is a significant increase in CI in mice treated with ABC plus clindamycin compared to clindamycin only treated animals. This suggests it is a combination of the ABC and high colonisation that

causes this increase in caecum weight which may be an indicator of inflammation

#### 3.2.2.2 Toxin levels in the caecum and colon

Tissue damage associated with *C. difficile* is largely attributed to the release and activity of TcdA and TcdB. Thus to determine if extent of damage could be attributed to differential production of toxin, luminal contents from mice were filtered and toxin activity measured. This was determined using a cell based assay using HT29 cells, which are sensitive to TcdA and Vero cells which are sensitive to TcdB (Figure 3.6). Using dilutions of these samples we were able to semi-quantify toxin activity by assessing the dilution at which toxin activity (as determined by cell rounding) was absent.

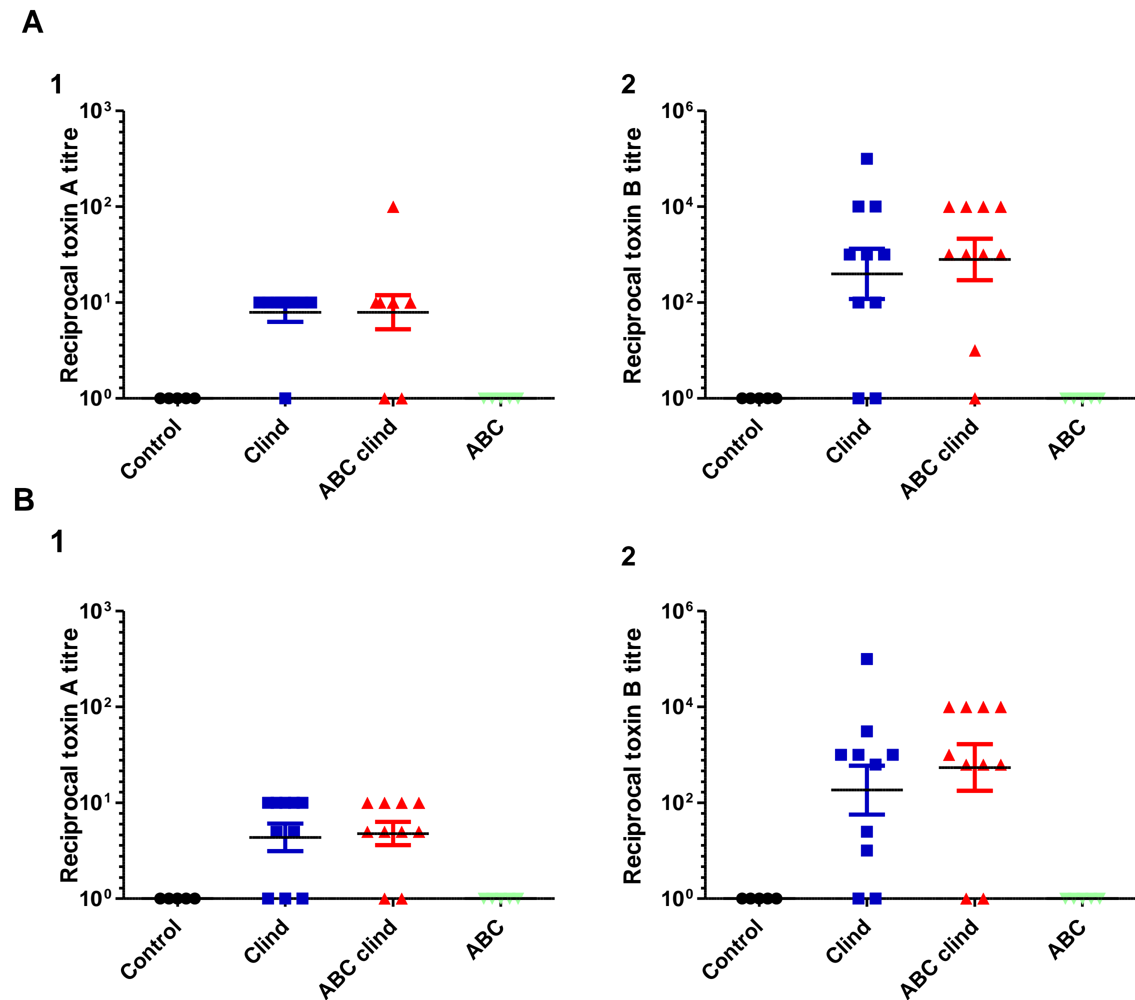



**Figure 3.6 Measurement of toxin in the caecum and colon of mice pre-treated with different combinations of antibiotics and infected with *C. difficile***

Samples were recovered from the caecum and colon of infected mice at 4DPI and were first filtered to limit bacterial contamination. Toxin titres were then calculated as the reciprocal of the first dilution of the sample that did not cause any cell rounding of HT29 cells (A1, B1) and Vero cells (A2, B2) reflecting TcdA and TcdB activity respectively. Figure 3.6.A reveals the measured activity in the caecum of animals from all 4 treatment groups, whilst Figure 3.6.B the toxin activity in the equivalent colon samples. The toxin activity measured for each animal sample is represented by a single symbol. Horizontal bars represent the mean toxin levels and error bar +/- the SEM. ABC and untreated (n=5) and ABC plus clindamycin and clindamycin (n=10). Statistical analysis was performed using Kruskal Wallis test with Dunn's multiple comparisons. This found no significant difference between clindamycin treated and ABC and clindamycin treated.

These data show that toxin activity was only detected in mice either pre-treated with clindamycin or the ABC plus clindamycin combination. This despite the fact that organisms were recovered at earlier time points from animals treated with ABC alone. In addition, the activity observed between the two groups was approximately equivalent and did not correlate with the level of tissue damage observed in animals from these different groups. In these assays, TcdA activity

was relatively low compared to TcdB. This is not unusual and reflects the differential sensitivity of these cell types to the individual toxins.

### 3.2.3 Culturable diversity of the microbiota

As microbiome diversity is known to be a major factor in *C. difficile* disease, we sought to determine whether the variety of culturable organisms varied between the different treatment groups. To facilitate this assessment, samples were taken from 2 animals from each treatment group and cultured to determine the diversity of the microbiota. This assessment was based on the number of unique colony morphologies that could be recovered from different types of agar/growth conditions used. Bacteria with unique colony morphologies were isolated and analysed further. In total 6 agar types were used both anaerobic and aerobic conditions. The use of this range of media types was to increase the diversity of organism recovered from the samples. This included multiple media types for the isolation of anaerobic bacteria including WCA and FAB. Several high nutrient media types including LB, BHI and CBM were used to enable the isolation of fastidious organisms. The final media type, MRS, was to aid in the isolation of *Lactobacillus* species. The identity of individual recovered bacteria was confirmed by amplification and analysis of a section of the 16SrRNA gene sequence. Although this is a very crude measure of diversity it does show the influence of different antibiotics upon the culturable members of the microbiota. These data confirmed that the greatest diversity of organisms were isolated from untreated animals. In contrast, several aerobic and anaerobic colony types were lost from animals treated with clindamycin and the ABC/clindamycin combination. Animals from both treatment groups had a >50% reduction in the number of unique colony types present after antibiotic treatment. In total 18 species were identified (Table 3-1) and retained for use in future experiments.

**Table 3-1 Identification of bacteria isolated from mouse faeces**

| Family | Isolate | Isolation conditions |
|---|---|---|
| **Dermacoccaceae** | *Dermococcus* | Aerobic |
| **Enterobacteriaceae** | *Citrobacter* | Anaerobic |
| | *E. fergusonii, Shigella sonnei or E.coli* | Anaerobic |
| | *E. fergusonii, S. sonnei or E.coli* | Anaerobic |
| **Enterococcaceae** | *Enterococcus faecium* | Aerobic |
| | *Enterococcus casseliflavus* | Aerobic |
| | *Enterococcus* | Anaerobic |
| | *Enterococcus* | Anaerobic |
| **Lactobacillaceae** | *Lactobacillus apodemi* | Aerobic |
| | *L. apodemi* | Anaerobic |
| **Micrococcaceae** | *Kocuria rhizophila* | Aerobic |
| | *K. rhizophila* | Aerobic |
| | *Micrococcus luteus* | Aerobic |
| **Pseudomonadale** | *Enhydrobacter or Moraxella* | Aerobic |
| **Staphylococcace** | *Staphylococcus lentus* | Aerobic |
| | *Staphylococcus warneri* | Aerobic |
| | *S. warneri* | Aerobic |
| | *S. warneri* | Aerobic |

### 3.2.4 Molecular assessment of microbiome diversity

As not all members of the microbiome can be directly cultured, the impact of antibiotic pre-treatment was further assessed by the amplification and analysis of the V4 region of the 16s rRNA gene. To determine the temporal changes in bacterial species, samples were taken from all animals in each group prior to antibiotic treatment (D-6), following 2 days of ABC treatment (D-3), post ABC and pre clindamycin (D-2), post clindamycin and pre-infection (D0) and at 3DPI

(D3). This allowed for comparisons of community structure throughout the experiment.

#### 3.2.4.1 Measurement of alpha diversity

By examining alpha diversity it is possible to determine changes in OTU diversity within each treatment group. This allows the impact of the different regimes on bacterial diversity to be determined. Here 3 measures of alpha diversity were used, Observed, Simpson and Shannon. The Observed diversity simply shows changes in the number of different OTUs present within samples but does not take into account the relative abundance of these individual OTUs. Both Simpson and Shannon measures of diversity take into account the abundance of individual OTUs. The effects of the different antibiotic treatments are reflected in the changes to alpha diversity shown in Figure 3.7.

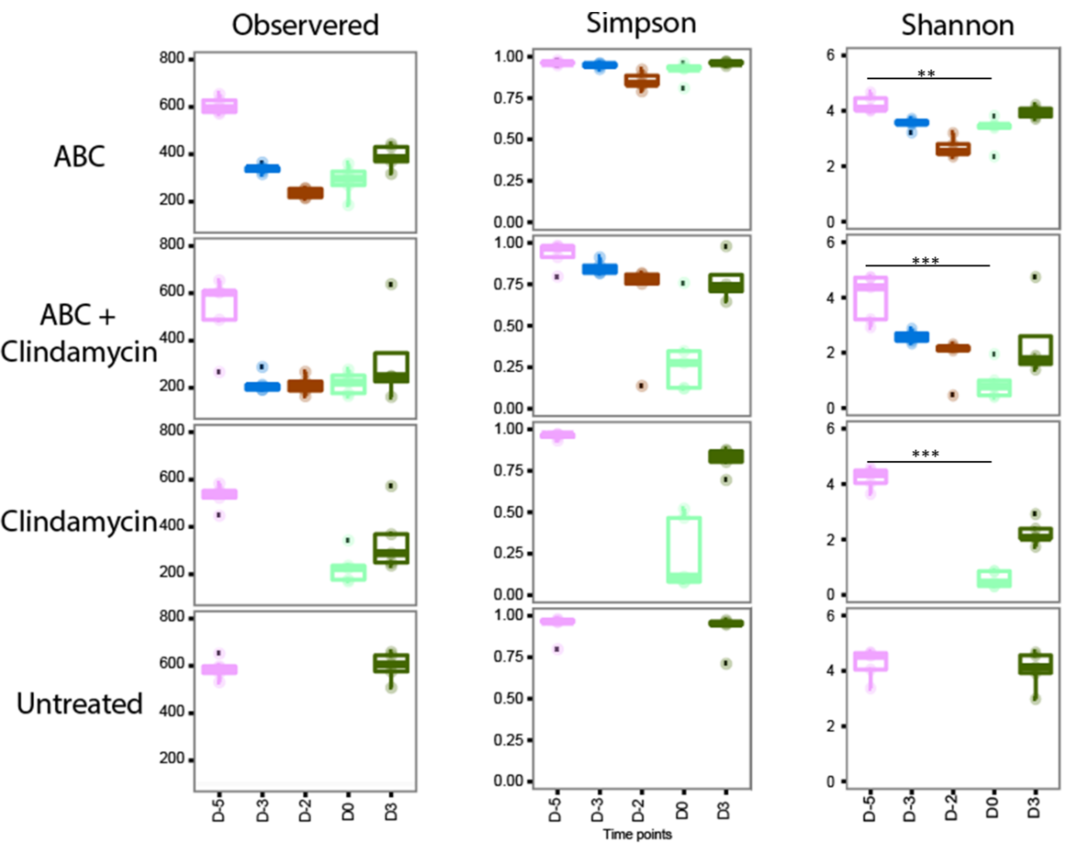



**Figure 3.7 Alpha diversity in the microbiota of mice from all treatment groups over antibiotic treatment and infection**
This reveals 3 different approaches to the measurement of alpha diversity following different antibiotic treatments. The Observed gives the number of OTUs present in the sample at each time point and only takes into account species number, both Simpson and Shannon indexes use OTU number and the abundance of OTUs (calculated using Phyloseq 's plot_richness() function). Each time point is represented by a box and whisker plot showing the minimum, maximum and mean values. Comparing mice treated with any of the antibiotic regimes at D-5 to the same mice at D0 show significantly lower Shannon diversity indexes compared to mice before the treatment. No sample is available at D0 for the untreated animals but there is no difference in diversity between D-5 and D3.

All antibiotic treatments resulted in a decline in the number of observed OTUs recovered. Use of ABC alone led to a transient reduction in the observed OTU number with the recovery of diversity seen immediately after of antibiotic treatment. Animals given clindamycin alone or the ABC/clindamycin combination suffered from dramatic loss of diversity that persisted until 3DPI with only minor recovery in the observed numbers of OTUs. This suggests that the most dramatic and persistent changes in community structure were due to the use of clindamycin.

Although useful, observed diversity does not take into account the relative proportion of OTUs within the microbiota so gives only a limited view. Both Simpson and Shannon take into account not just the number of different OTUs but also the relative abundance of these OTUs. A low Simpson or Shannon diversity measure signifies overrepresentation and unequal distribution of OTUs within the microbiota. In animals treated with the ABC alone there is a reduction in the Simpson and Shannon measures of diversity. This means that although there was a reduction in OTU numbers these were still fairly evenly distributed. However, statistical analysis of Shannon measures of diversity showed that treatment with the ABC results in a significant decrease in this measure ($p = 0.009$). However, clindamycin treatment results in an even more dramatic reduction in both Simpson and Shannon measures of diversity. Clindamycin treatment either alone ($p = 1x10^{-7}$) or in conjunction with the ABC ($p = 2x10^{-4}$) results in a significant reduction in Shannon diversity index. This significant reduction represents overrepresentation of a subset of OTUs. Examination of the Taxa plots confirms this finding as after clindamycin treatment there is a bloom of Enterobacteriaceae (Figure 3.10 and Figure 3.12). In both groups of animals receiving clindamycin the majority of the sequences identified within the sample belonged to OTU_1, *E. coli*. This highlights the importance of examining multiple measures of diversity when considering microbiome data as the observed diversity alone would not highlighted such differences. Untreated animals show no changes in any measure of alpha diversity in samples taken at the beginning and end of the experiment. Sadly, no other samples were taken during the course of this experiment so no information is available about changes in diversity at the time of infection.

#### 3.2.4.2 Measurement of beta diversity

Comparisons of alpha diversity give an indication of how the different antibiotic regimes alter diversity within a treatment group. However, they offer no direct comparison of the bacterial community structures between treatment groups. Unlike alpha diversity, beta diversity allows comparison between treatment groups rather than within treatment groups. Figure 3.8 shows PcoA plots using Weighted Unifrac measurement of beta diversity. Weighted Unifrac uses a combination of OTU abundance and phylogenetic distance to group samples. Before treatment at D-5 there is significant overlap between each of the treatment groups suggesting that the structure of each of the mouse microbiotas is similar at the start of the experiment. At D-3 post antibiotic treatment there is clear grouping of the treatment groups suggesting that there are unique community structures associated with each. There is significant overlap between animals treated with ABC plus clindamycin and clindamycin alone however there appears to be greater variation in animals receiving both the ABC and clindamycin.

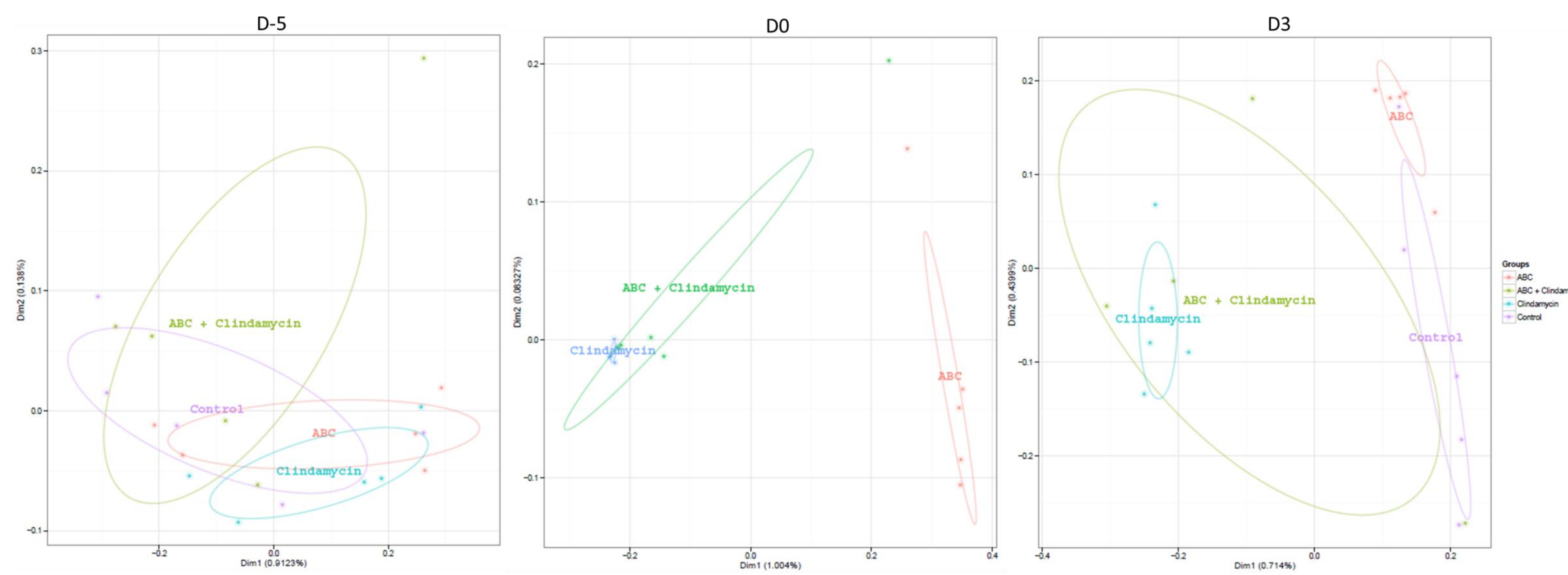



**Figure 3.8 Principle Co-ordinate analysis plots of all treatment groups at D-5, D0 and D3**
Samples are grouped according to treatment groups (Untreated, purple; clindamycin alone, blue, ABC plus clindamycin combination, green and ABC alone, red) using mean ordination values and spread of points. At D-5, before infection and antibiotic treatment, mice from all groups overlap suggesting similar structure of the microbiota at the beginning of the experiment. At D0, after the different antibiotic treatments the treatment groups begin to cluster together (There is no untreated sample at this time point). The ABC only treated group cluster separate from the other groups. Unsurprisingly the ABC plus clindamycin and clindamycin only treated groups overlap, although there is more variation in the ABC plus clindamycin group. At D3 the clear groupings are still clear although clustering is less tight than seen at D0, this is likely due to recovery and increased diversity of the microbiota at this time point.

### 3.2.5 Taxa plots of abundant OTUs

Comparisons of both alpha and beta diversity showed significant changes in the abundance and diversity of species present in the microbiota (Figure 3.7 and Figure 3.8). To more closely examine the specific changes associated with individual taxa plots of the most abundant phyla at each time point were constructed (Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12). These plots allow for easy visualisation of changes in the community structure.

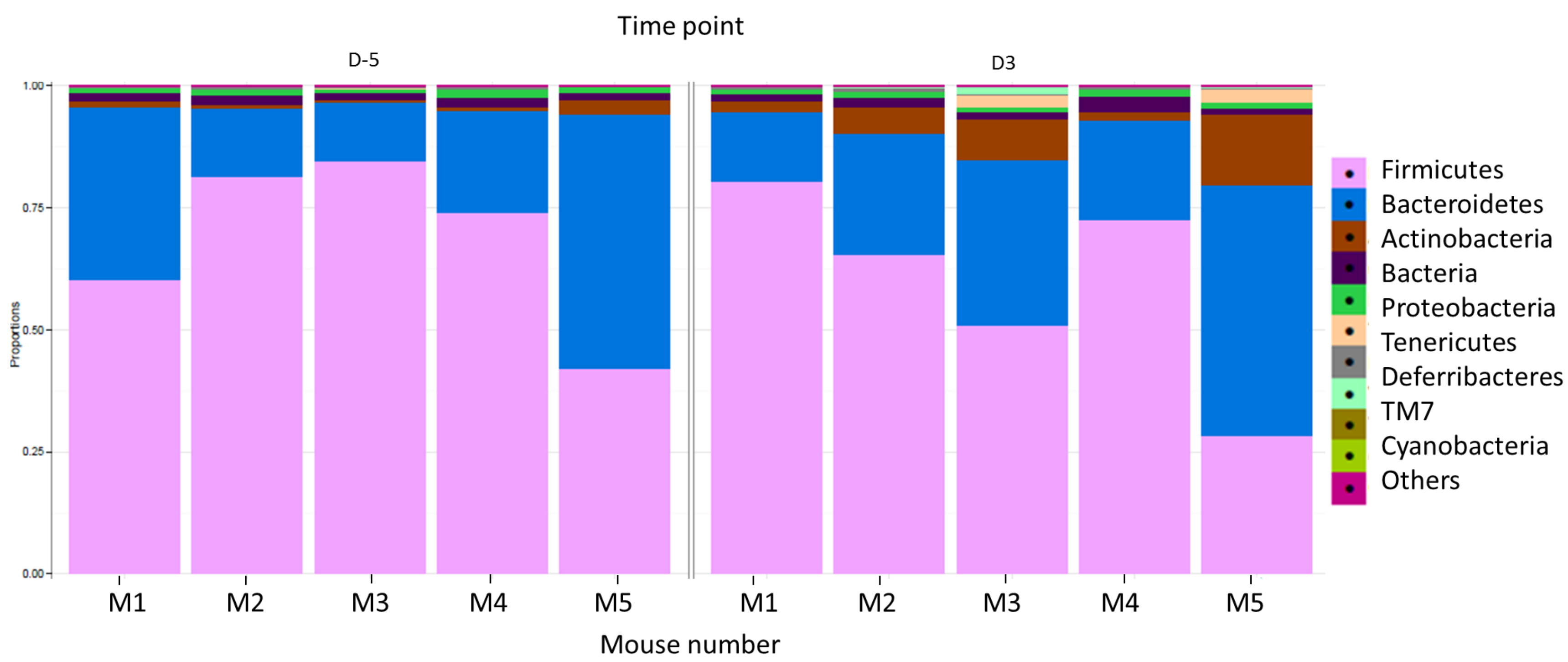



**Figure 3.9 Taxa plots of the most abundant phyla in untreated and infected mice**
Stacked bar plot of the most abundant phyla present at the start (D-5) and end (D3) of the experiment with all sampling points are displayed. Each bar plot represents an individual mouse denoted by the number at the base of the bar plot. Diversity and species richness remain throughout the course of the experiment. In these animals the most abundant phyla are represented by Firmicutes and Bacteroidetes with these phyla representing the majority of sequences recovered. Infection with *C. difficile* in the absence of antibiotic pre-treatment does not seem to have any impact the structure of the microbiota as it remains very similar to the initial sampling point.

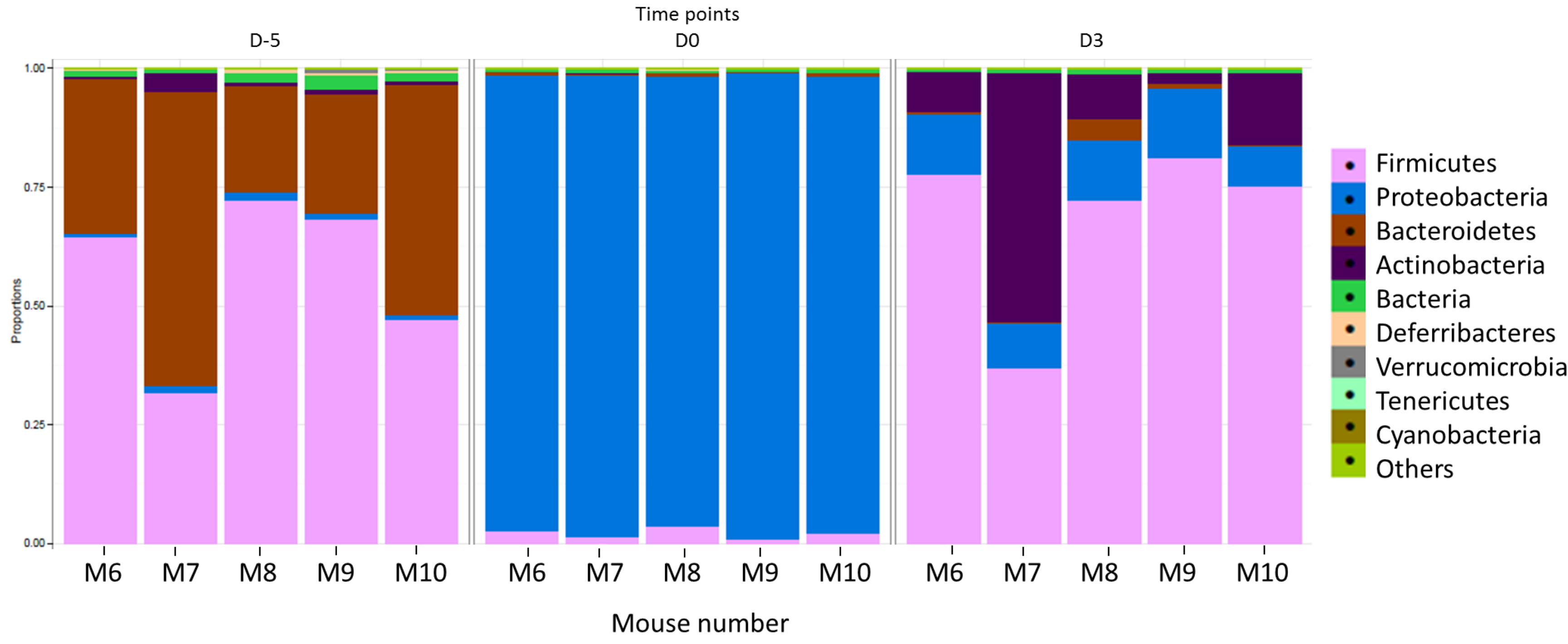



**Figure 3.10 Taxa plots showing the most abundant phyla identified in mice treated with Clindamycin alone**
Stacked bar plot of the most abundant phyla present at all sampling points are displayed. Each bar plot represents an individual mouse denoted by the number at the base of the bar plot. This reveals that clindamycin alters the community structure of the gut with a shift from a community dominated by Firmicutes and Bacteroidetes to one inhabited almost exclusively by Proteobacteria. Closer inspection show of this data shows that at D0 the majority of Proteobacteria sequences recovered belong to 2 OTUs both identified as Enterobacteriaceae. At D3 some recovery is present with an increase in the number of Firmicutes. However, a large proportion of the Firmicute sequences are derived from *Clostridium difficile* (OTU_13).

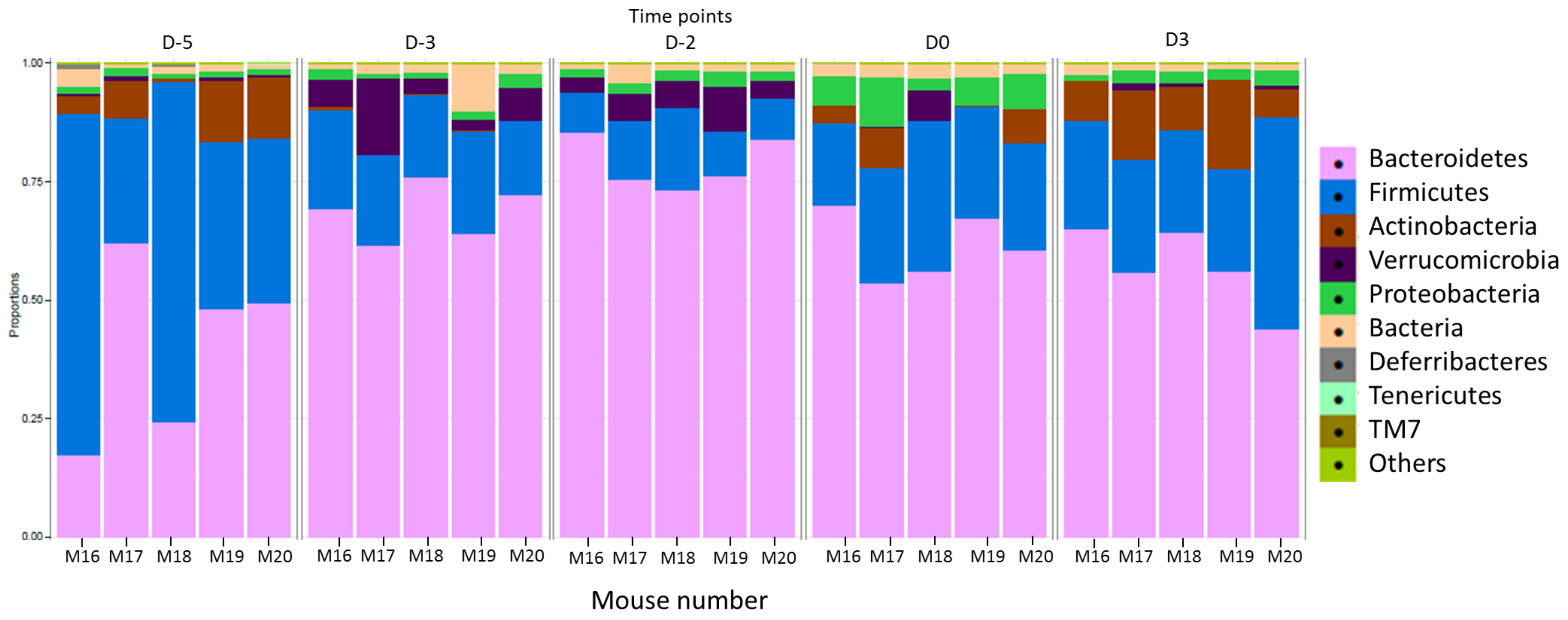



**Figure 3.11 Taxa plot of the most abundant phyla in mice treated with ABC alone**
The 20 most abundant phyla present at all sampling points are displayed. Each bar plot represents an individual mouse denoted by the number at the base of the bar plot. Treatment with the ABC causes changes to the community structure with increases in the proportion of Bacteroidetes to Firmicutes. The proportions start to resolve from D0. Treatment with the ABC does not cause sufficient changes to the microbiota to allow infection with *C. difficile.*

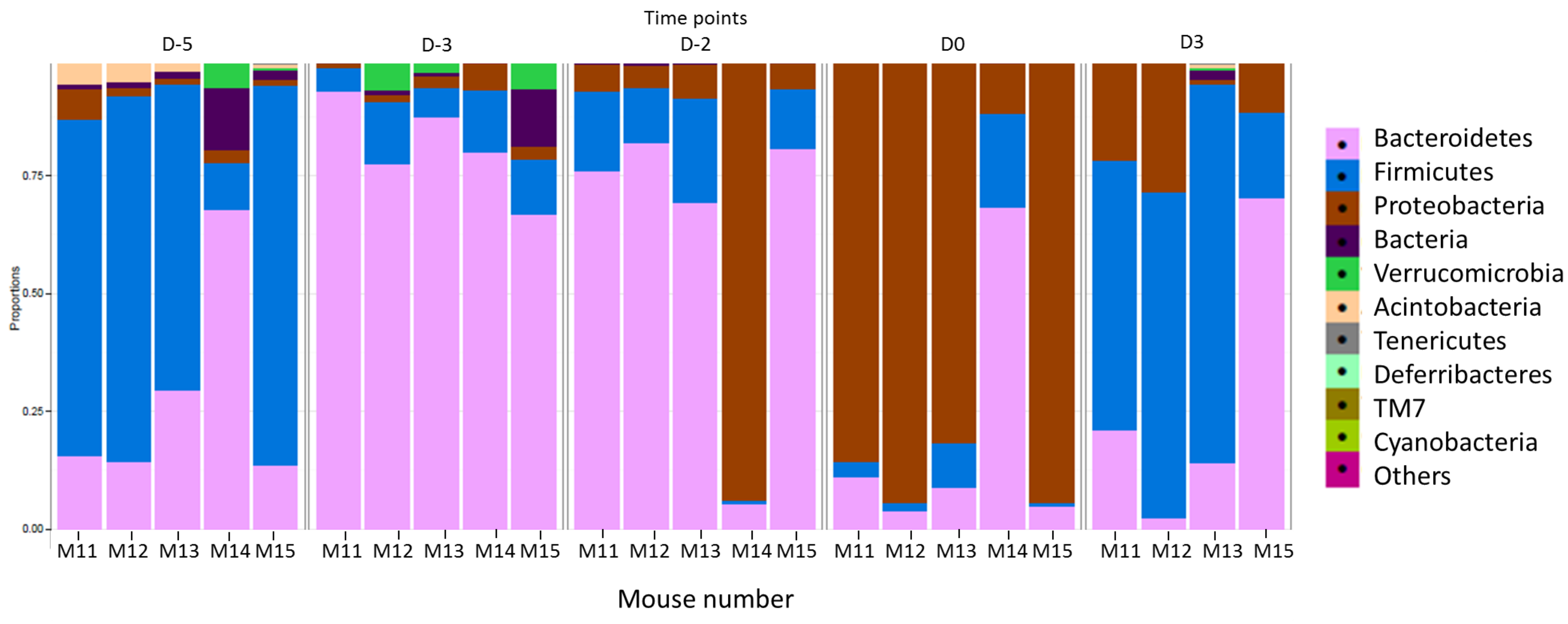



**Figure 3.12 Taxa plot of the most abundant phyla in mice treated with the combination of ABC and clindamycin.**
The most abundant phyla present at all sampling points are displayed. Each bar plot represents an individual mouse denoted by the number at the base of the bar plot. As was seen in ABC treated mice the ABC altered the ratio of Bacteroidetes to Firmicutes. Subsequent clindamycin treatment has a dramatic impact on diversity with large blooms of Proteobacteria. At D3 there is some recovery with decreases in the number of Proteobacteria. Closer inspection revels that a large proportion of the sequences at D3 are derived from *Clostridium difficile* (OTU_13).

These data reveal that whilst there is some variation between sampling points, the microbiota of untreated animals remained diverse (Figure 3.9). In contrast, animals given a single dose of clindamycin either alone or in combination with the ABC showed significant changes in community structure, with huge increases in Proteobacteria, found to represent 2 OTUs (Figure 3.10 and Figure 3.12). Treatment with the ABC alone results in an increase of OTUs associated with a higher proportion of Bacteroidetes to Firmicutes than is seen in mice before treatment (Figure 3.11 and Figure 3.12). In both groups in which clindamycin is used, OTU 13 (*C. difficile*) is one of the dominant OTUs present. In contrast, OTU 13 is not present in untreated animals or those given ABC alone, supporting the culture based data as *C. difficile* was not detected at high levels in these animals at 4DPI.

Please note that the colours representing specific phyla vary on each figure presented.

### 3.2.6 Tax4Fun assessment of metabolic potential of the microbiome

Whilst OTU abundance information is useful for determining which species are present within a sample and can show how antibiotics influence community structure, it provides limited insight into the functional changes within the microbiome. As functional capacity is more highly conserved than community structure (Figure 1.10), it is likely that significant changes to this may be important in infection. The use of new programs that allow the functional capacity of the microbiome to be evaluated using just 16s data are becoming popular. One such bioinformatics pipeline is called Tax4fun (Abhauer *et al.*, 2015). Application of this method allows the metabolic potential of a sample to be inferred through the use of reference genomes to indicate the potential metabolic activity of the OTUs identified within the sample. However, as many of the OTUs identified within these data sets are only resolved to genus level there will be limitations to this method. Although there are potential issues with these methods they can significantly increases the usefulness of these datasets as it helps to identify potential metabolic changes that may be important in the context of colonisation and infection of *C. difficile*.

#### 3.2.6.1 Abundance of BSH during antibiotic treatment

Due to the importance of primary bile salts in *C. difficile* spore germination, investigation of the microbiota was initially focused on determining the impact of antibiotic treatment on those organisms that express BSH and 7α-dehydroxylase enzymes. These two enzymes are involved in bile metabolism and have been proposed as potentially important enzymes in colonisation resistance due to their ability to modify BAs (1.3.2). Attempts were made to measure the abundance of both BSH and 7α-dehydroxylation enzymes within the Tax4fun datasets in the different treatment groups. The 7α-dehydroxylase enzymes were not found within the data sets although this is most likely due to poor annotation of these enzymes in reference genomes rather than actual absence. The abundance of BSH enzymes before and after antibiotic treatment was initially determined using data generated from Tax4fun analysis. As BSH are the first enzyme to act upon BAs during modification they were used as a proxy for the presence or absence of the pathway as these enzymes have to act to enable further BA modification (Figure 3.13).

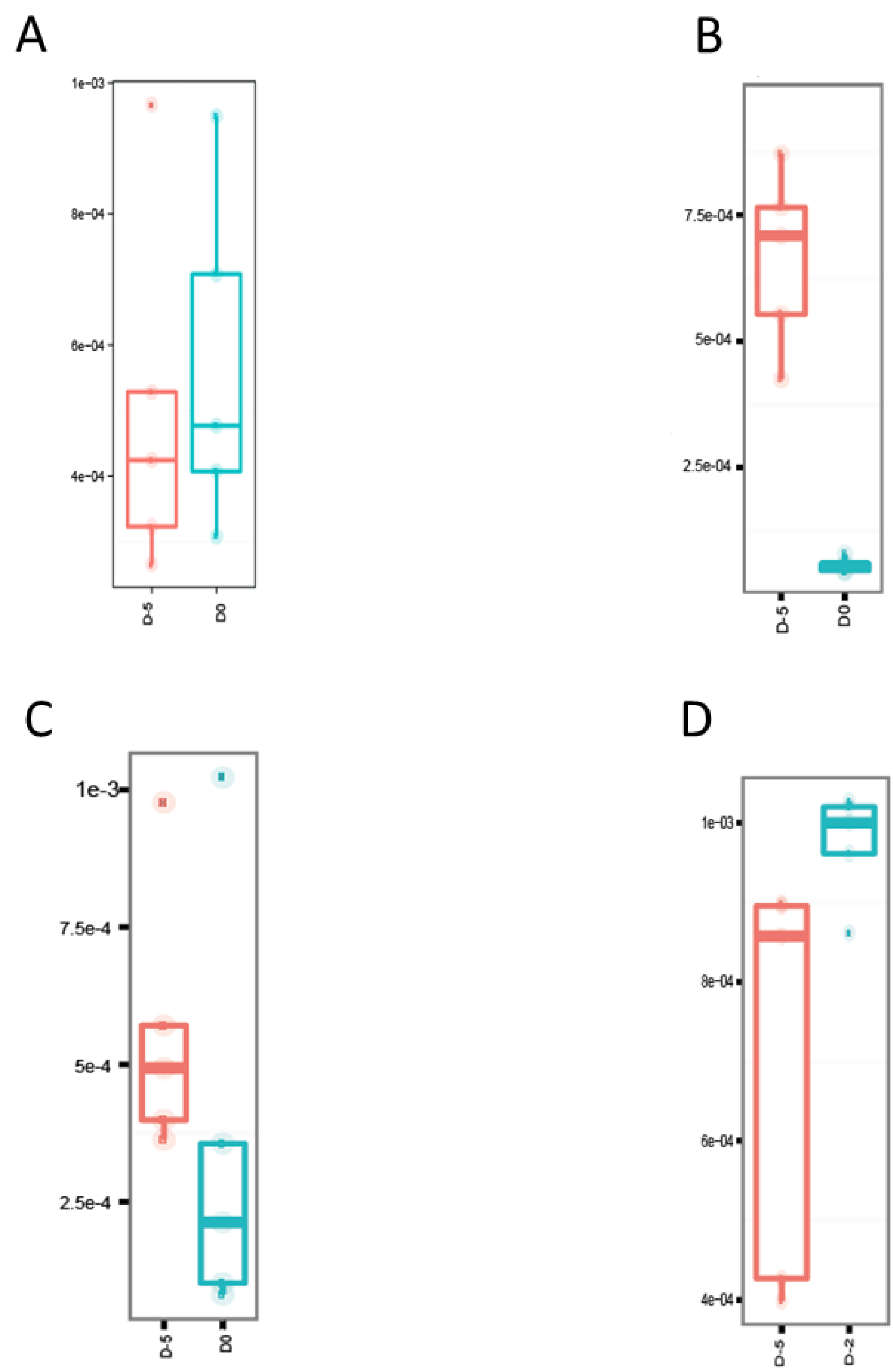



**Figure 3.13 Tax4fun assessment of BSH abundance in mice from different treatment groups** Kruskal-Walis test was used to compare the abundance of BSH in the microbiome before and after antibiotic treatment. A) In untreated animals there was no change in the abundance of these enzymes. B) After clindamycin treatment there was a significant decrease (p= 5.9e-5) in BSH abundance. C) In ABC plus clindamycin animals there was a decrease after clindamycin treatment but this is not significant. D) In animals treated with the ABC alone there was a significant (p= 0.048) increase in BSH abundance.

Using this approach, it was observed that BSH producing species showed significantly lower representation after use of clindamycin. Animals treated with

the ABC/clindamycin combination also have the same decrease after clindamycin is administered, although this is not significant. Use of the ABC alone results in an increase in the abundance of BSH enzymes which may explain why animals given this treatment were only transiently colonised. Untreated animals have no change in the abundance of BSH suggesting that this function is normally stable within the microbiome.

### 3.2.7 Impact of antibiotic treatment on presence of BSH enzymes and bile metabolism

As Tax4fun analysis showed significant changes to the abundance of BSH enzymes within the different antibiotic treatment groups (Figure 3.13) we used PCR amplification to determine if these enzymes could be directly detected in the microbiome of animals. As the BSH genes from different bacterial species have very divergent nucleotide sequences, CODEHOP primers that allow for the amplification of multiple BSH genes using one set of primers were used. The primers used to investigate these enzymes were designed in this manner and were a kind gift from Prof Julian Marchesi (CU) (Figure 3.14).

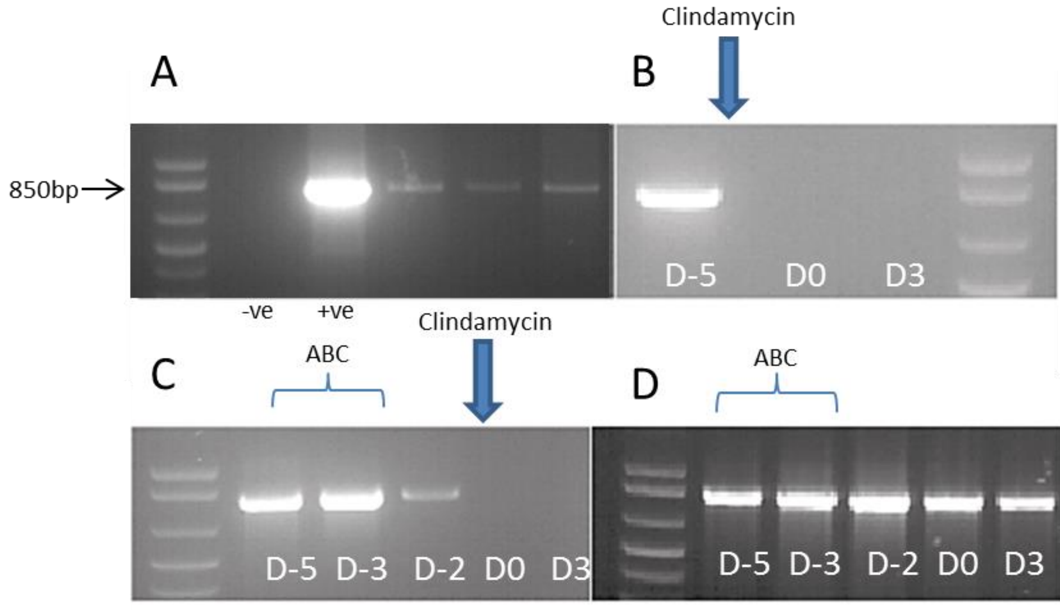



**Figure 3.14 Detection of BSH genes in the microbiome of mice treated with different antibiotic regimens**
BSH sequences were amplified using specific primers from microbiome samples collected from animals subject to different antibiotic treatment. Timing of ABC and clindamycin treatment is indicated in the figure. Each gel represents the typical amplified bands from untreated animals (A), those treated with clindamycin alone (B) those treated with a combination of ABC and clindamycin (C) and those given ABC alone (D).

The PCR results from this analysis confirmed that BSH genes could be amplified in all samples taken from untreated animals. Similarly, BSH could also be amplified in samples taken from animals treated with ABC. Conversely, there appeared to be a loss of BSH producing bacteria following treatment with clindamycin, either when used alone or in combination with ABC. Interestingly, animals in which the BSH genes could not be amplified were highly colonised whilst those in which BSH were detected were only transiently colonised (ABC) or not colonised at all (untreated group). These results support the initial Tax4fun analysis approach giving confidence to this approach in inferring metabolic potential. This additionally supports the idea that clindamycin is responsible for the loss of colonisation resistance and that this is mediated in part by bacteria involved in bile metabolism.

### 3.2.8 TOF-MS analysis of bile salts in the caecum

BSH activity represents the gateway reaction in BA metabolism with deconjugation of the amino acid allowing for further modification. As both the PCR amplification and Tax4fun analysis revealed that clindamycin treatment reduced BSH abundance, we wished to determine if these changes correlated with changes in the abundance of primary bile salts in the gut. This was evaluated using time of TOF-MS analysis on caecal content samples from all groups taken at 4DPI. The relative abundance of TC to DCA was then compared and revealed that higher levels of TC were present in their caeca of animals treated with clindamycin (either alone or in combination with ABC). In contrast, untreated animals had higher levels of deoxycholic acid in these samples (Figure 3.15-A, B). Direct comparison of samples from clindamycin only or ABC plus clindamycin showed no differences in the abundance of these bile salts (Figure 3.15-C). Investigation of the abundance of specific BAs in ABC only treated animals is underway but the data has not yet been received.

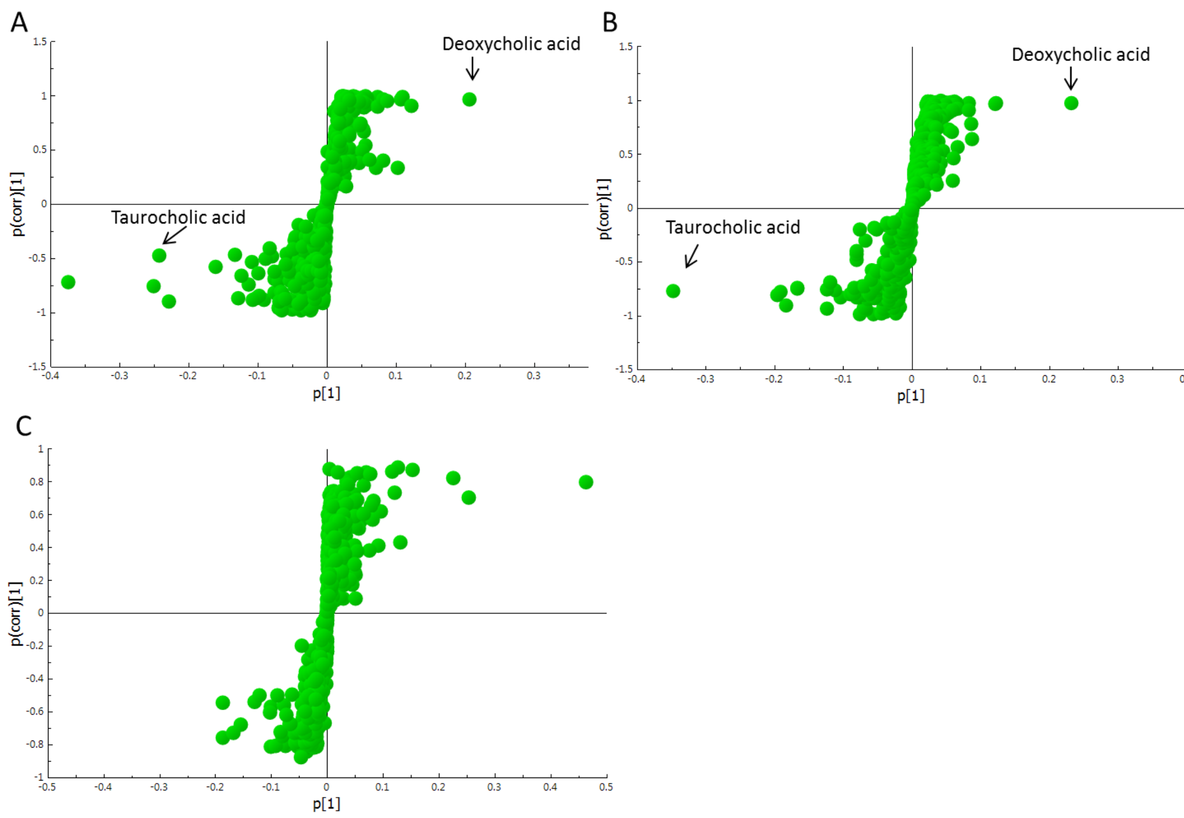



**Figure 3.15 Scatter plots of PLS-DA of faecal ions from mice in different treatment groups**

Each point represents a single faecal ion with ions identified as TC and DCA labelled. The p(corr)[1] p values show the differences between the groups and the p[1] values represent the abundance of ions A) Comparison of untreated animals and those given clindamycin. B) Comparison of untreated animals and those given ABC plus clindamycin C) Comparison of animals given clindamycin alone or ABC plus clindamycin. Clindamycin treatment, either alone or with ABC resulted in significantly higher levels of TC compared to untreated mice. Untreated mice had significantly higher levels of DCA compared to both clindamycin and ABC plus clindamycin treated animals. Clindamycin treated and ABC plus clindamycin treated animals showed no difference in the abundance of TC or DCA.

These data suggest that clindamycin treatment results in a loss of bacterial bile metabolism from the microbiome and that BSH can be used as a marker of this change. Comparison of DCA and TC abundance between clindamycin treated and ABC/clindamycin treated mice did not show any differences so it may be that whilst bile metabolism is important in colonisation resistance, it does not explain the enhanced tissue damage and weight loss observed when animals are given a combination of ABC and clindamycin.

### 3.2.9 Comparison of community structure before and after clindamycin treatment

To determine how the different antibiotic treatments may impact upon the microbiome it is important to compare the structure of the microbiota before and after the different treatments. Such comparison of the community structure before and after treatment with clindamycin showed that 120 OTUs were significantly altered in abundance by use of this antibiotic. In total, 8 OTUs (Table 3-2) were found to be increased after clindamycin with 102 significantly reduced (Table 3-3).

**Table 3-2; Comparatively increased OTUs following clindamycin treatment**
DESeqDataSetFromMatrix() function from DESeq package with a significance value cut-off of 0.001. This allows negative binomial GLM fitting and Wald statistics. Multiple testing corrections using (Benjamini and Hochberg 1995) to reduce false discovery rate.

| OTU | Adjusted P value |
|---|---|
| OTU_1 *Escherichia coli* | 4.3031e−237 |
| OTU_21 Enterobacteriaceae | 2.2604e−20 |
| OTU_233 *Lactococcus* | 1.1169e−16 |
| OTU_558 Enterobacteriaceae | 1.0536e−11 |
| OTU_547 *Escherichia/Shigella* | 2.4532e−10 |
| OTU_600 *Escherichia/Shigella* | 2.7872e−09 |
| OTU_781 *Escherichia/Shigella* | 7.5277e−09 |
| OTU_460 *Mannheimia* | 5.1012e−07 |

Of the 8 increased OTUs there are 3 that are identified as *Escherichia/Shigella* (OTU 547, 600 and 781). In addition to this OTU_1, *E. coli*, is shown to be the

most significantly increased confirming the bloom of Enterobacteriaceae following clindamycin treatment that was identified in the Taxa plots.

In total 102 OTUs were decreased after clindamycin treatment. For simplicity these have been assigned to bacterial families as several were represented by multiple OTUs.

**Table 3-3; Comparatively decreased OTUs following clindamycin treatment**
DESeqDataSetFromMatrix() function from DESeq package with a significance value cut-off of 0.001. This allows negative binomial GLM fitting and Wald statistics. Multiple testing corrections using (Benjamini and Hochberg 1995) to reduce false discovery rate.

| OTU | Adjusted P value |
|---|---|
| 5 Bacteroidales | 4.6671e−20 - 4.6216e−05 |
| 18 Porphyromonadaceae | 9.8556e−15 - 4.7587e−05 |
| 2 *Flavonifractor* | 1.1115e−11 - 5.1202e−05 |
| OTU_92 *Clostridium*_XlVb | 2.8024e−11 |
| 45 Lachnospiraceae | 2.6526e−10 - 8.8107e−05 |
| 3 *Oscillibacter* | 9.4232e−11 - 2.4789e−06 |
| 5 *Barnesiella* | 2.751e−10 - 3.6031e−06 |
| 10 Ruminococcaceae | 6.1919e−10 - 7.2647e−06 |
| 10 Clostridiales | 1.6439e−09 - 9.9022e−05 |
| 2 Bacteroidetes | 2.7872e−09 - 1.9396e−06 |
| OTU_50 *Alistipes* | 1.1706e−08 |
| OTU_18 Prevotellaceae | 4.7381e−08 |
| OTU_183 Clostridia | 9.4571e−08 |
| 3 Unclassified Bacteria | 4.8608e−07 - 1.4466e−05 |
| OTU_44 *Bacteroides acidifaciens* | 1.0783e−06 |
| OTU_69 *Clostridium*_XlVa | 2.477e−06 |
| 2 *Odoribacter* | 7.1769e−06 - 6.1494e−05 |
| OTU_5 *Allobaculum* | 9.1968e−06 |

Large numbers of OTUs were significantly decreased after clindamycin treatment which would be expected as it is a broad spectrum antibiotic. Closer observation of this data shows that huge numbers of the OTUs belong to the class of bacteria identified as Clostridia. This includes OTUs assigned as Lachnospiraceae, with

this family of bacteria the most affected by clindamycin treatment with 45 OTUs decreased after clindamycin treatment. Multiple Bacteroidetes were also decreased including OTU_44 *Bacteroides acidifaciens*, a known BSH producer. Other strains known to produce BSHs that were impacted were members of the Clostridia and Clostridiales. Specifically there was a decrease in OTU_69 a *Clostridium* XIVa, species in this cluster are known to produce 7α-dehydroxylase, suggesting that these enzymes may also be lost from the microbiome post clindamycin. A combination of microbiome, PCR, TOF-MS and Tax4fun data suggest that bacteria involved in bile metabolism are important in mediating colonisation resistance.

### 3.2.10 Comparison of significantly altered OTUs in colonisation and severe model

Although important in colonisation resistance, changes in BA metabolism seem to play no role in differences in disease severity. However, modification of the flora may be responsible by altering other functions that contribute to gut health. To determine if these changes contribute to disease severity, the organisms identified in samples from clindamycin alone and ABC plus clindamycin treated animals were compared. This revealed that 34 OTUs were significantly different between the two treatment groups, with 24 (Table 3-4)

**Table 3-4; Comparative increased OTUs in ABC plus clindamycin treatment group**
DESeqDataSetFromMatrix() function from DESeq package with a significance value cut-off of 0.001. This allows negative binomial GLM fitting and Wald statistics. Multiple testing corrections using Benjamini Hochberg.

| OTU | Adjusted P value |
|---|---|
| OTU_780 *Parabacteroides* | 1.77E-18 |
| OTU_ 11 *Parabacteroides goldsteinii* | 2.55E-18 |
| OTU_ 3 *Parabacteroides distasonis* | 2.51E-15 |
| OTU_ 2 *Bacteroides thetaiotaomicron* | 1.76E-14 |
| OTU_ 784 *Parabacteroides* | 9.94E-14 |
| OTU_772 *Parabacteroides* | 3.52E-13 |
| OTU_34 Firmicutes | 1.18E-10 |
| OTU_812 *Parabacteroides* | 2.92E-09 |
| OTU_ 22 *Erysipelotrichaceae* bacterium Alo17 | 7.83E-09 |
| OTU_850 *Parabacteroides* | 2.63E-08 |
| OTU_468 Bacteroides | 2.92E-08 |
| OTU_24 *Bacteroides intestinalis* | 3.07E-08 |
| OTU_7 *Enterococcus* | 1.15E-07 |
| OTU_5 *Allobaculum* | 1.25E-07 |
| OTU_10 *Barnesiella* | 3.44E-07 |
| OTU_122 Unclassified bacteria | 2.04E-05 |
| OTU_40 Porphyromonadaceae | 2.04E-05 |
| OTU_17 Burkholderiales | 2.65E-05 |
| OTU_733 *Parabacteroides distasonis* | 3.91E-05 |
| OTU_755 *Parabacteroides* | 3.95E-05 |
| OTU_750 *Parabacteroides* | 5.14E-05 |
| OTU_734 *Parabacteroides* | 5.25E-05 |
| OTU_756 *Parabacteroides* | 8.68E-05 |
| OTU_ 64 Betaproteobacteria | 9.65E-05 |

OTUs found to be decreased in the ABC plus clindamycin treatment group compared to the clindamycin alone group with 10 OTUs reduced (Table 3-5).

**Table 3-5; Comparatively decreased OTUs in ABC plus clindamycin treatment group**
DESeqDataSetFromMatrix() function from DESeq package with a significance value cut-off of 0.001. This allows negative binomial GLM fitting and Wald statistics. Multiple testing corrections using Benjamini Hochberg.

| OTU | Adjusted P value |
|---|---|
| OTU_15 *Lactobacillus johnsonii* | 7.87E-32 |
| OTU_ 36 Porphyromonadaceae | 8.14E-12 |
| OTU_223 *Lactococcus* | 2.37E-11 |
| OTU_21 Enterobacteriaceae | 2.31E-10 |
| OTU_45 *Lactobacillus intestinalis* | 2.92E-09 |
| OTU_91 *Mucispirillum schaedleri* | 2.92E-09 |
| OTU_181 *Enterorhabdus mucosicola* | 1.24E-05 |
| OTU_53 Porphyromonadaceae | 2.60E-05 |
| OTU_47 *Odoribacter* | 3.95E-05 |
| OTU_88 Lachnospiraceae | 5.0929e−05 |

In the severe disease model half of the 24 significantly altered OTUs were assigned to *Parabacteroides*. This included *Parabacteroides goldsteinii* (OTU_11) and *Parabacteroides distasonis* (OTU_3). Additionally there was an increase in OTU_2 identified as *Bacteroides thetaiotaomicron* a specialised mucin degrader. Interestingly there was a decrease in two other mucus dwelling species OTU_91, *Mucispirillum schaedleri*, and OTU_181, *Enterorhabdus mucosicola*. This suggests that ABC may somehow alter mucus homeostasis.

### 3.2.11 The impact of antibiotic treatments on the functional capacity of the microbiota

#### 3.2.11.1 Significantly altered KEGG pathways

Comparison of the metabolic potential of the microbiota between the clindamycin and ABC/clindamycin treatment groups provides an opportunity to associate specific metabolic functions with disease severity. Using Tax4fun we were able to map the majority of OTUs to KEGG metabolic pathways with significant differences between the clindamycin only and ABC plus clindamycin identified. In total 20 KEGG functions were significantly altered between the

two treatment groups with 14 functions increased (Table 3-6) and 6 decreased (Table 3-7) in the ABC plus clindamycin group.

**Table 3-6; KEGG functions that are significantly higher in the ABC plus Clindamycin compared to clindamycin alone**

Significantly altered pathways determined using a Kruskal-Wallis test with Benjamini Hochberg correction for multiple testing. Adjusted P values reported

| KEGG Information | Adjusted P value |
|---|---|
| K02014; iron complex outer membrane receptor protein | 0.045117 |
| K03296; hydrophobic/amphiphilic exporter−1 (mainly G− bacteria), HAE1 family | 0.045117 |
| K02004; putative ABC transport system permease protein | 0.045117 |
| K16089; outer membrane receptor for ferrienterochelin and colicins | 0.045117 |
| K01190; beta−galactosidase [EC:3.2.1.23] | 0.045117 |
| K05349; beta−glucosidase [EC:3.2.1.21] | 0.045117 |
| K07085; putative transport protein | 0.045117 |
| K03737; putative pyruvate−flavodoxin oxidoreductase [EC:1.2.7.−] | 0.045117 |
| K03088; RNA polymerase sigma−70 factor, ECF subfamily | 0.045117 |
| K01187; alpha−glucosidase [EC:3.2.1.20] | 0.045117 |
| K03585; membrane fusion protein | 0.045117 |
| K03654; ATP−dependent DNA helicase RecQ [EC:3.6.4.12] | 0.045117 |
| K03169; DNA topoisomerase III [EC:5.99.1.2] | 0.045117 |
| K01897; long−chain acyl−CoA synthetase [EC:6.2.1.3] | 0.045117 |

**Table 3-7; KEGG functions that are significantly lower in the ABC plus clindamycin compared to clindamycin alone**

Significantly altered pathways determined using a Kruskal-Wallis test with Benjamini Hochberg correction for multiple testing. Adjusted P values reported.

| KEGG Information | Adjusted P value |
|---|---|
| K00123; formate dehydrogenase, alpha subunit [EC:1.2.1.2] | 0.045117 |
| K01752; L−serine dehydratase [EC:4.3.1.17] | 0.045117 |
| K01581; ornithine decarboxylase [EC:4.1.1.17] | 0.045117 |
| K15580; oligopeptide transport system substrate−binding protein | 0.045117 |
| K07258; D−alanyl−D−alanine carboxypeptidase (penicillin−binding protein 5/6) [EC:3.4.16.4] | 0.045117 |
| K07306; anaerobic dimethyl sulfoxide reductase subunit A [EC:1.8.5.3] | 0.045117 |

Significantly altered KEGG functions include potential mucus degrading and utilising enzymes; beta−galactosidase, beta-glucosidase and alpha-glucosidase. This data is in agreement with the inferred increase in the mucin degrader *B. thetaiotaomicron* identified in the microbiome data and may represent a disruption of mucus homeostasis. In addition to these changes, there is also an upregulation of multiple transport proteins that may be involved in nutrient uptake, iron uptake and antibiotic efflux. This reflects adaptation of the resident microbiota to the niche created by the antibiotic treatment. Differences in inferred KEGG functionality between the clindamycin alone and ABC plus clindamycin group highlight the significant impact such treatments can have on nutrient and metabolite availability.

It is possible to use additional analysis techniques to determine the importance of specific metabolic functions in placing an animal into the treatment group it originated from. By doing this is it possible to determine if it is possible to use the changes identified to sort the samples into the correct groups. Using a Random Forest Classifier (RFC) it is possible to determine which metabolic functions identified by Tax4fun are important in classifying samples into the different antibiotic treatment groups. The plot shown in Figure 3.16 shows the mean decrease accuracy, which ranks the variables in order of importance in classifying a sample into its treatment groups. So the higher up the ranking the variable the more important it is in helping to classify samples. Figure 3.16 shows the 20 significantly altered KEGG pathways between the clindamycin

alone and ABC/clindamycin groups ranked in order of importance in placing samples into the ABC/clindamycin treatment group.

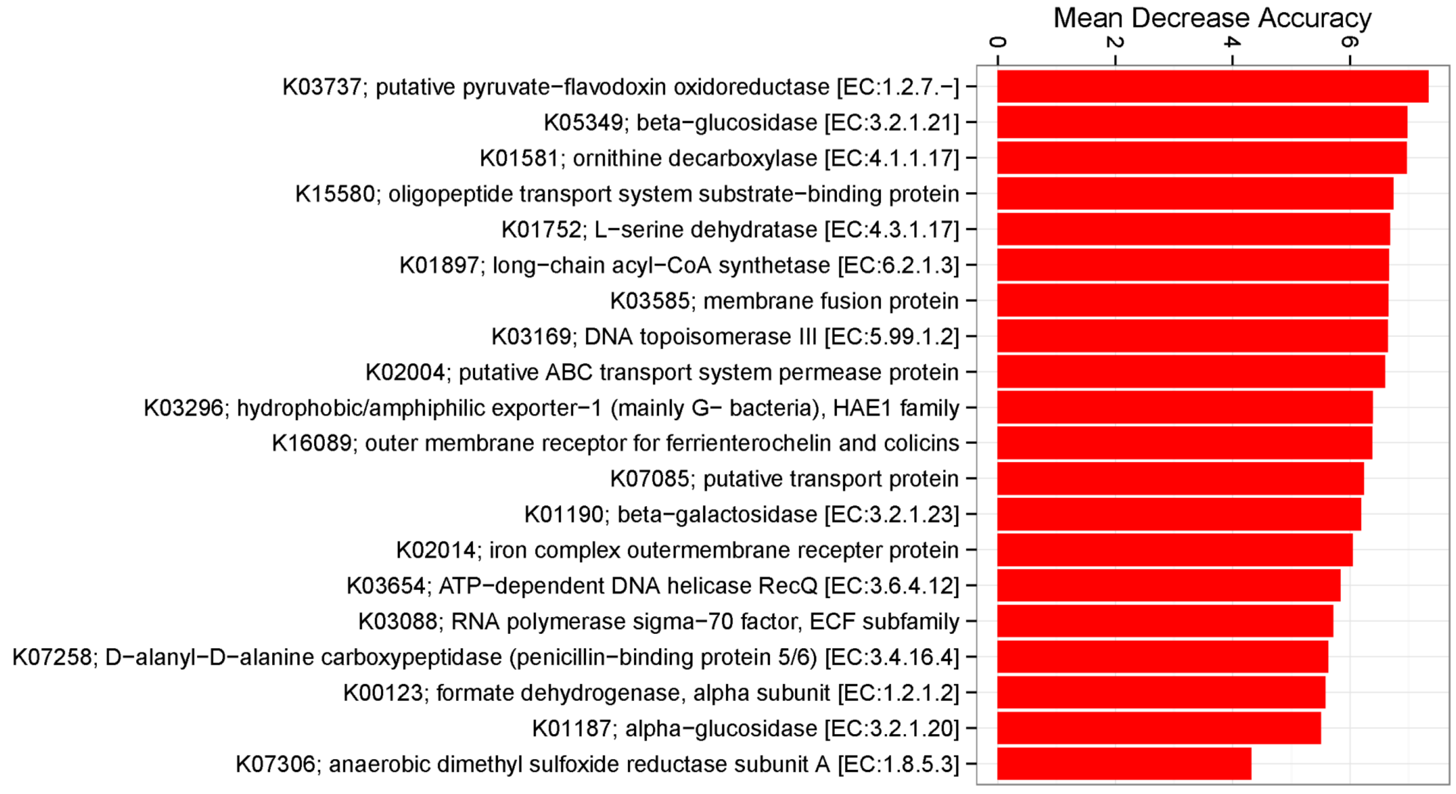



**Figure 3.16 Use of Random Forest Classifier on Tax4fun data to determine which metabolic functions correlate with different antibiotic regimes**
Metabolic functions that classify samples between ABC plus clindamycin and clindamycin only groups. Variables are ranked in order of importance in helping to correctly classify samples . Importance of variables calculated using randomForest() function. The higher the mean decrease accuracy score the more important the variable is in helping group the samples.

The mean decrease accuracy (Figure 3.16) shows that the 20 KEGG functions identified in tables 3-5 and 3-6 are sufficient to separate the ABC plus clindamycin from the clindamycin only treated mice. Use of this information can be successfully used to sort these samples into the correct treatment groups using a RFC (Figure 3.17).

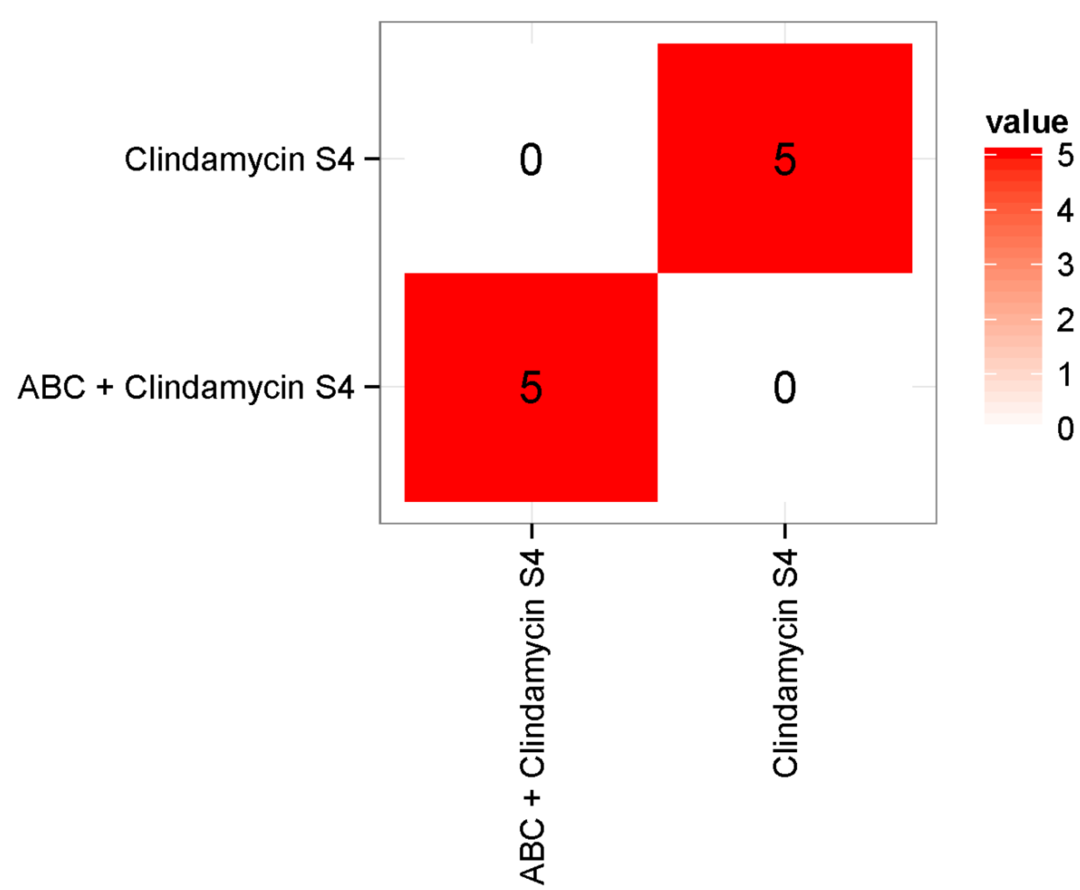



**Figure 3.17 Confusion matrix showing grouping of microbiome samples into treatment groups**
The Random Forest Classifier is able to successfully group all of the samples into the treatment groups.

Analysis of the OTUs present and the functions of these OTUs show that there are both structural and metabolic differences between the clindamycin only and ABC plus clindamycin treated animals. These differences have potentially important roles modulating disease severity and warrant further investigation. For example beta-glucosidase activity is ranked as the second most important variable in distinguishing mice from the clindamycin only group to the ABC plus clindamycin group. This suggests that further investigation of mucin degradation and homeostasis may be beneficial in increasing our understanding of disease.

## 3.3 Discussion

### 3.3.1 Infection Outcome

In this study analysis of how different antibiotic pre-treatments alter colonisation resistance and disease outcome were studied. As described in section 2.8.1, mouse models were established to ensure that the outcome of infection mirrored that previously reported in the literature including significant weight loss after infection and damage to tissue (Chen *et al.,*2008; Collins *et al.,* 2015; Sun *et al.,*2011). The mortality rate seen here was lower than in some of these studies; however as these studies often used the high toxin producer VPI 10463 this may provide an explanation for the differences observed. Additionally it has been noted that different *C. difficile* strains have varying levels of virulence in the mouse model (Theriot et al., 2011). It has recently been shown by Collins *et al.,* (2015) that severity can be increased further by extending the duration of the ABC to 5 days. It has been previously shown that the inclusion of metronidazole in drinking water results in a bitter and unpleasant taste, reducing the consumption of water by the mice (Hou *et al.,* 2007). This may mean that the dosage is not as high as expected which may explain why the ABC alone is not able to render the mice susceptible to infection and why the Simpson and Shannon diversity remains high. To ameliorate this some studies include a sweetener in the water when delivering antibiotics to encourage consumption.

In brief, in our experiments animals treated with clindamycin alone were colonised, with little weight loss or damage to tissue. In contrast animals treated with the ABC plus clindamycin combination suffered from significantly more weight loss, had significantly higher CIs and with epithelial damage present in tissue samples. Untreated but infected animals were not colonised, whilst those given ABC alone were only transiently colonised with very low numbers of *C. difficile* detectable 4 DPI, reflected in by both shedding and microbiome data.

### 3.3.2 Impact of the different treatment regimens on microbiome structure

Treatment with clindamycin had the biggest impact on the structure of the microbiome, with a bloom of Enterobacteriaceae observed in both groups treated with this antibiotic. Interestingly, the majority of the organisms recovered at D0 belonged to 2 OTUs from this phylum, one of which was *E. coli*. This bloom in Enterobacteriaceae is well documented in the literature and is commonly reported to be a consequence of antibiotic treatment in animals and people (Peniche *et al.,* 2013; Rea *et al.,*2012; Reeves *et al.,* 2011).

### 3.3.3 Clindamycin results in loss of colonisation resistance

In these experiments significant *C. difficile* colonisation was restricted to animals treated with clindamycin or ABC plus clindamycin. Mice treated with ABC alone were only transiently colonised suggesting that clindamycin removes those species that influence *C. difficile* persistence. In this work attention was focused on BSH enzymes, which act as the gateway reaction in bile metabolism. Tax4fun analysis supported this hypothesis by revealing that relative abundance of BSH enzymes was reduced in animals that became highly colonised with *C. difficile*. Amplification of BSH genes by PCR confirmed the loss of organisms expressing BSH after clindamycin treatment, which in turn was associated with long term colonisation. Untreated animals and those given ABC only retained these enzymes as part of the flora, and either did not become colonised or were only transiently affected suggesting rapid recovery of the microbiota. Additionally TOF-MS analysis of filtered caecum samples 4DPI revealed an increase in TC compared to untreated animals. This decrease in the abundance of BSH during *C. difficile* infection has recently been demonstrated in patients with recurrent infection with decreases in 10 OTUs accounting for a 67.3% decrease in BSH abundance in these patients compared to controls (Allegretti et al., 2016).

Another important process in this pathway is the multistep 7α-dehydroxylation pathway. Attempts to determine the abundance of the *bai*CD gene from this pathway using PCR and Tax4fun were unsuccessful. This may be because this pathway is much less abundant and restricted to a few members of the

microbiota, thus making detection in databases and amplification more difficult. Members of the *Clostridium* XIVa cluster are known to carry out this function. OTU_69, one member of this cluster, was significantly decreased after clindamycin treatment indirectly suggesting a potential decrease in this function. Loss of this OTU cluster has been associated with reduced colonisation resistance to *C. difficile* and has been described in previous studies (Collins *et al.,*2015). A recent study showed that a single bacterium from this cluster, *C. scindens,* was able to reduce *C. difficile* disease severity in mice as a consequence of DCA production (Buffie *et al.,*2014). Further experiments to validate this observation including the creation and testing of an isogenic *C.* scindens strain in which this gene is deleted are needed. Interestingly the use of *C. scindens* did not confer full protection, suggesting other members of the flora play a role in protection. Previously, BSH producing bacteria have been shown to restore 7α-dehydroxylase function to the microbiota, even though these bacteria do not harbour these enzymes. This is thought to reflect selection pressure created by the high concentration of CA, encouraging bacteria that are able to detoxify it to bloom (Gustafsson *et al.,*1998). Additionally the 7α-dehydroxylase enzymes are unable to act on conjugated bile salts, with upstream deconjugation by BSH required for these enzymes to function. This suggests that restoration of both functions may be required for colonisation resistance to be restored (Batta et al., 1990). Furthermore, the reduction of taurine to $H_2S$ has been found to be an essential substrate for 7α-dehydroxylase producing bacteria, suggesting that these bacterial populations interact with each other *in vivo* (Van Eldere *et al.,*1996). These studies show the importance of understanding bacterial interactions as multiple pathways are intertwined with production of some metabolic products directly influencing other functions in the gut.

It is clear that bacterial bile metabolism plays an important role in colonisation resistance but does not appear to be involved with increase severity. The work presented here suggests that BSH might be a good biomarker of bile metabolism which could indicate potential susceptibility to *C. difficile* colonisation.

### 3.3.4 The microbiome impacts upon disease severity

From our data, it would appear that disease severity is not pathogen driven as there were no significant differences in colonisation and toxin levels between clindamycin alone or the ABC plus clindamycin combination. However, there were significant differences in weight loss, CI and differences in damage to the guts of infected animals. Therefore additional severity may be due to the impact of the different antibiotic regimes on the microbiota. ABC treatment results in an increase in *B. thetaiotaomicron* a specialised mucin degrader with large portions of the its genome dedicated to utilising mucin (Martens *et al.,* 2009). An increase in *B. thetaiotaomicron* suggests that treatment with ABC may result in an increase in mucin degradation activity. Further investigation of Tax4fun data showed that increase in enzymes involved in mucin degradation were important in classifying mice from the severe group from those in which only colonisation was observed. However, as explained previously, the majority of OTUs could not be resolved to species level. This could potentially impact the ability of Tax4fun to accurately infer the metabolic potential. For this reason it would be useful to experimentally investigate changes in the abundance of mucin degrading enzymes. This could be done using reverse transcription PCR or by directly measuring the activity of these enzymes in the faecal samples of mice that have been treated with the ABC.

Previous studies have shown that *B. thetaiotaomicron* facilitates *C. difficile* infection in mice through the production of succinate which can be utilised as a carbon source by *C. difficile* promoting expansion of the organism in the colon (Curtis *et al.,* 2014; Ferreyra *et al.,*2014). Increased degradation of mucus leads to a spike in free sialic acid which can then be utilised by *C. difficile* (Ng *et al.,* 2013). Interestingly an increase in the abundance of mucosal bacteria has been found in patients in patients with IBD and is associated with more severe inflammation (Swidsinski et al., 2002). The impact of mucin degraders in this context is interesting as many organisms have previously been described as being beneficial organisms within the microbiome. For example, *B. thetaiotaomicron* has been shown to aid development of the postnatal gut (Xu & Gordon, 2003) and facilitate angiogenesis in the intestine (Stappenbeck *et al.,* 2002). This highlights how the influence of a particular organism can vary depending on individual circumstances.

The observed increase in mucin degraders seems to correlate with a loss of other mucus dwelling species. This most likely reflects disruption of mucus layer homeostasis with an increase in mucus degraders likely to disrupt the stable mucus layer, potentially reducing mucus thickness or preventing proper formation of the mucus layer. In mono associated rats *B. thetaiotaomicron* has been shown to increase goblet cell differentiation and expression of mucus related genes. This was ameliorated by administering the mice *Faecalibacterium prausnitzii* which then restored homeostasis of this system (Wrzosek et al., 2013). This suggests that *F. prausnitzii* may have some therapeutic potential, mediated by the restoration of mucus layer homeostasis and further investigation of this is warranted. It may be that in these mice there is increased mucus production which may prevent proper formation of the protective mucus layer. Interestingly, it was noted during the experiments that animals treated with the ABC had large quantities of mucus in their faeces. Additionally, disruption is supported by the observation that there is a significant decrease in two mucus dwelling species *M. schaedleri* and *E. mucosicola* following ABC plus clindamycin treatment. Such a change in mucus structure may allow *C. difficile* greater access to the epithelia resulting in more direct delivery of toxins, leading to increased weight loss and tissue damage. Dysregulation of the mucus layer may also mean greater activation of innate inflammatory responses through activation of TLR's on epithelial cell surfaces by bacterial antigens (Figure 3.18).

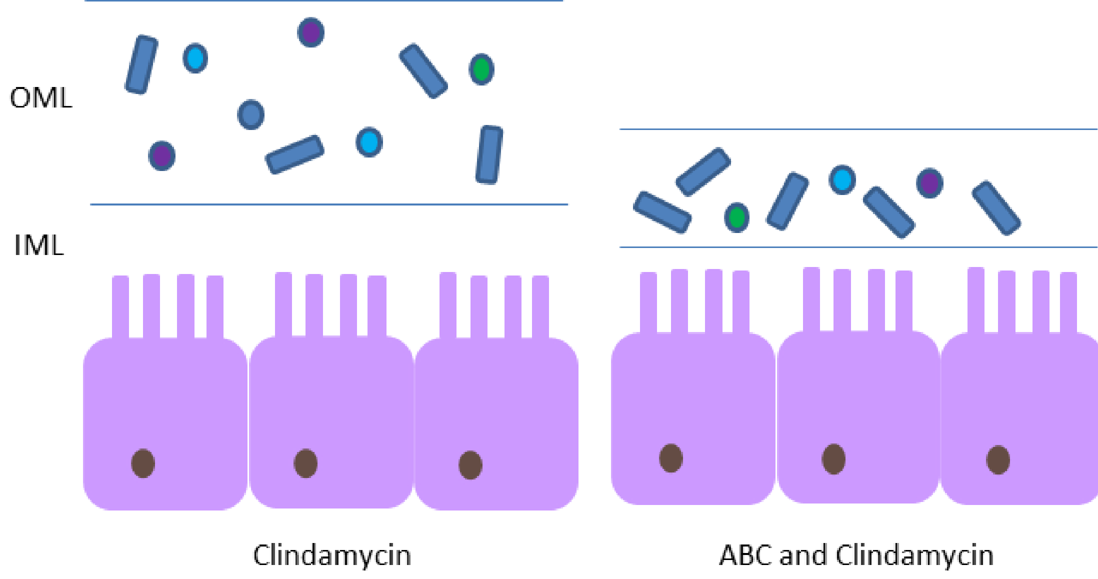


**Figure 3.18 Potential impact of the microbiota on mucus thickness**
The mucus is made up of two distinct layers the inner mucus layer (IML) and the outer mucus layer (OML). Increases in mucin degraders may result in a decrease in mucus layer thickness and stability allowing *C. difficile* cells and toxin to better access to the epithelial cell surface.

In addition to alterations in the abundance of mucus associated bacteria there were alterations in the number of *Parabacteroides*. Of the 24 OTUs significantly altered between the two disease models 12 belong to the *Parabacteroides*. This includes *P. goldsteinii* and *P. distasonis* which have the potential to invade epithelial cells so may also increase disease severity (Nakano et al., 2008). It has also been found that mice with increased numbers of *Parabacteroides* and *Bacteroides* have a more diffuse mucus layer (Jakobsson et al., 2015) which may explain the increased quantity of mucus in the faeces of these mice. Increases in *Parabacteroides* have been associated with super shedding mice (Lawley *et al.,* 2012) and also significantly associated with mice that succumb to *C. difficile* infection (Collins *et al.,* 2015). Similarly increases in *Parabacteroides* have been noted in humans infected with *C. difficile* suggesting a role for these bacteria in both mouse and human infection (Chang *et al.,* 2008; Rea *et al.,* 2012; Rea *et al.,* 2013).

This study suggests that both colonisation resistance and disease severity are modulated by the microbiota and can be altered by the antibiotics used before infection. The antibiotic regimes used in these experiments impact the microbiota in different ways, leading to changes in microbial diversity that alter metabolism. These alterations can favour pathogenesis by changing the availability of substrates that *C. difficile* can utilise and additionally by creating an environment primed for damage and inflammation. Knowledge of which antibiotics destroy bacteria involved in bile modification or alter mucus structure may be useful when prescribing to vulnerable patients. This data also offers the opportunity to use these microbial genes as markers of disease susceptibility. BSH in this study serve as a good marker for healthy bile metabolism in the gut, and could be used to highlight susceptibility to infection. Additionally increased proportions of mucus degrading enzymes seem to be a good indicator of disease vulnerability and it will be interesting to see if over representation of enzymes could predict onset of more severe disease in a clinical setting.

The work undertaken in this chapter has shown the importance of the microbiota in both colonisation resistance and disease severity. Severity seems to be mediated by an increase in invasive bacterial species and disruption of mucus homeostasis whereas colonisation resistance is controlled by changes to bacterial bile metabolism. Chapter 4 will focus on the ability of BSH to modify the efficiency of *C. difficile* spore germination *in vitro* and *in vivo*.

# 4 Inhibition of germination by Bile Salt Hydrolase producing strains

## 4.1 Introduction

In the previous chapter, a link between BA metabolism and resistance to *C. difficile* colonisation was established. These data, together with that presented in the literature, indicate that in particular loss of bacteria expressing BSH enzymes correlates with decreased colonisation resistance. This is because BSH producing bacteria modify BAs and open them up to further modification including dehydroxylation. Such modifications potentially limit the availability of TC to induce spore germination (1.3.2). Although this link has been alluded to in many publications, the capacity of the individual bacteria producing these enzymes to limit *C. difficile* spore germination has not been investigated formally. If such a link could be made, it would provide significant justification to the instillation of such bacteria therapeutically and would support the use of defined bacteria in colonic replacement therapies rather than the undefined mixtures used currently in faecal transplants.

In this chapter, the isolation and characterisation of several BSH producing bacteria is described. Their capacity to inhibit germination and outgrowth of *C. difficile in vitro* and *in vivo* was also determined.

## 4.2 Results

### 4.2.1 The impact of incubation with different bile salts on spore germination and growth

It has been suggested that modification of BA by intestinal bacteria from primary BA (TC) to secondary BA (CA and DCA) reduces the capacity of *C. difficile* to germinate and grow. To confirm this observation, *C. difficile* spores were exposed to different BAs and the growth within the culture observed. Spores were added to BHI with and without the addition of 0.1% TC, 0.1% CA or 0.1% DCA. Growth was determined by changes in $OD_{600}$ of the culture with samples taken for OD measurements every 3 hours for 12 hours and finally at 24 hours.

Using this approach we determined if incubation with different BAs had any impact on the time taken for spores to germinate and grow (Figure 4.1).

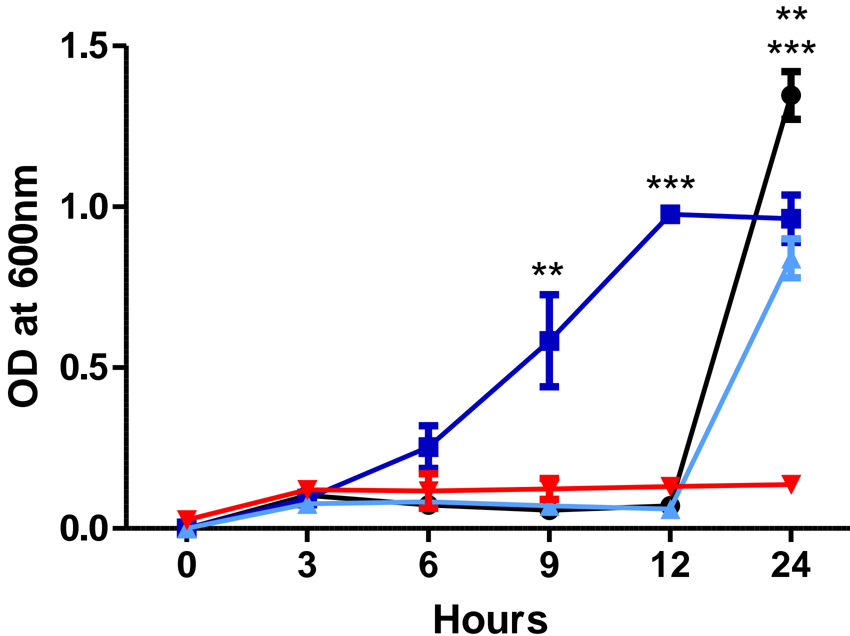



**Figure 4.1 Growth of *C. difficile* BI-7 spores after exposure to different bile salts**
Germination and growth of *C. difficile* in BHI broth alone (Black), BHI supplemented with 0.1% TC (Dark blue), CA (Light blue), and DCA (red). Spores incubated with TC had reached significantly higher ODs than all spores in all other growth conditions at 9 (** indicates p = < 0.01) and 12 hours (*** indicates p < 0.0001). At 24 hours spores from all growth conditions reached significantly high ODs than those incubated with DCA (*** indicates p = <0.0001). Additionally spores grown in BHI alone reached significantly higher ODs than those grown with CA or TC (** indicates p = 0.01). Error bars represent +/- the SEM of 3 biological replicates. Significance was determined using ANOVA with Tukey's post-test.

Incubation of spores in BHI with 0.1% TC resulted in rapid germination and growth of the bacteria with increases in OD observed from 6 hours post inoculation and significant differences in growth recorded at 9 (p = <0.01) and 12 hours (p= <0.0001). Growth reached stationary phase by 12 hours. In contrast no growth was observed within the first 12h when the spores were inoculated into BHI alone or BHI containing CA. This suggests that TC plays an active role in stimulating efficient spore germination. Inclusion of additional time points between 12-24 hours would have further helped to determine more precisely whether CA had any impact on germination. As expected, spores incubated with DCA did not germinate or outgrow within the 24 hour time period measured with the OD in this group significantly lower (p = < 0.0001) than those observed at 24 hours for all other growth conditions. As DCA is known to be a potent inhibitor

of *C. difficile* growth this is the outcome that was expected. The OD of cultures grown with TC or CA were significantly lower at 24 hours than those grown in BHI alone ($p = < 0.01$), suggesting some inhibition of growth by these BAs. These results suggest that germination and vegetative growth are most efficient after exposure to TC and reflect the huge differences that individual BAs appear to have on rates differential effects on germination and subsequent growth of the organism.

### 4.2.2 Detection of BSH activity in bacteria isolated from the mouse gut

As bacteria alone help to modify BAs, bacteria expressing BSH were chosen for further investigation. In chapter 3, several different species of bacteria were isolated from the colon of mice (Table 3-1). The exact identity of these strains was confirmed by sequencing the V4 variable region within the 16s rRNA gene. Subsequent analysis of the microbiome confirmed that clindamycin treatment of these animals resulted in a reduction of many of these bacteria.

A plate based assay was used to identify bacteria possessing BSH activity. This activity was determined by the precipitation of deconjugated BAs into the media resulting in the formation of white granular colonies. As TC is the most potent inducer of germination, strains were tested for their ability to deconjugate TC into CA. Of the 18 strains originally isolated, 5 were able to successfully to deconjugate TC to CA. This included a number of isolates identified as *Staphylococcal* species (Table 4-1).

**Table 4-1 BSH activity against TC in strains isolated from the mouse gut**

| Isolate | LB | LB + TC (0.5%) |
|---|---|---|
| *S. warnei* | | |
| *S. warnei* | | |
| *S. warnei* | | |
| *S. lentus* | | |

### 4.2.3 Impact of other BSH producing bacteria on *C. difficile* spore germination

To determine if BSH activity and its subsequent modification of TC affected spore germination the following experiment was performed. In brief, bacteria were grown in BHI containing 0.1% TC for 24 hours. The organisms were then removed by centrifugation and the media was filtered sterilised. This filtered material was then tested to determine if it influenced the efficient germination of *C. difficile* spores. Of these strains, 8/18 different bacterial isolates were able to inhibit *C. difficile* BI-7 spore germination (Table 4-2).

**Table 4-2 Ability of different bacterial strains to inhibit spore germination**

| Family | Organism | Inhibition of germination |
|---|---|---|
| Dermacoccaceae | *Dermococcus* | - |
| Enterobacteriaceae | *Citrobacter* | - |
| | *Escherichia fergusonii Shigella sonnei or E.coli* | - |
| | *E. fergusonii Shigella sonnei or E.coli* | - |
| Enterococcaceae | *Enterococcus faecium* | + |
| | *Enterococcus casseliflavus* | + |
| | *Enterococcus* | - |
| | *Enterococcus* | + |
| Lactobacillaceae | *Lactobacillus apodemi* | - |
| | *L. apodemi* | + |
| Micrococcaceae | *Kocuria rhizophila* | - |
| | *K. rhizophila* | - |
| | *Micrococcus luteus* | - |
| Pseudomonadale | *Enhydrobacter or Moraxella* | - |
| Staphylococcace | *Staphylococcus lentus* | + |
| | *Staphylococcus warneri* | + |
| | *S. warneri* | + |
| | *S. warneri* | + |

Of the 8 strains that could inhibit germination 5 had been previously shown to possess BSH activity against TC in a plate based assay (Table 4-1). These assays suggest that BSH activity may be able to decrease rates of spore germination *in vitro*. However, for 3 of these isolates inhibition of germination did not appear linked to BSH activity. This could either suggest that these strains were identified falsely as non BSH producers using the BSH plate assay or it may be that there are other bacterial factors that could be impacting on spore germination.

In addition to the *Staphylococcus* isolates shown in Table 4-1, a *L. murinus* isolate was identified with BSH against TC. Interestingly, a second *L. murinus* isolate displayed no equivalent BSH activity (Figure 4.2).

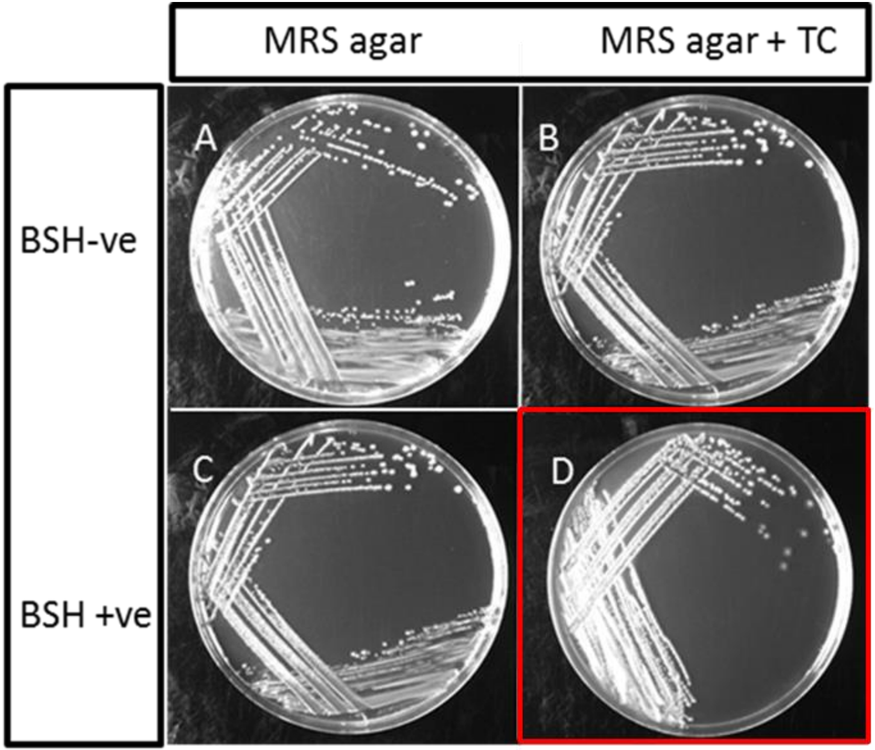



**Figure 4.2 Detection of BSH activity in *Lactobacillus murinus* isolates**
*L. murinus* strains were plated onto MRS (A and C) and MRS with 0.5% TC (B and D) and incubated for 72 hours. BSH activity was determined by the presence of white granular colonies and the precipitation of cholic acid into the media (D).

To further unravel the role of BSH production in inhibition of spore germination these two strains were studied in greater detail. Firstly to determine if differences in BSH production were readily attributable to specific genetic sequence differences, the whole genomes of both *L. murinus* strains were sequenced and compared.

#### 4.2.3.1 Genome sequencing of *Lactobacillus murinus* isolates

Genomic DNA was extracted from each *L. murinus* strain and sequencing carried out on the MiSeq at the Glasgow Polyomics Facility. The genomes of both *L. murinus* isolates were assembled and annotated as described in section 2.7.1.5. Once assembled, annotated genomes were mined for sequences annotated as BSH by the bioinformatic software PROKKA. Genomes were also analysed for the presence of the 7α-dehydroxylation genes (the *bai*CD gene of the *bai* operon), which did not appear to be present in either genome. Further inspection of the BSH specific sequences showed that these were almost identical, with the majority of the DNA and protein sequences conserved. However, the main difference between these two sequences was the absence of 39 nucleotides at

the start of annotated BSH gene, which is equivalent of a loss of the first 13 amino acids (Figure 4.3).

```
BSH-ve      -------------LGRNFDYEFSYGEKAVIIPRNYVFELRKLPALSKHYAMVGLTSVFDD
BSH+ve      MCTAVSFKTKDHYFGRNFDYELSYGEKAVIIPRNYVFELRKLPALSKHYAMVGLTSVFDD
                         :*******:**************************************

BSH-ve      TPLLYDAVNEHGLAMAGLNFEGNAVFYDFDPEMDNVTPFEFIPYILGQCKNVAEAKQMLT
BSH+ve      TPLLYDAVNEHGLAMAGLNFEGNAVFYDFDPEMDNVAPFEFIPYILGQCKNVAEAKQMLT
            ************************************:***********************

BSH-ve      KTNLVNEAFRADLPLSPLHWIISDSEDNTIVVETLADGMKVYDDPVDVMTNNPTFDKQLF
BSH+ve      KINLVNEAFSADLPLAPLHWIISDSEDNTIVVETLADGMKVYDDPVGVMTNNPTFDKQLF
            * ******* *****:****************************** *************

BSH-ve      NLNNYRGLSAKTPENTFDPTFELPAYSRGMGTLGLPGDLTSSSRFVKAAFVKAHSVCEPD
BSH+ve      NLNNYRGLSAKTPENTFDPTVELPAYSRGMGTLGLPGDLTSSSRFVKAAFVKAHSVCELD
            ********************.************************************* *

BSH-ve      ESSSVSQGFHILSAIEQQRGCCEVSEGKYEYTIYSACYNKDKGILYYKTYEDSQITALDM
BSH+ve      ESSSVSQGFHILSAIEQQRGCCEVSEGKYEYTIYSACYNKDKGILYYKTYEDSQITALDM
            ************************************************************

BSH-ve      HKVDLESDRFTLYPLSQTQHFNYAN
BSH+ve      HKADLESDRFTLYPLSQTQHFNYAN
            **.**********************
```

**Figure 4.3 Alignment of BSH protein sequences from BSH-ve and BSH+ve *L. murinus* strains**

Alignment of both *L. murinus* BSH protein sequences was carried out in Clustalw2. The important Cys1 catalytic domain is highlighted in yellow. The BSH from BSH-ve is missing the first 13 amino acids of the protein including both the methionine needed for expression and the Cys1 which is essential for function rendering the BSH inactive.

Amongst the missing amino acids is the one of the key cysteine residues, Cys1, which is essential for BSH activity (Ridlon et al., 2006) (Figure 4.4). As protein also lacks the methionine start codon it is unlikely that the protein is expressed. As a consequence, truncation of the BSH gene in the BSH-ve *L. murinus* strain is the most likely explanation for the lack of BSH activity in this isolate. To confirm the truncation of this gene, both the full length BSH and truncated BSH (tBSH) were amplified by PCR.

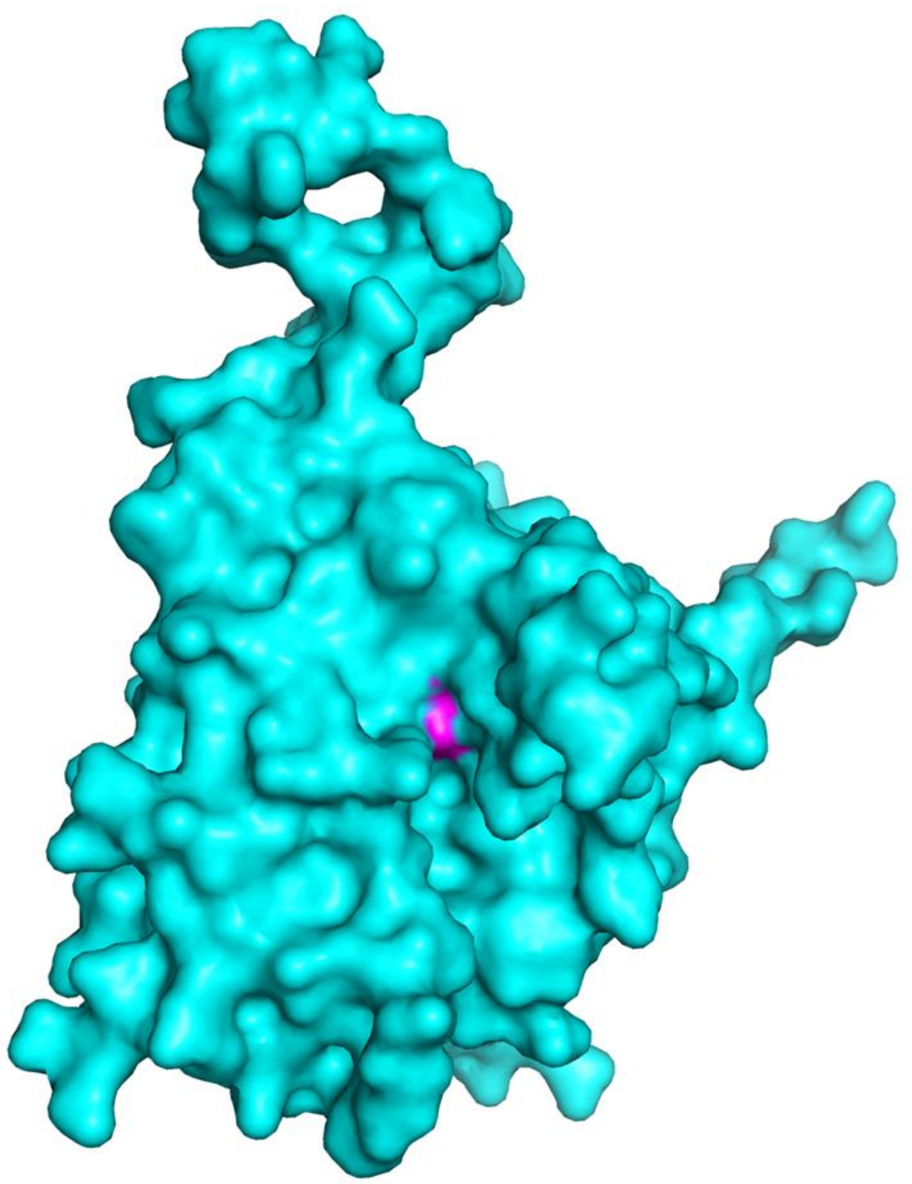


**Figure 4.4 Predicted structure of the full length BSH from *L. murinus***
The predicted structure of the full length BSH from *L. murinus* with the essential cysteine that forms the catalyst centre highlighted in purple.

#### 4.2.3.2 Amplification of BSH and truncated BSH genes from *L. murinus*

Primers were designed to allow amplification of both the full length BSH and tBSH from the *L. murinus* isolates. This analysis confirmed that the full length BSH could not be amplified from the strain lacking BSH activity, whereas primers designed for the amplification of the tBSH allowed amplification from both strains (Figure 4.5).

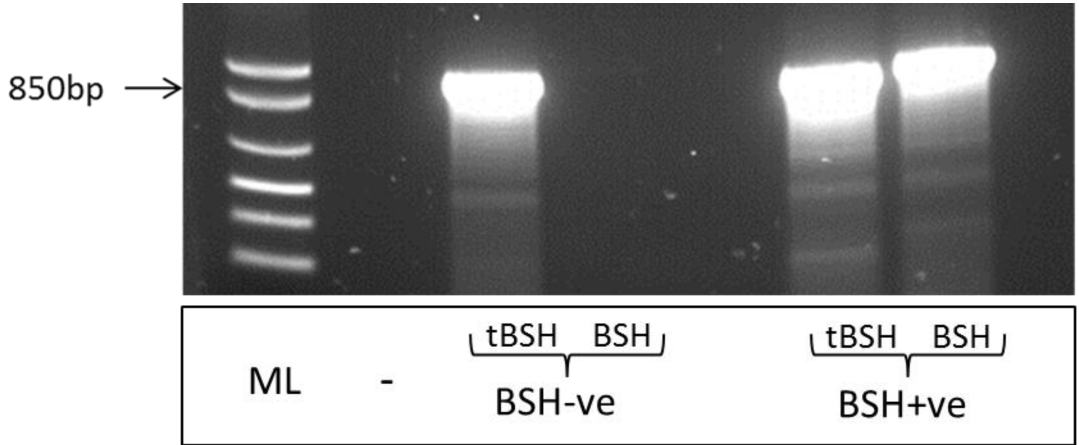



**Figure 4.5 Amplification of tBSH and BSH from both *L. murinus* strains**
Primers directed to the full length BSH and tBSH were used to detect BSH in both *L. murinus* isolates. Only the tBSH could be amplified from *L. murinus* that did not have BSH activity. Both the tBSH and full length BSH could be amplified from the *L. murinus* isolate with BSH activity. The BSH gene has an expected product size of 978bp and tBSH expected product size of 939bp.

This result suggests that this gene truncation in the BSH-ve *L. murinus* strain renders the gene inactive and explains the lack of BSH activity by this strain. As BSH are involved in resistance to high concentrations of BA *in vivo* it is likely that truncation of this gene may increase the sensitivity of this strain to BA. To investigate the impact of this truncation on the growth of this strain, cultures were grown in the presence and absence of TC.

### 4.2.4 The impact of TC on the growth rates of *L. murinus* strains

Analysis of the growth of both *L. murinus* strains was determined by inoculation of MRS broth with or without the addition of 0.1% TC. Viable counts were taken every 3 hours for 9 hours and viable counts performed to determine if TC had an impact on *L. murinus* viability and growth (Figure 4.6).

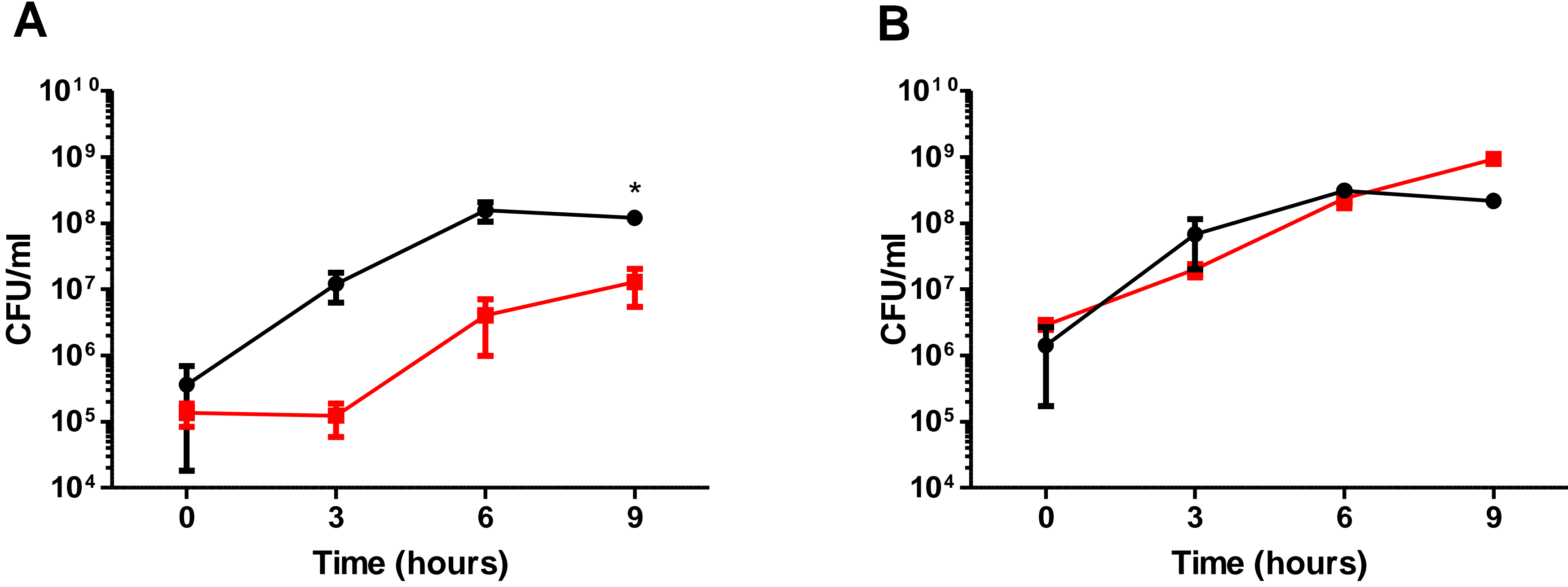



**Figure 4.6 Growth of *Lactobacillus murinus* isolates in MRS broth with and without the addition of 0.1% TC**
A) BSH-ve *L. murinus* isolate and B) BSH+ve *L. murinus* isolate were grown in MRS broth alone (black) and MRS broth with 0.1% TC (red). Data points represent the mean and error bars are +/- the SEM of 3 biological replicates. The BSH-ve *L. murinus* strain showed a growth defect in the presence of TC with a significant reduction at 9 hours. Significance was determined using a student's t test (* signifies p=0.0156).

From these data, it would appear that addition of 0.1% TC to the media dramatically slowed the growth of the BSH-ve strain of *L. murinus*. The difference in growth was only significant at 9 hours (p = 0.0156) potentially due to variation at the earlier time points. The strain was still able to grow to relatively high bacterial loads suggesting that BSH activity is only responsible in part to the bile tolerance observed within these strains. However, inactivation of this gene does have an impact on growth. Never the less, the ability to adapt to the presence of bile may mean that the strain carrying the intact BSH gene is more competitive within the host. However, this does not help explain why the seemingly low bile tolerance strain was originally isolated from the mouse faeces. One possible explanation is that *in vivo* the combinations of bacteria found in this niche are able to deconjugate BA more efficiently than a single strain. This may mean that the toxicity of these compounds overall remains low allowing BSH-ve strains to flourish.

### 4.2.5 BSH producing *L. murinus* can inhibit *C. difficile* spore germination

As previously described, BAs play an important role in *C. difficile* spore germination with modification of BAs decreasing their ability to induce germination. To determine if BSH activity and its subsequent modification of TC to CA affected spore germination the following experiment was performed. In brief, the two described *L. murinus* strains were grown in BHI containing 0.1% TC for 24 hours. The organisms were then removed by centrifugation and the media was filtered sterilised and adjusted to pH7. This filtered material was then tested to determine if it influenced the efficient germination of *C. difficile* spores (Figure 4.7).

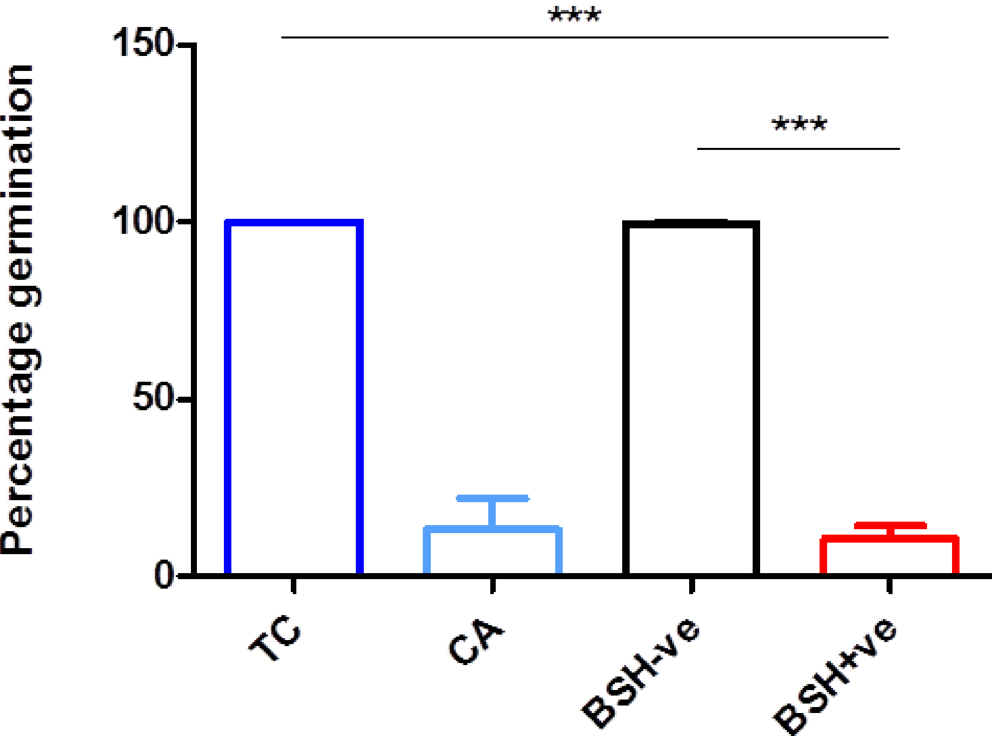



**Figure 4.7 Percentage spore germination of *C. difficile* BI-7 after incubation with supernatant from BSH-ve and BSH+ve *L. murinus***
Percentage *C. difficile* spore germination after incubation with 0.1% TC as a positive control of maximal germination (dark blue) and CA as a negative control of reduced germination (light blue). *L. murinus* strain were grown in BHI with 0.1% TC for 24 hours before being filtered and the supernatant used to stimulate spore germination. The BSH-ve *L. murinus* (black) and the BSH+ve *L. murinus* (red) showed differing capacities to inhibit spore germination. To calculate the percentage germination the number of vegetative cells and spores was determined using differential counts of heated and unheated samples. From this the percentage germination could be calculated as the percentage loss of bacteria after heating. Error bars represent +/- the SEM of 3 biological replicates. Statistical analysis was carried out with a ANOVA with Tukey's post-test. The BSH +ve *L. murinus* strain was found to significantly reduce germination compared to incubation with TC alone or incubation with a BSH –ve *L. murinus* strain (p= <0.001).

The media recovered from the BSH+ve isolate contained a substance that did not stimulate germination efficiently, with rates of germination mirroring those found after exposure to CA. This would suggest that modification of bile salts by bacteria has an important role in regulating the efficiency of *C. difficile* spore germination. However, it is also possible that *L. murinus* is able to impact upon *C. difficile* germination and outgrowth by other as yet undefined mechanisms. These could include the production of inhibitory compounds such bacteriocins that could inhibit *C. difficile* germination and growth (Y. S. Chen & Yanagida, 2006; Lee, Chung, & Seo, 2013; Weese et al., 2004; Yun, Oh, & Griffiths, 2014). Therefore, to further examine the impact of BSH on germination attempts were made to express both the full length BSH and tBSH in *E. coli*.

### 4.2.6 Cloning and expression of BSH from *L. murinus*

Primers were designed to contain the restriction sites Sph1 and Xba1 to allow cloning of both the BSH and tBSH gene into the pnz44 vector. This system allows for constitutive and high levels of expression of BSH in *E. coli*. To promote expression of the tBSH gene, an additional methionine start codon was added during the amplification step. Although restriction digest analysis of these plasmids suggested that cloning was successful, neither was found successfully expressing the BSH protein.

### 4.2.7 Germination assays with BSH expressing *E. coli*

Although BSH activity seemed to reduce the ability of *C. difficile* spores to germinate *in vitro*, we were aware that other factors expressed by the *L. murinus* strains may be influencing spore germination. However, as we had not been able to make this link directly through the expression of the BSH genes from this organism, we chose to indirectly establish this relationship using BSH expressing clones generated from the bacteria recovered from the human gut (Jones *et al.,* 2008). A library of BSH expressing clones was kindly provided by Prof Julian Marchesi (CU), which were generated by cloning the amplified BSH genes from the human gut microbiome into the constitutive pnz44 plasmid. Members of this library had been previously screened for BSH activity and clones selected that expressed functional BSH with differing substrate specificity (Jones *et. al.,* 2008). To identify clones with activity appropriate for study, a screen using TC was used. This identified two clones, one with high activity against TC and a second with undetectable levels of activity against TC (Figure 4.8).

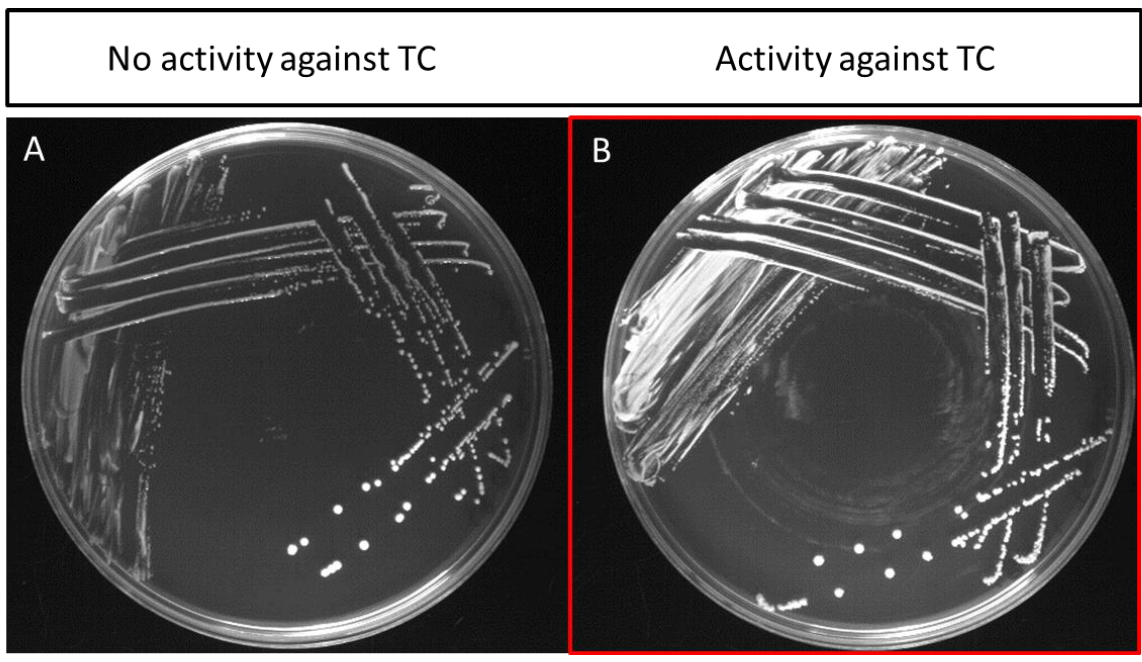



**Figure 4.8 Plate assay for BSH activity against TC from BSH isolated from the human gut microbiome**
*E.coli* containing BSH were plated onto LB agar containing 0.5% TC. Different BSHs have different substrate specificities. A) An *E. coli* clone containing a BSH with no activity against TC and B) an *E. coli* clone with high activity against TC as demonstrated by the formation of white granular colonies and the precipitation of TC into the media. These clones were used in subsequent germination assays.

These strains were utilised in germination assays, using the *E. coli* strain expressing a BSH with no activity against TC as a negative control. The BSH genes are expressed in the same plasmid from identical XL1-blue *E. coli*. This makes it easier to unravel the role of BSH expression on *C. difficile* spore germination, as the only factor that should differ between the clones is the activity of the BSH gene. It was found that the *E. coli* containing a BSH with activity against TC was able to inhibit spore germination with levels reduced to the levels seen when spores are incubated with CA alone. The *E. coli* containing the BSH with no activity against TC was unable to inhibit spore germination with levels the same as those seen in spore incubated with TC (Figure 4.9).

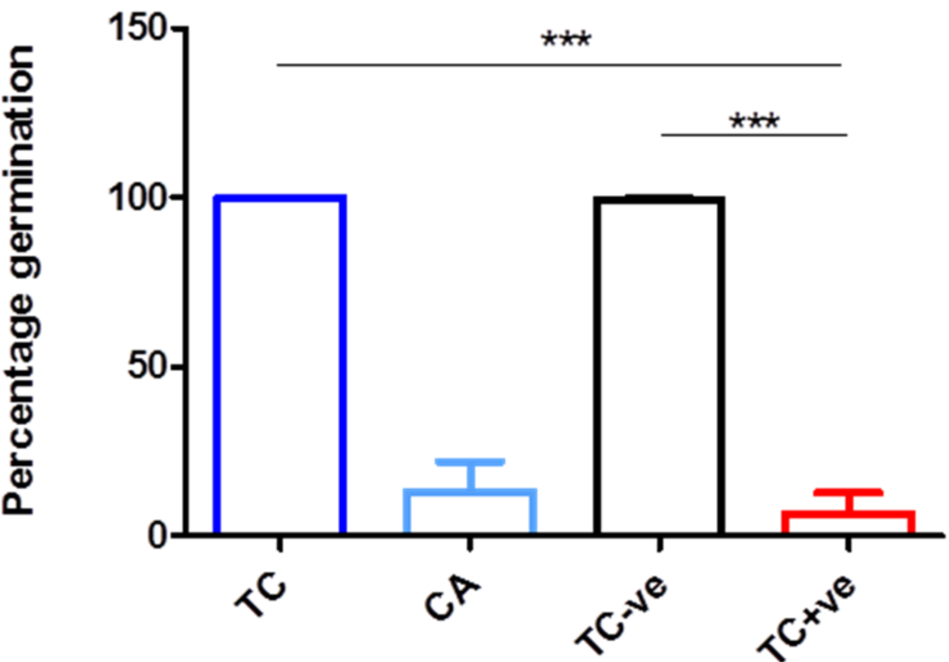



**Figure 4.9 Percentage germination of *C. difficile* BI-7 spores after growth with the TC-ve and TC+ve BSH clones**
Percentage germination of spores incubated with TC (dark blue), CA (blue) and TC-ve *E coli* (black) and TC+ve *E. coli* (red) BSH stains. The number of vegetative cells and spores was determined using differential counts of heated and unheated samples. From this the percentage germination could be calculated. Error bars represent +/- the SEM of 3 biological replicates. Statistical analysis was carried out with a ANOVA with Tukey's post-test. The *E. coli* with activity against TC (TC +ve) was found to significantly reduce germination compared to incubation with TC alone or incubation with the TC–ve *E. coli* clone (p= <0.001).

This supports the mechanism for inhibition of germination being the modification of TC to CA through BSH production.

### 4.2.8 Testing of *L. murinus* as a prophylactic treatment of *C. difficile*

Germination of spores is clearly a fundamental step in *C. difficile* pathogenesis. Consequently identification of a strain of *L. murinus* that is capable of decreasing spore germination of *C. difficile* offers a potential new prophylactic treatment. To determine if animals colonised with the BSH producing *L. murinus* and subsequently infected with *C. difficile* spores showed reduced colonisation, experiments using both strains of *L. murinus* were tested *in vivo*.

#### 4.2.8.1 Time line of prophylactic treatment experiment

For these experiments, 10 mice were placed into 3 different treatment groups, with 2 cages of 5 mice per treatment. The 3 groups were as follows, a control

group given no *L. murinus*, mice treated with the BSH–ve *L. murinus* and animals treated with the BSH+ve *L. murinus*. In all groups, animals were pre-treated with clindamycin to remove any members of the flora that naturally protect against *C. difficile* colonisation including those that produce BSH. The appropriate *L. murinus* strains were administered to the mice on 2 occasions 24h and 6h prior to *C. difficile* delivery. To determine the impact of this treatment on the gut flora, microbiome samples were taken from 6 mice (3 per cage) per group throughout the experiment. As the *L. murinus* strains were not specifically genetically marked (being originally isolated as members of the natural flora), it was hoped that use of the microbiome data may provide indirect evidence of successful colonisation.

#### 4.2.8.1 Shedding of *Clostridium difficile* in the faeces

During the course of these experiments, shedding of viable *C. difficile* in the faeces was used as an indicator of successful germination and growth within the animal (Figure 4.10).

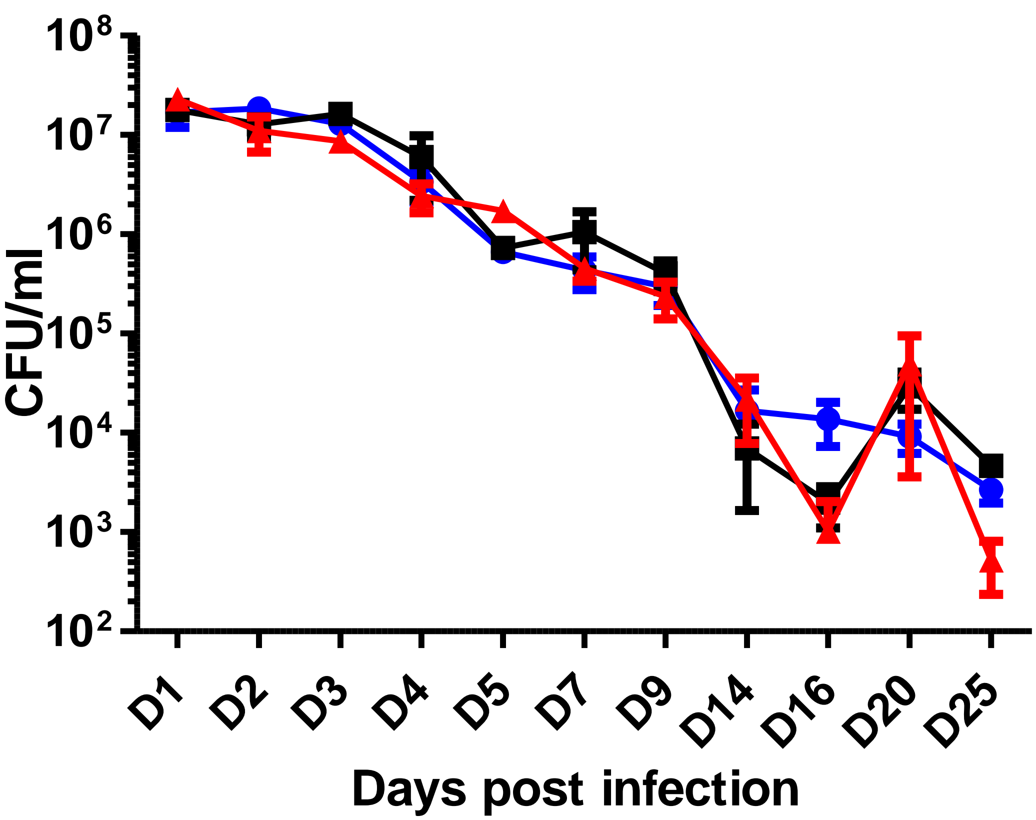



**Figure 4.10 Shedding of *C. difficile* in the faeces of mice from all experimental groups up to 25 DPI**
Mice were given 1x$10^9$ CFU of *L. murinus* on D-1 and 6 hours prior to *C. difficile* on D0. 1x$10^5$ CFU of *C. difficile* spores were given on D0. Faecal samples were collected daily and colony forming units (CFU) of *C. difficile* per gram of faeces were determined as a measurement of successful colonisation. Control mice given no *L. murinus* (blue), mice given BSH-ve *L. murinus* (black) and a final group given BSH+ve *L. murinus* (red) were shown to be equally colonised by *C. difficile* after infection. Error bars show +/- SEM (n=10).

As can be seen from the data, no differences were observed between any of the experimental groups with each showing very similar levels of *C. difficile* in the faeces at all time points tested. This high level of colonisation suggests that the BSH producing *L. murinus* strain was unable to prevent colonisation of these animals, as shedding in this group closely mirrored the control group and the BSH-ve group. This suggests that *C. difficile* spores were still able to germinate and grow effectively. However, closer inspection of the data revealed that of the two cages of animals given the BSH producing *L murinus*, all animals in one of the cages had levels of *C. difficile* below the limit of detection by D14, with no further shedding detected after this point. This would be a decrease in infection time of at least 11 days when compared to the other groups. In contrast, no other cage of animals reached this point during the 25 days of the experiment. It is not clear if this reduction is due greater efficiency of

colonisation by the BSH+ve *L. murinus* strain or whether other members of the flora from these mice are affecting *C. difficile* persistence. Clearly this is an area that requires more attention.

#### 4.2.8.2 Body weight of mice during *C. difficile* infection

As described previously, the extent of infection and indirectly the degree of suffering of the animals was monitored continually throughout the course of the experiment. In previous experiments we had observed that weight loss correlated with disease severity, so mice were weighed daily to determine if administration of *L. murinus* had any impact on this (Figure 4.11).

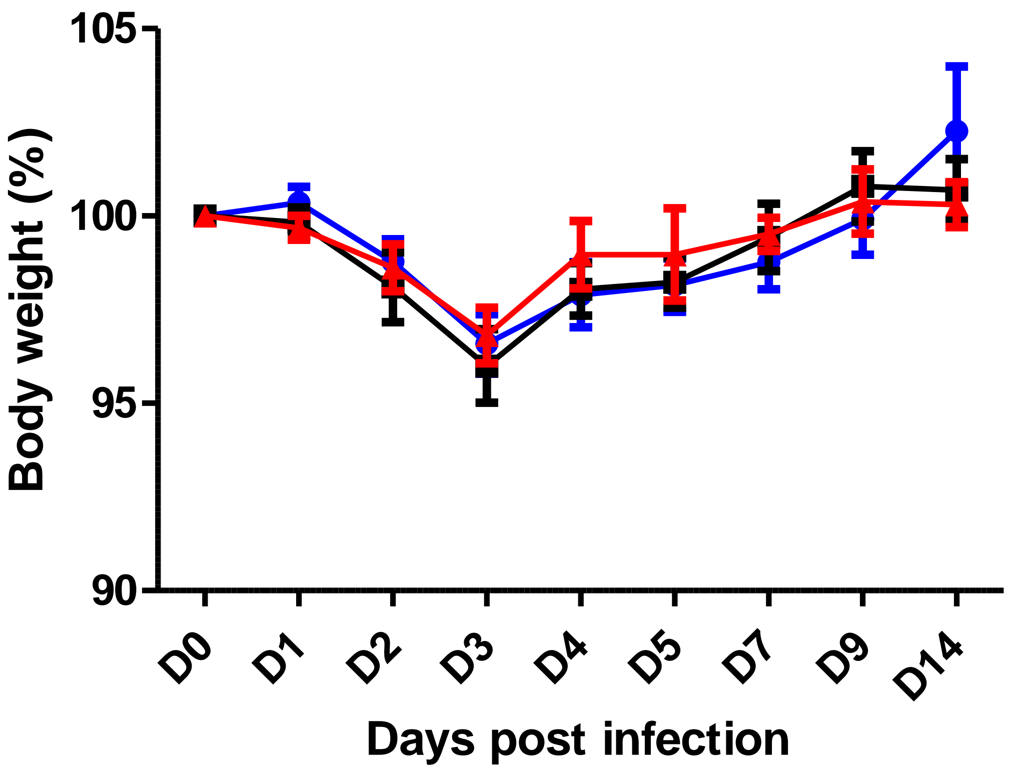



**Figure 4.11 Average percentage body weight of mice following *C. difficile* challenge**
Control animals (blue), BSH-ve animals (black) and BSH+ve animals (red) were weighed daily during the course of the experiment. Animals suffered from weight loss post infection with recovery by 4DPI. There were no differences in weight loss between the groups. Error bars represent +/- SEM of 10 mice per group.

Treatment with either strain of *L. murinus* had no impact on weight loss during infection, with the BSH-ve and BSH+ve strains closely mimicking the weight loss observed in the untreated animals. Whilst culture of *L. murinus* from the faeces was attempted, use of MRS agar was not selective enough to discriminate between the many different isolates recovered. To further investigate whether

animals were successfully colonised with *L. murinus,* PCRs for the BSH genes expressed by the strains were carried out on total bacterial DNA recovered from the faecal samples.

#### 4.2.8.3 Detection of BSH genes in the microbiome as a marker of *L. murinus* colonisation

Amplification of the BSH by PCR was performed using DNA recovered from the faecal samples at different time points during the experiment. Data is presented from samples collected prior to clindamycin treatment (D-5), post clindamycin treatment (D-3) and post treatment with *L. murinus* (D1) (Figure 4.12).

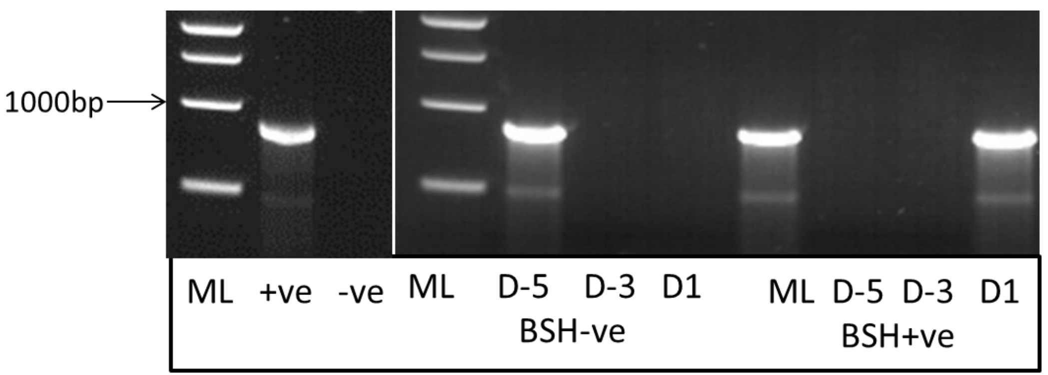



**Figure 4.12 Detection of *L. murinus* BSH in the microbiota of mice**
PCRs directed to the *L. murinus* BSH were carried out on samples collected before clindamycin treatment (D-5), after clindamycin treatment (D-3) and after both doses of *L. murinus* were administered (D1) from control, BSH-ve and BSH+ve groups (n=6). Each gel is representative of an animal from each group. Variations are explained fully in the text.

All animals in this experiment tested positively for the BSH gene prior to clindamycin treatment. As observed previously (Figure 3.14), this amplification was not possible post clindamycin suggesting that the organisms expressing this gene were sensitive to this antibiotic. After infection, animals given BSH+ve *L. murinus* appeared to have regained the BSH gene within the microbiome, in contrast to those treated with the BSH-ve strain which did not regain the gene. However, examinations of later time points during infection (7DPI, 10DPI and 14DPI) suggest that colonisation was only transient. This may explain why there were no apparent differences in the recovery of *C. difficile* from these animals. Further analysis of BA profiles *in vivo* would be needed to determine the full impact of the different treatment regimes. This would help to determine if the different *L. murinus* strains used in this experiment have an impact on BA metabolism *in vivo*.

## 4.3 Discussion

### 4.3.1 BSH producing bacteria can inhibit *C. difficile* spore germination *in vitro*

In the previous chapter, BSH activity was shown to play an important role in colonisation resistance. Loss of theses enzymes was linked to a change in BA metabolism (confirmed by TOF-mass spectrometry - Figure 3.15) resulting in increased germination and growth of *C. difficile in vivo*. In this chapter, several different bacterial species were identified as able to express BSH, the activity of which modified *C. difficile* spore germination *in vitro*. Further investigation revealed that *E. coli* clones expressing the BSH enzyme (Jones *et al.,*2008) were also able to inhibit spore germination, but only if this activity was specifically associated with the breakdown of TC.

Recovery of two *L. murinus* strains, which were genetically highly related, showed that only the isolate that expressed an intact and functional BSH was able to inhibit *C. difficile* germination. In mice, *Lactobacillus* are responsible for ~86% of BSH activity measured (Tannock *et al.,* 1989) making these strains a good candidates for prophylactic use *in vivo*. Whilst, experimental data outlined here would indicate that whilst the use of BSH+ve clones of *L. murinus* had little impact on *C. difficile* colonisation *in vivo*, there is increasing evidence that restoration of BSH function can help to recover BA metabolism both directly and indirectly. In particular, recovery of BSH activity has been linked to greater 7α-dehydroxylation activity within the microbiome after clindamycin treatment (Gustafsson *et al.,* 1998). This most likely reflects an increase in the amount of CA following BSH breakdown of TC, thus providing a selection pressure that allows bacteria that encode genes for the 7α-dehydroxylation pathway to thrive. Interestingly, another study, in which mice were fed CA showed a significant increase in the Clostridial composition of the microbiota. There was also a large increase in DCA levels suggesting an increase in the population that are capable of 7α-dehydroxylation, an activity linked mainly to Clostridial species (Saiful Islam *et al.,* 2011). A similar study carried out by Ridlon *et al.,* (2013) found that feeding mice 1% CA resulted in an increase in 7α-dehydroxylation and Clostridia. These studies strengthen the hypothesis that restoration of bile metabolism

could be achieved by introduction of BSH producing bacteria or through direct feeding of CA. However, such approaches must be performed with caution as changes in the relative composition and concentration of BAs can be harmful. This is because BAs and their derivatives are capable of damaging mammalian cell membranes and have been linked to increased risk of carcinoma (Barrasa *et al.,* 2013; Tsuei *et al.,* 2014), potentially making ingestion of these compounds unwise. Altering this pathway using bacteria may be a safer alternative as this it is more likely to be associated with microbiome homeostasis. Overall, it is the production of DCA that is ultimately required to inhibit *C. difficile* growth. Our data *in vitro* would suggest that BA metabolism is associated with different aspects of *C. difficile* pathogenesis with BSH activity linked to reduction in germination and 7α-dehydroxylation preventing vegetative growth.

### 4.3.2 *L. murinus* producing BSH are unable to inhibit spore germination *in vivo*

Use of *L. murinus in vivo* did not show any prove successful *in vivo*. However, shedding data showed that there was a difference between the 2 cages of mice in the BSH+ve group, with one cage having no detectable *C. difficile* after 14DPI. Evidence from both the literature and this thesis suggest that bacteria possessing BSH offer good prospects as potential probiotics. However, the evidence at least in the mouse experiments is less than compelling with no difference between the average shedding between control animals and those given the BSH expressing *L. murinus*. However, there could be several explanations for these disappointing data; firstly it may be that the dosage given was insufficient to restore the bacteria to the gut. PCR analysis for the BSH genes showed regained BSH in animals given the BSH+ve *L. murinus* strain but not those given the BSH-ve strain. PCR analysis of later time points showed that this colonisation was transient. Further study (including the direct marking of strains) would be required to directly confirm strains colonisation of the animals. This is important as it would allow the relative efficiency of colonisation by each strain to be determined. At present within this study it is unclear whether a sufficient number of bacteria survived passage through the acidic environment of the stomach to allow effective colonisation of the colon and restoration of BSH function. In the study by Gustafsson *et al.,* (1998) in which 7α-dehydroxylation was recovered following treatment with BSH producing strains, bacteria were

delivered for 5 days, in a freeze dried form at a concentration of $10^9$ CFU bacteria. This would have exposed the mice to much higher numbers of bacteria than used in these experiments. Therefore working out the appropriate dose of probiotic bacteria and timing of delivery is essential. However, as these experiments are dependent on pre-treatment with clindamycin to remove the existing microflora, extension of the dosing times could allow sufficient recovery of the animals own flora that could influence infection outcome.

Another explanation for the failure of this approach may relate to the rate at which the *L. murinus* was able to deconjugate the bile salts *in vivo*. If this was insufficient to influence the balance of bile salts in the gut, normal BA metabolism to the gut would not return. As such the activity of the BSH produced may be important and subsequent isolation of bacteria revealed other strains with higher activity for TC than the *L. murinus* tested *in vivo* (Figure 4.13).

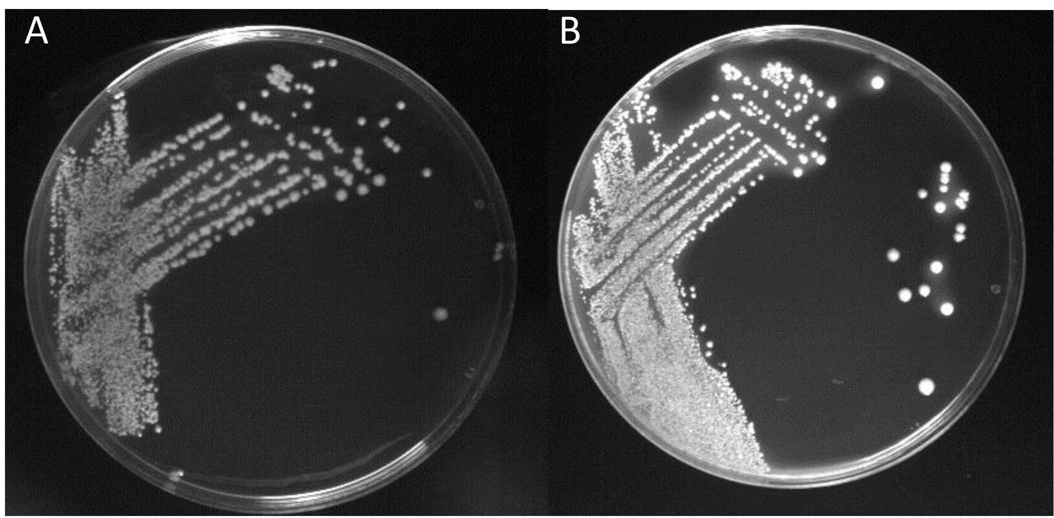



**Figure 4.13 BSH activity of *L. murinus* and an additional BSH producing strain**
BSH activity of A) BSH+ve *L. murinus* and B) an additional BSH producing bacterial strain isolated from MRS agar from a mouse faecal sample. Although both result in the precipitation of CA into the media there is a much higher concentration precipitated around the bacterial strain in B which suggests higher BSH activity in this strain.

In contrast to the situation *in vivo*, *in vitro* experiments use a finite amount of TC in the media, the majority of which can be deconjugated to CA. *In vivo* bile salts are released regularly, meaning very high levels of BSH activity are continually needed to efficiently and rapidly deconjugate these compounds. In addition to this, it has been shown that antibiotic treated animals produce higher levels or taurine conjugated bile salts than untreated animals (1.3). It

may be that a cocktail of BSH producing bacteria with varying substrate specificities are required to allow effective deconjugation of bile salts and therefore replacement of only one strain is insufficient to achieve this effectively.

As described in the introduction, several studies have been performed in which replacement of the normal flora has been shown to effectively treat *C. difficile* infection, both in mice and humans. Closer inspection of these mixtures reveals that many contain bacteria that are capable of BSH activity. In fact, one of the successful cocktails used by Lawley *et al.,* (2012) to treat mice contains one of the bacterium identified in this study, *S. warneri,* a BSH producer. Another bacteriotherapy is 'rePOOPulate' a mixture of 33 bacterial isolates successfully used to treat two patients suffering with relapsing *C. difficile*. Within this mix are several potential BSH producers, including several *Lactobacillus* and *Roseburia* species. It also contains bacteria that may be capable of 7α-dehydroxylation including several Clostridia and *Eubacterium*. So far one study has been published that claims to restore bile metabolism and therefore reduce colonisation and toxin levels *in vivo*. During this study mice were given *C. scindens,* a 7α-dehydroxylating species, before infection. Animals given *C. scindens* had a reduced death rate, lower rates of colonisation and lower faecal toxin titre. Although promising it may be necessary to repeat these experiments with a 7α dehydroxylase knock out strain of *C. scindens* to demonstrate that inhibition *in vivo* is mediated through the production of DCA and not by competition for the same niche. Similar studies have shown analogous decreases in disease severity when animals were treated with non-toxigenic *C. difficile* in both mice and humans, however, the mode of protection here is more likely to reflect niche competition (Gerding *et al.,* 2015; Zhang *et al.,* 2015). Unravelling bacterial interactions *in vivo* is challenging but the continued development of targeted microbiome therapeutics is showing promising signs.

This chapter has built upon the potential use of BSH producing bacteria as part of a therapeutic for difficult to treat *C. difficile* infections. Use of these bacteria warrants further investigation as an alternative treatment to FMT, in which undefined bacterial combinations are used to treat patients suffering from recurring disease. The data presented in this chapter and in chapter 3 show how important the microbiota is in determining the outcome of *C. difficile* infection.

In the next chapter the influence of TC on genetic regulation of germination and outgrowth will be investigated.

# 5 The influence of taurocholic acid on genetic regulation of germination and outgrowth

## 5.1 Introduction

BAs have been shown to influence a range of processes involved in virulence and BA resistance in a multitude of enteric bacteria (1.3.3). In *C. difficile* BAs, specifically TC, are known to be important in regulating spore germination (Sorg & Sonenshein, 2008) however, the influence of TC on genes expressed post germination is unknown. A range of new molecular techniques are making it easier to unravel the genetic regulation of these processes. In *C. difficile* the genes involved in germination are becoming more clearly defined after improvements in methods of genetic manipulation including ClosTron mutagenesis (Heap et al., 2010). In these situations, the importance of specific genes can now be established by mutation in those genes linked to (or inferred by association with homologous genes in other sporulating bacteria) germination. However, this approach does not allow identification of novel genes associated with this process. Transposon mutagenesis has traditionally allowed the rapid production of libraries of single gene mutants which can be tested phenotypically. Additional linkage of mutagenesis with whole genome sequencing (WGS) is a powerful tool that allows for rapid and high-throughput analysis of multiple mutants. However this technique is less useful in identifying genes involved in gene regulation post germination as it can only identify essential genes. BA resistance is a complicated process with many of the genes involved having functional redundancy. This means that loss of one gene involved in the process can result in upregulation of another that can compensate. This would make the construction of a mariner transposon (MT) library inappropriate for investigating this process. A technique that allows for the identification of genes whose expression is significantly altered under specific conditions is RNA-seq. Use of this technique allows for the identification of genes whose expression is significantly altered and may allow for the identification of genes in *C. difficile* that are important in modulating virulence and bile tolerance. These genes may yield important information on the infection process.

Analysis of RNA-seq data was carried out with the assistance and support of Dr James Connolly. Production of the transposon mutant library was carried out in collaboration with Kassie McLaren. Microscopy was performed using procedures and protocols optimised by Dr Anthony Buckley.

## 5.2 Results

Initially unique genes involved in spore germination were identified through the production of a random transposon library of *C. difficile* mutants.

### 5.2.1 Creation of a random transposon library of *C. difficile*

In order to identify genes that are involved in TC mediated spore germination it was first necessary to create a transposon mutant library.

#### 5.2.1.1 Conjugation of pRPF215 into *C. difficile*

The first step in creating the transposon mutant library required the introduction of the pRPF215 plasmid (a kind gift from Dr Robert Fagan, University of Sheffield). The pRPF215 plasmid contains the transposon and *erm*B gene required for the selection of mutants. To create a transposon mutant library the plasmid was first conjugated into *C. difficile* R20291 by conjugation using the donor strain of *E. coli* CA434 (Purdy et al., 2002) using the approach described in section 2.7.1.2. Successful transfer of the plasmid was confirmed by PCR amplification of a region of the *erm*B gene from *C. difficile* colonies recovered after conjugation experiments.

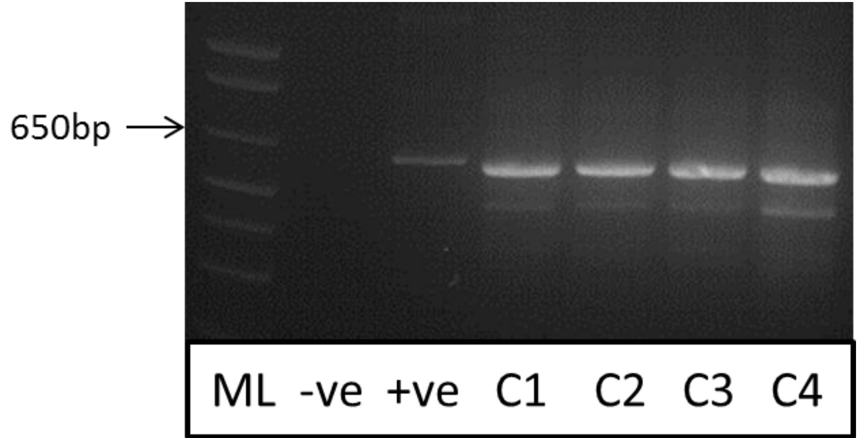



**Figure 5.1 Confirmation of the transfer of pRPF215 into *C. difficile* R20291 by conjugation**
Primers directed to a region of the *erm*B domain within the plasmid were used to confirm the successful conjugation of the plasmid from *E. coli* into *C. difficile*. DNA was extracted from colonies and amplified to confirm plasmid carriage. The expected fragment size was 600bp.

The transposon mutant library was subsequently generated from *C. difficile* isolates in which plasmid carriage was confirmed. In brief, a single colony of *C. difficile* containing the plasmid was grown to an $OD_{600}$ of 0.4 before being diluted and cultured on plates containing lincomycin and anhydrotetracycline. This allows for simultaneous induction of the MT and selection for mutants.The induction of the transposon is controlled under a pair of promoters with overlapping Tet operator sequence (TetO). One of these promoters (PtetR) controls the expression of TetR which acts as transcriptional regulator that represses both promoters. As expression of the MT is under the control of PTet, addition of anhydrotetracycline results in a conformational change in TetR ending repression of the system and allowing expression of the MT (Cartman & Minton, 2010). Incorporation of the transposon containing the *erm*B gene into the genome renders the mutant lincomycin resistant, allowing mutants to grow in the presence of this antibiotic. The vast majority of isolates will represent mutants with only 1 transposon present within the genome. This is because once the transposon has integrated into the genome the transposase inhibits further integration, although the precise mechanism of this is not understood (Cartman & Minton, 2010; Muñoz-López & García-Pérez, 2010). The growth from plates that contained well isolated colonies were scrapped and the mutants pooled for use in subsequent experiments.

## 5.2.2 Screening of the transposon mutant library for germination mutants

In order to screen the library for germination mutants it was first necessary to allow them to form spores. To select for germination mutants, spores from the mutant library were incubated in 10% TC for 24 hours, a concentration 100 times higher than would be typically used. To screen for those spores that were unable to germinate under these conditions the culture was heated at 65°C for 30 minutes killing vegetative cells and leaving spores. The remaining spores that had failed to germinate in the presence of TC were artificially germinated using a combination of thioglycollate and lysozyme.

Using this approach, we recovered hundreds of colonies that appeared only able to germinate in response to artificial germination. These colonies were all resistant to lincomycin suggesting that all contained the transposon within the genome. As such we presumed that these were germination deficient mutants that were unable to germinate in the presence of high concentrations of TC. To allow greater characterisation of these mutants, 20 were selected for further analysis.

### 5.2.2.1 Verification of germination deficient mutants

To verify that the mutants selected were unable to germinate in the presence of TC, spores of each mutant were plated onto BHI agar containing 0.1% TC, or artificially germinated using thioglycollate and lysozyme. True germination mutants should only form spores after artificial germination. Of the 20 mutants tested, only 6 (highlighted in red) were subsequently identified as ‘true’ germination mutants with the other 14 able to form small colonies in the presence of TC (Table 5-1).

**Table 5-1 Verification of germination null phenotype in 20 presumed germination mutants**

| | Artificial germination | Taurocholic acid |
|---|---|---|
| Colony | Colonies formed | |
| WT | + | + |
| 1 | + | + |
| 2 | + | + |
| 3 | + | + |
| 4 | + | + |
| 5 | + | + |
| 6 | + | + |
| 7 | + | + |
| 8 | + | + |
| 9 | + | - |
| 10 | + | - |
| 11 | + | + |
| 12 | + | - |
| 13 | + | - |
| 14 | + | + |
| 15 | + | - |
| 16 | + | + |
| 17 | + | + |
| 18 | + | + |
| 19 | + | - |
| 20 | + | + |

The high number of false positives suggests that only ~30% of the germination mutants were 'true' germination mutants. The reason for this poor level of discrimination is unclear and suggests that modification of the protocol would be

necessary to further increase the numbers of true germination mutants isolated. Traditionally the site of insertion of a transposon would be determined using Transposon Directed Insertion Sequencing (TraDIS). This system allows for rapid and high throughput identification of transposon insertion sites by allowing amplification from the transposons into the flanking gene (Barquist et al., 2016; Dembek et al., 2015; Langridge et al., 2009). However, as the selection of mutants appeared poorly discriminating, it was decided that whole genome sequencing (WGS) of the 6 'true' germination mutants would be used to determine the insertion location of the individual transposons.

#### 5.2.2.2 Genome sequencing and determining site of transposon insertion

Genomic DNA was extracted from each germination mutant (2.4.1.1) and sent for genome sequencing on the MiSeq platform at Glasgow Polyomics Facility (GPF). Assembly and annotation of mutant genomes was carried out as described in section 2.7.1.5. Annotated contigs were imported into CLC genomics work bench for visualisation. To identify the transposon insertion site an internal BLAST search was carried out for the MT in the annotated contigs. In all 6 genomes the MT was found in the *csp*BA gene with two different insertion positions within mutants identified.

| Germination mutants | Location from start of gene |
|---|---|
| 1 | 3757bp |
| 2 | 3757bp |
| 3 | 3757bp |
| 4 | 415bp |
| 5 | 415bp |
| 6 | 3757bp |

The *csp*BA gene produces CspB, a protease involved in the activation of SleC and CspA a catalytically inactive pseudoprotease that is involved in controlling levels

of CspC (1.2.2.1). Inactivation of CspB is known to halt germination in its first stage as it prevents activation of SleC, a spore lytic enzyme, preventing spore cortex hydrolysis and therefore halting germination at this stage (Figure 5.2).

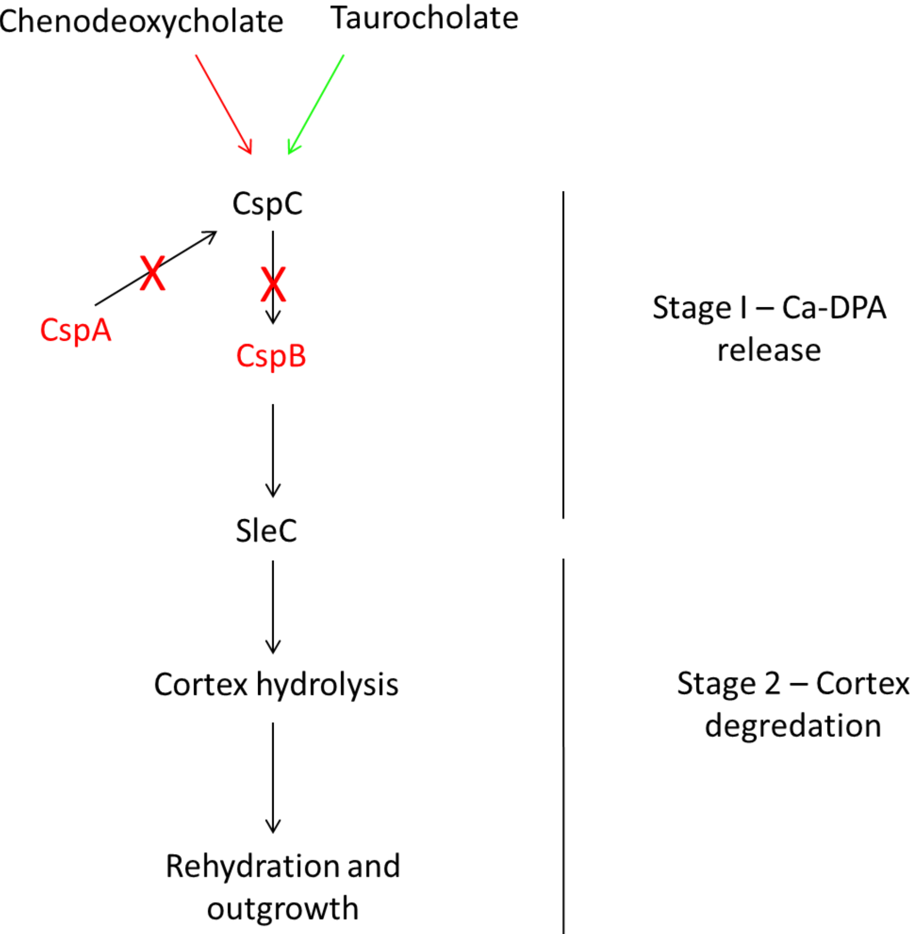



**Figure 5.2 Inhibition of germination by inactivation of cspBA genes**
The genes involved in *C. difficile* spore germination with the genes disrupted by insertion of the transposon shown. Disruption of the *csp*BA gene results in the halting of germination immediately after germinant sensing by CspC.

### 5.2.3 Modification of the transposon system with the addition of a phiLOV domain

The successful identification of a gene known to be involved in germination stimulated further interest in the use and extension of this technology. In recent years, work by this group has focussed on the use of a fluorescent reporter that can be used under the anaerobic growth conditions required by *C. difficile*. In particular, we have tested LOV domains as reporters of gene expression. LOV domains are found in plants, bacteria, fungi and algae. Excitation of these domains by blue light, results in a conformational change leading to the

formation of a covalent bond between an FMN chromophore and a cysteine. Decay of this bond results in the release of energy in the form of green fluorescence. Unlike other fluorescent reporters such as green fluorescent protein (GFP), LOV does not require molecular oxygen to function making it ideal for use in obligate anaerobes such as *C. difficile* (Figure 5.3). These domains have successfully been used as fluorescent reporters in plant viruses (Chapman et al., 2008) and bacteria (Gawthorne et al., 2012, 2016) and work by this group has demonstrated their use in *C. difficile* (Buckley et al., 2016).

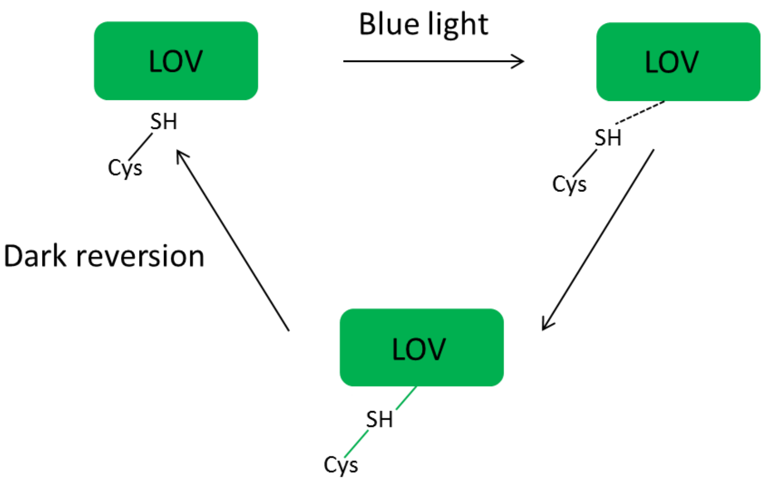



**Figure 5.3 Excitation of phiLOV domains.**
Schematic showing the reversible photo bleaching cycle of the phiLOV domain adapted from Christie (2007).

The LOV domain has been modified to enhance its photostability and fluoresce resulting in the creation of improved photostable LOV (phiLOV) (Christie et al., 2012). Transposon mutant libraries can be used to determine genes that are essential to a process and although incredibly useful, this approach provides little detail of genes that are significantly upregulated in particular circumstances but which are non-essential for that process. To try and address this issue, we attempted to harness the power of transposon mutagenesis and the phiLOV fluorescence. Modification of the pRPF215 plasmid to incorporate the phiLOV domain was carried out by Dr Rob Fagan (UoS). Incorporation of the LOV domain into the plasmid backbone was achieved by Gibson assembly of the amplified LOV domain into the plasmid. The LOV domain was modified to contain a ribosomal binding site to allow for expression. Expression of the LOV domain and fluorescence was dependent on the extent of expression of the gene in which the transposon was inserted (Figure 5.4).

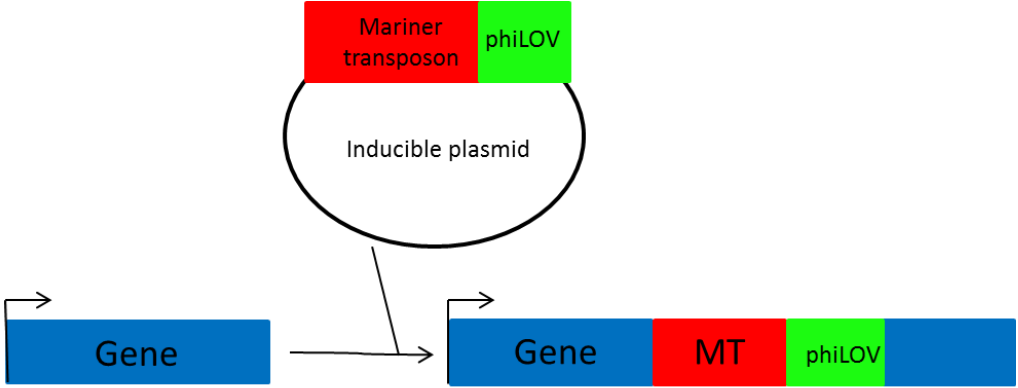



**Figure 5.4 Schematic of mariner-phiLOV system**
Diagram showing the structure of the MT-phiLOV pRPF215 plasmid and gene insertion.

The ultimate aim of this approach, was to allow identification of those mutants in which expression of particular genes were upregulated to particular stimuli (such as growth *in vivo*). As fluorescent light is the read out, it was hoped that such mutants could be selected and isolated using a Fluorescent Automated Cell sorter (FACs). It was hoped that by using this approach, several pools of mutants with differential levels of gene expression may be identified including those genes that are not essential but are highly upregulated under those conditions.

#### 5.2.3.1 Conjugation of PYAA023 into *C. difficile*

Conjugation of PYAA023 was carried out as described in 5.2.1.1. Successful conjugation of this plasmid was confirmed by PCR using primers that specifically amplified the phiLOV domain within the PYAA023 plasmid.

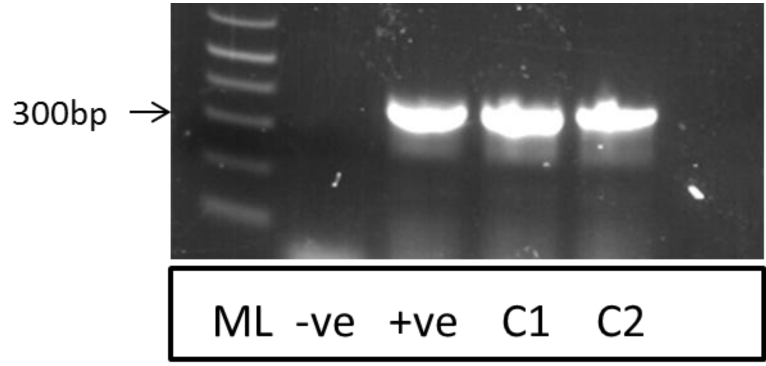



**Figure 5.5 Confirmation of the transfer of pYAA023 into *C. difficile* R20291 by conjugation** Primers directed to the LOV domain within the plasmid were used to confirm the successful conjugation of the plasmid from *E. coli* into *C. difficile.* DNA was extracted from colonies and amplified to confirm plasmid carriage. Expected fragment size of 336bp.

#### 5.2.3.2 Visualisation of phiLOV expressing transposon mutants

To test whether insertion of the transposon could result in differential expression of the LOV domain, the library was initially tested by inoculating the recovered mutants into BHI broth, which was cultured for 24 hours. Small volumes of this culture were then removed, washed with PBS and fixed in 10% formal saline. These fixed bacteria were then placed on a slide and air dried. Before use slides were stored in the dark to ensure maximal visualisation of LOV. Slides were visualised using an Axio-Zeiss Imager M1 light microscope to determine if fluorescent mutants displaying differential levels of phiLOV expression could be visualised (Figure 5.6).

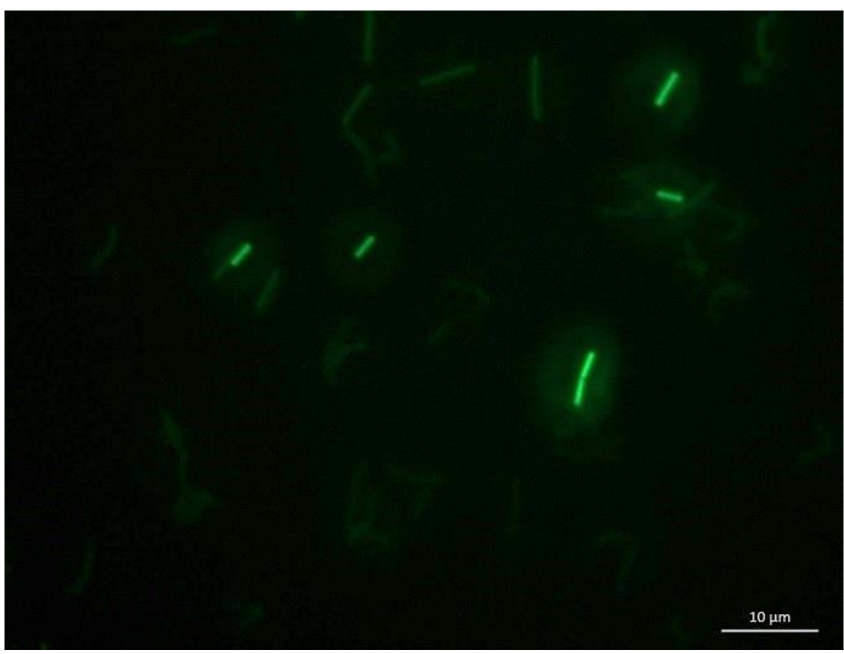



**Figure 5.6 Visualisation of library of modified mariner transposon mutants of *C. difficile* expressing the phiLOV domain**
10ul of spores from the mutant library were taken and inoculated into BHI broth and incubated for 24 hours. Differential expression of the LOV domain is apparent. Bacterial slides were examined using an Axio-Zeiss Imager M1 light microscope. Deconvolution of raw data was performed using a point-spread function with 30 rounds of iteration. Microscopy was carried out with the assistance of Dr Anthony Buckley.

Observation of these cultures by microscopy confirmed that phiLOV expressing bacteria were identifiable within the culture. Bacteria expressing differential levels of fluorescence were observed (Figure 5.6). This suggests that insertion of the phiLOV domain into different genes may allow identification of mutants with differing levels of gene expression. However, to allow enrichment and isolation of these high phiLOV expressers, it was essential that these cells could be differentiated by FACS. To determine whether this was feasible, the remaining culture was washed with PBS, fixed with formalin and subject to FACs (Aria III). As the optimal wavelength for excitation of phiLOV (450nm) was not available using the FACs used, cultures were subject to suboptimal stimulation using the wavelength used for GFP (525–550nm). Under these conditions, attempts to sort the library proved unsuccessful. While the lack of optimal excitation light may provide one explanation, others include the size and natural autofluorescence of *C. difficile*. Using the suboptimal settings, the fluorescence of the phiLOV domain was not sufficiently high to allow differentiation from the natural levels of green autofluorescence expressed by these bacteria. In addition, the small size of bacteria (*C. difficile* are approx. 5μm) makes sorting individual cells is very challenging. Without an appropriate high throughput screening approach, it

did not seem relevant to continue our attempts to use this technology. This still remains a valid approach and time might provide the improvements that will make this approach feasible in the future.

Focus was then moved to investigating the potential role of TC on regulation of genes expressed during outgrowth.

### 5.2.4 Investigation of the role of TC on gene expression during outgrowth

In multiple bacterial species including *Vibrio parahaemolyticus, Campylobacter jejuni* and *Salmonella typhimurium* bile salts have been found to regulate an array of virulence and resistance genes (1.3.3). Although TC plays an important role in regulation and induction of *C. difficile* spore germination, its impact on gene regulation post germination has not been previously investigated. To further explore the role of BAs in *C. difficile* pathogenesis, we chose to study the impact of TC on gene expression in the vegetative, post germination bacteria. This was undertaken using RNA-seq, which allows identification of genes whose expression is modified resulting in increased specific mRNA transcripts. This offers an opportunity to identify genes involved in bile tolerance and potentially virulence in *C. difficile*.

#### 5.2.4.1 Preparation of the RNA seq library

To analyse the impact of TC on expression of genes in *C. difficile*, the bacteria were grown to an $OD_{600}$ of 0.4. Once bacteria had reached this density TC was added to one sample to a final concentration of 0.1% whilst the other was adjusted to the same volume using fresh media. Bacteria were then harvested and RNA extracted as described in section 2.7.2. In total 3 biological replicates were prepared for each sample. In the first attempt the concentration of RNA was low and the quality poor so sample isolation was repeated with new reagents to ensure that no RNase contamination of the samples. To ensure that RNA samples were free from DNA contamination amplification of the 16s rRNA gene was performed. Lack of band formation suggested that the RNA was DNA free. Additionally, samples were checked for quality, DNA contamination and RNA degradation using a 2100 Bioanalyzer (Agilent). After RNA was found to be of good quality mRNA was reverse transcribed into cDNA before library

preparation and single ended sequencing on the Miseq platform. Analysis with the Bioanalyzer, cDNA production, library prep and sequencing was all carried out at GPF.

#### 5.2.4.2 Identification of significantly altered genes after exposure to taurocholate

Analysis to identify genes whose expression was significantly altered after TC exposure was carried out using CLC genomics workbench as described in section 2.7.3. In total 10 genes were found to be significantly altered after incubation with 0.1% TC compared to growth in BHI alone. Of these 10, 4 were rRNA subunit genes (data not shown). These rRNA subunits most likely represent differences in rRNA depletion efficiency between samples rather than anything of biological interest so were not investigated further. Other significantly upregulated genes are shown in Table 5-2.

**Table 5-2 Genes that are significantly upregulated after 1 hour exposure to 0.1% TC**

| Gene | Potential function | Adjusted P value |
|---|---|---|
| CDR20291_2128 | Efflux pump | 1.2477e−9 |
| CDR20291_2298 | Multidrug efflux pump | 6.5192e-4 |
| rpoD | RNA polymerase sigma factor | 3.9447e-3 |
| CDR20291_2296 | Efflux pump | 7.0659e-3 |
| CDR20291_2297 | Efflux pump | 7.5207e-3 |
| CDR20291_1886 | S-adenosyl-L-methionine -dependent methyltransferase (SAM dependent MTase) | 7.6637e-3 |

Unsurprisingly, 4 or the 6 genes reported here are efflux pumps. These efflux pumps are most likely involved in increasing *C. difficile* tolerance to bile salts, minimising the biological impact of these molecules. Bile salts are able to disrupt membranes and cause DNA damage so rapid efflux of these molecules can help to prevent damage to the cell. Upregulation of efflux pumps is a generalised response to various environmental stresses, including bile, and it

would be interesting to see if bile exposure increases *C. difficile* resistance to other environmental stresses such as antibiotic exposure.

Interestingly a S-adenosyl-L-methionine (SAM) dependent methyltransferase (MTase) was also upregulated following exposure to bile. These enzymes are important in a number of biological processes; using SAM as a methyl donor these enzymes methylate various biological molecules including proteins, lipids, DNA and RNA. The potential function of these enzymes with the cell is huge as they mediate many essential biological processes such as protein trafficking, signal transduction and gene expression. In this case, the role of SAM dependent MTase within the cell is not clear however it is likely it enables the bacteria to respond to bile salt exposure. In addition, there was also an increase in *rpo*D expression. This is an RNA polymerase sigma factor that is frequently linked to the transcription of housekeeping genes. This suggests protection from bile salts is mediated by rapid response to exposure and induction of genes that enable the cell to protect its DNA, RNA and membrane.

During collection of the samples for RNA extraction it was noted that exposure to TC resulted in a change in the way the cells interacted, with the bacterial pellet formed following exposure to TC much more difficult to resuspend (Figure 5.7).

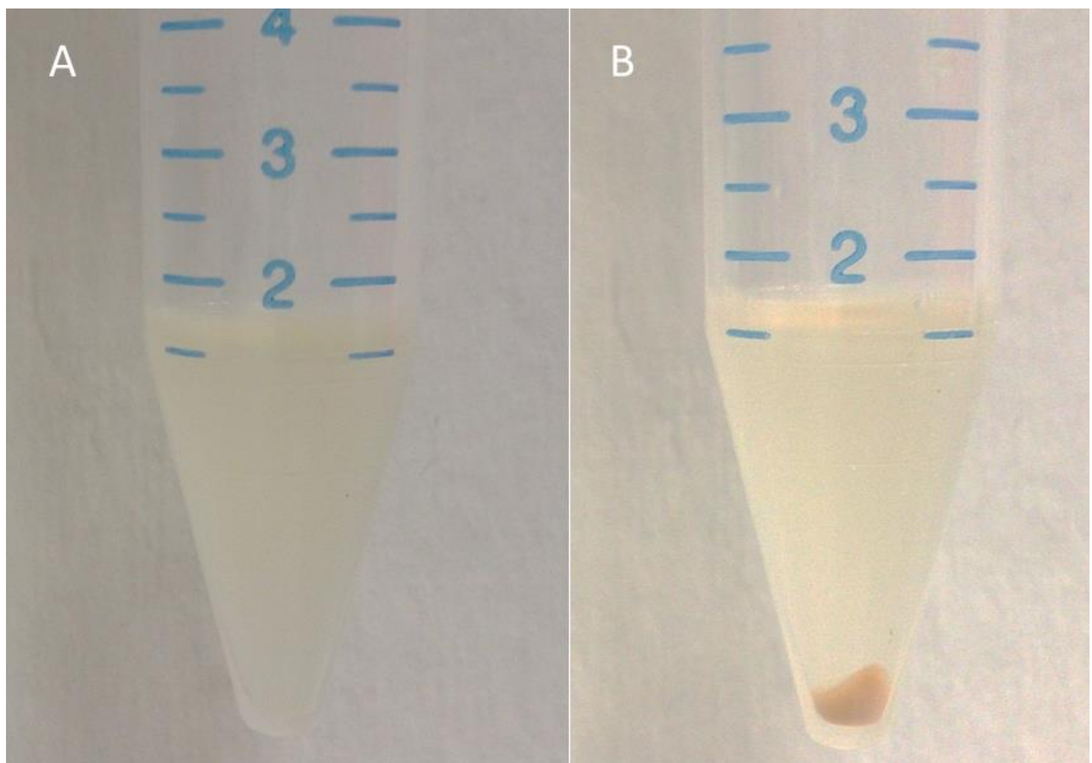



**Figure 5.7 Aggregative phenotype of *C. difficile* after exposure to 0.1% TC**
Resuspension of bacterial pellets grown in A) BHIS only and B) BHIS with 1 hour exposure to 0.1% TC. Exposure to TC results in increased aggregation of *C. difficile*.

As a consequence of this observation, we wished to determine if bile salt exposure altered the capacity of the bacteria to aggregate and form biofilms that can have relevance in clinical disease.

## 5.2.5 Biofilm formation at 24 and 48 hours

To further investigate the impact of TC on biofilm formation, two types of biofilm assay were performed. The first aimed to qualitatively determine the extent of biofilm formation in non-sedimentary liquid culture, whilst the second provided quantitative assessment of biofilms in sedimentary cultures using crystal violet staining.

### 5.2.5.1 Biofilm formation in non-sedimentary liquid culture

To investigate if exposure to TC affected the capacity of bacteria to interact, bacteria were grown in 25cm$^2$ tissue culture flasks, incubated upright and statically at 37$^o$C for 24 and 48 hours. The capacity of the bacteria to form biofilms was tested with and without 0.1% TC in four different growth media. These included samples with/without the addition of 0.1% TC to the overnight

culture or biofilm media. TC, either added during the preparation of an overnight culture used to seed the biofilm or in the media in which the biofilms were cultured, resulted in biofilms that were more adherent than those grown only with BHIS. Measuring adherence is challenging and in these experiments it was measured crudely by agitating the flasks to disrupt the biofilm (Figure 5.8).

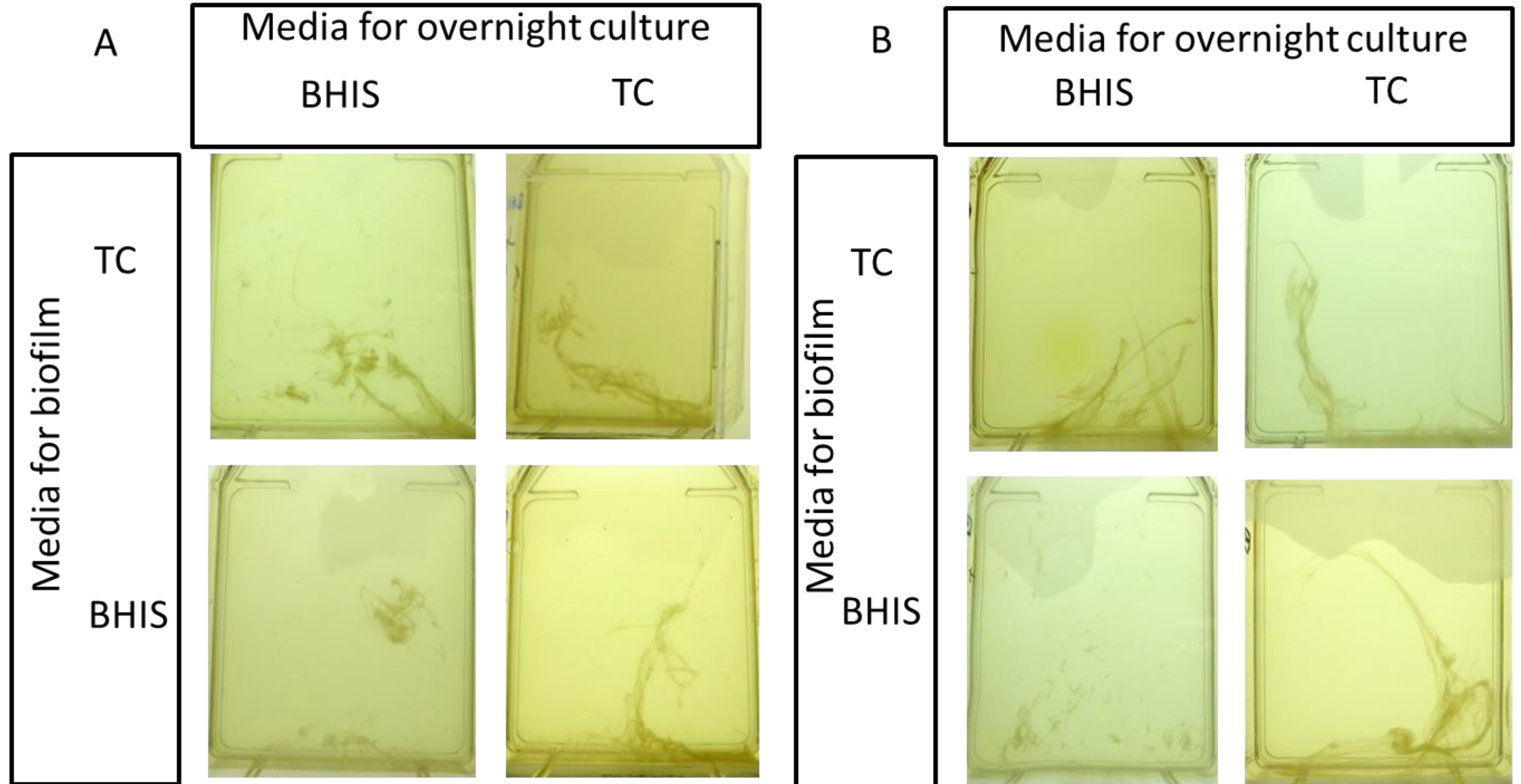



**Figure 5.8 Biofilm formation in non-sedimentary liquid culture**
Overnight cultures grown either in BHIS+C alone or with the addition of 0.1%TC were used to seed tissue culture flasks containing either BHIS+C alone or with 0.1%TC. These were incubated for A) 24 or B) 48 hours before the adherence of the biofilm to the flask was tested by mechanical disruption.

Biofilms grown in BHIS alone were easily dislodged from the base of the flask whereas biofilms grown with TC, either as part of the overnight or as part of the biofilm media, required vigorous shaking before the biofilm was dislodged. This suggests that TC may increase biofilm adherence although the mechanism for this is unclear.

#### 5.2.5.2 Crystal violet staining to quantify biofilms

Static biofilm assays show that the addition of TC increases biofilm adherence to an abiotic surface. However these assays cannot show any changes in the quantity of biofilm formed. To investigate this CV assays were carried out with the same growth conditions at 24 and 48 hours. These assays involve staining the biofilms with CV and then quantifying the amount of stain retained by the biofilms by measuring the OD. Measurements were taken at 24 and 48 hours (Figure 5.9).

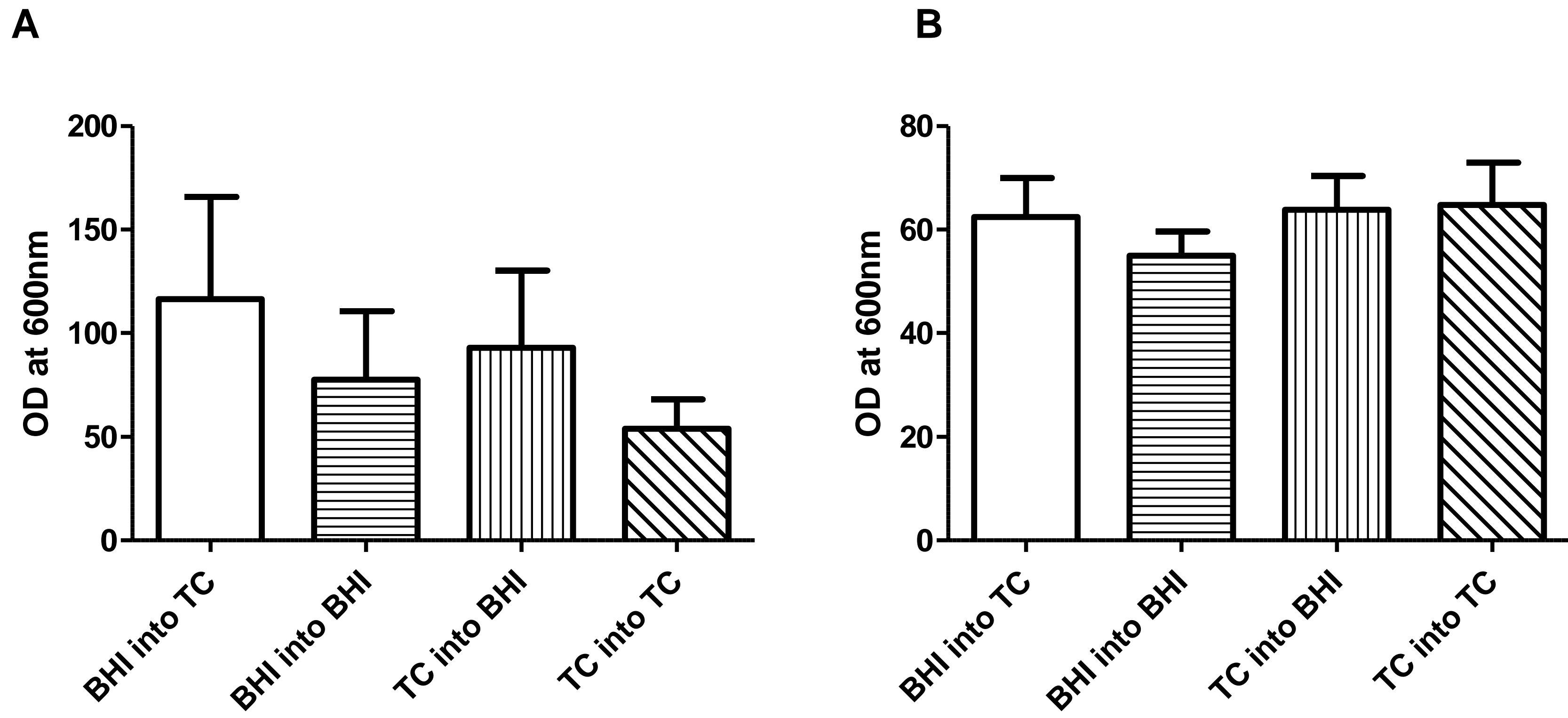



**Figure 5.9 *C. difficile* biofilm formation under different growth conditions**
Biofilms were grown in multiple different media compositions to determine the impact on biofilm formation at A) 24 hours and B) 48 hours. Overnight cultures to seed the biofilms were grown in the first media noted and biofilms in the second. BHI indicates BHIS+C media and TC is this media with the addition of 0.1%TC. 3 biological replicates, each with 3 technical replicates, were carried out for each condition. At 24 hours the addition of TC in the overnight of biofilm resulted in a slight increase in biofilm abundance although this was not significant. At 48 hours there were no differences in biofilm abundance.

At 24 hours there seemed to be higher levels of biofilm formed in growth conditions that used a combination of BHIS and TC, either as the media for the overnight culture or the biofilm itself. Although there was an increase in OD values this is not significant. Additionally, biofilm assays showed high variation making it difficult to determine if these changes represent any significant increase in biofilm abundance. At 48 hours all growth conditions show very similar levels of biofilm abundance suggesting that TC does not alter biofilm quantity. It is unclear how the inclusion of TC in the media increases adherence and the RNA seq data does not show any changes that can obviously associated with this. It may be that if any changes in expression that are responsible fall below the significance threshold or do not occur within the time frame examined. It is also possible that incubation with bile results in changes to membrane charge that may promote aggregation. It may also be that extracellular factors such as release of DNA may facilitate aggregation.

## 5.3 Discussion

### 5.3.1 Identification of germination mutants

The creation of a transposon mutant library was used to identify genes involved in bile mediated spore germination. Germination is an essential process in *C. difficile* infection so understanding genetic regulation of this process is important. Much of the pathway is now understood, however there are still some gaps in our understanding. Germination response varies between *C. difficile* isolates meaning studying the process in multiple isolates is important and may give more insight into the role germination plays in infection and disease outcome (Bhattacharjee *et al.,* 2016). Attempts to utilise a MT coupled with phiLOV fluoresce to identify non-essential but upregulated genes proved unsuccessful. Although the fluorescence of the phiLOV domain was above background levels (Figure 5.6) it was not sufficient to allow high throughput sorting of cells from those not expressing the domain. Unfortunately *C. difficile* has high levels of background green autofluorescence, making the use of fluorescence reporters for this kind of process challenging. For this reason

attempts to use this system for identifying genes involved in germination were not continued.

When the MT was used to identify mutants that were unable to germinate many hundreds of colonies were formed, however further investigation showed that only 30% of these were true germination mutants. The reason for overrepresentation of non-specific mutants may reflect to poor selection for spores from the vegetative cells. It may be that heating at 65°C for 30 minutes was insufficient to kill all of the vegetative cells. However this has always proved sufficient during other experiments in our lab. Insufficient heating would allow these cells to remain within the pool resulting in false positives after selection. In this study a total of 6 confirmed mutants were sent for WGS and the site of transposon insertion identified. All 6 mutants were found to have a mutation in the same gene, *csp*BA. This is unsurprising as it has previously been shown to play an essential role in spore germination. The small sample size of mutants dramatically reduced the likelihood of identifying multiple different genes. A similar study that used chemical mutagenesis to create germination mutants sequenced the genomes of 10 of these mutants. All of the mutants in their study were found to have mutations localised in the *csp*BAC operon suggesting that disruption of these genes are the most common in germination mutants. One advantage of the MT system of creating mutants is that there should only be one insertion site and therefore one gene disrupted per mutant whereas chemical mutagenesis may result in large numbers of mutations within many genes. If the phiLOV domain had enabled sorting it may have been possible to identify some of the non-essential but upregulated genes in this process. For example genes that may have functional redundancy such as CspB which is the main protease responsible for activation of SleC although potentially not the only one (Adams *et al.*, 2013).

The method here was further limited by the way the MT insertion was identified. Genome sequencing is cheap but selection of only a few mutants limits the possibility of identifying novel genes. Use of high throughput methods for identifying the transposon insertion site, such as TraDIS, were not possible here due to the high rate of false positives in the selection for germination mutants. However, the successful identification of genes involved in spore germination

means that this method is appropriate for investigating genes involved in spore germination and other biological processes which could be potentially utilised further.

## 5.3.2 The impact of TC exposure on *C. difficile*

### 5.3.2.1 Comparison of genes expressed with and without exposure to TC

Bile salts have previously been shown to play a number of roles in the expression of a host of genes including some involved in virulence in various bacterial species (1.3.3). Our investigation used RNA seq to identify changes in gene expression following exposure for 1 hour to 0.1% TC. In total 6 genes were found to be significantly upregulated. The reason for this low number of significantly differentially expressed genes is unclear as sequence coverage was acceptable in all samples. Within these 6 there were 4 efflux pumps. The role of efflux pumps in bile resistance has been well described in many bacterial species including both commensals (Fang *et al.,* 2009; Gueimonde *et al.,* 2009) and pathogens (Baucheron *et al.,* 2014 ; Quillin *et al.,* 2011). Response to environmental stressors is important for increasing *in vivo* survival. Increased expression of efflux pumps can result in increased resistance to other environmental stressors and in some cases exposure to bile has been shown to increase antibiotic resistance (Prouty *et al.,* 2004). One of the efflux pumps that is upregulated is CDR20291_2297, which has been identified as a LexA controlled efflux pump which is suggested to be involved in mediating antibiotic resistance in *C. difficile* (Walter *et al.*, 2014). LexA is an important stress response regulator and multiple genes involved in antibiotic resistance, sporulation, house-keeping and virulence controlled by LexA (Walter *et al.,* 2015; Walter *et al.,* 2014). Although only one gene from this operon was upregulated it shows that bile induces common stress response pathways within *C. difficile.* Further investigation of the response of *C. difficile* to bile salts that more closely mimic the range and levels seen in a healthy gut and antibiotic treated gut may be interesting and give a clearer picture of how *C. difficile* interacts with bile salts *in vivo*.

#### 5.3.2.2 Exposure to bile increases adherence

It was noted that after exposure to TC bacterial pellets became difficult to resuspened and seemed to aggregate more than the bacteria grown in BHIS alone. Biofilm quantity was measured and it was found that exposure to TC did not seem to increase the quantity of biofilm formed. It may be interesting to carry out viable counts of these biofilms in the future as this may give a more precise measure of biofilm quantity. Although there did not seem to be increased abundance of biofilm those that were formed seem to adhere more strongly to the plastic flasks with increased agitation required to disrupt these biofilms compared to those grown without TC. Interestingly this included samples where TC was only present in the overnight culture used to seed the biofilm. This suggests that addition of TC induces some phenotypic or morphological change that persists once TC is removed. Many bacterial species have been shown to form biofilms after exposure to bile salts (1.3.3.2). Increased aggregation has been noted as a precursor to biofilm formation in *Bifidobacteria* and this aggregation was linked to increased cell surface hydrophobicity (CSH). Increased CSH meant that cells were able to bind more tightly to mucin, something that would increase survival *in vivo* (Ambalam *et al.*, 2013). Measurement of CSH in *C. difficile* with and without exposure to bile may provide further insight to the role of aggregation and its role *in vivo*. In *Bacteroides fragilis* exposure to bile salts results in increased aggregation and also the production of fimbria like appendages (Pumbwe et al., 2007). It could be that in *C. difficile* similar phenotypic changes occur that result in the increased aggregation seen.

BAs, especially TC, play an important role in *C. difficile* germination and outgrowth, as described in chapters 3 and 4. Work here has shown that exposure of *C. difficile* to TC leads to an induction of multiple efflux pumps and an increase in bacterial aggregation. These changes may aid the survival of *C. difficile* within the host and further investigation of these mechanisms may help to increase understanding of the process of colonisation and persistence. Loss of colonisation resistance and dysbiosis are clearly essential for initiation of *C. difficile* disease, however, little is known about how the organism is able to maintain the gut in a dysbiotic state in order to persist. The next chapter will

investigate the production of the bacteriostatic compound *para-cresol*, and its potential role in limiting the capacity of the microflora to re-establish its complexity.

# 6 The role of *para-cresol* production in acute and relapsing infection

## 6.1 Introduction

The structure and diversity of the host microbiota has been shown to play a vital role in determining the extent and outcome of *C. difficile* infection (Chapter 3). Whilst dysbiosis is initially linked to the use of broad-spectrum antibiotics which can lead to a reduction in colonisation resistance, it is the long term maintenance of reduced diversity that is associated with susceptibility to relapsing disease (Chang et al., 2008). The microbiota consists of trillions of bacteria in a highly competitive and densely populated environment. Microorganisms must compete for resources, space and nutrients in order to thrive within the ecosystem. Competition between bacterial species is well documented and includes the production of antimicrobials and toxic by-products (1.4.5). A classical example of this is the production of bacteriocins. These typically narrow spectrum protein antibiotics inhibit closely related species, providing the producer with a competitive edge over closely related bacteria that may be competing for the same niche. The mechanism by which *C. difficile* can similarly suppress recovery of the microbiota is unknown but the production of the toxic metabolite *para*-cresol has been hypothesised the mechanism by which *C .difficile* may secure its niche (Dawson *et al.,* 2011; Dawson *et al.,* 2008; Selmer & Andrei, 2001). The ability of *C. difficile* to produce *para*-cresol seems to be rare, with the process not found many other bacterial species (Elsden *et al.,* 1976; Hafiz & Oakley, 1976). Supporting this hypothesis is the evidence that *C. difficile* can tolerate high concentrations of *para*-cresol , with the epidemic and virulent 027 strains able to produce and tolerate the highest concentrations (Dawson *et al.,* 2011; Dawson et al., 2008). These strains have been linked with more persistent dysbiosis in patients infected with *C. difficile* (Skraban et al., 2013).

*Para*-cresol is produced by the degradation of tyrosine via the intermediate para-hydroxyphenylacetate (*p*-HPA). The genes involved in the conversion of tyrosine to *p*-HPA are currently unknown, but the decarboxylation of *p*-HPA is

carried out by the *p*-HPA decarboxylase encoded by the *hpd*BAC operon (Figure 6.1)

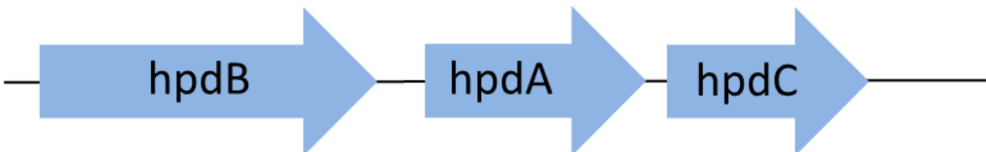



**Figure 6.1 The *hpd*BAC operon that is involved in the conversion of p-HPA to *para*-cresol in *C. difficile* adapted from Selmer & Andrei (2001)**
The *hpd*BAC operon consists of 3 genes *hpd*A, *hpd*B and *hpd*C. The function of *hpd*C is unknown. The *hpd*B gene encodes the pHPA decarboxylase that catalyses the conversion of *p*-HPA to *para*-cresol. The *hpd*A gene is thought to encode an activator of the *hpd*B gene. Independent mutants in all of these genes halt the production of *para*-cresol from p-HPA suggesting that all of the genes are essential for the production of *para*-cresol (Dawson et al., 2011).

Independent mutants in each of the genes described in this pathway have been generated by Dawson *et al.,* (2011) and result in the inability of these *C. difficile* strains to produce *para*-cresol in media supplemented with *p*-HPA. These modified strains were produced using ClosTron mutagenesis (Heap *et al.,* 2010; Kuehne *et al.,* 2011). ClosTron is a system that allows disruption of a gene of interest using a targeted intron system. This results in the insertion of an antibiotic resistance gene, in this case lincomycin resistance, into the gene of interest allowing for easy selection of mutants. All mutants used in this chapter are derived from the archetypal 027 ribotype parental strain, R20291. The mutant used in this chapter is in the *hpd*A gene and will be referred to as R20291_*hpdA*::CT, however similar observations have been observed using a second mutant R20291_*hpd*C::CT. A third independent mutant was also included in these experiments to ensure that experimental differences were not influenced by ClosTron mutagenesis. This third mutant was in a gene encoding an unrelated RNA polymerase sigma factor (CD*1754*) from the extra cytoplasmic function (ECF) subfamily, the specific function of which is unknown. The mutant in this sigma factor was used as a control in some of these experiments and will be referred to as R20291_*1754*::CT. This was required as insertion of the *erm*B cassette during ClosTron mutagenesis leads to increased resistance to clindamycin and potentially greater survival compared to the parental strain in the clindamycin treated gut. The strains R20291_*hpdA*::CT and R20291_*1754*::CT were kindly provided by Dr Lisa Dawson (London School of Hygiene and Tropical Medicine).

## 6.2 Preliminary phenotypic observations in vivo

The hamster model of *C. difficile* infection performed by Dr Anthony Buckley provided early intriguing data that warranted further investigation. This data is described below and provides the scientific foundations on which this chapter was based. The Golden Syrian Hamster has been widely used as a model for acute *C. difficile* infection and has proved useful for studying *in vivo* infection dynamics (1.6.1). In preliminary experiments, hamsters were treated with a single dose of clindamycin 5 days before oral administration of $1x10^4$ spores of *C. difficile*. Hamsters were challenged with R20291, R20291_*hpdA*::CT or R20291_*1754*::CT to determine the role of the production of *para*-cresol in acute infection (Table 6-1).

**Table 6-1; Times to death of Hamsters infected with R20291 and different ClosTron mutants**

| Strains | Time to death (Mean +/- SEM) | Number of animals |
|---|---|---|
| R20291 | 51h 21 min ± 4h 17m | 7 |
| R20291_*hpdA*::CT | 40h 30m ± 4.5h | 18 |
| R20291_*1754*::CT | 25h 56m ± 57m | 7 |

These initial experiments showed that animals infected with the R20291_*hpdA*::CT or R20291_*1754*::CT succumbed more rapidly to disease than those infected with R20291. As both mutants appeared to show increased virulence it is likely that the method of ClosTron mutation influences the survival and growth rate of the mutants *in vivo* in the hamster model. However, it was interesting to note that animals infected with the R20291_*hpdA*::CT mutant had significantly slower times to death than those infected with R20291_*1754*::CT (p=0.00375). This increased time to death suggests an important role for *para-cresol* production *in vivo* during acute infection.

# 6.3 Results

## 6.3.1 Optimising the relapsing model of infection

One key clinical feature of *C. difficile* infection is the capacity for the disease to reoccur (or relapse) in recovering patients, either as a consequence of reinfection from the environment or recovery and growth of the strain responsible for the initial infection. Understanding the mechanism of relapse is important as multiple bouts of relapsing disease are associated with increased patient morbidity and mortality (1.4.5.1).

As *para*-cresol production may be associated with maintenance of microbiome dysbiosis and thus allow *C. difficile* to persist and flourish in the gut, the hamster model in which initial colonisation and acute disease is determined, may not provide the best model in which this phenomenon can be assessed. Therefore, to study the impact of *para*-cresol production on long term persistence, the mouse model was used. Mice, in contrast to hamsters appear to be more resistance to toxin production by these strains, allowing longer term experiments to be conducted. However, initial attempts to test these mutants in a mouse model of infection proved difficult.

Early attempts to infect animals focussed on the use of the parental strain R20291 and the same infection schedule as had been used for hamster experiments (animals infected with spores 5 days post treatment with a single oral dose of clindamycin). Colonisation was determined by collection and plating of faecal samples from all animals (Figure 6.2).

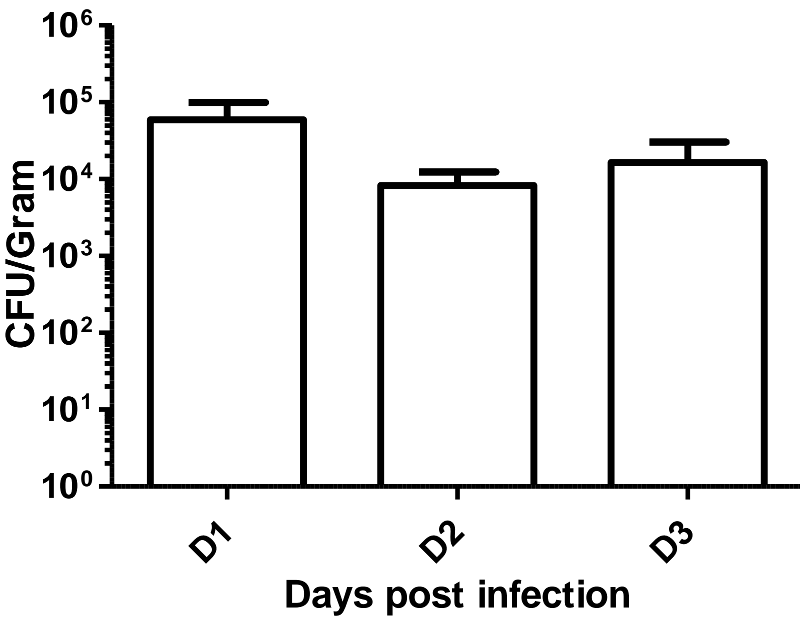



**Figure 6.2 Shedding of *C. difficile* in the faeces of mice infected with R20291 after a single dose of clindamycin 5 days before infection**
Recovery of colony forming units (CFU) of *C. difficile* per gram of faeces from animals infected with R20291 after a single dose of clindamycin 5 days before infection (n=10). Shedding was lower than expected and infection was not established in all mice. Error bars represent +/- the SEM.

Although some shedding was observed, recovery of bacteria was low and varied considerably between individual animals with only 3/10 found to shed the organism 1DPI. Whilst this increased to 8/10 animals 2DPI, the majority of these were at very low levels of $10^3$-$10^4$ CFU/ml, whilst data from the literature and from other studies carried out in this lab suggested levels should be closer to $10^7$CFU/ml. By 3DPI, bacteria were only recovered from 4 mice suggesting that the infection had not been stably established. This difference between susceptibility in mice and hamsters following the same treatment regimen is intriguing and may reflect differences in the composition of the microbiome between these animal species.

To more rationally determine the antibiotic treatment required to successfully initiate infection multiple regimes of clindamycin treatment were used. This is because longer antibiotic exposure is known to increase susceptibility to infection due to increased dysbiosis of the microbiota (Chapter 3). To determine the impact of this treatment we compared colonisation, in animals treated with a single oral dose of clindamycin, exposure to clindamycin in water for 7 days or

3 days respectively. The outcome of these experiments is summarised in Figure 6.3.

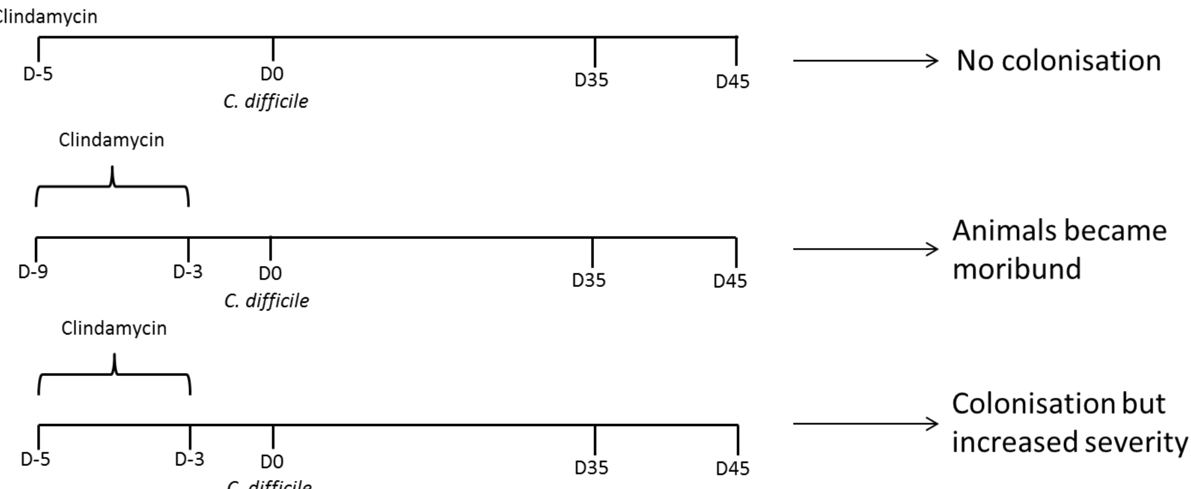



**Figure 6.3 Multiple antibiotic regimes were used to induce susceptibility to infection in mice**
Due to the problems with initiating stable colonisation in animals given the same clindamycin treatment used in hamsters multiple regimes were tested in parallel. A single oral dose of clindamycin was not sufficient to allow stable colonisation. Use of clindamycin in the water for 7 days resulted in animals infected rapidly becoming moribund. Use of clindamycin in the water for 3 days resulted in high levels of colonisation and lower levels of mortality.

This work revealed that a single oral dose of clindamycin 5 days prior to infection failed to modify the gut microbiome sufficiently to allow establishment of *C. difficile* infection, with no bacteria recovered from the faeces of any animals up to 3DPI. In contrast, inclusion of the antibiotic in the water increased susceptibility to infection, with animals highly colonised after infection. Animals that were treated for up to 7 days and infected with *C. difficile* developed very severe disease with high rates of mortality and weight loss (Figure 6.6). To determine the impact of longer-term treatment of clindamycin on disease outcome, animals were pre-treated with clindamycin for 3 or 7 days and then infected with either R20291 or R20291_*hpdA*::CT

## 6.3.2 Determining the impact of Δ*hpd*A mutation on colonisation and persistence in the mouse model

In these experiments, extent of infection and disease severity was determined by variation in the weight of individual animals and by recovery of bacteria from faeces. Plating of faeces is used to determine the levels of colonisation in mice post infection.

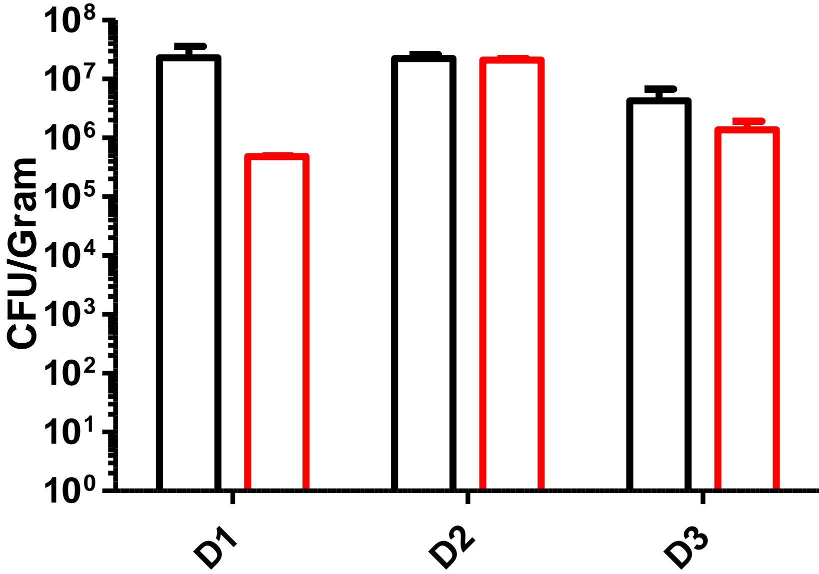



**Figure 6.4 Shedding of *C. difficile* in the faeces of animals infected with R20291 and R20291Δ*hpd*A after 3 days pre-treatment with clindamycin**
Recovery of colony forming units (CFU) of *C. difficile* per gram of faeces from animals infected with R20291 (black) and R20291_*hpdA*::CT (red) after 3 days of clindamycin pre-treatment. There is no difference in the recovery of *C. difficile* from either group of mice. Error bars represent +/- the SEM (n=5).

Data in Figure 6.4 suggests that although animals infected with R20291 appeared to shed the organism at higher levels at 1DPI infection, colonisation appeared equivalent 2DPI. This contrasted significantly with the weight loss experience by these animals with those infected with R20291_*hpdA*::CT suffering from significantly more weight loss than those infected with R20291 (Figure 6.5).

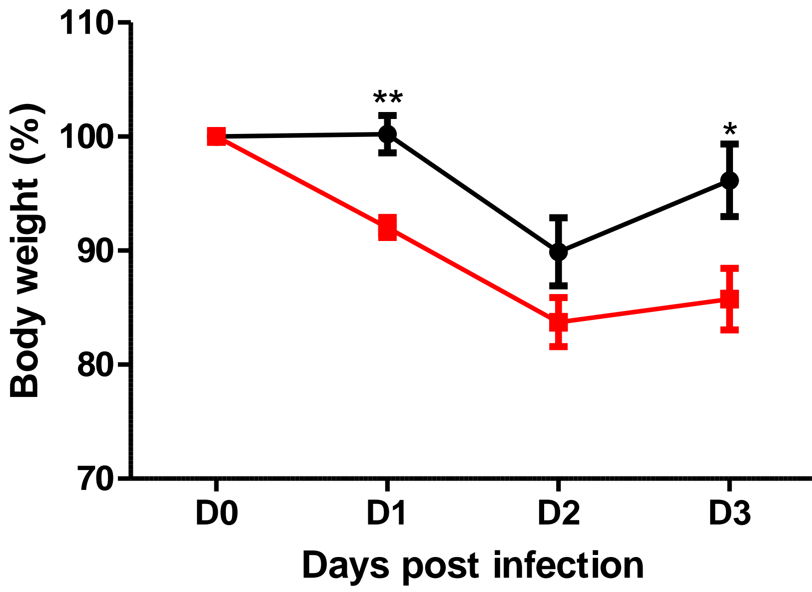



**Figure 6.5 Mouse body weight post infection with R20291 and R20291Δ*hp*dA after 3 days pre-treatment with clindamycin**
Body weights of animals infected with R20291 (black n=5) and R20291_*hpdA*::CT (red n=9). Error bars represent +/- SEM. One animal infected with R20291_*hpdA*::CT was culled as it had lost more than 15% body weight. Animals infected with R20291_*hpd*A::CT lost significantly more weight on D1 and D3 than those infected with R20291. Significance was determined with unpaired two tailed students t-test (* indicates p = 0.0376 ** indicates p= 0.0027).

In contrast to animals pre-treated with clindamycin for 3 days, animals given clindamycin for 7 days in the drinking water and infected with R20291_*hpdA*::CT experienced very severe disease symptoms with 7/9 animals reaching the experimental endpoint (loss of >15% body weight) at 2DPI. Interestingly animals infected with R20291 following 7 days of clindamycin did not suffer from as significant weight loss as those mice infected with R20291_*hpdA*::CT that survived (Figure 6.6).

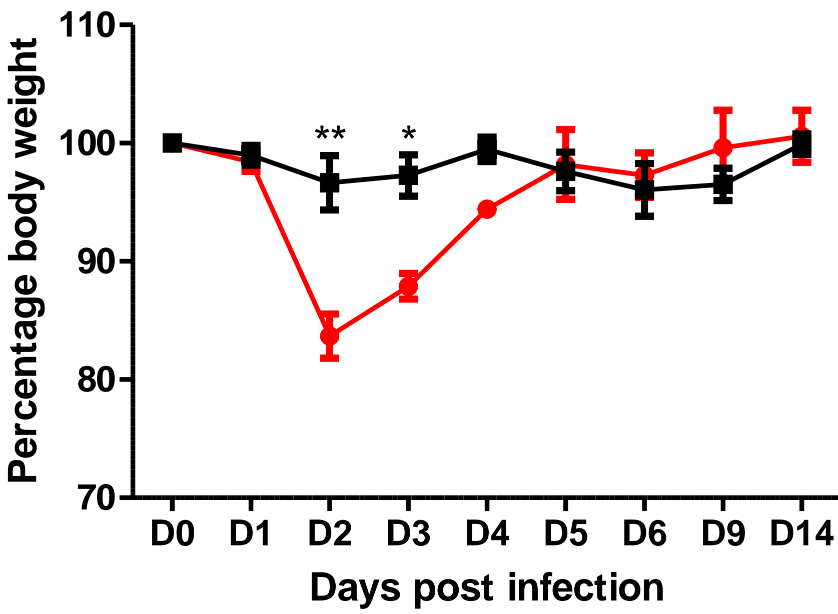



**Figure 6.6 Body weights of animals infected with R20291 and R20291Δ*hpd*A following after 7 days of pre-treatment of clindamycin**
Body weights of animals infected with R20291 (black n=5) and R20291_*hpdA*::CT mutant (red n=9). Error bars represent +/- SEM. At 2DPI, 7 of the animals infected with R20291_*hpdA*::CT were culled as they had lost more than 15% body weight. Animals infected with R20291_hpdA::CT suffered from more severe infection and lost significantly more weight that those infected with R20291 on D2 and D3. Significance was determined with unpaired two tailed students t-test (* indicates p = 0.0256 ** indicates p= 0.0012).

Differences in severity seen between these two models suggest that the extent of disease severity is linked to length of clindamycin exposure. It was also found that R20291_*hpdA*::CT is more virulent than R20291, mimicking what was observed in the hamster model of infection. In support of the data from the 3 day pre-treatment experiment, increased disease severity did not appear to be linked to increased colonisation as the bacterial counts of *C. difficile* recovered in the faeces did not differ significantly between these two groups (Figure 6.7).

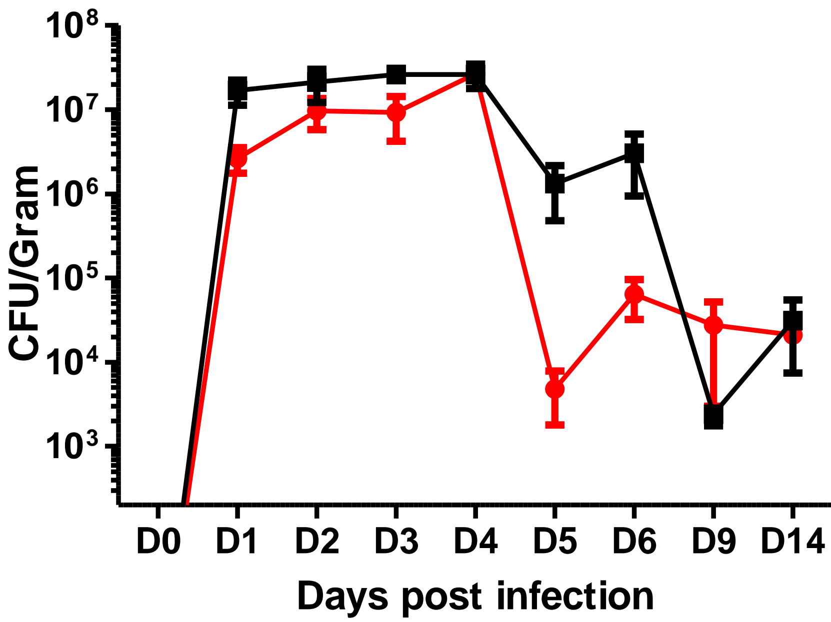



**Figure 6.7 Shedding of *C. difficile* in the faeces of animals infected with R20291 and R20291_*hpdA*::CT after 7 days pre-treatment with clindamycin**
Recovery of colony forming units (CFU) of *C. difficile* per gram of faeces from animals infected with R20291 (black n=5) and R20291_*hpdA*::CT (red n=9). At 2DPI 7 of the animals infected with R20291_*hpdA*::CT were culled as they had lost more than 15% body weight. After 2DPI only 2 mice remained in the R20291_*hpdA*::CT group, for this reason statistical analysis was not carried out after this time point due to the lack of statistical power. Error bars represent +/- the SEM.

As a result of these data sets, a regimen of 3 days of clindamycin treatment, delivered in the water was adopted as a standard treatment. This was considered sufficient to allow stable and robust colonisation of animals whilst limiting disease severity.

### 6.3.3 Is increased severity due to ClosTron mutagenesis

During this optimisation phase of the work, we became aware of a correlation between disease severity and infection with the mutant strain R20291_*hpdA*::CT. To determine if this increased severity was due to the mutation or as an indirect consequence of the insertion of the ClosTron cassette, the capacity of a second unrelated mutant R20291_*1754*::CT to initiate disease severity was assessed.

#### 6.3.3.1 Mouse body weight during infection

Following the previously used infection regimen, the body weight of animals infected with R20291, R20219_*hpd*A::CT and R20291_*1754*::CT were monitored (Figure 6.17). As observed previously, animals infected with R20291_*hpdA*::CT

lost significantly more weight than those infected with R20291. Interestingly, those infected with R20291_*1754*::CT showed similar weight loss to R20291 infected animals, suggesting that disease severity was not linked to the presence of the ClosTron cassette directly (Figure 6.8).

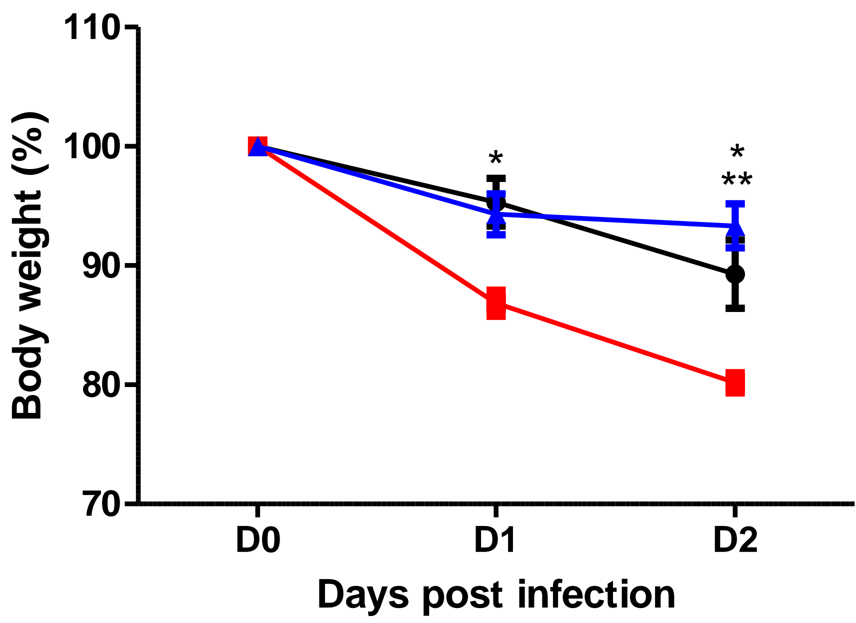



**Figure 6.8 Mouse body weight post infection**
Percentage body weights of mice infected with R20291 (black) R20291_*hpdA*::CT (red) and R20291_*1754*::CT. Animals infected with R20291_*hpd*A::CT suffered from significantly more wiehgt loss than those infected with R20291 or R20291_*1754*::CT. Error bars represent +/- SEM. Significance was determined using ANOVA with Tukey's post-test. (At D1 R20291 vs R20291_*hpd*A::CT * signifies p=<0.5). At D2 * indicates p=<0.05 (R20291 vs R20291_*hpdA*::CT) and ** indicates p=<0.001 (R20291_*hpdA*::CT vs R20291_*1754*::CT) There was no difference between R20291 and R20291_*1754*::CT). Error bars represent +/- SEM (n=5)

### 6.3.3.2 Shedding of *C. difficile* is not linked to disease severity

To determine if differences in weight loss could be linked to germination and growth rates of the bacteria, the number of bacteria recovered from the faeces 1DPI were quantified (Figure 6.9).

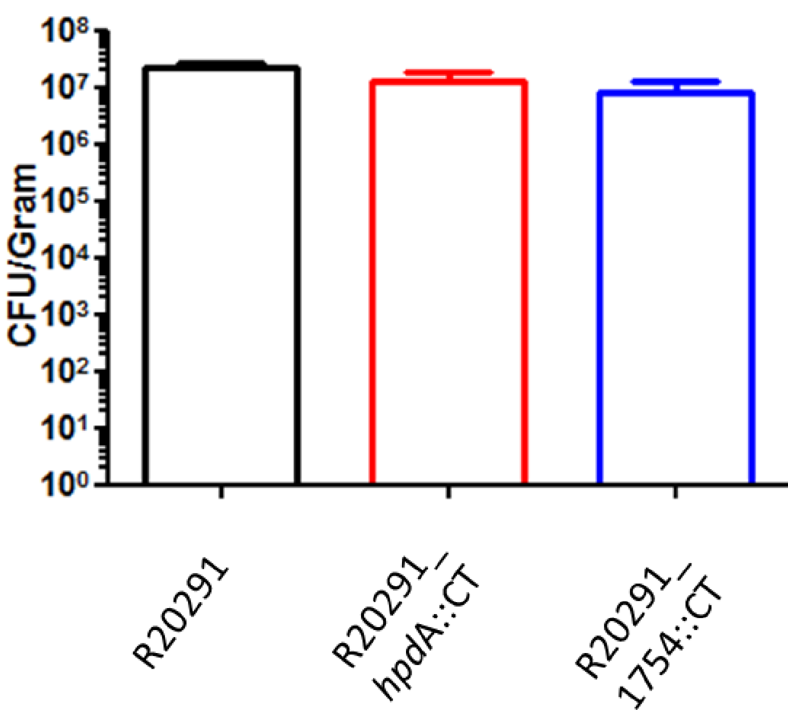



**Figure 6.9 Shedding of *C. difficile* in the faeces of animals infected with different strains of *C. difficile***
Recovery of colony forming units (CFU) of *C. difficile* per gram of faeces from animals infected with R20291 (black) R20291_*hpd*A::CT (red) and R20291_*1754*::CT (n=5). Shedding collected showed no difference between the animals from each infection group. Error bars represent +/- the SEM.

As observed previously, increased severity did not appear linked to differences in levels of colonisation with almost identical number of organisms recovered from each group of infected animals. However, measurement of bacterial numbers in the faeces can provide an inaccurate picture of colonisation directly on the mucosal surface. This is because faecal shedding can also represent growth and excretion of bacteria located within the lumen of the gut. As it is likely that weight loss reflects damage to the mucosal surface, most likely as a consequence of bacterial interaction, it became important to understand whether these strains showed differential interactions with the mucosal surface.

#### 6.3.3.3 Measurement of direct mucosal colonisation

To give a more complete picture of the *in vivo* dynamics of R20291, R20291_*hpdA*::CT and R20291_*1754*::CT, animals were sacrificed at 2DPI to determine the number of bacteria associated with the caecum and colon. Differential counts of heated and unheated samples allowed comparison of the

form of the bacteria (vegetative cell versus spore) within the sample and location of the bacteria (lumen or tissue associated) (Figure 6.10).

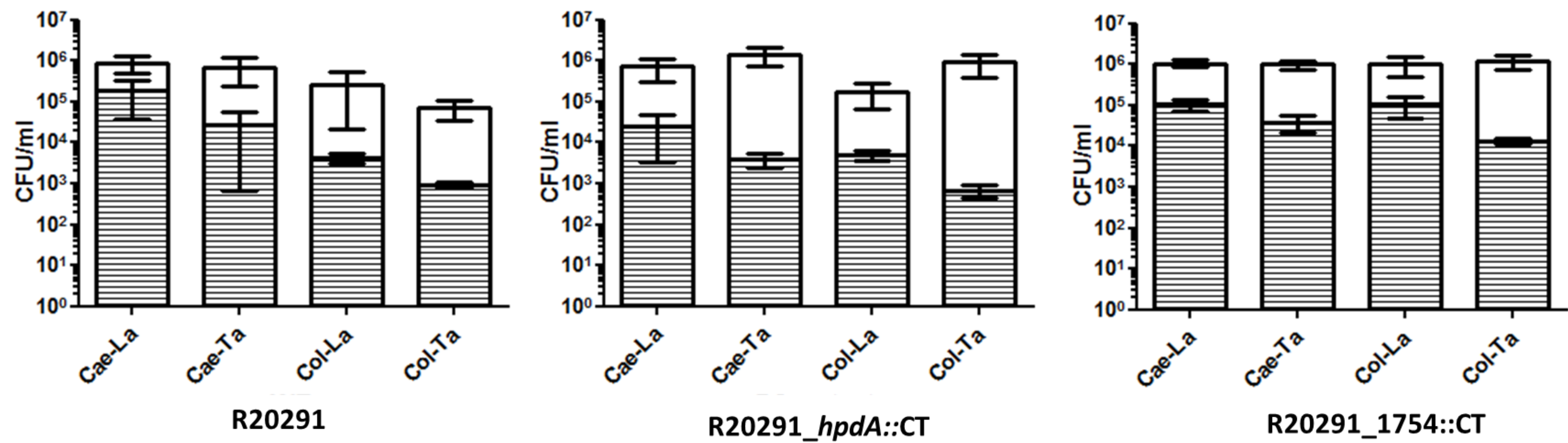



**Figure 6.10 Levels of *C. difficile* vegetative cells and spores in intestinal tissue 2DPI**
CFU/ml of bacteria recovered from the caecum (Cae) and colon (Col) at 2DPI. Both the lumen associated (La) and tissue associated (Ta) bacteria were enumerated as where the numbers of vegetative cells (No fill) and spores (horizontal stripes). Kruskal Wallis test were used to determine if the numbers of vegetative cells and spores in different sites differed between groups. It was found that mice infected with R20291_1754::CT there were significantly more spores in Col-La compared to R20291 ($p < 0.05$). Additionally mice infected with R20291_1754 had significantly higher numbers of spores in the Col-Ta than those infected with R20291_hpdA::CT ($p < 0.01$). The impact of these differences is unclear. Error bars represent +/- SEM (n=5)

These data revealed that colonisation of the caecum and colon did not differ significantly between any of the infection groups. A small difference in the number of spores produced by the R20291_*hpdA*::CT mutant was discernible, although the relevance of this observation to disease severity is unclear.

#### 6.3.3.4 Toxin production *in vivo*

Production of TcdA and TcdB by *C. difficile* results in the symptoms of disease. Therefore, it was important to determine whether these strains produced differing amounts of toxin *in vivo*. Toxin production by each strain was determined using the filtered extracts of luminal washes from caecal and colonic tissue. These samples were tested for toxin activity by their capacity to cause cell rounding *in vitro* (2.9.4) (Figure 6.11).

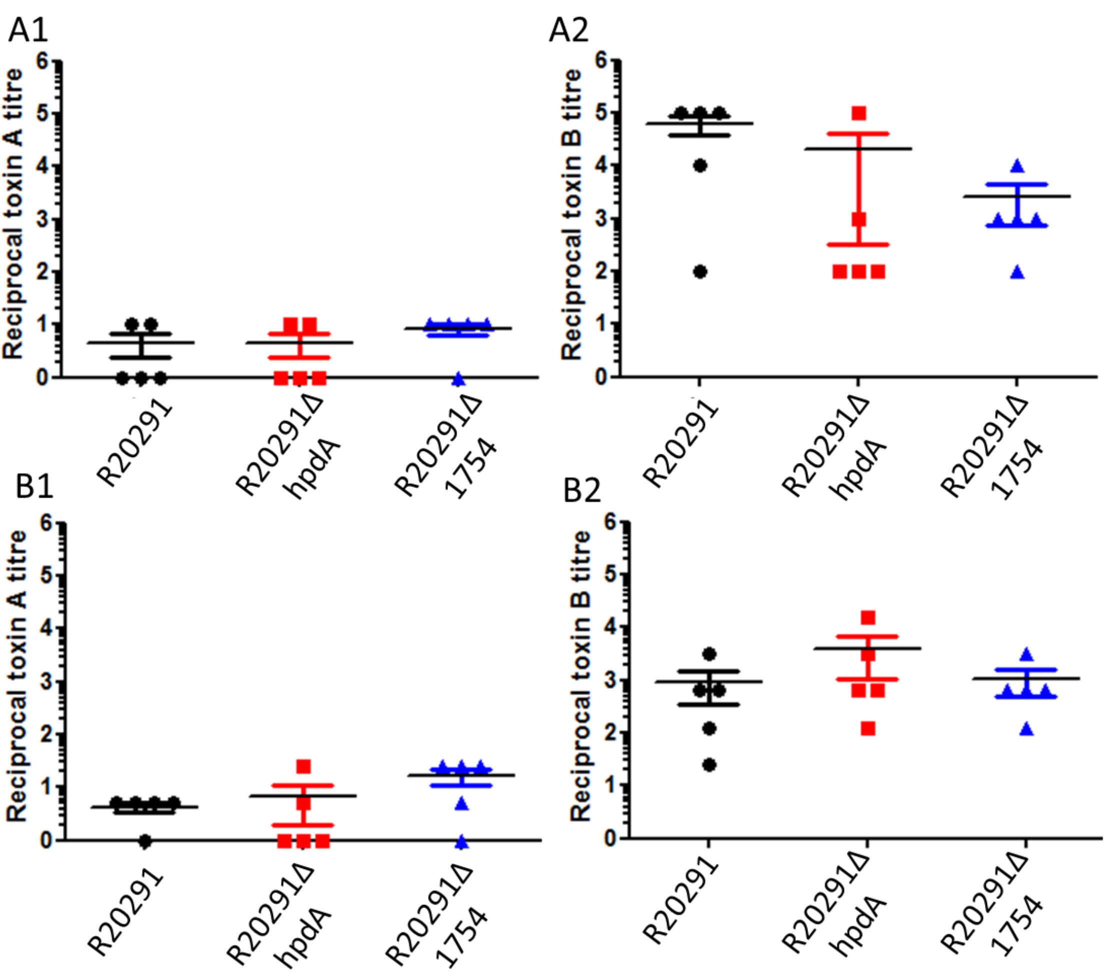



**Figure 6.11 Toxin production *in vivo***
Samples were recovered from the caecum and colon of infected animals 2DPI and filtered to limit bacterial contamination. Toxin titres were then calculated as the reciprocal of the first dilution of the sample that did not cause any cell rounding of HT29 cells (A1, B1) and Vero cells (A2,B2) reflecting toxin A and toxin B activity respectively. Figure 6.11.A reveals the measured activity in the caecum of animals, whilst Figure 6.11.B the toxin activity in the equivalent colon samples. The toxin activity measured for each animal sample is represented by a single symbol. Horizontal bars represent the mean toxin levels and error bar the calculated standard error of the mean of 5 samples for each group. ANOVA with Tukey's post-test showed no significant differences between the infection groups.

These data confirmed that toxin production did not differ significantly between the groups. Levels of TcdA were low in both the caecum and colon with very little variation between animals. Overall, TcdB measurements were higher in both the caecum and colon. This is typical of the pattern of toxin detection observed *in vivo* and is similar to that shown in chapter 3 when mice were infected with BI-7. Whilst small variations were observed between the groups these did not appear to correlate with disease severity *in vivo*.

#### 6.3.3.5 Histology

Assessment of pathology can further assist in determining disease severity and toxin production *in vivo*. Increased damage would suggest more severe infection and potentially higher toxin levels. Tissue sections were taken from the colon at 2DPI in order to assess pathology associated with each of the strains. Assessment of this tissue would suggest there is little difference between strains. There is some blood release into the tissue and the disruption of the epithelia does seem to be more severe in animals infected with R20291_*hpdA*::CT, however it would be necessary to examine more tissue sections from more animals to draw significant conclusions.

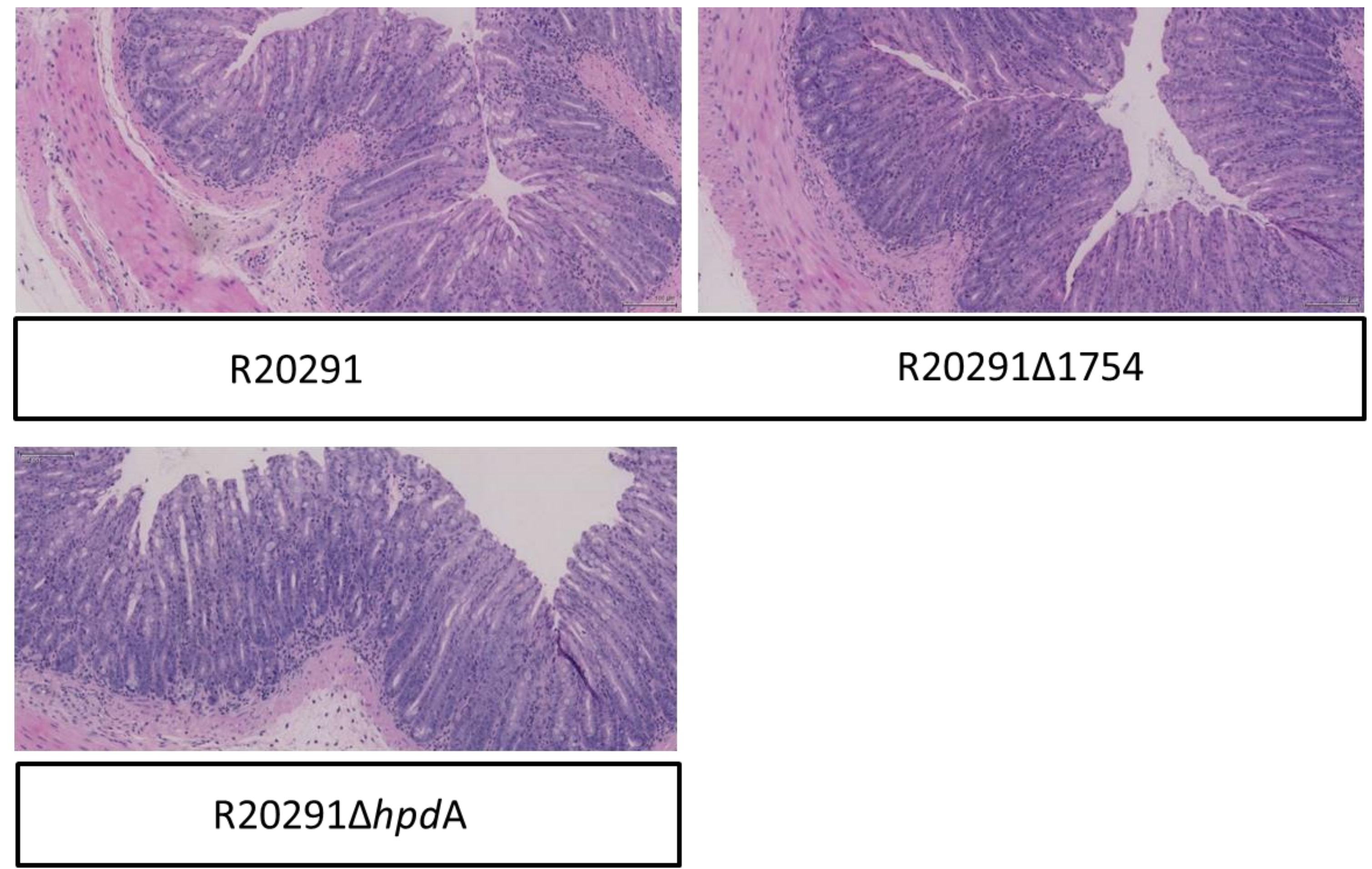



**Figure 6.12 H + E stained colon sections from mice 2DPI**
Tissue samples were taken 2DPI and immediately fixed in formalin. Sections were cut and H and E stained. Images represent typical histology in R20291, R20291_*1754*::CT and R20291_*hpdA*::CT infected animals. Sections at 10X magnification. Scale bar in bottom right corner shows 100μm

### 6.3.4 *In vitro* growth curves of R20291, R20291_*hpdA*::CT and R20291_*1754*::CT

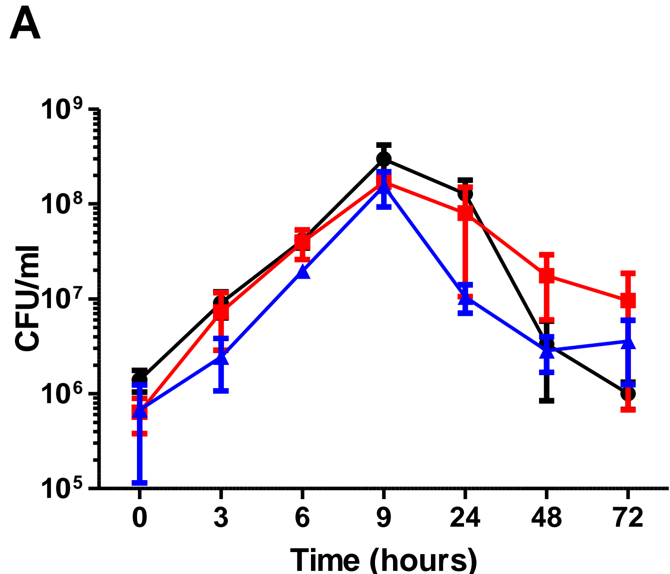
Whilst analysis of growth and toxin production *in vivo* is essential, the data is limited to a single time point. To extend understanding of the growth characteristics of these strains, further analysis was performed *in vitro*. This enabled multiple time points to be tested and analysis of parameters that may help to explain the differences observed in severity.

#### 6.3.4.1 Outgrowth, sporulation and toxin production in BHI broth

Vegetative growth, sporulation and toxin production was analysed over a 72 hour period in two different growth mediums, BHI and FAB, which were additionally supplemented with p-HPA to induced *para*-cresol production. This was included to determine if production of this compound negatively impacts growth of *C. difficile*.

Initial growth curves were carried out in BHI broth. Total counts were taken every 3 hours for 9 hours and then at 24, 48 and 72 hours. Spore numbers and toxin samples were taken at 24, 48 and 72 hours (Figure 6.13).

#### 6.3.4.1.1 Vegetative counts and spore production *in vitro*



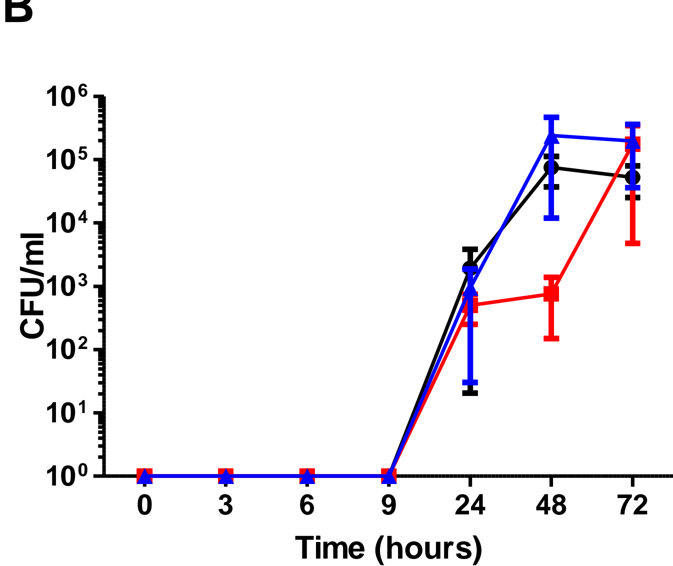




**Figure 6.13 Growth curves and sporulation rates in BHI Broth**
Growth curves were carried out and the number of vegetative cells and spores determined using differential counts of heated and unheated samples. Graph shows A) Vegetative cells and B) spore formation of R20291 (black), R20291_*hpdA*::CT mutant (red) R20291_*1754*::CT (blue). R20291)*hpd*A::CT showed a slight delay in sporulation at 48 hours but this was not statistically signifiacnt Error bars are +/- SEM of 3 biological replicates.

All strains exhibited similar growth rates with exponential growth observed over the first 9 hours after which sporulation was initiated. The R20291_*hpdA*::CT mutant showed a slight delay in sporulation at 48 hours, although this is not statistically significant, with a log less produced at this time point. Interestingly

animals infected with the R20291_*hpdA*::CT mutant also showed a similar reduction in spore numbers at 2DPI (Figure 6.10). It may be that the R20291_*hpdA*::CT strain is able to persist in the vegetative form for longer.

#### 6.3.4.1.2 Toxin production *in vitro* in BHI broth

To analyse toxin production, samples of culture were removed at 24, 48 and 72 hours, centrifuged to remove the majority of bacterial growth and then filtered before serial dilution and application to Vero cells to determine relative concentrations of TcdB (Figure 6.14).

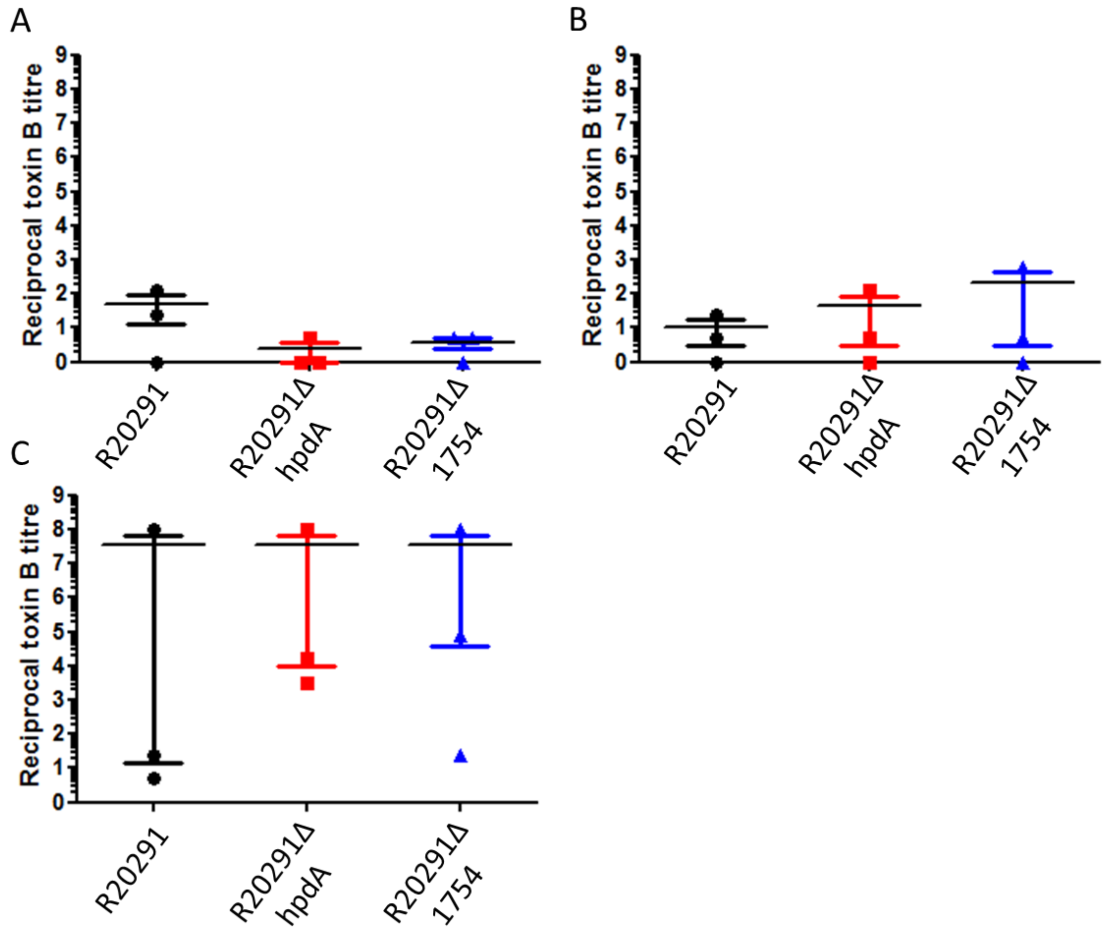



**Figure 6.14 Toxin B production in BHI broth**
Supernatant was collected after 24, 48 and 72 hours of growth and filter sterilised to remove bacteria before used in assays. Toxin B titres were determined after A) 24 hours B) 48 hours and C) 72 hours growth. Horizontal line represents the mean of 3 biological replicates. There were no significant differences recorded in toxin production between the strains. Error bars represent +/- the SEM of three biological replicates.

Production of TcdB in BHI was very low at 24 and 48 hours. At 72 hours TcdB production was low in 2 replicates and very high in the other, consequently, it difficult to draw any conclusions about the relative amounts of toxin produced by the strains. However, It is known that the concentration of glucose can influence toxin production (Karlsson *et al.*, 1999) and that these differences may reflect different concentrations of available glucose in the different batches of BHI used for this assay. For this reason, the experiments were repeated using fastidious anaerobe broth (FAB), which does not contain glucose.

#### 6.3.4.2 Outgrowth, sporulation and toxin production in FAB broth

Growth curves were carried out in FAB broth in exactly the same way as those carried out in BHI with vegetative cell outgrowth, sporulation and toxin production measured.

#### 6.3.4.2.1 Vegetative counts and spore production *in vitro*

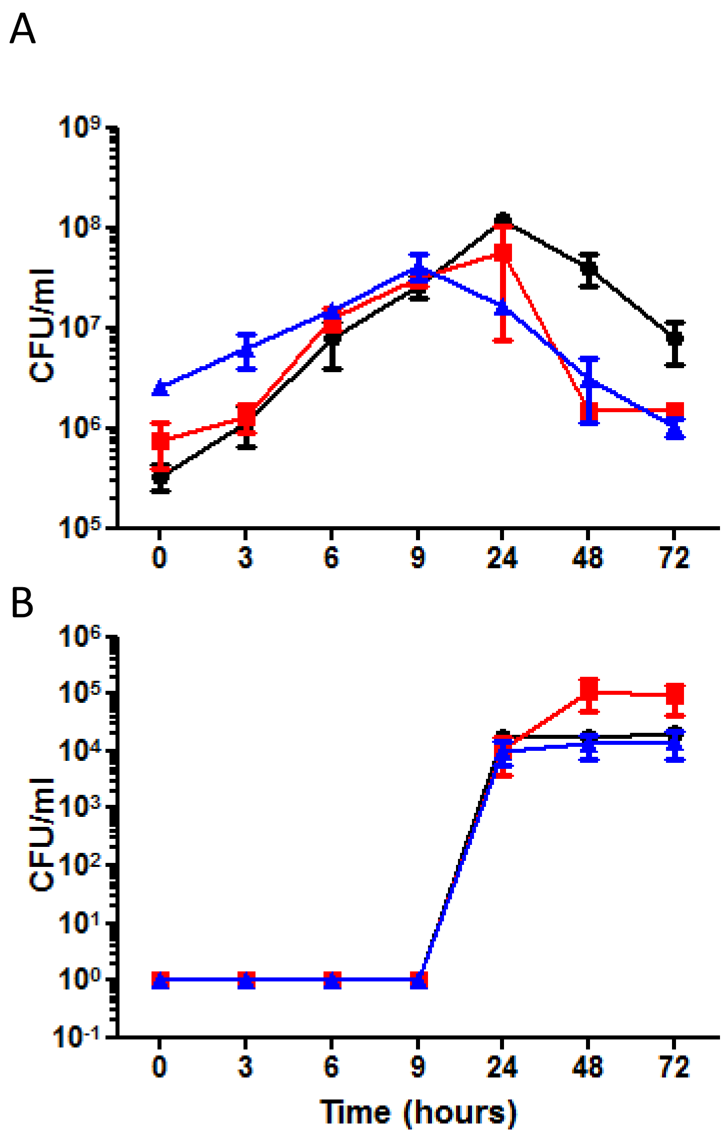



**Figure 6.15 Growth curves and spore production in FAB broth**
Growth curves were carried out and the number of vegetative cells and spores determined using differential counts of heated and unheated samples. Graph shows A) Vegetative cells and B) spore formation of R20291 (black), R20291_*hpdA*::CT mutant (red) R20291_*1754*::CT (blue). Error bars are +/- SEM of 3 biological replicates.

All *C. difficile* strains grew well in FAB reaching equivalent growth to that observed in BHI broth. In contrast to previous observations during *in vivo* (Figure

6.10) and growth in BHI broth (Figure 6.13), no delay in sporulation was observed with the R20291_*hpd*A::CT mutant.

#### 6.3.4.2.2 Toxin production *in vitro* in FAB broth

As described previously, samples were collected from cultures grown for 24, 48 and 72 hours respectively and analysed for production of TcdB (Figure 6.16).

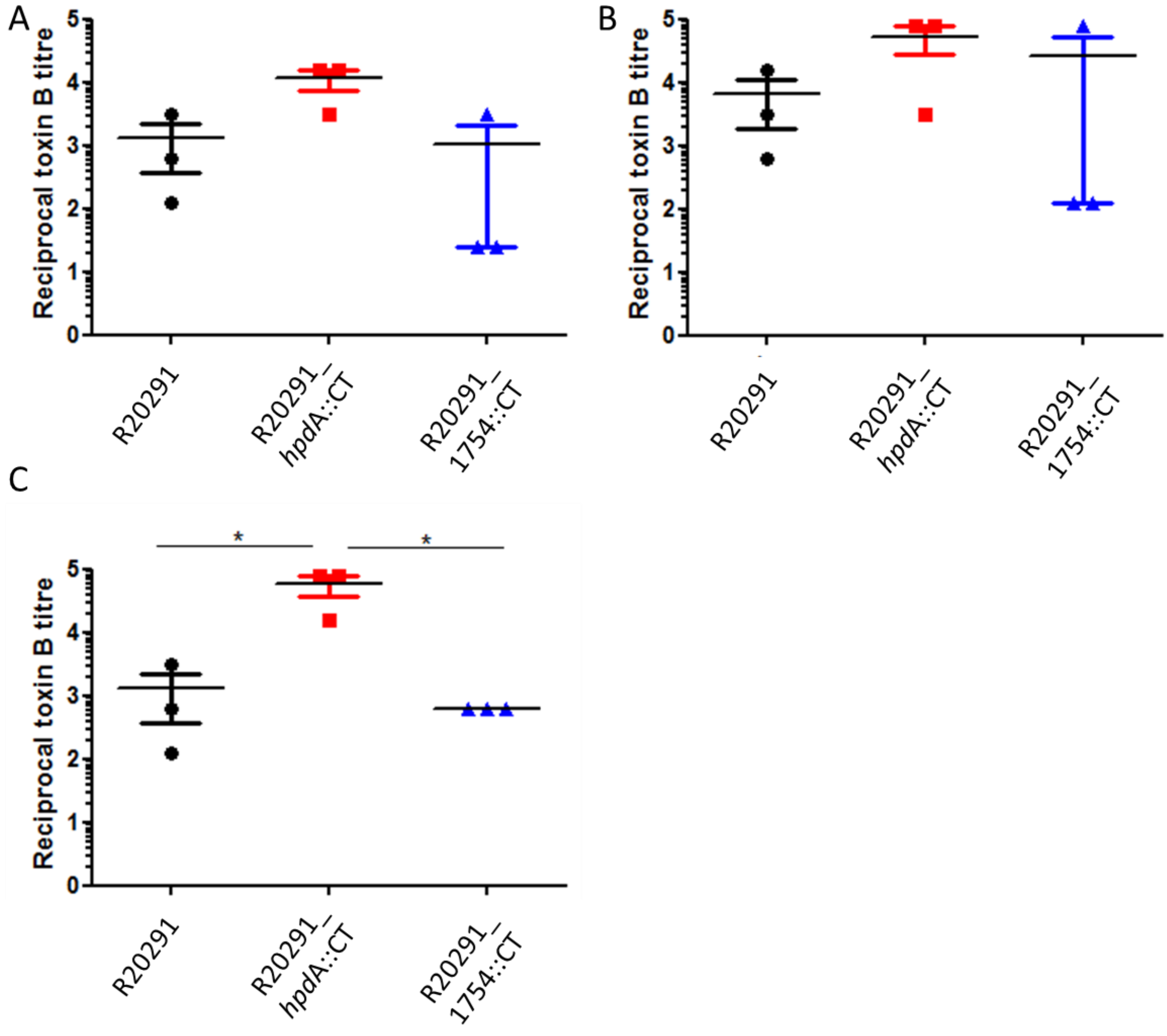



**Figure 6.16 Toxin production in FAB broth**
Supernatant was collected after 24, 48 and 72 hours of growth and filter sterilised to remove bacteria before used in assays. Toxin B titres were determined after A) 24 hours B) 48 hours and C) 72 hours growth. R20291_*hpd*A::CT produced more toxin on average at ever time point. This was only significant at 72 hours (p= < 0.05). Significance was determined using ANOVA with Tukey's post-test Horizontal line represents the mean. Error bars represent +/- the SEM of 3 biological replicates.

In agreement with the literature, production of toxins was much higher when the strains were grown in FAB broth, supporting the idea that the presence of glucose inhibits toxin production. Under these conditions R20291_*hpdA*::CT produced more toxin at every time-point measured. Whilst this increase is only statistically significant at the 72 hour time point, such incremental increases in production *in vivo* could have impact upon disease severity. Similarly elevated toxin production was only observed in the colon of animals infected with R20291_*hpdA*::CT so it is unknown if this increased production occurs *in vivo*. However it is important to consider that when examining toxin production *in vivo* only a very small snap shot in time is considered.

These data may suggest that increased severity is both linked to increased production of toxin and reduced diversity of the microbiome as a consequence of extended exposure to antibiotics. If antibiotic exposure modifies the microbiota associated with maintenance of mucus thickness (as described in chapter 3) this may increase sensitivity to toxin activity meaning increased production by this strain could be linked to more severe disease. Alternatively, the failure to produce *para*-cresol could modify the metabolism of tyrosine in the environment, the impact of which is unknown. At present, the only conclusion that can be drawn is that prevention of *para*-cresol production affects the severity of the acute phase of infection.

### 6.3.5 Investigating the role of *para*-cresol production in relapsing disease

As *para*-cresol is toxic to many members of the bacterial flora, it has been postulated that its production is required for long term maintenance of the dysbiotic gut. To investigate its role in relapsing disease, infected mice that were no longer shedding the organism within the faeces, were subject to a second, single oral dose of clindamycin. This is sufficient to stimulate the outgrowth of *C. difficile* that are persisting within the gut. Treated animals were monitored for relapse and disease severity through measurement of body weight and recovery of *C. difficile* from faecal samples.

#### 6.3.5.1 Mouse body weight during long term infection and relapse

Animals were weighed daily as a determinate of disease severity. As observed previously (6.3.2 and 6.3.3.1) in the initial stages of infection, animals infected with R20291_*hpdA*::CT suffered greater weight loss than those infected with R20291 (Figure 6.17).

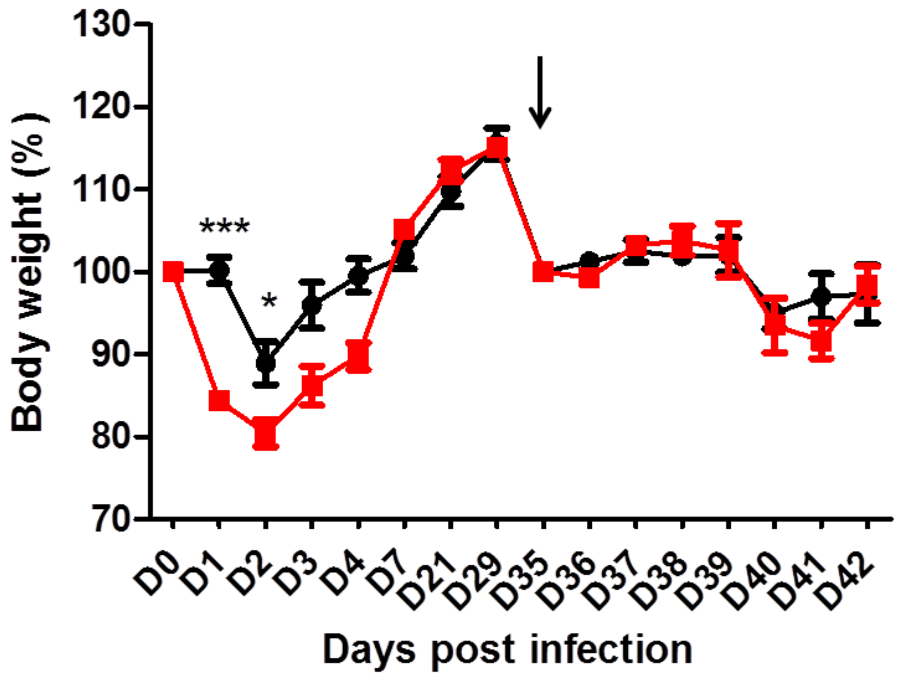



**Figure 6.17 Body weight of mice during infection and relapse**
Body weights of mice infected with the R20291_*hpdA*::CT mutant (red) and WT (black) during the initial infection and relapse. Error bars represent the +/- the SEM (n=5). One animal from the R20291_*hpdA*::CT group reached the experimental endpoint at 2DPI and was culled. 100% body weight was recalculated at D35 before relapse of infection. To induce relapse clindamycin was given at D35 (black arrow). Significance determined using an unpaired T-test (* represents p= 0.0376, *** represents p = < 0.0001).

As in the previous experiments, weight loss in these animals also occurred infected with R20291_*hpdA*::CT with animals losing significantly (p=< 0.0001) more weight than R20291 infected mice at 1DPI, t). After initiation of relapsed infection weight loss was reduced suggesting that either the animals were protected against bacteria induced pathology or that the microbiota was not modified sufficiently by a single dose of clindamycin to disrupt the mucus thickness and hence increase sensitivity to the toxin.

### 6.3.5.2 Shedding of *C. difficile* in the faeces during initial infection and relapse

To determine if there were any differences in initial colonisation levels and the ability of strains to persist in the gut, faecal samples were tested to determine the presence of R20291 and R20192Δ*hpd*A (Figure 6.18).

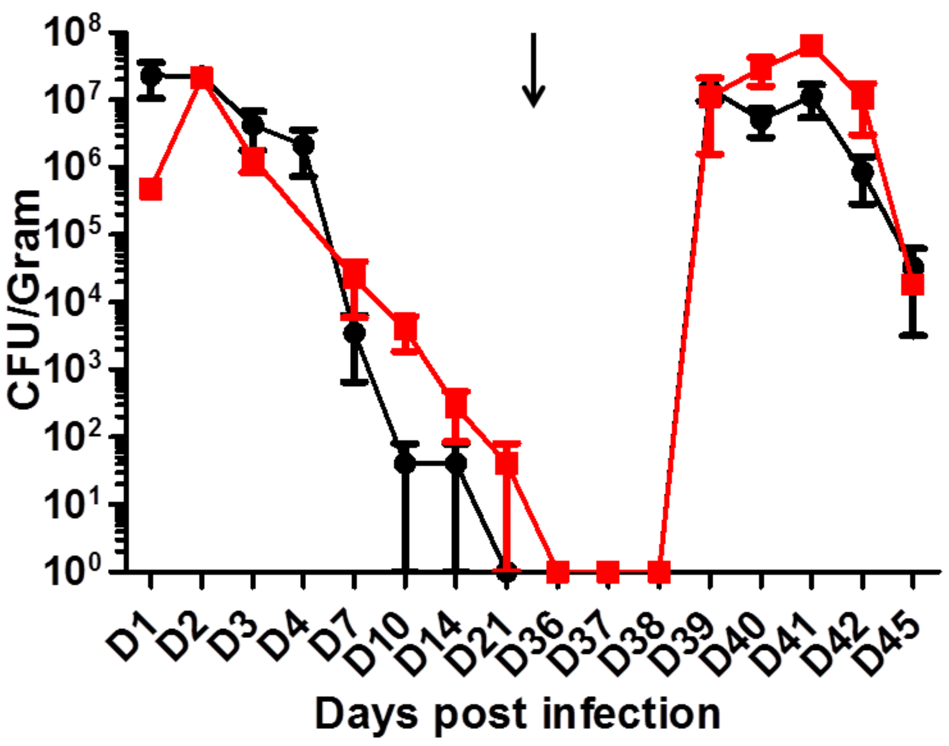



**Figure 6.18 Shedding of *C. difficile* in the faeces during initial infection and relapse**

CFU/g of *C. difficile* recovered from the faeces of animals infected with the R20291 (black) and R20291_*hpdA*::CT (red) during initial infection and relapse. One animal from the R20291_*hpdA*::CT group reached the experimental endpoint at 2DPI and was culled. 100% body weight was recalculated at D35 before relapse of infection. To induce relapse clindamycin was given at D35 (black arrow). Shedding showed no difference in the ability of the strains to cause relapsing infection. Error bars indicated the +/- SEM (n=5).

These data indicate that R20291 and R20291_*hpdA*::CT persist equally well in the mouse gut despite R20291_*hpdA*::CT being unable to make *para*-cresol. These data would suggest that *para*-cresol production is not involved in maintenance of dysbiosis within the gut. To confirm this observation, and to ensure that the animals were only infected with the appropriate *C. difficile* strains, samples taken on a weekly basis were subject to MVLA. This method, which can be used diagnostically, required the amplification of 7 independent repeat regions from the bacterial chromosome (Marsh et al., 2006). In this

context, this was undertaken to shown that bacteria recovered from the animals belonged to the R20291 parental strain background. The expected banding pattern of R20291 is shown in Figure 6.19.

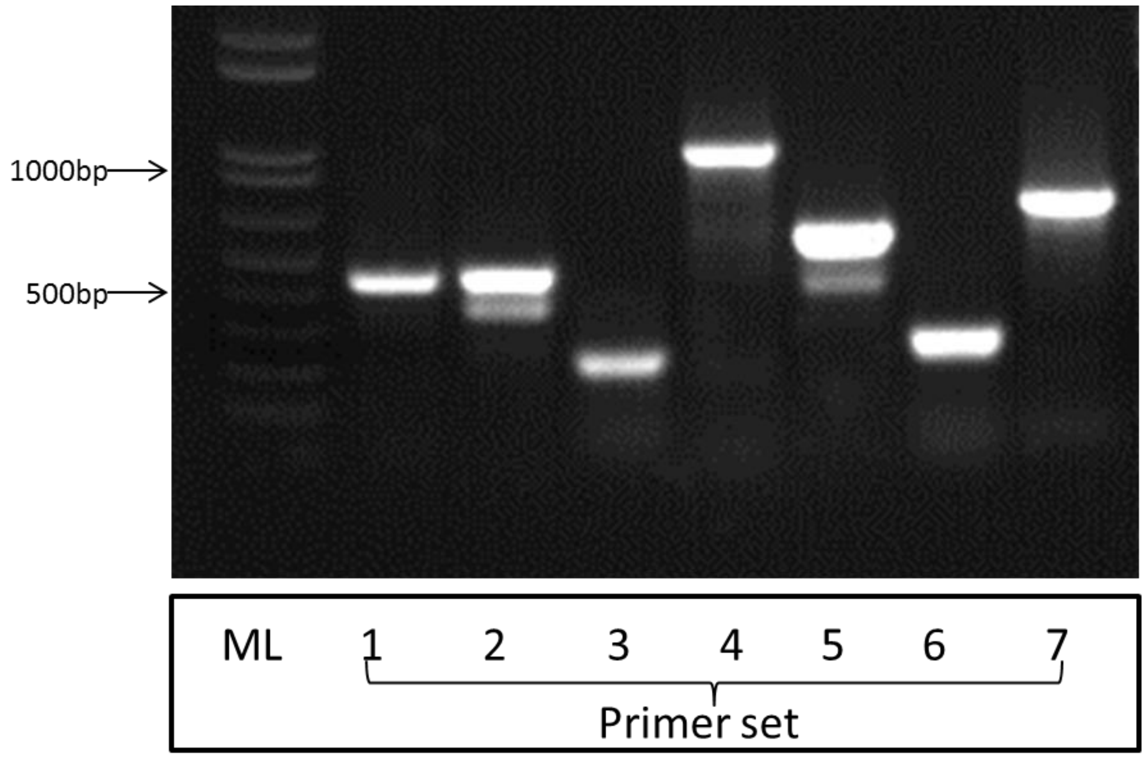



**Figure 6.19 MVLA banding pattern for R20291**
MVLA typing uses sets of 7 primers to differentiate between different strains of *C. difficile.* The above gel shows the expected banding pattern for R20291. Samples were taken from all cages weekly and MVLA analysis conducted. All *C. difficile* recovered from the mice throughout the experiment were found to be R20291. Expected band sizes are as follows; primer set 1 ~500bp, primer set 2~450bp, primer set 3 ~260bp, primer set 4 ~800bp, primer set 5 ~750bp, primer set 6 ~400bp and primer set 7~490bp.

MVLA analysis was conducted on multiple samples from each group weekly and confirmed that the correct strain of *C. difficile* was recovered from the mice throughout the experiment. As all of the strains used in these experiments are from the same parental background additional analysis was required to ensure that the correct strains were colonising the mice. Further amplification of sequences using specific primers was used to differentiate between the unmodified R20291 strain and R20291_*hpdA*::CT. Using this approach, we confirmed that mice were infected with the expected strain of *C. difficile*. As with MVLA analysis samples were taken weekly from multiple mice in each group to confirm presence of the correct strains in each group (Figure 6.20).

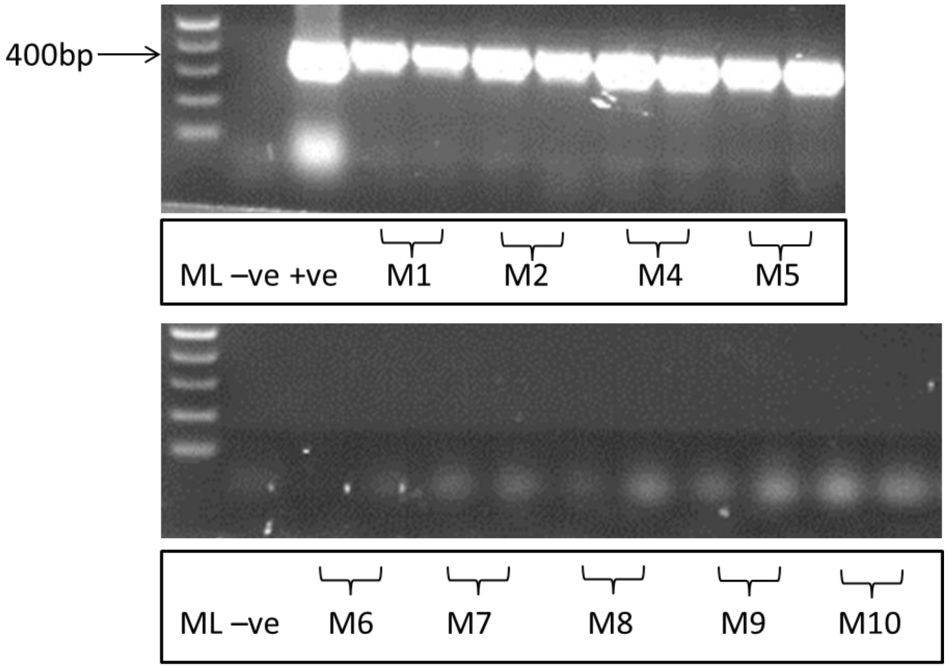



**Figure 6.20 Screening for the ClosTron intron sequence in bacteria isolated from faeces**
A PCR directed to a region of the ClosTron intron sequence and the *hpd*A gene for identification of R20291_*hpdA*::CT. WT yields no product whereas R20291_*hpdA*::CT gives a product of 408bp. Samples were taken weekly from multiple mice in each cage to confirm the presence of the correct mutants in each group. This gel represents samples taken at D40. Two colonies were taken from some of the animals to rule out the possibility of mixed infections.

These data confirmed that animals were infected with the correct strains during the course of the experiment.

# 6.4 Discussion

## 6.4.1 Increased disease severity

During the initial optimisation phase of this work, it became clear that animals infected with *para*-cresol mutant strain, R20291_*hpdA*::CT developed more severe symptoms of disease than those infected with the parental strain, R20291. Animals were subject to more significant weight loss and higher a mortality rate than those infected with R20291. Interestingly onset of weight loss was observed at least 24 hours earlier in R20291_*hpdA*::CT than R20291 infected animals. However, based on the data generated from these experiments, it is not possible to provide an explanation that directly explains this difference, as shedding, toxin production and sporulation rates between these two strains appear similar. The same observations were made when animals were culled 2DPI, with animals infected with R20291_*hpdA*::CT showing

increased weight loss compared those infected with R20291 or R20291_*1754*::CT. However it is important to highlight that analysis of these virulence traits only represents a very brief snap shot in time and that differences in toxin production may precede the peak of weight loss. Further experiments investigating toxin levels and colonisation earlier in infection could help to address these questions.

Previously, increased severity of ClosTron mutants in the hamster model has been linked to indirect increase in clindamycin resistance as a consequence of the ClosTron mutagenesis (Kelly et al., 2016). R20291 is naturally sensitive to clindamycin, with a minimum inhibitory concentration of 16 mg/ml (Buckley *et al.,* 2011) and inhibitory levels of clindamycin can be detected in the hamster gut up to 11 days post treatment (Larson & Borriello, 1990). Thus mutant strains resistant to this antibiotic are able to survive and proliferate more rapidly in this environment. However, this does not appear to be the explanation in mice as animals challenged with a second unrelated ClosTron mutant R20291_*1754*::CT, showed no enhanced virulence. This suggests that in mice and hamsters there are different selection pressures that impact the outgrowth of *C. difficile* strains. It may be that in mice levels of clindamycin in the gut are not high enough at the time of infection to impact upon outgrowth which would explain why there was no increase in severity in R20291_*1754*::CT. This suggests that ClosTron mutagenesis was not the cause of increased severity in the mouse model of infection.

As neither the *in vivo* or *in vitro* data could explain the increase in disease severity it may be that the increase in severity is not linked to the pathogen itself, as was seen in chapter 3. One factor that was not investigated was the host immune response. Interestingly, *para*-cresol has been linked to suppression of various aspects of the immune response including endothelial cell response to inflammatory cytokines and leukocyte migration (Dou *et al.,* 2002; Rie Faure *et al.,* 2006.). Thus, in its absence, the inflammatory response to infection could be enhanced. Therefore it may be interesting to investigate the host immune response to both the R20291 strain and the R20291_*hpdA*::CT mutant as it may be that increased immune response plays a role in increased severity. Additionally the structure of the microbiota was not investigated and it may be that differences in the microbiota may influence infection outcome in these

animals. As *C. difficile* is one of the few bacteria able to produce *para*-cresol it may be that in animals infected with the mutant R20291_*hpdA*::CT have an alteration in the metabolism of tyrosine or *p*ara-HPA. Changes in the levels of these compounds could alter the structure of the microbiota and increase virulence through modification of microbiota metabolism, although further investigation of this would be required.

### 6.4.2 Impact of *Para*-cresol production on relapsing infection

Whilst mutation in the *para*-cresol pathway appeared to modify the response to acute infection, its production did not appear to play a role in long term persistence of the intestinal niche. It was found that R20291_*hpdA*::CT mutant was able to successfully cause relapsing infection to the same extent as R20291. Relapse was observed in all animals with no difference in the levels of shedding in the faeces and no delay to relapse seen in R20291_*hpdA*::CT. The level of shedding was consistent between animals in each group with shedding observed at the temporal point, making it likely that this was true relapse and not reinfection of the mice. This suggests that *para*-cresol production may not be involved in enabling *C. difficile* to persist in the gut, but may be important in suppression of responses that may be involved in bacterial clearance to the initial infection.

### 6.4.3 Identification of a SNP in codY

Since the end of this project full genome sequencing has since been carried out on the R20291_*hpdA*::CT strain. Comparison of this to the parental R20291 strain has resulted in the identification of a single nucleotide polymorphism (SNP) in *cod*Y (Personal communication from Dr Lisa Dawson). This gene is known to play an important role in regulating toxin production (1.1.2.2). It may therefore be that this SNP results in the increased toxin production due to reduced function of codY. This increased toxin production may explain this increased virulence *in vivo*. The impact of this SNP means that it is likely that these experiments would need to be repeated as it is unknown what impact the increased toxin production would have on relapsing infection.

# 7 Final discussion

The initial aims of this thesis were to gain a greater understanding of the contribution played by host BA metabolism in *C. difficile* disease pathogenesis. This included evaluation of the contribution of the microbiota in the breakdown of these compounds, focussing particularly on the influence of organisms that express BSH enzymes. Additionally the role of *para*-cresol production on acute and relapsing disease was investigated. Within this final chapter, the influence of these factors on germination, colonisation and persistence will be discussed.

## 7.1 Investigation of the role of the microbiota in colonisation and severity

Previous work has shown that pre-treatment of animals with antibiotic combinations can significantly modulate the outcome of *C. difficile* infection (Chen *et al.*, 2008; Reeves *et al.*, 2011). The microbiota is important in *C. difficile* infection with changes in structure particularly associated with infection, relapse and severe disease (1.4.5). However, an understanding of the exact mechanisms involved in disease progression has not been thoroughly investigated. The work presented in this thesis (chapter 3) showed that specific microbiome profiles were linked to particular disease states (colonisation and severity), with loss of colonisation resistance associated with changes to bile metabolism. This correlated with a loss of organisms expressing BSH genes. This loss was confirmed by PCR and through examination of the abundance of these genes in the microbiota of animals following different treatments. A recent investigation of the abundance of these genes in patients infected with *C. difficile* echoed the findings here, with decrease in BSH abundance a marker of colonisation (Allegretti *et al.*, 2016). In addition to the loss of BSH producing bacteria there was also a significant reduction in OTU_69, a potential 7α-dehydroxylating bacteria. As this is the other important enzyme in this cascade, this suggests that antibiotic treatment of animals results in dysfunction of BA metabolism. This was confirmed using TOF-MS that showed clindamycin use was associated with loss of BA metabolism with significantly increased abundance of TC. This loss of BA metabolism is now known to be an important factor in *C. difficile* infection and much work is now focused on restoring this pathway as a

treatment for infection (Weingarden *et al.,* 2016; Weingarden *et al.,* 2014; Buffie *et al.,* 2014).

An area that is less well understood is that of disease severity. Comparison of the microbiome structures between animals that were colonised and animals that suffered from weight loss highlighted changes in the abundance of mucus degrading and mucus dwelling species. There were significant increases in the mucus degrading species *B. thetaiotaomicron* and decreases in two mucus dwelling species *M. schaedleri* and *E. mucosicola.* Taken together this could suggest that disruption of mucus homeostasis is significant and may result in increased accessibility to the epithelial surface resulting in greater toxin mediated damage. Increase in *B. thetaiotaomicron* has been previously shown to exacerbate both infection with *C. difficile* and *E. coli* by modifying the metabolic landscape to one that favours pathogen growth (Ferreyra *et al.,* 2014; Curtis *et al.,* 2014). This data taken in conjunction with that from the literature suggests that further investigation of this process is warranted. In order to examine this process further it may be worth placing more focus on changes in the mucosal associated microbiota as the composition is likely to be very different than that found in the lumen (Ouwehand *et al.,* 2004; Swidsinski *et al.,* 2002; Zoetendal *et al.,* 2002). This may allow association of specific changes in this subset of microbes with disease. Additionally further examination of the mucus layer may be useful in determining if these antibiotics directly or indirectly impact mucus layer thickness and mucus production. A recent study by Jakobsson *et al.,* (2015) found that mice displayed variable mucus layers. They utilised a method in which ex vivo sections were taken and mucus layer thickness directly measured. Bacterial diffusion into the mucus layer was also assessed with the use of fluorescent bacteria sized beads coupled with microscopy which allowed the depth of diffusion of these beads into the mucus layer to be measured. Performing such analysis on sections from animals treated with different antibiotic combinations would help to determine if the microbiome data does actually indicate changes in mucus layer structure and whether these changes do result in closer association of bacteria to the epithelia. It would also be interesting to use molecular and biochemical methods to investigate the expression of mucus degrading enzymes such as, beta galactosidase and beta glucosidase, in the faeces of mice. Various studies have

used API strips direct from faecal samples to detect this (Vlková *et al.,* 2005; Rada *et al.,* 2006). It may also be possible to detect expression of these genes using RT-PCR on RNA extracted from faecal samples. This could offer potential as a way of identifying patients that are at risk from more severe disease.

Another factor that should be considered is the observation that the anatomy of the mouse and human gut differ significantly and this may influence the composition of the microbiota. Key differences include caecum size and the relative ratio of intestine to colon length (Nguyen *et al.,* 2015) (Figure 7.1).

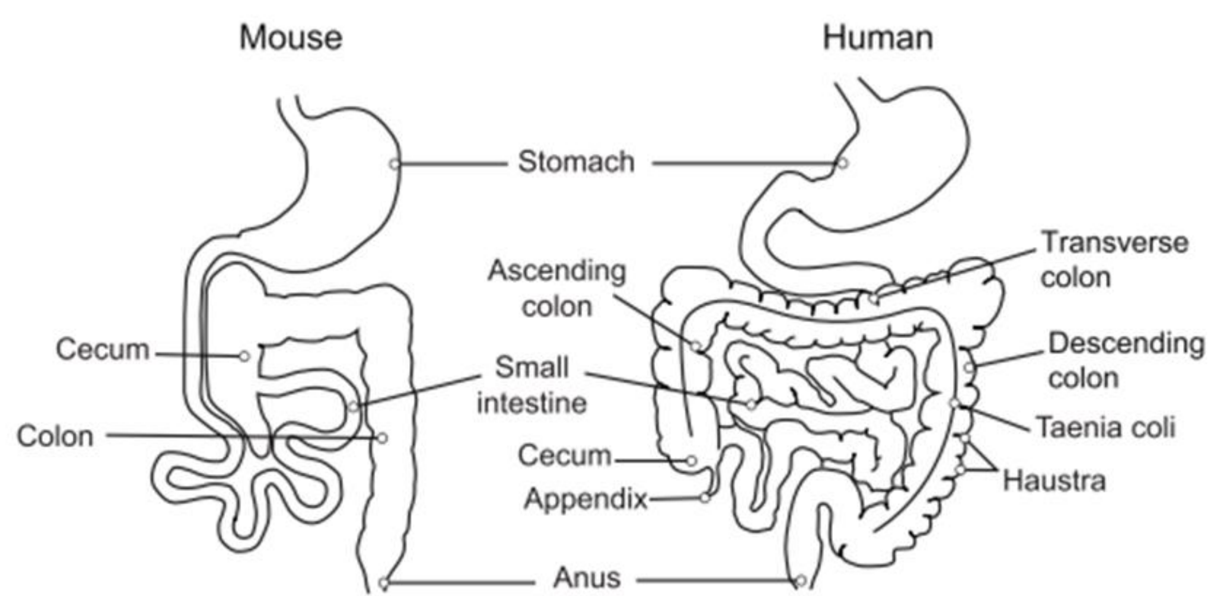



**Figure 7.1 The structures of both mouse and human GI tracts from Nguyen *et al.,* (2015)**
Although both mouse and human GI tracts have similar structures there are some key differences that may influence *C. difficile* infection. First the caecum of the mouse is much larger than that in humans and is the main site of *C. difficile* infection whereas in humans this is the colon.

In addition to these differences, variation in the distribution of important mucin producing goblet cells and antimicrobial secreting paneth cells exist. Goblet cells are abundant throughout the human GI tract whereas in the mouse distal colon these are found at the base of crypts in reduced numbers. In humans paneth cells are found in the colon and caecum (Cunliffe *et al.,* 2001) whereas in mice these cells are only present in the caecum (Ouelleyite *et al.,* 1994). Such differences may lead to the creation of species specific niches in which elected microbes may flourish. Although there are differences between the microbiota of humans and mice the two most abundant phylotypes in both species are the Bacteroides and Firmicutes (Eckburg *et al.,* 2005; Ley *et al.,* 2005). A study by

Krych *et al.,* (2013) found that mice and humans shared 90% of bacterial phyla and 89% of bacterial genera. Mice were reported to have one unique genus, *Mucispirillum*, which interestingly was one of the microbes identified as being significantly decreased during more severe infection. As this is likely a murine specific isolate it makes it more difficult to directly translate this observation into the context of human infection. This is why it may be preferable to examine the functional capacity of the microbiome rather than changes to specific organisms as it is likely that functional aspects are more highly conserved between the species.

In an attempt to improve the mouse model and to make it as representative of the human microbiota models are now being developed using GF mice that are colonised with microbiota from human donors. Such work has shown that the human flora successfully colonises the mouse and can transfer between successive generations (Collins *et al.,* 2015). Humanised mice show structural changes in the microbiota that make it more similar to that of a human than that of a naïve mouse, although some specific species are still not present (Turnbaugh *et al.,* 2009). A recent study found that a high number of bacterial species were enriched within the mouse gut compared to the human gut, suggesting host specific selection (Lagkouvardos *et al.,* 2016). One potential issue is that as these microbes have not co-evolved with the host the host microbiota interactions in this model may not be appropriate. This was confirmed by a study by Chung *et al.,* (2012) which found that immune systems of humanised mice did not mature properly compared to those inoculated with mouse microbiota. Although such drawbacks exist, these models do provide an opportunity to manipulate the human microbiota in the context of infection, a unique opportunity that deserves more consideration. Use of these models in studies such as this may allow specific hypothesis to be tested and help to confirm findings from previous experiments.

Another interesting difference is the make-up of the bile acid profile between these animals. As described previously BAs are of great importance in *C. difficile* infection, impacting on both spore germination and outgrowth (1.2.2). Mice are known to produce muricholic acids which can influence *C. difficile* germination and growth (Francis *et al.,* 2013). Additionally the proportion of different BAs in

mice is different to that seen in humans with a large abundance of muricholic acids (Figure 7.2).

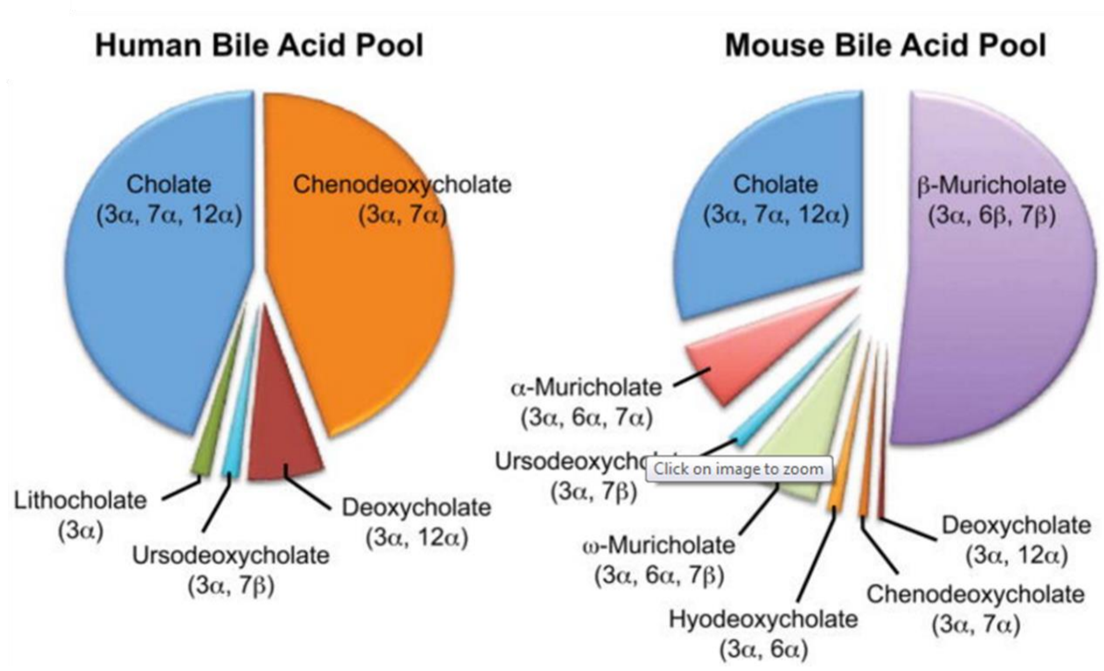



**Figure 7.2 BA profiles in mice and humans from De Aguiar Vallim *et al.,* 2013.**
There are several differences between the BA profiles of mice and humans. Mice produce a murine specific BA and lower quantities of CDCA. It may be that these differences impact upon *C. difficile* infection.

These differences in BA profiles are likely to reflect differences in diet and in BA production and regulation. Although nothing can be done to alter this it is important to take this into consideration.

Although differences between mice and humans exist, mouse models remain incredibly useful in helping to study *C. difficile* infection. Many of the symptoms of infection and varied disease outcomes can be replicated in this model making it invaluable for studying *C. difficile* infection dynamics and pathogenesis. Additionally the findings presented here have in part been observed in humans. Restoration of BA metabolism is known to be an important function of FMT and alterations in the abundance of BSH genes have now been shown to be predictive of disease. This shows that the use of mouse models, complemented with data from patients, are invaluable in aiding our understanding of infection.

## 7.2 Inhibition of germination by BSH

In this thesis, a role for bacteria that are able to modify BAs was established with particular focus placed on those that carry out the key first step in bile metabolism, the BSH producers. The ability of bacteria expressing BSH to influence spore germination *in vitro* was determined using a BSH producing *L. murinus* strain and *E. coli* expressing BSH from the human gut. Bacteria that were able to express BSH were found to limit *C. difficile* spore germination *in vitro* possibly by conversion of the effective germinant TC to the less potent germinant CA. This adds weight to the hypothesis that these bacteria may be important in limiting *C. difficile* colonisation by significantly reducing germination rates. The importance of this pathway has been reinforced recently with particular emphasis on restoration of bile metabolism as a treatment for *C. difficile* (Buffie *et al.,* 2014; Weingarden *et al.,* 2014, 2016). However, many of these studies focus on 7α-dehydroxylating bacterial species with the focus placed on inhibition of *C. difficile* growth rather than on spore germination. A study by Theriot *et al.,* (2015) found that *C. difficile* germination could be stimulated by the contents of the mouse small intestine, even in the absence of antibiotic treatment. This may suggest that inhibition of germination by bile alone is not sufficient to prevent infection with inhibition of outgrowth also essential. However it has been shown that restoration of BSH function alone can also rescue 7α-dehydroxylation function which is mediated through production of CA (Gustafsson *et al.,* 1998). Careful examination of several bacterial therapies described in the literature often confirms that each therapeutic combination contains at least one or more BSH producing species (Petrof *et al.,* 2013; Lawley *et al.,* 2012). Whilst in our experiments, direct use of a BSH producing bacteria was not sufficient to prevent colonisation, it may be that use of higher doses over a longer period of time were required to fully restore this function. The use of a cocktail of BSH and 7α-dehydroxylase producing bacteria may be preferable to restore colonisation resistance and diversity to the gut. Recovery of these microbes may then allow the rest of the microbiota to recover.

The work presented in these chapters highlights the importance of the microbiota in determining infection outcome. Understanding changes in the microbiota and bacterial functions that underlie colonisation and infection outcome may help with the development of a more effectively targeted

bacteriotherapy. There have been many studies published in the literature that highlight the importance of bacterial bile metabolism in colonisation resistance. Restoration of this pathway, either through FMT or with the use of bacterial therapies has been shown to either resolve or lessen colonisation of *C. difficile* (Weingarden *et al.,* 2014; Weingarden *et al.,* 2016; Buffie *et al.,* 2014). As BAs have a vital role in stimulating *C. difficile* germination (chapter 4) and controlling colonisation resistance (chapter 3) then greater understanding of genetic regulation of bile mediated germination and bile tolerance during growth is required. This is because, genes involved in these processes are important in mediating *in vivo* survival.

## 7.3 Genetic regulation of germination and bile acid resistance

BAs, particularly TC are closely involved in the induction of spore germination. Here, we attempted to identify novel genes involved in TC mediated spore germination but these were unsuccessful. However, the identification of a gene known to be essential for germination led to attempts to modify the MT mutagenesis system to allow screening of strains using a fluorescent reporter system. The aim of this approach was to identify genes that were upregulated but not essential for germination, in the hope that novel, but potentially functionally redundant genes in this process could be identified. This however was unsuccessful due to the small size and the high autofluorescence of *C. difficile*, which limited the capacity for high throughput screening using FACs. It was however useful to verify the use of such a system in the lab and it may be useful in investigating other biological processes in the future. It also has potential for identification of genes associated with germination through the creation and testing of mutant libraries. For example, it is known that the CspB protease, although the main, is not the only protease involved in the activation of SleC (Adams *et al.,* 2013). Therefore creation of a mutant library in a strain lacking SleC activity may allow this additional protease to be identified. Through understanding of the genetic regulation of these vital processes it may be possible to identify new treatment targets.

It is widely appreciated that bacteria that inhabit the gut have to adapt to the environmental challenges present. This includes contact with BAs, which are

present in high concentrations in the small intestine. The mechanisms by which many bacteria are able to resist BAs are becoming clearer (Gunn 2000; Begley *et al.,* 2005; Sistrunk *et al.,* 2016), but there are many aspects that require further investigation in Gram positive bacteria generally and in *C. difficile* in particular (Sistrunk *et al.,* 2016). As the balance of bile acids in the antibiotic treated gut is altered, with increased abundance of primary BAs it is likely that *C. difficile* has adapted to survive in these conditions. In the context of this work, we have revealed that possession of BSH genes is important for resistance to the effects of BAs, as the strain of *L. murinus* that expressed an inactive BSH grew poorly in media containing TC. In contrast, the strain expressing the intact BSH gene showed no equivalent growth impairment. Whilst the genome of *C. difficile* does not encode any BSH genes, the use of RNA seq allowed us to identify a number of genes involved in BA resistance. In total 6 genes were identified as being upregulated in the presence of 0.1% TC including a 4 efflux pumps, an RNA polymerase sigma factor and SAM dependent MTase. Upregulation of efflux pumps is a common response to bile exposure among many different enteric bacteria (Baucheron *et al.,* 2014; Quillin *et al.,* 2011; Gunn 2000; Gueimonde *et al.,* 2009). It would be interesting to investigate whether exposure to BA can increase resistance to antibiotics as this has been reported in other bacterial species. Further, it would be relevant to investigate whether the combination of BAs found in the antibiotic treated gut can influence the regulation of these genes.

During the preparation of cells for RNA extraction it was noted that bacterial aggregation frequently occurred following their exposure to TC. This characteristic has been described in a number of other bacteria, including *V. cholera* and *C. jejuni* (Hung *et al.,* 2006; Svensson *et al.,* 2014). As aggregation and biofilm formation may be important in persistence and survival *in vivo* this process was investigated further. Exposure of *C. difficile* to TC either as part of an overnight culture used to seed the biofilms, or as part of the biofilms growth media resulted in a slight increase in biofilm mass with increased aggregation and adherence. Further investigation of the mechanism of this may shed light on how these bacteria behave *in vivo.* Such investigation is important as the mechanisms involved in persistence are still poorly defined and understood.

## 7.4 *Para*-cresol production in acute and relapsing infection

*Para*-cresol is a bacteriostatic compound produced by a limited number of bacteria including *C. difficile* (Hafiz & Oakley, 1976). *C. difficile* strains that are most likely to cause relapsing and severe disease have the highest levels of production and tolerance of *para*-cresol (Dawson *et al.*, 2011; Dawson *et al.*, 2008). This hints at a role for its production in acute and relapsing infection and the work in chapter 6 aimed to investigate this further. Interestingly, animals infected with R20291_*hpdA*::CT, a mutant unable to produce *para*-cresol, suffered from more severe disease than those infected with the parental strain. The severity of infection was directly linked to the antibiotic treatment administered before infection with longer treatment with clindamycin resulting in increased weight loss and mortality. This increased severity however was not seen in those mice infected with parental the strain R20291 or those infected with an unrelated mutant, R20291_*1754*::CT. This suggests that the increased severity observed was not linked to increased clindamycin resistance as a result of ClosTron mutagenesis, although preliminary data suggests that this does impact upon severity in the hamster model. This most likely reflects differences in the microbiotas of these animals although this is yet to be investigated formally. It may also be that clindamycin concentrations in the hamster gut remain high for a longer period of time compared to in the mouse. This would explain why increased clindamycin resistance only seems to be an advantage in the hamster infection model.

To determine the cause of this increased severity, extensive *in vitro* experiments and short term 2 day infections were conducted to determine if there were any differences in growth rates and toxin production between the strains of *C. difficile*. Both studies showed a slight increase in the production of toxin by the *para*-cresol knockout strain R20291_*hpdA*::CT. Whilst this increase was not significant, it could be biologically relevant as these toxins are highly potent. Furthermore if the increased antibiotic exposure disrupts the mucus homeostasis as seen in chapter 3 it may be that this rise is sufficient to cause significant damage. An additional line of investigation that could be interesting to explore is the impact of *para*-cresol production on the immune system. Several papers have shown that this compound is able to dampen down various

aspects of immune response (Dou *et al.*, 2002; Faure *et al.*, 2006). It may be that animals infected with R20291_*hpdA*::CT generate a more significant inflammatory response to the infection. Investigation of this could be carried out in a number of ways including extraction of mRNA from the tissue to look at specific immune markers in the different models of infection.

## 7.5 Final conclusions

The work presented in this thesis has helped to further our understanding of the role that the microbiota plays in modulating *C. difficile* infection. The importance of BAs and bile metabolising bacteria has been further clarified and bacteria with potentially therapeutic traits have been identified. Potential therapeutic markers in the form of BSH genes and changes in BA profiles have also been identified and it may be that these could be utilised in a clinical setting to identify at risk patients. In addition, bacteria that potentially influence disease severity have been described and further investigation of the role of these microbes is warranted. Focus then turned towards the pathogen itself with the examination of the genetic regulation of germination and bile resistance investigated. Although attempts to identify novel genes involved in germination were unsuccessful, the identification of genes involved in BA resistance are the first described and more in-depth analysis of the regulation of these genes is warranted. Finally the production of a bacteriostatic compound *para*-cresol in acute and relapsing infection was examined. Increased disease severity seen in animals infected with R20291_*hpdA*::CT suggesting that *para*-cresol production may be involved in limiting severity in the acute infection.

Overall, this work has helped to improve our understanding of several important areas of infection including the modulation of infection by the microbiota and the importance of BA modifying bacteria in this. This work has also offered our first insight into genetic regulation of BA resistance in *C. difficile* opening up several interesting avenues of future work.

# Conference contributions

Presenting author underlined

Caitlin Jukes, Anthony Buckley, Janice Spencer, June Irvine, Denise Candlish, Umer Ijaz and Gillian Douce. The impact of bile acid modification on *Clostridium difficile* colonisation and severity. Poster presentation at the Young Microbiologist Symposium 2016, Dundee, **UK**

Caitlin Jukes, Anthony Buckley, Janice Spencer, June Irvine, Denise Candlish, Umer Ijaz and Gillian Douce. The role of the microbiota in *Clostridium difficile* colonisation and disease severity. Oral presentation at Microbiology Society conference 2016, Liverpool, **UK**

Caitlin Jukes, Anthony Buckley, Janice Spencer, June Irvine, Denise Candlish, Umer Ijaz and Gillian Douce .The impact of bile acid modification on *Clostridium difficile* colonisation. Poster presentation at Microbiology Society conference 2015, Birmingham, **UK**

Caitlin Jukes, Anthony Buckley, Janice Spencer, June Irvine, Denise Candlish, Umer Ijaz and Gillian Douce. The impact of bile acid modification on *Clostridium difficile* colonisation. Poster presentation at the International *Clostridium difficile* Symposium 2015, Bled, **Slovenia**

Caitlin Jukes, Anthony Buckley, Janice Spencer, June Irvine, Denise Candlish, Umer Ijaz and Gillian Douce The impact of bile acid modification on *Clostridium difficile* colonisation .Poster presentation at the Young Microbiologist Symposium 2014, Dundee, **UK**

Anthony Buckley, Lisa Dawson, Caitlin Jukes, Janice Spencer, Brendan Wren & Gillian Douce. A role for *para*-cresol in *Clostridium difficile* colonisation Poster presentation at Clospath 2013, Palm Cove, **Australia**

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