Exploiting Bacteriophages to Tackle *Clostridium difficile* Infection

Emma Jane Meader

A thesis submitted to the University of East Anglia for the degree of Doctor of Philosophy

The Institute of Food Research
Norwich Research Park
Colney Lane
Norwich, NR4 7UA

May 2013

Abstract

Clostridium difficile infection (CDI) currently affects around 20,000 people each year, in healthcare institutions and in the community, and will often follow disruption of the gut microbiome. Current treatment strategies call for the use of further antibiotics, of which there is a limited choice. There is a need for additional remedial and prophylactic solutions with greater specificity and low levels of toxicity and resistance.

This thesis describes the pathogenesis of CDI, the current treatment strategies and navigates the growing body of studies investigating the potential use of phage. The project involved extensive screening including faecal samples and environmental sources in an attempt to identify novel phages of *C. difficile* and documents efforts to improve the therapeutic capacity of a selected phage, ΦCD27, by mutagenesis. No exclusively lytic phages were isolated or obtained following mutagenesis with ethylmethane sulphonate, hydroxylamine or sodium pyrophosphate.

Batch fermentation models of CDI showed that a prophylactic approach to phage therapy of CDI offers a higher efficacy than a remedial regime. A continuous model of CDI in a colon model was successfully produced and demonstrated variable efficacy rates from no apparent decrease in the burden of *C. difficile* to a reduction to below the limit of detection by culture, with no detrimental effect on commensal microbiota. The lysogenic capacity of ΦCD27 appeared to prevent clearance of *C. difficile* in the models, but some strains containing the prophage exhibited reduced toxin production phenotypically. A possible mechanism of this altered phenotype included the action of ΦCD27 repressor proteins on the promoter regions of *C. diffiicle* toxin genes or regulatory elements, but affinity of a candidate repressor, ORF44, to PaLoc constituents was not demonstrated.

Studies have also demonstrated the ability of ΦCD27 to prevent outgrowth of germinating *C. difficile* spores, thus potential as an environmental decontaminant.

The findings of the project and the future prospects of phage therapy as an agent against CDI are discussed.

Outputs from this project

Publications

- Meader, E., M. J. Mayer, D. Steverding, S. R. Carding, and A. Narbad. 2013. Evaluation of bacteriophage therapy to control *Clostridium difficile* and toxin production in an *in vitro* human colon model system. Accepted for publication May 2013.
- Williams R, E. Meader, M. Mayer, A. Narbad, A.P. Roberts and P. Mullany. Determination of the *attP* and *attB* sites of ΦCD27 from *Clostridium difficile* NCTC 12727. Submitted to The Journal of Medical Microbiology 1.2.13. Under revision.
- Meader, E., M. J. Mayer, M. J. Gasson, D. Steverding, S. R. Carding, and A. Narbad. 2010. Bacteriophage treatment significantly reduces viable *Clostridium difficile* and prevents toxin production in an *in vitro* model system. Anaerobe 16:549-54.

Posters

- Williams R, M. Mayer, E, Meader, A. Narbad, A. Roberts and P. Mullany. Characterisation of a prophage of *Clostridium difficile* NCTC12727. 4th International *Clostridium difficile* Symposium European Society of Clinical Microbiology and Infectious Diseases. 20th-22nd September 2012. Bled, Slovenia.
- Meader E, M. Mayer, A. Narbad, S. Carding and D. Steverding. Prevention of *C. difficile* toxin production using bacteriophage therapy in an *in vitro* colon model. The Bacteriophage in Biology, Biotechnology and Medicine. 26th February 2010. BioPark, Hertfordshire, UK.

- Meader E, M. Mayer, A. Narbad, S. Carding, M. Gasson and D. Steverding. Bacteriophage treatment results in a significant decrease in viable Clostridium difficile and prevents toxin production in a disease model. RSC 2nd South and Eastern Regional Organic Chemistry Meeting, 2009. The John Innes Centre, Norwich, UK.
- Meader E, M. Mayer, S. Carding, M. Gasson, D. Steverding and A. Narbad. Bacteriophage treatment results in a significant reduction in viable *Clostridium difficile* and prevents toxin production in an *in vitro* colon model. Microbes in Norwich, 2009. The John Innes Centre, Norwich, UK.
- Mayer, M. N. Horn, R. Stentz, U. Wegmann, E. Meader, C. Shearman, A. Narbad and M. Gasson. Expression of bacteriophage endolysins in lactic acid bacteria and novel release mechanisms. International Phage Conference 2008. Edinburgh, UK.

Contents

List of figures

List of tables……

Acknowledgements

I am indebted to my supervisors Dr. Arjan Narbad, Dr. Dietmar Steverding, Professor Simon Carding and Professor Mike Gasson for the all support and guidance throughout my project and would like to say a special thank you to my supervisor Dr. Melinda Mayer for her expertise, sound advice and very high standards! I am also grateful to the Microbiology Staff at the Norfolk and Norwich University Hospital for allowing me to continue my clinical work throughout my studies and am particularly grateful to Professor Jim Gray for his assistance with the molecular aspects of the work and general brilliance.

During this project I have had the opportunity to visit and work with other research teams and would like to thank Dr. Rachel Williams (University College, London), for her help with the phage integration site studies and Dr. Pilar Garcìa (Instituto de Productos Lácteos, Asturias) for her help with the phage mutagenesis.

It's been a privilege to study at The Institute of Food Research. The many interesting discussions that I've had with researchers across many fields of science have helped to shape the project and the training and support has been exceptional. I especially want to thank Carmen Nueno-Palop for helping me to set up the *in-vitro* models, Maddie Houchen for her assistance with media preparation, Kathryn Cross and Dr. Mary Parker for helping me with the electron microscopy work, Dr. Martin Webb for overseeing the time lapse microscopy imaging and Jack Dainty for his statistical expertise. Finally, I would like to thank the staff at the University of East Anglia and the Institute of Food Research, past and present, for giving me the opportunity to carry out this work.

Abbreviations

AAC – Antibiotic-associated colitis
AAD – Antibiotic-associated diarrhoea
ADP – Adenosine diphosphate
ASL – Lysis buffer
ATCC – American Type Culture Collection
ATP – Adenine triphosphate
BHI – Brain heart infusion
bp – base pair
BSA – Bovine Serum Albumin
CCEY – Cefoxitin cycloserine egg yolk media
CCEYL – Cefoxitin cycloserine egg yolk media with lysozyme
CDI – *Clostridium difficile* infection
cDNA – Complementary DNA
CDT – *C. difficile* binary toxin
CFU – Colony forming units
DGGE – Density gradient gel electrophoresis
dNTP – Deoxyribonucleotide triphosphate
DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT – Dithiothreitol
EDTA – Ethylenediaminetetraacetic acid
EIA – Enzyme immunoassay
EMA – European medicines agency
EMS – Ethylmethane sulphonate
EMSA – Electrophoretic mobility shift assay
FDA – Food and drug administration
FISH – Fluorescence *in situ* hybridisation
GDH – Glutamate dehydrogenase
GI – Gastrointestinal
HIV - Human immunodeficiency virus
HNIg – Human normal immunoglobulin
HPA – Health Protection Agency
HX – Hydroxylamine
Ig – Immunoglobulin
IGR – Intergenic region
IL – Interleukin
IM - Intramuscular
IPTG – Isopropyl-β-D-1-thiogalactopyranoside
LREC – Local and Regional Ethics Committee
M – Mutated
mA – Milliamps
MAP – Mitogen-activated protein
MIC – Minimum inhibitory concentration
MOI – Multiplicity of infection
mV – Millivolts
NA – Not applicable

NaPP – Sodium pyrophosphate
NCBI – National Centre for Biotechnology Information
NCTC – National Collection of Type Cultures
ND – Not determined
NEB – New England Biolabs
NF-κβ – Nuclear factor kappa-light chain enhancer of activated B cells
NG – Nasogastric
NS – Not screened
OD – Optical density
ORF – Open reading frame
PaLoc – Pathogenicity locus
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PFU – Plaque-forming units
PMC – Pseudomembranous colitis
PVDF – Polyvinylidene fluoride
RCM – Reinforced Clostridia media
SD – Standard deviation
SDS – Sodium dodecyl sulphate
SEM – Scanning electron microscopy
SGL – Southern Group Laboratory
SM – Suspension media
SOC – Super optimal broth
STE – Sodium tris ethylenediaminetetraacetic acid
TBE – Tris borate ethylenediaminetetraacetic acid
TBS – Tris buffered saline
TE – Tris ethylenediaminetetraacetic acid
TEM – Transmission electron microscopy
TM – Toxic megacolon
TNFα – Tumour necrosis factor alpha
TUDCA – Tauroursodeoxycholic acid
U – Units
UK – United Kingdom
UV – Ultra violet
V - Volts
v/v – Volume to volume
WT – Wild-type
Wt/vol – Weight to volume
XGAL – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1 - Introduction

1.1. *Clostridium difficile* infection

Clostridium difficile is a Gram-positive spore-forming rod, ubiquitous in the environment but fastidious in nature, requiring strict anaerobic conditions for germination and growth. *C. difficile* was first described in 1935 as a component of neonatal faecal flora (Hall and O'Toole, 1935) and it was not until 1978 that two independent groups identified it as a causative agent of antibiotic-associated diarrhoea (AAD) (Bartlett *et al.*, 1978; George *et al.*, 1978). *C. difficile* infection (CDI) has since been implicated as the most common cause of AAD (Rupnik *et al.*, 2009), responsible for up to 30% of cases and accounting for 50-75% reports of antibiotic-associated colitis (Kelly *et al.*, 1994).

Despite an encouraging decline in the number of reports in England and Wales since 2007, 19,130 cases were reported in 2011, and 77% of these were in patients aged 65 years and over (Health Protection Agency surveillance data). *C. difficile* is predominantly transmitted via the faecal-oral route and it is estimated that 7-10% of adults are colonised without suffering from symptoms (Ryan *et al.*, 2010), but this incidence may be higher in hospitals and care institutions where exposure to *C. difficile* is more likely. Colonisation may also result from the ingestion of animal meat (Rodriguez-Palacios *et al.*, 2007) or contact with new-born mammals, including humans, as their gastrointestinal (GI) tract seems to serve as an important reservoir for *C. difficile* without causing symptomatic disease (Mandell *et al.*, 2010, Rodriguez-Palacios *et al.*, 2006; Rolfe and Iaconis, 1983).

There are few data detailing the financial burden of CDI in the UK but estimates suggest that it may run into billions of pounds each year (Dubberke and Olsen, 2012; Dubberke *et al.*, 2007; Kyne *et al.*, 2002; O'Brien *et al.*, 2007; Song *et al.*, 2008)

CDI normally follows a course of broad-spectrum antibiotic therapy due to the resulting collateral damage to the enteric microbiotia and the intrinsic antibiotic resistance profile typical of *C. difficile* (Starr *et al.*, 2003). This is particularly a problem in patients over the age of 65, as the numbers of

protective commensal species including *Bifidobacteria spp.* generally decrease with age regardless of antibiotic treatment (Hopkins and MacFarlane, 2002). In this compromised environment, the germination of *C. difficile* spores is permitted and *C. difficile* binds to intestinal mucus and epithelial cells via a mixture of high and low molecular weight surface layer proteins (Calabi *et al.*, 2002). During outgrowth, *C. difficile* produces two potent toxins – A and B, which give rise to colitis and pseudomembranous colitis (PMC), so called due to the formation and coalescence of neutrophilic, fibrinous, mucoid plaques throughout the colon (Mandell *et al.*, 2010). Patients experience abdominal cramping and diarrhoea and also suffer a loss of intestinal barrier function that can lead to dehydration and malnutrition. In severe cases of CDI, patients can progress to toxic megacolon (TM), a condition characterised by non-obstructive, radiologically confirmed dilatation of the colon greater than 6 cm in diameter with fever, leucocytosis, tachycardia or anaemia, sometimes with dehydration and a compromised mental state (Jalan *et al.*, 1969).

Prevention and management of CDI in a hospital environment can be difficult with many patients taking broad-spectrum antibiotics and being cared for together in wards. With an aging population, CDI is set to become one of the biggest challenges of the 21st century.

1.2. Pathogenesis of *C. difficile*

The Pathogenicity Locus (PaLoc) of the *C. difficile* genome, depicted in Fig. 1.1, contains genes required for the production and regulation of toxin A (*tcdA*) and toxin B (*tcdB*), which give rise to the virulent disposition of this organism (Voth and Ballard, 2005).

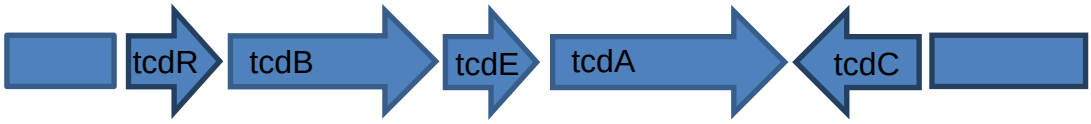


Figure 1.1. The pathogenicity locus (PaLoc) of toxigenic *C. difficile* strains. Approximately 20 kb of DNA containing toxin and regulatory genes.

Toxin A is a 308 kDa single-chain protein that binds to glycan receptors at the apex of the human colonocyte (Stubbe *et al.*, 2000) and is internalised by clathrin-mediated endocytosis (Papatheodorou *et al.*, 2010). It is a potent enterotoxin that contains four functional domains. The transmembrane domain allows the toxin to be transported to the cytoplasm after endosome acidification via pore formation (Zeiser *et al.*, 2011), at which point a cysteine protease cleaves the N-terminal glucosyltransferase domain (Egerer *et al.*, 2007). The glucosyltransferase is responsible for the glucosylation and subsequent inactivation of Rho, Rac, Cdc42, RhoG and TC10 – small GTP-binding proteins of the Rho family (Genth *et al.*, 2008). These proteins regulate the dynamics of the cellular actin and their dysfunction causes collapse of the cytoskeleton and disrupts the integrity of the tight junctions between the cells, allowing passage of the toxins through the epithelium (Rupnik *et al.*, 2009). Eventually the cells round up and die due to the ability of the toxins to activate the pro-apoptotic protein RhoB (Genth *et al.*, 2008). Macroscopic plaques comprised of neutrophils and fibrin may also be a feature (Gerding *et al.*, 1995) (Fig. 1.2).

Toxin B is also a glycosylating toxin, acting on the Rho proteins in the same manner as toxin A to cause cytotoxicity. It is smaller than toxin A at 270 kDa (Qamar *et al.*, 2001) and binds at the basolateral surface of enterocytes (Stubbe *et al.*, 2000). Although previously regarded as inferior to toxin A in terms of its capacity to induce disease (Lyerly *et al.*, 1985) it is now accepted that toxin B is a potent instigator of disease in its own right. Affinity to cardiac tissue has been demonstrated (Hamm *et al.*, 2006) and although extracolonic manifestations of CDI are rare, there have been cases of *C. difficile* bacteraemia (Lee *et al.*, 2010, Libby and Bearman, 2009) one of whom presented with a pericardial effusion (Byl *et al.*, 1996).

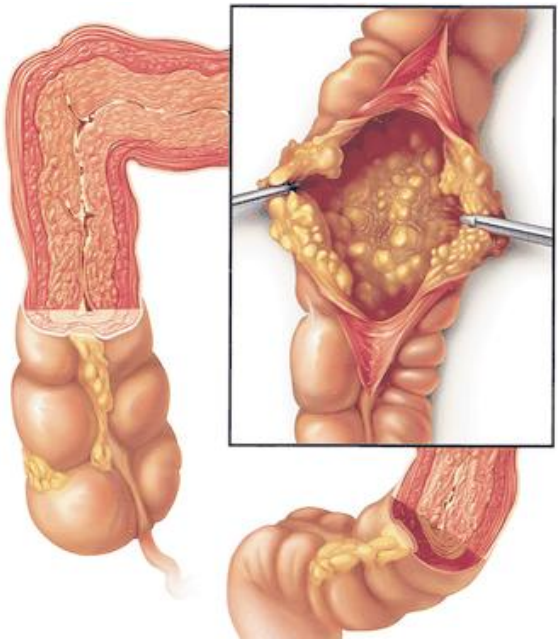


Figure 1.2. Pseudomembranous colitis showing neutrophilic fibrinous plaques. Image from CT evaluation of the colon: inflammatory disease (Horton *et al.*, 2000).

Past failures to demonstrate toxin B's ability to cause significant intestinal damage might have been due to the absence of specific toxin receptors (Carter *et al.*, 2010) inherent instability of the toxin (Reineke *et al.*, 2007), impure preparations (Krivan and Wilkins, 1987, Sullivan *et al.*, 1982) or self-aggregative properties (Mandell *et al.*, 2010).

While the vast majority of toxigenic strains will express both toxin A and B, some strains possess just one of these genes. The role of either toxin as the dominant virulence factor has been the subject of much debate with both A+B- and A-B+ strains implicated as capable pathogens and similarly A+B- and A-B+ strains with no apparent virulence capacity (Drudy *et al.*, 2007; Kuehne *et al.*, 2010; Lyerly *et al.*, 1985; Lyras *et al.*, 2009; Mitchell *et al.*, 1986, 1987). In a study using mutated strains of *C. difficile*, one with toxin A production disabled and another with toxin B production disabled, it was shown that those producing only toxin B caused death in 16/20 hamsters, whereas just 4/20 animals died due to infection with a toxin B deficient strain (Carter *et al.*, 2010). It has been proposed that the lack of either toxin

prevents an effective immune response to the pathogen (Drudy *et al.*, 2007), resulting in increased disease severity.

A third toxin, referred to as the binary toxin (or CDT) is encoded by three genes located at a different chromosomal locus – the CdtLoc. CDT can be found in strains that are also positive for toxins A and B (Perelle *et al.*, 1997; Stubbs *et al.*, 2000) including the hypervirulent NAP1/027 strain (McDonald *et al.*, 2005) but it is estimated that approximately 2% of A-B- strains also produce the binary toxin (Geric *et al.*, 2003). The specific binding capacity of the toxin is encoded by the *cdtB* domain, with the *cdtA* gene responsible for the enzymatic activity of the protein (Rupnik *et al.*, 2009). In cell culture, the addition of CDT has been shown to result in adenine diphosphate (ADP) ribosylation of G-actin causing breakdown of the actin fibres and a loss of cell integrity, plus in an ileal loop model it was sufficient to induce fluid accumulation and haemorrhage (Geric *et al.*, 2006). In a hamster model however, strains of A-B- *C. difficile* that produced CDT did not cause disease (Geric *et al.*, 2006). Its role in CDI pathogenesis remains unclear but in a comparison of 30-day fatality rates, patients infected with strains of *C. difficile* that produced binary toxin were more likely to die than those infected with a strain that did not produce binary toxin (relative risk 1.8, 95% confidence interval 1.2-2.7) (Bacci *et al.*, 2011).

1.3. The immune response to CDI

The immune response to *C. difficile* causes much of the intestinal damage characteristic of CDI (Savidge *et al.*, 2003) as it has been shown that disease severity in animal models can be significantly lessened by manipulation of the immune attack (Anton *et al.*, 2004; Cottrell *et al.*, 2007; Kelly *et al.*, 1994).

Both toxins A and B have cytotoxic activity, mediated by their glucosyltransferase function (Zeiser *et al.*, 2011) and cause massive recruitment of neutrophils (Kelly *et al.*, 1994) due to the stimulation of inflammatory mediators from colonocytes, mast cells and phagocytes including Tumour Necrosis Factor α (TNFα), interleukin (IL) 1β (Jeffrey *et al.*, 2010) and IL-8 (He *et al.*, 2002). Almost 100-fold increases in IL-8 production have been demonstrated following stimulation of human monocytes with just 1

nM toxin A, and this up-regulation is largely a result of enhanced *IL-8* gene transcription (Jefferson *et al.*, 1999). Expression of the pro-inflammatory cyclooxygenase-2 plus release of prostaglandin E2 and reactive oxygen species are triggered by toxin A (Kim *et al.*, 2005). The NF-κB and mitogen-activated protein (MAP) kinase pathways are also stimulated (Chae *et al.*, 2006; Chen *et al.*, 2006; Jefferson *et al.*, 1999; Kim *et al.*, 2005; Warny *et al.*, 2005). Toxin A has also been shown to activate and up-regulate protease-activated receptor 2 (Cottrell *et al.*, 2007) and corticotropin-releasing hormone as promoters of inflammation in ileal loop models. Long-term exposure to toxins A and B also leads to apoptosis and necrosis (Huelsenbeck *et al.*, 2007; Nottrott *et al.*, 2007) and the ensuing leakage of cytoplasmic contents results in further enrolment of immune mediators (Genth *et al.*, 2008). This inflammatory response is in stark contrast to the minimal reaction to other enteric toxins such as those produced by *Escherichia coli* (Erume *et al.*, 2012).

It is well documented that the magnitude of the immunoglobulin (Ig) A and G response to both toxin A and B can be used as a predictor for disease outcome, with a strong response lessening the chance of severe disease and recurrence (Kelly and Kyne, 2011; Kyne *et al.*, 2001; Leav *et al.*, 2010). However, it is important to note that this protective effect is not so evident in severely ill patients with multiple co-morbidities (Kelly and Kyne, 2011). Specific deficiencies in IgG2 and IgG3 subclasses have also been associated with an increased likelihood of CDI recurrence (Katchar *et al.*, 2007).

The antibody response to toxin antigens has been found to correlate with the levels of antibodies to non-toxin antigens, suggesting that a multi-faceted approach to immunomodulation may be required to achieve good protection (Kelly and Kyne, 2011). In response to *C. difficile*, antibodies are also generated against the surface layer proteins used by *C. difficile* to bind to mucus and intestinal epithelial cells (Calabi *et al.*, 2002). The levels of IgG directed against these proteins were found to be similar in asymptomatic carriers, CDI patients and control patients, but it is thought that the extent of the IgM response (the first antibody class to be produced in response to an antigen) may affect the course of disease, a lesser response being associated

with a higher chance of recurrence (Drudy *et al.*, 2004). Antibodies to flagellar proteins (such as FliC) and surface associated proteins (such as Cwp84) have also been detected in patients with CDI, but levels significantly decrease following resolution of symptoms (Pechine *et al.*, 2005 b).

1.4. Diagnosis of CDI

Due to the fact that patients may be colonised with *C. difficile* without suffering any symptoms of disease, it is essential to consider the results of diagnostic tests in light of clinical information. The detection of both toxins A and B (and it is important that an assay capable of detecting both of these toxins is used) in patients' stool is a good indicator of disease, but this is not suitable as a stand-alone test for CDI. Evidence of the presence of *C. difficile* should first be obtained through the use of an assay to detect glutamate dehydrogenase (GDH), which is present in both toxigenic and non-toxigenic *C. difficile* strains. Alternatively, a nucleic acid-based amplification method can be used – targeting either the toxin genes or a *C. difficile* housekeeping gene. Faecal lactoferrin, a neutrophil-derived protein, can also serve as a useful marker in assessing the extent of intestinal inflammation (Wren *et al.*, 2009).

In addition to laboratory testing, the identification of multiple plaques or pseudomembranes by endoscopy can offer or support a diagnosis when *C. difficile* is suspected (Fekety 1997).

1.5. Additional risk factors for CDI

Besides antimicrobial therapy, there are a myriad of documented and hypothesised factors thought to contribute to the development of CDI and in a recent study of community-acquired cases, approximately one third had not received antimicrobial therapy (or been hospitalised) in the 6 months prior to CDI development (Wilcox *et al.*, 2008). Increased awareness, frequency of testing and surveillance reporting can help to elucidate unconfirmed and unknown predispositions to CDI.

1.5.1. Patient demographics

Advanced age is a well-documented risk factor for CDI and recurrent CDI (Kim *et al.*, 2010; Loo *et al.*, 2011) and this is most commonly attributed to the changes in the gut microbiome including reductions in the numbers of *Bifidobacteria spp*. thought to offer colonisation resistance against *C. difficile* (Hopkins and MacFarlane, 2002). Elderly patients are also more likely to receive antimicrobial therapy and suffer the cumulative effects of previous courses plus an increased rate of hospitalisation and institutional admission is also to be expected, which raises the chance of *C. difficile* exposure.

1.5.2. Colonisation with *C. difficile* NAP1/027

The hypervirulent *C. difficile* NAP1/027 emerged in Canada, America and Europe in the late 1990s causing numerous outbreaks of severe CDI that responded less favourably to first-line treatment (Musher *et al.*, 2005; Pepin *et al.*, 2004; Warny *et al.*, 2005). This strain has been shown to produce toxin for a longer period relative to other strains (23 days as opposed to 13 days) (Freeman *et al.*, 2007), which may be due to an 18 base pair (bp) deletion in the *tcdC* gene responsible for down-regulation of toxin A production (Warny *et al.*, 2005). Detection of *C. diffiicle* NAP1/027 has been shown to be an independent risk factor for the development of CDI due to the increased virulence of this strain (Loo *et al.*, 2011). It has also been shown that colonisation with other strains (including non-toxigenic strains) may offer cross protection and enhance colonisation resistance against this pathogen (Loo *et al.*, 2011)

1.5.3. Use of Antacid Medications

The use of antacid medication including proton pump inhibitors and histamine receptor antagonists has also been identified as an independent risk factor for the development of CDI (Loo *et al.*, 2011). The reduced acidity of the stomach environment may weaken resistance against vegetative *C. difficile* cells and it has been shown that the survival rate of spores was enhanced at pH values approaching neutrality (Jump *et al.*, 2007). *C. difficile* colonisation in the large and small intestine has also been shown to be more successful *in vivo* when

gastric acid production is suppressed (Jump *et al.*, 2007; Lewis *et al.*, 1996; Verdu *et al.*, 1994; Wilson *et al.*, 1985)

Antacid treatments have been shown to reduce neutrophilic reactive oxygen production leading to decreased bacteriocidal action (Zedtwitz-Liebenstein *et al.*, 2002), which may further weaken defence against *C. difficile* colonisation and infection.

1.5.4. Feeding via a nasogastric (NG) tube

In addition to an increased need for antimicrobial therapy and long-term institutional care, some critically ill patients require nutritional support in the form of elemental feeds via a nasogastric tube. These feeds are completely absorbed in the small intestine, divesting colonic bacterial populations of the starch, dietary fibre, oligosaccharides and other nutritional compounds needed to support their growth (O'Keefe, 2010). In this state, numbers of *Bifidobacteria spp.* and butyrate-producers in the lower GI tract are suppressed, giving rise to the overgrowth of *C. difficile* (May *et al.*, 1994). This method of feeding also fails to stimulate the production of gastric secretions and gut motility, affecting turnover of the microbiota (Kaushik *et al.*, 2005; O'Keefe, 2010). In addition, the invasive nature of NG tube insertion can serve as an efficient inoculation mechanism straight into the GI tract, plus elemental feeds have been used as a culture medium for *C. difficile* with great success (Iizuka *et al.*, 2004). It has been demonstrated that by adding oligosaccharide supplements to the feeds, the numbers of intestinal *Bifidobacteria spp*. were boosted and the environment of the GI tracts of these patients were less permissive to the germination of *C. difficile*, causing clinical reduction in diarrhoea in NG fed patients with chronic relapsing CDI (Lewis *et al.*, 2005).

1.5.5. Other intestinal pathogens

It can be hypothesised that GI disruption due to infection by other pathogens could increase the risk of *C. difficile* colonisation and germination. *Salmonella spp.* infection has been shown to cause changes in the proportions and numbers of commensal intestinal bacteria (Santos *et al.*, 2009) and indeed CDI progressing to fatal PMC has been described following *Salmonella*

Saintpaul infection in a 20 year old female (Halvorson *et al.*, 2011). Uncomplicated cases of colitis associated with co-infections of *C. difficile* and *Salmonella spp*. or *Shigella spp*. have also been reported (Grinblat *et al.*, 2004).

Gastroenteritis is commonly of viral aetiology, with norovirus being most frequently implicated both in the community, in institutions and in hospitals. The virus can be transmitted easily from person to person due to the low infectious dose and hardy nature of the virus and it has been noted that increases in norovirus cases correlate positively with reports of CDI (Lopman *et al.*, 2004; Siebenga *et al.*, 2007). During a recent outbreak of norovirus, *C. difficile* toxins were demonstrated in the stool samples of 5/29 affected patients and a further 2/29 were *C. difficile* positive by culture alone (Koo *et al.*, 2009). The age range of the patients was not mentioned in this study but is of importance here since similar outbreaks in geriatric wards, where patients' microbiota may be more susceptible to colonisation and the development of fulminant CDI, may cause a higher proportion of patients to be affected.

1.5.6. Smoking

Cigarette smoking has also been proposed as a risk factor for CDI, with active smokers 75% more likely to develop it relative to people who have never smoked (Rogers *et al.*, 2012). This is possibly due to the very act of smoking as a route of spore ingestion, since *Clostridium spp*. are common isolates from cigarette samples and it is likely that the spores survive the effect of the smoke (Eaton *et al.*, 1995; Sapkota *et al.*, 2010). It may be that this increased risk reflects significant differences in the gut microbiome of smokers due directly to the effects of tobacco, given that cigarette smoke has been shown to decrease the levels of *Bifidobacteria spp*. (and organic acids) in the cecum of rats (Tomoda *et al.*, 2011). Other factors could include the different dietary tendencies and higher alcohol consumption reported in studies of smokers (Cade and Margetts, 1991) and the anticipated higher risk for hospitalisation and antimicrobial therapy.

1.6. The genome of *C. difficile*

The adaptive and resilient nature of *C. difficile* strains is further evidenced by their genomes, which show a high degree of heterogeneity across all functional categories and a core genome of only 16% - predicted to be the lowest of any bacterial species known to date (Janvilisri *et al.*, 2009; Scaria *et al.*, 2010). Some additional conservation can be observed in strains infecting a particular host species, demonstrating their adaptation to survive in different environments (Janvilisri *et al.*, 2009). When compared to other sequenced Clostridial genomes *C. acetobutylicum, C. botulinum, C. perfringens* and *C. tetani*, a homology of just 15% is observed, with up to 61% of the predicted coding sequences being exclusive to *C. difficile* (Sebaihia *et al.*, 2006). Further details of the relatedness of some Clostridial species to *C. difficile* can be found in 4.2

The continual evolution of *C. diffcile* can be seen by comparing sequence data from historical strains right up to the hypervirulent NAP1/027 strain and includes the acquisition of transcriptional regulators, restriction modification genes, a multi-antimicrobial extrusion family drug/sodium antiporter, genes permitting the utilisation of different carbohydrate sources and antibiotic resistance elements. Antimicrobial resistance patterns are a likely reflection of various antibiotic policies of hospitals across the world but now typically include tetracycline, erythromycin, bacitracin, beta-lactamase and fluoroquinolones, confirmed by antimicrobial assays (Sebaihia *et al.*, 2006; Stabler *et al.*, 2009). The hypervirulent strain NAP1/027 also has 14 genes that have been disrupted by an insertion and 10 frameshift mutations including one that results in a truncated version of tcdC – a negative regulator of toxin A production. Variability in the toxin B gene sequence is a feature of many strains, but in NAP1/027 it has been shown to correlate with increased cytotoxicity in cell cultures (Stabler *et al.*, 2009). Variations in the flagella region also seem to confer greater motility – another important virulence factor since it allows easier traversal of the mucus layer on its way to gut epithelial cells. All of these features are compatible with the increased virulence and fatality rate associated with NAP1/027 strain types (Loo *et al.*, 2011).

C. difficile genomes commonly harbour prophages and prophage elements (Fortier and Moineau 2007; Goh *et al.*, 2005, 2007; Govind *et al.*, 2006; Horgan *et al.*, 2010; Janvilisri *et al.*, 2009; Mahony *et al.*, 1985; Nagy and Foldes, 1991; Sebaihia *et al.*, 2006; Sell *et al.*, 1983; Stabler *et al.*, 2009) and this may to some extent account for the inexorable uptake of new genetic elements driving the progressive nature of this pathogen.

The importance of re-annotating genome sequences was recently illustrated for CD630 (NCTC 11307) (Monot *et al.*, 2011). New database submissions over 5 years allowed the function of over 500 previously unclassified genes to be assigned, and using updated techniques, 127 new coding sequences were identified. This information is imperative to our understanding of *C. difficile* pathogenesis both to support previous findings and to discover new virulence mechanisms and potential therapeutic targets.

1.7. Therapies used for the treatment of CDI

1.7.1. Current antibiotics

1.7.1.1. Metronidazole

Patients presenting with uncomplicated *C. difficile* for the first time are treated with oral metronidazole – a nitroimidazole antibiotic with a high specificity for anaerobic bacteria and a range of parasites (Muller, 1983). Upon reduction of the nitrile group under anaerobic conditions, cytotoxic intermediate products are produced that interact directly with the bacterial DNA to inhibit synthesis and cause cell death. The usual dosage for an adult is 400 mg orally every 8 h for 10-14 days, which has few side effects (Joint Formulary Committee, 2012). Metronidazole treatment of *C. difficile* carriage in the absence of diarrhoea and/or abdominal symptoms is not effective (Johnson *et al.*, 1992).

Clinical improvement is normally observed within 2 or 3 days following treatment initiation, but the frequency of resistant strains, defined as those with a minimum inhibitory concentration (MIC) of >16 µg/mL, has increased in recent years. Some institutions have reported rates as high as 6.3% (Pelaez *et al.*, 2008). The frequency of recurrences following metronidazole therapy (not associated with resistance) has also been reported to occur in 10-35% of

cases (Barbut *et al.*, 2000; Do *et al.*, 1998; McFarland *et al.*, 2002) and these are normally seen within 8 weeks post treatment.

1.7.1.2. Vancomycin

Vancomycin is an effective agent in the treatment of CDI but its use is tightly restricted to prevent the development of resistance in enteric bacteria, particularly in *Enterococcus spp*. and *Staphylococcus spp.* (Sujatha and Praharaj, 2012). It is reserved for patients who have severe CDI or those who have suffered a relapsed or re-infection with *C. difficile*.

Vancomycin is a glycopeptide antibiotic that acts primarily by disrupting bacterial cell wall synthesis through binding to the D-Ala-D-Ala terminus of the peptidoglycan precursors. This prevents the enzymatic cross-linking required to complete the cell wall formation (Watanakunakorn, 1984). Oral administration allows a greater concentration of the drug to reach the gut and the poor absorption efficiency means that the toxicity concerns can be minimised. Its spectrum of activity includes both aerobic and anaerobic Gram-positive bacteria.

Relapses of CDI are also common following vancomycin treatment (Louie *et al.*, 2011) but proposed preventative strategies include tapering the treatment frequency after a course of 10-14 days to twice daily dosing, then once daily and a ‘pulse’ every other day after symptom resolution (Tedesco, 1982) or by giving another antimicrobial such as rifaximin immediately after the treatment course (Johnson *et al.*, 2009). The aim here is to eliminate the spores that have survived the antimicrobial treatment and are germinating in the compromised enteric microbiome.

Interestingly, genetic elements thought to impart resistance to vancomycin (58-77% similar to VanG-type resistance of Enterococci) have been demonstrated in *C. difficile* 630 (Sebaihia *et al.*, 2006). Phenotypic resistance however, was not demonstrated by antimicrobial assay and it is not clear if these genes are not expressed or are not sufficient to impart resistance to the effects of the antibiotic.

1.7.1.3. Nitazoxanide

Nitazoxanide is a synthetic thiazolide compound that interferes with electron transfer in anaerobic energy metabolism (Mandell *et al.*, 2010). The few small studies that have compared the efficacy of nitazoxanide versus metronidazole or vancomycin have suggested a comparable cure rate (Musher *et al.*, 2006 a, 2006 b, 2009) and it has even achieved clinical responses in cases of initial treatment failure following metronidazole therapy (Musher *et al.*, 2007).

1.7.1.4. Rifaximin

Rifaximin is a narrow-spectrum rifamycin drug that acts via steric inhibition of the beta subunit of the bacterial DNA-dependent ribonucleic acid (RNA) polymerase causing abortive transcription (Mandell *et al.*, 2010). Mammalian polymerases are unaffected at the concentrations required to prevent bacterial DNA replication. Due to the mode of action, high level resistance to rifamycins can be achieved by single amino acid substitutions (O'Connor *et al.*, 2008) and so it is always preferable to use these agents in combination with other drugs. Rifaximin is not active against all clinical strains of *C. difficile*, for example the hypervirulent strain NAP1/027 is resistant, but in sensitive isolates it has been shown to be as effective as vancomycin in an animal model (Kokkotou *et al.*, 2008). Other studies have demonstrated a reduced rate of CDI recurrence when a 2 week course of rifaximin is administered after treatment with vancomycin (Johnson *et al.*, 2007).

1.7.1.5. Rifalazil

Rifalazin is also a rifamycin known to have activity against a wide range of Gram-positive, Gram-negative organisms and Mycobacteria (Luna-Herrera *et al.*, 1995). Good activity versus a range of *C. difficile* strains was recorded, plus the authors demonstrated superiority to vancomycin in a hamster model of CDI both in terms of symptom resolution and reduction of recurrence rates (Anton *et al.*, 2004).

1.7.1.6. Tigecycline

Tigecycline is a third generation tetracycline that reversibly inhibits protein synthesis by binding to the 30S ribosomal subunit (Mandell *et al.*, 2010). Its use in the treatment of CDI is limited to a handful of cases refractory to metronidazole and vancomycin therapy, but has shown promise in these patients with 6 out of 6 patients reporting treatment success (Larson *et al.*, 2011).

1.7.1.7. Bacitracin

Bacitracin is a polypeptide antibiotic produced by *Bacillus subtilis*, which complexes with cell wall components to impede cell wall formation (Mandell *et al.*, 2010). It is active against many Gram-positive bacteria and is often used as a topical application (Joint Formulary Committee, 2012). Early studies have suggested a clinical efficacy equal to that of vancomycin therapy, although a high number of patients continued to excrete *C. difficile* (Dudley *et al*., 1986). More recently, high level resistance has been demonstrated in *C. difficile* strains (Citron *et al.*, 2003), including the NAP1/027 hypervirulent strain (Bourgault *et al.*, 2006), thus bacitracin is of diminishing clinical use for the treatment of CDI.

1.7.1.8. Fusidic acid

Fusidic acid is produced by *Fusidium coccineum*, and blocks the elongation phase of protein synthesis in a select range of anaerobic and aerobic bacteria (Mandell *et al.*, 2010). In a small double-blind, randomised controlled trial, fusidic acid was shown to be equally as effective as metronidazole in the treatment of CDI (Wullt and Odenholt, 2004), but the bacteriostatic nature of the drug means that high recurrence rates can be expected.

1.7.1.9. Teicoplanin

Derived from *Actinoplanes teichomyceticus*, teicoplanin is a mixture of glycopeptide analogues that inhibit cell wall synthesis in a similar manner to vancomycin (Mandell *et al.*, 2010). It has a long half-life and is listed as an alternative treatment of CDI and PMC in the British National Formulary

(Joint Formulary Committee, 2012). In a study of 47 patients allocated to vancomycin or teicoplanin treatment, it was shown that those who received teicoplanin experienced resolution of symptoms and no recurrences, with all but one apparently clear of *C. difficile* colonisation (de Lalla *et al.*, 1989). In the vancomycin group, 3 of 23 patients suffered a relapse and a further asymptomatic patient remained culture positive for *C. difficile*. Superiority to vancomycin has also been demonstrated in more recent studies (Wenisch *et al.*, 1996) but in practice it is rarely used in hospitals in the UK.

1.7.1.10. Oritavancin

Oritavancin is a second generation lipoglycopeptide antibiotic that is very similar to vancomycin in terms of structure and mode of action (Laverty *et al.*, 2011). The side chains of this drug also bind to the bacterial cell membrane and induce profound changes in the membrane potential giving rise to increased permeability (Domenech *et al.*, 2009). There are currently no clinical recommendations relating to the use of oritavancin in CDI, but high efficacy has been demonstrated in an *in vitro* human gut model study and superior activity was observed due to the increased activity against *C. difficile* spores (and hence reduced scope for recurrences) relative to vancomycin (Baines *et al.*, 2008).

1.7.1.11. Fidaxomycin

Fidaxomicin has recently been approved for use by the FDA (Food and Drug Administration) and EMA (European Medicines Agency) as a treatment for CDI (Cornely, 2012). It is a novel macrocyclic antibiotic (previously referred to as OPT-80) and works by inhibition of bacterial RNA polymerases (Cruz, 2012) via the active metabolite hydrolysed in the intestine. It is poorly absorbed and has a narrow spectrum of activity, making it ideal for use in CDI. The clinical efficacy is greater than that of vancomycin based on phase III clinical trials, with a greatly diminished rate of recurrence ($p \leq 0.005$) (Louie *et al.*, 2011) even despite concomitant antimicrobial use (Mullane *et al.*, 2011), but in patients suffering from CDI caused by the NAP1/027 strain, no improvement in the recurrence rate was observed. Synergistic action of fidaxomicin with rifaximin and rifampicin has also been reported (Ackermann

et al., 2004; Babakhani *et al.*, 2004). The drug has been tolerated with no major safety concerns although 5.9% treated with fidaxomicin withdrew from the trial due to adverse events, primarily vomiting (Louie *et al.*, 2011). The price of fidaxomicin is currently three times that of vancomycin but it represents a much needed addition to the repertoire of treatments against refractory CDI (Lancaster and Matthews 2012).

No fidaxomicin resistance has yet been reported but *C. difficile* isolated from a treated patient who suffered a recurrence, gave an elevated MIC of 16 μg/mL (Goldstein *et al.*, 2011).

1.7.2. Novel antimicrobial agents currently in development

1.7.2.1. RBx14255

A novel ketolide, RBx14255, has recently been described and shown to have potent activity against C*. difficile in vitro* and in a hamster model of CDI (Kumar *et al.*, 2012). Ketolides inhibit bacterial protein synthesis and ribosome assembly but show poor activity against Gram-negative anaerobes. The effect on the resident gut microbiota was not examined in this study but with MIC results for *C. difficile* spores comparable to those of metronidazole and vancomycin, the risk of CDI recurrence still persists.

1.7.2.2. RBx11760

RBx11760, a novel oxzolidinone and a potent inhibitor of bacterial protein synthesis was shown to exhibit bacteriocidal activity against *C. difficile*, significantly reduce toxin production and lessen sporulation relative to both metronidazole and vancomycin in a hamster model of CDI (Mathur *et al.*, 2011).

1.7.2.3. CB-183,315

This new lipopeptide antibiotic has also shown promise against clinical *C. difficile* strains in the hamster model and sustained cure rates highly comparable to those obtained following vancomycin treatment. It is currently in phase III clinical development (Mascio *et al.*, 2012). The mode of action seems to be very similar to that of daptomycin, and causes depolarisation of

the cell membrane without increasing permeability (Mascio *et al.*, 2012). It may be that this results in the efflux of cytoplasmic ions such as potassium, which ultimately leads to cell death.

1.7.2.4. MBX-500

MBX-500 is a hybrid antibiotic comprised of an anilinouracil DNA polymerase inhibitor fused with a fluoroquinolone DNA gyrase/topoisomerase inhibitor. This agent has shown good activity against *C. difficile* in a hamster model relative to vancomycin with relative sparing of many groups of anaerobic and aerobic commensals. Unfortunately, relapses were still observed in 30% of the animals (Butler *et al.*, 2012).

1.7.2.5. LFF571

This agent is a semi-synthetic derivative of dicarboxylic acid with potent activity against *C. difficile* (LaMarche *et al.*, 2012) brought about by inhibition of protein synthesis. In hamster model experiments, this agent has been shown to give rise to fewer CDI relapses post-treatment relative to vancomycin (Trzasko *et al.*, 2012). Encouragingly, LFF571 has low activity against *Bifidobacterium spp*. and *Lactobacillus spp.* but the effect on other Gram-positive organisms is variable (Citron *et al.*, 2012).

1.7.2.6. Tauroursodeoxycholic acid (TUDCA)

TUDCA is a hydrophilic bile acid and is used therapeutically to treat cholestasis (interruption in the production and excretion of bile) and biliary cirrhosis (inflammation of the liver characterised by obstruction of the bile duct) (Paumgartner and Beuers, 2002; Saksena and Tandon, 1997). A recent study found that bile acid signalling can affect the activity of Rho proteins. It was shown that phosphorylation of the RacI/Cdc42 by TUDCA protects these proteins from toxin B-catalysed glycosylation and caused a reduction in the cytopathic effect observed in HepG2 cells (Brandes *et al.*, 2012).

1.7.3. Antimicrobial peptides

Antimicrobial peptides include host defence peptides produced by humans and other higher organisms that exhibit direct antibacterial activity on

pathogenic bacteria, and post-translationally modified peptides (lantibiotics) are produced by Gram positive bacteria to give them competitive advantages in mixed populations. They act on (often multiple) membrane and cell wall targets and thus the incidence of resistance is rare due to the extensive mutations required for evasion (Dawson and Scott, 2012). Their therapeutic use in the gut is challenging owing to the presence of proteolytic enzymes that degrade the peptides (Lai *et al.*, 2007), but they seem to play an important role in homeostasis of the microbiota and the control of inflammation (Gersemann *et al.*, 2009; Hua *et al.*, 2010). Peptides can be chemically manipulated in attempt to sidestep degradation, improve activity or modify the specificity of the antimicrobial action (Wetzler and Barron, 2011)

The lantibiotic lacticin 3147 has shown promising activity against *C. difficile* but suffers the effects of degradation in the gut environment and is also active against groups of commensal bacteria (Gardiner *et al.*, 2007; Rea *et al.*, 2007). Another lantibiotic, NVB302, has shown good stability and selective activity in the GI tract and resolution of CDI in a hamster model (Johnson, 2010). Clinical development of this agent is now being carried out by Novacta Biosystems Ltd.

Cathelicidin peptides are expressed in some human epithelial cells of the gut including epithelial cells and this is up-regulated in response to inflammation such as that seen in cases of colitis (Koon *et al.*, 2011). The major cathelicidin, LL-37, has been shown to significantly reduce inflammation resulting from CDI in mice after colonic administration (Hing *et al.*, 2012). When studied in human monocytes it was found to inhibit the activation of the NF-κB pathway and reduced toxin A mediated TNFα expression (Hing *et al.*, 2012). No significant antibacterial activity was demonstrated against *C. difficile* suggesting that recurrence following withdrawal of treatment is likely to be high.

Thuricin is a two-component bacteriocin produced by a strain of *Bacillus thuringiensis* isolated from a human faecal sample, which exhibits activity against clinical strains of *C. difficile* but spares many groups of gut commensals (Rea *et al.*, 2011). Thuricin compared favourably to the

performance of metronidazole in the reduction of *C. difficile* in an *in vitro* human colon model and it is a promising candidate for potential therapeutic use. The limitation impeding development is the difficulty in delivering the peptides through the upper GI tract to the colon.

1.7.4. Probiotics

Several probiotic bacterial strains thought to impart increased colonisation resistance to CDI have been proposed and these include *Bacteroides spp.* and *Bifidobacteria spp.* (Freeman *et al.*, 2003; Hopkins and MacFarlane, 2002) on the basis that these strains are mainly absent from patients suffering with CDI and present in those that do not develop CDI. *Bacteroides spp.* numbers have been shown to decrease with age, which correlates with the increased risk of CDI in later life (Hopkins and MacFarlane, 2002).

In addition to competitive exclusion, probiotic strains can actually modulate the host's response to pathogens such as *C. difficile* (1.2), via inhibition of the NF-κB pathway (the first responder to stressful stimuli that regulates DNA transcription and gene expression). This observation has been reported for mixtures of *Streptococcus thermophilus, Lactobacillus spp* and *Bifidobacterium spp.* (Petrof *et al.*, 2004). Conversely, activation of the NF-κB pathway has been shown to improve cell survival following *C. difficile* toxin A exposure via a reduction in apoptosis (Chae *et al.*, 2006).

Despite being one of the most studied of the probiotics and having been used as such for the prevention of CDI both in animal studies (Kaur *et al.*, 2011) and in humans (Biller *et al.*, 1995; Gorbach *et al.*, 1987; Siitonen *et al.*, 1990), *Lactobacillus spp.* have been shown to increase in numbers during CDI (Hopkins and MacFarlane, 2002). Specific species, which were not scrutinised, are likely to exhibit different properties and probiotic features, for example some *Lactobacillus gasseri* strains have been demonstrated to adhere to intestinal mucus, compete with and displace gut pathogens including *C. difficile* (Ferreira *et al*., 2011). *Lactobacillus rhamnosus* has also been shown to inhibit MAP kinases, which regulate apoptosis amongst other roles (Yan and Polk, 2002).

Saccharomyces boulardii has also been proposed as a potential probiotic strain to help prevent the onset of CDI (Elmer *et al.*, 1996; Sullivan and Nord, 2002). *S. boulardii* has been shown to have anti-inflammatory activity via inhibition of toxin-mediated IL-8 production and IL-1β in a colonocyte cell line (Chen *et al.*, 2006). It also blocked the activation of Erk 1/2 (a MAP kinase), and when tested in a mouse ileal loop model resulted in normalised fluid secretion relative to a control (Chen *et al.*, 2006). Even some glycan fractions from yeasts including *S. boulardii* have potent anti-inflammatory action in a mouse model (Jawhara *et al.*, 2012). *S. boulardii* also stimulated the intestinal Ig A response to *C. difficile* in a mouse model (Qamar *et al.*, 2001). Despite this, a single centre, randomised double-blind, placebo-controlled trial was recently carried out, in which patients (over 65 years of age) due to receive broad-spectrum antibiotic therapy were started on twice daily capsules containing *S. boulardii* throughout their treatment. No reduction was observed in the number of antibiotic-associated diarrhoea cases or the frequency of CDI (Lewis *et al.*, 1998).

The use of *S. boulardii* is not without issue however, as highlighted in a recent case report (Thygesen *et al.*, 2012). An elderly, immunocompromised female patient was given *S. boulardii* in addition to antimicrobial treatment and subsequently developed *S. boulardii* fungemia, requiring a course of Ambisome.

Colonisation with non-toxigenic strains of *C. difficile* has been shown to offer protection from CDI both in hamsters (Borriello and Barclay, 1985) and humans (Seal *et al.*, 1987; Wilson and Sheagren, 1983). This approach is currently being investigated in a phase II clinical trial (Villano, 2010).

1.7.5. Prebiotics

Prebiotics are non-digestible carbohydrates that support the growth and activity of probiotic strains in the gut (Delzenne *et al*., 2011). Certain prebiotics can also be expected to aid in the prevention of progression of CDI due to the anticipated boost to enteric species that impart *C. difficile* colonisation resistance, for example non-digestible oligosaccharides can be used to boost numbers of *Bifidobacteria spp.* (Hopkins and MacFarlane, 2002). Inulin has

also been used to the same effect (Petry *et al.*, 2012) but other studies have found that *C. difficile* can also ferment this prebiotic (Terada *et al.*, 1992).

In a fascinating and as yet unsubstantiated study of *C. difficile* dynamics, the concentration of bacterial metabolic products in the gut of experimental mice was raised by increasing the amount of fermentable fibre in their diet from 2% to 20%. This was associated with exclusion of *C. difficile* in 6 out of 7 colonised mice whereas mice fed 2% fermentable fibre continued to excrete the organism (Ward and Young, 1997). This implies that a poor diet may be a further contributing factor to CDI and represents an additional approach to tackling the problem.

1.7.6. Faecal transplantation

1.7.6.1. The concept

Effective antimicrobial therapy does nothing to restore the integrity of the gut microbiota that gave rise to the CDI in the first place and indeed, relapses and reinfections are commonplace. It is generally accepted that a mature diverse intestinal flora itself imparts resistance against *C. difficile* colonisation and it is this concept that gave rise to the idea of transplanting healthy gut flora by means of faecal matter from a donor to a patient suffering from CDI in order to resolve the dysbiosis and replenish the gut with the normal enteric bacteria needed to help resist further attacks.

The transfer of enteric flora by transplanting faecal material from healthy to diseased individuals has long been established in veterinary medicine. Transfaunation is the ingestion of manure from healthy adult horses and can resolve diarrhoea in foals. Also, anorexia in cattle brought about by indigestion can be relieved with a cud transplant from healthy cows (Radostis *et al.*, 2000). More recently, experiments in mice have shown that faecal transplantation can resolve relapsing CDI caused by the hypervirulent NAP1/027 strain (Lawley *et al.*, 2012).

1.7.2.1. Typical protocols

Faecal instillation is a simple and inexpensive procedure that can be performed at care institutions with combined efforts of the Gastroenterology, Radiology and Microbiology departments. It is currently performed in a small number of specialist research centres throughout the world and the individual methods vary somewhat. Donor faeces can be infused into either the proximal or distal end of the GI tract and, taking into account the patient's clinical situation, clinical history and receptiveness to the method, it is the choice of the patient and clinician as to which route is used.

Basic generalised protocols are indicated in Fig. 1.3 a-b. For upper GI tract instillation, a single small volume (about 25 mL) of stool is required and a nasoduodenal or nasojejunal tube must be fitted (checked by X-ray imaging). NG tubes are used extensively in hospitals and are generally regarded as safe provided the correct procedure and checks are in place (Hutchinson *et al.*, 2008). Rare complications may include perforation of the oesophagus (Lahdes-Vasama *et al.*, 2009), trauma and complications arising from incorrect positioning, for example into the lungs (Lo *et al.*, 2008; Zausig *et al.*, 2008) or the brain (Geissler, 2007) and the risk of aspiration of stomach contents giving rise to pneumonia (Metheny *et al.*, 2006). With regards to instillation of the lower GI tract, colonoscopies and retention enemas are also routine practice in the hospital setting. Their use in patients with severe recurrent CDI with or without pseudomembranous colitis however, is a more challenging and risky proposition due to the extremely friable nature of their colonic epithelium. The risk of bowel perforation is even greater given that this protocol calls for a large volume of stool suspension (250-1500 mL) and invariably requires several consecutive attempts before resolution of diarrhoea is reported (Bakken, 2009). Lower GI administration is certainly less desirable from the perspective of the provider but the prolonged leakage of material following instillation is also a significant drawback in terms of the patient's experience. Despite this, some studies have reported a strong patient preference for colonoscopy rather than NG tube instillation (Rohlke *et al.*, 2010). There is the additional risk that colon lavages may pose a significant infection control hazard and necessitate clinical deep cleaning (as

opposed to routine clinical cleaning) of the endoscopy suite following each patient – an additional cost implication for the provider.

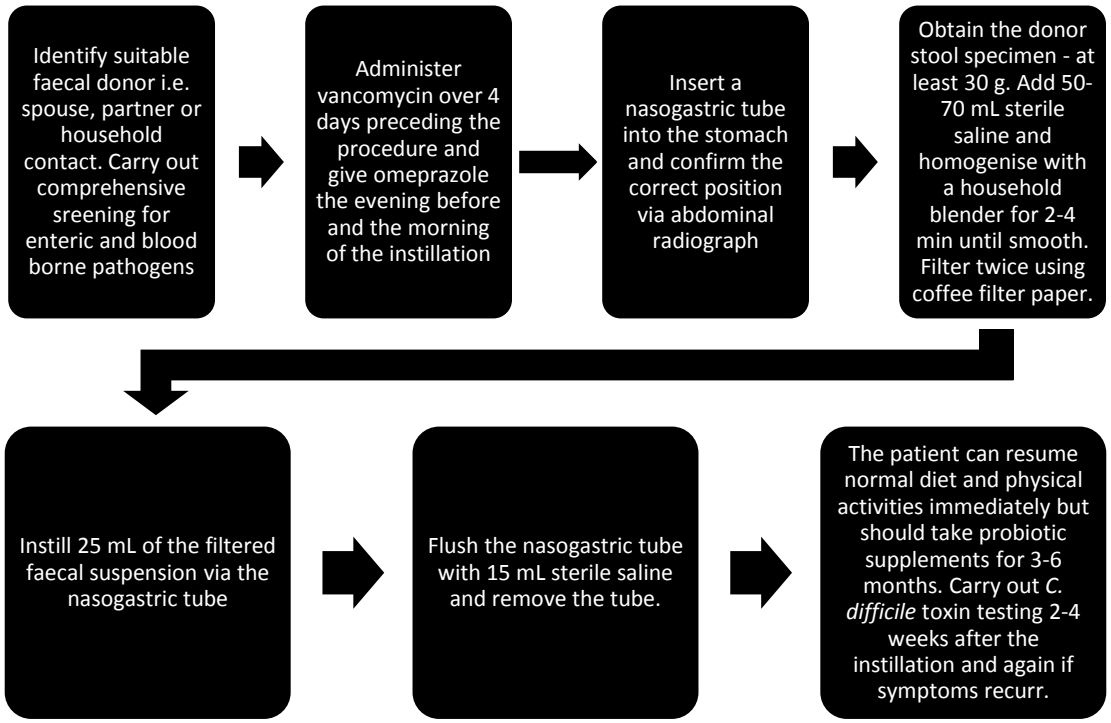


Figure 1.3 (a). The basic protocol for faecal bacteriotherapy via nasogastric instillation.

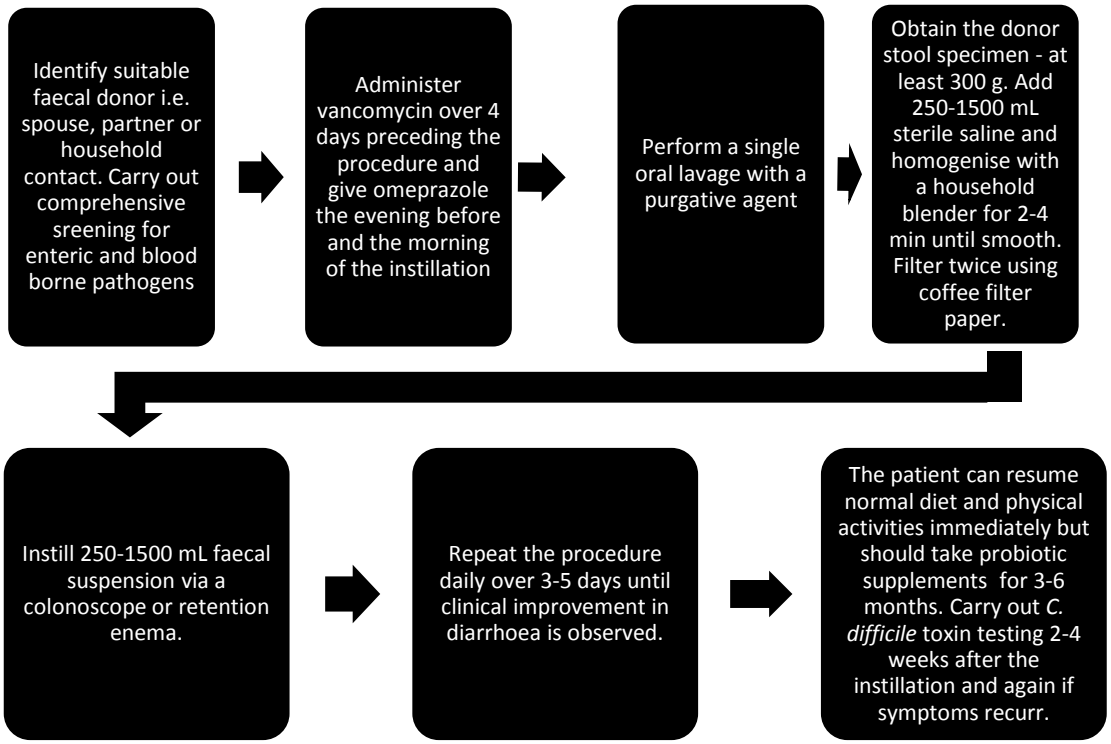


Figure 1.3 (b). The basic protocol for faecal bacteriotherapy via colonoscopic or retention enema instillation.

Donor faeces can be blended with saline or 4% milk (Aas *et al.*, 2003; Lund-Tonnesen *et al.*, 1998) The collection time must be as close to the time of the procedure as practicably possible and certainly within 24 h prior to instillation (Bakken, 2009). In some studies the prepared faecal slurry was frozen at -20°C in aliquots for later use (Flotterod and Hopen, 1991).

As part of the preparatory process of faecal bacteriotherapy, treatment with oral vancomycin is required to reduce the burden of *C. difficile* cells for 4-7 days prior to the time of instillation - 250 mg three times a day (Aas *et al.*, 2003) or 500 mg twice per day (Borody *et al.*, 2003). In addition, patients receiving via the upper GI route require a treatment to increase the pH of the proximal small intestine, for example omeprazole to decrease hydrochloric acid production, given the evening before and the morning of the instillation procedure (Aas *et al.*, 2003). Some studies have also employed the use of a colonic lavage prior to upper GI infusion. For patients receiving their bacteriotherapy via enema, some studies have recommended an oral lavage

with a purgative agent, e.g. polyethylene glycol (PEG) plus electrolytes on the day of the therapy (Nieuwdorp *et al.*, 2008).

1.7.6.3. Efficacy of faecal bacteriotherapy for CDI

The success rate of faecal transplantations is high, but few studies have compared the relative efficacy of upper and lower GI instillation (accepting that lower GI treatments require multiple attempts). The reported success rates of a comprehensive range of studies are detailed in Tables 1.1 a-b. There are over twice the number of reports for lower GI instillation as opposed to upper GI administration, but favour for this route is supported by an average success rate of 93% relative to 84% for upper GI infusions. This is particularly impressive since eligible patients had already suffered repeated bouts of CDI and multiple treatment failures. No studies have yet reported on the adoption of both these approaches to ensure complete reconstitution of enteric flora in recipients.

The retrospective nature of some case report publications may suggest a degree of bias in favour of procedures that give rise to successful outcomes and there is certainly a need for controlled trials on faecal bacteriotherapy. There may also be long-term sequelae that are currently unrecognised but considering the acute life-threatening nature of CDI in eligible patients, these are of questionable significance.

Reference	Number of patients	Site of instillation	Author-reported success rate (%)
Eiseman *et al.*, 1958	1	Upper GI	1 (100%)
Flotterod and Hopen, 1991	1	Upper GI	1 (100%)
Lund-Tonnesen *et al.*, 1998	1	Upper GI	1 (100%)
Aas *et al.*, 2003	16	Upper GI	15 (94%)
Nieuwdorp *et al.*, 2008	4	Upper GI	4 (100%)
MacConnachie *et al.*, 2009	15	Upper GI	11 (73%)
Garborg *et al.*, 2010	38	Upper GI	31 (82%)
Russell *et al.*, 2010	1	Upper GI	1 (100%)
Upper GI overall success rate: 84%			

Table 1.1 (a) Studies reporting outcomes of upper GI faecal instillation.

Reference	Number of patients	Site of instillation	Author-reported success rate (%)
Eiseman *et al.*, 1958	3	Lower GI	3 (100%)
Schwan *et al.*, 1984	1	Lower GI	1 (100%)
Tvede and Rask-Madsen, 1989	6	Lower GI	6 (100%)
Paterson *et al.*, 1994	7	Lower GI	7 (100%)
Lund-Tonnesen *et al.*, 1998	17	Lower GI	14 (82%)
Gustafsson *et al.*, 1998	13	Lower GI	13 (100%)
Persky and Brandt, 2000	1	Lower GI	1 (100%)
Faust *et al.*, 2002	6	Lower GI	6 (100%)
Jorup-Ronstrom *et al.*, 2006	5	Lower GI	5 (100%)
Nieuwdorp *et al.*, 2008	3	Lower GI	3 (100%)
Garborg *et al.*, 2010	2	Lower GI	2 (100%)
Mellow and Kanatzar, 2011	13	Lower GI	11 (85%)
Rohlke *et al.*, 2010	19	Lower GI	18 (95%)
Yoon and Brandt, 2010	12	Lower GI	12 (100%)
Silverman *et al.*, 2010	7	Lower GI	7 (100%)
Kelly *et al.*, 2012	26	Lower GI	24 (92%)
You *et al.*, 2008	1	Lower GI	1 (100%)
Khoruts *et al.*, 2010	1	Lower GI	1 (100%)
Fenton *et al.*, 1974	1	Lower GI	1(100%)
Bowden *et al.*, 1981	16	Lower GI	13 (81%)
Lower GI overall success rate: 93%			

Table 1.1 (b) Studies reporting outcomes of lower GI faecal instillation.

1.7.6.4. Selecting patients for faecal transplantation

In a review of faecal bacteriotherapy case reports (Bakken, 2009), the eligibility criteria was defined as follows:

a) Recurrent CDI confirmed by the presence of toxins A and/or B in stool and the absence of other treatable causes of diarrhoea.
b) A suitable donor must be available, preferably a spouse or partner but household contacts can also be considered
c) Potential donors must be screened for transmissible enteric pathogens including *C. difficile* and also blood-borne pathogens including hepatitis B, hepatitis C and human immunodeficiency virus (HIV). Donors must not be undergoing treatment for pre-existing medical conditions such as bowel disease or malignancy and cannot have received antibiotic treatment in the preceding 3 months.

1.7.6.5. Adverse events associated with faecal transplantation

In the majority of studies, the potential adverse events following faecal transplantation cannot directly be associated with the procedure, for example, a case of pneumonia that developed 14 days post faecal transplantation (Aas *et al.*, 2003) and one upper GI haemorrhage most probably related to concomitant use of non-steroidal anti-inflammatory drugs and the fitting of a nasogastric tube rather than the faecal transplantation itself (MacConnachie *et al.*, 2009). One case of peritonitis however, which developed 3 days after the faecal transplant cannot be excluded as being caused by the infusion and possible leakage of faecal material following the procedure (Aas *et al.*, 2003).

1.7.6.6. Synthetic stool instillation

The success of faecal bacteriotherapy is put down to the resolution of dysbiosis by acquisition of normal enteric flora from the healthy donor (Grehan *et al.*, 2010). Major concerns associated with the method include the potential transmission of pathogens (both documented and as yet unknown) despite the comprehensive screening prior to donation. The notion of transplanting faecal material is also quite unappealing to patients and some reluctance could be expected (Bakken, 2009). Both these concerns could be addressed if the key components within the faecal material that give rise to the resolution could be identified and prepared synthetically. Cocktails of some of the probiotic strains regarded as being protective against *C. difficile* colonisation have been used in place of faecal material. Mixtures comprising the commensals *Enterococcus faecalis, Clostridium bifermentans and Peptostreptococcus productus* have been used successfully to treat refractory CDI via rectal instillation (Petrof *et al.*, 2013; Tvede and Rask-Madsen, 1989).

1.7.7. Passive immune products

In animal studies, it has been shown that concentrated specific immunoglobulin (prepared from the colostrum of toxoid-vaccinated cows) given orally or parenterally to hamsters colonised with *C. difficile* was protective against diarrhoea and prevented death due to CDI (Kelly *et al.*, 1996; Lyerly *et al.*, 1991). Application of this work in humans is limited to a

study by a different group, who demonstrated that orally administered 'Mucomilk' (bovine IgA concentrate from the milk of vaccinated cows) was not as effective as metronidazole in a randomised double-blind study (Mattila *et al.*, 2008).

Human monoclonal antibodies against toxins A and B, generated in transgenic mice and administered intra-peritoneally, have also been shown to reduce mortality associated with CDI in the hamster model (Babcock *et al.*, 2006). Safety in humans has also been demonstrated and in a multi-centre, randomised, double-blind, placebo-controlled trial, these antibodies (administered intravenously) were given alongside the routine antibiotic schedule with the purpose of establishing a potential role in the prevention of recurrent CDI (Leav *et al.*, 2010; Lowy *et al.*, 2010). Co-administration of anti-toxin A and B monoclonal antibodies brought about a 72% reduction in the CDI recurrence rate but it was noted that there was no difference in the severity of the infections (Lowy *et al.*, 2010). It is hypothesised that these antibodies may pass through the inflamed colon due to the increased porosity of the epithelium (Giannasca *et al.*, 1999; Riegler *et al.*, 1995) but this is confounded by the observation that the protective effect of antibodies is less marked in severe disease (Kelly and Kyne, 2011). Active transport of the antibodies via the FcRn receptor in the epithelium is an alternative mechanism (Yoshida *et al.*, 2006).

For very severe cases of CDI, human normal Ig (HNIg) pooled from blood donations is sometimes used, particularly when patients show no or very little clinical response to vancomycin treatment. It has been used with successful outcomes (Shahani and Koirala, 2012) and the presumed mode of action is the neutralisation of toxin A by specific antibodies. So far there have been no randomised clinical trials of the use of HNIg in CDI.

1.7.3. Vaccination

Since high levels of serum Ig G have been shown to offer protection against CDI (Kyne *et al.*, 2000; Leav *et al.*, 2010), developing a vaccination to help patients avoid infection is an appealing prospect and economically valuable (Lee *et al.*, 2010). Vaccine-induced protection against fatal CDI was first

demonstrated in hamsters (Libby *et al.*, 1982) using a toxoid preparation. In a similar study it was also shown that these antibodies could be transferred to provide protection to non-immune hamsters via serum or ascitic fluid (Giannasca *et al.*, 1999). Recombinant vaccines (Ryan *et al.*, 1997) and DNA vaccines (Gardiner *et al.*, 2009) have also proved effective. Historically there has been more emphasis on developing immunity directed against toxin A, since this was thought to be the primary virulence factor. An increasing understanding of the role of toxin B in CDI has substantiated the need to incorporate it into the vaccination strategy and indeed it has been shown that vaccinations encompassing antigenic determinants to both toxin A and B afford better protection than those to toxin A only (Babcock *et al.*, 2006; Fernie *et al.*, 1983; Kim *et al.*, 1984; Kink and Williams, 1998; Libby *et al.*, 1982).

A recent study details the results of a phase I randomised, placebo-controlled, double-blind clinical trial to evaluate the immunogenicity and safety of a formalin-inactivated adjuvant toxin A and B candidate vaccine (Greenberg *et al.*, 2012). Two age groups were included in the trial – participants aged between 18-55 and those aged over 65. After two doses of either 2 µg, 10 µg or 50 µg of toxin A (administered intramuscularly), IgG seroconversion was observed in 100% of the 18-55 age group, although the neutralising capacity of the antibodies was not measured. The production of IgG directed against toxin B was less relative to IgG targeting toxin A as measured by enzyme immunoassay (EIA). For those aged over 65 years, 50 µg was required to achieve 100% seroconversion. These antibody levels were shown to decrease in the months following vaccination but remained positive (above the assay cut-off value) for at least 236 days afterwards. No safety concerns were reported. The production of IgA to impart mucosal immunity is also important and it has been suggested that co-administration of the probiotic yeast, *S. boulardii,* with vaccination may help to enhance the intestinal IgA response (Qamar *et al.*, 2001).

Vaccinations based on toxins are unlikely to affect rates of colonisation or help in the reduction of transmission and so other research groups have opted to work on the development of non-toxoid based vaccines in order to prevent

the acquisition of *C. difficile*. Surface-associated proteins including the flagellar cap protein FliD and Cwp84 protease (Pechine *et al.*, 2005 a, 2005 b), and surface layer proteins used for attachment (Eidhin *et al.*, 2008). Despite a reduction in the levels of *C. difficile* in mice (Pechine *et al.*, 2005 a, 2005 b) and hamsters (Eidhin *et al.*, 2008) the response was not sufficient to prevent colonisation.

1.7.9. Toxin-binding proteins

Based on the protective effect of neutralising antibodies against *C. difficile* toxins A and B, the potential of agents that bind and therefore inactivate these toxins has been explored. Tolevamer is a toxin-binding polymer that has shown significant reductions in cytotoxicity in cell monolayers (Hinkson *et al.*, 2008) and in hamster models of CDI, where 80% protection from mortality associated with CDI was observed in addition to a zero relapse rate relative to metronidazole (Kurtz *et al.*, 2001). Promising results were also seen in phase II clinical trials, with rates of diarrhoea resolution comparable to those following vancomycin treatment (Louie *et al.*, 2006). No serious adverse events were reported but tolevamer-treated patients were at increased risk of hypokalaemia. In phase III clinical trials, however, inferiority to both metronidazole and vancomycin was demonstrated (Bouza *et al.*, 2008; Louie *et al.*, 2007) and these findings were also corroborated by the results of tests using a human *in vitro* colon model (Baines *et al.*, 2009), despite reporting good activity in the presence of faecal material *in vitro*.

1.7.4. Other novel strategies

Some essential oils including nerolidol (found in ginger, lavender, tea tree and lemongrass), thymol (common thyme oil), eugenol (found in clove, nutmeg and cinnamon) and geraniol (from rose, geranium and citronella oil) have shown inhibitory action against *C. difficile* growth *in vitro* in a dose dependent fashion (Thapa *et al.*, 2012). Although this action was not generally observed to the same extent in commensal organisms, *Faecalibacterium prausnitzii* was very sensitive to the effects of the oils in *in vitro* tests. *F. prausnitzii* has been shown to have anti-inflammatory properties and its absence has been linked to the relapse of Crohn's disease (Sokol *et al.*, 2008). Further investigations

into the specific effects of essential oils on *C. difficile* and commensal organisms in the context of the gut are required before considering their use as supplements.

1.8. Bacteriophage biology

Bacteriophages (hereafter referred to as phages), are viruses that specifically infect bacteria and are typically specific to a single species or individual strains. Phages are the most abundant life form on earth and are ubiquitous in the environment with particularly high numbers found in soil, sewage and lakes (Serwer *et al.*, 2007). It is estimated that the total weight of phages in the oceans is greater than that exerted by all of the humans on earth (Parfitt, 2005) and consequently human contact with phages and ingestion is an everyday occurrence that has so far not been associated with any known harmful effects (Sulakvelidze *et al.*, 2001). In fact, it has been suggested that they may play a role in the control of inflammation in response to gut flora and normalise levels of TNFα and IL-6 (Gorski and Weber-Dabrowska, 2005).

Tailed phages can assume either a lytic or a lysogenic life cycle. Lysogenic phages contain genes that instruct the integration of the phage genome into that of the bacterial host thus replicating with each new bacterial generation without causing damage to the host cell (Lamont *et al.*, 1993). When a phage has integrated into the host genetic material, it is referred to as a prophage.

Lytic phages however, (the life cycle of which is illustrated in Fig. 1.4) take over host cell machinery to replicate independently of the bacterial genome and rapidly induce cell death by lysis. This liberates new virions that can then go on to infect other target bacteria until they have all been eliminated (Kutter and Sulakvelidze, 2005).

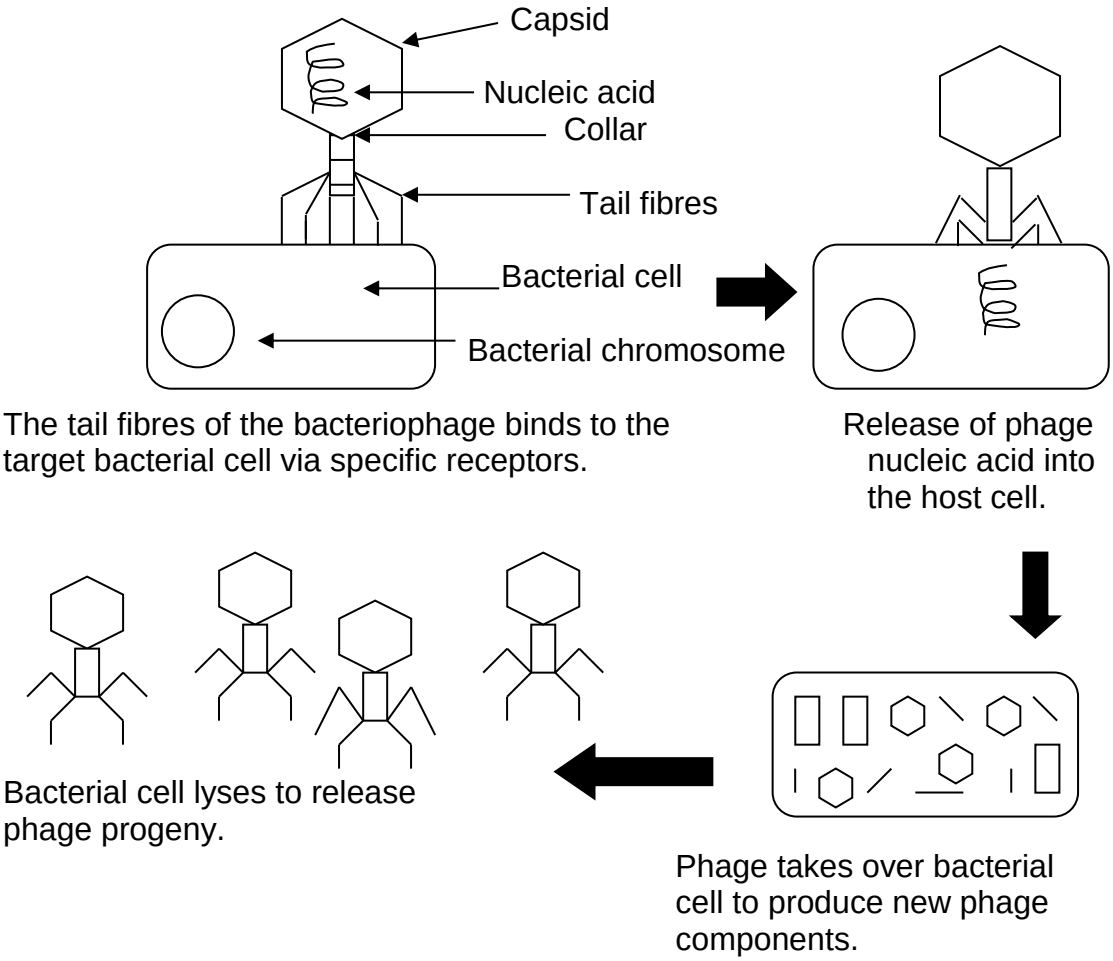


Figure 1.4.The life cycle of lytic phages.

Prophages may be released spontaneously or can be induced to adopt the lytic life cycle when the host bacteria are treated with ultraviolet (UV) radiation or mitomycin C (Goh *et al.*, 2005; Liu *et al.*, 2005). Mitomycin C is a quinone-containing alkylating agent produced by *Streptomyces caespitosus*. It causes breaks in single-stranded DNA and the cross-linkage of adenine and guanine, which results in the complete inhibition of DNA synthesis. This mode of action is very comparable to that of ionising radiation and although non-cell specific, these effects are seen more frequently in rapidly dividing cells (Kopp and Seregard, 2004).

1.9. Phage therapy

1.9.1. History and early research

The first observation of phage activity was recorded by Ernest Hanbury Hankin, a British biologist who in 1896 reported that the water of the Ganges in India and Bangladesh had antimicrobial activity against *Vibrio cholera* (Parfitt, 2005). Twenty-one years later this observation was attributed to phage activity by Felix d'Herelle at The Pasteur Institute in France and work on their use to treat bacterial infections began (d' Herelle, 1917). The results of early work into phage therapy were likely to have been misled by the lack of knowledge of phage acid sensitivity, exquisite specificity, lytic and lysogenic life-cycles and the very crude experimental preparations that were administered, which may have contained infectious agents and toxic compounds (Sulakvelidze *et al.*, 2001). Indeed, there are reports of treatment failures and adverse reactions such as diarrhoea (Hanlon, 2007). This, coupled with the widespread availability of penicillin, led phage therapy to fall out of favour in the West, but countries such as Georgia and Poland continued to pursue this approach to antimicrobial treatment and there are many Russian and Polish publications demonstrating excellent clinical efficacy for bacterial dysentery (Babalova *et al.*, 1968; Miliutina and Vorotyntseva, 1993), skin and soft tissue infections (Cislo *et al.*, 1987), pulmonary infections (Ioseliani *et al.*, 1980) and intestinal dysbiosis (Litvinova *et al.*, 1978) to name a few. These reports were brought to the Western audience in a recent review (Sulakvelidze *et al.*, 2001).

In 1923 Dr Felix d'Herelle and his co-worker George Eliava founded the Eliava Institute in Tblisi, Georgia. This institute has been working on and manufacturing phage preparations for routine therapeutic use ever since.

1.9.2. Recent research in the West

Phage therapy has recently attracted the attention of clinicians, scientists and patients trying to tackle the problem of antibiotic resistant bacterial infections (Parfitt, 2005). Work on the reproduction of phage therapy studies in accordance with European regulations and standards has begun (Harper *et*

al., 2011) and includes success against the high-profile target, *Staphylococcus aureus* (and Methicillin-resistant *S. aureus* - MRSA) in a mouse model of septicaemia (Matsuzaki *et al.*, 2003). Phage therapy has also been demonstrated as an effective agent to reduce the burden of specific pathogens in animal guts. A bacteriophage isolated from the faeces of sheep that were resistant to colonisation by *E. coli* O157:H7 resulted in a 2 $\log_{10}$ reduction in the number of intestinal *E. coli* O157:H7 when administered to colonised sheep (Raya *et al.*, 2006). Similarly, a 0.5-5 $\log_{10}$ reduction in the number of *Campylobacter jejuni* in the cecum of broiler chickens was reported when specific phage therapy was administered (Loc Carrillo *et al.*, 2005).

One concern that remains, both for antibiotic and phage treatment, is the release of toxic components when the bacterial cells are lysed. *C. difficile* associated disease is brought about by the action of enterotoxin A and cytotoxin B (Voth and Ballard, 2005), and the lysis of *C. difficile* could liberate quantities of toxin that may exacerbate the patient's symptoms. This concept is commonly referred to as the Jarisch-Herxheimer reaction and is typically seen in patients treated for syphilis (Li *et al.*, 2012). Heat stable proteins are released faster than the body can remove them, leading to a massive inflammatory response that resembles bacterial sepsis. The risk of the Jarisch-Herxheimer reaction and the extent of the reaction has been shown to increase with the burden of organisms (Yang *et al.*, 2010). This issue has been addressed in a novel approach to phage therapy using a genetically engineered non-replicating phage to tackle *Pseudomonas aeruginosa* (Hagens *et al.*, 2004). A phage export protein gene was replaced by a gene coding for a restriction endonuclease, rendering the phage incapable of escape from the host cell, yet effectively killing it. In mice heavily infected with *P. aeruginosa* it was found that this tactic improved the survival rates relative to the use of the wild-type lytic phage.

Studies have also included investigations into the safety of phage therapy. Orally administered doses of up to 10^5 plaque forming units (PFU)/mL of the coliphage T4 to healthy volunteers resulted in no adverse reactions and phage was detectable for no more than 2 days post phage ingestion (Bruttin and Brussow, 2005).

1.9.3. Phage therapy of *C. difficile*

Over seven different lysogenic bacteriophages of *C. difficile* have been described to date and these are detailed in Chapter 3. Induced prophages of *C. difficile* have previously been used to investigate the ability of phages to prevent ileocecitis in hamsters (Ramesh *et al.*, 1999). Twenty-four hours after clindamycin treatment, gastric acid was neutralised with bicarbonate and hamsters were challenged with *C. difficile* followed immediately by a phage suspension containing 10^8 PFU/mL. Some hamsters also received further doses at 8 hour intervals. All of the control animals in this study who did not receive any dose of phage died within 72 hours of *C. difficile* administration, however 17 of the 18 hamsters who did receive phage survived until the end of the study. The *C. difficile* strain isolated from the hamster that died was shown to be resistant to the phage used, although the mechanism was not elucidated. Later studies of phage administration in a hamster model of CDI have shown *in vivo* lysogenisation, which may explain this finding (Govind *et al.*, 2011).

1.9.4. Administration of phage preparations

The efficacy of phage therapy following differing routes of administration can be variable, although this is based mainly on data from veterinary studies for which quick and simple mass treatments are sought. In a study comparing the relative recovery of different phages in poultry after oral administration, beak spray or intra-muscular (IM) inoculation, it was found that IM delivery resulted in the most comprehensive dissemination with phage particles being recovered from the lungs and air sacs, liver, duodenum and spleen (Oliveira *et al.*, 2009). Oral and spray administration was still effective at delivering phage to the respiratory tract. Differences in recovery rates between the types of phages were observed but this was attributed to the presence of some phage-sensitive hosts in the gut resulting in amplification of the phage

For the purposes of treating gut pathogens, oral administration or delivery via colonoscope or enema offers the best access for remedial therapy. Treatment via the upper GI tract requires pre-neutralization of stomach acid in order to preserve the viability of the phage particles as they pass through. Removal

(albeit temporary) of this important barrier to GI pathogens carries its own risk of infection (including CDI) and this should be taken into account in terms of post-treatment care and advice. The short and longer-term impact of stomach acid neutralisation on the gut microbiome is not clear but significant changes in pH can be expected to bring about shifts in bacterial populations (Kim *et al.*, 2010).

1.9.5. Phage resistance

Host bacteria are able to develop resistance to phage attack (besides lysogeny), for example via the production of repressors, the loss of receptors or the masking of phage receptors with a capsule (Hyman and Abedon, 2010). This is an important consideration when assessing the potential of phage therapy and some studies have reported on this phenomenon. Phages specific for various serovars of *Salmonella enterica* were isolated from environmental samples including wastewater and poultry excreta and used to treat infected poultry (Atterbury *et al.*, 2007). This resulted in up to a 4.2 $\log_{10}$ reduction in colony forming units of the target *Salmonella* species in the cecum after 24 hours but the authors also noted the development of resistance to the phages used in proportion to the dose that was administered (a higher dose of phage causing a higher proportion of the bacteria to be resistant). Contrary to this however, another study demonstrated that 12 out of 13 bacteriophage populations examined were able to overcome the expression of specific translational repressor proteins produced by the host bacteria (Lindemann *et al.*, 2002). The frequency of fermentation failures reported during the production of dairy products would suggest that phages have the edge in terms of their capacity to maintain the ability to infect host strains (Kutter and Sulakvelidze, 2005). Phage populations will usually contain a considerable proportion of highly altered sequences relative to the original virus (Domingo *et al.*, 1985) and the likelihood of the target bacteria developing resistance to predatory phages is no doubt affected by species-specific factors.

1.9.6. The immune response to phage therapy

The development of neutralising antibodies post parenteral administration of a range of phages has been demonstrated (Kucharewicz-Krukowska and Slopek, 1987), which requires thorough investigation in terms of the potential impact on host damage and the efficacy of future rounds of therapy. The possibility of a weak innate response to phage treatment could also not be ruled out in a recent study of pulmonary *P. aeruginosa* infection in a mouse model, based on a heightened cytokine profile relative to controls treated with phosphate buffered saline (PBS) (Morello *et al.*, 2011). Despite these reports, no treatment failures were attributed to this matter in a comprehensive review of phage therapy in former Soviet Union and Eastern Europe (Sulakvelidze *et al.*, 2001).

1.9.7. Overview of phage therapy

There are many features of phages that theoretically make them superior to the anti-infective drugs currently in use for the treatment of CDI. The exquisite specificity offered by phage therapy is a significant benefit relative to broad-spectrum antibiotic therapy and could be expected to slash the risk of relapse and secondary bacterial infections due to the preservation of commensal microbiota. It does however, mean that new preparations would be required on a regular basis to ensure that common strains are covered, and the limited repertoire of *C. difficile* phages described to date highlight the possibility that not all strains may be treated in this way. Dosing frequencies may be lessened as phage replication will be maximised in areas where there is a high burden of target and discontinue only when all of the target bacterial cells have been eliminated. Environmental sources offer an endless resource of new phages and propagation is quick, straightforward and inexpensive. This, in addition to the encouraging experience and success in treating GI pathogens ensures phage therapy as a valuable candidate for further work into the prevention and remediation of CDI, but the extent of the problems associated with lysogenisation, resistance and immune stimulation still remains to be determined.

1.10. Outline of the thesis

1.10.1. Background

CDI represents a major challenge to healthcare services, affecting patients in hospital and in the community. Worryingly, the efficacy of our first line treatments seems to be diminishing and we are in need of alternative therapies. The use of phage has shown potential in the treatment of GI infections (Atterbury *et al*., 2007; Sulakvelidze *et al.,* 2001; Kutter and Sulakvelidze, 2005), and the high degree of specificity is particularly appealing as limiting the damage to commensal gut microbiota would be expected to minimise the risk of CDI recurrence - a major challenge of treatment (Kamboj *et al*., 2011). There is little data exploring the use of phage to treat CDI and this is likely to be due to the relative scarcity of phages known to infect *C. difficile* strains and the ability of all those isolated so far to lysogenise with their host (Govind *et al*., 2006; Mahony *et al*., 1985; Nagy and Foldes, 1991; Sell *et al*., 1983; Horgan *et al.,* 2010; Shan *et al*., 2012). Despite the lysogenic capacity, *C. difficile* phages haves been shown to reduce numbers of *C. difficile* in an *in vivo* model (Ramesh *et al.,* 1999).

1.10.2. Aims

The initial aims of the study were to carry out screening for novel phages of *C. difficile*, chiefly those with no lysogenic capacity, to search for candidates for use as therapuetic agents against CDI. Using a *C. difficile* phage isolated previously (Mayer *et al*., 2008) and its sensitive hosts, the project set out to investigate its impact on *C. difficile* in simulated gut environments and whether the *in vitro* activity could be improved by phage mutagenesis. Further objectives developed during the study were concerned with determination of the mechanisms responsible for phenotypic changes in *C. difficile* toxin production as a result of lysogeny.

This project has aimed to contribute to the foundations of Western phage therapy research, which may one day lead to the development of phage therapy regimes to treat bacterial infections and reduce our dependency on antibiotics.

1.10.3. Hypothesis

The use of bacteriophages will prove effective as a method of controlling CDI with minimal damage to the colonic microbiota.

Chapter 2 – General methods

The following methods and consumables were used throughout the project. A full table of all the bacterial strains used is provided in Appendix 1.

2.1 Bacteriology

2.1.1. Propagation of *C. difficile*

2.1.1.1. Liquid culture

Stocks of *C. difficile* were maintained in Robertson's cooked meat media (Southern Group Laboratory, Corby). One hundred µL of the stock was used to inoculate 25 mL pre-reduced BHI (with complements) or RCM media, which was then transferred immediately to the anaerobic cabinet and incubated at 37°C for 12-18 h. At least one further subculture (but no more than 4) was made prior to the culture being used experimentally.

2.1.1.1.1. Measuring the optical density of liquid cultures.

The Cecil CE 2041 (2000 series) spectrophotometer and semi-micro cuvettes (Fisher Scientific) were used to measure the optical density of cell suspensions at a wavelength of 600 nm (OD_{600}). After automatic calibration, 1 mL of sterile media was used to obtain a blank reading. When anaerobic cultures were to be measured, pre-reduced blank media was used as the broth contained a colorimetric resazurin indicator. Samples that were clearly turbid were always diluted 1 in 10 prior to OD_{600} measurement to obtain a more accurate reading and duplicate readings were taken.

2.1.1.2. Solid media

2.1.1.2.1. Non-selective media

Plates containing approximately 20 mL of BHI or RCM agar were pre-reduced in the anaerobic cabinet and cultures were processed in the anaerobic cabinet when possible. Twenty µL of pure liquid culture was dispensed in triplicate onto the surface of the plates and allowed to sink into the media (for the purposes of enumeration) or streaked around the edge of the plate with a sterile loop to check for purity.

2.1.1.2.2. Selective media

2.1.1.2.2.1. Enumeration of *C. difficile*

Plates containing approximately 20 mL of cefoxitin cycloserine egg yolk agar (CCEY) or CCEY with 5 mg/mL lysozyme (CCEYL) were pre-reduced in the anaerobic cabinet and cultures were processed in the anaerobic cabinet when possible. Three drops of 20 µL liquid sample were dispensed onto the surface of the agar and allowed to sink into the media in the anaerobic cabinet prior to inversion and incubation for 2 days. Characteristic colony morphologies (Fig. 2.1) were counted to enumerate the levels of *C. difficile*.

2.1.1.2.2.2. Isolation of *C. difficile*

Selective agar was also used for the isolation of *C. difficile* from faecal samples. A pea-sized portion of stool was emulsified in 2 mL sterile saline and treated with an equal volume of 100% ethanol for 45 min. A 10 µL aliquot of the suspension was streak-plated on pre-reduced CCEY or CCEYL agar plates and incubated for up to 48 h in the anaerobic cabinet at 37°C. Colonies of *C. difficile* were grey with diffuse edges and a ground-glass appearance as shown in Fig. 2.1.

Figure 2.1. Colonies of *C. difficile* on CCEY agar after 30 h incubation.

2.1.1.3. Spore stocks

2.1.1.3.1. Production of *C. difficile* spore stocks for culturing

One hundred µL of an overnight culture of *C. difficile* in BHI or RCM was used to inoculate pre-reduced Robertson's cooked meat media (Southern Group

Laboratory, Corby). The media was incubated in the anaerobic cabinet at 37°C for 18-24 h and the stocks were then stored at room temperature.

2.1.1.3.2. Production of pure *C. difficile* spore stocks for experimentation

Five-hundred µL aliquots of *C. difficile* culture grown in BHI or RCM media were used to inoculate the centre of 10 pre-reduced blood agar plates. The inocula were spread around using a sterilised glass rod and the plates were incubated in the anaerobic cabinet. After 24 h the plates were placed inside a plastic bag to prevent drying and incubated for a further 12 days. On day 13 the plates were removed from the anaerobic cabinet and exposed to air for 24 h. After this the plates were checked for contamination and all *C. difficile* growth was scraped off aseptically using sterile loops, and transferred to 10 mL of pre-chilled PBS on ice. An equal volume of chilled ethanol (96%) was added, mixed by gentle inversion and the suspension incubated on ice for 1 h. The spore stocks were then washed 4 times with chilled PBS and centrifuged at 3220 x g for 10 min at 4ºC. Serial dilutions in pre-reduced PBS were enumerated using CCEY agar.

2.1.1.3.2.1. Purification of *C. difficile* spore stocks

Urografin (Schering Healthcare Ltd) is a prescription medication used as contrast in medical imaging, but can also be used for centrifugal separation of bacterial spores from cell debris and vegetative cells. The concentration and centrifugation parameters were optimised for *C. difficile* cells. Spore stocks were sonicated for 15 min in a chilled bath. Five-hundred µL chilled Urografin (35% v/v) was added to a microtube and 400 µL spore stocks was layered on top. The microtubes were centrifuged at 11,000 x *g* for 1 h, after which the top layer of liquid was removed. The bottom layer (containing the spores) was washed twice in chilled PBS and re-suspended in 250 µL chilled PBS. A 10 µL aliquot was examined under a light microscope to determine the quantity of spores. The preparation was concentrated or diluted to give approximately 5 spores per visual field.

2.1.2. Media for culturing commensal bacteria

A range of commensal gut bacteria were enumerated by culture using selective media as shown in Table 2.1**.**

Organism	Media	Pre-reduced	An/aerobic	Duration of incubation
Lactobacillus spp.	Rogosa (2.5.6)	Yes	Anaerobic	3 days
Bifidobacterium spp.	Beeren's (2.5.7)	Yes	Anaerobic	5 days
Bacteroides spp.	Bacteroides (2.5.8)	Yes	Anaerobic	5 days
Enterobacteriaceae	MacConkey (2.5.9)	No	Aerobic	1 day
Total anaerobes	Wilkens-Chalgren(2.5.10)	Yes	Anaerobic	3 days
Total aerobes	Nutrient (2.5.11)	No	Aerobic	2 days
Total Clostridium spp.	Clostridia (2.5.12)	Yes	Anaerobic	5 days
Gram-positive cocci	Slanetz-Bartley (2.5.13)	No	Aerobic	2 days

Table 2.1. Media and conditions for culture of commensal gut bacteria.

2.1.3. *C. difficile* toxin A and B quantification

The Premier Toxins A and B EIA (Meridian BioSciences Inc) was used for detection of *C. difficile* toxins A and B in the batch fermentation and colon model vessels. Briefly, 200 µL sample diluent was measured into a sterile microtube and 50 µL of sample added to it before thorough vortex mixing for 15 s. One well was used for each sample plus one for a negative control, a positive control and an in-house control (fresh culture of NCTC 11204). One-hundred µL of the controls and diluted samples were added to the corresponding wells. Fifty µL of enzyme conjugate was added to each well before sealing the plate with the covers provided and shaking gently to mix the samples and reagents. The plates were incubated at 37°C for 50 min. Next, 6 plate washes were carried out manually with 1 x wash buffer, tapping the plate over tissue paper to completely remove well contents between each wash. Two drops of substrate (~50 µL) were added to each well before shaking firmly and incubating in the dark at ambient temperature for 10 min.

Finally, 2 drops of stop solution were added to each well and the plate was carefully agitated to mix the contents. The absorbance values were measured using a plate reader (Molecular Devices, California, USA) at a single wavelength of 450 nm as instructed by the manufacturer. The approximate concentration of toxins relative to the absorbance reading obtained using this assay has previously been determined (Novak-Weekley and Hollingsworth, 2008) and this is illustrated in Fig. 2.2.

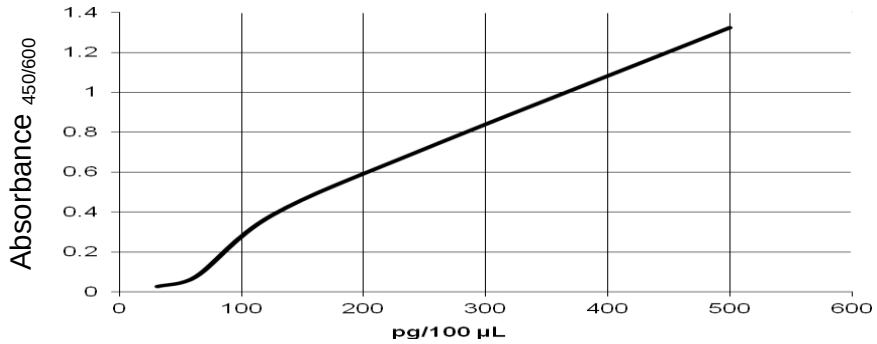


Figure 2.2. Absorbance measurements (450nm) using the Meridian Premier Toxins A and B assay, determined from a range of *C. difficile* toxin concentrations. (Novak-Weekley and Hollingsworth, 2008).

2.2. Phage biology

2.2.1. Mitomycin C induction of phages

Mitomycin C induction was performed by the method of Sell *et al*. (1983). Fresh turbid cultures of *Clostridium spp*. (OD_{600} 0.6-1) were used for inductions. Mitomycin C (Sigma) at a final concentration of 3 mg/mL was added to the cultures and mixed by inversion before further incubation for 4 h. Decreases in the OD_{600} could often be demonstrated at this time if the strain contained an inducible phage. Cultures were centrifuged at 3320 x *g* for 10 min and the resulting supernatant was passed through a 0.45 µm syringe filter (Millipore) to a sterile glass container. Aliquots of the filtered supernatant were used in spot or overlay plaque assays to check for the presence or to build up stocks of phage.

2.2.2. Plaque assays

2.2.2.1. Overlay plaque assays

One-hundred µL of an active *C. difficile* culture in BHI or RCM broth and 100 µL of the phage-containing liquid at the required dilution was added to cooled molten soft BHI or RCM agar and poured over the surface of pre-reduced BHI or RCM agar plates. This was allowed to set for 5 min in air before transferring to the anaerobic cabinet for incubation. After 18-24 h plates were examined for distinct plaques, as shown in Fig. 2.3. The number of plaques can be enumerated to calculate the number of PFU per mL of sample.

A plate without added phage was also set up with each test to verify that the culture conditions were suitable for *C. difficile* growth.

Figure 2.3. Overlay plaque assay showing phage activity.

2.2.2.2. Spot plaque assays

One-hundred µL of an active *C. difficile* culture in BHI or RCM broth was added to cooled molten soft BHI or RCM agar, which was poured over the surface of a pre-reduced BHI or RCM agar plate and allowed to set for 5 min in air before transferring to the anaerobic cabinet. Twenty µL of the required

dilution of phage-containing liquid was dispensed onto the surface of the agar and allowed to sink into the media prior to inversion and incubation for 18-24 h at 37°C. Plates were examined for zones of clearing or distinct plaques as pictured in Fig. 2.4.

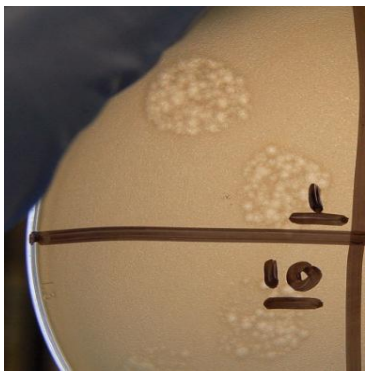


Figure 2.4. Spot plaque assays showing phage activity.

2.2.2.3. Production of lysogens

Lysogenic strains of NCTC 11204 containing ΦCD27 as a prophage were made by carrying out spot plaque assays as described above, only with one spot of 500 µL highly concentrated (greater than 10^7 PFU/mL) phage suspension to give what appears to be confluent lysis within the zone. By incubating the plate for 24-36 h and sub-culturing material from the centre of the zone, lysogens were isolated, which were confirmed by mitomycin C induction (2.2.1).

2.2.3. Propagation of phages

2.2.3.1. Plate harvests

Overlay plaque assays were carried out as previously described (2.2.2.1). Plates containing phage produced semi-confluent plaques. Phage progeny were harvested by overlaying the agar with 15 mL of BHI and incubating at room temperature on an orbital shaker (Stuart SSL1, 30 rpm) for 3 h. The BHI was harvested and filtered through a 0.45 µm membrane and stored at 4-8°C. Fresh phage stocks were made for each experiment.

2.2.3.2. Propagation of phage in broth cultures

Cultures of susceptible *C. difficile* strains were grown in 20 mL BHI broth (with complements) to approximately 0.4 OD_{600} as previously described (2.1.1.1). Five mL of fresh induced phage was added to the culture and returned to incubation in the anaerobic cabinet for 2-5 h. Decreases in OD_{600} were measured and the phage was harvested by centrifugation at 3320 x *g* and passing the phage-containing supernatant through a 0.45 µm syringe filter.

2.2.3.3. Storage of phage stocks

Phage stocks were kept at 4°C for up to 3 months.

2.2.4. Concentration of phages

Bacteriophages were concentrated by PEG precipitation as described previously (Sambrook *et al.*, 1989). Using a sterile Pasteur pipette, 20 mL of phage-containing liquid was dispensed into a sterile 50 mL centrifuge tube. DNase and RNase (2.5.20/2.5.21) were added to a final concentration of 10 µg/mL each and the tubes were incubated for 1-2 h in a 37ºC water bath. Sodium chloride was added to a final concentration of 1 M and the tubes were kept on ice for 1 h, agitating at frequent intervals. The tubes were centrifuged at 11,000 x *g* at 4ºC for 10 min and the supernatant was transferred to a new sterile 50 mL centrifuge tube. PEG molecular weight 8000 g was added to obtain a final concentration of 10%. This was agitated to aid dissolution and then incubated on ice for 2 h. The tubes were centrifuged again for 10 min at 11,000 x *g* at 4ºC. The supernatant was removed and the pellet re-suspended in 1 ml of BHI broth (with complements) or suspension media (SM). Finally the suspension was filtered through a 0.45 µm pore membrane into a sterile container and stored at 4ºC.

2.2.5 Microscopy

2.2.5.1. Light microscopy

Light microscopy was used to verify cell morphology from pure cultures and in the preparation of spore suspensions. Fifteen µL of the sample to be viewed

was added to clean glass microscope slide. A cover slip was placed over the liquid and slides were viewed at x400 magnification.

2.2.5.2. Electron microscopy

2.2.5.2.1. Transmission Electron Microscopy (TEM)

Formvar/carbon grids (Agar Scientific) were processed in duplicate. A grid was picked up using tweezers and bent slightly at the edge by holding the dark shiny (coated) side against the bottom of a petri-dish and turning the tweezers. This ensured that the grids were level and were held in place in an upright position. Approximately 30 µL of sample was pipetted onto each grid and incubated for 1 min at room temperature. The grids were held vertically over a waste container and 5 drops of uranyl acetate (saturated solution in water) (Sigma) were pipetted over to wash off the sample and stain the adhered material. Excess stain was blotted from the edge of the grids using a piece of filter paper and they were placed face-up onto a steel wire mesh platform in a petri dish, taking note of the positions relating to each sample. When all the grids were prepared, the dish was transferred to a fume cabinet and 5 mL of 25% glutaraldehyde (Fisher Scientific) was pipetted into a plastic cap placed next to the wire mesh in the petri dish. The lid of the dish was immediately replaced and the grids were incubated in the cabinet for 30 min. After 1 h, the grids were turned over and left for a further 30 min before being turned back and allowed to incubate for 12-15 h. The grids were transferred to a new clean petri dish and submitted to the electron microscopy department for analysis.

2.2.5.2.2. Scanning electron microscopy (SEM)

One mL of turbid bacterial suspensions (concentrated by centrifugation if required) was transferred to a sterile 1.5 mL microtube. One-hundred and ten µL of 25% glutaraldehyde was added and mixed well into the bacterial suspension. Samples were left to fix for 1 h before being submitted for SEM analysis.

2.3. Molecular methods

Molecular biology methods were conducted according to protocols in (Sambrook *et al.*, 1989) unless stated otherwise.

2.3.1. Equating CFU/PFU with DNA quantity

The total amount of DNA (ng) based on the yield of viable organism was calculated using the following equation to indicate the number of PFU/mL required to obtain sufficient DNA for extraction:

$$\left(\frac{\left(\text{Size of genome (bp)} \times \frac{\text{Molecular weight of DNA}}{(660)} \right) \left(10^{9} \right)}{6.023 \times 10^{23} \text{mol}^{-1}} \right) \times \text{PFU (total)} = \text{DNA (ng)}$$

In order to recover up to 60 µg using the Qiagen Lambda Midi kit (detailed below), approximately 1 x 10^{12} PFU must be extracted. (The genome of ΦCD27 is 50930 bp meaning the expected yield would be 55.8 µg).

2.3.2. Nucleic acid extraction

2.3.2.1. DNA extraction

Samples (1.5 mL) of cultures and colon model samples were transferred to a microtube and centrifuged for 5 min at 12,000 x *g*. The supernatant was removed and the pellets were frozen at -20°C prior to DNA extraction using the methods described below.

2.3.2.1.1. Qiagen stool mini kit

Nucleic acid from defrosted pellets was extracted as instructed by the manufacturer. ASL lysis buffer (1.4 mL) was added and the tube was vortex mixed thoroughly until completely homogenised. The tube was then heated for 5 min at 95°C, after which the sample was vortex mixed and centrifuged for 1 min at 12,000 x *g*. Supernatant (1.4 mL) was carefully transferred to a new sterile tube. An inhibitEX tablet was added using sterile tweezers and the

tube was vortex mixed until the tablet had dissolved. After incubation for 1 min at ambient temperature the tube was centrifuged for 3 min at 12,000 x *g*. The supernatant was transferred to a new sterile microtube and centrifuged again. Fifteen µL of proteinase K was added to a new sterile microtube. Two-hundred µL of the second supernatant was added followed by 200 µL buffer AL. The tube was vortex mixed for 15 s and incubated at 95°C for 10 min. Next, 200 µL of 100% ethanol was added. The lysate was transferred to a QIAamp spin column in a 2 mL collection tube and centrifuged at 12,000 x *g* for 1 min. The flow-through was discarded and the spin column was placed into a new collection tube. Five-hundred µL of buffer AW1 (wash buffer) was added to the spin column before centrifuging again at 12,000 x *g* for 1 min. Again the flow-through was discarded and the column placed into a new collection tube. Five-hundred µL of AW2 (wash buffer) was added to the column, after which it was centrifuged at 12,000 x *g* for 3 min. The flow-through was discarded and the spin column was transferred to a new collection tube. The spin column was centrifuged for 1 min at 14,000 x *g* to dry the membrane, and then transferred to a new sterile 1.5 mL microtube. One-hundred µL of elution buffer was added directly onto the membrane and incubated for 5-10 min. Finally, the tube was centrifuged at 14,000 x *g* for 1 min and the resulting eluate was stored at 4°C.

2.3.2.1.2. Phenol chloroform method

Pellets were defrosted and re-suspended in 1 mL lysis mix (1 units/100 µL mutanolysin and 166 mg lysozyme dissolved in 20 mL TE, pH 7.5), vortex mixed vigorously and incubated at 37°C for 1 h. Next, 20 µL 10 mg/mL RNAse (2.5.21) was added followed by 84 µL clearing mix (49 µg/mL proteinase K, 9.5 mM EDTA pH 8, 0.24 M NaCl and 0.8% sodium dodecyl sulphate (SDS) dissolved in sterile deionised water) and incubated at 60°C with occasional inversion for 1 h or until the mixture had cleared. The mixture was divided into 2 clean microcentrifuge tubes and mixed with 500 µL phenol, inverted for 30 s and centrifuged for 5 min at 11,000 x *g*. The aqueous layer was transferred to a clean tube and the process was repeated with phenol:chloroform:isoamylalcohol (25:24:1) and then chloroform. Next, 1 mL of ice cold ethanol was added, mixed thoroughly and centrifuged for 30 min at

13,000 x *g*, after which the supernatant was removed and the pellet washed with 1 mL 70% ethanol. After removing the ethanol, the pellet was air dried for 10 min and finally re-suspended in 200 µL Tris EDTA (TE) buffer, pH 8 (2.5.26).

2.3.2.1.3. Boom method

The Boom method was carried out as previously described (Health Protection Agency, 2008). Briefly, the pellets were defrosted and re-suspended in 200 µL sterile distilled water. This was added to a tube containing 900 µL L6 buffer (Severn Biotech) and 20 µL isoamyl alcohol (Sigma). The tube was vortex mixed for 1 min and centrifuged for 15 s at 12,000 x *g*. The supernatant was transferred to a new sterile 1.5 mL microtube. Extraction matrix (100 µL silica beads from Severn Biotech) was added. The mixture was vortex mixed for 15 s and then shaken on a rocking platform for 15 min. The tube was centrifuged at 12,000 x *g* for 15 s and the supernatant discarded. Five-hundred µL of L2 buffer (Severn Biotech) was added and mixed. The tube was centrifuged at 12,000 x *g* for 15 s and the supernatant was discarded. This washing step was repeated with L2 buffer, then with 500 µL 70% ethanol twice and finally with 500 µL acetone. The silica pellet was dried by placing the opened tubes into a heating block set at 50°C for 5-10 min. The nucleic acid was re-suspended by adding 150 µL PCR-grade water, mixed well and heating at 56°C for 5 min. The tube was then centrifuged at 12,000 x *g* for 20 min and 100 µL of the supernatant carefully withdrawn to a new sterile tube. In the event of any silica becoming disassociated from the pellet, the tube was centrifuged again to ensure that no silica was transferred. Extracted nucleic acids were stored at 4°C and diluted 1 in 10 prior to use in PCR assays. These extractions were not suitable for spectrophotometric quantification due to the potential interference from the silica beads.

2.3.2.1.4. Freeze-thaw cell disruption

When required, frozen pellets were thawed at ambient temperature, mixed thoroughly using a vortex mixer and centrifuged for 1 min at 3320 x *g* to

clarify. Material was then used directly as a PCR template for density gradient gel electrophoresis (DGGE) analysis.

2.3.2.1.5. Phage DNA extraction

Reagents were kit components unless otherwise stated. Lambda DNA kits (Qiagen) were used to extract phage DNA. One hundred µL of buffer L1 (containing RNase and DNase) was added to 50 mL of the phage-containing plate harvest (~1 x 10^{11} PFU). Next, 19 mL ice-cold L2 was added, mixed gently by inversion and incubated on ice for 60 min. The tubes were centrifuged at 11,000 x *g* for 10 min at 4°C and the supernatant was carefully discarded. The pellet was completely re-suspended in 3 mL L3 before adding 3 mL L4 and immediately inverting 6-8 times to mix. The suspension was then incubated at 70°C for 20 min and cooled on ice. Three mL of L5 buffer was added and promptly mixed by inversion. The suspension was centrifuged at 4°C for 30 min at 17,000 x *g* and the supernatant was carefully transferred to a fresh tube. The centrifugation step was repeated for 10 min to obtain a clear supernatant. A Qiagen tip-100 was equilibrated with 4 mL QBT and allowed to empty by gravity flow before applying the supernatant. When this had passed through, the column was washed with 10 mL buffer QC. DNA was eluted with 5 mL buffer QF and precipitated by mixing with 3.5 mL isopropanol at ambient temperature. The DNA was centrifuged at 17,000 x *g* for 30 min at 4°C and the supernatant was carefully removed and discarded. The DNA pellet was washed with 2 mL 70% ethanol and centrifuged at 17,000 x *g* for 10 min. The supernatant was discarded as before and the pellet was air-dried for 5-10 min before re-suspension in 60-100 µL nuclease-free water (Promega).

2.3.2.2. RNA extraction

Cultures of *C. difficile* were prepared as described previously (2.1.1.1). Samples of turbid cultures were taken after 10 h growth in order to evaluate the differential expression of the toxins of *C. difficile*.

Before all work with RNA, clean surfaces and pipettes were wiped with RNase AWAY (Sigma) to inactivate RNase enzymes and gloves were replaced

regularly throughout the protocol. All consumables that were not certified RNase-free were double-autoclaved.

The SV Total RNA Isolation System (Promega) was used for RNA extractions. Reagents were kit components unless otherwise stated. The protocol adapted for Gram-positive organisms was used, with some modifications. A 1.5 mL aliquot of culture was transferred to a microtube and centrifuged at 11000 x *g* for 2 min to pellet the cells. The supernatant was removed and this was repeated twice to obtain the equivalent of 4.5 mL culture. The pellet was re-suspended in 100 µL freshly prepared TE buffer containing 3 mg/mL lysozyme (Sigma) and tapped to mix. The re-suspended pellet was incubated at ambient temperature for 8 min before 75 µL of RNA lysis buffer was added followed by 350 µL RNA dilution buffer and mixed by gentle inversion. Next, 200 µL ethanol was added and pipetted up and down several times. This mixture was transferred to an assembled spin column and centrifuged at 13,000 x *g* for 1 min. Flow-through from the collection tube was discarded and 600 µL wash solution was added. The spin columns were centrifuged at 13,000 x *g* for 1 min. To a separate tube 40 µL yellow core buffer, 5 µL 0.09 M $MnCl_2$ and 5 µL DNase I was combined and mixed by pipetting. The entire mix was applied to the spin column membrane and incubated at 20-25°C. After 15 min, 200 µL DNase stop solution was added and the spin column was centrifuged at 13,000 x *g* for 1 min. Another wash step with 600 µL wash solution followed, after which the columns were centrifuged at 14,000 x *g* for 2 min to dry the membrane. The column was removed from the collection tube and placed into a 1.5 mL microtube. One-hundred µL of nuclease-free water was added and the column was centrifuged at 13,000 x *g* for 1 min. The resulting eluate was used immediately or flash-frozen in liquid nitrogen before being stored at -80°C.

The DNase step included in the SV Total RNA Isolation System was not sufficient to remove all traces of contaminating DNA. Frozen samples (50 µL) were defrosted gently on ice and 5 µL TURBO DNase buffer (Invitrogen) and 1 µL TURBO DNase (Invitrogen) was added, mixed gently and incubated at 37°C. After 30 min, 5 µL DNase inactivation reagent (Invitrogen) was added. The tube contents were mixed well at regular intervals during a 5 min

incubation at 25°C. The tubes were centrifuged at 10,000 x *g* for 90 s at 4°C and the RNA was transferred to a fresh tube. In order to verify that all DNA had been eliminated, 1 µL was used as a template for PCR with Hotmaster Taq (2.3) using the following primers (Eurofins MWG) targeting the 16S v3 region:

- V3_F: 5'-CCTACGGGAGGCAGCAG-3'
- V3_R: 5'-ATTACCGCGGCTGCTGG-3'

2.3.2.2.1. Production of cDNA

Superscript® III Reverse Transcriptase (Invitrogen) was used to produce complementary DNA (cDNA) copies of pure extracted RNA. Reagents were kit components unless otherwise stated. To 13.9 µL of the RNA extract, 0.3 µL of random primers was added and incubated at 65°C for 5 min. The reaction was cooled slowly to ambient temperature for 15 min. Two microliters of reverse transcription buffer, 2 µL 100 mM dithiothreitol (DTT), 0.8 µL 100 mM deoxyribonucleotide triphosphate (dNTP) mix and 1 µL reverse transcriptase were added. The reaction was incubated at 25°C for 10 min and then at 48°C for 1 h. The transcriptase was inactivated by incubating at 70°C for 15 min and the reations were cooled before use as a template for PCR.

2.3.3. Concentration of nucleic acid

To a starting volume of 1 mL in an 50 mL centrifuge tube, 100 µL of 3 M sodium acetate at pH 5.2 (Sigma) was added and mixed by inversion. Next, 800 µL iso-2-propanol (Fluka) was added, mixed again and left on ice for 30 min. The tubes were centrifuged at 13,000 x *g* for 30 min and the pellet washed in 1 mL 70% ethanol (Sigma). After centrifugation and removal of the supernatant, the pellet was re-suspended in 200 µL nuclease-free water (Promega).

2.3.4. Quantification of nucleic acid

The Nanodrop instrument and software (Thermo Scientific) was used according to the manufacturer's instructions. The type of nucleic acid (DNA or RNA) to be measured was selected and the instrument was cleaned with lint-

free tissue moistened with ultra-pure water. It was calibrated by taking a blank measurement with 1 µL ultra-pure water and dried with tissue. One µL of sample was used to obtain a reading, cleaning with tissue between each. Nucleic acid peaks were typically at 260 nm. Peaks at 280 nm were indicative of protein contamination and samples with high absorption at 280 nm were re-extracted. An absorbance ratio (260/280) of between 1.7 and 2 indicated a pure extraction of good quality.

The approximate quantity of nucleic acid could also be estimated by running a 1 and 5 µL aliquot on a 1% agarose gel (Melford Laboratories Ltd) and comparing the intensity of the bands obtained with the known quantities of the Hyper I DNA ladder (BioLine Ltd., Fig. 2.5).

2.3.5. Polymerase chain reaction (PCR)

PCR was performed using PCR Sprint (Thermo Scientific) and the TProfessional Basic (Biometra) thermal cyclers, with the polymerases GoTaq (Promega) for colony PCR, Hotmaster (5 Prime) for DGGE (see 6.4.3) and Phusion (New England Biolabs (NEB)) for high fidelity applications including amplification of genes for protein expression. Protocols were as per manufacturer's instructions. Primers were obtained from Sigma Genosys and annealing temperatures were calculated using the website http://www.basic.northwestern.edu/biotools/oligocalc.html (Kibbe, 2007).

2.3.5.1. Go Taq DNA Polymerase (Promega)

Amplification of DNA using Go Taq Polymerase was carried out with the reaction compositions detailed in Table 2.2 a**.** Amplification of DNA with Go Taq Polymerase was carried out using the cycling parameters given in Table 2.2 b.

a

Component	Quantity
DNA template	1 µL (~500 ng)
5 x GoTaq reaction buffer (Promega)	10 µL
dNTP 100 mM (Bioline)	0.4 µL
Forward primer 20 µM	1 µL
Reverse primer 20 µM	1 µL
Deionised water	36.35 µL
Go Taq polymerase (Promega)	0.25 µL

b

Temperature	Duration	Number of cycles
95°C	2 min	1
95°C	30 s	25-30
55°C*	30 s	
72°C	1 min per kb	
72°C	5 min	1

Table 2.2. (a) Go Taq Composition and (b) cycling parameters. *Adjusted depending on primer melting temperature (T_m).

2.3.5.2. Phusion DNA Polymerase (NEB)

Phusion is a high fidelity polymerase enzyme. Amplification of DNA using Phusion Polymerase was carried out with the reaction compositions detailed in Table 2.3 a. Amplification of DNA with Phusion Polymerase was carried out using the cycling parameters given in Table 2.3 b.

a

Component	Quantity
DNA template	1 µL (~ 500 ng)
5 x Phusion reaction buffer (NEB)	10 µL
dNTP 10 mM (Bioline)	1 µL
Forward primer 20 µM	1.25 µL
Reverse primer 20 µM	1.25 µL
Deionised water	34.5 µL
Phusion polymerase (1 U/µL) (NEB)	0.5 µL

b

Temperature	Duration	Number of cycles
98ºC	30 s	1
98°C	10 s	25-30
Tm or Tm+3°C for >20 bp primers	30 s	
72°C	15-30 s per kb	
72°C	5 min	1

Table 2.3. (a) Phusion composition and (b) cycling parameters.

2.3.5.3. Hotmaster Taq DNA Polymerase (5 PRIME)

Amplification of DNA using Hotmaster Taq Polymerase was carried out with the reaction composition described in Table 2.4 a**.** Amplification of DNA using Hotmaster Taq Polymerase was carried out with the cycling parameters detailed in Table 2.4 b.

a

Component	Quantity
DNA template	2 µL
5 x Hotmaster reaction buffer (5 PRIME)	5 µL
dNTP 2.5 mM (Bioline)	4 µL
Forward primer 10 µM	1 µL
Reverse primer 10 µM	1 µL
Bovine serum albumin (BSA) (10 mg/mL New England BioLabs)	1 µL
Deionised water	35.8 µL
Hotmaster polymerase (1 U/µL) (5 PRIME)	0.2 µL

b

Temperature	Duration	Number of cycles
94ºC	2 min	1
94°C	20 s	29
58°C*	10 s	
65°C	20 s	
65°C	5 min	1

Table 2.4. (a) Hotmaster composition and (b) cycling parameters. *Adjusted depending on primer melting temperature.

2.3.6. Gel electrophoresis of nucleic acid

Agarose gels were made at a concentration of 0.8-1.5% (Melford Laboratories Ltd) and run in 0.5 x Tris borate EDTA (TBE) (Fisher Scientific). A positive and a negative control were tested on each gel and the sample volume was typically 5-10 µL. If samples required a loading dye, 1 µL spots of loading buffer (0.015% bromethyl blue (Sigma), 10% glycerol (Sigma) in 0.5 x TBE buffer) were dispensed onto parafilm (M Laboratory) and mixed with samples prior to loading into the gel.

The gels were stained in 1 mg/L ethidium bromide for 30 min, briefly rinsed in deionised water and visualised with transilluminating UV light using the AlphaImager software (ProteinSimple).

2.3.6.1. Hyperladder I

Hyperladder I (Bioline) was used as a DNA ladder in every electrophoretic gel. The quantities and sizes of the fragments are shown in Fig. 2.5.

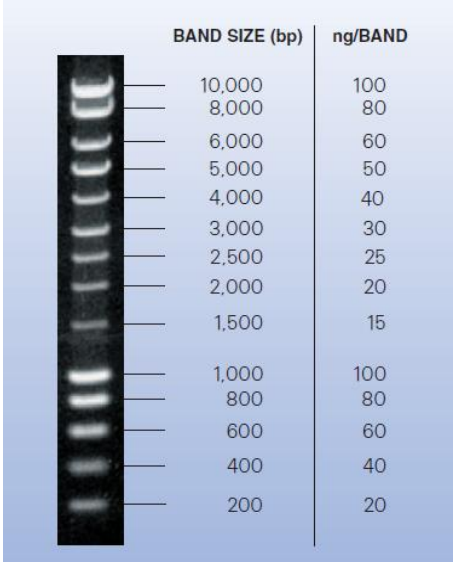


Figure 2.5. Hyperladder I fragment sizes and quantities based on 5 µL loaded onto a 1% agarose gel stained with ethidium bromide.

2.3.7. PCR clean-up

Nucleic acids (greater than 100 bp) were cleaned with SureClean (Bioline) to remove primers and restriction enzymes in preparation for sequencing and other sensitive downstream applications. Microtubes (0.5 mL) (Amicon) suitable for PCR clean-up were used for each sample to be purified. An equal volume of SureClean was added to the PCR product. The samples were vortex mixed for 30 s and incubated at ambient temperature for 30 min before being centrifuged at 13,000 x *g* for 30 min. The supernatant was carefully removed by aspiration and a volume of 70% ethanol 2 times that of the original PCR product volume was added. The tubes were vortex mixed for 30 s and centrifuged as before. The supernatant was air-dried at 55˚C for approximately 5 min to ensure complete removal of the ethanol, and re-suspended in 10-20 µL nuclease-free water (Promega).

2.3.8. Restriction digestion of nucleic acid

DNA was purified with SureClean to remove proteins, primers and dNTPs. The concentration of DNA to be digested depended on the size of the smallest fragment to be obtained and the intended downstream application. Digests were carried out according to the NEB guide with water baths used

for the incubations. Restriction enzymes were heat-inactivated after the incubations and products were purified with SureClean to remove the enzymes.

2.3.9. Phosphatase treatment of nucleic acid

Antarctic Phosphatase (NEB) was used to remove 5' phosphate groups from plasmid vectors after restriction digestion and prevent self-ligation as per manufacturer's instructions. The reactions were comprised of 1 x Antarctic Phosphatase buffer (NEB), up to 1 µg restricted DNA in any buffer, sterile ultra-pure water (if dilution was required) and 10 units of Antarctic Phosphatase. This was incubated for 15 min at 37°C in a water bath for 5' extentions or blunt ends or 60 min for 3' extensions. Reactions were then inactivated at 65°C for 5 min. Products were purified using SureClean to remove the enzyme.

2.3.10. Sequencing

Purified PCR products and plasmid preps were sequenced using BigDye v. 3.1. (Applied Biosystems). DNA was quantified using the Nanodrop spectrophotometer and diluted according to the size of the product to be sequenced as shown in Table 2.5.

Template	**Quantity**
PCR product	
100-200 bp	3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng
1000-2000 bp	10-40 ng
>2000 bp	40-100 ng
Double stranded	30-40 ng/kb
Bacterial genomic DNA	2-3 µg

Table 2.5. Guide of nucleic acid quantities required for BigDye 3.1. sequencing.

The reaction components were as shown in Table 2.6 a and were added in this order**.** The cycling parameters are given in Table 2.6 b.

a

Component	Quantity
DNA template	13.5 µL
Water	
5 x BigDye 3.1 reaction buffer	3.5 µL
Forward or reverse primer 1.6 pmol/µL	2 µL
BigDye v. 3.1 thawed on ice	1 µL

b

Temperature	Duration	Number of cycles
96°C	2 min	1
96°C	30 s (ramp 1°C/s)	25
50°C	15 s	
60°C	4 min	1

Table 2.6 (a) BigDye v. 3.1 composition and (b) cycling parameters prior to capillary sequencing

Samples were submitted to Genome Enterprise Ltd for capillary sequencing using the 3730XL DNA analyser (Life Technologies).

The capillary sequencing carried out as part of the phage integration site determination was performed by the Wolfson Institute for Biomedical Research, UCL.

2.3.11. Subcloning

2.3.11.1. Adding 3’ Adenosine-overhangs onto PCR products

Many polymerase enzymes such as Phusion produce blunt ended products and for the purposes of cloning it was necessary to add adenosine overhangs to the 3’-ends. This was achieved by incubating 39.35 µL DNA (diluted in nuclease-free water if required) with 10 µL 5 x GoTaq buffer, 0.4 µL 100 mM dNTP and 0.25 µL GoTaq polymerase for 20 min at 72°C. Samples were then stored at 4°C and cloning was carried out within 2 days of adding the overhangs.

2.3.11.2. Ligation

2.3.11.2.1. Cloning of products for sequencing using pCR®2.1

Cloning of PCR products was performed using the TA cloning kit (Invitrogen). A map of pCR®2.1 is shown in Fig. 2.6. The following was used to calculate the amount of PCR product (X) needed for each ligation reaction based on a 1:1 molar ratio.

X ng PCR product = (Y base pairs PCR product) x (50 ng pCR®2.1)/3900*
* The size in base pairs of the pCR®2.1 vector.

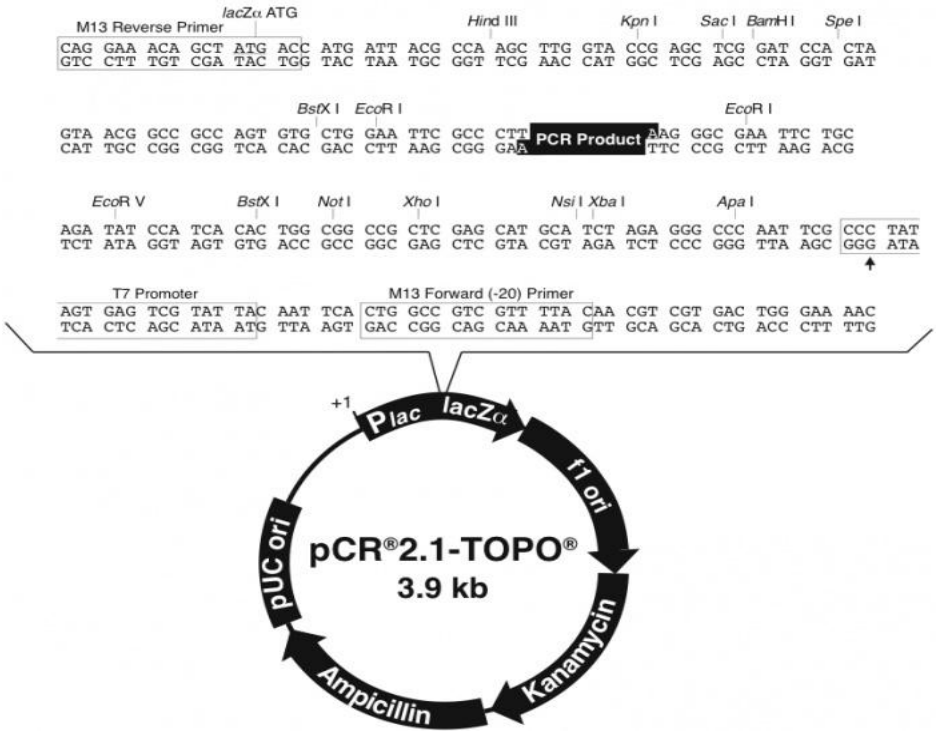


Figure 2.6. The plasmid vector pCR®2.1 (reproduced from the Invitrogen manual).

A 3:1 ratio (insert:vector) often gave a better transformation efficiency but no more than 3 µL of the PCR product was added to the ligation reaction mixture due to the inhibitory effect of the salts on the ligase. Dilutions of the PCR products were made using nuclease-free water (Promega) where required.

The plasmid vector pCR®2.1(Invitrogen) was defrosted on ice and centrifuged briefly to collect all liquid at the bottom of the vial. The ligation reactions were

set up as follows: X µL fresh PCR product (with 3'-A overhangs); 1 µL 10 x ligation buffer; 2 µL 25 ng/µL pCR®2.1 vector and water to make a total volume of 9 µL. Finally, 1 µL of T4 DNA ligase (4 Weiss units) was added and the reactions were incubated at 14°C for 12-18 h. To confirm that a product of the correct expected size was achieved, 1 µL of the ligation mixture was used as a template in PCR using GoTaq (2.3.5.1) and the following primers (Sigma GenoSys):

- M13_F: 5'-CTG GCC GTC GTT TTA C-3'
- M13_R: 5'-GTC ATA GCT GTT TCC TG-3'

Products were visualised by gel electrophoresis and successful ligations could then be used for transformations (2.3.11.3).

2.3.11.2.2. Cloning of products for protein expression using pET15b

A map of pET15b is shown in Fig. 2.7. The pET15b vector and the fragments to be ligated were digested with restriction enzymes *NdeI* and *XhoI*. One µg in 6 µL was incubated at 37°C for 1 h with 1 µL buffer 4 (NEB), 2 µL nuclease-free water (Promega) and 1 µL *NdeI* (NEB). A second digestion under the same conditions was carried out by adding 1 µL buffer 4, 8 µL nuclease-free water and a further 1 µL *NdeI.* Next, 1 µL buffer 4 was added, 5 µL nuclease-free water and 3 µL BSA (NEB) with 1 µl *XhoI* (NEB). The reaction was incubated for 1 h at 37°C before a further digestion with 1 µL buffer 4, 1 µL BSA, 7 µL nuclease-free water and 1 µL *XhoI*. The vector was also treated with Antarctic phosphatase (2.3.9) after restriction digestion (2.3.8). The vector and the fragments were purified with SureClean (Bioline, 2.3.7) and the yields and quality of the products assessed using the Nanodrop spectrophotometer. A ratio of 3:1 insert: vector was used, calculated as previously described. The ligation reactions were set up using Fastlink ligase (Epicentre) as described by the manufacturer: X µL digested cleaned PCR product (diluted in nuclease-free water); 1.5 µL 10 x Fastlink ligation buffer; 1.5 µL 10 mM ATP; 3 µL (~150 ng) restricted phosphotased pET15b vector; 1 µL of Fastlink DNA ligase made up to 15 µL with nuclease-free water. The mixture was mixed gently, centrifuged briefly to draw liquid to the bottom of

the tube and incubated at ambient temperature for 2.25 h. To confirm that a product of the correct expected size was achieved, 1 µL of the ligation mixture was used as a template in PCR using GoTaq and the following primers (Sigma GenoSys):

- T7P2: 5'-TGA GCG GAT AAC AAT TCC C-3'
- T7_T: 5'-GCT AGT TAT TGC TCA GCG G-3'

PCR product sizes were checked by gel electrophoresis and successful ligations could then be used for transformations (2.3.11.3).

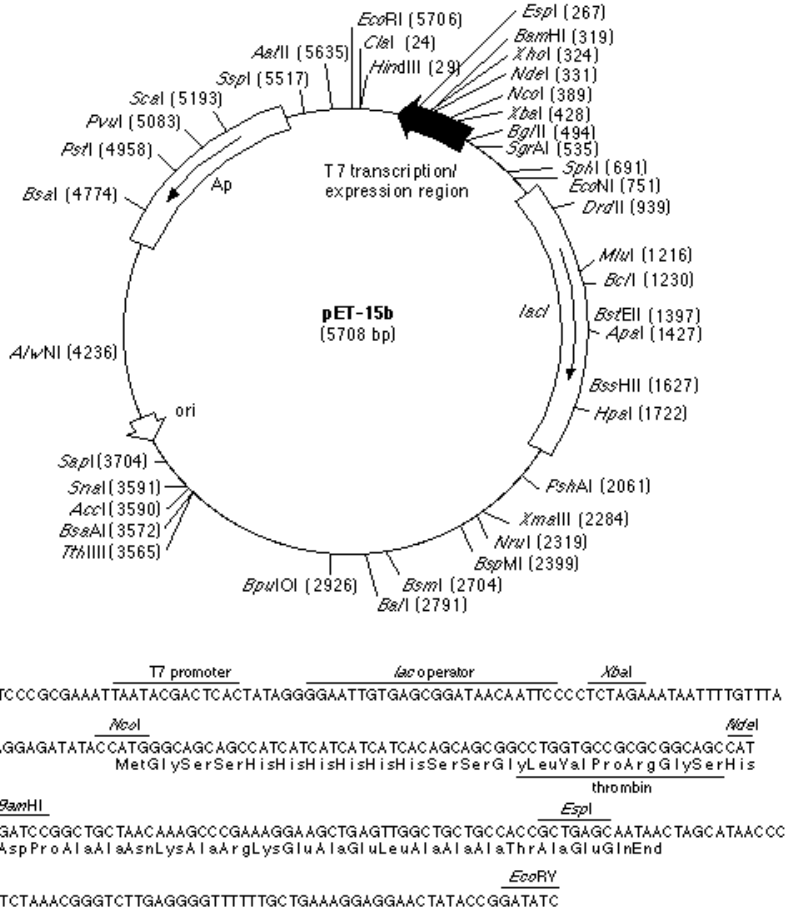


Figure 2.7. The plasmid vector pET15b (reproduced from the Novagen datasheet).

2.3.11.3. Transformation of *E.coli*

2.3.11.3.1. Basic protocol for TOP10 and BL21 (DE3)

Chemically competent *Escherichia coli* TOP10 cells (Invitrogen) were used for transformations for basic cloning and *E. coli* BL21 (DE3) cells (Invitrogen) were used for transformations with pET15b for protein expression. One vial of One Shot® cells (sufficient for 2-4 transformation reactions) was transferred from -80°C to an ice bucket and allowed to defrost for 8 min. Cells aliquots of 12 µL were dispensed into pre-chilled microtubes for each transformation reaction. Empty vector controls were included to permit calculation of transformation efficiency. Five to 10 ng of chilled ligated DNA (in a volume between 1-5 µL) was transferred to the cells, tapped twice to mix and immediately returned to the ice for 30 min. The tubes were then placed in a 42°C water bath for exactly 30 s without mixing and immediately returned to the ice for 2 min. Two hundred and fifty µL pre-warmed (37°C) SOC broth was added to the cells and the tubes were taped in a rack and placed in a shaking incubator at 37°C for 1 h at 225 rpm. Fifty and 100 µL of each transformation reaction was spread onto 2 L agar plates containing 100 µg/mL ampicillin (plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGAL, 2.5.19) for pCR®2.1), and incubated at 37°C for 18-24 h.

2.3.11.3.2. Confirmation of positive transformants

Where possible, a minimum of 10 (white or pale blue for pCR®2.1 transformations) colonies were tested per transformation reaction. A sterile toothpick was used to touch the colony and transfer the material to 10 µL of sterile ultra-pure water in a microtube, which was then mixed and briefly centrifuged. The choice of primers depended on the vector used for the transformations, as described previously (section 2.3.11.2). GoTaq polymerase and 1 µL of the suspended colony material as the template were used for PCR. The presence and size of a product was confirmed by gel electrophoresis.

2.3.11.3.3. Transformation efficiency

Empty vector controls were carried out to calculate the efficiency of the transformation reactions using the following equation:

(Numbers of colonies) x (1000 ng/µg)/(ng of control DNA) = transformants/µg

2.3.11.4. Plasmid extractions

Reagents were prepared as instructed in the kit insert. Confirmed positive transformants were grown in L broth containing ampicillin (100 µg/mL) for 8 h in a shaking incubator at 37°C, sub-cultured to fresh L broth and grown for a further 12-16 h. A 1.5 mL aliquot of culture was transferred to a microtube and centrifuged at 11000 x *g* for 2 min to pellet the cells. The supernatant was removed and this was repeated twice to obtain the equivalent of 4.5 mL culture.

2.3.11.4.1. Qiagen plasmid mini kit

Reagents were kit components from the plasmid mini kit (Qiagen) unless otherwise stated. Cell pellets were thoroughly re-suspended in 0.3 mL of buffer P1 containing LyseBlue. Buffer P2 (0.3 mL) was added and the tubes were inverted 4-6 times and incubated at room temperature for 5 min. Chilled buffer P3 (0.3 mL) was added and the tubes were vigorously inverted 4-6 times and incubated on ice for 5 min to precipitate the genomic DNA, proteins, cell debris and potassium dodecyl sulphate. The mixtures were centrifuged for 10 min and the supernatant containing the plasmid DNA was promptly transferred to a fresh tube. A QIAGEN-tip was equilibrated by adding 1 mL of buffer QBT and allowing it to empty by gravity flow. When all of the buffer QBT had drained, the supernatant was added to the resin and again allowed to empty by gravity flow. Next the QIAGEN-tip was washed twice with 2 mL buffer QC and the DNA eluted with 0.8 mL buffer QF. The DNA was precipitated by adding 0.7 volumes of isopropanol at room temperature, mixing and centrifuging at 14,000 x *g* for 30 min. The supernatant was removed and the DNA pellet was washed with 1 mL 70% ethanol before being centrifuged at 14,000 x *g* for 5 min. The supernatant was carefully removed,

taking care not to disrupt the pellet. The pellet was air-dried for 10 min and re-dissolved in 20 µL nuclease-free water (Promega).

2.3.11.4.2. Omega E.Z.N.A. Plasmid Mini Kit

Reagents were kit components unless otherwise stated. The spin protocol was used according to manufacturer's instructions and reagents were prepared as instructed in the kit insert. Pellets were re-suspended in 250 µL solution I containing RNase A and vortex mixed until no cell clumps were visible. The suspension was transferred to a fresh 1.5 mL tube and 250 µL solution II was added to lyse the bacterial cells. The tube was mixed gently by inversion and after 4 min 350 µL of solution III was added and immediately mixed by gently inversion. A white flocculent precipitate formed and was pelleted by centrifugation at 13,000 x *g* for 10 min at ambient temperature. A HiBind DNA Mini Column was placed into a collection tube and equilibrated with 100 µL of equilibration buffer. The column was centrifuged at 13,000 x *g* for 30 s and the filtrate was discarded. The cleared supernatant was applied to the column ensuring not to disturb the white pellet, and the column was centrifuged again. The flow-through was discarded and the column was placed back in the same collection tube. Five hundred µL of buffer HB was added to the column and centrifuged as described previously to remove residual protein contamination. The flow-through liquid was again discarded. Next 700 µL of DNA wash buffer was added to the column, which was centrifuged as before, discarding the flow-through. The wash buffer step was repeated and a further centrifugation at 13,000 x *g* for 2 min was carried out to dry the column completely. The column was placed into a fresh 1.5 mL tube and 30 µL of nuclease-free water (Promega) was added directly to the centre of the matrix. The column was incubated at ambient temperature for 1-2 min and centrifuged at 13,000 x *g* for 1 min. A second elution with a further 30 µL nuclease-free water was carried out to increase the yield of DNA.

2.4. Protein analysis

2.4.1. Protein induction

2.4.1.1. Protein induction from *E. coli* BL21(DE3) to optimise induction and harvest conditions.

With each induction an empty vector control was also tested to serve as a negative control. A colony from a fresh *E. coli* BL21 (DE3) transformation was added to 10 mL L broth containing 100 µg/mL ampicillin and incubated for 12-18 h at 37°C. Two-hundred µL of this culture was added to 3 fresh 10 mL aliquots of pre-warmed L broth with ampicillin and incubated at 37°C in a shaking incubator. When cultures reached OD_{600} 0.4-0.5 (approximately 2-3 h post inoculation) indicating mid-log phase, isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega) at a final concentration of 0.5 mM was added to induce β-galactoside production and hence protein production due to the shared promoter. Cultures were returned to the 37°C shaking incubator. Cultures were harvested at 3 and 4 h (to assess differences in protein yield at the different times) by centrifugation at 3320 x *g* for 15 min at 4°C. The supernatants were removed and the pellet immediately frozen at

-20°C.

2.4.1.2. Protein induction from *E. coli* BL21 (DE3) for downstream applications

His-tagged protein production was induced as recommended by the Qiaexpress Ni-NTA FastStart handbook (Qiagen). Ten mL of an overnight culture was added to 250 mL pre-warmed L broth containing 100 µg/ml ampicillin. When cultures reached OD_{600} 0.6 (approximately 3-4 h post inoculation) indicating mid-log phase, IPTG at a final concentration of 1 mM was added to induce protein production. Cultures were returned to the 37°C shaking incubator and after 4 h they were harvested by centrifugation at 3320 x *g* for 20 min at 4°C. The supernatants were removed and the pellet immediately frozen at -20°C.

2.4.2. Protein extraction

2.4.2.1. Crude protein extraction

Frozen pellets were defrosted on ice for 15 min and 500 µL of extraction buffer (20 mM Tris, 50 mM sodium chloride, pH 7.5 or 50 mM sodium phosphate, 300 mM sodium chloride and 20 mM imidazole) was added. The cell pellet was vortex mixed rigorously to completely re-suspend the cells and this suspension was then transferred to a fresh 2 mL screw-top tube containing 100 µg sterile acid washed 0.1 mm glass beads (Sigma) on ice. The suspension was bead-beaten using the FastPrep FP 120 cell disruptor for 4 cycles of 30s with 5-10 min on ice in between each. The sample was centrifuged at 13,000 x *g* for 30 min at 4°C and the supernatant was carefully transferred to a fresh tube and stored at 4°C until further use.

2.4.2.2. Purification of His tagged proteins under native conditions for downstream applications.

His-tagged proteins were partially purified using the Ni-NTA Fast Start kit (Qiagen). A frozen pellet from a 250 mL culture of induced *E.coli* BL21(DE3) cells was defrosted on ice for 15 min, re-suspended with 10 mL native lysis buffer supplemented with 1 mg/mL lysozyme and 3 U/mL benzonase and incubated on ice for 30 min. The cell suspension was gently swirled to mix every 10 min. The lysate was centrifuged at 14,000 x *g* for 30 min at 4°C to pellet the cellular material and the supernatant was transferred to a fresh, chilled container.

The resin in a Fast Start column was re-suspended by inverting several times and the storage buffer drained from the column. The lysate was applied to the column and 5 µL of the flow-through was saved for analysis by gel electrophoresis. The column was washed twice with native wash buffer and 5µL of each flow- through was saved for analysis by gel electrophoresis. The 6xHis-tagged protein was eluted with 3 consecutive 1 mL aliquots of native elution buffer (50 mM Na_2HPO_4, 300 mM NaCl and 250 mM imidazole, pH 8). All 3 fractions were analysed by gel electrophoresis.

2.4.3. Bradford assays

Assay standards were prepared using Purified BSA (NEB) at concentrations of 200, 180, 160, 140, 120, 100, 80, 60, 40, 20 and 0 µg/mL. Dilutions were made with the buffer used for the protein extraction or purification. Dilutions of sample were also made depending on the anticipated concentration of the neat extraction. Ten µL of the standard concentrations and the samples in duplicate were pipetted into designated wells of a sterile, flat-bottomed, 96-well microtitre plate (Sterilin). The colorimetric indicator was freshly prepared using 4 mL Bradford's reagent (Biorad) with 16 mL H_20, mixed by gentle inversion. Next, 190 µL of the diluted Bradford's reagent was dispensed into each well and the samples were mixed on the SoftMax plate reader (Molecular Devices). After mixing, any bubbles were removed and the plates were incubated at ambient temperature for 5 min before reading at 600 nm (single wavelength). A standard curve of the standards was made and the concentration of protein was calculated using the equation generated by the curve. The mean concentration of the duplicate samples was taken.

2.4.4. Protein gels

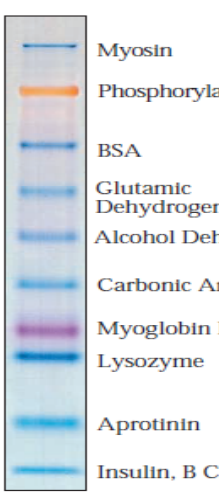
NuPage Novex Bis-Tris gels and reagents were purchased from Invitrogen and the protocol was carried out according to the manufacturer's instuctions. A protein concentration of between 5 and 10 µg (based on results of the Bradford assay) was required for clear visualisation on the gel. Samples were prepared in 0.5 mL tubes in a total volume of 10, 15 or 20 µL as shown in Table 2.8.

Component	µL	µL	µL
Total volume of sample + H_20	6.5	9.75	13
Reducing agent	1	1.5	2
LDS sample buffer	2.5	3.75	5
Total volume	10	15	20

Table 2.7. Composition of protein samples prior to running on NuPage Novex gels.

Prepared sample mixtures were heated to 70°C for 10 min and chilled on ice. The tubes were briefly centrifuged. Gels were rinsed with deionised water and secured in the XCell SureLock system (Invitrogen). Two-hundred mL of MES

buffer (Invitrogen) was added to the inner chamber to submerge the wells and 500 µL of antioxidant was added and mixed well. Ten microlitres of SeeBlue Plus 2 pre-stained standard (Fig. 2.8) (Invitrogen) and the heated and chilled samples were loaded on to the gel. The remaining 400 mL MES buffer was poured into the outer chamber. Gels ran for 35 min at 200 V (start 110-125 mA, end 70-80 mA). The gel was removed from the outer casing and stained with Simply Blue Safestain (2.4.5) or were used for Western blot analysis (2.4.6).



Protein	Approximate Molecular Weights (kDa)				
	Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS	NuPAGE® Tris-Acetate
Myosin	250	210	188	191	210
Phosphorylase	148	105	98	97	111
BSA	98	78	62	64	71
Glutamic Dehydrogenase	64	55	49	51	55
Alcohol Dehydrogenase	50	45	38	39	41
Carbonic Anhydrase	36	34	28	28	n/a
Myoglobin Red	22	17	17	19	n/a
Lysozyme	16	16	14	14	n/a
Aprotinin	6	7	6	n/a	n/a
Insulin, B Chain	4	4	3	n/a	n/a

NuPAGE® Novex
Bis-Tris 4-12% Gel

Figure 2.8. The See Blue Plus 2 pre-stained protein standard.

2.4.5. Staining protein gels.

Gels were washed 3 times with deionised water with gentle shaking and were then submerged in SimplyBlue SafeStain (Invitrogen) and placed on a rotary shaker (Stuart SSL1) at 40 rpm for 1 h. Gels were then de-stained in deionised water for 1 h on the shaker, placed between 2 acetate sheets and imaged on a flatbed scanner (Hewlett Packard).

2.4.6. Western blot analysis.

A protein gel was run as previously described, ensuring that the order of the samples was the same as on the initial stained gel. Proteins were transferred to membranes as described by the Invitrogen Western transfer protocol.

NuPAGE transfer buffer was prepared by adding 25 mL 20 x NuPAGE transfer buffer (Invitrogen), 50 mL methanol (Fisher Scientific) and 500 µL NuPAGE antioxidant (Invitrogen), made up to 500 mL with deionised water. The 4 blotting pads in the XCell II Blot Module (Invitrogen) were soaked in approximately 350 mL of the transfer buffer (prepared as above). A polyvinylidene fluoride (PVDF) transfer membrane was submerged in methanol for 30 s. It was then placed into deionised water to wash briefly before transferring to 50 mL NuPAGE transfer buffer for several minutes. When the protein gel had finished running, it was removed from the outer casing, rinsed briefly in deionised water and placed into a plastic dish. The blot was prepared in the order shown in Fig. 2.9.

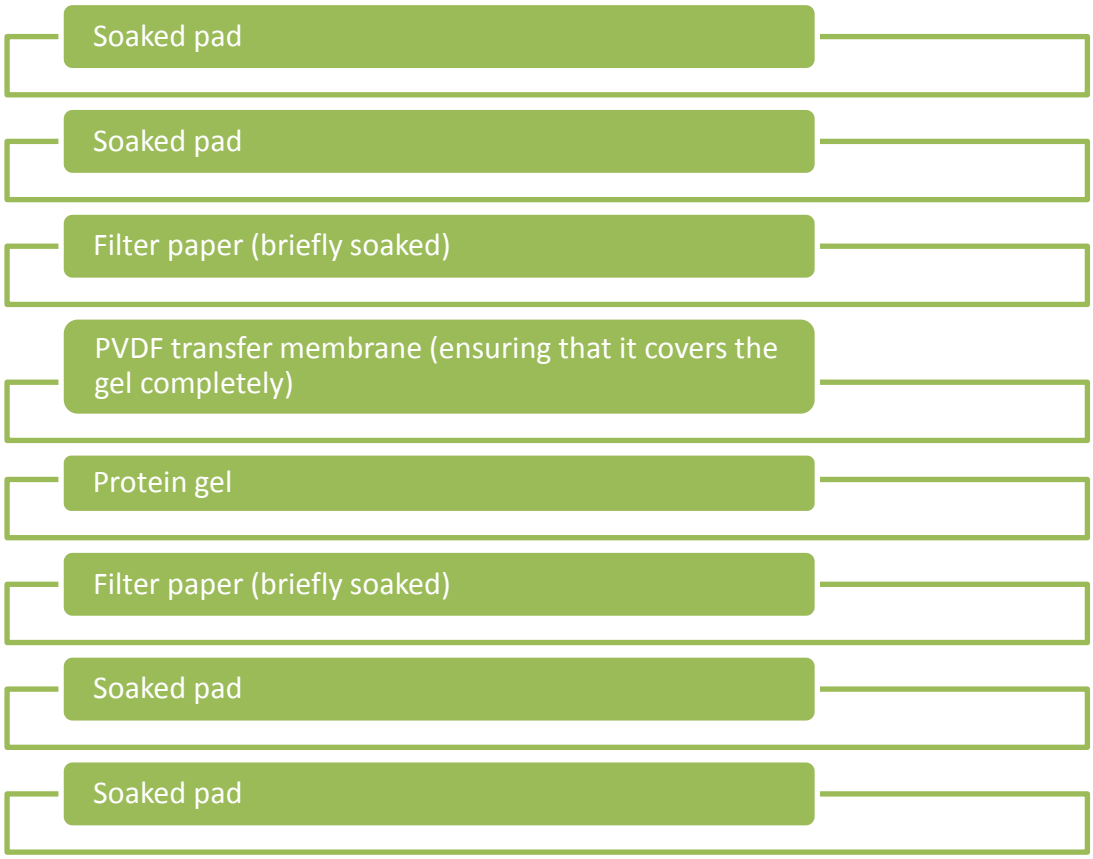


Figure 2.9: Assembly of the Western blot

The assembled blot was placed directly into the XCell II module and secured. Transfer buffer was carefully added to the inner chamber of the blot module until the membrane was covered. Approximately 650 mL of deionised water

was used to fill the outer chamber. Transfer was carried out at 30 V for 1 h (170 mA start, 110 mA end). The blot module was then removed from the gel tank and the PVDF transfer membrane was lifted into a plastic dish containing 50 mL Tris buffered saline (TBS) buffer (2.5.29) to wash for 10 min. After 10 min the wash was repeated with fresh TBS. A 3% BSA (Sigma) solution was made in TBS buffer and 10 mL was added to the PVDF transfer membrane in a fresh plastic dish to block non-specific binding. This was incubated for 15-18 h at 4°C on a tilting platform. The remaining BSA stock and the transfer membrane was brought to room temperature and the membrane was placed into the larger washing dish with 50 ml of TBS with tween (TBS-Tween) with Triton (2.5.30). This was incubated on a rotating platform for 10 min at room temperature. After 10 min the wash was repeated with fresh TBS-Tween/Triton. After 10 min the TBS-Tween/Triton was replaced with TBS and incubated on the rotating platform for 10 min. Anti-His-TAG antibody (Novagen) was prepared as a 1 in 2000 dilution (2.5 µL in 5 mL 3% BSA). The PVDF transfer membrane was placed in a fresh plastic dish with the antibody dilution and incubated on a tilting platform for 1 h. The PVDF transfer membrane was then washed twice in TBS-Tween/Triton and once in TBS as before. Anti-Murine IgG whole molecule-alkaline phosphatase (Invitrogen) was also prepared as a 1 in 2000 dilution (2.5 µL in 5 mL 3% BSA) and incubated with the transfer membrane as before.

A *FAST*™ BCIP/NBT alkaline phosphatase substrate tablet (Sigma) was defrosted 20 min prior to the end of the incubation. The PVDF transfer membrane was washed in 50 ml TBS-Tween/Triton 4 times for 10 min on a rotating platform, each with fresh buffer. After the second wash, the BCIP/NBT tablet was re-suspended in 10 mL sterile ultra-pure water and vortex mixed to completely re-suspend the tablet. After the washing steps the membrane was carefully placed into a fresh plastic dish on a level surface. The dissolved BCIP/NBT development solution (10 mL) was poured evenly over the membrane and incubated at ambient temperature. The reaction was stopped by submerging both sides of the PVDF transfer membrane in deionised water when the expected bands became visible (up to 15 min).

2.4.7. Protein purification

2.4.7.1. Protein dialysis

Protein samples were dialysed using International dialysis membrane/visking tubing (Medicell), cut according to the sample volume. The tubing was sterilised by boiling in ultra-pure water for 15 min and then cooled in fresh deionised water for 10 min. One end of the tubing was sealed with a plastic clip and the sample was dispensed inside. The other end was secured with a clip and the tubing was checked for leaks. The sample was placed into 5 L of chilled ultra-pure water with a magnetic stirrer for gentle agitation and dialysed for 3 h at 4°C. The sample was then placed into fresh chilled ultra-pure water (with a magnetic stirrer) for a further 3 h at 4°C. After a final change of water, the dialysis was carried out for further 8-12 h at 4°C. The samples were carefully removed from the tubing and incubated at 37°C for 1 h to dissolve any precipitates.

2.4.8. Protein concentration

Dialysed proteins were concentrated using a 10 K 0.5 mL ultra-centrifugal filter (Amicon). Five-hundred µL was loaded into the device, which was centrifuged at 14,000 x *g* for 3 min. The filter device was removed, placed inverted into a fresh 1.5 mL microtube and centrifuged again for 2 min at 1000 x *g*. The concentrated protein was stored at 4ºC.

2.5. Media and supplements

2.5.1. Cefoxitin cycoserine egg yolk media (CCEY)

Forty-eight g CCEY media (Bioconnections) was suspended in 1 L deionised water, allowed to soak for 10 min before thoroughly mixing and sterilising by autoclaving at 121°C for 15 min. The medium was cooled to 47°C and 2 vials of supplements (containing 250 mg cycloserine and 8 mg cefoxitin), 40 mL egg yolk emulsion and 10 mL laked horse blood were added. Plates of 25 mL were poured and cooled. For some experiments 5 mg/mL lysozyme was added to promote spore germination (CCEYL).

2.5.2. Brain Heart Infusion (BHI) broth with complements

Thirty-seven g BHI broth (Oxoid) was dissolved in 1 L deionised water. Complements (10 µL 0.5% v/v vitamin K, 5 mg/L hemin, 1mg/L resazurin and 0.5g/L L-cysteine) were added and the medium was autoclaved at 121°C for 15 min and cooled ready for use. Hemin stock was prepared by dissolution of 100 mg hemin and 0.28 g potassium hydroxide in 25 mL ethanol (96%) followed by the addition of ultra-pure water to 200 mL and autoclaving at 121°C for 15 min.

2.5.3. Reinforced Clostridial Media (RCM)

Thirty-eight g RCM (Oxoid) was dissolved in 1 L deionised water and autoclaved at 121°C for 15 min. The media was then cooled ready for use.

2.5.4. Agar plates

To make plates, medium was prepared as described, with 15 g agar base (Oxoid). Plates of 25 mL were poured at 50°C.

2.5.5. Soft agar

BHI, RCM or L broth were made as previously described but with agar base (Oxoid) or agarose (Melford Laboratories) at a concentration of between 0.6 and 0.7%. Medium was mixed and autoclaved at 121°C for 15 min and used once cooled to 50°C.

2.5.6. Rogosa agar

Eighty-two g Rogosa medium (Oxoid) was disolved in 1 L deionised water and autoclaved at 121°C for 15 min. Plates of 25 mL were poured at 50°C.

2.5.7. Beeren's agar

Fifteen point six g Columbia base agar (Oxoid), 2 g glucose (Sigma), 0.2 g cysteine HCl (BDH Prolabo), 2 g agar base (Oxoid), 2 mL propionic acid (Sigma) and 16 mL 1 M NaOH (Sigma) were added to 400 mL deionised water and autoclaved at 121°C for 15 min. Plates of 25 mL were poured at 50°C.

2.5.8. Bacteroides agar

Eleven point two g Brucella broth (BD) and 6 g agar base (Oxoid) were dissolved in 400 mL deionised water and autoclaved at 121°C for 15 min. The medium was cooled to 50°C and 4 mL 0.5 mg/mL hemin solution (Sigma), 80 µL 5% v/v vitamin K solution (Sigma), 1.2 mL 25 mg/mL kanamycin, 3 mL 1 mg/mL vancomycin and 20 mL laked horse blood (Oxoid) were added. Plates of 25 mL were poured.

2.5.9. MacConkey agar

Fifty-two g MacConkey agar (Oxoid) was dissolved in 1 L deionised water and autoclaved at 121°C for 15 min. Plates of 25 mL were poured at 50°C.

2.5.10. Wilkens-Chalgren agar

Forty-three g Wilkens Chalgren agar (Oxoid) was dissolved in 1 L deionised water and autoclaved at 121°C for 15 min. Plates of 25 mL were poured at 50°C.

2.5.11. Nutrient agar

Thirteen g Nutrient broth (Oxoid) and 15 g agar base (Oxoid) were dissolved in 1 L deionised water and autoclaved at 121°C for 15 min. Plates of 25 mL were poured at 50°C.

2.5.12. Clostridia agar

Seventeen point two g Wilkens Chalgren agar (Oxoid) was dissolved in 400 mL deionised water and autoclaved at 121°C for 15 min. The media was cooled to 50°C and 3.2 mL 1 mg/mL novobiocin and 3.2 mL 1 mg/mL colistin were added. Plates of 25 mL were poured.

2.5.13. Slanetz Bartley agar

Sixteen point eight g Slanetz-Bartley agar (Oxoid) was dissolved in 400 mL deionised water, autoclaved at 121°C for 15 min and cooled to 50°C. Plates of 25 mL were poured.

2.5.14. Batch fermentation media

Batch fermentation medium was made according to previous published experiments (Mandalari *et al.*, 2007) as shown in Table 2.9.

Component	g/L
Peptone water (Oxoid)	2
Yeast extract (Oxoid)	2
NaCl (Sigma)	0.1
K_2HPO_4 (Fisher Scientific)	0.04
KH_2PO_4 (Fisher Scientific)	0.04
$MgSO_4.7H_2O$ (Riedel-de-Haën)	0.01
$CaCl_2.2H_2O$ (Riedel-de-Haën)	0.01
$NaHCO_3$ (Fisher Scientific)	2
Tween 80 (Sigma)	2mL
Vitamin K 5% v/v aqueous solution (Sigma)	10uL
Cysteine.HCl (BDH Prolabo)	0.5
Bile salts (Oxoid)	0.5
Hemin (Sigma) dissolved in 300 µL 1M NaOH (Sigma)	0.02

Table 2.8. Composition of the batch fermentation media

The components were dissolved in 1 L deionised water and autoclaved at 121°C for 15 minutes.

2.5.15. Colon model media

The medium composition for the colon model system was made as described in previous studies of CDI (Baines *et al.*, 2005) and is shown in Table 2.10.

Component	g/L
Starch	3
Pectin (Sigma)	2
Arabinogalactan (Sigma)	1
Peptone water (Oxoid)	2
Bile salts (Oxoid)	0.5
Yeast extract (Oxoid)	2
NaCl (Sigma)	0.1
KH_2PO_4 (Fisher Scientific)	0.04
$MgSO_4.7H_2O$ (Riedel-de- Haën)	0.01
$CaCl_2.6H_2O$ (Riedel-de- Haën)	0.01
$NaHCO_3$ (Fisher Scientific)	2
Cysteine HCL (BDH Prolabo)	0.5
Hemin (Sigma) dissolved in 300 µL 1M NaOH (Sigma)	0.005
Tween 80 (Sigma)	2 (mL)
K_2HPO_4 (Fisher Scientific)	0.04
Vitamin K 5% v/v aqueous solution (Sigma)	10 (µL)

Table 2.9. Composition of the colon model media

The components were dissolved in 1 L deionised water and autoclaved at 121°C for 15 minutes. When cooled to 50°C 0.4 g glucose (Sigma) was added to the medium.

2.5.16. LB broth

Ten g tryptone (BD), 5 g yeast extract (Oxoid) and 10 g NaCl (Sigma) were dissolved in 1 L deionised water, adjusted to pH 7 and autoclaved at 121°C for 15 min.

2.5.17. L broth

Ten g tryptone (BD), 5 g yeast extract (Oxoid) and 5 g NaCl (Sigma) were dissolved in 1 L deionised water and autoclaved at 121°C for 15 min. When cooled to 50°C, 1 g glucose was added.

2.5.18. Blood agar

Forty g blood agar base (Oxoid) were dissolved in 1 L deionised water and autoclaved at 121°C for 15 min. When cooled to 50°C, 5% oxalated horse blood was added and mixed. Plates of 25 mL were poured.

2.5.19. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)

Forty mg was dissolved in 1 mL dimethyl formamide, wrapped in foil and stored at -20°C. For blue-white selection, 40 µL was spread onto L agar and dried 40 min prior to inoculation with transformation reactions.

2.5.20. DNase

In a sterile glass bottle, 100 µL of 1 M Tris, pH 7.5 was added, followed by 30 µL of 5 M NaCl. This was made up to 20 mL with sterile ultrapure water to make the nuclease buffer. Ten milligrams of DNase I (Sigma) was weighed into a sterile screw-top vial and 1 mL of the nuclease buffer was added. This was allowed to dissolve completely, divided into aliquots of 200 µL and stored at -20ºC.

2.5.21. RNase

RNase A (Sigma) was made as described in 2.5.20 (10 mg dissolved in 1ml of nuclease buffer), but the solution was boiled for 15 minutes prior to storage at -20°C.

2.5.22. Super optimal with catabolite repression (SOC) broth

Twenty g tryptone (BD), 5 g yeast extract (Oxoid) and 0.5 g NaCl (Sigma) were dissolved in 1 L deionised water and autoclaved for 15 min at 121°C. When cooled, 10 mL autoclaved 1 M $MgCl_2$ (Fisher Scientific), 10 mL autoclaved 250 mM KCl (Sigma) and 27.8 mL filter sterilised 2 M glucose (Sigma) was added.

2.5.23. Phosphate buffered saline (PBS) buffer

KCl (0.2 g), 0.2 g KH_2PO_4, 8 g NaCl and 1.15 g Na_2HPO_4 were dissolved in 1 L deionised water and adjusted to pH 7.3. Aliquots were autoclaved for 10 min at 115°C.

2.5.24. Antibiotic stocks

2.5.24.1. Ampicillin (50 mg/mL stock)

Ampicillin (0.05 g) (BioGene Ltd) was dissolved in 1 mL deionised water, filter sterilised and stored at -20°C.

2.5.24.2. Clindamycin (9.4 mg doses)

Clindamycin (0.0094 g) (Melford Laboratories Ltd) was dissolved in 500 µL sterile, ultra-pure water and used immediately.

2.5.24.3. Colistin (1 mg/mL stock)

Colistin (0.2 g) (Duchefa Biochemie Ltd) was dissolved in 20 mL deionised water, filter sterilised and stored at -20°C.

2.5.24.4. Kanamycin (25 mg/mL stock)

Kanamycin (0.5 g) (Sigma) was dissolved in 20 mL deionised water, filter sterilized and stored at -20°C.

2.5.24.5. Metronidazole (150 mg doses)

Metronidazole (0.015 g) (Duchefa Biochemie Ltd) was dissolved in 500 µL sterile, ultra-pure water and used immediately.

2.5.24.6. Novobiocin (1 mg/mL stock)

Novobiocin (0.02 g) (Sigma) was dissolved in 20 mL deionised water, filter sterilised and stored at -20°C.

2.5.24.7. Rifampicin (25 mg/mL stock)

Rifampicin (0.5 g) (Duchefa Biochemie Ltd) was dissolved in 20 mL DMSO, wrapped in foil and stored at -20°C.

2.5.24.8. Vancomycin (1 mg/mL stock)

Vancomycin (0.02 g) (Duchefa Biochemie Ltd) was dissolved in 20 mL deionised water, filter sterilized and stored at -20°C.

2.5.25. T2 buffer

Seven point five g K_2SO_4, 6 g NaCl, 2.25 g KH_2PO_4 and 4.5 g Na_2HPO_4 were dissolved in 1 L of deionised water. A further 1.5 L deionised water was added, mixed and the solution was dispensed into 100 mL aliquots. The aliquots were autoclaved at 121°C for 15 min and cooled. One hundred µL 1 M $MgSO_4$, 1 mL 0.1% gelatine and 1 mL 0.01 M $CaCl_2$ were added to each aliquot.

2.5.26. Tris EDTA (TE)

A solution of 10 mM Tris and 1 mM EDTA pH 8 was made in deionised water. The buffer pH was adjusted depending on requirments, autoclaved and stored at ambient temperature.

2.5.27. Sodium Tris EDTA (STE)

A solution of 0.1 M NaCl, 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8) was made in deionised water, autoclaved and stored at ambient temperature.

2.5.28. Suspension media (SM)

A solution of 50 mM Tris, 0.1 M NaCl, 8 mM $MgSO_4$ and 0.01% gelatin was made in deionised water. The pH was adjusted to 7.5 before autoclaving at 121°C for 15 min and cooling prior to use.

2.5.29. Tris buffered saline (TBS)

A solution of 10 mM Tris and 150 mM NaCl was made in deionised water. The pH was adjusted to 7.5 before autoclaving at 121°C for 15 min and cooling prior to use.

2.5.30. Tris buffered saline with Tween ± Triton (TBS/Tween/Triton)

A solution of 20 mM Tris and 500 mM NaCl was made in deionised water. Tween 20 (Sigma) at 0.05% v/v was added and when required, 0.05% v/v Triton was also included. The solution was autoclaved at 121°C for 15 min and cooled prior to use.

2.5.31. De Man Rogosa Sharpe (MRS) media

Sixty-two g MRS media (Oxoid) was dissolved in 1 L deionised water, brought to the boil, autoclaved at 121˚C for 15 min and cooled prior to use.

Chapter 3 - Bacteriophages of *C. difficile*

3.1. Abstract

C. difficile genomes contain a high frequency of prophages and phage elements. Prophages can be induced using agents such as mitomycin C and norfloxacin, with different chemicals sometimes giving rise to the induction of different phages. All *C. difficile* phages characterised to date contain elements that indicate the potential to lysogenise with their host and so far no successful attempts at disabling this capacity have been reported. This chapter describes the range of *C. difficile* phages that have been characterised and documents efforts to mutate ΦCD27 (a temperate phage induced from *C. difficile* National Collection of Type Cultures 12727) using ethylmethane sulphonate, hydroxylamine and sodium pyrophosphate in order to hinder or eliminate its ability to lysogenise.

3.2. Introduction

The earliest report of a bacteriophage active against a species of *Clostridium* was in 1934 by Cowles, who isolated a phage of *C. tetani* from sewage. Since then, a variety of both exclusively lytic and lysogenic phages of this genus have been described (Dolman and Chang, 1972; Grant and Riemann, 1976; Nieves *et al.*, 1981; Roseman and Richardson, 1969; Sebald and Popovitch, 1967; Shimamura *et al.*, 1974). Some clostridial phages have been shown to cross react with different species, such as the *C. sordellii* phages P50, PL73, PL78 and PL81 (Schallehn, 1985), for which activity against *C. difficile* (strain 2) was also demonstrated.

3.2.1. Phages of *C. difficile*

With regards to *C. difficile,* a limited number of phages have been described and characterised to date, despite the high frequency of prophage carriage (Goh *et al.*, 2007; Horgan *et al.*, 2010; Sebaihia *et al.*, 2006; Shan *et al.*, 2012; Stabler *et al.*, 2009). All of the *C. difficile* phages discovered so far have demonstrated lysogenic capacity and or contain typical elements associated with lysogeny control, including repressors, anti-repressors and integrases (Govind *et al.*, 2006; Mahony *et al.*, 1985; Nagy and Foldes 1991; Sell *et al.*, 1983; Shan *et al.*, 2012). Goh *et al* (2005) carried out the first molecular characterisation of 4 temperate *C. difficile* phages, 3 of which were from the *Myoviridae* family (ΦC2, ΦC5 and ΦC8) and 1 *Siphoviridae* (ΦC6), obtained via mitomycin C induction of 56 clinical isolates of *C. difficile*. All 3 of the *Myoviridae* phages produced clear plaques when tested with sensitive *C. difficile* hosts. In 2007, Fortier and Moineau reported another 3 phages of the *Myoviridae* family, one of which was induced from the hypervirulent NAP1/027 strain, and a further 5 Siphoviruses from other strains. Both Nagy and Foldes (1991) and Fortier and Moineau (2007) also observed numerous tail-like proteins in induced concentrated supernatants of *C. difficile* cultures when examined by TEM, even in the absence of intact phage particles. This may indicate a high incidence of phage elements in *C. difficile* genomes rather than complete prophages. The first *C. difficile* phage of the *Siphoviridae* family to be sequenced was by Horgan *et al.* (2010), and revealed many distinctions

relative to the genomes of *Myoviridae* phages (Goh *et al*., 2007; Govind *et al*., 2006; Mayer *et al.,* 2008).

More recently a novel molecular approach has been taken for prophage screening in *C. difficile* (Shan *et al.*, 2012). Using degenerate primers targeting the phage holin gene, 15 of 16 *C. difficile* strains were found to harbour a Myovirus prophage, 12 of which were confirmed by TEM analysis following prophage induction of the host. Two of these strains also contained a Siphovirus, both of which were confirmed by subsequent TEM analysis. Interestingly, both norfloxacin and mitomcycin C were used as inducing agents and the high frequency of recovery suggests that the use of multiple agents increases the chances of phage recovery by prophage induction in *C. difficile*.

All of the *C. difficile* phages that have been fully characterised so far have been propagated in a sensitive host following a considerable screening effort to identify susceptible *C. difficile* strains. The scarcity of hosts and the apparent narrow range of phage activity has somewhat limited the pace of study, but by applying molecular screening techniques and sequencing technology that can utilise small amounts of target nucleic acid, we can begin to explore those that are as yet uncharacterised and correlate this data with the phenotypic and virulence traits of the host.

Much of the interest in *C. difficile* phages has been concerned with phage encoded virulence factors since prophages, like all mobile genetic elements, help to drive the evolution of pathogens like *C. difficile.* The acquisition of phage elements can lead to the emergence of hypervirulent strains and threaten the efficacy of therapies designed to suppress or eliminate it. Identical prophages from inductions have been described in a range of hypervirulent NAP1/027 isolates (Fortier and Moineau, 2007), which were significantly different from other non-epidemic strains tested. This suggests that the carriage of certain prophages may be used to predict the clinical severity or pathogenicity of *C. difficile* strains (Shan *et al.*, 2012) and that phage-encoded virulence factors are associated with different phenotypes,

despite failure to identify known virulence determinants in any of the *C. difficile* phage genomes.

In the literature so far, there are no reports of exclusively lytic *C. difficile* phages despite significant efforts to isolate them (Goh *et al.*, 2005; Shan *et al.*, 2012). The apparent absence of phages with a solely lytic lifestyle could be due to the high incidence of prophages in *C. difficile* genomes, which may impart resistance to further infection refered to as phage immunity (Kutter and Sulakvelidze, 2005). Also, a lysogenic relationship is likely to be favoured when the host exists for long periods of time as spores, which are not amenable to phage infection, as is the case with *C. difficile*.

3.2.1.1. ΦCD27

Phage ΦCD27 was isolated by Mayer *et al.* (2008), after prophage induction of *C. difficile* NCTC 12727 with mitomycin C. It has a long straight tail and an icosahedral capsid (Fig. 3.1**)**, consistent with other viruses of the *Myoviridae* family (King *et al.*, 2012; Mayer *et al.*, 2008). Four sensitive hosts (out of 38 tested) were identified – NCTC 11204, NCTC 11205, NCTC 11207 and NCTC 11209.

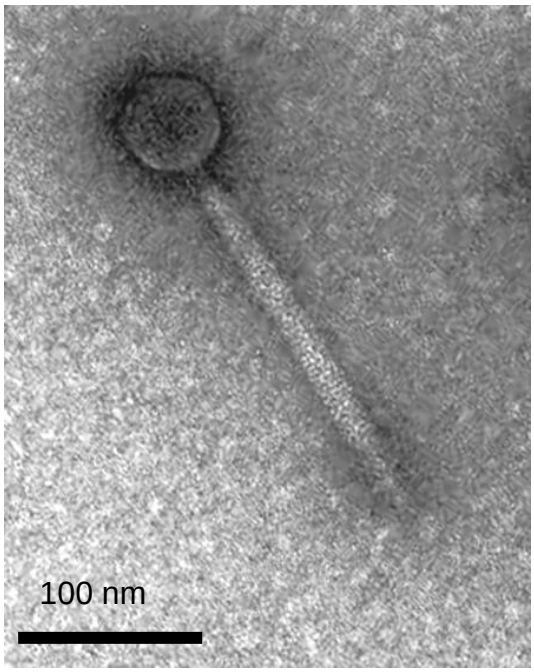


Figure 3.1 TEM image of ΦCD27 (Mayer *et al*., 2008).

3.2.2. Manipulation of phage characteristics

Some researchers have described mutagenic techniques that have successfully disabled the lysogenic capacity of phages. Ethylmethane sulphonate (EMS) is an alkylating agent that produces point mutations by nucleotide substitution (Masker *et al.*, 1985). The ethyl group of the compound interacts with guanine nucleotides to form 0^6-methylguanine, to which polymerases will pair a thymidine rather than a cytosine (Coulondre and Miller, 1977). Mutants have previously been described following treatment with EMS, including *Clostridium spp*. (Bowring and Morris, 1985). The resulting phenotypes were highly varied across a range of bacterial species, (van der Vijver *et al.*, 1975; Zablotny and Fraenkel, 1967).

Hydroxylamine is another mutagenic agent that acts in a similar manner, altering cytosine nucleotides to uracil and resulting in guanine-cytosine residues becoming adenine-thymine residues (Levisohn, 1970). Mutations of phage using hydroxylamine have been described and include the reversion of T4 rII mutants (Levisohn, 1970).

Sodium pyrophosphate (NaPP) is another agent which, despite extensive use in food products and no apparent mutagenic activity (Kim *et al.*, 2010), has proved effective at producing deletions in phage genomes. Mutant phages of *Bacillus subtillis*, defective in their ability to lysogenise with their host, were identified as clear-plaque phenotypes following treatment with NaPP (Kroyer and Dean, 1979). Similarly, lytic derivatives of temperate phages of *S. aureus* and *Lactobacillus spp*. were isolated following sequential exposures to 100 mM NaPP (García *et al.*, 2007, 2009; Ladero *et al.*, 1998).

Mutations that give rise to changes in the bacteriophage components that confer specificity, such as the endolysin (Mayer *et al.*, 2011) and tail proteins (Scholl *et al.*, 2002) are known to alter host specificity. Phages that infect multiple strains, especially common clinical isolates, have greater therapeutic potential and this factor may represent a particular problem for *C. difficile* since all the phages described to date exhibit narrow host ranges (Goh *et al.*, 2007; Goh *et al.*, 2005; Horgan *et al.*, 2010; Mahony *et al.*, 1985; Mayer *et al.*, 2008; Shan *et al.*, 2012). Mutagenesis using hydroxylamine has previously

been successful at altering the host ranges of *E. coli* phages (Scholl *et al.*, 2001).

3.3. Objectives

- To demonstrate the activity of wild type ΦCD27 vs germinating spores of NCTC 11204 using time lapse microscopy
- To mutate ΦCD27 using hydroxlyamine, EMS and NaPP, and to generate phenotypes defective in lysogeny.
- To mutate ΦCD27 using hydroxlyamine, EMS and NaPP, and to generate phages with increased host range specificity.

3.4. Materials and methods

3.4.1. The potential of ΦCD27 to prevent *C. difficile* spore outgrowth

The lytic activity of ΦCD27 can be observed by spot and overlay plaque assays (2.2.2). In this section, experiments were also set up to observe this action on germinating spores using time lapse microscopy as previously described for *Clostridium botulinum* (Stringer *et al.*, 2005). Spores of *C. difficile* were prepared and purified by density separation using urografin (2.1.1.3). Twenty µL was placed onto a defined area of a Superfrost Plus electrostatically charged microscope slide and incubated at 2-8˚C for 30 min. Slides were then immersed in sterile distilled water to remove any unbound spores, air-dried and transferred to the anaerobic cabinet for at least 16 h before use. At the beginning of each experiment, 0.6% soft agar in 0.45 µm filtered BHI+C broth was melted and cooled to 45˚C. The soft agar was then mixed with ~ 1 x 10^6 PFU/mL ΦCD27 in BHI+C or blank BHI+C in the anaerobic cabinet. A coverslip was placed over the agar and was sealed with aluminium tape to prevent drying and to make the slide impermeable to oxygen. The slides were then transferred to an XZY stage (Marzhauser, Wetzlar-Steindorf, Germany) with a PE60 temperature controller held at 37°C (Linkham Scientific Instruments, UK) linked to a Leica DMRB optical microscope with a stage mounted peltier device (Linkham). Fields were observed by phase contrast microscopy using a Leica X40/0.70 numerical

aperture and a PL FLUOTAR objective. Stills were captured every 5 min using Image Pro Plus image analysis software (Media Cybernetics, USA) and a JVC KY-F70 coupled device digital camera. Images for each field were compiled to produce an animated sequence. The experiments were carried out 3 times, each with a control slide to verify the viability of the spores.

3.4.2. Mutagenesis

3.4.2.1. Ethylmethane sulphonate (EMS)

The method was adapted from procedures previously described (Buxton, 1976). Thirty µL EMS solution (1 M, Sigma), was added to 2 mL NCTC 12727 (harbouring ΦCD27) in the exponential phase of growth. The mixture was incubated at 37˚C on a rotating platform for 2 h. The cells were harvested by centrifugation at 1500 x *g* for 10 min and washed twice with 10 mL pre-reduced BHI+C before re-suspending in 2 mL pre-reduced BHI+C. The 2 mL of washed cells were added to 50 mL pre-reduced BHI + C, mixed gently and incubated for 3 h or until turbid. The culture was induced with mitomycin C as previously described (2.2.1) and the supernatant was serially diluted before testing in overlay plaque assays with NCTC 11204. Plaque assays with the wild-type ΦCD27 were carried out in parallel for comparison of plaque phenotype.

3.4.2.2. Hydroxylamine

The method used was obtained from The John Roth Laboratory Protocols (http://rothlab.ucdavis.edu/) with some modifications. Concentrated phage preparations (by PEG precipitation – 2.2.4) containing greater than 10^7 PFU/mL re-suspended in suspension medium (SM) (2.5.28) were used for the mutagenesis. One mL of the concentrated bacteriophage suspension was combined with 2 mL phosphate buffer (0.45 M potassium phosphate and 5 mM ethylene glycol tetraacetic acid (Sigma) adjusted to pH 5.5 with hydrochloric acid (Riedel-de Haën) and 1 mL of the hydroxylamine solution (0.35 g hydroxylamine hydrochloride (Sigma), 4.44 mL sterile water and 0.56 mL of 4 M sodium hydroxide (Sigma)). Every 8 h, phage titres were determined by removing a 500 µL portion, centrifuging at 11,000 x *g* for 150

min, re-suspending in 500 µL SM buffer and allowing to re-suspend for a further 8 h at room temperature before making serial dilutions and performing overlay plaque assays with NCTC 11204. Enumerations were repeated approximately every 8 h to determine the point at which 1-1.5% of the original titre remain. Mutated phage from the appropriate time point, re-suspended in SM buffer, was added to an exponentially growing culture of NCTC 11204. After 8 h, the cultures were clarified and the supernatant was passed through a 0.45 µm filter. The filtrate was used to carry out overlay plaque assays with NCTC 11204 to check for the presence of clear-plaque phenotypes. Plaque assays with the wild-type ΦCD27 were carried out in parallel for comparison.

3.4.2.3. Sodium pyrophosphate (NaPP)

This method was carried as described previously (Ladero *et al.*, 1998) with some modifications suggested by Dr. Pilar Garcia, Instituto de Productos Lácteos de Asturias (personal communication). NaPP (0.2 M) was made in Tris-HCl, pH 8. *C. difficile* NCTC 11204 was propagated in RCM broth and fresh inductions of ΦCD27 were made from NCTC 12727 grown in RCM broth. For these experiments, anaerobic jars were used for the incubations. The ΦCD27 preparation was enumerated and diluted to achieve a concentration of approximately 1 x 10^5 PFU/mL. ΦCD27 was exposed to 0, 10, 25, 50, 75, 100 or 200 mM NaPP for 40 min. Concentrations beyond 200 mM were not used due to poor solubility. A variety of different incubation temperatures were used, ranging from 37°C-51°C. Buffer-only controls were carried out with every test to ensure that any reduction in the PFU value was due to the NaPP and treatments that gave rise to 5-10% ΦCD27 survival were selected. Eight rounds of exposure were carried out, recovering phage by plate harvests (2.2.3.1) and enumerating the PFU value after each one. Plaque assays with the wild-type ΦCD27 were carried out in parallel for comparison.

3.4.2.4. Identification of clear-plaque phenotypes

Plaque assays of ΦCD27 were carried out following each round of mutagenesis with hydroxylamine, EMS and NaPP. Plates were incubated for

5 days and scrutinised using a 10 x magnification lens. Only plaques with absence of turbidity were followed up.

3.4.2.5. Verification of disabled lysogenic capacity

Clear plaques were excised using a glass Pasteur pipette and incubated in 1 mL BHI with complements broth for 1 h at ambient temperature with occasional gentle mixing. The broth was passed through a 0.45 µM syringe filter and the entire filtrate was added to the centre of an agar plate seeded with *C. difficile*. Material from within the zone of lysis was subcultured onto BHI agar prior to culture in BHI plus complements broth and induction with mitomycin C (2.2.1). Supernatants were tested for the presence of induced phage by spot plaque assays (2.2.2.2).

3.4.2.6. Verification that mutagen exposure gave rise to mutated genotypes

Genomic DNA extracted from wild-type ΦCD27 and ΦCD27 post NaPP exposure (1.5 µg) was subjected to restriction digestion using *NdeI* and *HindIII* (NEB) using the protocol described in 2.3.8. The predicted fragment sizes based on the ΦCD27 genome are indicated in Table 3.1. Digestion profiles were visualised on a 0.8% agarose gel (65 V for 6.5 h) stained with ethidium bromide.

Restriction enzyme	Number of sites	Fragment sizes (bp)
NdeI	12	8049, 4060, 5027, 1262, 40, 6345, 1238, 6246, 1451, 1347, 9078, 6787.
HindIII	46	755, 438, 65, 607, 992, 251, 307, 601, 33, 243, 472, 91, 137, 249, 24, 113, 346, 24, 507, 735, 1491, 162, 252, 138, 1276, 84, 3470, 946, 210, 54, 4315, 1803, 178, 362, 7520, 5419, 214, 222, 349, 11, 502, 5689, 639, 5057, 1762, 1815.

Table 3.1 Predicted restriction profiles of wild-type ΦCD27.

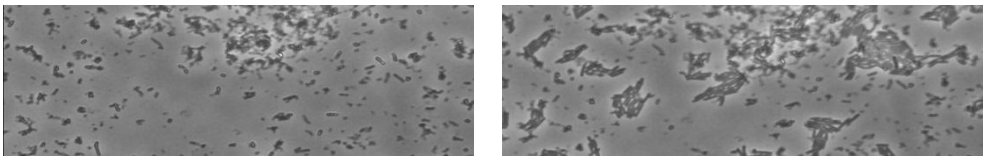
3.4.2.7. Screening for mutated ΦCD27 with an extended host range

Mutated phage recovered from plate harvests were also tested neat in spot plaque assays with a selection of *C. difficile* strains that were not susceptible to wild-type ΦCD27. Strains R23 613 and R23 737 (clinical strains kindly provided by Dr. Jonathan Brazier, Anaerobe Reference Unit, University Hospital of Wales, Cardiff), DSMZ 12056, NCTC 12730 and environmental isolates NNUH-4, NNUH-7 and PIG-ENV (4.5.2) were tested.

3.5. Results

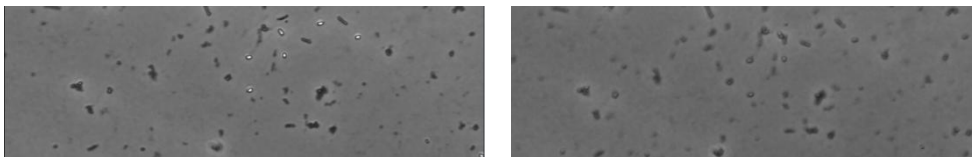
3.5.1. The potential of ΦCD27 to prevent *C. difficile* spore outgrowth

Time lapse microscopy experiments of *C. difficile* spores in the presence and absence of ΦCD27 showed that the phage was able to prevent outgrowth of germinating cells. Examples of the still images obtained are shown in Fig. 3.2 a-b but the animated sequence was able to show the un-coating of the spores just before the cells appeared to lyse.



Frame 1 (5 min) **Frame 200 (16.6 h)**

Figure 3.2 (a). Time lapse microscopy frames showing the germination of *C. difficile* NCTC 11204 spores over 16.6 h in the absence of ΦCD27 treatment.



Frame 1 (5 min) **Frame 200 (16.6 h)**

Figure 3.2 (b). Time lapse microscopy frames showing no outgrowth of *C. difficile* NCTC 11204 spores over 16.6 h when treated with ΦCD27 (~1 x 10^6 PFU).

3.5.2. Mutagenesis

Random mutagenesis techniques on *C. difficile* NCTC 12727 containing ΦCD27 as a prophage were employed in addition to random direct mutation of ΦCD27 in the absence of bacterial cells.

3.5.2.1. EMS

Induced ΦCD27 from NCTC 12727 mutated with EMS produced plaques of varying morphology. These included clear-plaque phenotypes and plaques that appeared larger than normal, which were evenly distributed and therefore unlikely to be due to variations in the agar thickness. All were followed up but reverted to the standard plaque phenotype upon secondary propagation in all cases. The capacity for lysogeny was demonstrated by culture of *C. difficile* from the centre of lysis zones and the induction of phage that gave plaques with NCTC 11204. No mutated ΦCD27 that were able to infect *C. difficile* R23 613, R23 737, DSMZ 12056, NCTC 12730, NNUH-4, NNUH-7 or PIG-ENV were recovered.

3.5.2.2. Hydroxylamine

The viability of ΦCD27 decreased rapidly in the presence of hydroxylamine relative to the buffer-only control. The exposure time that gave rise to 1-1.5% of the original titres was approximately 21-28 h. Hydroxylamine mutagenesis was carried out 3 times, during which numerous query clear plaque phenotypes were followed up. Material that was cultured from the centre of the zones was found to contain *C. difficile* with inducible ΦCD27 in all cases. No mutated ΦCD27 that were able to infect *C. difficile* R23 613, R23 737, DSMZ 12056, NCTC 12730, NNUH-4, NNUH-7 or PIG-ENV were recovered.

3.5.2.3. Sodium pyrophosphate

During the first round of mutagenesis, ΦCD27 recovery notably decreased after treatment with sodium pyrophosphate at concentrations of 100 mM and above. The recovery rates for 100 mM and 200 mM treatments were similar (6% and 8.5% respectively) and ΦCD27 treated with 100 mM was selected for the second round of mutagenesis. It was expected that recovery rates for a

given concentration would increase with each round of treatment, so 50 mM, 100 mM and 200 mM treatments were used for all 8 rounds and phage from the concentration that gave the most appropriate recovery rate was selected for the subsequent round (Table 3.2). The incubation temperature was also increased for some rounds and the incubation times were extended to up to 80 min (Table 3.2). Lysogenic *C. difficile* was isolated from the centre of lysis zones confirming that the ability to lysogenize had been retained. No mutated ΦCD27 infecting *C. difficile* R23 613, R23 737, DSMZ 12056, NCTC 12730, NNUH-4, NNUH-7 or PIG-ENV were recovered.

Round	Concentration of NaPP	Incubation temperature	Incubation time	Recovery of treated ΦCD27	Recovery of untreated ΦCD27
1	100 mM	37°C	40 min	6.00%	123.13%
2	200 mM	42°C	40 min	17.00%	79.91%
3	200 mM	48°C	40 min	3.30%	62.77%
4	100 mM	37°C	45 min	2.00%	96.53%
5	100 mM	37°C	30 min	2.02%	99.68%
6	100 mM	40°C	45 min	2.77%	82.63%
7	50 mM	40°C	60 min	3.80%	108.00%
8	100 mM	40°C	80 min	1.80%	98.00%

Table 3.2. ΦCD27 recovery over 8 rounds of sequential exposure to sodium pyrophosphate.

3.5.2.4. Verification that mutagen exposure gave rise to mutated genotypes

Differing restriction profiles were obtained for wild-type ΦCD27 and mutated ΦCD27 genomic DNA following digestion with *NdeI* and *HindII* (Fig. 3.3). For wild-type ΦCD27, many of the bands match predicted fragment sizes (detailed in Table 3.1) and many of these were not visualised in the mutated ΦCD27 DNA, hinting at multiple mutation sites (indicated in Fig. 3.4 a-b).

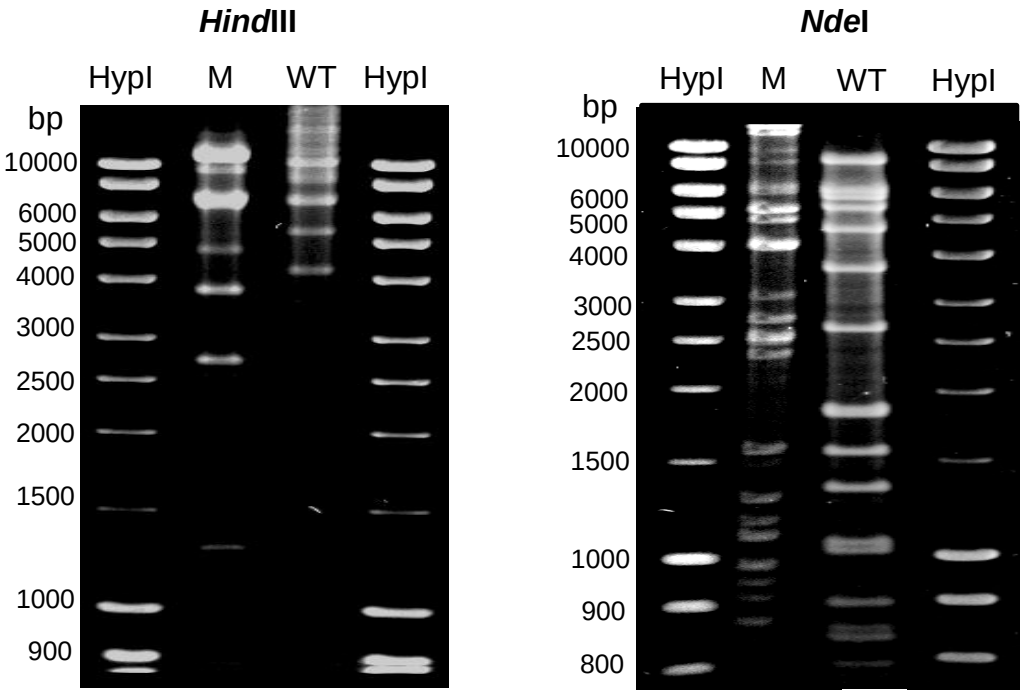


Figure 3.3. *Hind*III and *Nde*I restriction profiles for NaPP treated ΦCD27 (M) and untreated ΦCD27 (WT) with Hyperladder I (HypI).

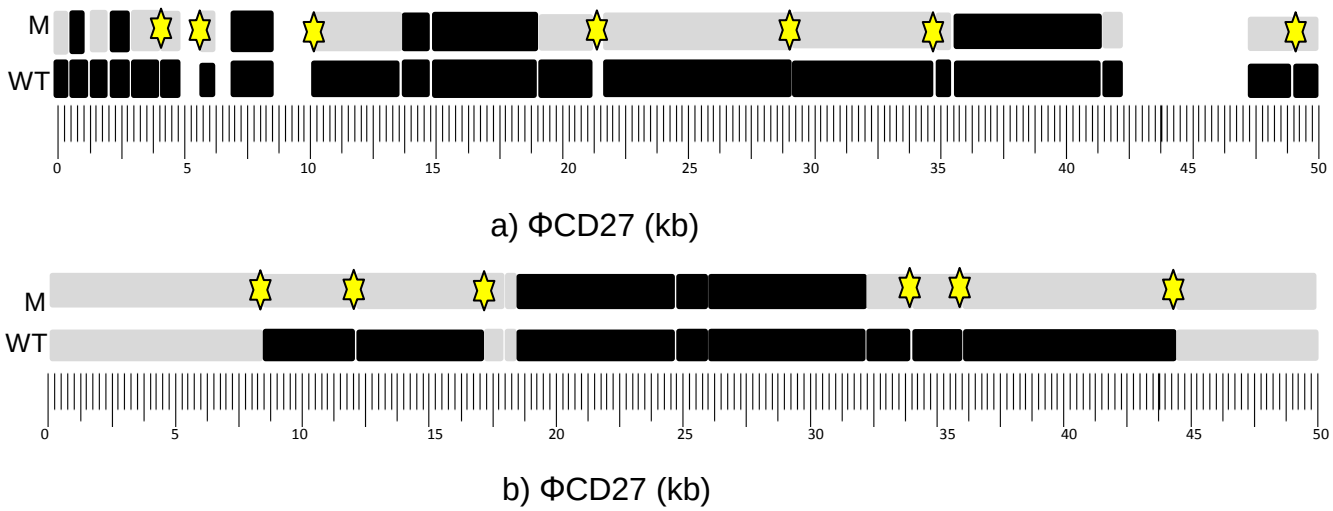


Figure 3.4 Diagrammatic representation of a) *Hind*III digests and b) *Nde*I digests of wild-type (WT) and NaPP treated (M) ΦCD27. Predicted fragments are in grey, with visualised predicted fragments in black. Potential sites of mutation are highlighted with a yellow star.

3.6. Discussion

To date, exclusively lytic phages of *C. difficile* have not been described, and possible issues and changes relating to the novel phage isolation are discussed in Chapter 4.

C. difficile spores represent a major challenge for healthcare providers – their abundance from patients suffering from CDI and their hardy nature require aggressive, continual cleaning to control their spread. In addition to use as a therapeutic agent, phages may also be useful as environmental decontaminants. The lytic activity of ΦCD27 on spores of NCTC 11204 was demonstrated by time lapse phase contrast microscopy and appeared to prevent the outgrowth of germinated cells in the 3 replicates tested. Although these spores were immobilised in agar and their behaviour may have been different in other situations, it can be suggested that phages in the environment may be a useful preventative agent against *C. difficile* outgrowth. Also, since the spores were observed to have germinated (rather than remained dormant) they would not be expected to be viable following this treatment. These observations may have useful applications for environmental decontamination in combination with agents that promote germination, such as sodium taurocholate (Paredes-Sabja *et al.*, 2008; Sorg and Sonenshein, 2008), but also suggests that phage use may be effective in the prevention of *C. difficile* spore outgrowth following ingestion.

Despite the lytic activity of ΦCD27 against NCTC 11204, lysogens were repeatedly isolated from the centre of lysis zones. This feature ultimately leads to phage resistance and *C. difficile* containing the ΦCD27 prophage quickly dominate susceptible strains. The mutagenic techniques applied to ΦCD27 did not yield any phages that were deficient in their capacity to lysogenise with their host. Despite the recovery of plaque phenotypes that appeared clear or larger than those previously obtained, secondary propagation gave rise to the standard phenotype and all of those investigated were able to form lysogens with NCTC 11204.

Similarly, the mutagenic techniques did not yield any extension of host range that permitted infection of DSMZ 12056, NCTC 12730, the clinical strains R23

613 and R23 737 or the environmental isolates NNUH-4, NNUH-7 and PIG-ENV. The decreases in ΦCD27 viability relative to the buffer-only controls, observed following treatment with hydroxylamine and sodium pyrophosphate were useful indicators of mutagenic action. The demonstration of differing restriction profiles of phages have been used by other researchers to confirm the presence of mutants (Ladero *et al.*, 1998; McLaughlin *et al.*, 1986), and clear differences were observed between wild-type and (sodium pyrophosphate) mutated ΦCD27 after *NdeI* and *HindIII* digestion, the extent of which suggests multiple point mutations following exposure to sodium pyrophosphate.

Lytic mutants were screened for by looking for clear-plaque phenotypes, which is an approach taken by many researchers for the detection of phages deficient in their lysogenic abilities (Garcia *et al.*, 2009; Kroyer and Dean, 1979; Ladero *et al.*, 1998; McLaughlin *et al.*, 1986). However, this approach may have caused some lytic mutants to escape detection since the emergence of phage-resistant *C. difficile*, via mechanisms other than lysogeny, could have caused turbidity within the plaques and masked the lytic phenotypes. PCR-based methods to identify disrupted genes of interest may offer an alternative screening strategy.

Due to licencing limitations it was not possible to take a directed mutagenesis approach, but this may have increased the likelihood of isolating suitable mutants. The regions associated with the establishment and maintenance of lysogeny have been well described. The immunity repressor, *cI*, causes suppression of genes associated with lytic growth when it binds to its promoter (Trun and Trempy, 2003). The pro-lytic protein, *Cro,* competes for the same promoter site. Other genes within the phage-encoded lysogeny 'cassette' include *cII*, the product of which is readily degraded by the bacterial protease HflA and acts as an indicator of host nutritional status, promoting the production of *cI* (and therefore lysogeny) in times of nutritional depletion. Both *cI* and *cII* mutants have been identified or produced and are devoid of the ability to lysogenise with their host (Pedersen *et al.*, 2010). A phage-encoded integrase enzyme is also required for the establishment of lysogeny (Trun and Trempy, 2003) and similarly, mutations within this gene give rise to

exclusively lytic phenotypes (Durmaz *et al.*, 2002). Other genetic elements that affect lysogeny have been described. Disruption of gene '*d*' of *Bacillus subtilis* by site-directed frameshift mutagenesis, gave rise to clear-plaque mutants. Interestingly, this gene also conferred host immunity when cloned into *B. subtilis* (McLaughlin *et al.*, 1986).

The successful disabling of lysogenic capacity, with extension of the host range to cover clinically relevant strains, would considerably increase the therapeutic potential of ΦCD27. Site-directed mutagenic techniques would permit minimal manipulation of the phage by affecting only key genes involved in lysogeny and or receptor binding, but practical use may still be limited by public resistance to genetically engineered viral particles.

Chapter 4 – In Search of Novel Phages

4.1. Abstract

Phages represent the richest and most diverse life form on earth and the majority of them are yet to be discovered and characterised. This chapter details the screening processes adopted and samples tested in an attempt to recover and identify new phages infecting *C. difficile.* No novel *C. difficile* phages were found but recovery methods were validated by isolation of phages infecting *E. coli* and a new phage infecting *C. sporogenes* was also discovered. Potential future modifications to *C. difficile* phage screening methods are discussed.

4.2. Introduction

Phages represent the most abundant life-form known to man and are particularly plentiful in soil, rivers, lakes and sewage (Kutter and Sulakvelidze, 2005). It would seem that only a fraction of bacteriophages that currently exist have been unearthed when the results of plaque assays are compared to the abundant phage-like particles that are seen using electron microscopy (Hendrix, 2002). This is a heartening thought for researchers and clinicians that advocate the use of phages for the prevention and remediation of bacterial infections, but does indicate that modifications to current isolation methods will be necessary to yield those that remain uncharacterised. Indeed, double-stranded DNA phages account for approximately 96% of phages that have been isolated (Serwer *et al.*, 2004), which may suggest that the current methods being used for phage isolation are not appropriate for the detection of RNA phages. In addition, plaque assays will fail to identify filamentous, non-lytic phages.

Many phages isolated and characterised to date have been obtained from environmental sources such as sewage effluent, sea water or clinical samples (Cantalupo *et al.*, 2011; Imamovic *et al.*, 2010; Morales *et al.*, 2012). Up to 10^7 phage particles per gram can be found in soil and the faeces of ruminants (Furuse *et al.*, 1978) and hundreds of different phage genotypes have been obtained from the human gut (Breitbart *et al.*, 2003). Enrichment techniques are often employed to amplify low numbers of phage prior to screening via spot plaque assays and this approach is recommended when searching for *C. difficile* phages due to the anticipated narrow host range (Nagy and Foldes, 1991).

Phages are often sensitive to extremes of pH, making them unsuitable for many of the reported virus isolation methods (Seeley and Primrose, 1982), but a pH neutral method for the recovery of mammalian viruses and phages from water samples using beef extract and ammonium sulphate to recover at least 83% of the original phage titre has been described (Shields and Farrah, 1986). Many other elution buffers have been used, including sodium chloride (Ishihara *et al.*, 2006), albumin (Ostle and Holt, 1979), glycine (Hurst and

Gerba, 1979) and beef extract (Guzman *et al.*, 2007; Hurst *et al.*, 1991; Williamson *et al.*, 2003).

Prophages can be induced from host bacterial strains (2.2.1), and thus provide another source of novel phages. All C*. difficile* phages characterised to date are derived from a host strain based on their lysogenic capacity (Fortier and Moineau, 2007; Goh *et al.*, 2005, 2007; Govind *et al.*, 2006; Horgan *et al.*, 2010; Mahony *et al.*, 1985; Mayer *et al.*, 2008; Nagy and Foldes, 1991; Nale *et al.*, 2012; Sell *et al.*, 1983; Shan *et al.*, 2012). Some cross-reactivity between Clostridial hosts has been observed. For example, phages of *C. sordellii* have previously been shown to infect *C. difficile* (Schallehn, 1985), and therefore efforts to find novel *C. difficile* phages should also include inductions from other closely related Clostridial species. Based on the phylogenetic relatedness of clostridial 16S rRNA (Collins *et al.*, 1994), this should also include *C. bifermentans* (Fig 4.1).

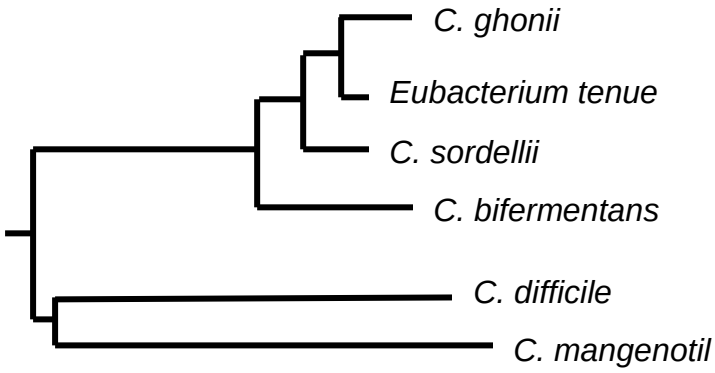


Figure 4.1. Dendrogram of the phylogenetic relatedness of clostridial species most similar to *C. difficile* based on 16S rRNA gene sequences (Collins *et al.*, 1994).

Despite the lysogenic capacity of these phages, induced prophages have been used successfully in experimental models to prevent and treat bacterial infections including *C. difficile* (Meader *et al.*, 2010; Ramesh *et al.*, 1999). The possibility of disabling their integrative ability as part of further development ensures them as worthy candidates for investigation.

4.3. Objectives

- To compare the efficacy of a range of buffers for the elution of phage from environmental samples.
- To isolate novel phages infecting *C. difficile* from a range of environmental samples.
- To isolate novel phages infecting *C. difficile* from cultures of *C. difficile, C. sporogenes* and *C. sordellii* induced with mitomycin C.

4.4. Materials and methods

4.4.1. Sample sources utilised for phage isolation

Environmental sources from which the isolation of *C. difficile* could be expected were chosen for phage screening. Faecal samples from humans and animals represented the majority of samples tested since phage yielded from this source would be more likely to survive the conditions of the gut and be suitable for therapeutic development. Table 4.1 shows the types and numbers of samples screened.

Sample type	Location	Number of samples
Crude strained sewage	Norfolk	1
Crude settled sewage	Norfolk	7
Digested anaerobic sewage	Norfolk	1
Untreated sewage (hospital waste)	Norfolk	6
Water from a duck pond	Norfolk	3
Water from a duck pond	Kent	1
Giraffe faeces	Norfolk	1 (pooled)
Zebra faeces	Norfolk	1 (pooled)
Antelope faeces	Norfolk	1 (pooled)
Sheep faeces	Norfolk	1 (pooled)
Cat faeces	Norfolk	1 (pooled)
Chicken cecum content	Yorkshire	5 (pooled)
Horse faeces	Norfolk	1
Pig faeces and ceca	Bristol	7 (pooled)
Soil	Norfolk	13
Lake water	Norfolk	9
Human faecal samples	Norfolk and Norwich University Hospital	42
Compost liquor	Norfolk	2

Table 4.1. Samples tested for the presence of *C. difficile* phages

4.4.2. Isolation of new *C. difficile* strains and sample enrichment

Solid environmental samples (approximately 20 g) were reconstituted using 200 mL pre-reduced BHI broth with complements, (protecting phage from osmotic shock), and warmed to 37°C for 10 min prior to use to disaggregate particulate matter. Liquid samples (up to 250 mL) were re-constituted with pre-reduced BHI with complements (3 parts sample, 1 part quadruple strength BHI with complements to achieve the correct final concentration). Vigorous vortex mixing was avoided at all stages of processing as this is known to significantly reduce phage viability (Kutter and Sulakvelidze, 2005). Similarly, samples were kept out of direct sunlight during processing to preserve viability as much as possible.

Untreated and alcohol shocked samples (2.1.1.3) were plated on pre-reduced CCEY agar to isolate *C. difficile* strains. In the latter stages of the environmental screening process, the CCEY agar was supplemented with 5 mg/L lysozyme as this has been shown to enhance spore germination and recovery of *C. difficile* (Wilcox *et al.*, 2000). Environmental samples were kept

in the anaerobic cabinet (Don Whitley Scientific) overnight to maintain anaerobic conditions and, where possible, separate aliquots were stored at 2-8˚C. The following day the environmental samples were enriched with any putative *C. difficile* strains yielded from that particular source. In addition, strains NCTC 11204, NCTC 11207 (derived from neonatal meconium), DSMZ 12056 (derived from the rumen of a lamb) and R23 613 (a clinical strain of ribotype 027 kindly donated by Jonathan Brazier, Anaerobe Reference Unit, University Hospital of Wales, Cardiff) were inoculated into each sample to be enriched. It has been documented that phages may not replicate if the population of host bacteria is below 10^3-10^5 cells per mL (Kutter and Sulakvelidze, 2005), and so environmental samples were inoculated with at least 10^6 CFU/mL *C. difficile* based on OD_{600} readings for NCTC 11204. It was estimated that an incubation period of 48 h should be sufficient to permit good growth of the host *C. difficile* strains, allow the phage to adsorb, infect and liberate the progeny (Kutter and Sulakvelidze, 2005).

An overview of the methods used in an attempt to isolate novel phages is summarised in Fig. 4.2.

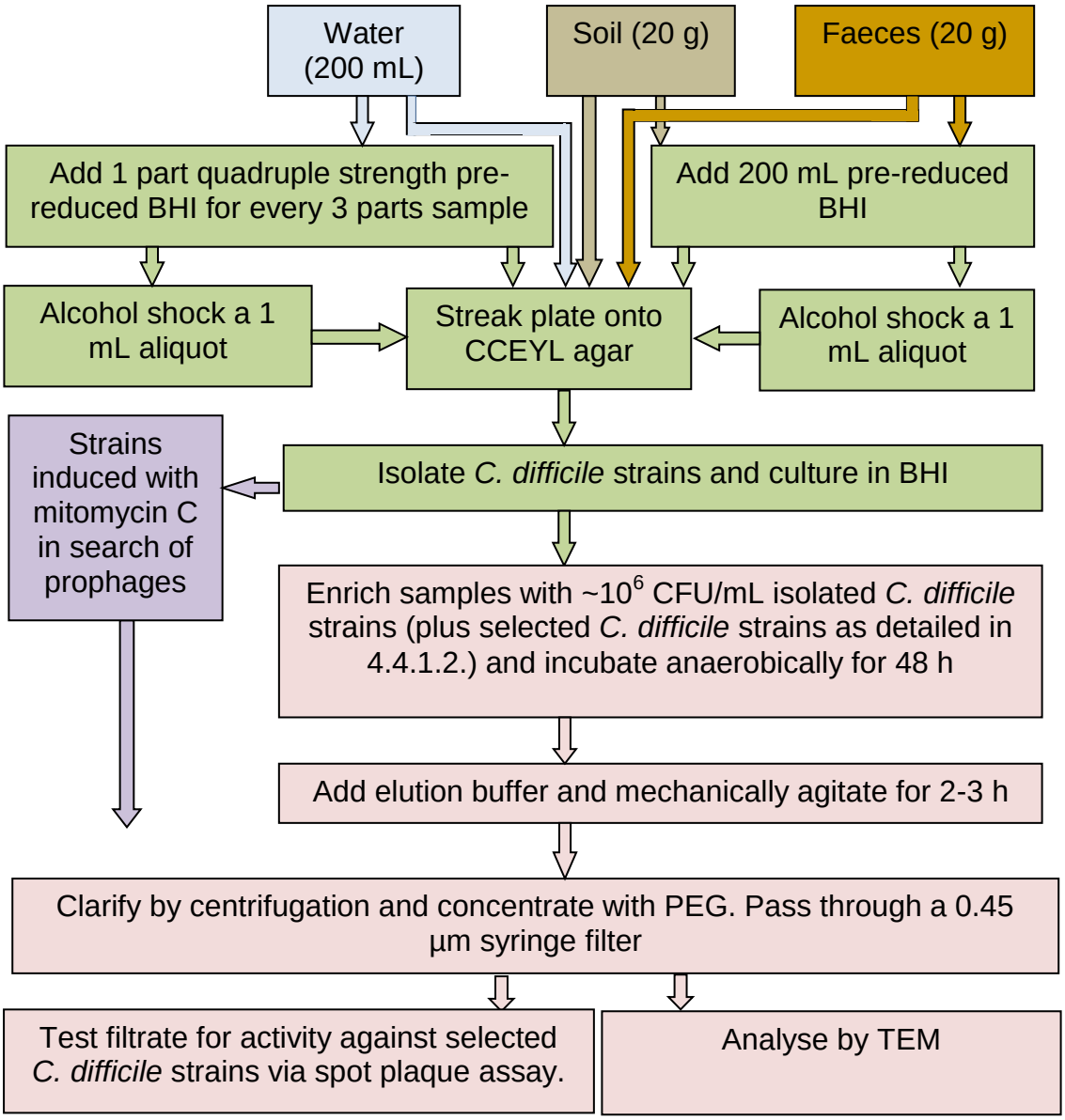


Figure 4.2. An overview of the methods used to isolate novel phages.

4.4.3. Enhancing phage adsorption

Following the inoculation of *C. difficile* enrichment strains, phage adsorption was enhanced by the addition of 10 mM calcium chloride and magnesium chloride to the environmental samples, as previously reported (Havelaar and Hogeboom, 1983).

4.4.4. Elution of phages

In order to determine the method that would elute the greatest proportion of phages from environmental samples, 12 g portions of soil were sterilised in

Duran bottles and allowed to cool before being spiked with 2 mL of a suspension containing 1.275 x 10^6 PFU/mL of ΦCD27. The samples were then stored at 4°C for 24 h, and then equilibrated to ambient temperature for 30 min. One hundred mL of each test elution buffer was added to the spiked soil samples, and the mixtures were left to agitate on a magnetic stirrer (medium speed) for 1 h 45 min at room temperature.

Phage elution buffers documented in previous work were selected for comparison (listed in Table 4.2). Concentrated buffers were added to give the required final concentration and stirred gently with a magnetic stirrer or on a rotating platform for 1 h 45 min.

The elution protocol for buffer 11 required sequential additions to bring about the formation of a floc (Shields and Farrah, 1986) rather than simple agitation and recovery. Beef extract (to give a final concentration of 2% w/v) was added followed by 2 volumes of saturated ammonium sulphate to give rise to a floc containing phage. Centrifugation was carried out to pellet the floc at 14,000 x *g* for 20 min (at 4˚C). The pellet was re-suspended in 20 mL sterile distilled water before PEG precipitation (2.2.4).

Elution buffer	Buffer composition	pH	Reference	Reported recovery rate
1	0.025 M glycine	2	Modified from Hurst & Gerba, 1979	66%
2	0.025 M glycine	7	This work	NA
3	0.025 M glycine	10	This work	NA
4	2% beef extract	7	Williamson *et al*., 2003*; Hurst *et al*., 1991 and Guzman *et al*., 2007	26%*
5	2% beef extract & 0.025 M glycine	7	This work based on the findings of Williamson *et al*., 2003	NA
6	Saline	ND	This work	NA
7	Sterile distilled water	ND	This work	NA
8	0.05 M magnesium chloride	ND	This work	N/A
9	0.05 M calcium chloride	ND	This work	NA
10	1% albumin	ND	Modified from Ostle and Holt, 1979	0.2%
11	10% beef extract & saturated ammonium sulphate	7	Shields and Farah, 1986	>83%
12	0.06 M ammonium acetate	ND	Modified from Ostle and Holt, 1979	0.2%
13	0.001 M sodium chloride	ND	Modified from Ishihara *et al*., 2006	NA
14	0.1 M sodium chloride	ND	Modified from Ishihara *et al*., 2006	NA
15	0.5 M sodium chloride	ND	Modified from Ishihara *et al*., 2006	NA
16	1 M sodium chloride	ND	Modified from Ishihara *et al*., 2006	NA
17	1.25 M sodium chloride	ND	Modified from Ishihara *et al.,* 2006	NA
18	2.5 M sodium chloride	ND	Modified from Ishihara *et al*., 2006	NA
19	BHI + complements	ND	This work	NA

Table 4.2. Elution buffers used to evaluate ΦCD27 recovery. Samples were plated in triplicate using overlay plaque assays. ND: not determined. NA: not applicable.

4.4.5. Recovery of eluted phages

The samples were clarified by centrifugation at 1500 x *g* for 5 min, concentrated by PEG precipitation (2.2.4) in 20 mL aliquots and filtered using a 0.45 µm syringe filter before being tested undiluted in spot plaque assays.

In the latter stages of the environmental screening period, samples were also tested directly following clarification in spot plaque assays. For phage recovery and survival experiments, serial dilutions of the filtered supernatants were made to 10^{-4} in BHI + C, and 100 µL aliquots of these were used for PFU enumeration in an overlay plaque assay.

In order to determine if there were any detrimental effects of PEG precipitation on phage viability, a 20 mL sample of ΦCD27 at a known titre (5 x 10^6 PFU/mL) was subjected to PEG precipitation (2.2.4) and then reconstituted to 20 mL in BHI + C broth. The viable titres of the original and PEG precipitated samples were determined by serial dilution and overlay plaque assays in triplicate.

4.4.6. Survival and recovery of phages.

In order to evaluate the likely survival rates of phages in sewage samples, 10 mL aliquots of crude strained sewage, crude settled sewage and anaerobic digested sewage were spiked with ΦCD27 and mixed gently but thoroughly. One of each sample type was kept at room temperature. Plaque assays (overlay method) were carried out at time 0, 3 days, 1 week, 10 days and 2 weeks post inoculation. These experiments were designed to ascertain an approximate maximum period of time that phages would remain viable during storage in the original sample.

The pH stability of ΦCD27 was determined by adjusting the pH of 10 SM aliquots to pH 1-10, adding 1 x 10^6 PFU ΦCD27 and incubating at room temperature for 5 h. Aliquots were then tested for viable phage by triplicate overlay plaque assay (2.2.2.1).

4.4.7. Validation of phage recovery methods

In order to verify that the methods employed resulted in the isolation of viable phage from the environmental samples, the levels of *E. coli* phage (expected in abundance) were quantified in parallel with the screening tests using *E. coli* K12 (kindly provided by D. Hatziioanou). Samples that yielded no or very few *E. coli* phages were re-tested if possible. Environmental samples were not enriched with *E. coli* K12.

4.4.8. Induction of *Clostridium* species to search for novel prophages

Strains of *C. difficile* from the culture collection and those isolated from environmental and clinical sources, were grown to mid-log phase and induced with mitomycin C (2.2.1). The resulting supernatants were concentrated by PEG precipitation, submitted for TEM analysis and tested for phage activity on lawns of *C. difficile* strains from the collection.

Laboratory strains of *C. sordellii* ATCC 9715 and NCTC 13356 were also induced and included in the screening as it was considered that phages induced from closely related strains may infect *C. difficile*. In separate experiments which focused on screening for phage endolysin activity against a range of *Clostridium spp,* a strain categorised as *C. sporogenes* in the laboratory culture collection (C22-10) was found to be sensitive. This prompted the screening of induced supernatants of other *C. sporogenes* strains in search of phages that may cross-infect *C. difficile.* These comprised of strains BL81-04, BL84-18, BL84-17 and BL02-01. It was subsequently found, following 16S RNA sequencing, that C22-10 was in fact *C. bifermentans*.

4.4.9. Enhancing plaque clarity and plaque size

Calcium chloride (10 mM) and glycerol (5% v/v) were added to agar plates and soft top agar, used for environmental and prophage screening tests. It has previously been demonstrated that the addition of calcium chloride (10 mM) increases the size and clarity of coliphage plaques in the overlay plaque assay method (Havelaar and Hogeboom, 1983). Similarly, the addition of 5% (v/v) glycerol to solid media has also been shown to increase the size of phage plaques, making them more clearly discernible (Santos *et al.*, 2009). Although this study also reported that the use of sub-inhibitory levels of antibiotics could also improve plaque clarity and viral yield, it was decided not to adopt this approach as the sensitivity of environmental strains was undetermined and may be very low due to the lack of antibiotic exposure.

4.4.10. Ethical approval

Since this study used human clinical faecal samples, advice as to whether this project required full ethical approval was sought from the Local and Regional Ethics Committee (LREC). As no information regarding the patients' demographics or clinical history was used and the samples were discarded after the testing period, no approval was required. The LREC advice can be found in Appendix 2.

4.5. Results

4.5.1. Validation of phage isolation methods

Environmental samples processed for the detection of novel *C. difficile* phages were first tested for the presence of phages infecting the *E. coli* strain K12 in order to verify that the sample contained viable phages. Phages infecting *E. coli* K12 were not isolated from all processed environmental samples. Two samples of lake water, two soil samples and one human faecal sample were subjected to repeat attempts at isolation but were not successful. Fig. 4.3 shows a typical K12 spot plaque assay and Fig. 4.4 illustrates the range of recovery rates obtained from the environmental samples.

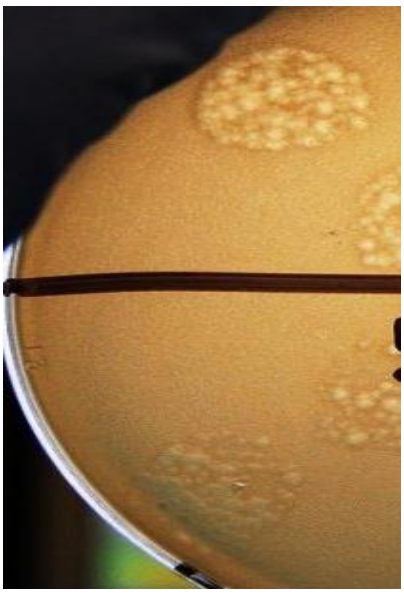


Figure 4.3. Spot plaque assay of crude settled sewage samples using *E.coli* K12 as the indictor.

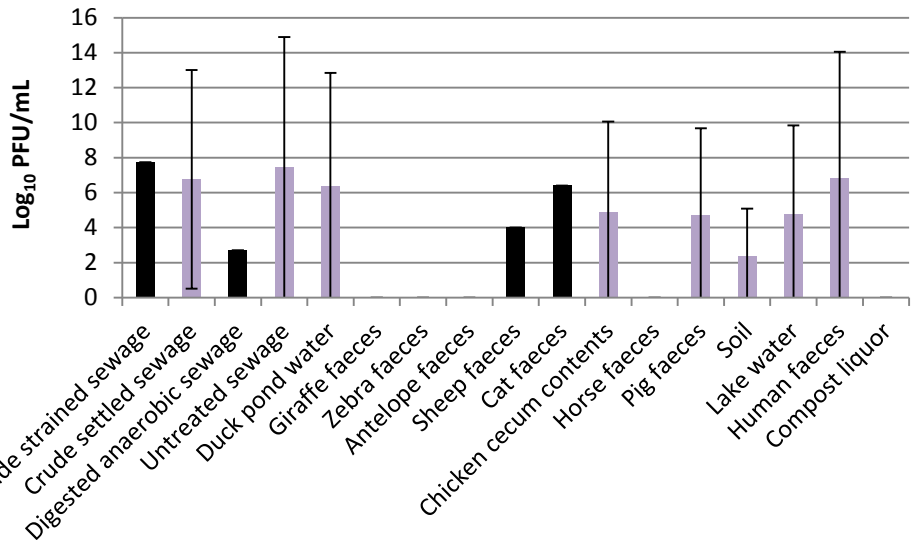


Figure 4.4. Mean recovery of *E.coli* K12 phages from environmental samples ± SD. Bars in black represent sources for which only one sample was tested.

It is not clear if negative samples did not contain any K12 phages, or that the isolation methods used affected the viability of these phages. A processed crude settled sewage sample (negative for K12 phages) was submitted for TEM analysis and showed the presence of numerous phage particles (Fig. 4.5).

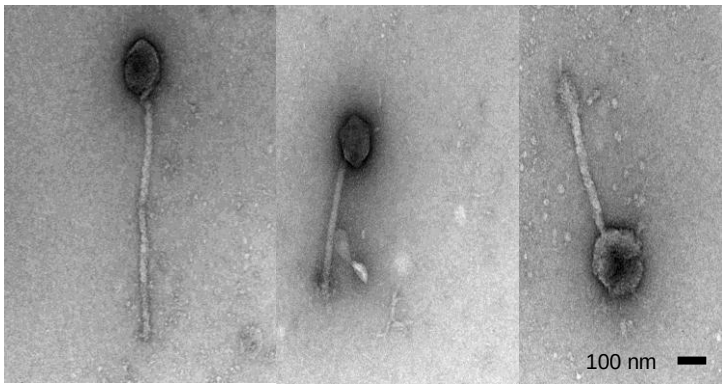


Figure 4.5. Phage particles seen in a processed sample of crude settled sewage

4.5.2. Recovery of *C. difficile* strains

C. difficile strains were successfully isolated from alcohol shocked human faecal samples and pig faeces, as shown in Table 4.3. Their identity was confirmed by 16S rRNA gene sequencing and matching to the NCBI database using BLAST (Altschul *et al.*, 1990; Zhang and Madden, 1997; Zhang *et al.*, 2000).

***C. difficile* strain isolated**	**Source**
NNUH-1	Human faecal sample
NNUH-2	Human faecal sample
NNUH-3	Human faecal sample
NNUH-4	Human faecal sample
NNUH-5	Human faecal sample
NNUH-7	Human faecal sample
NNUH-8	Human faecal sample
PIG-ENV	Pig faeces

Table 4.3. *C. difficile* strains isolated from environmental and faecal samples

4.5.3. Elution of phages from particulate matter

Fig. 4.6 shows the results of the phage elution experiments using samples spiked with ΦCD27. This indicates that the overall recovery rate is low but generally better at neutral pH values. Sodium chloride (1 M) gave the greatest recovery level, with a mean yield of 65.1% (n=3). The elution experiment incorporating the use of beef extract and ammonium sulphate failed to yield a floc that could be harvested and was therefore not included in the elution results.

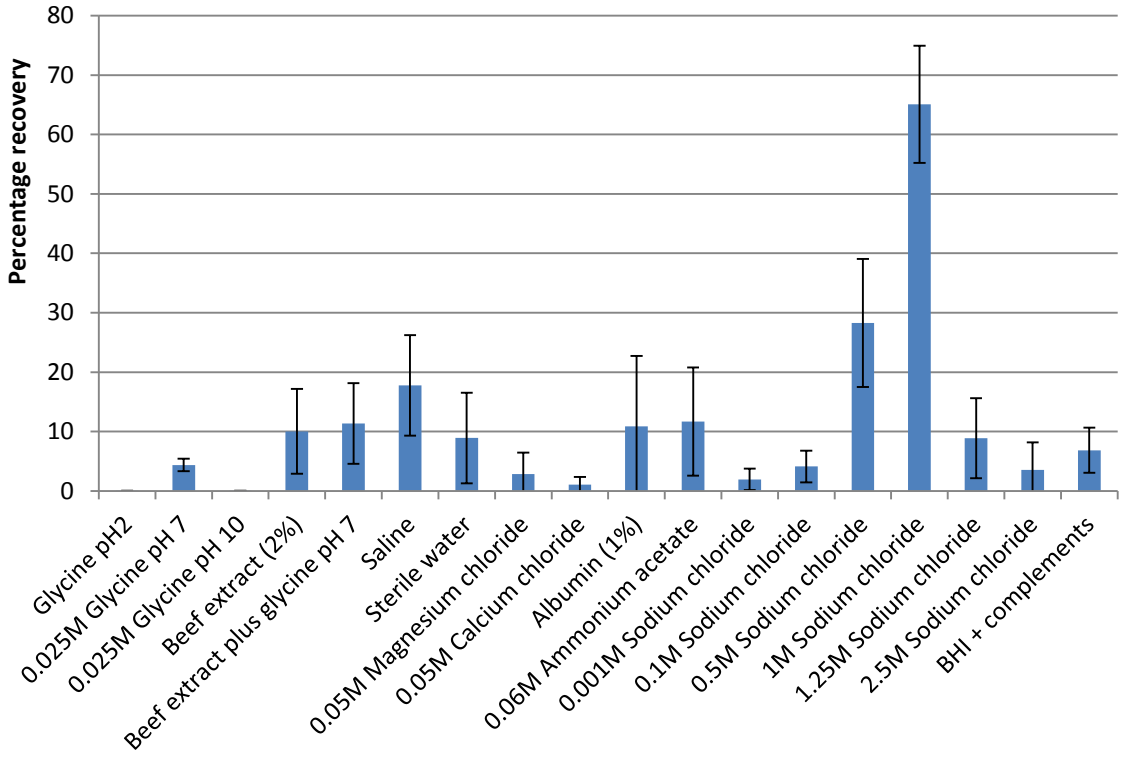


Figure 4.6. Elution of ΦCD27 from spiked soil samples with a range of elution buffers ± SD.

4.5.4. Survival and recovery experiments

Levels of ΦCD27 in spiked sewage samples decreased rapidly over 20 days relative to the control BHI with complements, with undetectable levels in crude settled sewage after this time period (Fig. 4.7). In the sewage samples, the mean recovery plummeted to 0.18% of the original titre by day 12. Although the purpose of these experiments was to assess viability of phage in these samples, it is not known to what extent the elutive capacity decreased over the 20 days.

After 5 h at pH 1-2 no ΦCD27 could be recovered, and only low recovery (mean 14.1%) was demonstrated at pH 3. At pH 4 81% of phage was recovered and 100% was recovered between the range 5-8. At pH 9-10, no ΦCD27 could be isolated. These results are illustrated in Fig. 4.8.

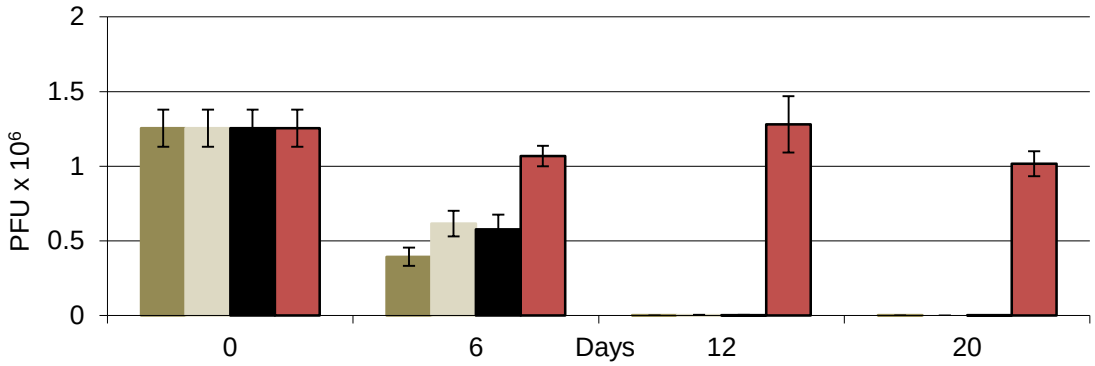


Figure 4.7. Recovery of ΦCD27 from spiked sewage and BHI media over 20 days at room temperature. = Crude sewage, = crude settled sewage, = anaerobically digested sewage, = BHI.

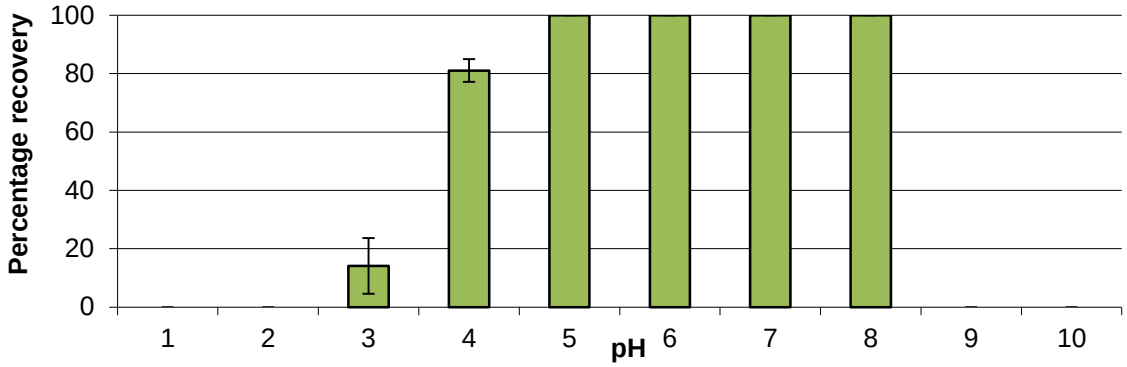


Figure 4.8. Recovery of ΦCD27 between pH 1-10. Result are means of triplicate counts ± SD.

4.5.5 Enhancement of plaque size and clarity

The plaques that were observed after testing with 10 mM $CaCl_2$ added to the molten agar in spot and overlay assays appeared larger and were of improved clarity relative to the other plaque assays. Concentrations above this caused precipitation in the media and actually obscured the visibility of lytic activity. The addition of 5% glycerol was also useful for enhancing the visibility of phage plaques.

4.5.6. The effect of PEG precipitation on phage viability

Concentration of phages by PEG precipitation was found to reduce the recovery and detection efficiency by 41.5% (± 8.3) (n=3).

4.5.7. Recovery of phages infecting *C. difficile*

No free-living lytic phages specific for *C. difficile* NCTC 11204, NCTC 11207, DSMZ 12056, R23 737 or any of the environmental strains were recovered, although many putative plaques from spot assays of neat clarified environmental samples were followed up. Defined plaques were subsequently not identified in overlay plaque assays, and in all cases, any antimicrobial activity against *C. difficile* was nullified following neutralisation of the supernatant, indicating that any activity may be due solely to organic acids.

4.5.8. *C. difficile* prophage inductions

Table 4.4. shows the OD_{600} values of *C. difficile* cultures before and after induction with mitomycin C. The release of prophages was anticipated to cause a decrease in the OD_{600} due to lysis of the host cells as phage particles are liberated. With the exception of NCTC 12727, from which ΦCD27 of the *Myoviridae* family is derived (as shown in Fig. 4.9 a), no decreases were observed. NCTC 11307 (strain 630) has 2 known prophages described in the literature (Goh *et al.*, 2007; Sebaihia *et al.*, 2006), but did not show a decrease in OD_{600} following mitomycin C induction. Despite this, the level of growth post induction was minimal, indicated by an increase in OD_{600} of only 0.02 and was consistent with the release of phage particles as shown by TEM analysis (Fig. 4.9 b). The phage particles observed are consistent with *Myoviridae* morphology, as previously described (Fortier and Moineau, 2007; King *et al.*, 2012).

C. difficile strain	OD_{600} before induction with mitomycin C	OD_{600} 2 hours post induction with mitomcyin C	Electron microscopy observations
12727 (1 known prophage)	0.200	0.110	Phage particles seen
11209	0.894	1.536	No phage particles
12733	0.748	1.382	No phage particles
11208	0.555	1.092	No phage particles
12731	0.397	0.937	No phage particles
12730	0.615	1.098	No phage particles
11205	0.691	1.302	No phage particles
12734	0.264	0.561	No phage particles
11382	0.585	1.064	No phage particles
11206	0.462	0.750	No phage particles
12732	0.246	0.820	No phage particles
12728	0.427	1.179	No phage particles
12726	0.210	0.963	No phage particles
12729	0.615	1.412	No phage particles
R23 720	0.711	1.378	No phage particles
R23 635	0.233	0.890	No phage particles
R23 524	0.395	1.040	No phage particles
R23 613	0.380	1.022	No phage particles
R23 521	0.346	0.908	No phage particles
R23 621	0.545	1.239	No phage particles
R23 639	0.337	0.964	No phage particles
R23 614	0.655	1.169	No phage particles
R23 642	0.385	0.972	No phage particles
R23 737	0.186	0.756	No phage particles
R23 732	0.651	1.05	No phage particles
R23 727	0.671	1.118	No phage particles
G83/07	0.211	0.757	No phage particles
1787	0.107	1.193	No phage particles
11207	0.320	1.111	No phage particles
11223	0.145	0.812	No phage particles
11307 (2 known prophages)	1.297	1.299	Phage particles seen
DSMZ 12056	0.324	1.341	No phage particles
DSMZ 12057	0.467	1.111	No phage particles
NNUH-1	0.366	0.722	No phage particles
NNUH-2	0.450	0.620	No phage particles
NNUH-3	0.450	0.869	NS
NNUH-4	0.692	0.785	NS
NNUH-5	0.332	0.963	NS
NNUH-7	0.568	0.959	NS
NNUH-8	0.566	0.758	NS
PIG-ENV	0.411	0.771	NS

Table 4.4. $0D_{600}$ values of *C. difficile* cultures before and after induction with mitomycin C. (Samples were diluted 1 in 10 prior to reading and corrected thereafter.) NS = not screened by electron microscopy.

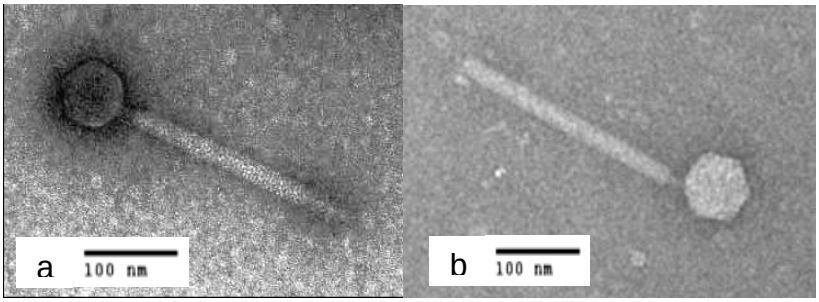


Figure 4.9. Phage particle of the *Myoviridae* family observed by TEM after induction of a) *C. difficile* NCTC 12727 and b) *C. difficile* NCTC 11307.

4.5.9. Plaque assays using concentrated induced supernatants of *C. difficile.*

Only ΦCD27 gave plaques when added to *C. difficile* NCTC 11204 (shown in Fig. 4.10), NCTC 11205, NCTC 11207 and NCTC 11209. No other susceptible hosts were identified. No phage activity in any other induced supernatants (including 11307) was observed when cross tested with all 12 *C. difficile* isolates in the collection.

Figure 4.10. Plaques caused by ΦCD27 on a lawn on *C. difficile* NCTC 11204.

4.5.10. Prophage inductions from other *Clostridium spp.*

4.5.10.1. *C. sporogenes*

Table 4.5. shows the optical density results obtained when 5 *C. sporogenes* strains were induced with mitomycin C. As previously described, strain C22-10 was found to be a *C. bifermentans* strain erroneously categorised as *C. sporogenes*.

C. sporogenes	**OD_{600} before induction with mitomycin C**	**OD_{600} 2 h post-induction with mitomycin C**	**Electron microscopy observations**
BL81-04	1.1	1.478	No phage particles
BL84-18	0.834	1.446	No phage particles
BL84-17	1.074	1.474	No phage particles
BL02-01	1.2	1.9	Phage particles seen

Table 4.5. OD_{600} values of *C. sporogenes* strains before and after induction with mitomycin C.

The phage yielded as a result of mitomycin C induction of *C. sporogenes* BL02-01 (ΦCS01) is shown in Fig. 4.11. The approximate length and width of the prolate particle head was 110 nm and 40 nm respectively. The tail appeared to be quite flexible with transverse striations, and measured 300 nm in length and 12 nm in width. This morphology indicates that the phage could be a member of the *Siphoviridae* family (Fortier and Moineau, 2007; King *et al.*, 2012).

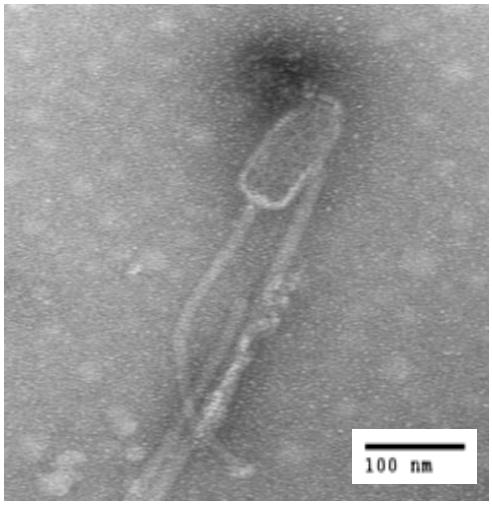


Figure 4.11. TEM of ΦCS01, induced from *C. sporogenes* BL02-01.

Only ΦCS01 produced plaques when the induced supernatant was tested in plaque assays with *C. sporogenes* BL81-04. No phage activity was observed with any *C. difficile* strains in the collection.

4.5.10.2. *C. sordellii*

Two strains of *C. sordellii* were also induced in search of novel prophages that may infect *C. difficile*. The pre- and post-induction OD_{600} values are given in Table 4.6).

C. sordellii	OD_{600} before induction with mitomycin C	OD_{600} 2 hours post-induction with mitomycin C	Electron microscopy observations
ATCC 9715	0.679	1.983	N/A
NCTC 13356	0.721	1.417	N/A

Table 4.6. The OD_{600} values of *C. sordellii* strains before and after induction with mitomycin C.

No phage activity was observed with any of the *C. sordellii* induced supernatants versus all of the *C. difficile* strains in the collection.

4.5.10.3. *C. bifermentans*

No phage particles were observed upon induction of *C. bifermentans* C22-10 with mitomycin C (Table 4.7).

C. bifermentans	OD_{600} before induction with mitomycin C	OD_{600} 2 h post-induction with mitomycin C	Electron microscopy observations
C22-10	1.2	1.350	No phage particles

Table 4.7. The OD_{600} values of the *C. bifermentans* strain before and after induction with mitomycin C.

No phage activity was observed with the *C. bifermentans* induced supernatant versus all the *C. difficile* strains in the collection.

4.6. Discussion

C. difficile strains were successfully isolated from 7 out of 42 human faecal samples and 1 out of 7 pig faeces/cecum samples. This identification was initially based simply on the morphology of the colonies on CCEY to expedite processing in the interest of preventing significant sample deterioration, owing to the poor survival of phage demonstrated in separate experiments, but was later confirmed by 16S sequencing.

Enrichment of environmental samples with selected laboratory strains and any strains derived from the sample itself was believed to be the best strategy for phage recovery, but no novel lytic *C. difficile* phages infecting any strain in the collection were isolated. Enrichment may not support all phages found in the environment, as previously observed (Ashelford *et al.*, 2003). Some researchers omit any enrichment or purification step when searching for novel phages and simply introduce environmental samples directly into overlay plaque assays (Serwer *et al.*, 2004). Despite some obscurity and ambiguity of plaques due to the particulate material, this approach proved successful for the isolation of phages infecting *Bacillus subtilus* and other bacterial genera, whilst the same sample tested by liquid culture enrichment yielded no phages (Serwer *et al*., 2004). This finding, together with the poor survival rates demonstrated for phage in sewage samples (which also validate the need for expedited testing), suggests that unprocessed samples used promptly in screening assays may have yielded better results. However, to date no non-lysogenic phages infecting *C. difficile* have been described in the literature, which does seem to suggest scarcity, absence, extremely narrow host ranges or the need to modify isolation techniques.

In the majority of cases, the processing and concentration of environmental samples yielded viable phage, as demonstrated by the detection of *E. coli* K12 phages. The isolation of K12 phages was not successful in all cases, even despite repeat attempts. This may reflect a genuine absence of this type of phage or highlight a problem with the isolation methods applied to the sample, therefore invalidating negative *C. difficile* phage screening results. Indeed, the recovery experiments indicate that despite best efforts, only

65.1% of phage from spiked samples could be recovered, and this may be further lessened in samples for which strong interactions between phage and particulate material have occurred. Significant batch to batch variations in the reagents used for phage recovery have previously been demonstrated (Katzenelson *et al.*, 1976; Payment *et al.*, 1984; Shields and Farrah, 1986). In particular, the use of some batches of beef extract did not result in the formation of a floc, while with other batches a thick floc rich in bacteriophages could be observed (Shields and Farrah, 1986). The beef extract used to reproduce this work did not give rise to a floc and may therefore be explained by this observation. In addition to reagent variability, it was not feasible to agitate (gently on a rotating platform) the enriched environmental samples during incubation due to the limited electrical supply inside the anaerobic cabinet used for these experiments. By introducing sample agitation, the chances of phage-host interaction may have been increased and with it the possibility of isolating a novel phage. Changes to the method of phage concentration such as extending the PEG incubation period to 12-15 h may also have improved phage yield (Kutter and Sulakvelidze, 2005).

Several other observations relating to environmental screening techniques have come to light that may go some way to explain the lack of *C. difficile* phage recovery. In addition to the inefficiencies of elution, filtration has been shown to reduce the yield of phage by up to 20% (personal communication with Chloe James, University of Liverpool). Similarly, a closer look at the effect of PEG precipitation indicated that a reduction of up to 45.1% in the titre of viable phage could be seen. Future tests should also include a chloroform (and/or an ether) exposure step to break open infected cells in case the conditions of the environmental sample are not permissive to lysis (Kutter and Sulakvelidze, 2005). This step would also reveal any phages that trigger an abortive infection system, instructing bacterial cell death in response to infection, which may have hidden positive results in the screening tests. Some groups have shown that, depending on the conditions, infecting phage particles may not be able to lyse and release new phage particles (Kutter and Sulakvelidze, 2005). Although this adaptation would sacrifice phages

containing a lipid envelope, these would be of negligible use in a gut environment.

The environmental screening was carried out from October 2007 to March 2008. The proportion of phage particles relative to the levels of bacteria in environmental samples has been shown to vary depending on the season, with up to ten-fold fewer in winter (Kutter and Sulakvelidze, 2005). This may be due to the induction of prophages in summer as a result of the UV light. Despite the abundance of phage in environmental sources such as soil, these samples are comprised of multiple microenvironments, which vary depending on the level of rainfall, temperature and the presence of different biofilms and binding agents such as clay. No evaluation as to the presence or abundance of such compounds was undertaken but may have influenced phage recovery.

The environmental screening methods were phenotypic and so quite time consuming. If it were possible to identify the presence of phage genes permitting attachment to (and subsequent infection of) *C. difficile* hosts based on sequence similarity to known *C. difficile* phages, the screening rate could be considerably increased.

In addition to environmental sampling, induced strains of *C. difficile* were concentrated and examined by electron microscopy for evidence of phages, plus they were screened against the entire laboratory collection of *C. difficile* strains for signs of lytic activity. This approach was successful in obtaining a prophage of *C. sporogenes* (ΦCS01) which infects *C. sporogenes* BL81-04 but no phages infecting *C. difficile* were detected, despite the high incidence of prophages in *C. difficile* genomes (Fortier and Moineau, 2007; Goh *et al.*, 2005; Govind *et al.*, 2006; Horgan *et al.*, 2010; Mahony *et al.*, 1985; Mayer *et al.*, 2008; Nagy and Foldes, 1991; Nale *et al.*, 2012; Sell *et al.*, 1983; Shan *et al.*, 2012). Successful alternative inducing agents have been described including UV light (Rambler and Margulis, 1979) and norfloxacin (Shan *et al.*, 2012). Other studies have used temperature, pressure (Kutter and Sulakvelidze, 2005) and even plant extracts (Erskine, 1973). Reports show that by using a variety of inducing agents, different profiles of phages can be induced from the same collection of host strains (Shan *et al.*, 2012).

Adaptations to the sample processing protocol prior to analysis by electron microscopy could have aided the screening process and increased the chances of finding novel phages. Wash steps using ammonium acetate followed by centrifugal sedimentation has been recommended by Kutter and Sulakvelidze (2005). This may have reduced the background artefacts, which may have impeded clear identification of phage particles. It may also be possible that unusual phage morphologies could have been missed in the screening process.

In summary, no novel phages infecting *C. difficile* were discovered despite testing a large number of samples from different sources and attempts to improve phage isolation protocols. This work has highlighted the need for new approaches to environmental screening for *C. difficile* phages and the incorporation of alternative inducing agents when searching for prophages.

Chapter 5 – Modelling phage therapy of *C. difficile* infection using batch fermentations.

5. 1. Abstract

An *in vitro* batch fermentation model of acute *C. difficile* infection was used to evaluate the effect of a prophylactic and a remedial bacteriophage treatment regime. It was demonstrated that the prophylaxis regime was effective at restraining the growth of *C. difficile* ($p = <0.001$) and considerably suppressed the production of toxins A and B. The remedial treatment regime caused a less profound and somewhat transient decrease in the number of viable *C. difficile* cells ($p = <0.0001$), but still resulted in a lower level of toxin production relative to the control. The numbers of commensal bacteria including total aerobes and anaerobes, *Bifidobacterium spp.*, *Bacteroides spp*., *Lactobacillus spp*., total *Clostridium spp*. and *Enterobacteriaceae* suffered no detrimental effect as a result of phage therapy, whereas significant decreases were observed following metronidazole treatment, the current recommended treatment for uncomplicated *C. difficile* infection.

5. 2. Introduction

The human gut hosts a rich, diverse population of organisms and disruption of these complex communities is associated with the onset of *C. difficile* infection plus a wide range of other pathologies including allergies and colorectal cancer (Prakash *et al.*, 2011). The dynamics and functionality of these organisms are imperative to the health of the human host and evidence on specific groups and their associated benefits is mounting (Chassard *et al.*, 2008; Freeman *et al.*, 2003; Hopkins and Macfarlane, 2002; Parkes *et al.*, 2009; Rehman *et al.*, 2012). These functions include vitamin synthesis (Chassard *et al.*, 2008) and immunomodulatory effects (Schiffrin *et al.*, 1995) and are described in more detail in 1.7.4. It is therefore crucial to assess the impact of any proposed therapeutic agent on the commensal microbiota and this is particularly poignant for CDI, in which the diseased state arises following antimicrobial treatment and the subsequent disruption to the gut flora. It is also essential to assess the activity of therapeutic agents and their effect on the target pathogen in conditions simulating those *in vivo* as the abundance of microbial flora, associated metabolites, neutralising proteins and surfactants may impede or inhibit activity.

Batch fermentations offer a simple preliminary *in vitro* model system for observing the performance of therapies in the distal colon (Possemiers *et al.*, 2005; Salminen *et al.*, 1998). Culture of the major bacterial groups from these models has been used to enumerate the effects of interventions on the microbiota (Olano-Martin *et al.*, 2000), the prebiotic effects of some functional foods (Mandalari *et al.*, 2007, 2008; Saulnier *et al.*, 2008; Stewart *et al.*, 2008) and also the fate of enteric pathogens such as *Salmonella spp.* (Martin-Pelaez *et al.*, 2008; Meimandipour *et al.*, 2009). They have also been used previously to represent CDI and to investigate potential therapeutic agents. (Rea *et al.*, 2007). This group used simple faecal fermentations in an anaerobic chamber to determine the activity of the lantibiotic lacticin 3147 against *C. difficile* strains, and demonstrated a decrease to below the limit of detection by culture. They also monitored the effect on the commensal microbiota by culture and recorded a 3 $\log_{10}$ decrease in the numbers of *Bifidobacterium spp*. and *Lactobacillus spp*. as a result of the lacticin

treatment. These groups are thought to confer colonisation resistance against *C. difficile* (Freeman *et al.*, 2003) and so these results are not conducive to the prevention of CDI recurrence. No pH control was included in these fermentations and since clostridia are known not to grow well in acidic conditions (Gibson and Roberfroid, 1995) and the growth of lactic acid bacteria would be expected to cause a reduction in the pH, this may have contributed somewhat to the inhibition of *C. difficile* under these conditions.

ΦCD27 is a bacteriophage of the *Myoviridae* family, which produces semi-clear plaques on a lawn of *C. difficile* NCTC 11204 (2.2.2; Mayer *et al.,* 2008). Despite having lysogenic capacity, the lytic activity of ΦCD27 has therapeutic potential and is amenable to investigation using a batch fermentation model of *C. difficile* infection. The batch fermentation culture models were designed to represent a short window of active *C. difficile* infection, during which vegetative cells are producing high levels of toxins A and B.

There is a clinical need for the selective elimination of gut pathogens without compromising the integrity of the microbiota. The specificity of phage therapy warrants investigation as to its ability to infect and kill target hosts in the gut environment. Due to the nature of CDI onset, prophylactic agents could be strategically instigated alongside any course of antimicrobial therapy that is likely to lead to the suppression of normal commensals and subsequent germination of *C. difficile*. Various studies have also demonstrated differing efficacies depending on the dosing intervals, and with these factors in mind, a prophylactic phage regime was tested in addition to a remedial schedule.

5. 3. Objectives:

- To determine the relative efficacy of a prophylactic and a remedial phage therapy schedule to treat CDI.
- To determine any significant changes to commensal gut microbiota as a result of phage therapy, relative to an un-treated control and a metronidazole-treated control.

5. 4. Methods

5. 4. 1. Bacteriophage propagation

Clostridium difficile strain 11204, obtained from the NCTC culture collection, was used for the batch fermentation experiments. Culture conditions and media details are described in 2.5.14.

Bacteriophage ΦCD27 was induced from a turbid culture of *C. difficile* NCTC 12727 using mitomycin C at 3 µg/mL as described in 2.2.1. In an attempt to minimise the concentration (and possible effects of) mitomycin C from the phage preparation, lysates were prepared from plate harvests (2.2.3.1).

5. 4. 2. Bacterial culture

5. 4. 2. 1. *Clostridium difficile* inoculum

C. difficile was cultured as described (2.1.1.1) and material from the second subcultures was used to inoculate the batch fermentation vessels. Growth curves were used to determine the inoculum volume required in advance, and this was confirmed by colony counts of serial dilutions post inoculation.

5. 4. 2. 2. Enumeration of gut bacteria

Five mL samples from the batch fermentation vessels were taken at each time point and enumerations of *C. difficile* and a range of gut bacteria were made (2.1.2). Growth curves were plotted and the area under the curve was calculated to represent the overall level of growth. After processing, samples were stored at -20°C.

5. 4. 3. The batch fermentation system

The batch fermentations were carried out over 48 h according to the procedure described previously (Mandalari *et al.*, 2007). The apparatus is pictured in Fig. 5.1. Culture media was prepared as described previously (2.5.14). Aliquots of 135 mL were dispensed into the fermentation vessels, which were then autoclaved at 121°C for 15 minutes. The pH of each vessel was adjusted and maintained at 6.8 ± 0.2. Oxygen-free nitrogen was bubbled through the sterile batch fermentation vessels overnight to create anaerobic

conditions prior to the inoculation of fresh faecal slurry. Ten percent slurry was made with freshly voided faecal sample from a volunteer aged under 60 years, diluted with pre-reduced PBS. Fifteen mL of this slurry was then used to inoculate each vessel (230 mL capacity). At time 0, approximately 2 x 10^6 cells of *C. difficile* NCTC 11204 from an overnight culture, were added to vessels 2 and 3. No *C. difficile* was added to vessel 1, which served as a control. Vessel 4 received a dose of ΦCD27 at 0, 6, 24 and 36 h, and was challenged with approximately 2 x 10^6 cells of *C. difficile* culture at 6 h. The dosing schedule is summarised in Table 5.1, and was designed to represent a snapshot of acute *C. difficile* infection.

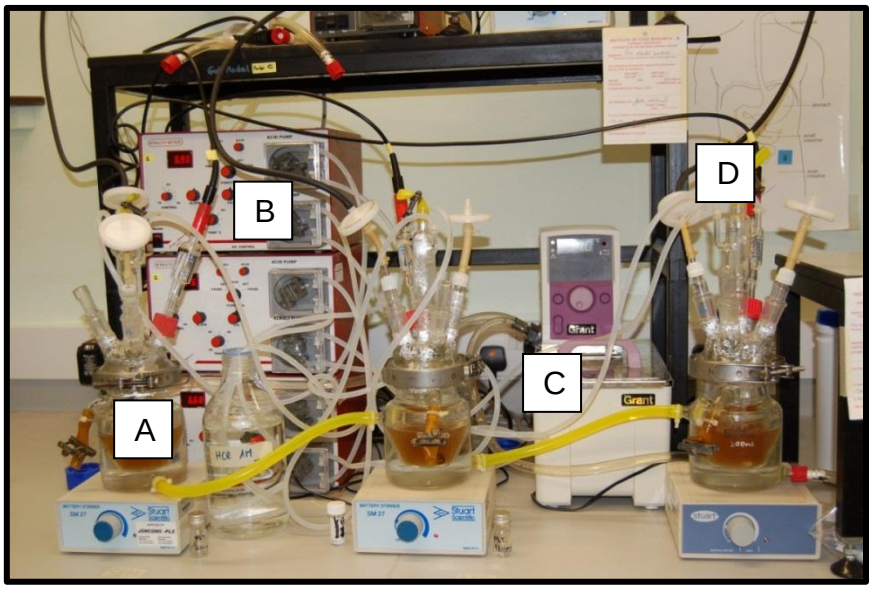


Figure 5.1.The batch fermentation system. Each vessel (A) is pH controlled (B), maintained at 37°C using water jackets (C) and kept anaerobic via a continuous nitrogen purge (D).

Vessel	0 h	6 h	24 h	36 h	48 H
Control	-	-	-	-	-
Control *C. difficile*	*C. difficile* added	-	-	-	-
Remedial regime	*C. difficile* added	ΦCD27 added	ΦCD27 added	ΦCD27 added	ΦCD27 added
Prophylactic regime	ΦCD27 added	*C. difficile* and ΦCD27 added	ΦCD27 added	ΦCD27 added	ΦCD27 added

Table 5.1. The dosing schedules for batch fermentation vessels.

Phage preparations were stored in sterile BHI broth plus complements, and dosed at a multiplicity of infection (MOI) value of 7 in the first experiment and 10 in the two subsequent repeats. The dose volume was 10 mL. Vessel 3 received phage treatment at 6, 24 and 36 h. Samples for analysis were taken at 0, 6, 24, 36 and 48 h. At the sampling time points, 5 ml of sample was extracted using a Pasteur pipette, and transferred immediately to the anaerobic cabinet for enumeration of commensal bacterial groups (5.4.2.2) Samples were then used for toxin quantification (2.1.3).

In the prophylactic schedule, ΦCD27 dosing began prior to the addition of vegetative *C. difficile* cells. It was therefore necessary to exclude the possibility that it could replicate in faecal flora and subsequently compromise the ability of the experiment to fairly evaluate both the prophylactic and remedial regime in parallel. Three bottles of 25 ml batch culture media were pre-reduced in the anaerobic cabinet overnight after which 10 g freshly voided faecal sample was added to each. The same faecal sample was also used for the 3rd batch fermentation experiment. ΦCD27 was harvested from a semi-confluent plaque assay (2.2.3.1) and 1 ml was added to each of the faecal cultures before returning to the anaerobic incubator. At time 0, 24 h and 48 h the cultures were mixed well and a 1 ml sample removed for ΦCD27 enumeration by overlay plaque assay.

In a separate experiment demonstrating the effect of metronidazole on *C. difficile* and other microflora in the batch fermentations, the antibiotic doses were based on the normal human therapeutic dose of 500 mg every 8 h and the approximate volume of the human colon (0.5 L). The total volume contained in each batch vessels was 150 mL, meaning that 150 mg metronidazole powder comprised each dose.

5. 4. 4. Fluorescence *in situ* hybridisation (FISH)

A fluorescence *in situ* hybridisation (FISH) probe (Bloedt*, et al.*, 2009), 5'-CATCCTGTACTGGCTCAC-3' labelled with Cy5, was tested in an attempt to achieve specific *C. difficile* detection with a sensitivity level superior to that of culture.

Samples were prepared from an 8 h culture of *C. difficile* NCTC 11204, centrifuged for 2 min at 500 x *g* to remove particulate debris. The supernatant (375 µl) was added to 1125 µl pre-chilled 4% paraformaldehyde solution in a 1.5 ml microcentrifuge tube and kept at 4°C overnight to fix. After this the fixed samples were centrifuged at 500 x *g* for 5 min and the supernatant removed and replaced with 1 ml PBS (filtered through a 0.45 µm syringe filter and subsequently autoclaved). After another centrifugation step and removal of the supernatant, the pellet was washed again with another 1 ml of sterile filtered PBS. When this supernatant was removed, the pellet was thoroughly resuspended in 150 µl sterile filtered PBS containing 2 mg/ml lysozyme and 50 U/ml mutanolysin. Next, 150 µl of ethanol (96%) was added. The sample was mixed well and stored at -20°C for at least 1 h.

For hybridisation, 16 µL of the prepared cells were added to 264 µl of filtered hybridisation buffer (40 mM Tris-HCl pH 7.2; 1.8 M NaCl and 2% SDS), pre-warmed to 50°C. In a separate microcentrifuge tube, 15 µl of the probe at a concentration of 50 ng/µl was added. Immediately, 135 µl of the hybridisation mix was added to the probe mix at a ratio of 1:10, vortex mixed and placed in a 50°C oven for 15 h to hybridise. To wash, 5 ml of washing buffer (pre-warmed to 50°C), was added to a sterilin tube, followed by 20 µl of 4,6-diamidino-2-phenylindole. Next, 100 µl of the hybridised sample was added and the tubes were returned to the 50°C oven for 30 min.

Filter apparatus was assembled with wet filters (0.2 µm diameter) matt side facing upwards. The sample mixtures were poured onto the filters and a vacuum pump was used to draw the liquid through. The sample tube was rinsed with warmed washing buffer and passed through the filter, which was then removed and placed onto a glass slide. A drop of SlowFade® (Invitrogen) was added to the filter prior to placing a coverslip over the slide. The slides were then transferred to the dark room for analysis.

The Nikon Eclipse 90i fluorescent microscope was used to analyse the FISH samples. First the UV light was used to visualise 4,6-diamidino-2-phenylindole-stained bacteria. Next the green light filter was used to visualise

C. difficile cells. Fifteen fields of view were counted, and the mean of these (corrected for the dilution) denoted the quantity.

5. 4. 5. *C. difficile* toxin detection

Samples from the batch fermentation vessels were stored at 4°C for up to 2 days following the bacterial enumerations, and tested for *C. difficile* toxin using the Premier Toxins A & B assay kit (Meridian Bioscience Incorporated, Ohio, USA) according to the manufacturer's instructions and as described in 2.1.3.

5. 4. 6. Statistical analysis of data

Data from the bacterial enumerations at each sampling interval were plotted as growth curves and the area under the curve was calculated. Repeats were grouped together and tested for significant differences relative to the control tests by means of a one-way ANOVA using a Tukey's Honestly Significant Difference post-hoc test.

5.4.7. Measuring the effect of *C. difficile* toxins and lysed *C. difficile* cells on commensal faecal bacteria.

Enumerations were carried out from cultures of faecal sample inoculated with *C. difficile* NCTC 11204 (a toxigenic strain) and NCTC 12726 (a non-toxigenic strain) to ascertain possible effects of *C. difficile* toxins on the growth of common groups of commensal bacteria. Ten g of fresh faeces was suspended in 100 ml pre-reduced PBS and transferred to the anaerobic cabinet. One mL was added to pre-reduced BHI plus 200 µL from an overnight culture of the relevant *C. difficile* strain. Experiments were carried out in replicates of 3. After 24 h, the numbers of *Bifidobacterium spp*., total *Clostridium spp*., *Lactobacillus spp., Bacteroides spp*., total anaerobes, total aerobes, *Enterobacteriaceae* and Gram-positive cocci were enumerated by culture as described in 2.1.2. and 5.4.2.2.

Faecal cultures with and without lysed *C. difficile* cells were carried out to investigate the possible benefits of liberated nutritional compounds to other groups of bacteria. An overnight culture of *C. difficile* NCTC 11204 was

autoclaved and bead beaten 4 times with glass beads <106 μm (Sigma) for 30 s with 10 min rests on ice between each (Fast Prep 120 BIO 101 Thermo). One ml was added to a 1% faecal fermentation (in pre-reduced batch fermentation media – 2.5.14) and incubated overnight. One mL of an overnight *C. difficile* culture and 1 mL of PBS (not pre-reduced) served as controls. Experiments were carried out in replicates of 3. After 24 h, the numbers of commensal gut bacteria were enumerated by culture (2.1.2).

5. 5. Results

5. 5. 1. Bacterial culture

A batch fermentation system was used to model the effect of ΦCD27 phage therapy on the levels of viable *C. difficile*, toxins A and B, and a range of faecal organisms. Two different treatment schedules were tested – remedial and prophylactic. Levels of *C. difficile* in the remedial schedule were on average less than 1 $\log_{10}$ lower than the untreated control but still reached levels greater than 1 x 10^6 CFU/mL after initiation of phage treatment. When the mean area under the curve values are considered, the reduction was significant ($p = <0.0001$). The prophylactic treatment schedule resulted in a greater than 4 $\log_{10}$ reduction in the numbers of *C. difficile* relative to the control when an MOI of 7 was used (experiment a), and suppression to below the limit of detection by culture at 48 h when an MOI of 10 was used ($p=<0.0001$; experiment b and c). These results are shown in Fig. 5.2.

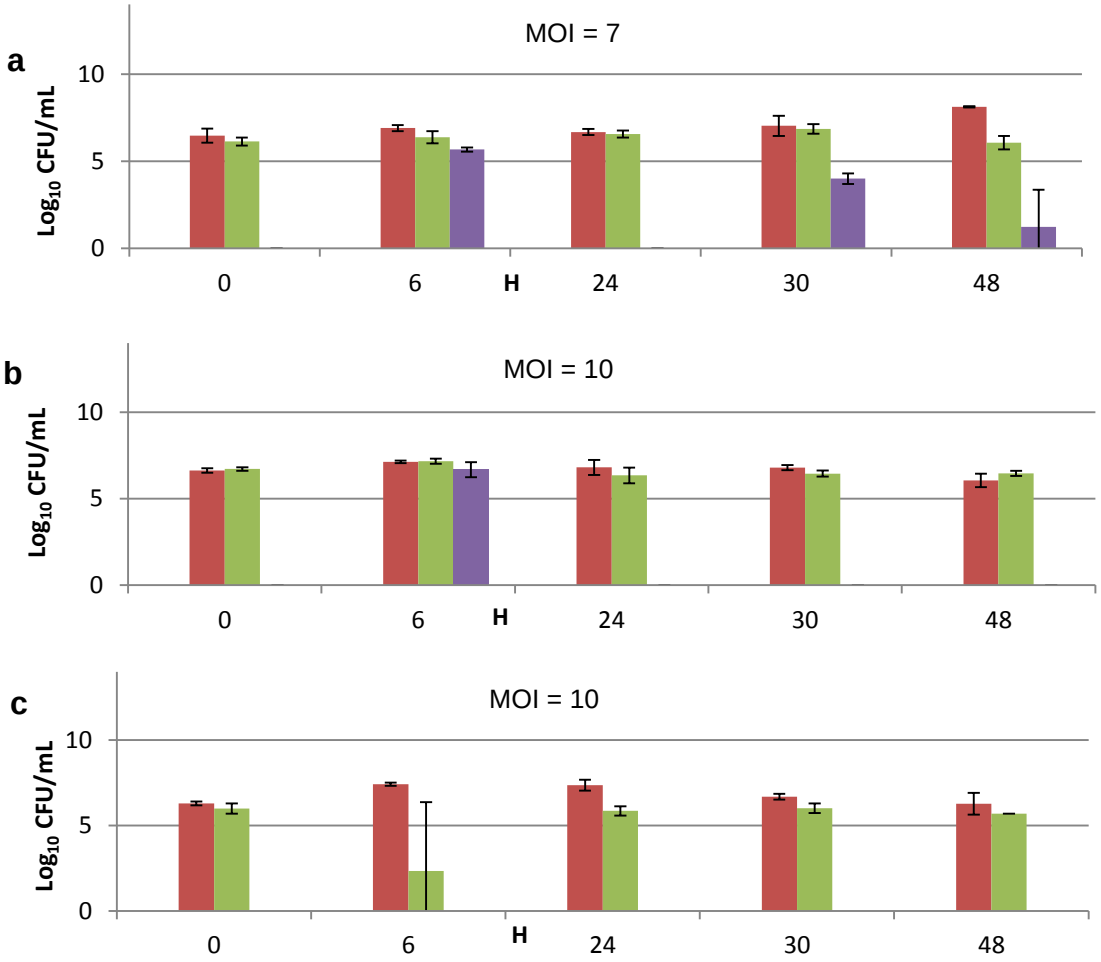


Fig 5.2. a-c. The effect of phage therapy on levels of *C. difficile*. Figures represent plate counts of *C. difficile* from replicate experiments of 4 vessels. No cells were detected in the control vessel with no *C. difficile*. = control vessel (plus *C. difficile*), = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are the means of triplicates ± SD.

The possible adverse effect of phage therapy on other commensal gut bacteria was also monitored in this batch model by viable enumerations at each sampling point. No significant reduction in the number of *Bifidobacterium spp.*, total anaerobes, *Bacteroides* spp., *Lactobacillus* spp., or total *Clostridium* spp. was observed in the phage treated vessels relative to the control vessels. There was however, a 0.79 $\log_{10}$ increase in the number of total aerobes ($p = 0.017$) and a 1.43 $\log_{10}$ increase in Enterobacteriaceae ($p = 0.021$) (Fig. 5.3-5.10).

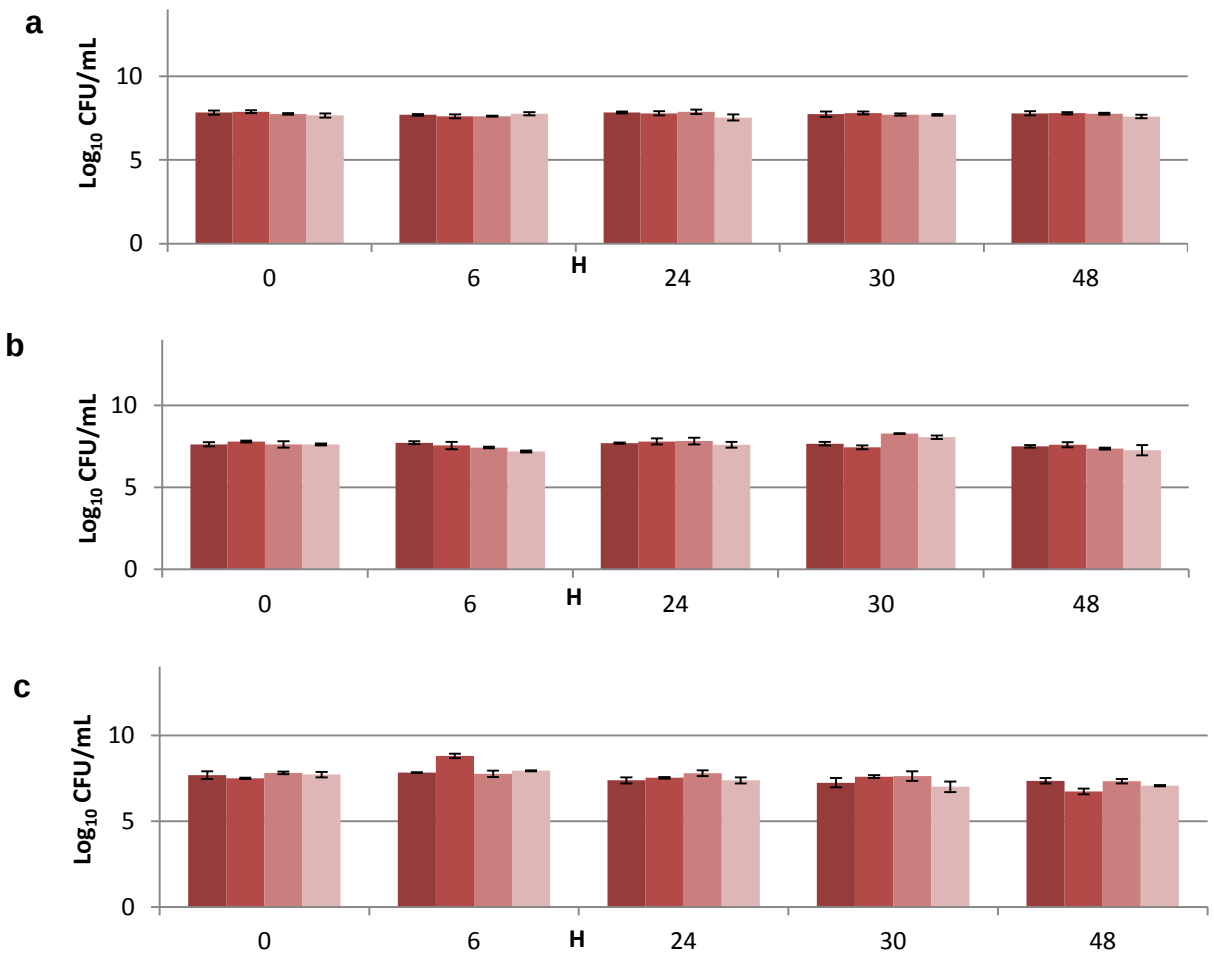


Figure 5.3. a-c. The effect of phage therapy on *Bifidobacterum spp*. Figures represent plate counts of replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are means of triplicates ± SD

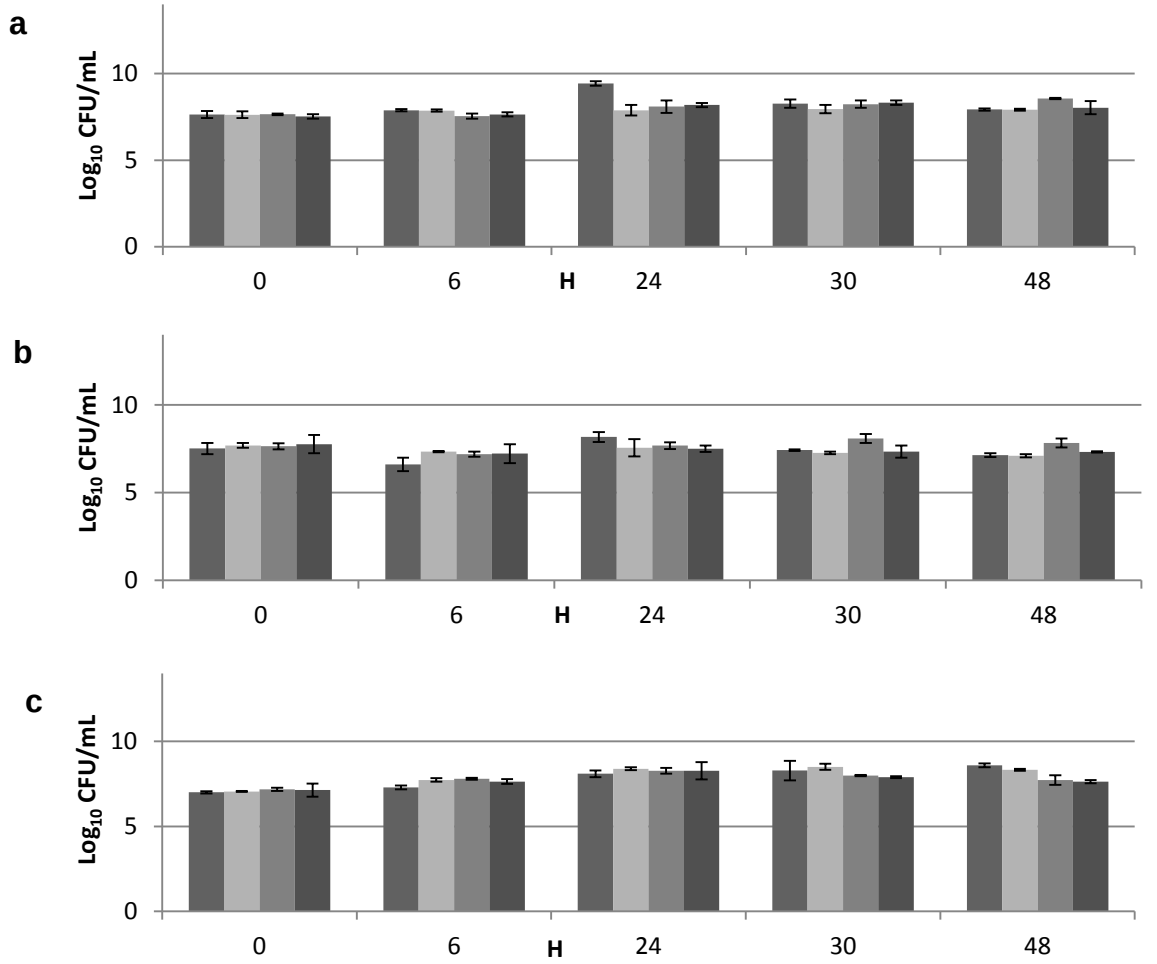


Fig 5.4. a-c. The effect of phage therapy on levels of total *Clostridium spp*. Figures represent plate counts from replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are the means of triplicates ± SD.

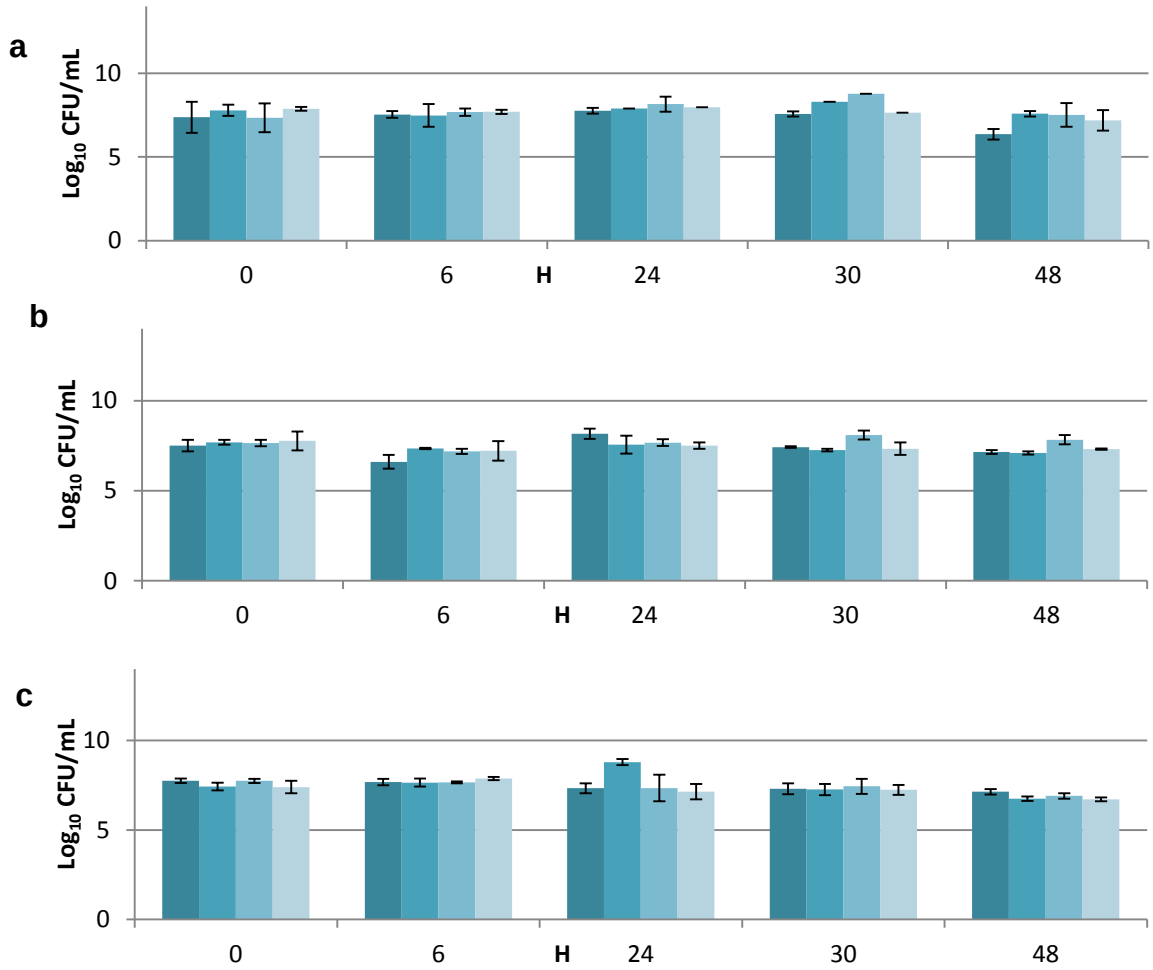


Fig 5.5. a-c. The effect of phage therapy on levels of *Lactobacillus spp*. Figures represent plate counts from replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are the means of triplicates ± SD.

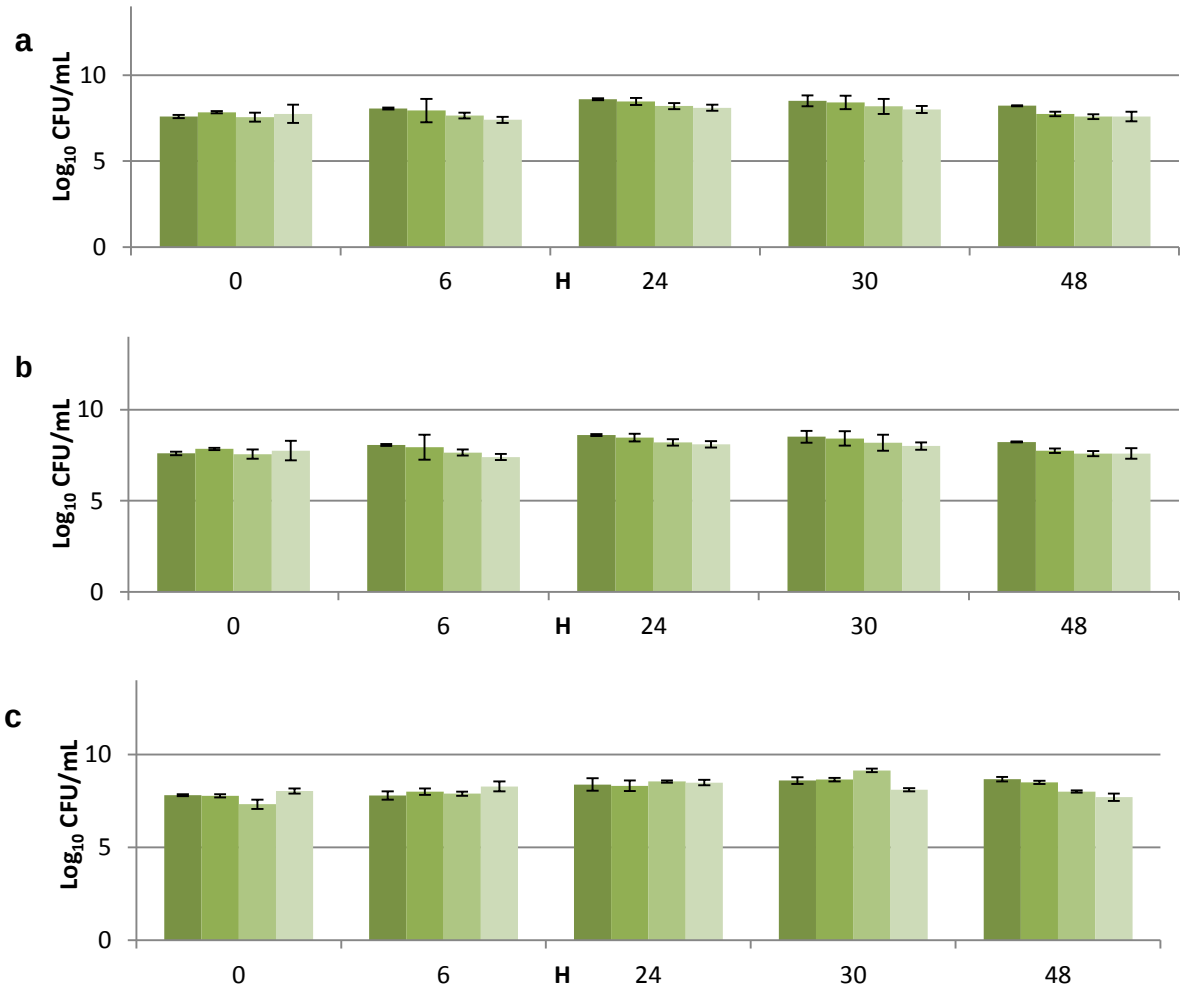


Fig 5.6. a-c. The effect of phage therapy on levels of *Bacteroides spp.* Figures represent plate counts of replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are the means of triplicates ± SD.

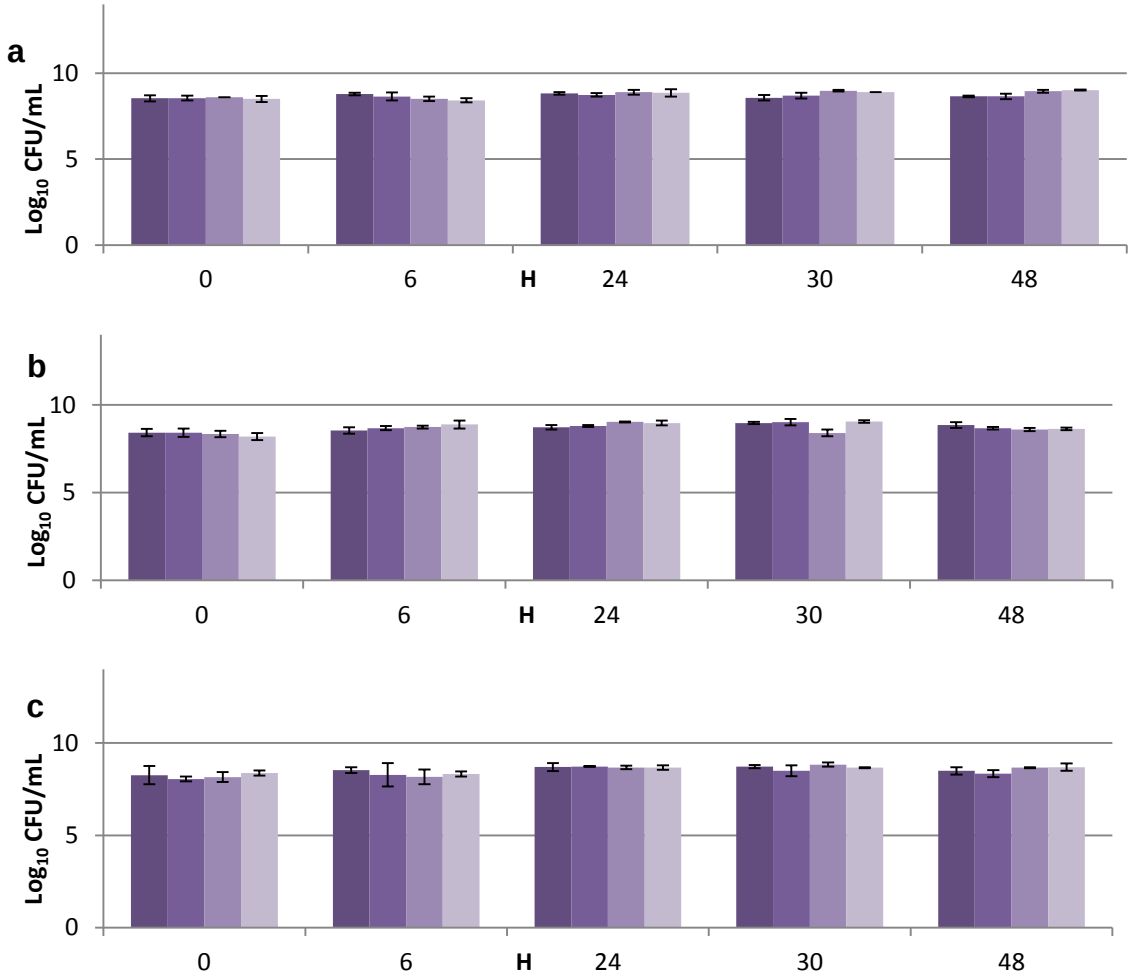


Fig 5.7. a-c. The effect of phage therapy on levels of total anaerobes. Figures represent plate counts of replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are means of triplicates ± SD.

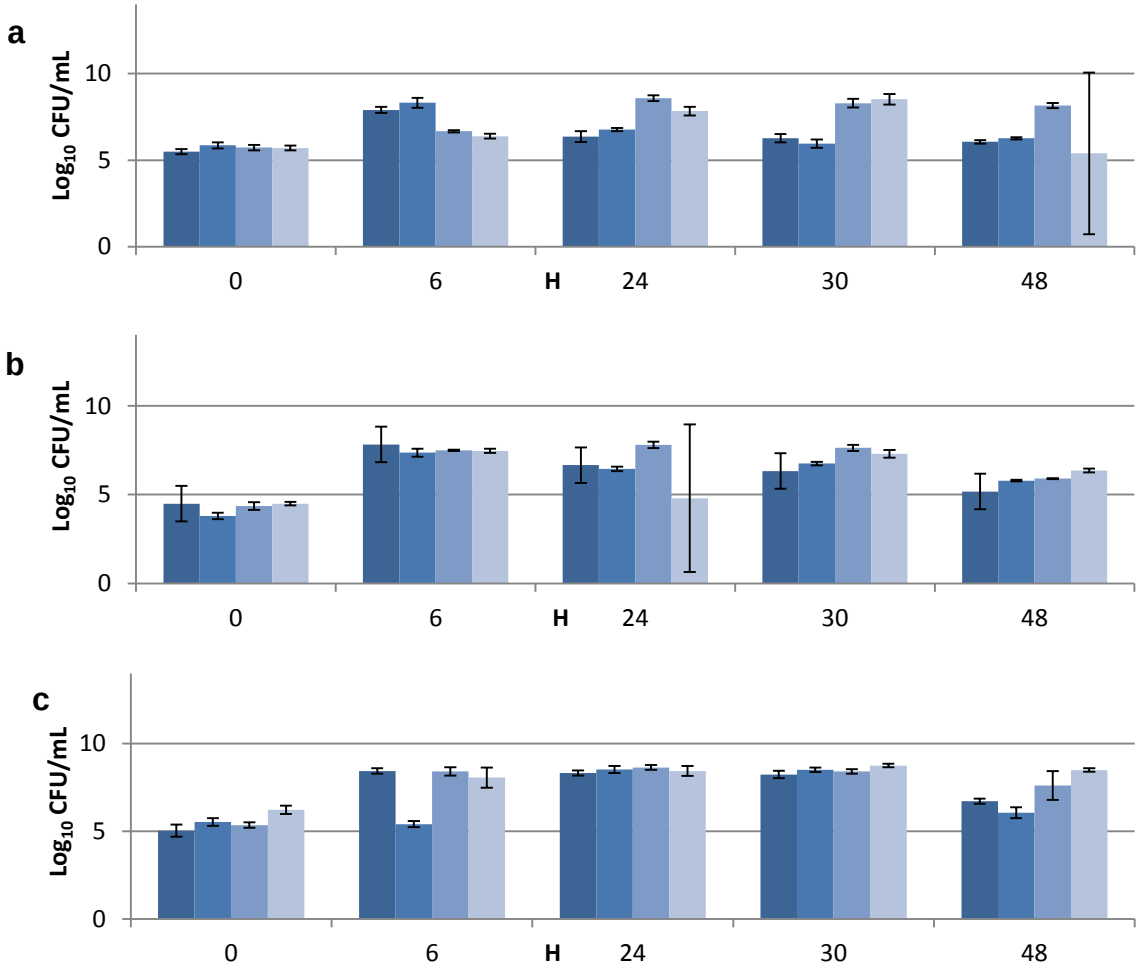


Fig 5.8. a-c. The effect of phage therapy on levels of total aerobes. Figures represent plate counts of replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are means of triplicates ± SD.

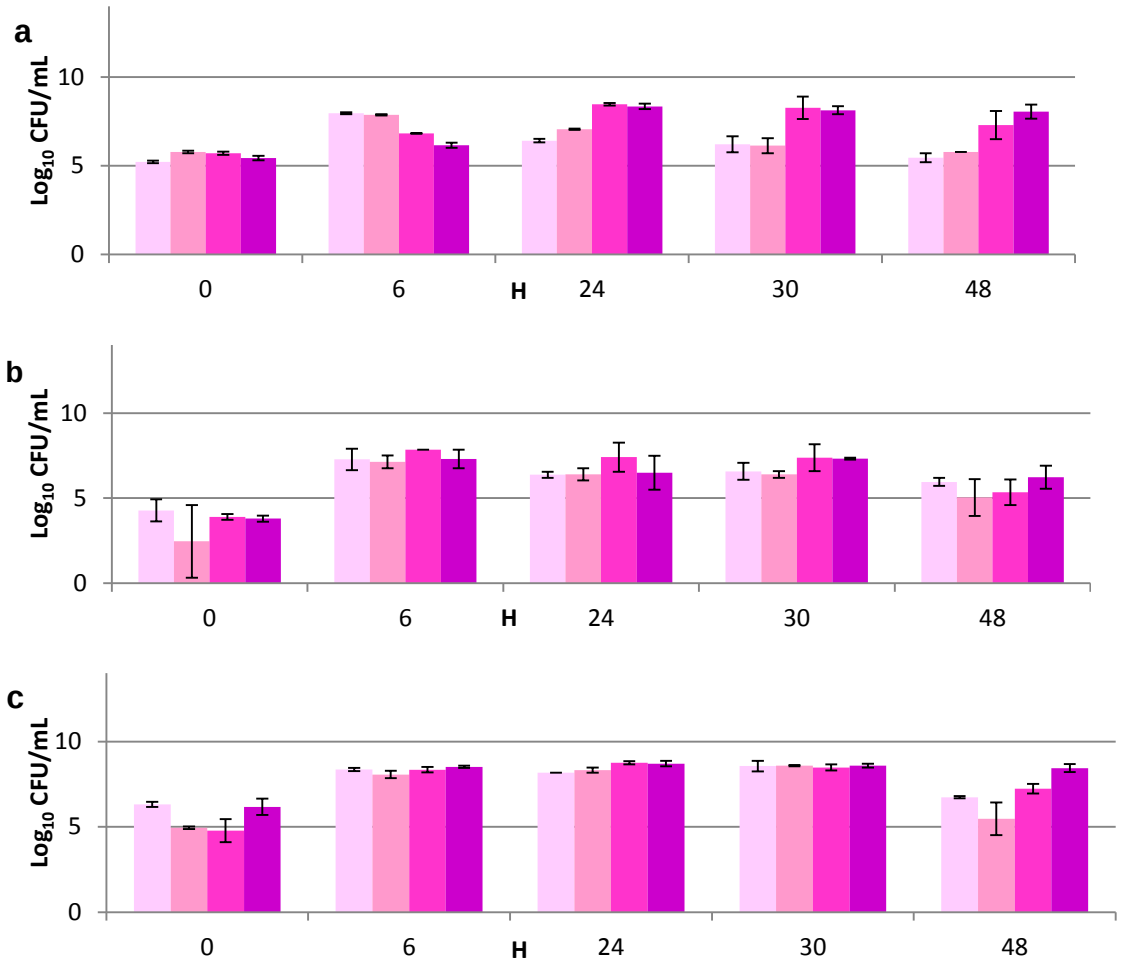


Fig 5.9. a-c. The effect of phage therapy on levels of *Enterobacteriaceae*. Figures represent plate counts of replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are means of triplicates ± SD.

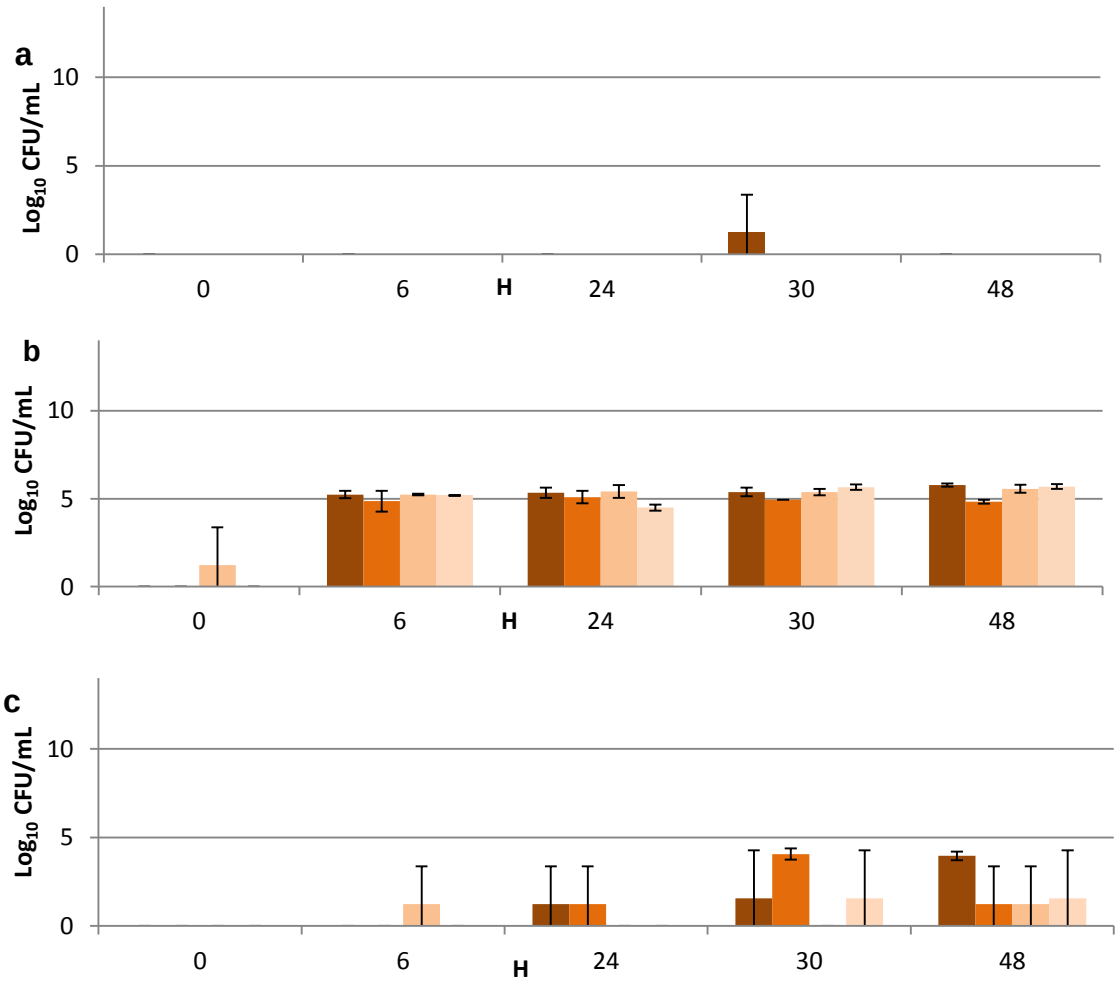


Fig 5.10. a-c. The effect of phage therapy on levels of total Gram-positive cocci. Figures represent plate counts of 4 replicate experiments of 4 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are means of triplicates ± SD.

The batch fermentation system was also used to model the effect of therapeutic doses of metronidazole on *C. difficile* and the gut flora over 48 h (Fig.5.11-19). The results show eradication of *C. difficile* after addition of the antibiotic, but also demonstrate a profound detrimental effect on some of the other bacterial populations. These findings are in agreement with those described previously (Freeman *et al.*, 2007). Levels of total *Clostridium spp*. were undetectable by viable counts after the first dose of metronidazole (given at 6 h after inoculation), and at the end of the fermentation (48 h) were at least

2 log_{10} lower than in the control vessel (p = <0.0001). *Lactobacillus spp*. were reduced by between 1 and 2 log_{10}, with *Bacteroides* spp. and *Enterobacteriaceae* being completely eliminated from the system (p = < 0.0001). The level of total anaerobes decreased by approximately 1 log_{10} throughout the 48 h fermentation compared to the controls, while aerobic populations were increased by about 1 log_{10} (p = <0.0001). Gram-positive cocci, which were present only transiently in the control vessels (and at 1 x 10^4 CFU/mL at most), showed a marked increase and finished at levels of 1 x 10^7 CFU/mL. Statistically, this increase was highly significant (p = <0.0001).

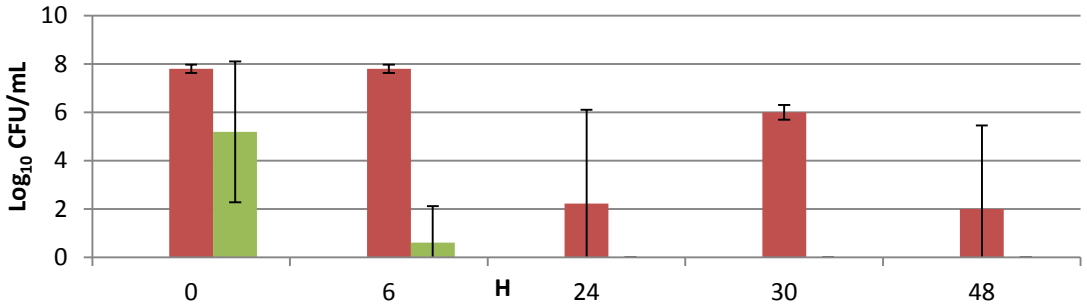


Fig 5.11. Effect of metronidazole therapy on levels of *C. difficile*. Figures represent plate counts of 2 replicate experiments of 2 vessels = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are the means of triplicates ± standard deviation.

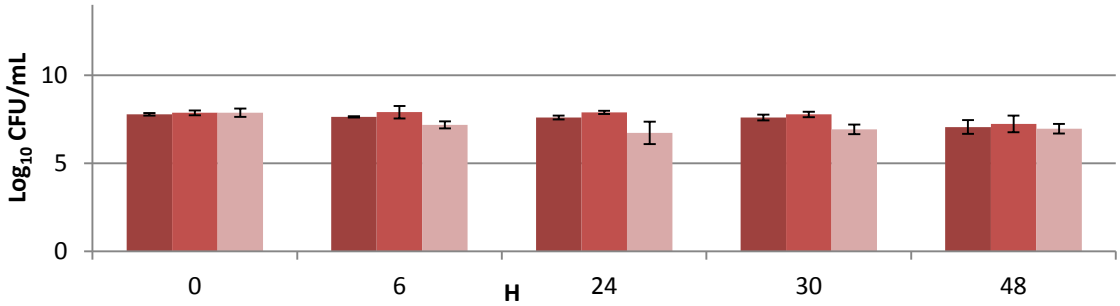


Fig 5.12. The effect of metronidazole therapy on levels of *Bifidobacterium spp*. Figures represent plate counts of 2 replicate experiments of 3 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) and = metronidazole treated vessel. Values are means of triplicates ± SD.

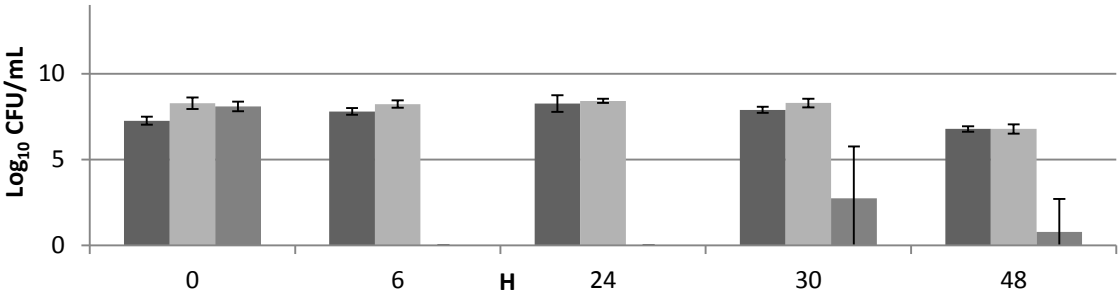


Fig 5.13. The effect of metronidazole therapy on levels of total *Clostridium spp*. Figures represent plate counts of 2 replicate experiments of 3 vessels = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are means of triplicates ± SD.

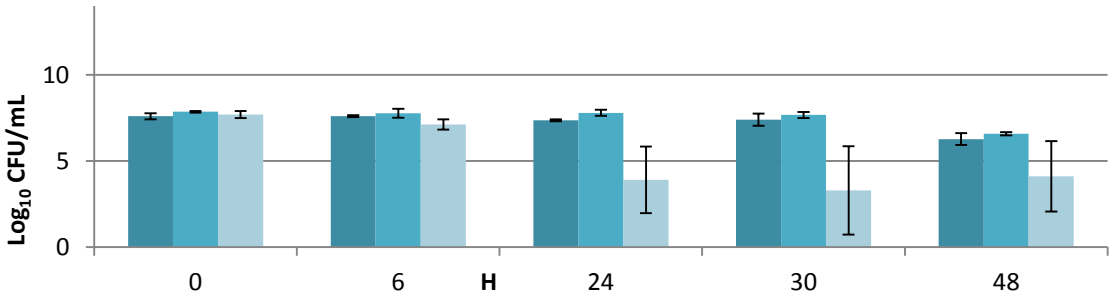


Fig 5.14. The effect of metronidazole therapy on levels of *Lactobacillus spp*. Figures represent plate counts of 2 replicate experiments of 3 vessels = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are means of triplicates ± SD.

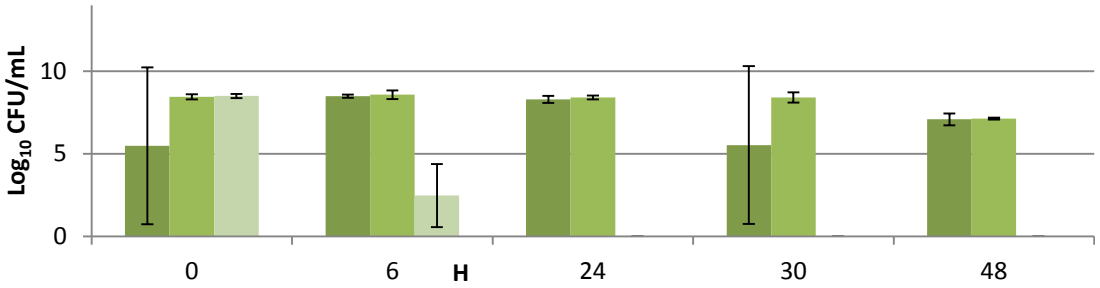


Fig 5.15. The effect of metronidazole therapy on levels of *Bacteroides spp*. Figures represent plate counts of 2 replicate experiments of 3 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are means of triplicates ± SD.

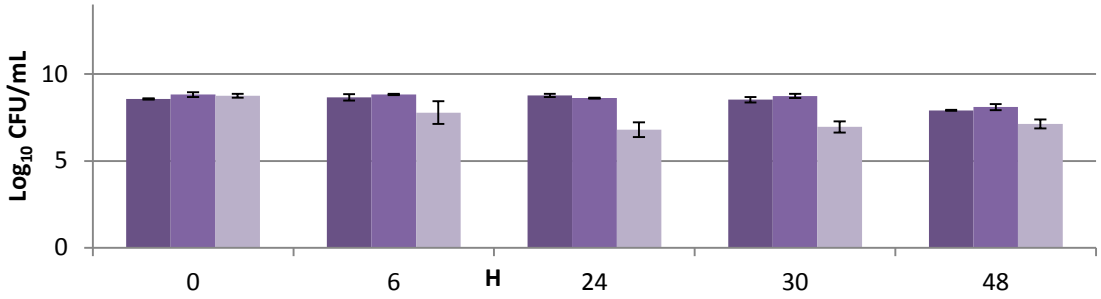


Fig 5.16. The effect of metronidazole therapy on levels of total anaerobes. Figures represent plate counts of 2 replicate experiments of 3 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are means of triplicates ± SD.

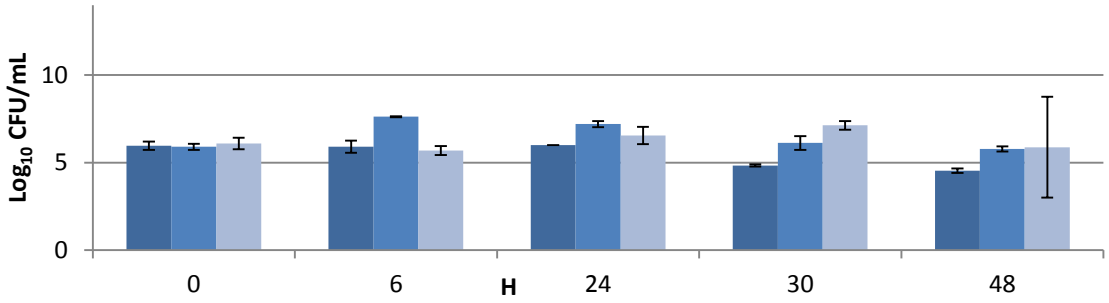


Fig 5.17. The effect of metronidazole therapy on levels of total aerobes. Figures represent plate counts of 2 replicate experiments of 3 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are means of triplicates ± SD.

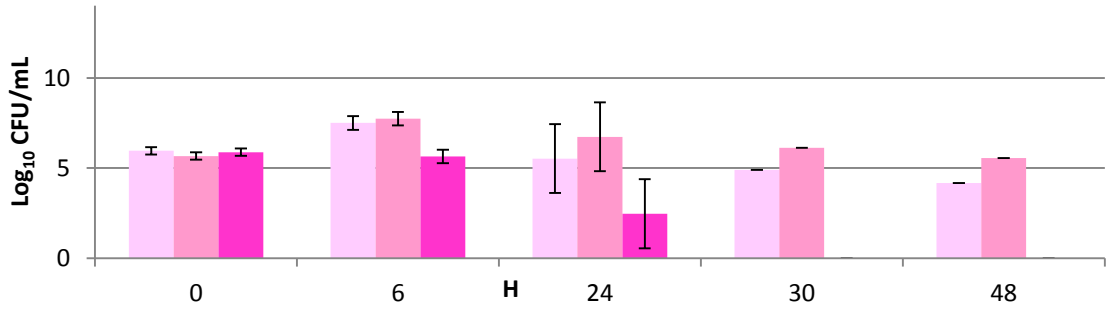


Fig 5.18. The effect of metronidazole therapy on levels of *Enterobacteriaceae.* Figures represent plate counts of 2 replicate experiments of 3 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are means of triplicates ± SD.

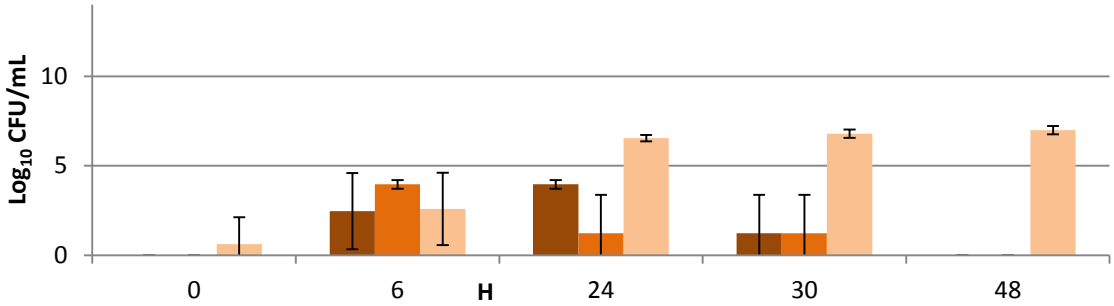


Fig 5.19. The effect of metronidazole therapy on levels of Gram-positive cocci. Figures represent plate counts of 2 replicate experiments of 3 vessels. = control (no *C. difficile*) vessel, = control (plus *C. difficile*) vessel and = metronidazole treated vessel. Values are means of triplicates ± SD.

5. 5. 2. *C. difficile* toxin production

The production of *C. difficile* toxins A and B were measured during replicate experiments 2 and 3 (Fig 5.20). As expected, no toxins were detected at any point in the experiment in the control vessel that was not inoculated with *C. difficile*. In the control vessel inoculated with *C. difficile* positive toxin results were obtained after 24 h fermentation, remaining until the end of the 48 h fermentation. Samples of batch fermentations from the remedial therapy schedule taken at 6 h onwards were all weakly positive for *C. difficile* toxin. In the prophylactic therapy schedule, toxin levels remained below the cut-off value of the assay throughout the experiment.

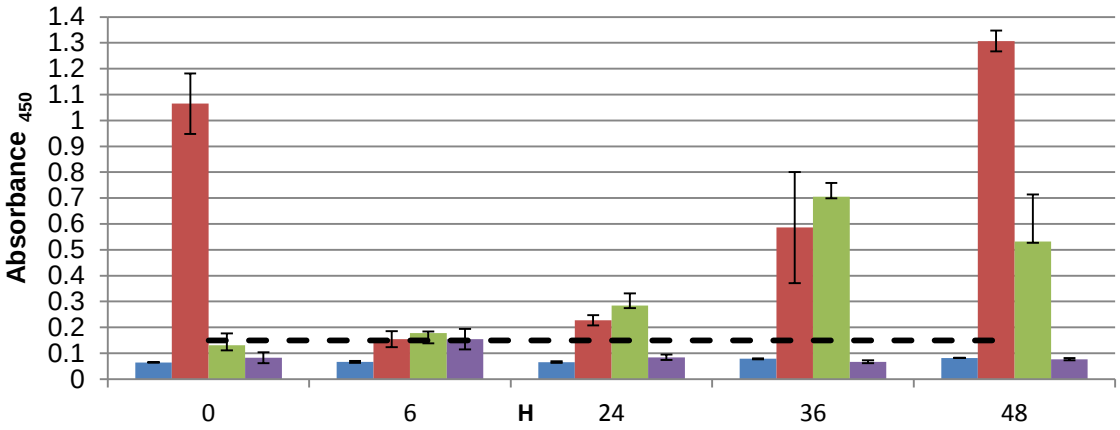


Figure 5.20. *C. difficile* toxin measurements during phage treatment (experiments 2 and 3). = Control (no *C. difficile)* vessel, = control (plus *C. difficile)* vessel, = remedial phage treatment schedule vessel and = prophylactic phage treatment schedule vessel. Values are means of triplicate readings ± SD. The dashed line represents the cut-off value of the assay.

In the metronidazole-treated fermentations, toxin production decreased following the commencement of the therapy but remained weakly positive throughout the experiment (Fig. 5.21).

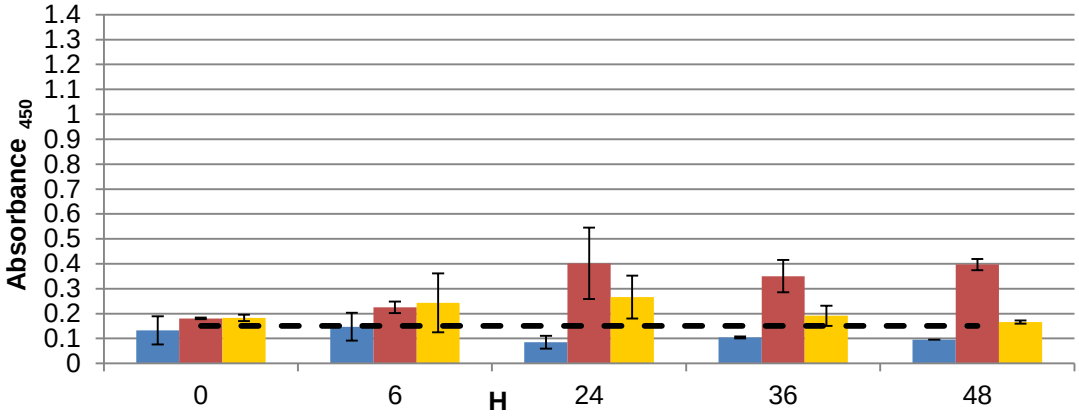


Figure 5.21. *C. difficile* toxin measurements during metronidazole treatment. = Control (no *C. difficile)* vessel, = control (plus *C. difficile)* vessel and = metronidazole treated vessel. Values are means of triplicate readings ± SD. The dashed line represents the cut-off value of the assay.

5.5.3. The effect of toxin and lysed *C. difficile* cells on commensal microbiota.

The numbers of faecal commensal bacterial groups isolated in the presence of a toxigenic strain of *C. difficile* and a non-toxigenic strain of *C. difficile* are shown in Fig. 5.22. A significantly higher number of *Lactobacillus spp.* were isolated 24 h after growth in a faecal fermentation containing non-toxigenic *C. difficile* relative to a toxigenic strain ($p = 0.003$), although this difference was less than 1 $\log_{10}$. A decrease of greater than 1 $\log_{10}$ was observed for total *Clostridium spp*. ($p = 0.002$) in the toxigenic culture. The group that seemed most sensitive to the *C. difficile* toxins was *Bacteroides spp*., for which a greater than 2 $\log_{10}$ reduction was recorded. These results suggest that *C. difficile* toxins may have a degree of antimicrobial activity, which has not been previously reported in the literature.

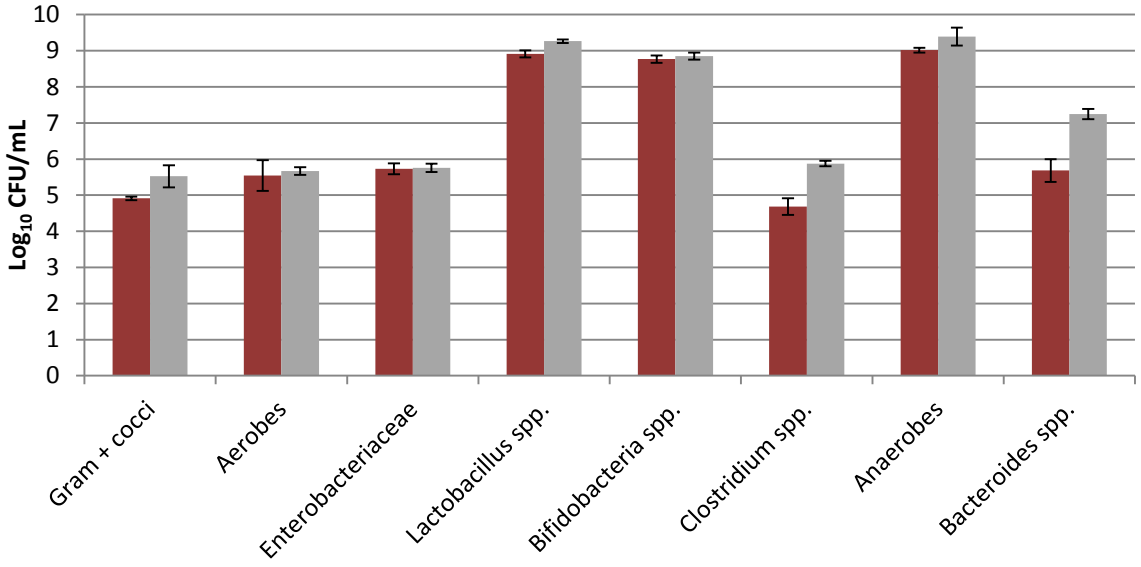


Figure 5.22. Growth of major bacterial groups grown in the presence of toxigenic *C. difficile* ( ) and non-toxigenic *C. difficile* ( ). Values are means of 3 replicates ± SD.

There were no significant differences in the numbers of commensals recovered from a 24 h faecal fermentation in the presence of live *C. difficile* cells, lysed *C. difficile* cells and PBS. These results are shown in Fig. 5.23.

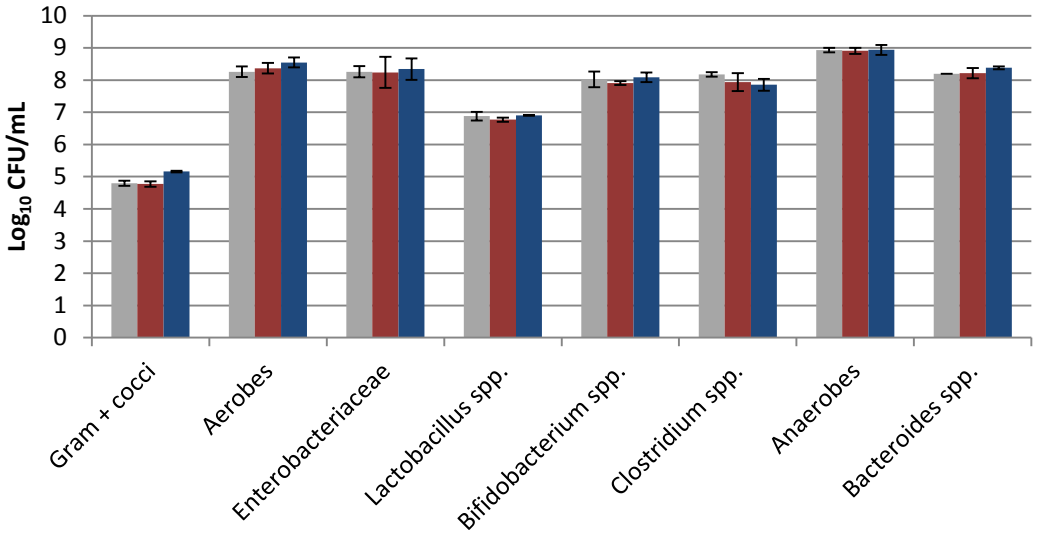


Figure 5.23. Growth of major bacterial groups grown in the presence of PBS ( ), live *C. difficile* cells ( ) and lysed *C. difficile* cells ( ). Values are means of 3 replicates ± SD.

5.5.4. Tests for lysogeny

In order to investigate the possible risk of the bacteriophage switching to a lysogenic life cycle during the treatment, 5 isolates of *C. difficile* from each batch fermentation vessel (in which *C. difficile* could be recovered), were induced using mitomycin C and tested for the presence of the phage. No phage could be found in the parent strain of *C. difficile* NCTC 11204 used for initial colonisation of the batch vessels. In all of the phage treatment vessels, 100% of the *C. difficile* strains isolated were found to contain ΦCD27 as a prophage (after mitomycin C induction). This rendered the *C. difficile* resistant to further treatment with ΦCD27, and is a likely explanation for the regrowth of *C. difficile* following the initial decline at the beginning of the treatment.

5.5.5. Phage replication in faecal samples

In order to verify that the ΦCD27 was not replicating in other faecal bacteria, approximately 1 x 10^7 PFU/ mL was added to a 10% suspension of faecal sample in batch fermentation broth and incubated anaerobically for 48 h. Enumeration of ΦCD27 throughout this period confirmed that there had been no significant increase or decrease in the phage titre (Fig. 5.24).

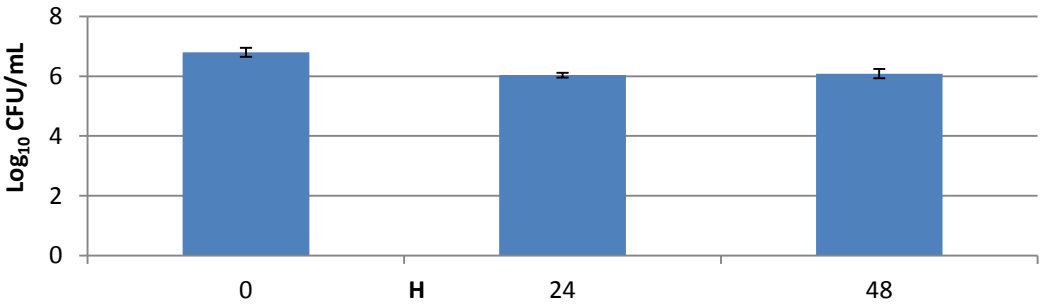


Figure 5.24. Enumerations of ΦCD27 recovered from a spiked faecal sample. Values are means of triplicate plaque assays ± SD.

5.5.6. Fluorescence *in situ* hybridisation

Cells of *C. difficile* labelled with the specific probe were visible under the fluorescent microscope in the neat culture suspension but not in any of the dilutions. This sensitivity was not acceptable for the purposes of the study and not superior to that of culture. It was therefore not pursued further.

5.6. Discussion

In this study we used an *in vitro* batch fermentation model to test the efficacy of specific bacteriophage treatment regimes in controlling the levels of *C. difficile*. Our results demonstrate that a prophylactic phage treatment regime resulted in a significant reduction in the levels of viable pathogens recovered. In addition, this reduction in *C. difficile* cell numbers also resulted in the suppression of toxin production. To a lesser extent, phages may help in the remediation of *C. difficile*-associated disease as this treatment regimen resulted in a less profound decrease in viable *C. difficile* and weak toxin production relative to an untreated control. It was interesting to note that metronidazole-treated vessels also retained weak toxin production throughout the therapy. Patients suffering from acute *C. difficile* infection are reported to excrete up to 10^4 to 10^7 organisms per gram of faeces (Rea *et al.*, 2007), although to our knowledge, there are no published reports of the levels expected within the colon. Enumerations from the control vessels show that levels surpassed 10^7 CFU/mL, indicating that these results have some clinical relevance, however, the perceived success of ΦCD27 in the reduction of *C.*

difficile numbers in the prophylactic regime may have been due to the changes in the growth phase and or gene expression as it adjusts to the batch fermentation media and fermentation by-products. The burden of *C. difficile* spores has not been investigated here (and this was also omitted from previous investigations of lacticin 3147 using batch fermentations (Rea *et al.*, 2007)). This is an important consideration as the presence of spores permits recrudescence of disease.

The actively growing *C. difficile* cells were added to a culture of faecal flora with no prior antibiotic treatment. The onset of *C. difficile* infection is normally preceded by the effects of a broad-spectrum antibiotic and so the numbers and proportions of commensal flora did not truly represent those of the diseased state. As with previous batch fermentation studies, the donor faecal sample was also from a healthy adult under the age of 65 years and therefore not in the risk group for development of CDI. This factor may have enhanced any therapeutic action of the phage, since normal commensal flora is itself associated with CDI resolution. The batch fermentations are maintained at pH 6.8 ± 0.2 and do not incorporate the acidic and proteolytic conditions of the upper gastrointestinal tract, which can be expected to have a detrimental effect on the viability of phages (Ryan *et al.*, 2011). Advances in microencapsulation (Cook *et al.,* 2012) and also the administration of neutralising agents may help to address this problem and aid delivery of the phage to the lower gastrointestinal tract. *In vitro* pH tolerance tests (4.5.4) have shown no reduction in the viability of ΦCD27 at pH 5 to 8, which encompasses the range encountered in the human gastrointestinal tract from the duodenum to the rectum (Fallingborg, 1999).

In both the prophylactic and remedial treatment models, the efficacy of phage treatment was improved by increasing the phage infection dose from 7 to 10 MOI. Integration of ΦCD27 into *C. difficile* NCTC 11204 was demonstrated for 5/5 of the *C. difficile* colonies isolated from the phage treated batch fermentations at the end of the experiment, when an MOI value of 7 was used. At higher MOI values no *C. difficile* was recovered at 48 h and therefore possible lysogens could not be observed. The lysogenic switch renders the host bacteria resistant to further phage treatment and this probably explains

the observed re-growth of the pathogen in the remedial treatment regime. The presence of a prophage may also protect the host from infection by other different phages (Serra-Moreno *et al.*, 2006). The ability of phages to lysogenise with bacterial hosts is a major disadvantage with regards to their therapeutic potential, and these data demonstrate evidence of this. Conversely, the rapid release of antigenic determinants and endotoxins brought about by *in vivo* lysis of pathogenic bacteria can sometimes initiate an overwhelming systemic immune response. This is known as the Jarisch-Herxheimer reaction and has been observed following antibiotic treatment of syphilis (Kobayashi *et al.*, 2011), Q fever (Aloizos *et al.*, 2008), Leptospirosis (Leblebicioglu *et al.*, 2003) and group B Streptococcal infection (Rac *et al.*, 2010). There is concern about the risk of similar reactions in the context of phage therapy (Dixon, 2004) and some efforts have focused on the production of lysis-deficient phages for therapeutic use (Hagens and Blasi, 2003; Paul *et al.*, 2011) with encouraging results. However, studies using lytic phages to treat canine ear infections caused by *Pseudomonas aeruginosa* have not reported any systemic reactions (Hawkins *et al.*, 2010). An alternative explanation for the incomplete elimination of *C. difficile* from the treatment vessels is the emergence of resistance to phage infection, as observed in other previous studies (Atterbury *et al.*, 2007; 1.9.5). It is probable that different phages and their bacterial hosts have a diverse range of optimal dosing schedules, and consideration should also be given to possible adherence of the phage to non-specific matter and how this affects the dynamics and interactions between phage and host.

Despite dosing with the same number of plaque-forming units, the prophylactic and remedial treatment routines used in this study gave dissimilar results in terms of the recovery of viable *C. difficile* and the production of toxin. In further support of the prophylactic regime, it should be considered that, *in vivo*, the increase in *C. difficle* numbers post-germination would actually be more gradual than the large inoculum of rapidly growing cells that was added to the vessels. *In vivo*, resolution of *C. difficile* would also be aided by inflammatory reactions and antibody responses to immunogenic determinants.

As expected, due to the relatively high host-specificity, the *C. difficile* phage did not have any significant detrimental effect on any of the other measured commensal bacterial populations. However, based on the large diversity of bacterial species in the human gut it can be expected that the growth and survival of many bacterial groups were not supported by the batch fermentation culture media. It should also be noted that many low G-C organisms have been found to be under-represented in *in vitro* incubations (Macfarlane and Macfarlane, 2007) and despite continuous nitrogen purges, no anaerobic indicator was used in the vessels. Also, it is not known to what extent the immunomodulators produced in CDI could be expected to have a suppressive effect on commensal flora, but this is another factor to consider when interpreting *in vitro* data. (Also see 6.6.4 for further discussion of *in vitro* testing).

In the phage-treated batch fermentations, significant increases in the numbers of *Enterobacteriaceae* and total aerobes were recorded but despite this the magnitude of deviation from the control was less than 1 $\log_{10}$ based on the mean counts. This extent of variation has been observed between sequential replicates of the batch fermentation models (5.5) and for other bacterial counts using these models (Gibson and Wang, 1994, Olano-Martin *et al.*, 2000). Differences may have been brought about by the liberation of nutritional factors from lysis of the *C. difficile* cells although this effect was not reproduced in separate experiments (Fig. 5.24). A reduction in competition for nutrients in the enclosed batch system could also have been a factor which may not occur *in vivo*. Separate studies have suggested a significant antimicrobial effect of the *C. difficile* NCTC 11204 toxin on *Lactobacillus spp*, total *Clostridium spp*. and *Bacteroides spp*. but not *Enterobacteriaceae* or total aerobes (Fig. 5.23). The apparent antimicrobial activity of the toxins may be due to strain-specific factors such as bacteriocins.

In comparison to the effects of phage on commensal microbiota, the levels in the metronidazole treated batch fermentations were significantly reduced, presumably due to its broad antibacterial spectrum. The dramatic rise in Gram-positive cocci observed in metronidazole-treated vessels may have serious implications for patients colonised with vancomycin-resistant

Enterococcus spp. and may predispose to associated diseases and nosocomial spread (Al-Nassir *et al.*, 2008; Sethi *et al.*, 2009).

Other studies have used FISH to quantify components of the microbiota (Chassard *et al.*, 2008; Hidalgo *et al.*, 2012; Mandalari *et al.*, 2007, 2008), which also detects non-viable organisms. Detection of *C. difficile* by FISH was attempted but gave levels of sensitivity inferior to that of culture. This may have been due to lack of cell penetration due to ineffective lysozyme action or complete disruption of cell integrity due to over-treatment. Probe permeation is notoriously problematic in Gram-positive organisms (Franks *et al.*, 1998) but cell association with masking proteins, hybridisation temperature or strain specificity may have also prevented detection.

Batch fermentations have significant temporal limitations as these are closed systems and the nutritional value of the media is spent within 48 h. In the distal bowel however, substrate availability is limited and the conditions are similar to that of a batch culture (Macfarlane and Macfarlane, 2007). While these models lack the effects of metabolic, secretory and immunological factors of the gut, the media and conditions are carefully controlled and these studies are generally less variable than *in vivo* experiments, allowing associations and effects of interventions to be more easily identified.

Pre-emptive selective treatments such as phage therapy could dramatically reduce the burden of *C. difficile*-associated disease in health care settings, with no disruption to patients' normal gut microbiota and therefore no anticipated predisposition to secondary complications. The high specificity of phage therapy however, is a limiting factor in terms of the range of *C. difficile* strains that are affected. Bacteriophage cocktails could be used to defend against multiple strains and resistant bacteria, plus there is the possibility of synergistic use with other agents to enhance current treatment strategies.

In summary, this work has shown that: (i) prophylactic bacteriophage therapy resulted in a significant decrease in the number of viable *C. difficile* cells and gave negative toxin results (ii) remedial treatment of a colonised system resulted in a short-lived reduction in the number of viable *C. difficile* CFU's and reduced toxin production (iii) integration of ΦCD27 into the genome of *C.*

difficile NCTC 11204 was demonstrated after it was used for therapeutic treatment; (iv) ΦCD27 treatment did not detrimentally affect the abundance and proportions of selected groups of commensal flora, whereas (v) treatment of *C. difficile* with metronidazole in the batch fermentation system caused significant reductions in total *Clostridium spp*., *Lactobacillus* spp., *Bacteroides spp*. and *Enterobacteriaceae* plus a significant increase in the proportion of Gram-positive cocci relative to an untreated control. Supplementary studies also suggested that (vi) *C. difficile* toxins may exhibit some antibacterial activity.

Many of the limitations in this study have been addressed in subsequent experiments using a three-component continuous model colon.

Chapter 6 – Modelling phage therapy of *C. difficile* infection using an *in vitro* colon model

6.1. Abstract

The potential of ΦCD27 as a prophylactic agent was investigated using a three-component human colon model of *C. difficile* infection. This work builds on previous batch fermentation studies, which suggested that a prophylactic approach was more effective than a remedial regime. Phage treatment resulted in elimination of vegetative *C. difficile* cells in 2 of the 3 experiments, with clinically relevant suppression of toxin production relative to an untreated control in all experiments. No detrimental effect on commensal flora as measured by culture and molecular profiling techniques was observed. Despite this, an increase in the number of *C. difficile* spores was recorded as well as a strong predilection for phage integration, demonstrating the potential for recrudescence and acquisition of virulent genetic elements.

6.2. Introduction

Phage therapy has the potential to offer a highly specific method of reducing the burden of vegetative *C. difficile* cells. In batch fermentation models of CDI (Chapter 5), it was demonstrated that prophylactic treatment with ΦCD27 (as opposed to a remedial-based approach) resulted in a significant reduction of viable *C. difficile* cells and a reduction in toxin production with no detrimental impact on the main bacterial groups comprising commensal gut flora (Meader*, et al.*, 2010). These models were designed to represent a short window of acute CDI but in order to better evaluate the efficacy of a prophylactic phage-treatment regime, a more representative and temporally relevant experiment that can model the disease from colonisation through to active disease was required. Many *in vitro* and *in vivo* models of CDI development have been described to date:

6.2.1. The golden Syrian hamster model

This model is widely accepted as an accurate representation of human CDI and has been used extensively in the evaluation of promising therapeutic agents since it was first described (Small, 1968). Both male (Bartlett *et al.*, 1977) and female (Dvoskin *et al.*, 2012; Goulding *et al.*, 2009) hamsters have been used and are pre-treated with clindamycin at 15 mg/kg by subcutaneous injection (Freeman *et al.*, 2005) or oral gavage (Goulding *et al.*, 2009). After 24 h a spore suspension of 1 x 10^2-1 x 10^7 CFU/mL is administered by oral gavage and it is from this time that therapeutic agents are often given. Untreated animals typically develop diarrhoea (wet tail) and become moribund between 30-50 h post inoculation, at which point they are sacrificed. Histological evidence of inflammation in the ceca and colons can be observed post mortem (Goulding *et al.*, 2009), although disease predominantly affects the cecum (Abrams *et al.*, 1980). Using this model, different strains of *C. difficile* have shown significant variations in the severity and pathogenesis of disease (Goulding *et al.*, 2009).

The results of hamster model generally correlate well with data from clinical trials, as demonstrated by recent work on the novel therapeutic agent OPT-80 (Louie*, et al.*, 2009 a, 2009 b) although some agents have proved not to be

efficacious despite promising results in hamster models, as was the case with the toxin–binding protein, Tolevamer (Baines *et al.*, 2009). Hamsters also lack a cytokine response. In humans, particular cytokines, such as interleukin 1β, tumour necrosis factor α and interleukin 8 are released in response to *C. difficile* toxins and contribute to the pathogenesis of CDI. Differences in the profiles of these cytokines can have a considerable effect on the severity of disease in humans (Kelly and Kyne, 2011).

6.2.2. The mouse model

Murine models have also been used extensively to reproduce CDI and have the advantage of affecting both the cecum and colon of the animals, which is more representative of human disease. They also allow the generation of cytokine profiles, although there have been some differences between murine levels and the results of human studies with regards to levels of tumour necrosis factor alpha (TNFα) and interferon gamma (IFN-γ) (Ishida *et al.*, 2004; Pawlowski *et al.*, 2010), both important mediators of inflammation.

Various murine lines have been used including gnotobiotic C57BL/6 mice, CF1 and CD1 mice. In the majority of studies, animals are pre-treated with an antibiotic cocktail, typically including kanamycin, gentamicin, colistin and metronidazole (Kang *et al.*, 2011) before clindamycin, given intraperitoneally (Jarchum *et al.*, 2011; Kang *et al.*, 2011), or by subcutaneous injection (Jump *et al.*, 2011). In some studies, no pre-treatment with antibiotics was employed (Pawlowski *et al.*, 2010).

Mice can also be used for a relapse model of CDI (Sun *et al.*, 2011) as the infection is less likely to be lethal, the severity being dependent on the dose of *C. difficile* with co-administration of antibiotics. Mice that recover suffer further episodes when subsequently challenged with antibiotics (although *C. difficile* was not proven as the cause in all cases) and antibiotics plus a *C. difficile* spore inoculum. Immunosuppressed patients are at a greater risk of severe and relapsing CDI, which has also been demonstrated in this model by adding dexamethasone to the drinking water. The results of this study indicate that neutralising antibodies to toxins A and B protect against CDI relapse and this has also been shown to be the case in humans (Kelly and Kyne, 2011).

6.2.3. Piglet models

The anatomical similarities between human and swine organs make them ideal candidates for models of human disease. Swine are also susceptible to CDI and it is regarded as an important aetiological agent of porcine neonatal diarrhoea (Post *et al.*, 2002; Songer, 2004; Waters *et al.*, 1998; Yaeger *et al.*, 2002). Pseudomembraneous colitis can also be induced in piglets following antimicrobial therapy (Steele *et al.*, 2010). Porcine models are not frequently used to evaluate novel therapies but have been used in competitive exclusion experiments where pre-colonisation with a non-toxigenic strain of *C. difficile* ameliorated the growth retardation associated with CDI in piglets after challenge with a toxigenic strain (Songer *et al.*, 2007).

Murine, hamster and piglet models all permit the assessment of stool consistency – an important marker of CDI in humans.

6.2.4. Guinea pig models

One of the first studies of antibiotic-associated enterocolitis, which provided evidence of a toxin-mediated effect, used guinea pigs that were given clindamycin with no prior inoculation of *C. difficile* (Knoop, 1979). Similar effects were observed with penicillin treatment (Lowe *et al.*, 1980), but these models were not subsequently used to the same extent as hamster or mouse models.

6.2.5. Ligated ileal loop models

Ileal loop models using rabbits and mice have previously been adopted for the study of *C. difficile* toxins and potential toxin antagonists (Carneiro *et al.*, 2006; Ketley *et al.*, 1987; Warren *et al.*, 2012). Ileums are typically flushed with PBS before loops are created with double ties. Toxin A with and without test agents is injected into the loops, which are then returned to the abdominal cavity. Further treatments are generally given by the intravenous route and assessments are typically achieved via toxin quantification of samples and detection or observation of inflammatory markers.

With these *in vivo* models, only the short-term effects on cells can be

investigated, whilst implications on the gut microbiota remain undetermined. Long-term effects cannot be evaluated and experiments may not even exceed the proposed treatment duration. Clearly, all model systems offer both advantages and disadvantages and the use of a range of models is required to demonstrate therapeutic potential and highlight some possible limitations.

6.2.6. The 3-stage continuous *in vitro* colon model

The 3 stage continuous system represents conditions in the large intestine from the proximal to the distal bowel. It was validated using human intestinal contents from the cecum/ascending colon and the sigmoid colon from 2 patients who died suddenly from coronary heart disease (Macfarlane *et al.*, 1998). The pH of these regions was measured and microbiological profiles were generated by culture of commensal bacterial groups. These continuous colon model systems correlate well with results from *in vivo* and human studies (O'May *et al.*, 2005 a, 2005 b) and have previously been used to evaluate the efficacy of therapeutic agents to eradicate intestinal pathogens such as *Campylobacter jejuni* and *Salmonella enterica* (Fooks and Gibson, 2003; Zihler *et al.*, 2010).

The colon model system was validated to model CDI (Freeman *et al.*, 2003). This group investigated the effect of various antimicrobial agents in relation to the risk of CDI onset (Baines *et al.*, 2005, 2006, 2009; Saxton *et al.*, 2009), the efficacy of current treatment strategies (Baines *et al.*, 2009, 2011; Freeman *et al.*, 2007) and novel therapeutics (Baines *et al.*, 2009). The model was shown to maintain a diverse microbial community throughout the experimental period, concordant with those from an *in vivo* Syrian golden hamster model (Freeman *et al.*, 2005) and serves as an excellent forerunner to animal experiments. It may be regarded as more temporally relevant to human disease, since the onset of toxin production and *C. difficile* proliferation in both the colon model and patients is generally 5-10 days after clindamycin therapy has ceased as opposed to 2 days in the animal models. This model has also been shown to correlate with clinical trials better than the hamster model in some cases (Baines *et al.*, 2009). Further to successful preliminary *in vitro* studies, hamster model results and phase II clinical trials of Tolevamer

(a toxin binding styrene derivative), the phase III clinical trials demonstrated inferiority to vancomycin. This poorer efficacy was subsequently demonstrated in the colon model and the reduced activity was proposed to be due to binding interference from the particulate matter (Baines *et al*., 2009).

The colon model offers an excellent simulation of the gut environment and it is of paramount importance to assess the activity of novel therapeutic agents in such conditions where the action of digestive enzymes, metabolites, surfactants or simply the abundance of bacterial flora can render a drug ineffective despite the results of promising *in vitro* tests, as demonstrated in a study of the lantibiotic lacticin 3147-producing *Lactococcus lactis* (Dobson *et al.*, 2011).

6.3. Objectives

- To determine the efficacy of a prophylactic phage treatment regime in the prevention of CDI using a 3-stage continuous culture *in vitro* model.
- To determine any significant changes to commensal gut microbiota as a result of phage therapy relative to an untreated control by culture and molecular profiling.

6.4. Methods

6.4.1. *C. difficile* strains and bacteriophage induction.

C. difficile strains 11204 and 12727 were obtained from the NCTC culture collection and maintained in Robertson's cooked meat media (SGL, Corby) at room temperature and grown anaerobically at 37°C in pre-reduced BHI (Oxoid, UK) with complements (2.5.2). Spore preparations of NCTC 11204 and NCTC 12727 were made as previously described (2.1.1.3).

Bacteriophage ΦCD27 was induced from *C. difficile* NCTC 12727 using mitomycin C and phage stocks were propagated by recovering phage particles from semi-confluent plaque assays (2.2.3.1). For the phage-free control broth, 15 mL BHI with complements was added to uninfected lawns of *C. difficile*, incubated and harvested in the same way as phage stocks.

6.4.2. The Colon Model

6.4.2.1. Apparatus

The colon model is a three-component continuous system designed to replicate the spacial, temporal, nutritional, physiochemical and microbiological composition of the human large intestine and is shown in Fig 6.1. Each model system was comprised of 3 linked vessels positioned in a cascade arrangement, pH controlled to 5.5, 6.2 and 6.8 respectively (± 0.2) to simulate conditions in the ascending, transverse and descending sections of the colon. The capacity of vessel 1 was 280 mL and the capacity of vessels 2 and 3 was 300 mL. Nitrogen was continuously bubbled through each vessel to maintain an anaerobic environment. Media was fed into vessel 1 at a constant rate, to give a retention time of approximately 66.7 h and waste from the last vessel was collected in an additional receptacle.

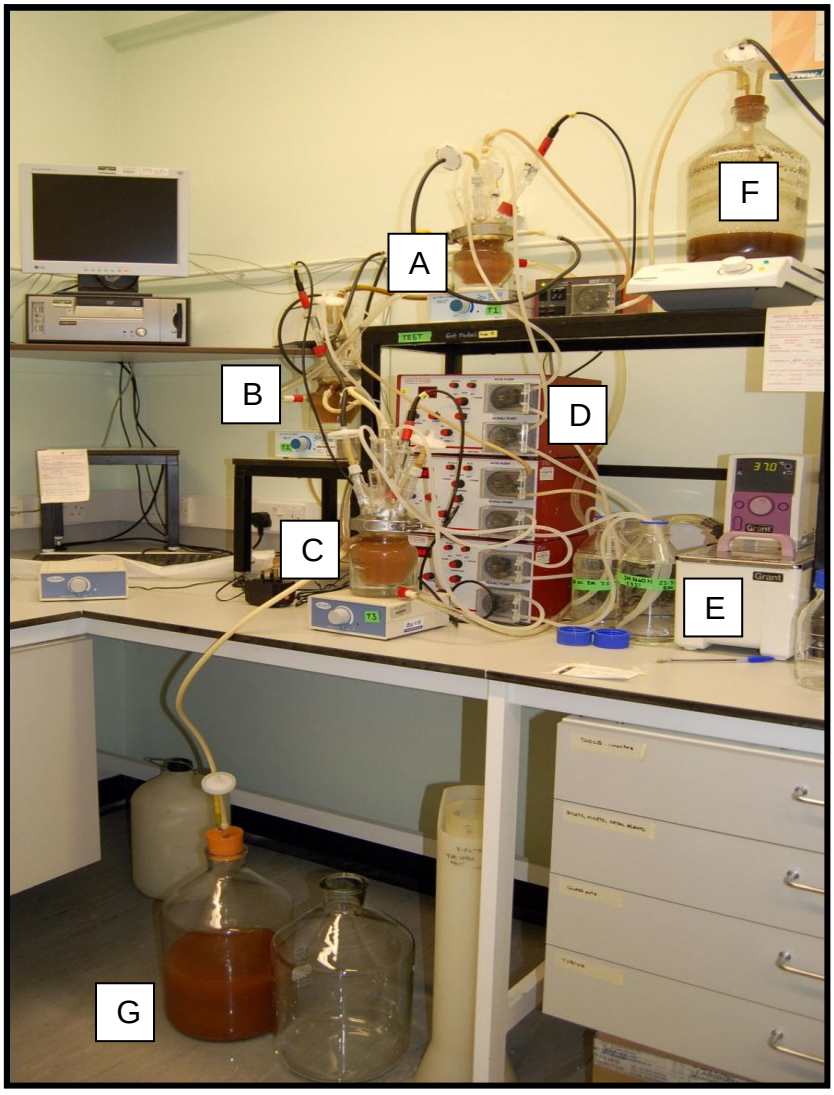


Figure 6.1. The colon model system. A. Vessel 1 (representing the ascending section). B. Vessel 2 (representing the transverse section). C. Vessel 3 (representing the descending section). Each vessel was pH controlled (D), maintained at 37°C using water jackets (E) and kept anaerobic via a continuous nitrogen purge. Media (F) is fed in to vessel 1 and waste is collected at the end of the system (G).

Freshly voided faeces from the same male volunteer aged over 60 years, was used to prime the system in each replicate experiment. Each experiment was comprised of 2 model systems – one to serve as a control and one to receive the prophylactic phage therapy and replicate experiments were carried out sequentially. The intervention points are indicted on the timeline below (Fig. 6.2). In the prophylactic phage treated system, phage preparations were

dosed at an MOI of 10 (calculated on the basis of the initial *C. difficile* inoculum) and were dispensed into vessel one (ascending section) every day for 42 days. The same volume of phage-free broth was administered to the control system. Samples (5 mL) were taken from all vessels of each system approximately every 3 days.

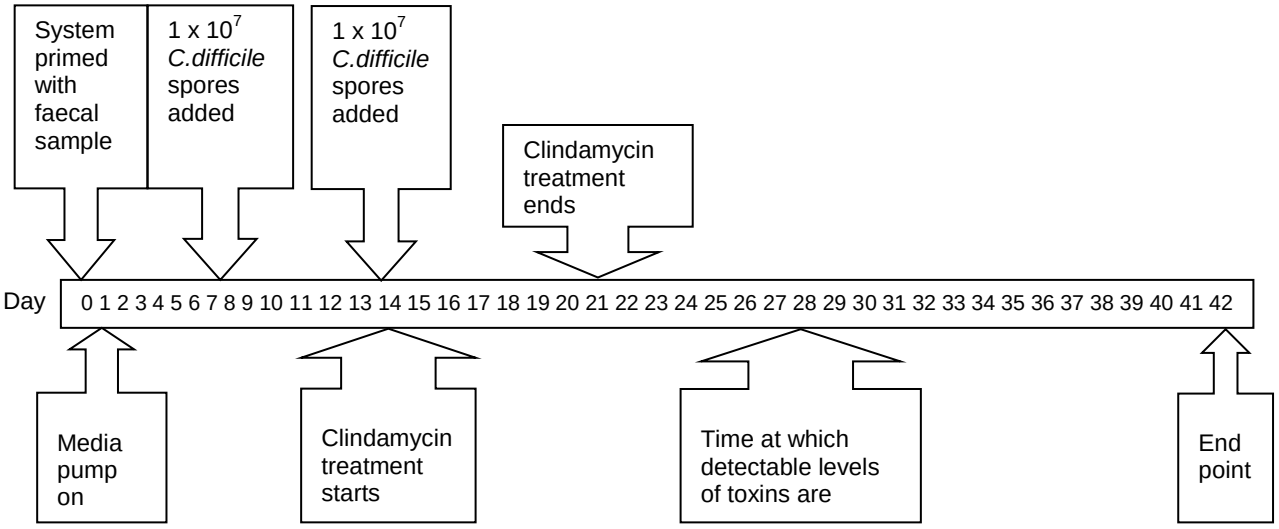


Figure 6.2. Timeline of colon model interventions.

6.4.2.2. Antibiotic treatments

Colon model systems were treated with 33.9 mg/L clindamycin every 6-8 h between day 14 and day 21 to disrupt the normal levels of faecal bacteria and thus permit the germination and outgrowth of *C. difficile*. The doses simulated the concentration expected in the human gut after a 600 mg oral dose (Baines *et al.*, 2008).

To ensure the correct concentration of clindamycin was reaching each part of the system, bioassays were carried out on filtered supernatants on day 1, 3 and 7 of the antibiotic course, using FI10640 *Micrococcus luteus* (kindly provided by D. Hatziioanou) as the indictor strain. Assays were performed in duplicate. 500µL of colon model samples were centrifuged for 2 min at 12,000 x *g* to remove bacterial cells and debris. The supernatant was removed, diluted in PBS and filter sterilised before adding 50 µL to a bored-out well in an MRS agar plate seeded with *M. luteus* (100 µL overnight liquid culture per 100 mL agar). Standard concentrations of 0.64, 1.25 and 1.56 µg/ml

(undiluted) of clindamycin were also tested with each batch of samples. Plates were kept upright for 15 min to allow the liquid to be absorbed and then incubated aerobically overnight. Zones of clearing around the wells were measured the next day and clindamycin concentrations were estimated using a calibration curve obtained from the known standards.

6.4.2.3. Enumeration of *C. difficile* cells and spores

Triplicate samples of 20µL from the colon model vessels were serially diluted to 10^{-7} in PBS and bacterial enumerations were carried out (2.1.1.2.2.1).

Growth curves were plotted and the area under the curve was used to represent the overall level of growth for the purposes of statistical analysis. All replicates of the control and the phage treated system were tested by means of a one-way ANOVA using a Wilcoxon Rank Sum test.

Spores of *C. difficile* were enumerated on CCEY as previously described (2.1.1.3.2).

6.4.2.4. Toxin quantification

Premier Toxins A & B enzyme immunoassay kits (Meridian Bioscience Incorporated), used according to the manufacturer's instructions were used to quantify the production of toxin in each vessel of each system. The method is described in 2.1.3.

6.4.3. Denaturing gradient gel electrophoresis (DGGE)

Bacterial population profiles from the control and phage-treated systems were obtained using DGGE, as previously described (Muyzer *et al.*, 1993) with some modifications. A range of methods to extract nucleic acid (2.3.2) were tested to determine the most suitable protocol.

6.4.3.1. Amplification of 16S rDNA

Primers targeting the V3 region of 16S rDNA (forward (including a GC clamp): 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTA CGGGAGGCAGCAG-3' and reverse: 5'-ATTACCGCGGCTGCTGG-3') were used. The composition per 50 µL reaction was as follows: 35.8 µL Sterile

water, 5 µL 10 x Hot Master Buffer, 4 µL dNTPs (2.5 Mm each), 1 µL each primer (10 µM), 0.2 µL Hot Master Taq Polymerase (5 PRIME), 1 µL BSA (10 mg/ml) and 2 µL DNA template. The presence of a product was confirmed by visualising on a 0.8% agarose gel.

6.4.3.2. Gel preparation, running conditions and investigation of discordant bands.

The BioRad D Code Universal Mutation Detection System was used to run the DGGE gels. PCR samples and DNA ladders (made from pooled amplified PCR products from a faecal sample) were applied directly to 8% (wt/v) polyacrylamide gels containing a gradient from 40% to 60% formamide (v/v) plus 0.08% (wt/v) ammonium persulphate (Sigma) and 0.14% NNN'N'-tetramethylethylenediamide (Fisher Scientific). Electrophoresis was carried out in 0.5 x TBE buffer (Fisher Scientific) at 59°C at a constant voltage of 80 V. The DNA molecules are immobilised at differing points in the gel depending on domain with the lowest melting temperature, as partial separation of the DNA strands prevents further migration. After 14 hours the gels were stained using SYBR Green (Invitrogen) for 30 min, rinsed with distilled water and visualised using the BioRad Pharos FX Plus Molecular Imager with an external laser at 65% voltage. Images were captured using Quantity One software and exported to Phoretix 1D Pro for band profile analysis. PAST software (version 2.14) was used to make statistical comparisons (Hammer *et al.*, 2001).

A representative sample of well-separated, discrepant bands (those present in the control vessels and absent in the phage-treated vessels or those present in the phage-treated vessels but not the control vessels) were excised using a UV transluminator (Dark Reader, Clare Chemical Research Ltd), re-amplified and re-run on a further 3 DGGE gels to obtain pure bands. Amplicons were cleaned using SureClean Plus (Bioline) (2.3.7), sub-cloned into pCR2.1 vectors using the TA cloning kit (Invitrogen) (2.3.11) and sequenced with the forward M13 primer (2.3.10). Good quality sequences were submitted to the Ribosomal Database Project 10 (Cole *et al.*, 2009), to obtain probable identities of the bacterial species corresponding to the band.

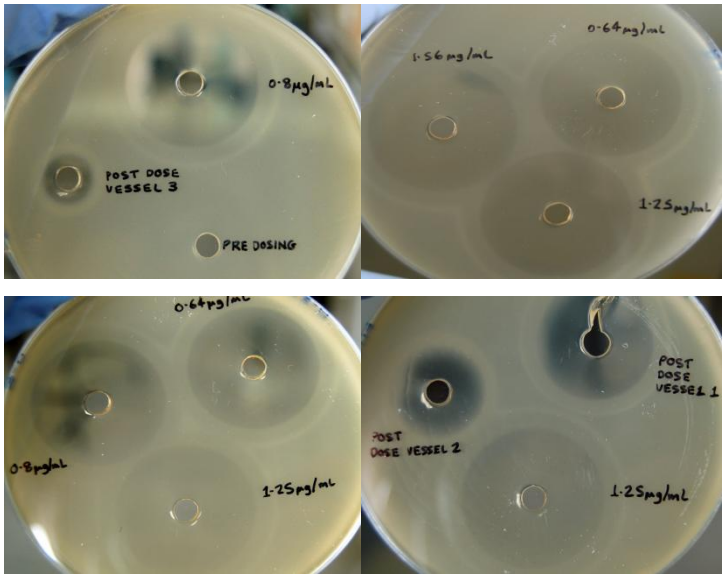
6.5. Results

A continuous colon model system was used to investigate the effect of phage therapy on *C. difficile* growth and toxin production.

6.5.1. Validation of colon model performance

Bioassays were carried out to determine the concentration of clindamycin in each colon model vessel. The use of *M. luteus* and clindamycin standards permitted the calculation of clindamycin concentration as shown in Fig. 6.3 a-b. The system required adjustments and extra doses to achieve levels approximating 33.9 mg/mL within the treatment timeframe as indicated in Table 6.1. The growth of *C. difficile* and commensal gut bacteria may have been affected by differing exposures to clindamycin despite efforts to control the concentration.

a



b

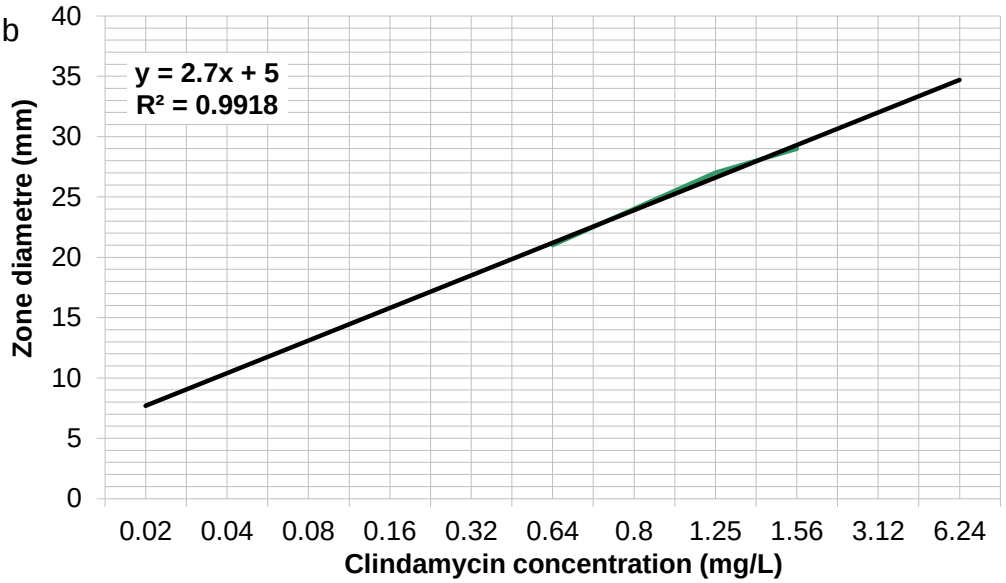


Figure 6.3 a) Example of bioassay results from experiment 2 and b) Calibration line made using measurements from the clindamycin bioassays.

	Vessel	72 h (mg/mL)	Adjustment	118 h (mg/mL)
Experiment 1	Ascending	0.0616	Decrease retention time (increase pump speed).	48.2
	Transverse	0.00575	None	29.9
	Descending	0.00075	Extra 9.4 mg dose	35.6
Experiment 2	Ascending	25.6	Extra 5 mg dose	29.3
	Transverse	5.6	Extra 5 mg dose	30.9
	Descending	1.2	Extra 5 mg dose	34.2
Experiment 3	Ascending	37.3	Extra 5 mg dose	35.9
	Transverse	16.7	None	32.3
	Descending	31.9	None	29.9

Table 6.1. Assessment and modulation of clindamycin concentrations of the colon model.

6.5.2. Reproducibility of results in the absence of phage treatment.

In each control system, vegetative *C. difficile* growth was detected after the clindamycin treatment. In order to determine the significance of reductions in *C. difficile* numbers due to phage therapy, it was necessary to establish the natural variability of the control systems observed in replicate experiments. In the ascending section (vessel 1), a significant difference in the numbers of *C. difficile* vegetative cells was recorded. A significant difference was also observed in the numbers of *C. difficile* spores from the ascending and transverse vessels, as shown in Table 6.2.

	P values (student's t test)		
	Experiment 1 and 2	**Experiment 1 and 3**	**Experiment 2 and 3**
C. difficile (vegetative) recovered from the ascending vessel	0.026437	0.062758	**0.002754**
C. difficile (vegetative) recovered from the transverse vessel	0.067053	0.15196	0.021408
C. difficile (vegetative) recovered from the descending vessel	0.132195	0.210368	0.151343
C. difficile (spores) recovered from the ascending vessel	0.011598	**0.003135**	0.011245
C. difficile (spores) recovered from the transverse vessel	0.007132	0.075667	**0.002219**
C. difficile (spores) recovered from the descending vessel	0.03411	0.016097	0.033666

Table 6.2: Extent of variation in *C. difficile* numbers between replicate control experiments. P values were calculated from total numbers throughout the experiment (area under the curve).

The numbers of *C. difficile* cells that were recovered are similar to the quantities that can be isolated from patients with active CDI and therefore have some clinical relevance (Mutters *et al.*, 2009). The pattern of germination post clindamycin treatment is comparable with the work of previous groups that have used the colon model to simulate CDI and the enumerations of vegetative *C. difficile* cells are also similar (Freeman *et al.*, 2003, 2007). Spore numbers differed significantly between replicate experiments (Table 6.2) and were generally present at levels approximately 1 $\log_{10}$ lower than previous reports (Freeman *et al.*, 2003, 2007).

6.5.3. Efficacy of phage therapy

A significant decrease in the total number of *C. difficile* vegetative cells was recorded in the phage-treated systems relative to the untreated controls ($p = < 0.001$). In the first replicate, phage treatment precluded the growth of *C. difficile* whereas in the control system, high numbers ($>10^5$ CFU/mL) were recorded less than 24 h after the clindamycin treatment ceased (Fig.6.4 a, 6.5 a and 6.6 a). The absence of vegetative *C. difficile* cells in the phage-treated systems was confounded by the significant increase in the number of spores relative to the control ($p = < 0.001$) (Fig. 6.7 a, 6.8 a and 6.9 a). In replicate 2,

phage treatment was not successful at preventing growth of *C. difficile* and levels comparable to that of the control system were recorded between days 24 and day 28 (Fig 6.4 b, 6.5 b and 6.6 b). Interestingly, on this occasion the phage-treatment yielded significantly fewer spores relative to the control system ($p = <0.001$) (Fig. 6.7 b, 6.8 b and 6.9 b). In the third replicate, phage treatment again precluded the proliferation of *C. difficile* while in the control system, vegetative *C. difficile* was detected at levels greater than 10^7 CFU/mL from day 28 (Fig. 6.4 c, 6.5 c and 6.6 c). As with replicate 1, the successful suppression of vegetative cells in the phage-treated system gave way to a significant rise in the overall levels of spore counts ($p = <0.001$) (Fig. 6.7 c, 6.8 c and 6.9 c).

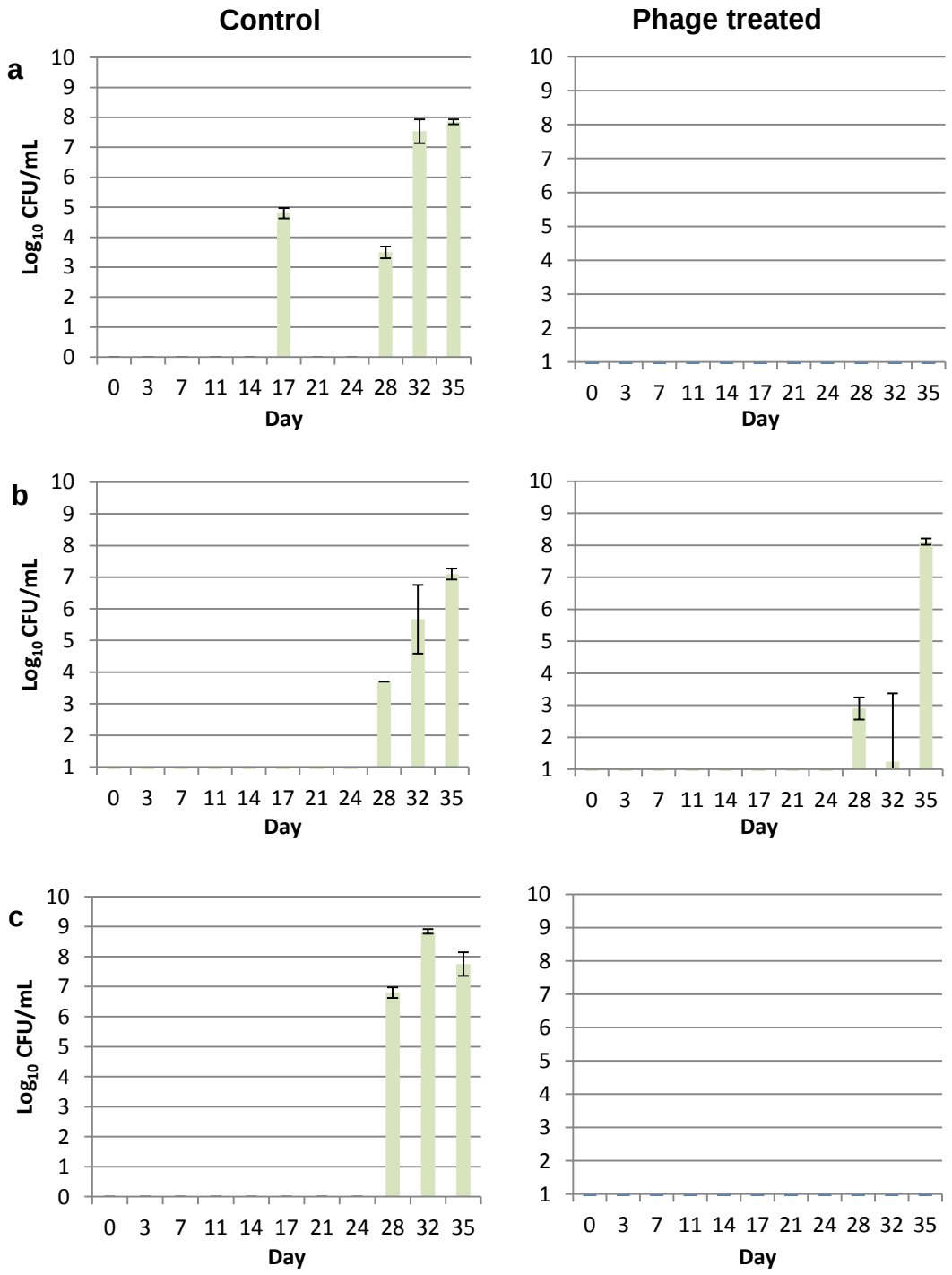


Figure 6.4 a-c. Vegetative *C. difficile* cell counts from the ascending vessels of the colon model replicates 1-3 respectively. Figures represent the means of triplicates ± SD.

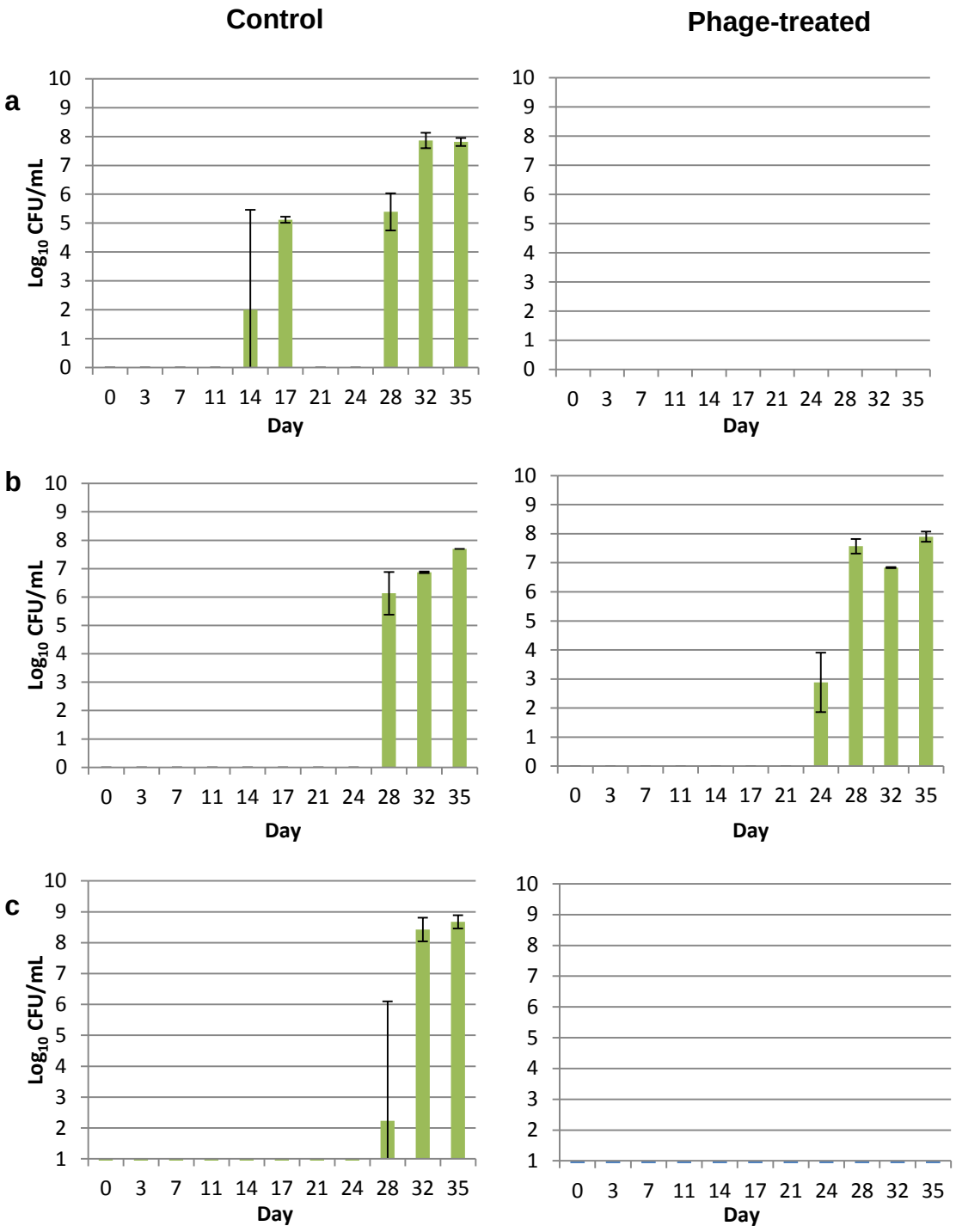


Figure 6.5 a-c. Vegetative *C. difficile* cell counts from the transverse vessels of the colon model replicates 1-3 respectively. Figures represent the means of triplicates ± SD.

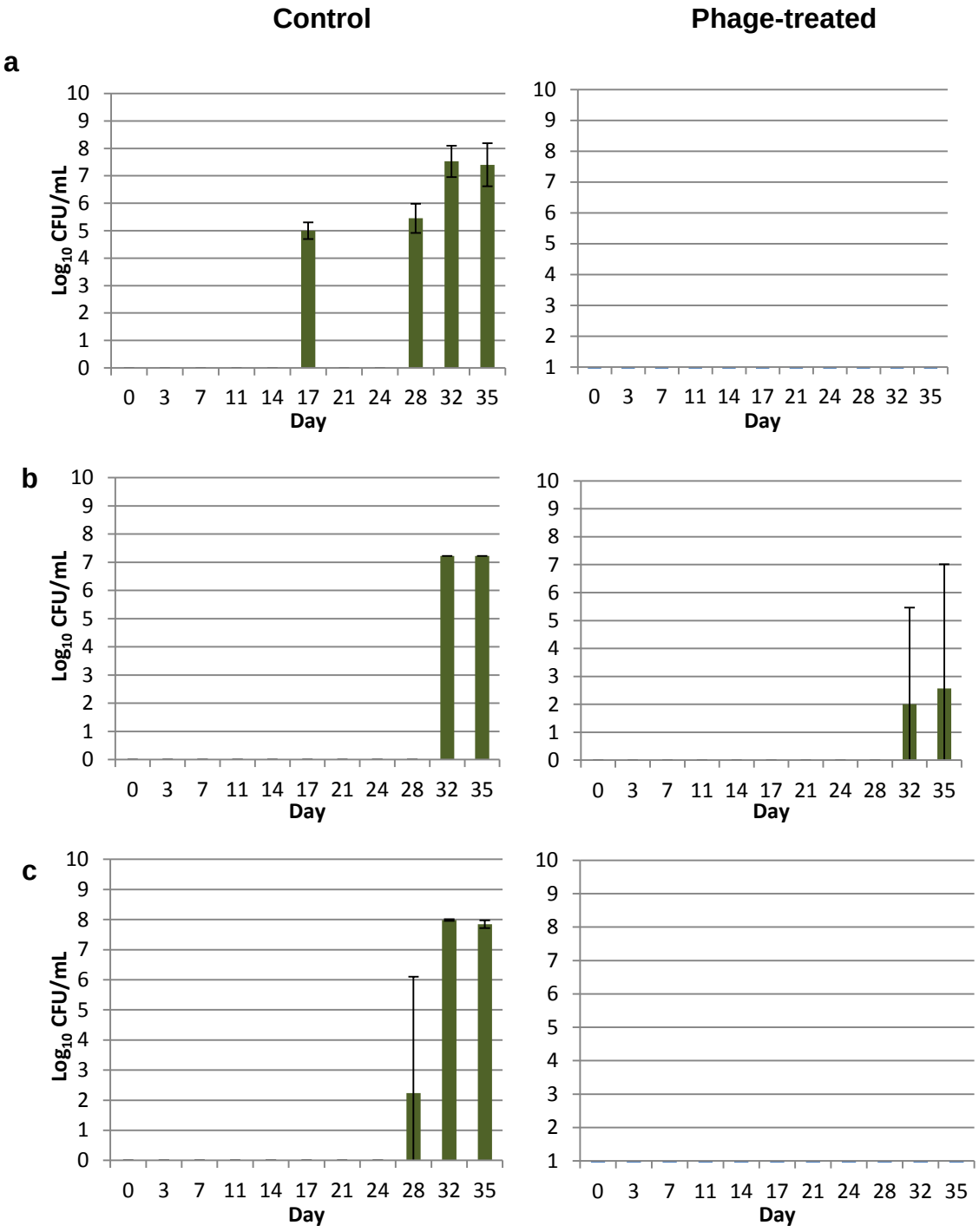


Figure 6.6 a-c. Vegetative *C. difficile* cell counts from the descending vessels of the colon model replicates 1-3 respectively. Figures represent the means of triplicates ± SD.

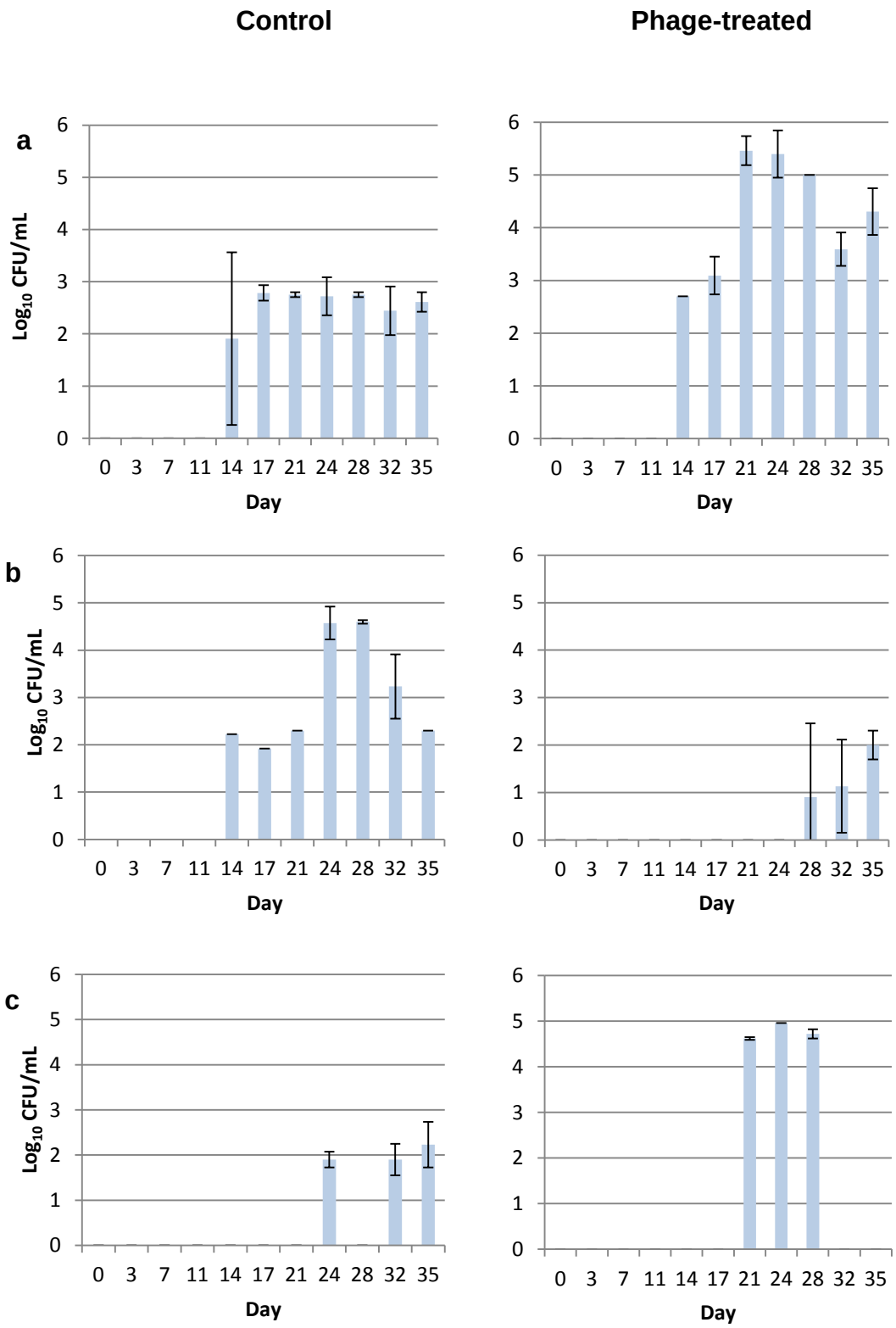


Figure 6.7 a-c. *C. difficile* spore counts from the ascending vessels of the colon model replicates 1-3 respectively. Figures represent the means of triplicates ± SD.

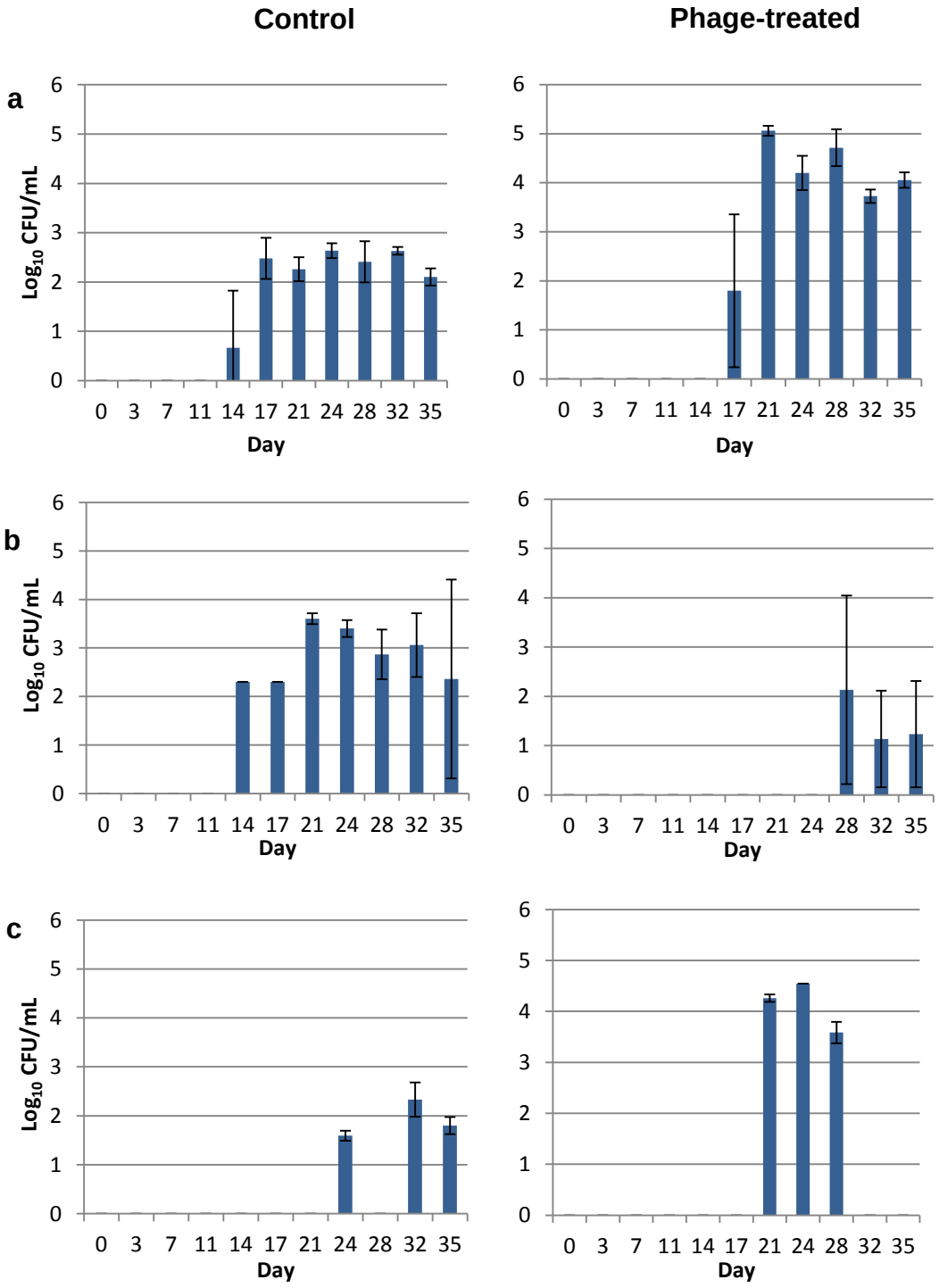


Figure 6.8 a-c. *C. difficile* spore counts from the transverse vessels of the colon model replicates 1-3 respectively. Figures represent the means of triplicates ± SD.

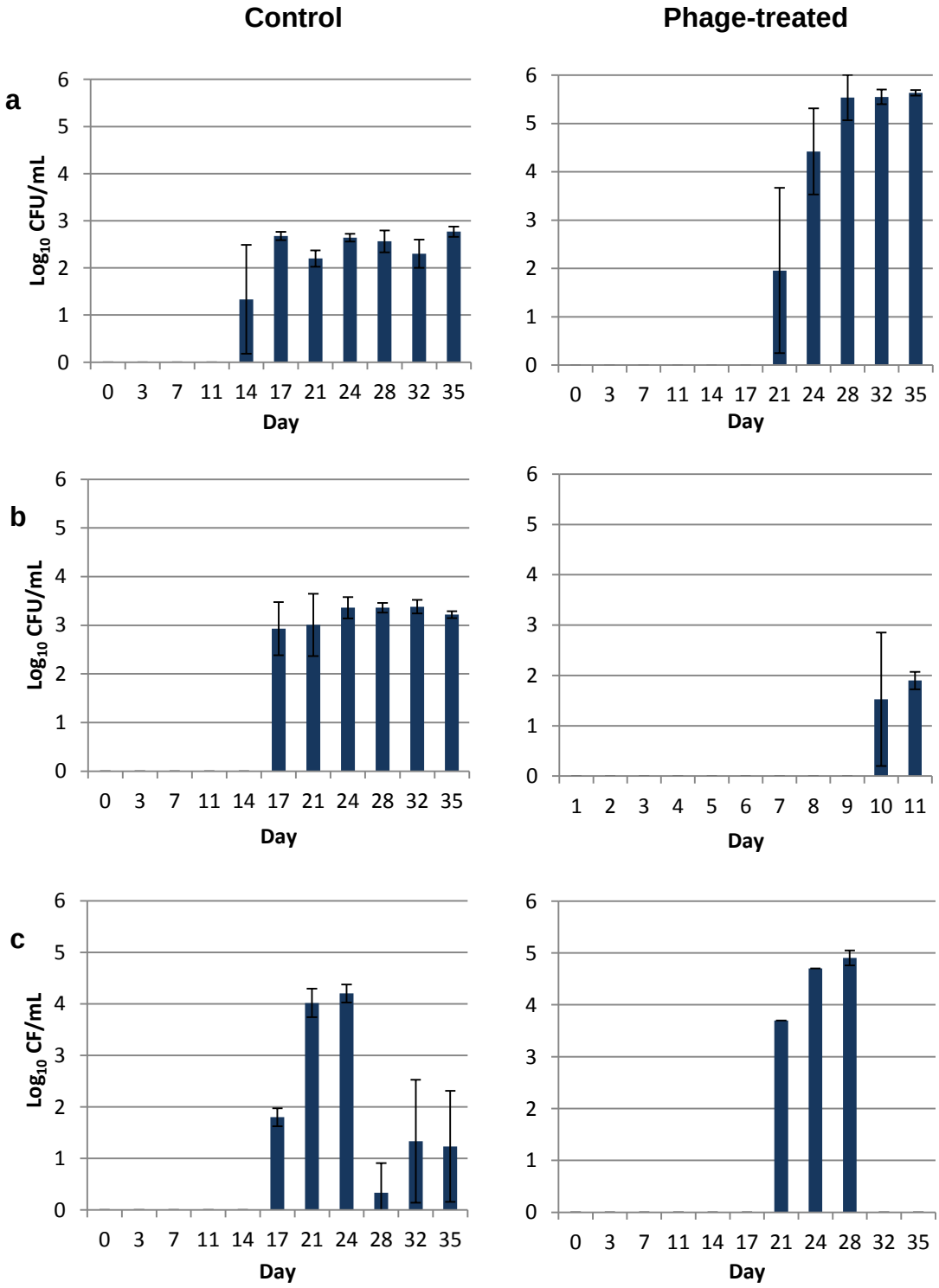


Figure 6.9 a-c. *C. difficile* spore counts from the descending vessels of the colon model replicates 1-3 respectively. Figures represent the means of triplicates ± SD.

6.5.4. Toxin measurements

Toxin production in the control system was strongly positive approximately 10 days following the cessation of clindamycin therapy (Fig. 6.10) and this correlated well with the spike in *C. difficile* numbers at this time. In the phage-treated systems however, toxin levels remained below the cut-off value of the enzyme immunoassay throughout the experiment, despite the presence of vegetative *C. difficile* at levels exceeding 10^8 CFU/mL.

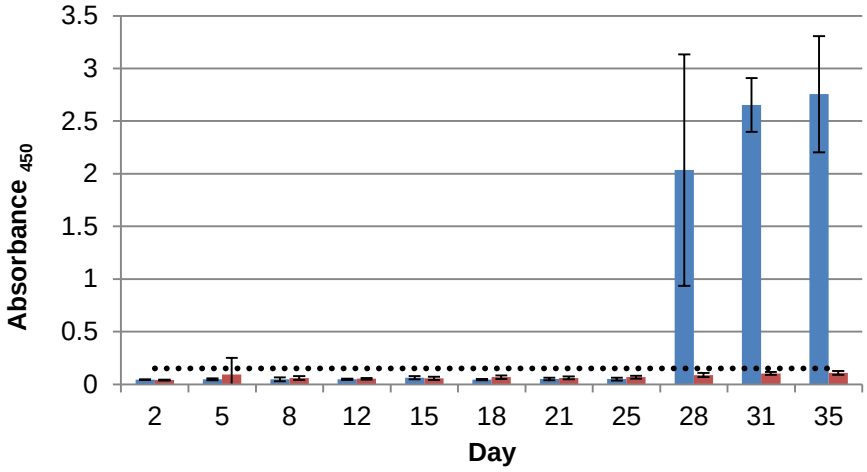


Figure 6.10: Mean toxin results from the descending sections of the control and phage-treated vessels ± SD. The cut-off value of the Meridian toxins A and B assay is indicated by the dashed line. = the control systems and = results from the phage-treated systems.

In order to investigate the high levels of vegetative *C. difficile* cells detected in experiment 2 (phage-treated), ten *C. difficile* colonies isolated from the transcending section of the phage-treated system were taken on day 35. Ninety percent of these were shown to harbour ΦCD27 as a prophage. The transition to a lysogenic life cycle is a likely explanation for the survival of *C. difficile* throughout the treatment and was investigated further by producing a lysogenic strain of NCTC 11204 *in vitro* by sub-culturing material from within lysis zones on a spot plaque assay, growing in BHI broth and confirming the presence of a prophage after mitomycin C induction. Relative to the wild-type strain, the lysogen produced less toxin *in vitro* (Fig. 6.11). The mean absorbance values (450 nm) of the toxin assay for 10 cultures of the lysogens and 10 cultures of the wild type were 0.75 (± 0.12) and 1.32 (± 0.19)

respectively (p = <0.001). *C. difficile* cells were not recoverable from experiments 1 and 3 at day 35 and could therefore not be examined for evidence of lysogeny.

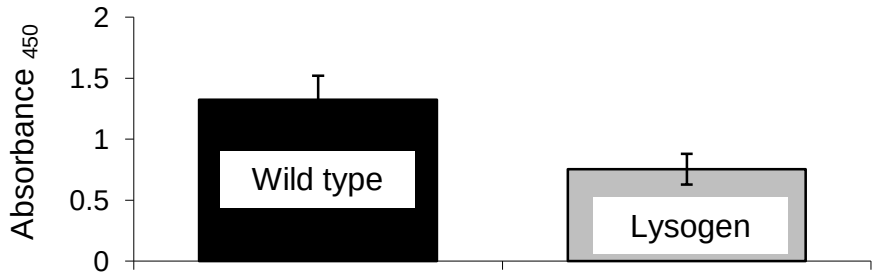


Figure 6.11: Toxin assay results of the supernatants of wild type and lysogenic cultures. Values are means of 10 replicate cultures ± SD.

6.5.5. The effect of phage treatment on commensal flora.

The effect of phage treatment on commensal flora was investigated by bacterial culture and molecular profiling using DGGE analysis. The culture results revealed no detrimental effect of phage therapy on any of the groups that were measured, but did show a significant increase in the number of *Bacteroides spp.* (p = 0.011) **(**Fig. 6.12-6.14**),** total aerobes (p = 0.001) (Fig. 6.15-6.17), and *Enterobacteriaceae* (p = 0.009) (Fig. 6.18-6.20) in the phage-treated vessels. A significant increase in the numbers of *Lactobacillus spp*. were also observed following phage treatment (p = 0.05), but this deviation seems to be due to the results of one replicate of the control system, in which numbers of *Lactobacillus spp.* had fallen to below the limit of detection by day 35 (Fig. 6.21-6.23). The figures illustrating the numbers of *Bifidobacterium spp*., total anaerobes, *Clostridium* spp. and Gram-Positive cocci (for which no significant changes were observed) can be found in Appendix 3.

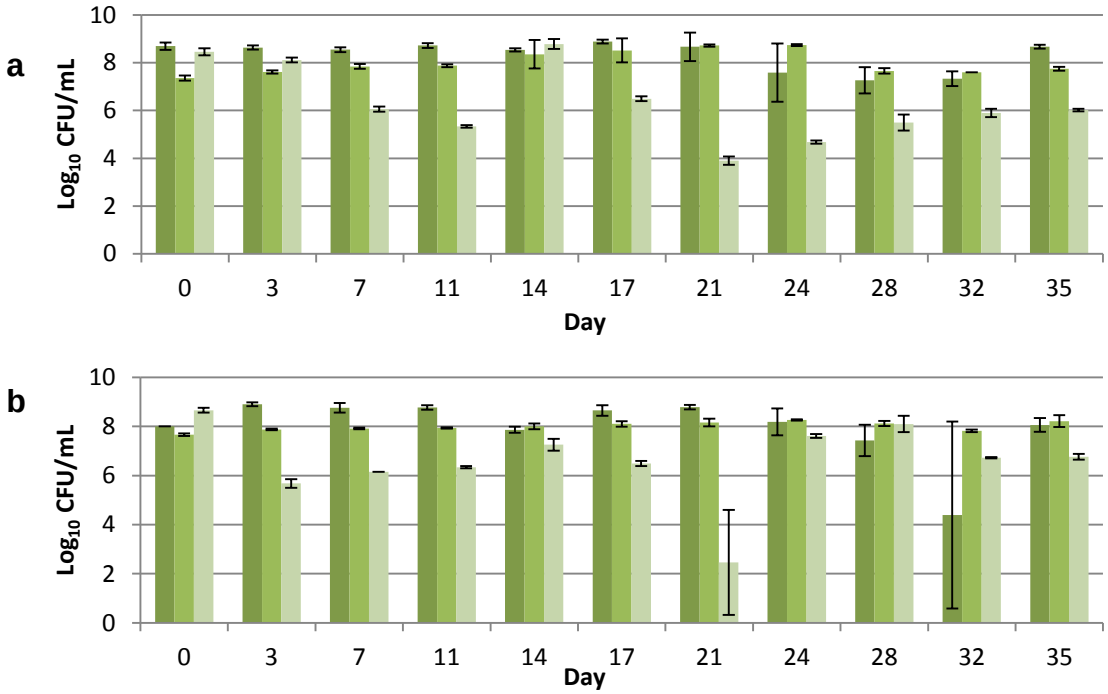


Figure. 6.12. *Bacteroides spp*. recovered from the control (a) and the phage-treated (b) ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3.

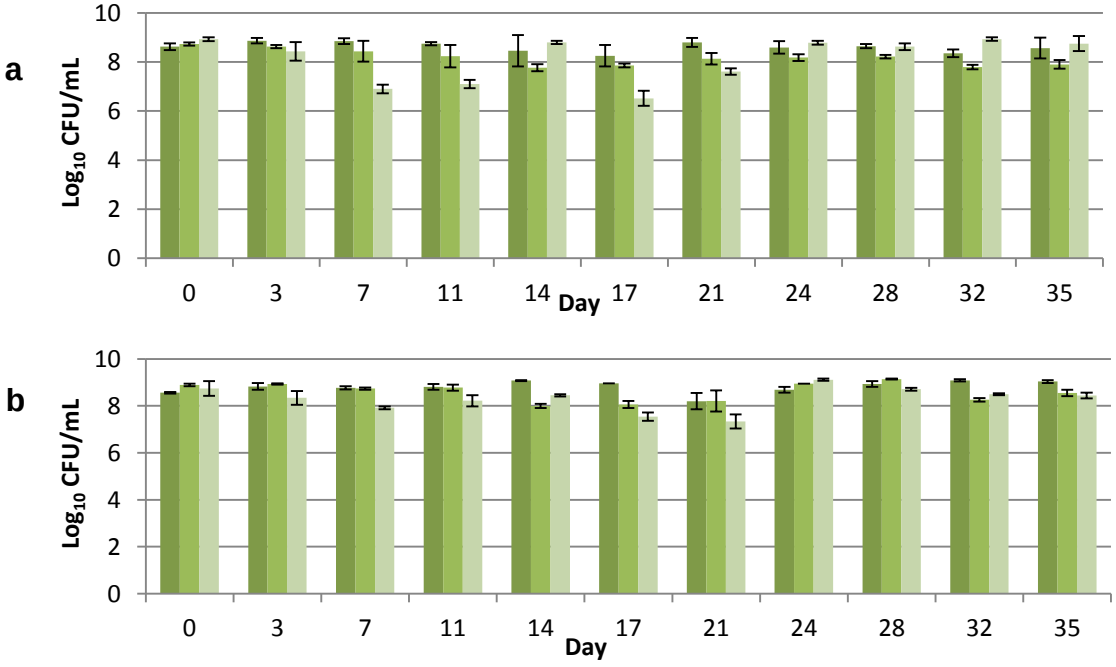


Fig. 6.13. *Bacteroides spp*. recovered from the control (a) and the phage-treated (b) transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

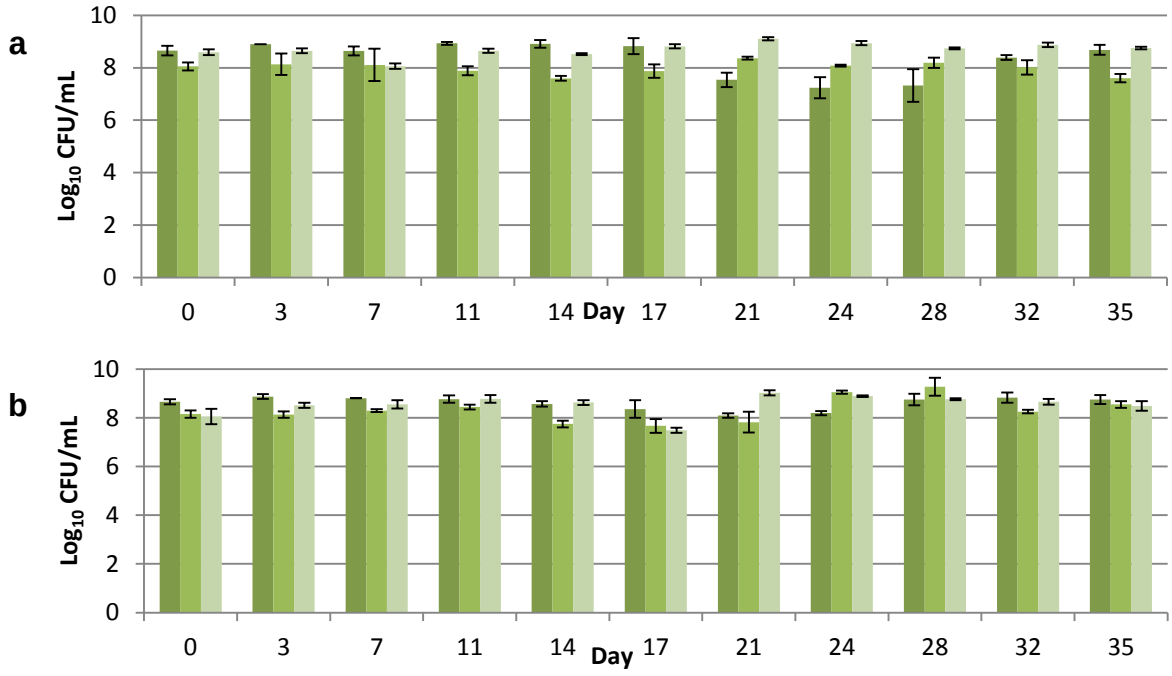


Fig. 6.14. *Bacteroides spp*. recovered from the control (a) and the phage-treated (b) descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

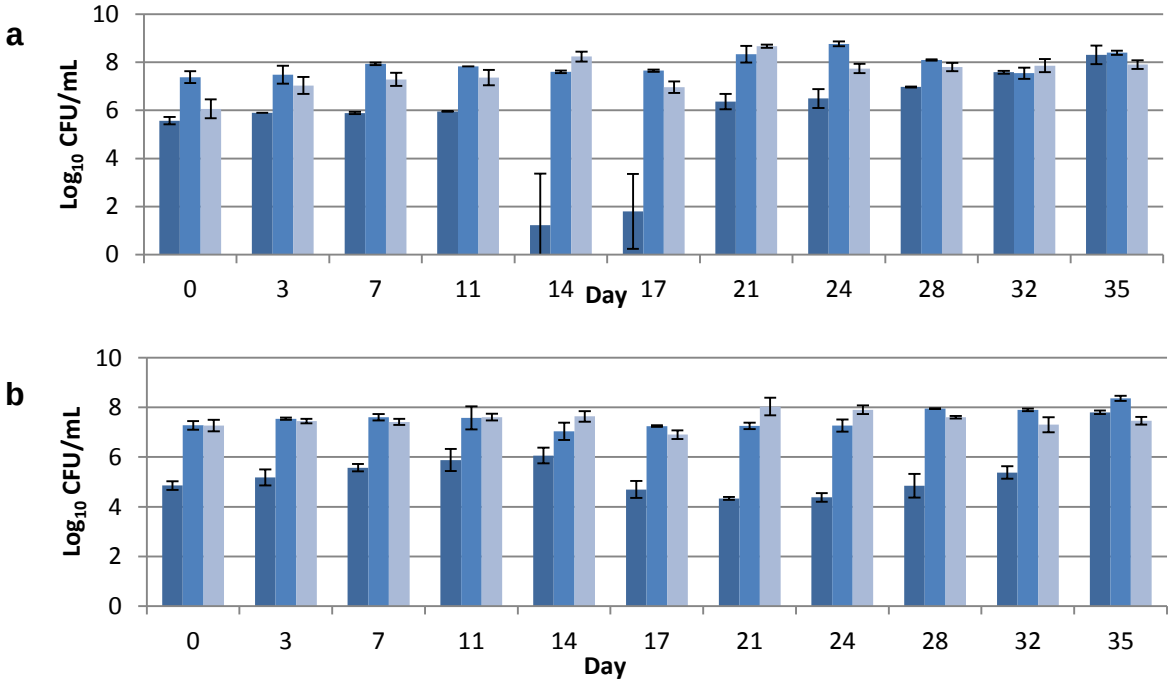


Figure 6.15. Total aerobes recovered from the control (a) and the phage-treated (b) ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

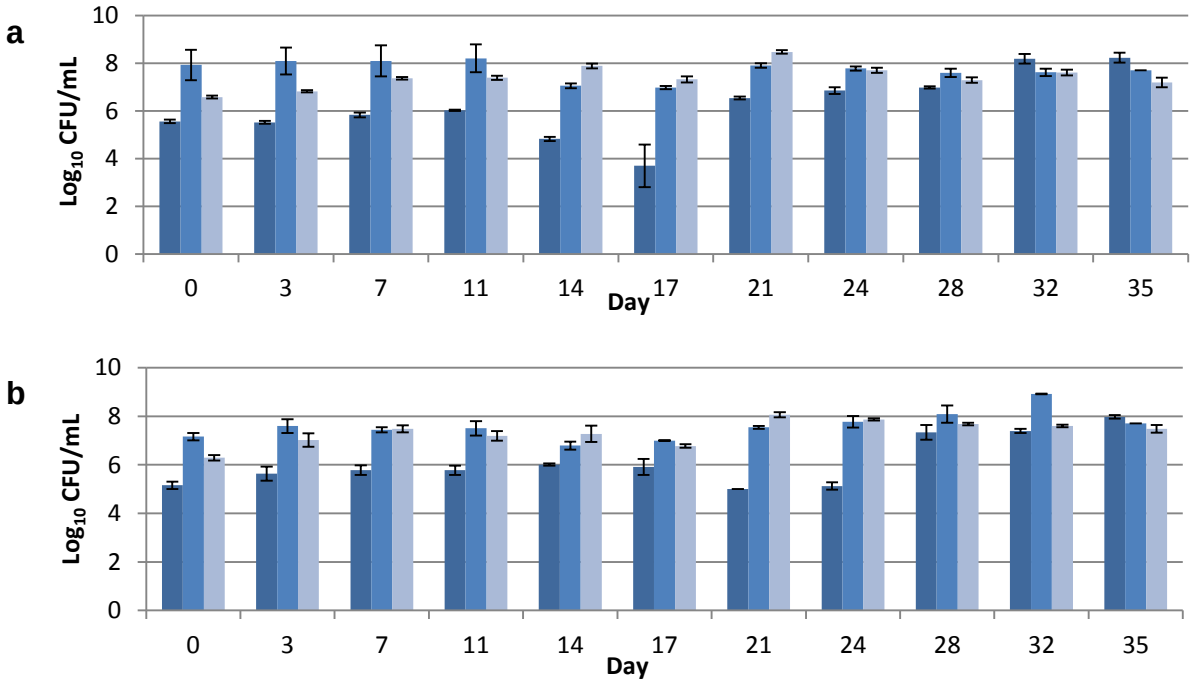


Figure 6.16. Total aerobes recovered from the control (a) and the phage-treated (b) transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

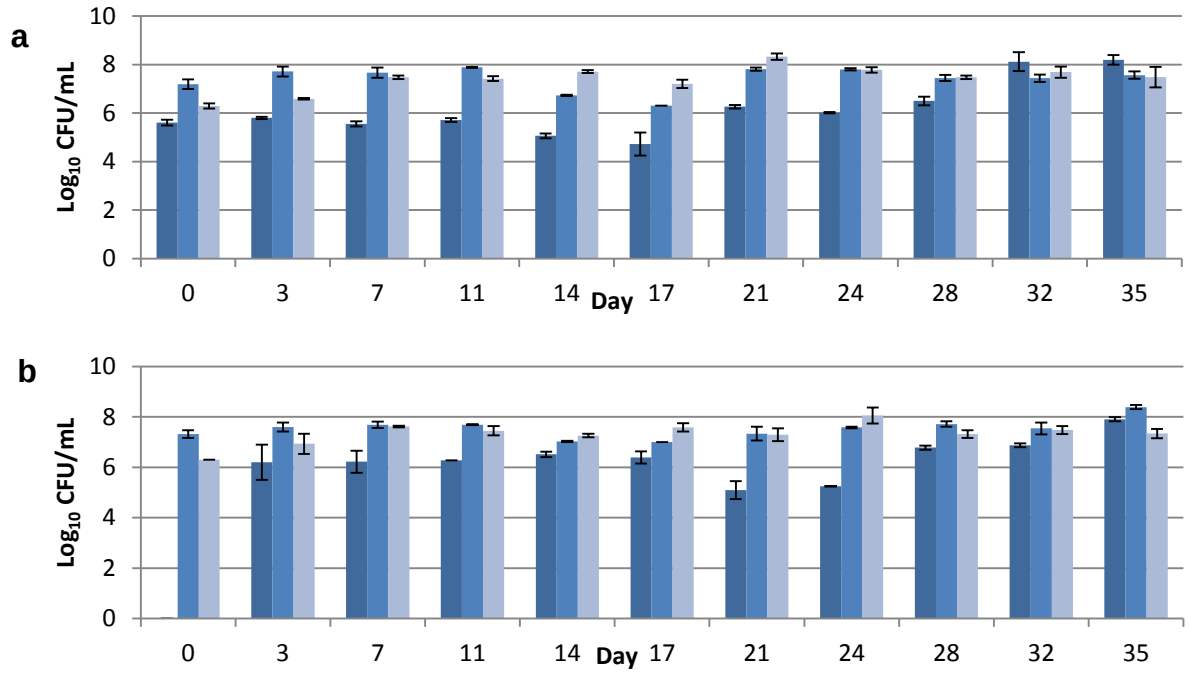


Figure 6.17. Total aerobes recovered from the control (a) and the phage-treated (b) descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

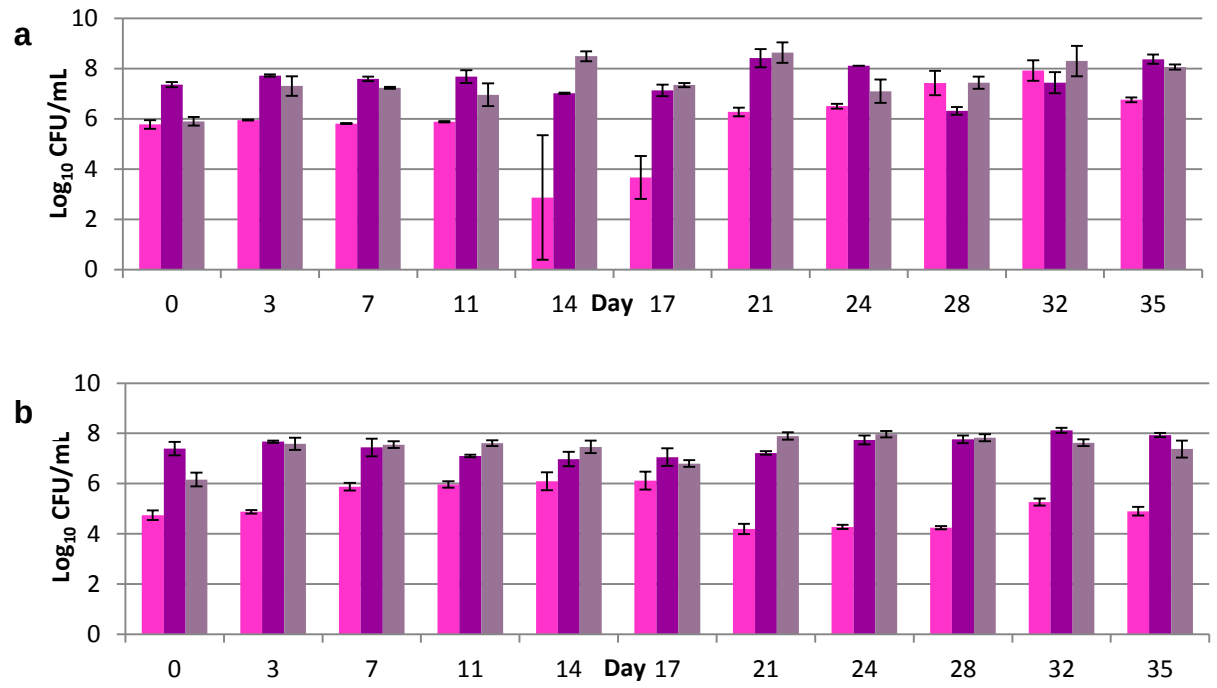


Figure 6.18. *Enterobacteriaceae* recovered from the control (a) and the phage-treated (b) ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

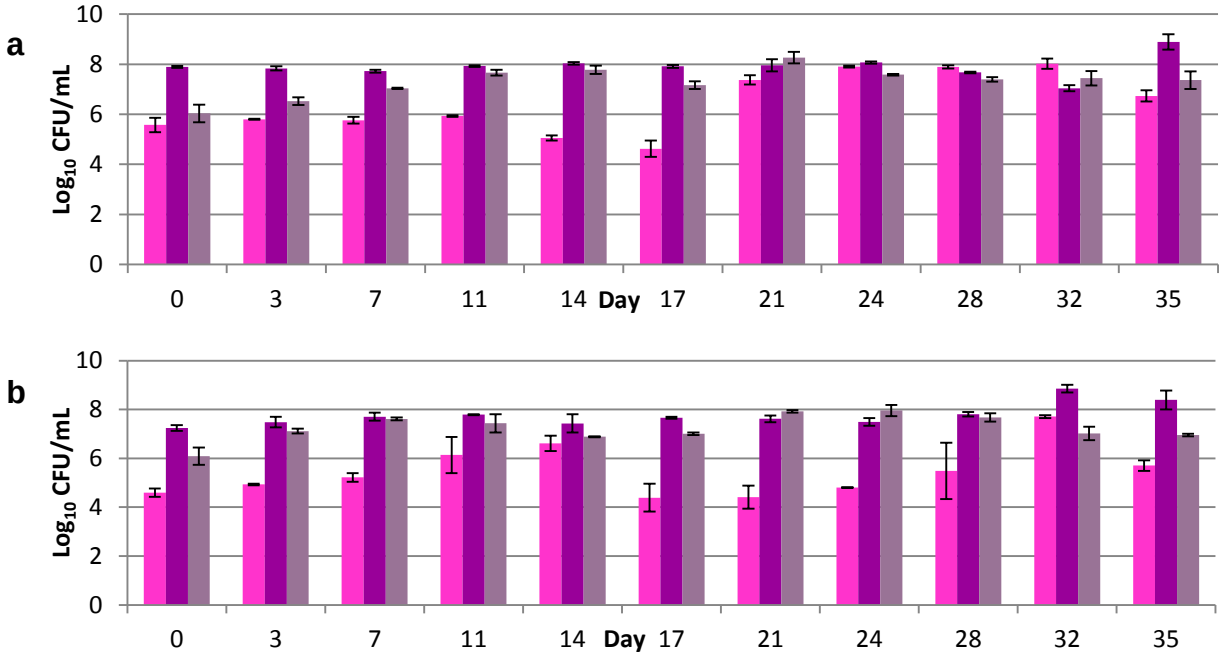


Figure 6.19. *Enterobacteriaceae* recovered from the control (a) and the phage-treated (b) transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

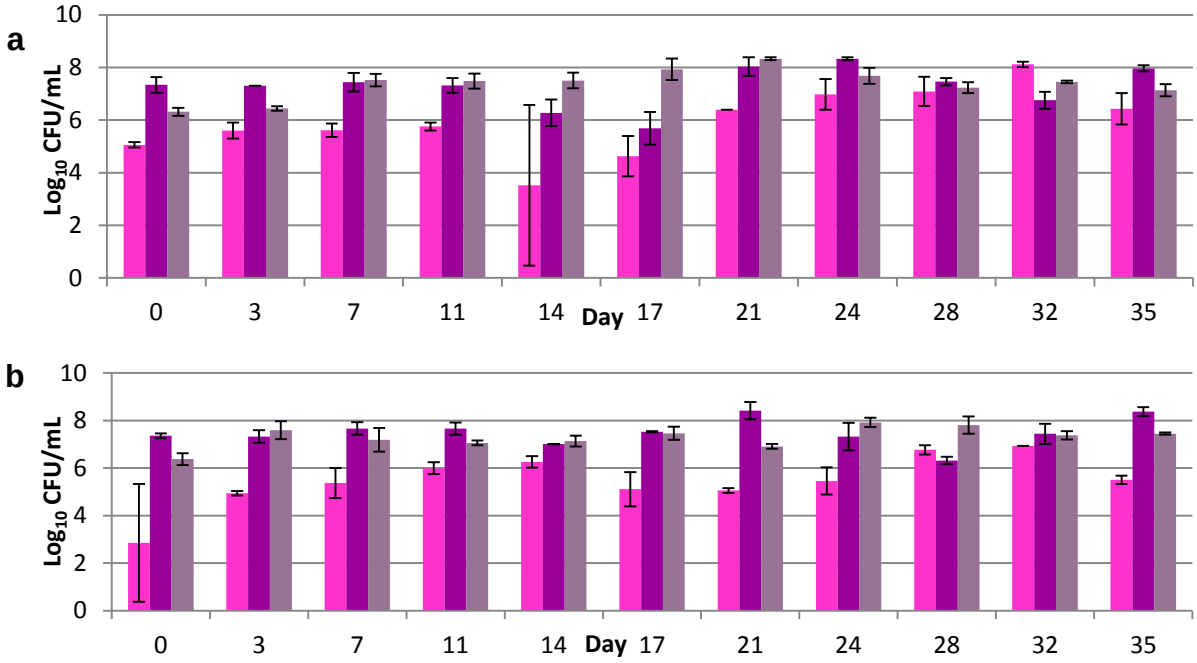


Figure 6.20. *Enterobacteriaceae* recovered from the control (a) and the phage-treated (b) descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

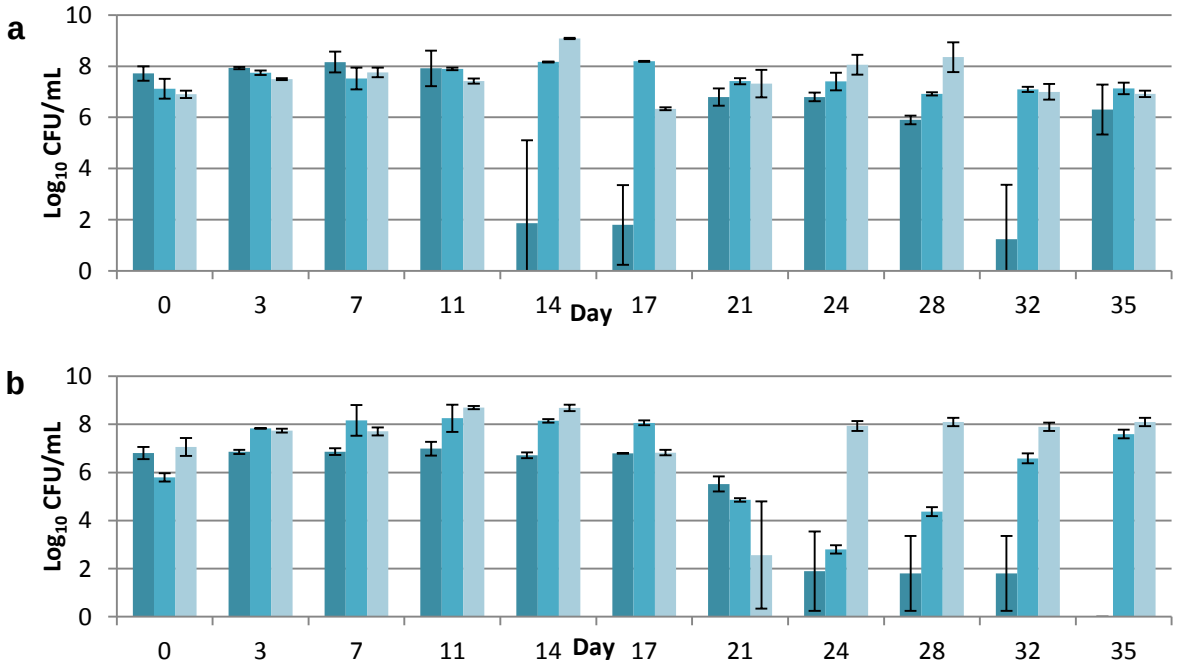


Figure 6.21. *Lactobacillus spp*. recovered from the control (a) and the phage-treated (b) ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

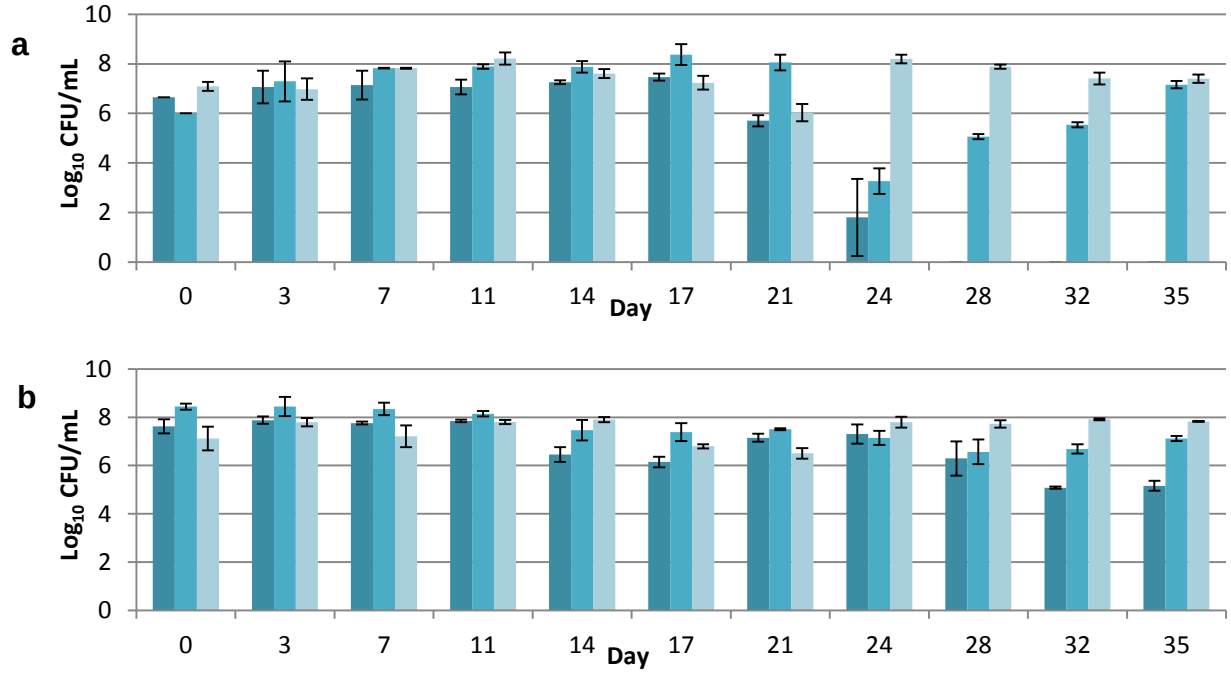


Figure 6.22. *Lactobacillus spp*. recovered from the control (a) and the phage-treated (b) transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

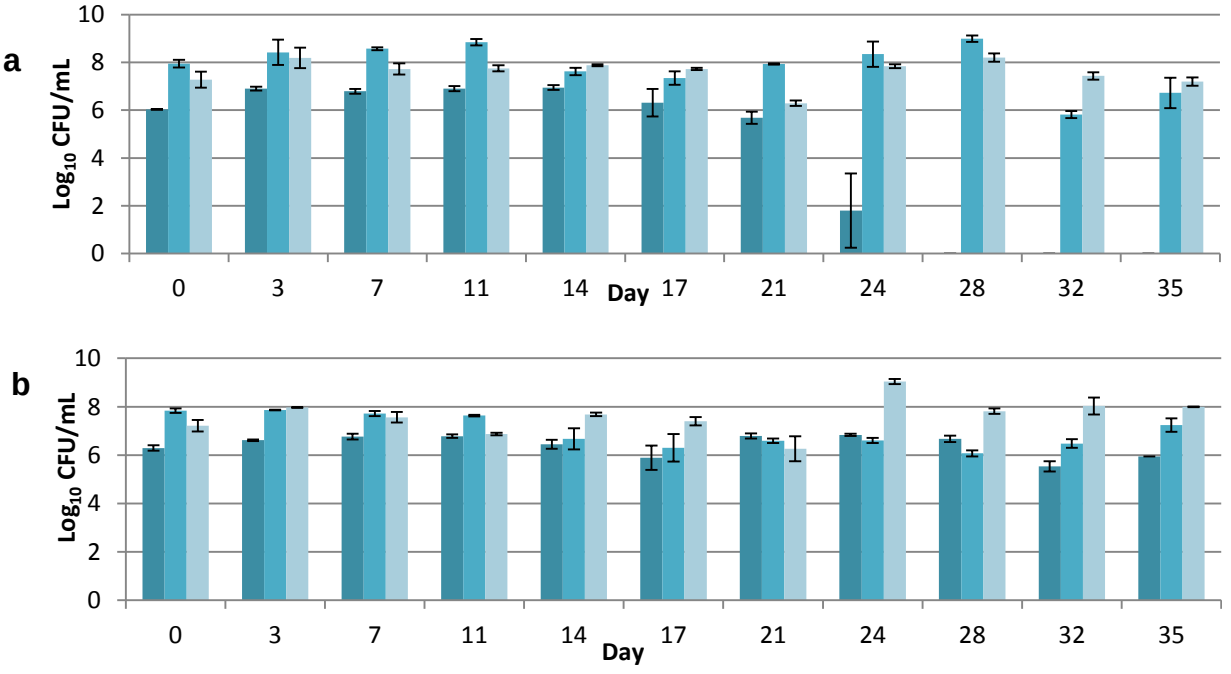


Figure 6.23. *Lactobacillus spp*. recovered from the control (a) and the phage-treated (b) descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

6.5.6. Nucleic acid extraction efficiencies.

In order to determine the optimal method for nucleic acid extraction, the Qiagen Stool mini kit, the Boom method and the modified phenol chloroform method in addition to freeze-thaw disrupted samples were compared. The nucleic acid yields from samples of *C. difficile* NCTC 11204 vegetative cultures, NCTC 11204 spores and faeces were determined. The PCR products from these extracts were also compared by using V3 primers (6.4.3.2) minus the GC clamp (since it was found that *C. difficile* NCTC 11204 could not be amplified using the V3 primers with the GC clamp present). The highest and most consistent yields were obtained following freeze-thaw disruption (Table 6.3).

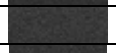
Extraction method	Sample	DNA yield (µg/mL)	V3 PCR product	DGGE profile
Qiagen Stool Mini kit	*C. difficile* cells (vegetative)	34		None
	C. difficile (spores)	9		None
	Faecal sample	1		None
	Faecal sample spiked with *C. difficile* cells (vegetative)	0.043		None
	V3 PCR positive control (ladder)	29		Good
Boom method	*C. difficile* cells (vegetative)	Cannot measure due to use of silica matrix		Not tested
	C. difficile (spores)			
	Faecal sample			
	Faecal sample spiked with *C. difficile* cells (vegetative)			
	V3 PCR positive control (ladder)	29		N/A
Modified phenol chloroform method	*C. difficile* cells (vegetative)	13		None
	C. difficile (spores)	21		None
	Faecal sample	9		None
	Faecal sample spiked with *C. difficile* cells (vegetative)	13	Not tested	None
	V3 PCR positive control (ladder)	29		Good
Freeze-thaw extraction	*C. difficile* cells (vegetative)	Not measured		Good
	C. difficile (spores)			Good
	Faecal sample			Good
	Faecal sample spiked with *C. difficile* cells (vegetative)			Good
	V3 PCR positive control (ladder)	29		Good

Table 6.3. Comparison of 4 methods of nucleic acid extraction/cell disruption from faecal samples and *C. difficile* NCTC 11204 vegetative cells and spores.

6.5.7. Density gradient gel electrophoresis (DGGE) analysis

In the analysis of band profiles generated by DGGE, a comparison of the Shannon Diversity Indices of the control and phage-treated vessels **(**Fig. 6.36) show no significant differences ($p = 0.07$; correlation coefficient 0.56). Despite this, some clear discrepancies were observed in the banding patterns on the DGGE gels. Twenty-five well isolated bands, unique to either the control or

phage-treated samples (Fig. 6.37), were picked and sub-cloned. Three replicates of each were sequenced and good quality sequences were submitted to the Ribosomal Database Project 10 to obtain probable identities. The replicate picks gave highly variable results with all 3 picks giving different identifications in some cases. For those that gave 2 or more identifications in agreement with each other, 3 further picks were sequenced. The results are shown in Table 6.4 with the 'seqmatch' score. This is the number of unique 7-base oligomers shared between the sequence and the given RDP sequence divided by the lowest number of unique oligomers in either of the two sequences (Cole *et al.*, 2009). Only bands 2, 8, 16, 19, 22 and 25 gave identities that were in agreement.

All these identified bacteria represented normal commensal flora with the exception of band 2 (control vessel). Five of 6 sequences for these clones gave a >0.721 seqmatch score for *Veillonella spp*. No species discrimination was achieved and although this group comprises normal mammalian commensals, it should be noted that *Veillonella spp*. are sometimes reported as human pathogens (Houston *et al.*, 1997).

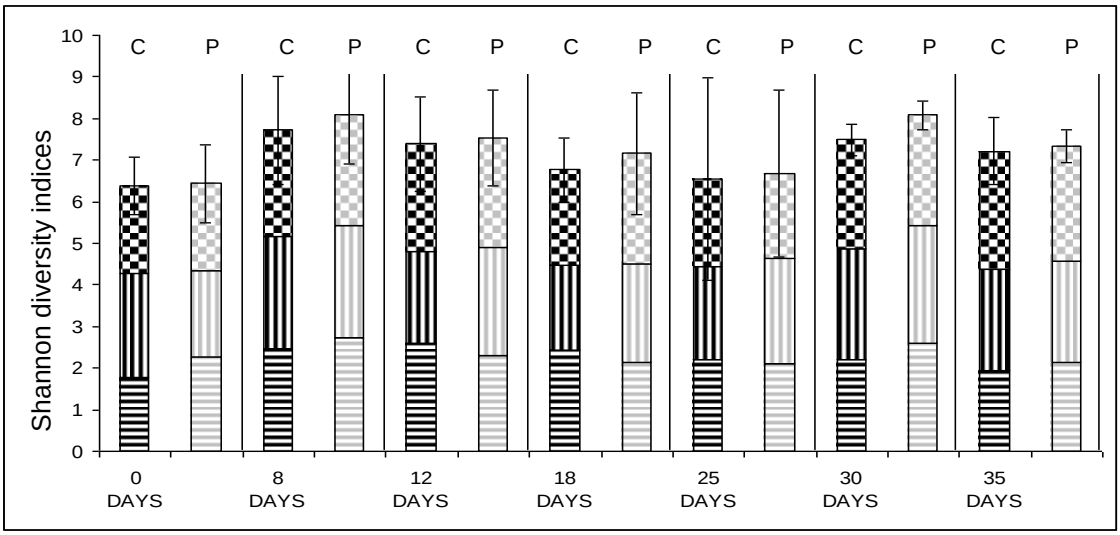


Figure 6.24: Comparisons of the Shannon Diversity Indices obtained from DGGE profiles ± SD. Higher scores represent higher bacterial population biodiversity. Black patterns illustrate the control systems (C). Grey patterns illustrate the phage-treated systems (P). Horizontal lines = ascending vessels; vertical lines = transverse vessels and chequered squares = descending vessels.

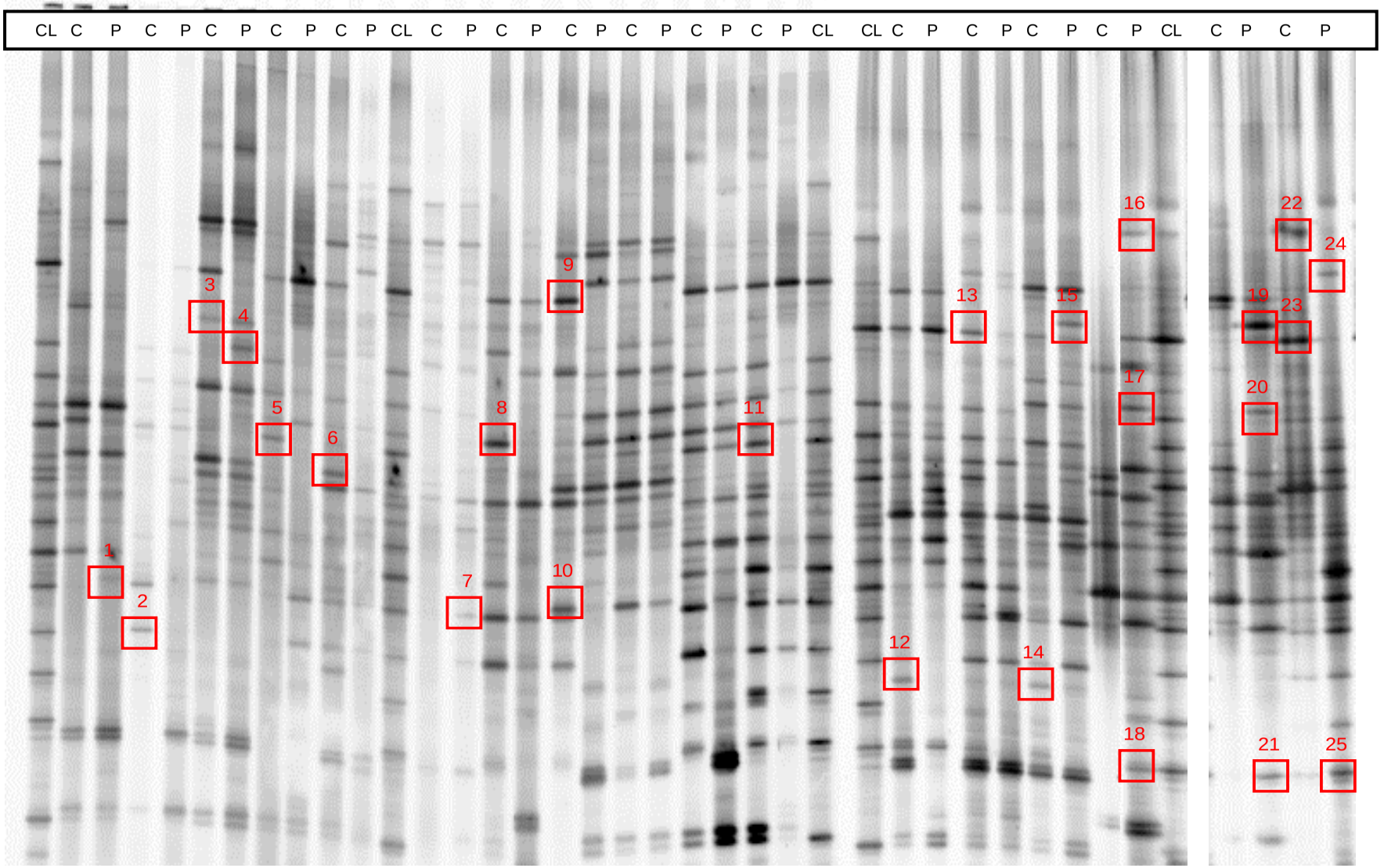


Figure 6.25: DGGE gels showing discordant banding profiles between control system samples (C) and phage-treated systems (P). Highlighted samples were picked, purified and sequenced (results shown in table 6.4). Clone Ladder samples are denoted by CL.

Band number	Control/ Phage-treated system	Length (bp)	Closest match	RDP 10. Seqmatch score
1	Phage-treated	168 173	*Prevotella spp.* *Roseburia spp.*	0.956 0.795
2	Control	189 173 170 169 162	*Veillonella spp.* *Veillonella spp.* *Veillonella spp.* *Veillonella spp.* *Prevotella spp.*	1.000 0.942 0.721 0.969 0.949
3	Control	138 142 162	*Parabacteroides spp.* *Anaerovorax spp.* *Bacteroides spp.*	0.700 0.881 0.784
4	Phage-treated	359* 185 211 415* 518* *Double insert	*Faecalibacterium spp.* *Lachnospiracea spp.* *Bacteroides spp.* *Alistipes spp.* *Bacteroides spp.*	0.595 0.892 0.717 0.569 0.398
5	Control	268 216 195	*E.coli* *Megasphaera spp.* *Megasphaera spp.* No other query transformants on plate	0.799 0.907 0.894
6	Control	195 224 215	*Bacteroides spp.* *Lactobacillus gasseri* *Clostridium hathewayi*	0.471 0.917 0.901
7	Phage - treated	219 175 221	*Prevotella spp.* *Clostridium hathewayi* *Veillonella spp.*	0.939 0.941 0.980
8	Control	330 220 180 279 220	*Enterococcus faecalis* *Enterococcus faecalis* *Shigella sonnei* *Enterococcus faecalis* *Enterococcus faecalis*	0.955 0.949 1.000 0.876 0.954
9	Control	267 194	*Prevotella spp.* *Lactobacillis gasseri*	0.827 0.962
10	Control	No sequences returned		
11	Control	228 224 190	*Megasphaera spp.* *Enterococcus faecalis* *Megasphaera spp.*	0.869 0.877 0.703
12	Control	195 169 215	*Clostridium hathewayi* *Laconifactor spp.* *Lactobacillus casei*	0.855 1.000 0.961
13	Control	189 169 246	*Prevotella spp.* *Anaerostipes spp.* *Lactobacillus gasseri*	0.873 0.982 0.887

14	Control	189 169 189	*Prevotella spp.* *Clostridium hathewayi* *Bacteroides spp.*	0.994 0.884 0.929
15	Phage-treated	169	*Laconifactor spp.* (Remaining clones gave poor quality sequences).	0.878
16	Phage treated	227 194 194 164 171	*Enterococcus faecalis* *Enterococcus faecalis* *Enterococcus durans* *Lactobacillus gasseri* *Enterococcus faecalis*	0.937 1.000 1.000 0.615 1.000
17	Phage-treated	225 85 270	*E.coli/Shigella spp.* *Sarcina maxima* *Dorea spp.*	0.923 0.762 0.801
18	Phage-treated	169 165 180	*Clostridium spp.* *Enterobacter cloacae* *Prevotella spp.*	0.931 0.956 0.989
19	Phage treated	163 189 189 168 148	*Bacteroides spp.* *Bacteroides spp.* *Bacteroides spp.* *Bacteroides spp.* *Dorea spp.*	0.956 1.000 1.000 1.000 0.951
20	Phage-treated	189	*Prevotella spp.*	0.917
21	Phage-treated	95 194 187	*Enterobacter spp.* *Lactobacillus casei* *Prevotella spp.*	0.744 0.904 0.665
22	Control	413 245 189 210	*Bacteroides spp.* *Bacteroides fragilis* *Bacteroides fragilis* *Lactobacillus gasseri* (Poor sequence returned for 5th pick)	0.777 0.852 0.880 0.696
23	Control	237 218 254 242	*Lactobacillis gasseri* *Bacteroides spp.* *Bacteroides spp.* *Candidatus megasphaera micronucliformis* (Poor sequence returned for 5th pick)	0.862 0.909 0.902 0.770

24	Phage-treated	177 195 195	*Dialister invisus* *E.coli/Shigella spp.* *Candidatus megasphaera micronucliformis*	0.799 0.847 0.588
25	Phage treated	281 113 241 193 150	*Lactobacillus caesei* *Lactobacillus caesei* *Lactobacillus caesei* *Lactobacillus casei* *Lactobacillus casei*	0.912 0.972 0.901 0.941 0.942

Table 6.4. Results of the unique band identities according to the Ribosomal Database Project 10. Green shading highlights bands for which a majority agreement was observed and further picks were sequenced to confirm the designation.

6.6. Discussion

6.6.1. Efficacy of phage therapy to treat CDI.

This study has shown that prophylactic phage treatment of *C. difficile* in an *in vitro* colon model can result in a significant reduction in the number of viable *C. difficile* cells relative to an untreated control, and suppresses toxin production to below levels that are regarded as clinically significant by current diagnostic tests. The number of *C. difficile* cells recovered from the control systems were comparable to reported levels in other studies that have used the colon model to assess pathogenesis of *C. difficile* strains and the efficacy of treatment regimens (Baines *et al.*, 2009; Freeman *et al.*, 2007) and are also clinically relevant (Mutters *et al.*, 2009). The levels of *C. difficile* vegetative cells isolated from phage–treated systems however, were highly variable between replicate experiments despite the highly controlled conditions. Phage treatment was sufficient to suppress the levels of vegetative cells of *C. difficile* to below the limit of detection by culturing (60 µL plated undiluted) in 2 of the 3 experiments. In 1 replicate however, levels of *C. difficile* approached those of the untreated system, although these samples were negative for *C. difficile* toxins A and B. It was hypothesised that lysogeny between the NCTC 11204 host and ΦCD27 gave rise to phage resistance in the host and rendered treatment with ΦCD27 ineffective in terms of cell lysis. It might be that a component of the faecal inoculum affected the phage activity although no

major differences in the numbers of commensal bacteria were recorded. This observation highlights the delicate balance of the microbiota and its significance with regards to therapeutic outcome. The suppression of toxin production was presumed to be a result of lysogeny between the *C. difficile* strain and ΦCD27 and this was also shown in previous batch fermentation models of CDI (5.5.4;(Meader *et al.*, 2010)) Lysogenisation of phage with the host has also been demonstrated *in vivo* during remedial therapy in a hamster model when a single dose of ΦCD119 was added 1 h after *C. difficile* 602 challenge (Govind *et al.*, 2011). It was found that despite recovering reduced numbers of *C. difficile* cells and recording suppressed levels of toxin production in lysogens produced both *in vitro* and *in vivo*, all phage-treated hamsters died within 5 days. Also, all *C. difficile* strains were shown to contain ΦCD119 as a prophage and were resistant to further infection. The outcome may have been improved with additional phage doses but this demonstrates a significant pitfall of using bacteriophages that have the capacity to lysogenise with their host, especially since all *C. difficile* phages isolated to date have this ability (Goh *et al.*, 2005; Govind *et al.*, 2006; Horgan *et al.*, 2010; Mahony *et al.*, 1985; Mayer *et al.*, 2008). The effect of a prophylactic phage treatment regime is yet to be investigated *in vivo*.

The relative increase in the number of *C. difficile* spores in the phage-treated colon model systems is a cause for concern. This observation has been recorded previously *in vitro* and in hamster models of vancomycin-treated CDI (Freeman *et al.*, 2005). Relapse rates of CDI in humans exceed 70% in some studies (Kamboj *et al.*, 2011) and the persistence of spores following treatment permits the recurrence of CDI at any time. With regards to phage treatment, these spores may harbour the therapeutic phage as a prophage and so future rounds of preventative or remedial therapy could have little or no effect. Transmission to other patients via spore ingestion would also still be possible and these factors represent considerable restraints on the potential therapeutic use of phages against *C. difficile* and possibly other spore-forming organisms.

Despite efforts to minimise the influence of variables in the experiments, some aspects to consider when interpreting this data include the possible different

antimicrobial effects of a phage-infected *C. difficile* culture supernatant (used to harvest the phage particles) and an un-infected *C. difficile* supernatant (used for the control systems). Changes in gene expression in response to phage infection may have given rise to chemical factors that could have affected the growth of *C. difficile* in the colon model. The colon model system lacks the influence of metabolic (Freeman *et al.*, 2003), secretory (Yadav *et al.*, 2011) and immunological factors (Modi *et al.*, 2011) that are likely to play an important role in CDI.

6.6.2. Characteristics of ΦCD27 during treatment.

Phage repressor proteins produced during lysogeny can directly regulate toxin production in *C. difficile* (Govind *et al.*, 2011; Govind *et al.*, 2009) and although manipulation at this level could be an avenue to pursue for therapeutic benefit, this finding has considerable bearing on the safety of phage therapy, particularly since the acquisition of phages by *C. difficile* does not always give rise to a reduction in the amount of toxin produced. Sekulovic and colleagues (2011) noted a 1.6- fold increase in the amount of toxin A production and a 2.1-fold increase in the quantity of toxin B as a result of ΦCD28-2 integration. Phage proteins may also interact with other bacterial genes in its target host and bacteria in the surrounding environment with unknown consequences for normal commensal organisms. Aside from the effect on bacterial genes, the integration of phages into their target host also has the potential to introduce new virulent genetic elements, indeed some significant virulence genes of *C. difficile* are themselves thought to be phage-derived (Canchaya *et al.*, 2002; Goh *et al.*, 2005).

Toxin C is an ADP-ribosylating binary toxin present in some hypervirulent strains of *C. difficile* (Davies *et al.*, 2011), which has been associated with an increased CDI fatality rate (Bacci *et al.*, 2011). NCTC 11204 is not known to produce toxin C, but the effect of phage-therapy on this virulence factor is an important factor to consider as any increase could enhance the severity of CDI.

A significant advantage of the colon model is that these systems are less prone to the natural variations often seen with *in vivo* models as the retention

time, which is known to have a considerable effect on colonic bacteria (Cummings *et al.*, 1979) and nutritional components can be controlled. It was particularly beneficial to be able to use the same donor inoculum for each experiment, but should be considered that the efficacy of phage treatment could be quite different if faecal material from another donor was used to prime the model, as individual variations in microbiota composition, metabolites and nutritional factors can be expected to impact on resilience against *C. difficile* colonisation and proliferation.

6.6.3. Impact of ΦCD27 therapy on commensal bacterial populations. Due to the specificity of the phage, no detrimental impact on commensal bacteria was expected and indeed no statistically significant decreases in these groups or overall diversity were observed relative to controls by culture or DGGE. The use of molecular profiling permitted the inclusion of bacterial genera and species that cannot be cultured, however, these methods are not without issue. PCR inhibition, contamination and differing template affinities would give rise to variation in the profiles generated. A variety of nucleic acid extraction methods were tested in preparation for DGGE, which gave highly variable results with generally very poor yields. The decision to process samples for PCR following a single freeze-thaw cycle was made in order to achieve more consistent and reproducible results but has associated flaws. Of particular concern is the possibility of under-representing the Gram positive organisms that may require a rigorous lysis step to liberate associated nucleic acid. It is possible that material from non-viable or decaying organisms might comprise much of the DNA that is available for PCR.

Considerable variations in the results of unique band identification were observed and this highlights reproducibility issues of the DGGE method and difficulties in the determination of unique bands.

Phage treatment gave rise to significantly higher numbers of *Lactobacillus spp., Bacteroides spp.,* aerobes and *Enterobacteriaceae* by culture relative to the controls. Only an increase in numbers of *Enterobacteriaceae* and aerobic bacteria in the phage-treated system was observed during our previous study of CDI in a batch fermentation model (Meader *et al.*, 2010). The apparent

antimicrobial effect of C. *difficile* toxins also has some support from supplementary studies, which show significantly poorer recovery of *Lactobacillus spp*., *Bacteroides spp*. and also total *Clostridium spp*. growing in the presence of a toxigenic *C. difficile* strain versus a non-toxigenic strain (5.5.3). The promotion of these bacterial groups is of unknown clinical significance but can be expected to be of benefit to the host. In similar colon model studies of antibiotics that are active against *C. difficile*, major groups of commensal bacterial including *Bacteroides* spp, *Bifidobacterium spp.* and *Lactobacillus spp*. were reduced to levels below the limit of detection by culture (Freeman*, et al.*, 2005). In a study by Rea *et al.* (2011), which used a similar colon model to investigate *C. difficile* therapy using Lacticin 3147 (a lantibiotic produced by *Lactococcus lactis*) and employed solely molecular methods to enumerate commensal bacteria, significant reductions in the *Firmicutes* and *Bacteroidetes* phyla were reported. As part of the same work, this group also reported that the novel lantibiotic, Thuricin CD, achieved a 3 $\log_{10}$ reduction in the burden of *C. difficile* with no significant detrimental effect on commensals. Despite the impact on *C. difficile*, no significant increases in the proportions of commensal bacterial were reported.

This work has concentrated on the importance of quantifying bacterial profiles, but other studies have focused on assessing shifts in the metabolic activity within bacterial populations in response to nutritional factors and pathogens (Nuenen *et al.*, 2003). Some short chain fatty acid products of fermentation are damaging to human health and are associated with the onset of AAD, liver problems (Mortensen and Clausen, 1996) and cancer (Gill and Rowland, 2002). Further models of CDI interventions should consider this as an additional measure to assess the safety of novel therapies, but it should be taken into account that due to the model's lack of mucosal absorption, these profiles are not likely to represent *in vivo* levels.

The colon model is not expected to accurately reproduce the biofilms observed in the lumen and coating food particles (Macfarlane and Macfarlane, 2007). It is unknown to what extent this would affect the clinical relevance of the study, but the adherence of *Bifidobacterium* spp. to intestinal mucus has been documented (Ouwehand *et al.*, 1999) meaning that these groups may

have been under-represented in the model. *Bifidobacterium spp.* are regarded as protective against *C. difficile* colonisation, and so it is possible that these conditions were more permissive to *C. difficile* growth than *in vivo* conditions. The addition of mucin to future colon models may help to address this issue.

6.6.4. Conclusion

This work has shown that in a human colon model, prophylactic phage therapy of CDI can considerably reduce the burden of *C. difficile* vegetative cells and caused a clinically relevant decrease in toxin production. Disappointingly, the phage has also shown a strong predilection for integration with its host, demonstrating the risk of virulence gene acquisition and prevention of *C. difficile* elimination. In this system, integration led to reduced levels of toxin production, but this may have the opposite effect in other strains. This, together with the observed increase in spore production, illustrates the possibility of CDI recrudescence post phage treatment. Further work into the use of phages to prevent and treat CDI should include phages with disabled capacity for lysogeny and those with a greater range of susceptible *C. difficile* strains. The specificity of phage therapy, benign nature and ease of production still maintains their worth as candidates for alternative therapeutic agents and co-treatments in the battle against CDI.

Chapter 7 - The effect of lysogeny on *C. difficile* toxin production

7.1. Abstract

The integration of bacteriophages into *C. difficile* host chromosomal DNA has previously been shown to affect the expression of toxin A by the action of phage repressor proteins. Differential toxin production was determined in 4 wild-type *C. difficile* strains and their lysogenic counterparts showing a clear decrease in phenotypic toxin production upon lysogeny in 3 strains but an increase in the fourth strain. Transcriptional analysis of toxin and toxin regulatory genes was also carried out and showed apparent weaker expression of toxin A in lysogens relative to their wild-type counterparts. A putative repressor of ΦCD27, encoded in open reading frame (ORF 44), was shown to be expressed in the lysogenic state, but the affinity of the purified protein to promoters regions for *C. difficile* toxins and toxin regulatory genes could not be demonstrated by Electrophoretic Mobility Shift Assay. The integration site of the phage was determined and found to be the same in all 4 laboratory-made lysogens and the host from which the prophage derives.

7.2. Introduction

After infection, bacteriophages can either adopt a lytic life cycle or become lysogenic (Kutter and Sulakvelidze, 2005; Lamont *et al*., 1993). The integration of phage elements into host DNA during lysogeny is beneficial to both the phage and the host bacterium. In lysogenic infection the phage can replicate at the rate of the bacterial strain and persist until the end of its life, before releasing progeny that can go on to infect other cells. Genetic elements encoding virulence factors such as toxins and antibiotic resistance are often embedded in phage DNA (Chen *et al.*, 2011; Frobisher and Brown, 1927), and acquisition can impart significant competitive advantages enabling the host bacterium to survive in changing environments. In some clostridial species, toxins are also encoded by phage sequences (Eklund *et al.*, 1971) and in the genome of *C. difficile* some putative phage elements have also been identified including the toxin A gene, *tcdA*, a negative regulator of toxin A, *tcdC* (Goh *et al.*, 2005) and the *tcdE* gene homologous to holins (Tan *et al.*, 2001).

Phage genes may encode regulatory factors that enhance or repress the expression of some elements, such as the repression of Shiga-toxin production in *E. coli* by Stx prophages (Serra-Moreno *et al.*, 2008) and the impairment of DNA repair systems in *Streptococcus pyogenes* due to the influence of the SF370.4 prophage (Scott *et al.*, 2008), which gives rise to increased mutation rates in competitive conditions. Studies have also demonstrated the effect of prophage-encoded repressors on the expression of toxin and regulatory genes located in the PaLoc region of the *C. difficile* genome. Phenotypic observations and transcriptional analysis of *C. difficile* containing ΦCD119 as a prophage revealed a reduction in the expression of *tcdA, tcdB, tcdR, tcdE* and *tcdC* relative to the phage-free counterparts (Govind *et al.*, 2009). This was also observed *in vivo* following experimental treatment of susceptible *C. difficile* with ΦCD119 in a hamster model (Govind *et al.*, 2011). Upon examination of the ΦCD119 genome a putative repressor denoted as *repR* was identified and found to be expressed in all the lysogenic strains (Govind *et al.*, 2009). Purified, recombinant repR protein showed binding affinity for the promoter region of the positive *tcdA* (toxin A) regulator,

tcdR, in gel shift assays, thus offering an explanation for the observed reduced toxigenic phenotype of the lysogens, since this protein is an RNA polymerase sigma factor that augments toxin A production (Mani and Dupuy, 2001). Phage infection does not always lead to a reduction in toxin production. It was found that levels of toxin B were significantly increased in 4 of 6 *C. difficile* strains after phage integration and an increase in toxin A in one strain was also reported (Goh *et al.*, 2005). This was demonstrated not to be via acquisition of further copies of phage-encoded toxin genes but hypothesised to be due to the action of phage-encoded regulators. A similar study (Sekulovic *et al*., 2011) demonstrated increased extracellular toxin A and B in NAP1/027 strains containing a ΦCD38-2 prophage relative to strains without the phage. Up-regulation of all PaLoc genes was noted, in particular *tcdE*, indicating that toxin export was facilitated by the production of this gene. The authors note varying effects on toxin production when the same phage infected different *C. difficile* strains.

Apart from the genes of the PaLoc region, other constituents of the *C. difficile* genome have been associated with the control of toxin production. The known global gene expression regulator and energy sensor CodY, has been shown to reduce the expression of *C. difficile* toxins A and B by binding to the promoter region of *tcdR* to cause down regulation (Dineen *et al.*, 2007). The affinity for DNA binding is evidenced by the helix-turn-helix region of this dimeric protein (Brennan and Matthews, 1989). In addition, CodY has also been shown to interact directly, albeit with a low affinity, with the promoters of *tcdA* (toxin A) and *tcdB* (toxin B). For reasons that remain unclear, binding to the promoter region of *tcdC* (a negative regulator of toxin production) can also be observed (Dineen *et al.*, 2007). In a similar manner the transcription factor, CcpA, can act as both a positive and negative regulator (Kim *et al.*, 2002; Lorca *et al.*, 2005; Moir-Blais *et al.*, 2001; Moreno *et al.*, 2001) and has previously been implicated in the regulation of enterotoxin production in *C. perfringens* (Varga *et al.*, 2004) and the repression of *C. difficile* toxins A and B in the presence of high glucose concentrations (Antunes *et al.*, 2011). A protein encoded by a gene responsible for the initiation of sporulation, Spo0A, has also been shown to reduce toxin production by more than 90%

(Underwood *et al.*, 2009) as demonstrated by a knock-out model of *C. difficile* 630. This down regulation is hypothesised to occur at the level of toxin transcription or translation in a similar manner to *Clostridium perfringens,* where Spo0A is thought to bind to a region upstream of the toxin gene to up regulate production (Huang *et al.*, 2004; Underwood *et al.*, 2009).

The genome of ΦCD27 has been sequenced previously (Mayer *et al.*, 2008). When this genome was scrutinised, several genes that were homologous to known phage repressor protein genes were identified – *orf44, orf45* and *orf72,* as shown in Table 7.1. The DNA binding potential of the translated proteins was recognised by the helix-turn-helix domains with homology to the XRE family of repressors (Brennan and Matthews, 1989), as seen with other transcriptional regulators such as repressors of phage lambda (Jordan and Pabo, 1988) and the *trp* repressor of *E. coli* (Otwinowski *et al.*, 1988).

ORF	Location on genome	Homology of translated protein (NCBI E value on 29.7.12)	NCBI Accession number
44	35729-35274 reverse	Phage repressor of *C. difficile* 630 (6e-105)	YP_001089466.1
45	35883-36104	Phage repressor of *C. difficile* 630 (5e-44)	YP_001089465.1
72	48448-49611	Phage DNA-binding protein of *C. difficile* 630 (0.0).	YP_001089442.1

Table 7.1. Putative phage repressors of ΦCD27

The identification of transcriptional regulators that inhibit the production of toxin from *C difficile* could lead to a novel therapeutic approach in which such proteins are administered *in vivo* to specifically repress the virulence of the infecting pathogen without the risk of a Jarisch-Herxheimer reaction (1.9.2) or disruption of the commensal flora.

7.3. Objectives

- To establish differential expression of the toxin-encoding genes of *C. difficile* (*tcdA* and *tcdB*), and other elements concerned with regulation of these genes (*tcdC, tcdE, tcdR, CodY, CcpA* and *Spo0A*) in wild types and laboratory-made lysogenic strains of *C. difficile* NCTC 11204, 11205, 11207 and 11209.

- To express a putative repressor protein of ΦCD27 and investigate the affinity of the purified protein to promoter regions of the *C. difficile* PaLoc genes *tcdA*, *tcdB*, *tcdC*, *tcd E* and *tcdR*.

- To determine the integration site of ΦCD27 into susceptible *C. difficile* strains NCTC 11204, 11025, 11207 and 11209 to investigate potential disruption of putative toxin regulatory genes.

7.4. Methods

7.4.1. Measuring differential toxin production

Lysogens of *C. difficile* NCTC 11204, 11205, 11207 and 11209 were produced as previously described (2.2.2.3). Triplicate liquid cultures were made in BHI broth with complements and grown for 10 h. Supernatants were harvested and immediately tested using the Meridian Bioscience *C. difficile* Tox A and B assay (2.1.3). The toxin assay results of the wild type strains relative to the lysogenic strains were compared and statistically analysed using a Student's t test.

Gene	Primer sequences (melting temperatures)	Product size	Reference
16S	F: 5'-CCT ACG GGA GGC AGC AG-3' (60°C) R: 5'-ATT ACC GCG GCT GCT GG-3' (57.6°C)	193 bp	(Muyzer *et al.*, 1993)
Toxin A (*tcdA*)	F: 5'- GCA GCT ACT GGA TGG CAA AC -3' (64.7°C) R: 5'- ATC TCG AAA AGT CCA CCA GC -3' (63.2°C)	1783 bp	(Govind *et al.*, 2009)
Toxin B (*tcdB*)	F: 5' - GCA GTT GAA TAT AGT GGT TTA G -3' R: 5' – TCA CTA ATT GAG CTG TAT GAG – 3'	538 bp	This work
Negative regulator of toxin A production (*tcdC*)	F: 5'- GTT CAA AAT GAA AGA CGA C -3' (54.5°C) R: 5'- CTA TCC CTG GTA TGG TTA TT -3' (54.5°C)	385 bp	This work
Positive regulator of toxin A production (*tcdD/R*)	F: 5'- TCA AAG TAA GTC TGT TTT TGA GGA A -3' (61.9°C) R: 5'- TGC TCT ATT TTT AGC CTT ATT AAC AGC -3' (62.6°C)	177 bp	(Govind *et al.*, 2009)
Protein of unknown function, showing homology to phage holins (*tcdE*)	F 5'- TGG AGG AAT CAG AAA AGT AGC A -3' (62.6°C) R 5'- CAT TTC ATC TGT CAT TGC ATC T -3' (61.5°C)	273 bp	(Govind *et al.*, 2009)
Gene responsible for the initiation of sporulation (*Spo0A*)	F 5'-GCTAAGGATGGAATTGAAGCA-3' (51.7°C) R 5'-GCTCCTAGATTTATTGCGCTTT-3' (54.0°C)	181 bp	This work
Known transcriptional regulator (*CcpA*)	F 5'-GTGATGAGGCTGCCATAGGT-3' (53.5°C) R 5'-TTGCTACTGCTCCCATATCGT-3' (54.3°C)	144 bp	This work
Known transcriptional regulator (*CodY*)	F 5'-GGAAGCGGTCAAAGATTAGG-3' (51.4°C) R 5'-GCTTCAAGCTCGGAGTAGGA-3' (55.0°C)	186 bp	This work

Table 7.2. Primers used to assess differential expression of PaLoc genes and other regulatory elements.

7.4.2. Determination of differential toxin production and regulatory gene expression in *C. difficile* between wild types and lysogens.

Total RNA from stationary phase cultures (approximate OD_{600} 1.5) of NCTC *C. difficile* 11204, 11205, 11207 and 11209 and the corresponding lysogenic strains were extracted using the SV Total RNA Extraction System and treated with Turbo DNase as described previously (2.3.2.2). Complementary DNA copies of the RNA were made using the Invitrogen Superscript Reverse Transcriptase kit (2.3.2.2.2) and these products were used as templates for PCR using primers for toxin and regulatory genes as shown in Table 7.2. The PCR products (5 µL) were analysed by electrophoresis on a 1.5% agarose gel as previously described (2.3.4.2). After staining with ethidium bromide the band intensities were visually determined and used to estimate differential expression.

7.4.3. Production of a putative repressor of ΦCD27

Using the sequence information of *orf44*, primers were designed to amplify a product covering the coding region and flanking sequence, with some mismatches to incorporate an *NdeI* restriction site (in the forward primer) and *XhoI* (in the reverse primer), as shown in Fig. 7.1.

5’TATTTTATAGAGG**TGATAAATTTGAATACTGAAAATGA**-3’

ORF44_*NdeI*: 5’-GG**TGATACATATGAATACTGAAAATGA**-3’

Figure 7.1. (a) Original ΦCD27 sequence (blue font) and forward primer (black font) with *NdeI* site highlighted in yellow and base-pair mismatches highlighted in red font. Start site of *orf44* is underlined.

5’-TTTCATAACTA**TTCATAAGTTCACCTACCTATTTTAC**CAATAGACG-3’

ORF44_*XhoI*: 5’-**TTCTCGAGTTCACCTACCTATTTTAC**-3’

Figure 7.1. (b) Original ΦCD27 sequence (blue font) and forward primer (black font) with *XhoI* site highlighted in yellow and base-pair mismatches highlighted in red font. Stop site of *orf44* is underlined.

Total nucleic acid from ΦCD27 was extracted from lysates of phage propagated in BHI broth plus complements using the Qiagen Lambda Mini kit (2.3.2.1.5). Concentrations of 50 ng and 100 ng were used as a template for PCR with Phusion Polymerase (2.3) and the ORF44_*NdeI* and ORF44_*XhoI* primers as detailed above (Fig. 7.1). Five cycles of PCR with an annealing temperature of 49°C were carried out first to permit extension of the mismatched bases, with 20 cycles at 64°C thereafter.

PCR products from putative phage repressors were digested with *NdeI* and *XhoI* (2.3.8) and subcloned for protein expression into the restricted, dephosphorylated pET15b vector, placing the sequence downstream of the T7 promoter, the IPTG-inducible *lac* operator, and an in-frame 6xHis tag. The estimated size of the 6xHis-tagged product was 19.63 kDa, as predicted from the translated sequence (EditSeq 5.06, DNA STAR Inc). The pET15b-*orf44* ligated products were used to transform *E. coli* TOP10 cells for sequence confirmation then *E. coli* B21(DE3) cells for expression (2.3.11.3). Positive transformants were confirmed with T7P2 and T7_T primers (2.3.5.1) and protein inductions were carried out using 0.5 mM IPTG to determine the optimum harvest time. *E.coli* cells with empty vector controls were also

induced and extracted in parallel. Two buffers for protein extraction were tried in order to optimise the yield: Tris buffer (20 mM Tris, 50 mM NaCl, pH 7.5) and sodium phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8). The protein concentration was estimated using a Bradford assay (2.4.3) and the crude extracts were run on a 10% NuPage Bis-Tris Mini Gel (2.4.4), stained using SimplyBlue Safe Stain (2.4.5). Putative ORF 44 protein was identified as an additional band (relative to the empty vector control) in the expected position. The presence of the ORF44 protein was confirmed by Western blot, utilising labelled anti-His antibodies to visualise the product (2.4.6).

Once expression had been confirmed, protein inductions were carried out for the purposes of downstream applications, and proteins were extracted using the Qiagen Qiaexpress Ni-NTA FastStart Kit (2.4.2.2). Aliquots of each elution and wash flow-through were applied to an SDS-PAGE protein gel to assess purity. Dialysis was used to further purify the protein (2.4.7.1) and concentration (when necessary) was via ultra-centrifugal filters (Amicon) (2.4.8).

7.4.4. Identification of toxin gene promoters and amplification of PaLoc intergenic regions.

Promoter regions of PaLoc genes were estimated by submitting intergenic region sequences to the Softberry BPROM Bacterial Sigma 70 Promoter Recognition Programme. Primers were designed to give a product that spanned these predicted regions and as much of the intergenic region as possible in less than 600 base pair fragments, to ensure suitability for use in the electrophoretic mobility shift assays (EMSAs). PCR products were subcloned into pCR2.1 (2.3.11) and confirmed by sequencing (2.3.10).

7.4.5. Purification of PCR products of intergenic regions by gel extraction

The Qiagen QIAEX gel extraction kit was used according to the manufacturer's protocol. All reagents were kit components unless otherwise stated. PCR products of the subcloned intergenic regions were applied to a

1.5% agarose gel as described previously (2.3.4.2) with some modifications. Extra wide gel combs were used to allow up to 30 µL to be loaded into each well and a one lane gap separated each sample. Bands were visualised on a UV transilluminator and excised from the stained gel with a clean scalpel. Three times the volume of QX1 buffer (relative to the weight of the band) was added and the band was re-suspended by vortex mixing. QIAEX II solution was added (30 µL) and the tubes were incubated for 10 min at 50ºC to dissolve the agar and bind the DNA. Tubes were vortex mixed every 2 min and centrifuged for 30 s at 13,000 x *g* after incubation. The supernatant was carefully removed and 500 µL of buffer QX1 was added. The tubes were vortex mixed to re-suspend the pellet, centrifuged for 30 s at 13,000 x *g* and the supernatant was carefully removed. Pellets were washed twice with buffer PE in the same way and the pellet was air-dried for 15 min. The DNA was eluted by adding 20 µL of nuclease-free water, vortex mixing and incubating at ambient temperature for 5 min. This elution was transferred to a new microtube to allow a second elution with a further 20 µL incubated at 50ºC for 5 min. The yields of the 2 separate elutions were compared by gel electrophoresis.

7.4.6. Electrophoretic mobility shift assays (EMSAs)

Molecular Probes Electrophoretic Mobility Shift Assay (EMSA) kits from Invitrogen Detection Technologies were used to assess the binding affinity of a putative phage repressor protein, ORF 44, to toxin and regulatory gene promoters in the PaLoc region of *C. difficile* NCTC 11205 and 11209. Reagents were kit components unless otherwise stated. Concentrations of proteins used were similar to those used in previous studies of phage repressor protein interactions and are considered to be physiologically relevant (Govind *et al*., 2009). Binding reactions were comprised of 50-500 ng protein (170-1700 nM) with dilutions made in sterile ultra-pure water if necessary. The binding buffer (50 mM Tris, pH 7.8, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol stored at -20°C) was selected from previous published studies of EMSAs using a phage repressor protein (Govind *et al.*, 2009) and 3 µL of this was added to the reactions with 40 ng of DNA. DNA-only controls and protein controls of the highest concentration

were also tested. Calf thymus DNA (Sigma) at 40 ng was used to check for non-specific binding (added before the promoter-containing DNA). Binding reactions were incubated for 20 min at ambient temperature and 2 µL of 6 x EMSA gel loading buffer was added to each before applying the whole volume to wells of a 6% Novex TBE polyacrylamide gel (Invitrogen) mounted in the XCell SureLock module. Gels ran in 0.5 x TBE buffer for 35-60 min (depending on the DNA fragment size) at 100 V (12-15 mA start, 6-15 mA end) and were stained by submerging in TBE buffer (2.5 x) containing 5 µL 10,000 x SYBRGreen, with gentle rocking on a rotating platform (Stuart, 30 rpm) for 20 min.

7.4.7. Determination of the site of ΦCD27 integration.

It was considered that integration of the phage into the host genome could disrupt a gene involved in the regulation of toxin production. The site of integration (the *attB* site) was determined and compared between *C. difficile* NCTC 12727 (the original host of the ΦCD27 prophage) and each lysogenic strain that was studied. Genomic DNA extractions from ΦCD27, NCTC 12727 and the 11204LYS (NCTC 11204 containing ΦCD27) were made (2.3.2.1.3). Approximately 700 ng of genomic DNA and 600 ng of the plasmid vector pUC19 were separately digested with *HindIII* and *SacI* restriction enzymes at 37˚C for 1-3 h. Genomic DNA was also digested with *Sau3AI* under the same conditions and pUC19 digestion with *BamHI* was also carried out. Two µL aliquots were visualised on a 1.5% agarose gel to confirm that digestion had occurred, prior to inactivation of the restriction enzymes. The digested DNA (approximately 500 ng) was ligated into pUC19 vectors (approximately 100 ng) using T4 DNA ligase (NEB) for 2-15 h at 16˚C (2.3.11.2).

Ligation reactions (2.5 µL) were used as templates for PCR reactions with primers designed to amplify pUC19 and regions up/down stream of identified *HindIII, SacI* and *Sau3AI* restriction sites to give products for which the expected size (for phage-only DNA) could be calculated based on the ΦCD27 sequence. Red Mix (Bioline) containing Taq polymerase, reaction buffer, dNTPs and ions was used according to manufacturer's instructions. Templates 1-6 (Table 7.3) were each tested with primer pairs A-H (Table 7.4)

to give a total of 48 reactions, plus 1 control without template for each primer pair. Primer sequences are shown in Table 7.5.

Template designation	Vector	Genomic DNA source	Restriction enzyme used
1	pUC19	12727	*HindIII*
2	pUC19	11204LYS	*HindIII*
3	pUC19	12727	*SacI*
4	pUC19	11204LYS	*SacI*
5	pUC19	12727	*BamHI/Sau3AI*
6	pUC19	11204LYS	*BamHI/Sau3AI*

Table 7.3. Details of ligation mixtures used as PCR templates for the identification of the *attB* site

Primer combination designation	Primer 1	Primer 2
A	pCC1F	ORF41_F
B	pCC1R	ORF41_F
C	pCC1F	ORF42_R
D	pCC1R	ORF42_R
E	pCC1F	ORF42_F
F	pCC1R	ORF42_F
G	pCC1F	ORF43_R
H	pCC1R	ORF43_R

Table 7.4. Primer combinations used with ligation mixture templates for the identification of the *attB* site.

Primer	Sequence (melting temperature)
ORF42_R	AAT ATA ACT GAA GAA CAG GAG AGT C (57.7°C)
ORF42_F	TAA CGC CAC GAC TTA GTC TAT C (59.9°C)
ORF41_F	GAA ATG AGC GAA TTT TTA CTA GG (59.7°C)
ORF43_R	GGC TTA CAA CAA GTT AAT TGG TC (60.3°C)
pCC1F	GGA TGT GCT GCA AGG CGA TTA (56.3°C)
pCC1R	CTC GTA TGT TGT GTG GAA TTG (50.0°C)

Table 7.5. Sequences of the primers used for the identification of the *attB* site.

Products of a larger than expected size were estimated to contain genomic DNA from the bacterial host genome and these fragments were submitted for

sequencing with the appropriate ORF primer to elucidate the site of phage integration in the host genome.

The inferred site was then confirmed by designing specific primers flanking this integrated region, incorporating both phage and host sequence. Amplification was carried out with GoTaq (2.3.5.1) with an annealing temperature of 60°C, a 30 s extension and 25 cycles. *C. difficile* NCTC 11204, 11205, 11207 and 11209 were tested with the corresponding lysogenic strains. The identity of the fragments was confirmed by subcloning the products (2.3.11) and sequencing using Big Dye version 3.1 (2.3.10).

7.5. Results

7.5.1. Differential toxin production in wild type and lysogenic strains

When the mean absorbance values of the toxin assay were considered, the lysogenic strains of *C. difficile* NCTC 11204, 11205 and 11209 produced considerably less toxin than their wild type counterparts ($p = < 0.05$). For 11207 however, a significant increase relative to the wild type was observed ($p = < 0.05$). These results are shown in Fig. 7.2. The toxin measurements were made from cultures when they reached an OD_{600} of 1.5 (approximately 1 x 10^8 CFU/mL) and it was noted that lysogenic strains required an incubation time of 1-3 h longer than wild types in order to reach this cell density.

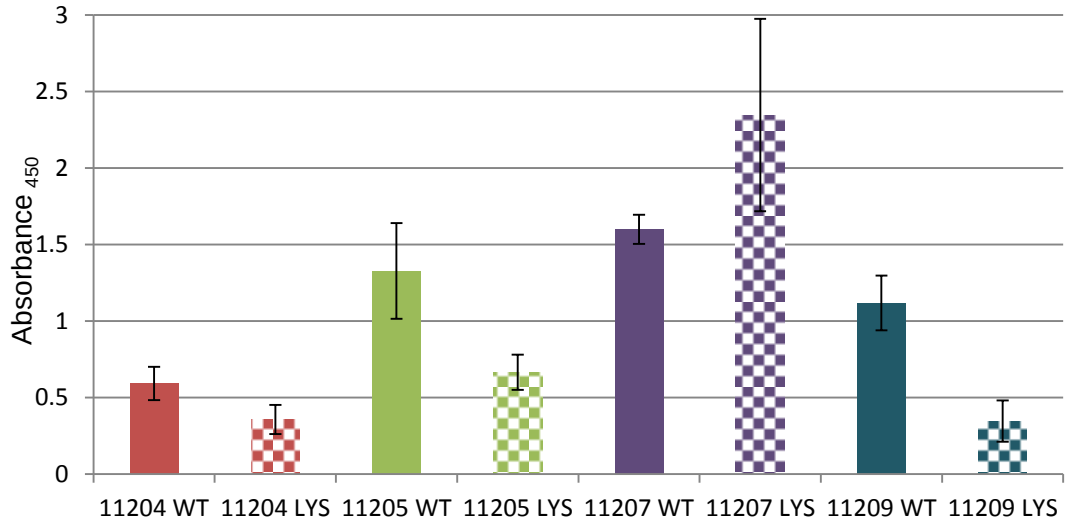


Figure 7.2. Differential toxin production in wild type (WT) and lysogenic (LYS) strains of *C. difficile.* Values are means of 5 replicate cultures ± SD.

7.5.2. Expression of PaLoc and regulatory genes in wild-type and lysogenic strains of *C. difficile*

Genomic DNA extractions (2.3.2.1.3) of all *C. difficile* strains were used as positive controls for all the primers designed to amplify the genes indicated in Table 7.3. Synthesis of cDNA was carried out on DNase-treated total RNA extracted samples and was used as a template for PCR to assess the differential expression of genes involved in *C. difficile* toxin regulation. Expression of putative phage repressor proteins was also investigated. After DNase treatment, no PCR products were obtained in amplification reactions of the control target (16S DNA), indicating that all genomic DNA in the samples had been degraded. After cDNA synthesis, amplification using primers for the 16S region was positive in all extracts and similar in terms of band intensity (Fig. 7.3 and Table 7.6). Toxin A (*tcdA*) expression was found to be slightly lower in lysogenic strains compared to corresponding wild-types with the exception of NCTC 11209, which was higher in the lysogenic strain (Fig. 7.3). For toxin B *(tcdB*), no expression was observed in NCTC 11204 or 11207, wild types and lysogens. Weak expression was observed for the wild type of NCTC 11205, which was absent in the lysogen. Very weak *tcdB*

expression was recorded for the wild type of NCTC 11209, which was slightly stronger in the lysogen (Fig. 7.3). No expression of *tcdC* was seen in any strains, wild types and lysogens (Fig. 7.3), which was to be expected since cultures were harvested in the stationary phase (corresponding to peak levels of toxin production). For *tcdE*, no expression was observed in NCTC 11204 and 11207, wild types and lysogens. Strong expression in the wild type NCTC 11205 was observed, with no expression recorded for the lysogenic strain. In NCTC 11209 however, expression was stronger in the lysogenic strain relative to the wild-type (Fig. 7.3). For *CodY,* expression was essentially equal apart from a slight reduction in 11207LYS compared to 11207 wild-type (Fig. 7.3). *CcpA*, which was not expressed at all in 11204LYS or wild-type, showed similar expression in 11205LYS and 11205 wild-type. For 11207 and 11209 wild-types, no expression was detected but their lysogenic counterparts demonstrated weak expression (Fig. 7.3). Finally, *Spo0A* expression was generally lower in lysogens relative to wild-types, but no expression was detected in either 11207LYS or 11207 wild-types (Fig. 7.3).

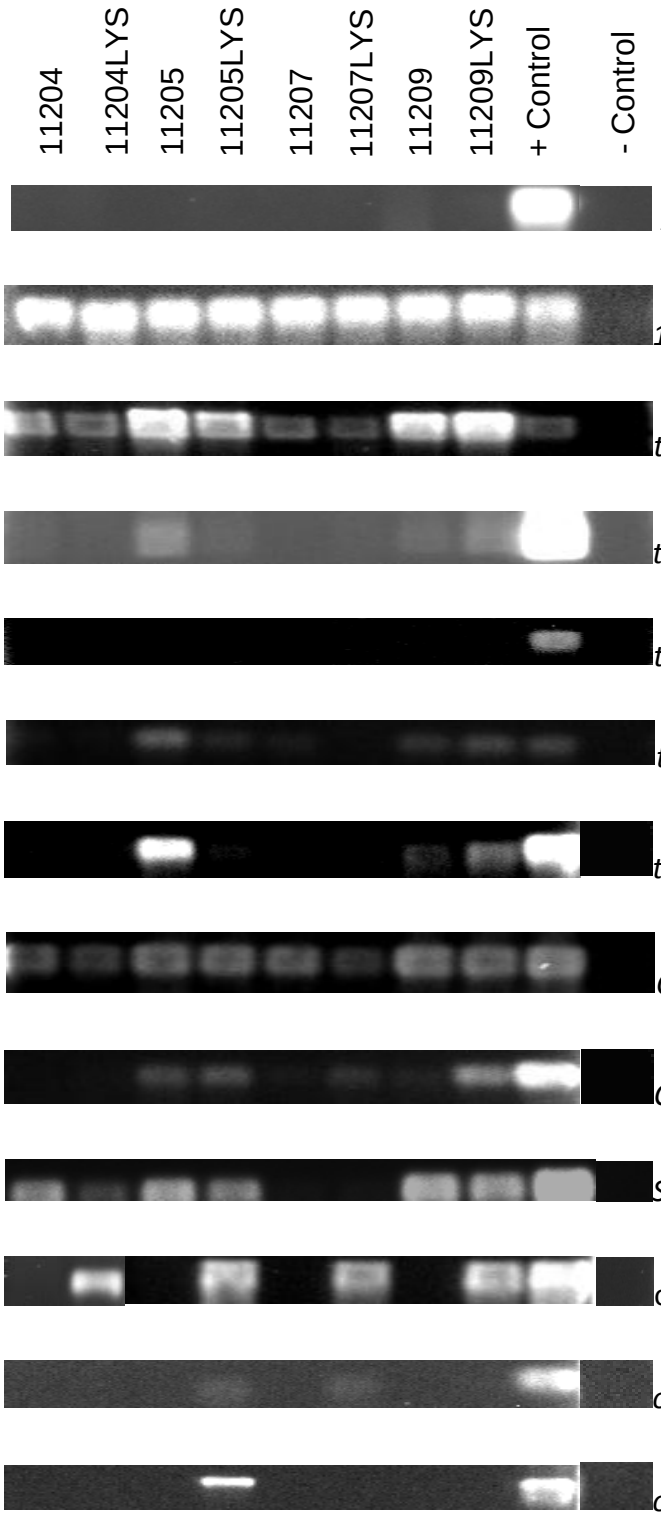


Figure 7.3. Band intensity of amplified cDNA for wild-type and lysogenic strains of *C. difficile*. 11204LYS genomic DNA was used for the positive control. Sterile ultra-pure water was used for the negative control.

* The negative control was pooled with others in a separate lane on this gel.

	16S (Post DNAse)	*16S* (Post cDNA synthesis)	*tcdA*	*tcdB*	*tcdC*	*tcdE*	*tcdR*	*CodY*	*CcpA*	*Spo0A**
Positive control	+++	++	+	++++	+	+++	(+)	+	++	+++
Negative control	-	-	-	-	-	-	-	-	-	-
11204	-	++	++	-	-	-	-	(+)	-	++
11204LYS	-	++	+	-	-	-	-	(+)	-	+
11205	-	++	+++	+	-	++	+	+	+	+++
11205LYS	-	++	++	-	-	(+)	(+)	+	+	++
11207	-	++	++	-	-	-	(+)	+	-	-
11207LYS	-	++	+	-	-	-	-	(+)	(+)	-
11209	-	++	++(+)	(+)	-	(+)	(+)	+	-	+++
11209LYS	-	++	+++	-	-	+	+	+	+	++

Table 7.6. Differential expression of PaLoc genes and toxin regulatory genes of *C. difficile* in wild type strains and lysogens (- = no expression; (+) = very weak; + = weak; ++ = strong and +++ = very strong).* PCR product was diluted 1 in 10 prior to electrophoresis due to high band intensity.

7.5.3. Putative repressors of ΦCD27

The expression of phage repressors is required to maintain the lysogenic state of prophages (Kutter & Sulakvelidze, 2005). The results of putative phage repressor gene expression in the wild-type and lyosgenic *C. difficile* strains are shown in Fig. 7.3 and Table 7.7. No PCR products were obtained with *orf44*, *orf45* or *orf72* primers when used to test wild-type genomic DNA, confirming absence of ΦCD27, and genomic DNA from lysogenic strains (containing ΦCD27) was used for the positive controls. Mitomycin C induction of ΦCD27 from all lysogenic strains was demonstrated.

Expression of *orf44* was observed in all lysogenic strains. Negligible expression of *orf45* was observed. *Orf72* was expressed in 11205LYS but no

other lysogenic strains. These results point to *orf44* as the candidate ΦCD27 repressor.

	orf44	*orf45*	*orf72*
Positive control (genomic DNA of corresponding lysogens)	++	++	++
Negative control (genomic DNA of wild-type strains)	-	-	-
11204	-	-	-
11204LYS	++	-	-
11205	-	-	-
11205LYS	++	-	++
11207	-	-	-
11207LYS	++	-	-
11209	-	-	-
11029LYS	++	-	-

Table 7.7. Expression of putative phage repressor genes in wild type and lysogenic strains of *C. difficile* (- = no expression; ++ = strong expression).

7.5.4. Purification of a putative repressor protein of ΦCD27

The *orf44* gene was amplified (2.3.5.2), subcloned (2.3.11.2.2) and expressed (2.4.1.2) and the His-tagged protein was purified as previously described (2.4.2.2). All fractions obtained from the Qiagen Ni-NTA columns (3.25 µL samples) were run on a protein gel (Fig. 7.4 a) and the presence of a 6xHis tagged product was confirmed by Western blot (Fig. 7.4.b).

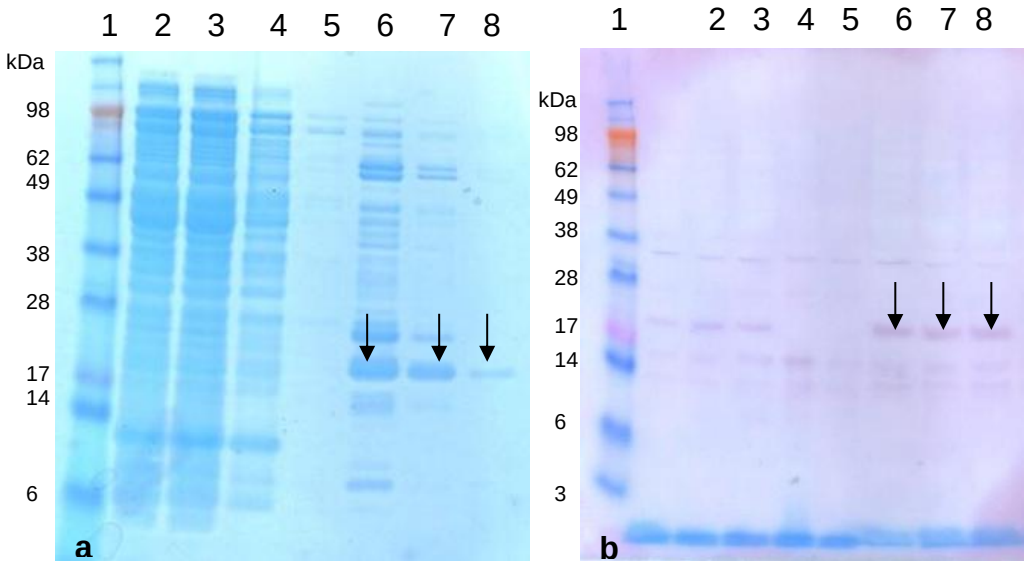


Figure 7.4. Protein gel after Ni-NTA column extraction (a) and Western blot (b). SeeBluePlus2 marker (1), lysate (2), column flow-through (3), wash 1 (4), wash 2 (5), elution 1 (6), elution 2 (7) and elution 3 (8). The arrows highlight the ORF44 protein.

The first and second elutions contained many other protein bands in addition to ORF44. The third elution had fewer visible secondary products and so was selected for dialysis (2.4.7.1)and concentration by ultra-centrifugal filtration (2.4.8). A protein gel (Fig. 7.5) and a Bradford assay were carried out to verify the product size and estimate the concentration prior to use in the EMSAs.

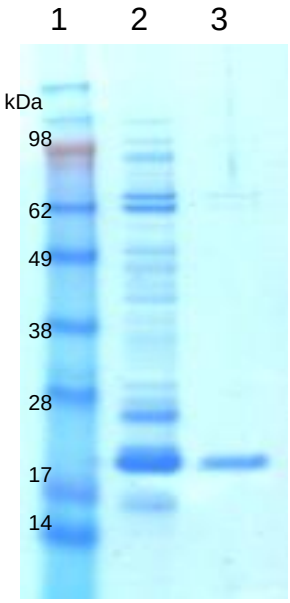


Figure 7.5. Dialysed ORF44 protein extracts. SeeBluePlus2 ladder (1), elution 1 (2) and concentrated elution 3 (3).

7.5.5. Amplification and preparation of PaLoc intergenic regions.

Intergenic regions (IGR) were successfully amplified and the recovery post QIAex gel extraction gave yields suitable for EMSA testing as shown in Fig. 7.6. Quantities were estimated based on band intensity relative to bands of the Hyperladder I.

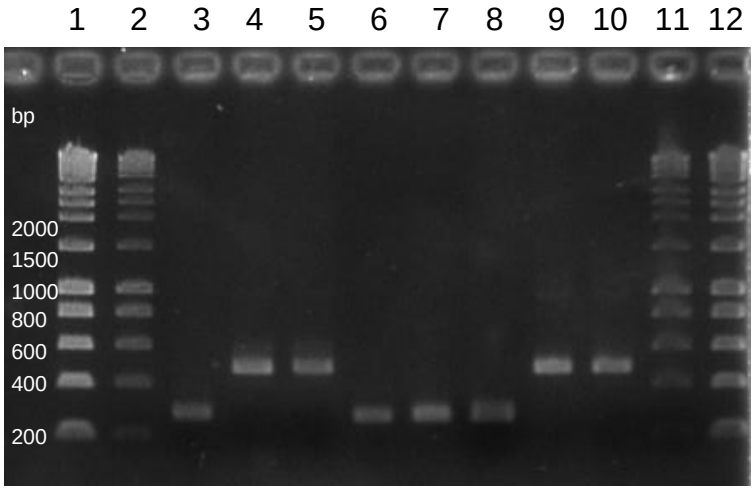


Figure 7.6. PCR products of PaLoc intergenic regions Hyperladder I, 5 µL (1 & 12), hyperladder I, 1 µL (2 &11), PCR product from IGR 1 containing NCTC 11205 promoter for *tcdD* (3). PCR products from IGR 2 containing NCTC 11205 promoter region of *tcdA* (4-5). PCR products from IGR 1 containing NCTC 11209 promoter region of *tcdD* (6-8) and PCR products from IGR 2 containing NCTC 11209 promoter region of *tcdA* (9-10).

7.5.6. Electrophoretic Mobility Shift Assays (EMSAs)

ORF44 purified protein at concentrations of 50-500 ng (above the 30 ng sensitivity limit of the EMSAs) was tested with 40 ng DNA from *C. difficile* NCTC 11205 and 11209 (containing promoter regions of PaLoc genes). DNA bands were visualised with SYBRGreen but for unknown reasons no protein bands could be detected after staining with SYPRO Ruby. This was investigated with a series of repeat tests and modification of conditions (protein concentration, run duration and binding buffers used), but with no success. Only non-denatured products were tested by EMSA as the protein was required to be in its native state to study the DNA binding affinities.

Denatured products from the same preparation could be visualised on a 10% NuPage Novex Bis-Tris gel, with no indication of dimer formation. The observation was presumed to be a result of retro-phoresis due to the intrinsic nature of the protein, which has an isoelectric point of 6.193 (and therefore a net negative charge at pH 7). The interpretation of the EMSAs was confounded by this inability to visualise protein since protein-DNA complexes would also be subjected to retro-phoresis. Weakening of bands with increased protein concentration may indicate the formation of complexes, but only in the absence of non-specific interactions (judged by results of calf thymus DNA-containing samples)

7.5.6.1. Intergenic regions containing the promoter for *tcdD.*

No band retardation was observed following binding reactions with the ORF44 protein and the intergenic region upstream of *tcdD* (11205 and 11209 regions were tested as shown in Fig. 7.7).

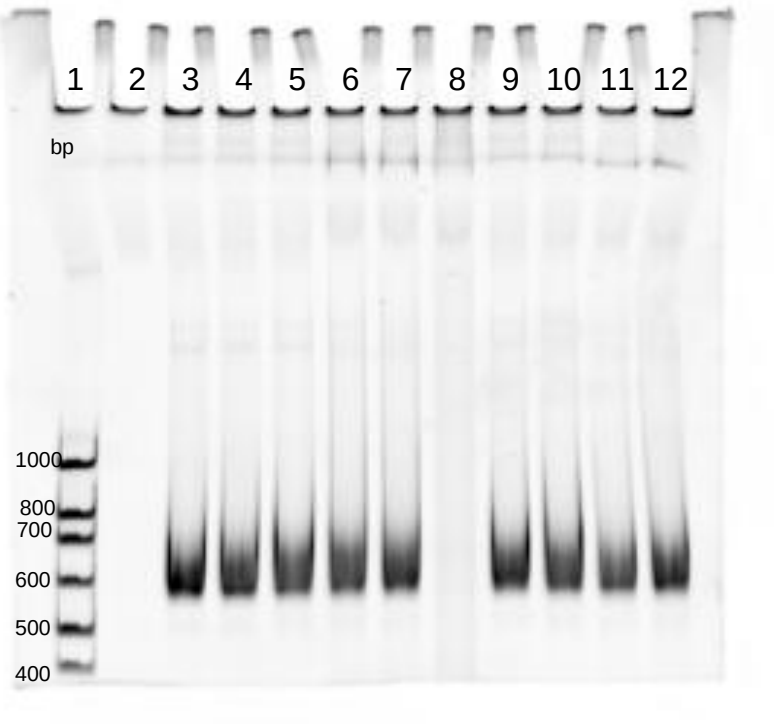


Figure 7.7. EMSA of ORF44 protein with 40 ng *tcdD* promoter containing DNA. DNA ladder Alpha Innotech, 1µL (1), 273 ng ORF 44 only (2), 40 ng DNA (11205) only (3), DNA (11205) plus 50 ng protein (4), DNA (11205) plus 100 ng protein (5), DNA (11205) plus 250 ng protein (6), DNA (11205) plus 273 ng protein (7) and 273 ng protein plus 40 ng calf thymus DNA (8); DNA (11209) plus 50 ng protein (9), DNA (11209) plus 100 ng protein (10), DNA (11209) plus 250 ng protein (11) and DNA (11209) plus 273 ng protein (12).

7.5.6.2. Intergenic regions containing the promoter for *tcdA.*

Despite an apparent weakening in the intensity of the band observed with *tcdA*-promoter containing DNA from *C. difficile* NCTC 11205 and 250/500 ng ORF 44 protein relative to 50 ng, no actual shift in the position of the band was observed and this weakened intensity could also be observed in the presence of calf-thymus DNA (Fig. 7.8). Similarly, no shift was observed with DNA of this region from NCTC 11209.

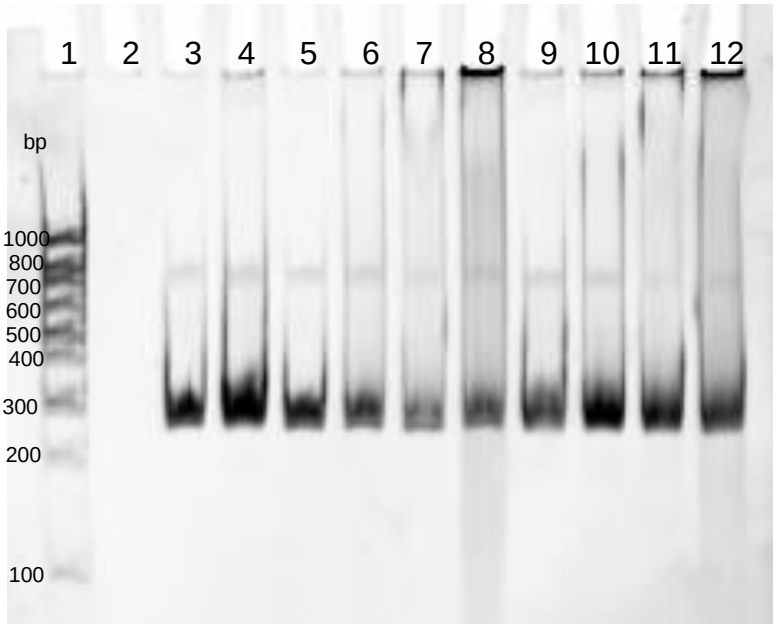


Figure 7.8. EMSA of ORF44 protein with 40 ng *tcdA* promoter containing DNA. DNA ladder Alpha Innotech, 1µL (1), 500 ng protein only (2), 40 ng DNA (11205) only (3), 40 ng DNA (11209) only (4), DNA (11205) plus 50 ng protein (5), DNA (11205) plus 250 ng protein (6), DNA (11205) plus 500 ng protein (7), 40 ng calf thymus DNA plus 500 ng protein and DNA (11205) (8); DNA (11209) plus 50 ng protein (9), DNA (11209) plus 250 ng protein (10), DNA (11209) plus 500 ng protein (11) and 40 ng calf thymus DNA plus 500 ng protein and DNA (11209) (12).

7.5.6.3. Intergenic regions containing the promoter for *tcdE.*

No significant mobility shifts or weakening in the intensity of the bands were seen following binding reactions with increasing concentrations with ORF44 and putative *tcdE* promoters, as shown in Fig. 7.9.

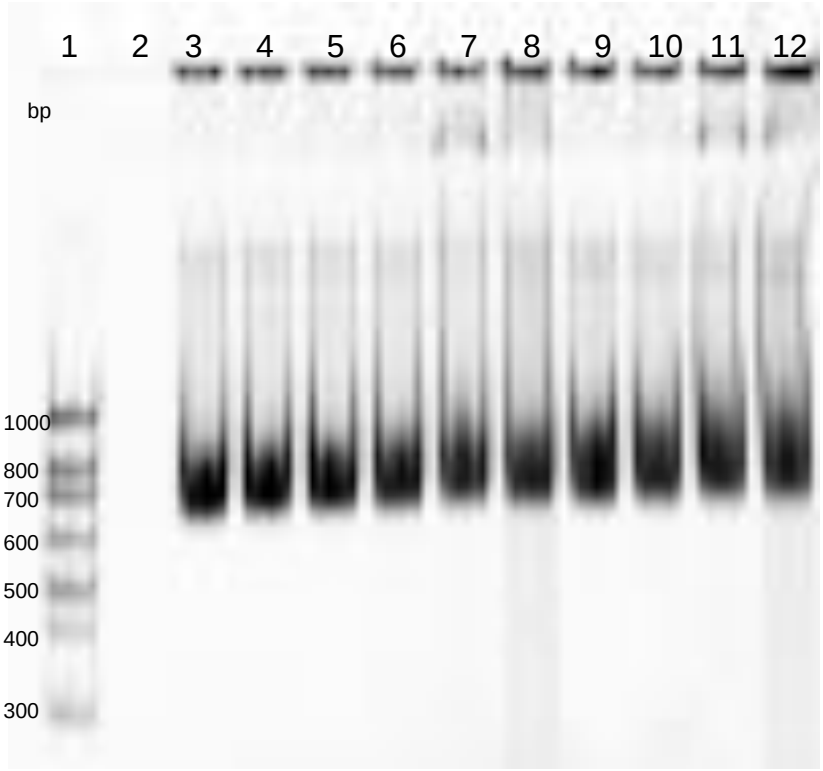


Figure 7.9. EMSA of ORF44 protein with 40 ng *tcdE* promoter containing DNA. DNA ladder Alpha Innotech, 1µL (1), 500 ng protein only (2), 40 ng DNA (11205) only (3), 40 ng DNA (11209) only (4), DNA (11205) plus 50 ng protein (5), DNA (11205) plus 250 ng protein (6), DNA (11205) plus 500 ng protein (7), 40 ng calf thymus DNA plus 500 ng protein and DNA (11205) (8); DNA (11209) plus 50 ng protein (9), DNA (11209) plus 250 ng protein (10), DNA (11209) plus 500 ng protein (11) and 40 ng calf thymus DNA plus 500 ng protein and DNA (11209) (12).

7.5.7. Phage integration site

Restriction digestions of genomic DNA from the lysogenic strains were successful. These digests were ligated into pUC19 and used as templates for PCR. The resulting fragments of the primer and target combinations (from Tables 7.2-7.4) are illustrated in Fig. 7.10 and show the presence of products of the expected size (Table 7.5) and some that are larger than expected from phage DNA alone (Table 7.6), indicating the presence of bacterial DNA. These latter fragments were sequenced to identify the integration site (2.3.10).

***HindIII* Restriction site on ΦCD27 genome**	**Expected size of fragment (bp)**	**Expected size of ligated fragment (bp)**	**Seen on gel**
34670	214	417	No
34884			
35106	222	425	No
35455	349	552	Yes (C1, **Fig. 7.10**)
35466	11	214	Yes (GI, **Fig. 7.10**).
SacI restriction site on ΦCD27 genome	**Expected size of fragment (bp)**	**Expected size of ligated fragment (bp)**	**Seen on gel**
10526	2203	2406	N/A
12729			

Table 7.5. Expected fragment sizes of ligated restriction digests. No *Sau3AI* restriction sites can be found on the ΦCD27 genome.

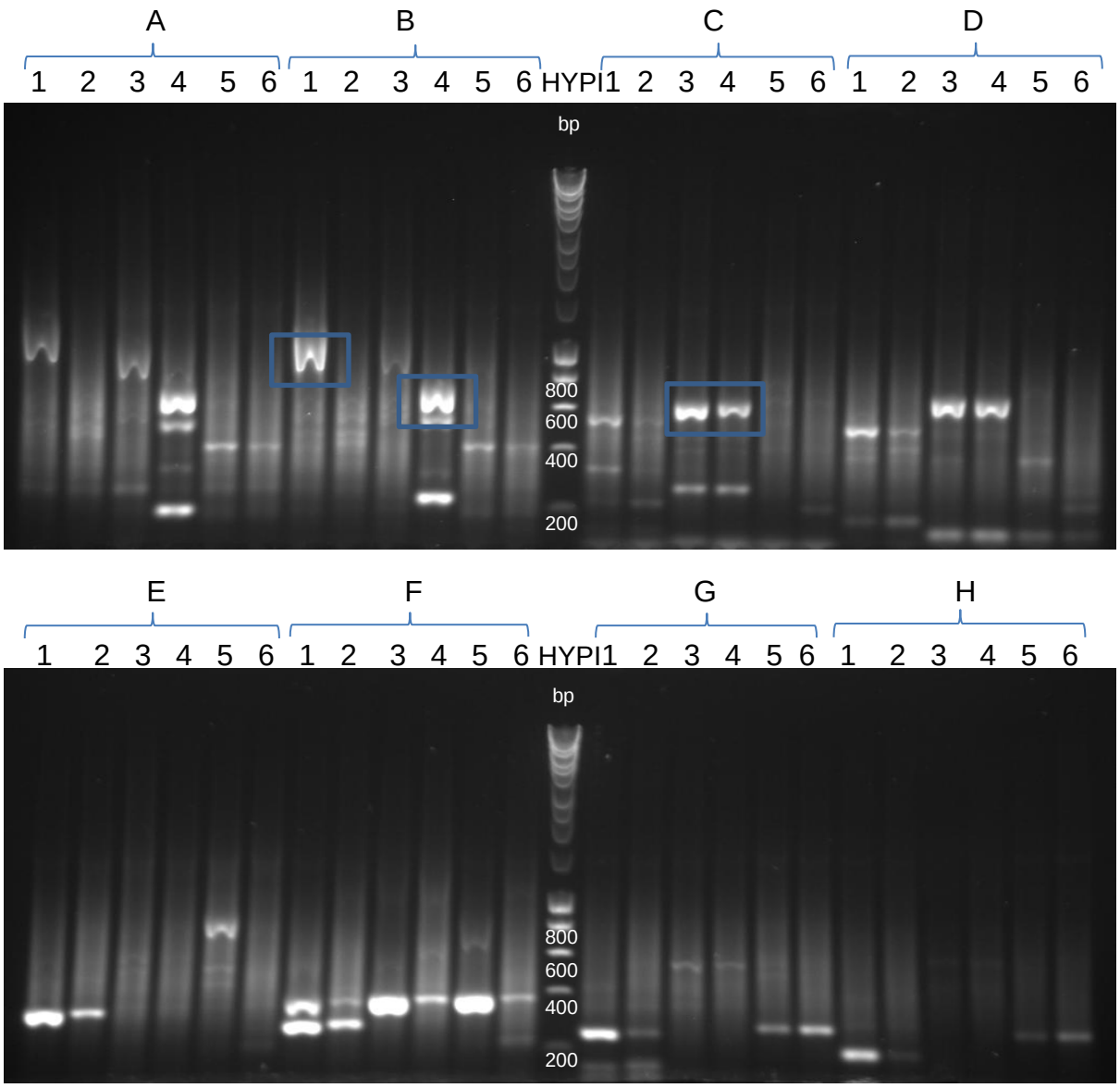


Figure 7.10. PCR products from templates of a *HindIII* digest of *C. difficile* NCTC 12727 (1), *HindIII* digest of 11204LYS (2), *SacI* digest of 12727 (3), *SacI* digest of 11204 LYS (4), *Sau3AI* digest of 12727 (5) and *Sau3AI* digest of 11204LYS (6) amplified with pCC1_F & ORF41_F primer pairs (A), pCC1_R & ORF41_F primer pairs (B), pCC1_F & ORF42_R primer pairs (C), pCC1_R & ORF42_R primer pairs (D), pCC1_F & ORF42_F primer pairs (E), pCC1_R & ORF42_F primer pairs (F), pCC1_F & ORF43_R primer pairs (G), pCC1_R & ORF43_R primer pairs (H). The fragments of unexpected size are highlighted by the blue boxes.

Designation (Lane and sample number, Fig. 7.10)	Size
B1	~1.1 kb
B4 (small)	~500 bp
B4 (large)	~600 bp
D3	~600 bp
D4	~600 bp

Table 7.6. Fragments of unexpected size, indicating the presence of bacterial DNA.

Fragments from B4 were of poor quality and fragments of D3 and D4 gave no homology to phage DNA and were therefore rejected. Fragment B1 however, gave both ΦCD27 sequence and sequence homologous to *C. difficile* 630 (in the absence of *C. difficile* 12727 sequence data) as illustrated in Fig. 7.11. These results show that ΦCD27 integrates at a position identical to base pair 3379694 in the genome of *C. difficile* 630.

CTTTATTACATATATTATTTCCAGAAAAGTAAAAAGCCACTCTGGCAGGAGTGACTTTGAGC
TTGATGTAAAAATCAAGTTTAATAAAAAACGACATTAATATTTAGTTAATGAAACTTCACTC
TAGGCTAATAGATTGTAGTTTCTTTTTTTGTTTTTGATACGAATTTACATCTTTATTATATC
GCATTTTGAGAAAAAATAAAACTATGAATATTAGAAATATATTTTATTTTATCTCTAAGTAT
ATATATTATTAGCCACCTAAAAAACTTTCATAAAAACAAAAAGAACAATATCTTTTTGGCAA
CTGGCTGGCATAATTTATAAGAATTTTATAAATCTTAGTCTGCGTCACTAATTTTCTCTAGT
TTTGCCGATTTAGTTTTGTTATACAACTAAAATAATACAATAGAATTAGTTAATGTTCAACA
AGGTTGTTTGAAAATTAAATAAATTTATAGGTATGAATAGTATTTATTCCTAAAAAGGAACA
GAGTATTTAAAGGTCAAGGTAAGTTAGAATAGTATTGTGAACTAACATTTGATTATGCGAAA
TTCTATGAAACAGGAACCCTGTGACTTTAGTCATGGGAGGTTCAGTGTATATCGCGTATTTC
AATTGTTATAAGTATTTTATTTATTTGGACTAACTAATAATAGCCAAGTGGATGTGGTAAAA
CTACATTACTTAGAATAATAGCAGGTCTTGAAGATGTAAATAGTGGAACTATAATTCTCCAG
GATAAAGATATTACAAATTTAGAGCCATCAAAGAGAGGTTTTGGTATAGTATTTCAATCATA
TGCATTATTTCCGAATATGACTGCTTATAACAATATAGCTTTTCCACTAAAAGAGAGAAAAG
TTTCAAAAGAAAAAATAGATAATAAAGTTAAGGAAGTACTTGAAACAGTTGGATTGACTAAT
GAAGCTCATAAATACCCTAAAGCATTATCAGGTGGACAACAACAGAGGATTGCAATAGCAAG
AGCATTAGCTCTTGAACCAAAGTTTTTACTTTTAGATGAACCAATGTCTGCATTAGATGCAA
AGGTTAGACACAAGCTTGGCGTAATCATGGTCATAG

Figure 7.11. Sequence product of fragment B1 (NCTC 12727 digested with *HindIII*). Red type shows sequence matching ΦCD27 genome, blue sequence shows sequence matching to *C. difficile* 630 DNA. The *HindIII* site is underlined. Black sequence represents vector (pUC19) DNA.

In order to confirm this integration site of ΦCD27 in 12727 (and all ΦCD27 lysogens), primers up- and down-stream of the integration site were designed:

- INTGRTN_ORF41_F: 5’-AGT CCC TAT AGC TTT GCG TCA C-3’ (Tm 63.3°C)
- INTGRTN_CD630_R: 5’-TGC ATC TAA TGC AGA CAT TGG-3’ (Tm 63.6°C)

The primers gave a product of 717 bp in size (as shown in Fig. 7.12) only from the lysogens. The PCR products were subcloned and sequenced. This confirmed the same integration site in all 4 lysogens.

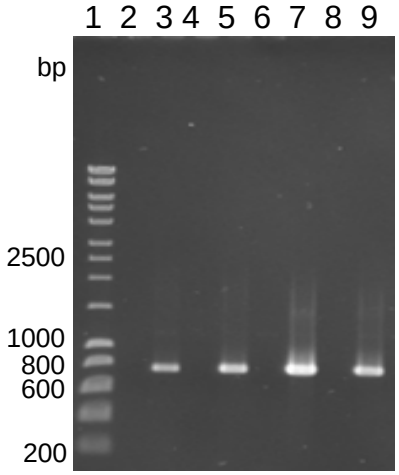


Figure 7.12. PCR products using primers to confirm integration site of ΦCD27 (INTGRTN_ORF41_F and INTGRTN_CD630_R). Hyperladder I (1), 11204 (2), 11204LYS (3), 11205 (4), 11205LYS (5). 11207 (6), 11207LYS (7), 11209 (8) and 11209LYS (9).

7.6. Discussion

Differential toxin production in wild type and lysogenic *C. difficile* strains was successfully determined, and showed reduced levels for 3 of the 4 lysogenic counterparts, and stronger production for 1 relative to the wild-types. Differential expression of toxin genes and toxin regulator genes was also assessed, and was found to be discordant from the phenotypic profiles. Despite stark reductions in toxin production in lysogens relative to wild-types as measured by EIA (in agreement with previous reports of levels of up to 50% lower (Govind *et al.*, 2009)), the trends in *tcdA* and *tcdR* expression were

only very slightly lower in the lysogen of 11205 relative to the wild type. A decrease in *tcdA* expression for 11207LYS was also observed even though phenotypic toxin expression was significantly higher in this lysogen relative to the wild-type and expression in 11209LYS was higher relative to the wild-type despite comparatively less toxin production phenotypically. Other studies have reported increases and decreases in phenotypic toxin A and B production with no corresponding change in the transcription of the implicated gene (Goh *et al.*, 2005). This observation could imply effects on the release of toxin rather than being directly involved in the regulation of toxin gene transcription, but in these cases it does not appear to be due to changes in *tcdE* expression.

The results of cDNA PCR must be interpreted with caution since gene expression in bacteria is greatly affected by the growth rate and nutritional status (Lodish *et al.*, 2000). Despite every effort to control the conditions prior to RNA extraction, subtle variations between cultures may have given rise to the differences observed. In addition to this, visual estimation of band intensity is a rather crude method of analysis and replicate experiments were not carried out to assess the degree of variation between tests. Also, previous studies have suggested that secondary structures, which may have formed in the extracted RNA, can prevent primer binding and target transcription (Braham *et al*., 2009). Having said this, the purpose of this investigation was to highlight clear differences in expression in advance of EMSA testing with the putative phage repressor ORF44, and this method has also been adopted by previous studies (Govind *et al.*, 2009). Real-time PCR is an alternative method of analysis that could be used to support these results. Expression levels may have shown clearer differences (or similarities) at different times during the growth phase. The stationary phase was selected as this is the time when peak toxin production is expected and therefore differences were more likely to be apparent. Extractions from other time points would add considerable value to this work since the duration of toxin production may have been affected. This observation has been reported previously for the highly virulent *C. difficile* 027 strain (Freeman *et al.*, 2007). While peak toxin amounts remained similar to strain 001, the period of toxin production was

significantly extended and this feature is thought to explain the severe disease seen in infected patients. Also of note is that lysogenic strains required an incubation time 1-3 h in excess of their wild-type counterparts to reach the same optical density. This observation opposes the findings of previous work, showing no difference in the growth rate of lysogens and wild types (Govind *et al.*, 2009; Sekulovic *et al.*, 2011).

In other studies of phage-mediated toxin production, cells of *C. difficile* were lysed prior to measuring the quantity of toxin produced to include intracellular amounts (Govind *et al*., 2009). This approach was not considered here since it was initially thought not to impact greatly on the virulence of the pathogen (at least until cell death when the pathogen degrades) but data for this may have helped to elucidate the effect on mediators of toxin production, for example down-regulation of *tcdE*, which may play a role in toxin export due to the homology to phage holins.

Many strains of *C. difficile* show variations within PaLoc, namely the *tcdC* region (Spigaglia and Mastrantonio, 2002) and these variations impact on strain toxigenicity. A frameshift mutation in *tcdC* in the highly virulent *C. difficile* 027 is thought to play a role in the hypertoxigenicity of this pathogen (Dupuy *et al.*, 2008). Subtle strain-to-strain variations were observed in terms of toxin A production and this may in part be explained by sequence differences in *tcdC* that were unfortunately not investigated.

The putative phage repressor ORF44 was successfully cloned and expressed in *E. coli*. No significant interactions between ORF44 and the promoters of *tcdA*, *tcdB*, *tcdC*, *tcdE* and *tcdR* were demonstrated by EMSA. Similarly, ORF44 showed no affinity to the estimated promoters of *CodY*, *CcpA* or *Spo0A*. The results are inconclusive in that the ORF44 protein may interact with genes elsewhere in the *C. difficile* genome that are currently not regarded as regulators of toxin production. It may also be that a different repressor may be interacting with any of these regions. The protein extract used for these experiments appeared relatively pure when visualised by gel electrophoresis but it must be considered that contaminating proteins from the cell extracts could cause interference with the binding reactions, particularly since the

protein could not be visualised on the EMSA gels. Despite absence of evidence for dimerisation after denaturing gel electrophoresis, the possibility of multimer formation in the protein's native form, a feature of many DNA-binding proteins (Jia *et al.*, 2005), cannot be ruled out and could be an alternative explanation for its absence from the gel images. This may also have impeded the binding interactions with the promoter-containing DNA molecules.

The affinities of the 2 other putative repressor proteins of ΦCD27, ORF45 and ORF72, were not determined in this study due to time limitations. Expression of ORF45 was negligible in the *C. difficile* lysogens and ORF72 expression was detected only in 11205LYS. Investigation of these proteins using the same methods employed for ORF44 may help to elucidate the mechanisms of toxin regulation in these lysogens. All three putative repressors ORF44, ORF 45 and ORF 72, may be involved in the lysogenic switch and may therefore still be useful targets in terms of therapeutic manipulation.

The integration site of ΦCD27 was determined successfully and has been shown to be the same in all the lysogenic strains tested and strain 12727, from which the phage originally derives. This position is the same as prophage 2 of CD630 (Sebaihia *et al*., 2006). It is in a region containing repeat sequences of DNA with no known toxin or regulatory genes within an ORF encoding a putative ATP binding protein of an ABC transporter (based on alignment to the published *C. difficile* 630 genome and nucleotide Blast of the sequence). This may indicate a detrimental effect on toxin export in the lysogenic strains. There is of course the possibility for multiple integration sites, as has been described previously for the Stx phage Φ24B (Fogg *et al.*, 2007). This may cause the disruption of genes with known (and unknown) roles in toxin regulation.

In summary, the phenotypic expression of toxin has been shown to vary considerably between wild types and lysogenic strains of *C. difficile*, in agreement with previous work (Govind *et al*., 2009; Goh *et al*., 2005). ORF44 was identified as a putative phage repressor proteins based on its expression

in the lysogenic state, but its role in toxin regulation has not yet been determined.

Chapter 8 – Concluding remarks

The pace of research into the use of phages to manage bacterial infections in humans is increasing, driven by the diminishing efficacy of current antibiotic strategies. There are numerous studies demonstrating *in vitro* and *in vivo* remediation of a host of bacterial infections including *Staphylococcus aureus* (Seth *et al*., 2013), *Campylobacter jejuni* (Loc Carillo *et al*., 2005) and *Pseudomonas aeruginosa* (Vieira *et al*., 2012). Some phage studies have now come as far as phase II clinical trials (Harper *et al*., 2011).

There are very few studies that have investigated the control of CDI using phage, but all have demonstrated improvements in features of CDI, such as increased survival rates in animal models (Govind *et al.*, 2011; Ramesh*, et al.*, 1999) and reductions in *C. difficile* toxin production (Meader *et al.*, 2010). The *in vitro* model investigations performed in this study to examine the efficacy of phage treatment are the first to evaluate a prophylactic approach using phage for the prevention of CDI. Prophylaxis for CDI is feasible since we are aware of many of the risk factors that give rise to CDI, such as broad spectrum antibiotic therapy (Starr *et al.*, 2003) or proton pump inhibitor use (Loo *et al.*, 2011). In the preliminary batch fermentation studies it was indicated that this strategy may be more effective than a remedial regime and this might also be true for other treatments, such as Tolevamer – a *C. difficile* toxin binding protein (1.7.9). The observation that ΦCD27 is effective at preventing outgrowth of germinating spores (3.5.1) is another argument for prophylactic use

The colon model has previously been shown to give results consistent with the inferences of clinical trials (Baines *et al.*, 2009) and bearing this in mind, this initial assessment of the performance of phages in the prevention of CDI demonstrates some potential for use in humans. ΦCD27 seemed to survive well in the simulated gut environments but neither of the models assessed the viability and subsequent activity of the phage after oral administration and traversal of the (neutralised) acidic barrier of the stomach.

One of the problems highlighted in both the batch fermentation and colon model experiments was the tendency for ΦCD27 to lysogenise with the host,

causing numbers of *C. difficile* to reach levels of an untreated control. In conditions where energy is in short supply, bacterial cells have high levels of cyclic AMP and this is known to promote lysogeny following phage infection. Similarly, plentiful energy supplies are conducive to low levels of cyclic AMP and this state promotes the lytic cycle (Kutter and Sulakvelidze, 2005). Despite the comprehensive validation of the colon model by MacFarlane *et al*. (1998) the abundance of nutrients at the start of the experiment and gradual depletion of nutrients towards the end could affect the lysogenic and lytic tendencies of the phage. This may also help to explain the apparent differing efficacies of phage treatment in prophylactic and remedial regimens since the phage lytic cycle would be favoured earlier at the start of the experiment before the media becomes spent. These observations suggest that manipulation of the environment via the availability of nutrients in order to favour the lytic cycle might be possible but is also likely to have an impact on the commensal flora of the gut. Nutrient depletion has been shown to pre-dispose to *C. difficile* infection, based on findings from patients fed via naso-gastric tubes (Iizuka *et al.*, 2004). This observation hints at the possibility that in plentiful energy supplies, available in the guts of healthy individuals, spontaneous lytic induction of phage may help to maintain normal turnover of gut bacteria.

The failure of prophylactic phage treatment to control *C. difficile* numbers in one of the three colon model replicates led to the finding that lysogenisation of ΦCD27 gave rise to reduced levels of toxin production relative to the wild-type strain. This finding was later supported by Govind *et al.* (2009), who demonstrated the action of a phage repressor protein, RepR, on the promoter region of *tcdR* to cause a reduction in the expression of toxin A. In a reproduction of this work, the putative phage repressor of ΦCD27, ORF44, was not demonstrated to function in this way (or have an affinity to any of the PaLoc promoter regions), but other putative repressors of ΦCD27 (ORF 45 and ORF 72) are yet to be investigated. In addition to this mechanism, the possibility of multiple integration sites cannot be ruled out, leading to disruption of a gene or genes that contribute to the regulation of toxin production.

The annotation of the ΦCD27 genome (carried out in 2008) was based on comparisons between other sequences submitted to NCBI for which functions had been experimentally determined or putatively assigned. The rate of sequence submission to the NCBI database is over 200,000 every month (http://www.ncbi.nlm.nih.gov) and the high value of re-annotating sequences several years after the initial searches has previously been demonstrated (Stabler *et al.*, 2009). Regular re-annotation of the ΦCD27 genome might allow the identification of additional known and putative repressors that have since been presented to the database.

C. difficile's highly mosaic genome is a testament to its remarkable ability to acquire new genetic elements (Sebaihia *et al.*, 2006) and studies of all novel therapies should consider the potential for resistance. Throughout the studies of ΦCD27 detailed in this manuscript, this feature was difficult to assess since *C. difficile* from the batch fermentations and colon models that were not killed by phage treatment were subsequently found to contain ΦCD27 prophage. The use of phages that are unable to lysogenise would permit better evaluation of resistance emergence. In the evaluation of the antimicrobial peptide lacticin 3147 as a potential treatment for CDI (Rea *et al.*, 2007), the possibility of using *Lactobacillus spp*. which are resistant to the agent in combination with it to counteract the effect on the commensal *Lactobacillus spp.* was suggested. No data relating to the preservation of the sensitivity of *C. difficile* in these circumstances was presented, but it would be interesting to check for instances of transferred resistance.

There are many other aspects of phage therapy that require attention to fully assess its potential, which are beyond the scope of this thesis. The preparation of phage stocks for administration is one such area and should be taken into account for *in vitro* and *in vivo* work-up studies, since treatment and purification steps might alter the viability and/or behaviour of the phage. Purification of phage according to buoyant density by caesium chloride gradient centrifugation is most commonly employed and is effective at removing bacterial cell debris and endotoxins (Biswas *et al.*, 2002), thought to be associated with many of the treatment failures in early reports of phage therapy that used basic methods such as filtration (Gill and Hyman, 2010).

One consideration however, is that the antigenic determinants in these more primitive preparations may have stimulated the immune system and contributed to apparent treatment successes. It remains to be seen if ΦCD27 efficacy as a therapeutic phage is altered by purification processes such as CsCl gradient centrifugation - some researchers have stated the need for higher $MgCl_2$ concentrations afterwards in order to maintain the phage's ability to form plaques (Kutter and Sulakvelidze, 2005).

Attempts were made to disable the lysogenic capacity of ΦCD27 but the desired phenotype was not obtained (3.5.2). Similarly, efforts to isolate exclusively lytic phages infecting *C. difficile* from environmental and clinical samples were not fruitful (4.5.7). Isolation of phages from environmental samples remains an unpredictable technique, which has so far only resulted in the isolation of phages with lysogenic capacity. Phages that can integrate into the host genome are unlikely to be considered for use as a therapeutic agent since their use carries a risk of acquisition of phage-derived virulence factors, although no genetic regions encoding known virulence determinants have been identified in any of the *C. difficile* phages characterised to date. Despite this, the association between *C. difficile* virulence and the carriage of specific prophages suggests that some genetic determinants of virulence are yet to be characterised (Shan *et al*., 2012).

Summary

Given the profound effect of broad-spectrum antimicrobial activity on commensal bacteria and the host of detrimental implications that we are now realising, specific treatments such as phage therapy should be considered as a priority, not just in answer to the problem of antibiotic resistance.

The models of CDI used in this study clearly demonstrate potential for phage therapy and highlight the need to further work to overcome the problems encountered, namely disabling the capacity for lysogeny and widening the susceptible host range. Genetic manipulation has the potential to alleviate these issues but would subject this therapeutic approach to another level of scrutiny given the public hostility to such techniques.

This study investigated phage therapy as a sole treatment for the prophylaxis of *C. difficile*, but improved results could be anticipated if it were to be used in conjunction with other strategies for prophylaxis and remediation. Phage therapy used with probiotics and prebiotics could be enough to selectively control the burden of *C. difficile* cells and the associated toxins, while supporting the growth of bacterial groups that offer colonisation resistance against *C. difficile*. Similarly, phage therapy could work synergistically with toxin-neutralising agents such as Tolevamer (1.7.9).

Standardisation of the techniques and governance of phage therapy appears to be one of the greatest challenges preventing this approach from moving forward. Regulations will most certainly require a certain degree of flexibility to allow for the use of phages with differing specificities, in a similar manner to the directives in place for seasonal influenza vaccines, which allow for the use of different antigenic determinants produced using a standard method (World Health Organisation Technical Report Series No. 963, 2011).

Phages have provided a wealth of information and resources to molecular biologists since their discovery. This study contributes to the rapidly growing body of data that may one day lead to an established role of phage therapy in Western medicine for the treatment of bacterial infections including CDI.

References

Aas, J., C. E. Gessert, and J. S. Bakken. 2003. Recurrent *Clostridium difficile* colitis: Case series involving 18 patients treated with donor stool administered via a nasogastric tube. Clinical Infectious Diseases **36:**580-585.

Abrams, G. D., M. Allo, G. D. Rifkin, R. Fekety, and J. Silva, Jr. 1980. Mucosal damage mediated by clostridial toxin in experimental clindamycin-associated colitis. Gut **21:**493-9.

Ackermann, G., B. Loffler, D. Adler, and A. C. Rodloff. 2004. *In vitro* activity of OPT-80 against *Clostridium difficile*. Antimicrob Agents Chemother **48:**2280-2.

Al-Nassir, W. N., A. K. Sethi, Y. Li, M. J. Pultz, M. M. Riggs, and C. J. Donskey. 2008. Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of *Clostridium difficile*-associated disease. Antimicrob Agents Chemother **52:**2403-6.

Aloizos, S., S. Gourgiotis, K. Oikonomou, and P. Stakia. 2008. Recurrent Jarisch-Herxheimer reaction in a patient with Q fever pneumonia: a case report. Cases J **1:**360.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J Mol Biol **215:**403-10.

Anton, P. M., J. Gay, A. Mykoniatis, A. Pan, M. O'Brien, D. Brown, K. Karalis, and C. Pothoulakis. 2004. Corticotropin-releasing hormone (CRH) requirement in *Clostridium difficile* toxin A-mediated intestinal inflammation. Proc Natl Acad Sci U S A **101:**8503-8.

Anton, P. M., M. O'Brien, E. Kokkotou, B. Eisenstein, A. Michaelis, D. Rothstein, S. Paraschos, C. P. Kelly, and C. Pothoulakis. 2004. Rifalazil treats and prevents relapse of *Clostridium difficile*-associated diarrhea in hamsters. Antimicrob Agents Chemother **48:**3975-9.

Antunes, A., I. Martin-Verstraete, and B. Dupuy. 2011. *CcpA*-mediated repression of *Clostridium difficile* toxin gene expression. Mol Microbiol **79:**882-99.

Ashelford, K. E., M. J. Day, and J. C. Fry. 2003. Elevated abundance of bacteriophage infecting bacteria in soil. Appl Environ Microbiol **69:**285-9.

Atterbury, R. J., M. A. Van Bergen, F. Ortiz, M. A. Lovell, J. A. Harris, A. De Boer, J. A. Wagenaar, V. M. Allen, and P. A. Barrow. 2007. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Appl Environ Microbiol **73:**4543-9.

Babakhani, F. K. S. S., N. Robert, P. Sears, and Y. K. I. Shue. 2004. Abstract E-2047 Resistance Development, Cross-Resistance, and Synergy Studies of OPT-80, 44th ICAAC, Washington.

Babalova, E. G., K. T. Katsitadze, L. A. Sakvarelidze, N. S. Imnaishvili, T. G. Sharashidze, V. A. Badashvili, G. P. Kiknadze, A. N. Meipariani, N. D. Gendzekhadze, E. V. Machavariani, K. L. Gogoberidze, E. I. Gozalov, and N. G. Dekanosidze. 1968. Preventive value of dried dysentery bacteriophage. Zh Mikrobiol Epidemiol Immunobiol. **2:**143–145.

Babcock, G. J., T. J. Broering, H. J. Hernandez, R. B. Mandell, K. Donahue, N. Boatright, A. M. Stack, I. Lowy, R. Graziano, D. Molrine, D. M. Ambrosino, and W. D. Thomas, Jr. 2006. Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamsters. Infect Immun **74:**6339-47.

Bacci, S., K. Molbak, M. K. Kjeldsen, and K. E. Olsen. 2011. Binary toxin and death after *Clostridium difficile* infection. Emerg Infect Dis **17:**976-82.

Baines, S. D., J. Freeman, and M. H. Wilcox. 2005. Effects of piperacillin/tazobactam on *Clostridium difficile* growth and toxin production in a human gut model. J Antimicrob Chemother **55:**974-82.

Baines, S. D., J. Freeman, and M. H. Wilcox. 2009. Tolevamer is not efficacious in the neutralization of cytotoxin in a human gut model of *Clostridium difficile* infection. Antimicrob Agents Chemother **53:**2202-4.

Baines, S. D., A. R. Noel, G. S. Huscroft, S. L. Todhunter, R. O'Connor, J. K. Hobbs, J. Freeman, A. M. Lovering, and M. H. Wilcox. 2011. Evaluation of linezolid for the treatment of *Clostridium difficile* infection caused by epidemic strains using an *in vitro* human gut model. J Antimicrob Chemother **66:**1537-46.

Baines, S. D., R. O'Connor, G. Huscroft, K. Saxton, J. Freeman, and M. H. Wilcox. 2009. Mecillinam: a low-risk antimicrobial agent for induction of *Clostridium difficile* infection in an *in vitro* human gut model. J Antimicrob Chemother **63:**838-9.

Baines, S. D., R. O'Connor, K. Saxton, J. Freeman, and M. H. Wilcox. 2008. Comparison of oritavancin versus vancomycin as treatments for clindamycin-induced *Clostridium difficile* PCR ribotype 027 infection in a human gut model. J Antimicrob Chemother **62:**1078-85.

Baines, S. D., K. Saxton, J. Freeman, and M. H. Wilcox. 2006. Tigecycline does not induce proliferation or cytotoxin production by epidemic *Clostridium difficile* strains in a human gut model. J Antimicrob Chemother **58:**1062-5.

Bakken, J. S. 2009. Fecal bacteriotherapy for recurrent *Clostridium difficile* infection. Anaerobe **15:**285-9.

Barbut, F., A. Richard, K. Hamadi, V. Chomette, B. Burghoffer, and J. C. Petit. 2000. Epidemiology of recurrences or reinfections of *Clostridium difficile*-associated diarrhea. J Clin Microbiol **38:**2386-8.

Bartlett, J. G., N. Moon, T. W. Chang, N. Taylor, and A. B. Onderdonk. 1978. Role of *Clostridium difficile* in antibiotic-associated pseudomembranous colitis. Gastroenterology **75:**778-82.

Bartlett, J. G., A. B. Onderdonk, R. L. Cisneros, and D. L. Kasper. 1977. Clindamycin-associated colitis due to a toxin-producing species of *Clostridium* in hamsters. J Infect Dis **136:**701-5.

Biller, J. A., A. J. Katz, A. F. Flores, T. M. Buie, and S. L. Gorbach. 1995. Treatment of recurrent *Clostridium difficile* colitis with *Lactobacillus* GG. J Pediatr Gastroenterol Nutr **21:**224-6.

Biswas, B., S. Adhya, P. Washart, B. Paul, A. N. Trostel, B. Powell, R. Carlton, and C. R. Merril. 2002. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. Infect Immun **70:**204-10.

Bloedt, K., M. Riecker, S. Poppert, and N. Wellinghausen. 2009. Evaluation of new selective culture media and a rapid fluorescence *in situ* hybridization assay for identification of *Clostridium difficile* from stool samples. J Med Microbiol **58:**874-7.

Borody, T. J., E. F. Warren, S. Leis, R. Surace, and O. Ashman. 2003. Treatment of ulcerative colitis using fecal bacteriotherapy. J Clin Gastroenterol **37:**42-7.

Borriello, S. P., and F. E. Barclay. 1985. Protection of hamsters against *Clostridium difficile* ileocaecitis by prior colonisation with non-pathogenic strains. J Med Microbiol **19:**339-50.

Bourgault, A. M., F. Lamothe, V. G. Loo, and L. Poirier. 2006. *In vitro* susceptibility of *Clostridium difficile* clinical isolates from a multi-institutional outbreak in Southern Quebec, Canada. Antimicrob Agents Chemother **50:**3473-5.

Bouza, E., M. Dryden, R. Mohammed, J. Peppe, S. Chasan-Taber, J. Donovan, D. Davidson, and G. Short. 2008. Results of a phase III trial comparing tolevamer, vancomycin and metronidazole in patients with *Clostridium difficile*-associated diarrhoea. Presented at the Eighteenth Eur. Congr. Clin. Microbiol. Infect. Dis. Barcelona, Spain.

Bowden, T. A., Jr., A. R. Mansberger, Jr., and L. E. Lykins. 1981. Pseudomembraneous enterocolitis: mechanism for restoring floral homeostasis. Am Surg **47:**178-83.

Bowring, S. N., and J. G. Morris. 1985. Mutagenesis of *Clostridium acetobutylicum*. J Appl Bacteriol **58:**577-84.

Braham, S., M. Iturriza-Gomara, and J. Gray. 2009. Optimisation of a single-primer sequence-independent amplification (SP-SIA) assay: detection of previously undetectable norovirus strains associated with outbreaks of gastroenteritis. J Virol Methods **158:**30-4.

Brandes, V., I. Schelle, S. Brinkmann, F. Schulz, J. Schwarz, R. Gerhard, and H. Genth. 2012. Protection from *Clostridium difficile* toxin B-catalysed Rac1/Cdc42 glucosylation by tauroursodeoxycholic acid-induced Rac1/Cdc42 phosphorylation. Biol Chem **393:**77-84.

Breitbart, M., I. Hewson, B. Felts, J. M. Mahaffy, J. Nulton, P. Salamon, and F. Rohwer. 2003. Metagenomic analyses of an uncultured viral community from human feces. J Bacteriol **185:**6220-3.

Brennan, R. G., and B. W. Matthews. 1989. The helix-turn-helix DNA binding motif. J Biol Chem **264:**1903-6.

Bruttin, A., and H. Brussow. 2005. Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. Antimicrobial Agents and Chemotherapy **49:**2874-2878.

Butler, M. M., D. L. Shinabarger, D. M. Citron, C. P. Kelly, S. Dvoskin, G. E. Wright, H. Feng, S. Tzipori, and T. L. Bowlin. 2012. MBX-500, a hybrid antibiotic with *in vitro* and *in vivo* efficacy against toxigenic *Clostridium difficile*. Antimicrob Agents Chemother **56:**4786-92.

Buxton, R. S. 1976. Prophage mutation causing heat inducibility of defective *Bacillus subtilis* bacteriophage PBSX. J Virol **20:**22-8.

Byl, B., F. Jacobs, M. J. Struelens, and J. P. Thys. 1996. Extraintestinal *Clostridium difficile* infections. Clinical Infectious Diseases **22:**712.

Cade, J. E., and B. M. Margetts. 1991. Relationship between diet and smoking-is the diet of smokers different? J Epidemiol Community Health **45:**270-2.

Calabi, E., F. Calabi, A. D. Phillips, and N. F. Fairweather. 2002. Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. Infect Immun **70:**5770-8.

Canchaya, C., F. Desiere, W. M. McShan, J. J. Ferretti, J. Parkhill, and H. Brussow. 2002. Genome analysis of an inducible prophage and prophage remnants integrated in the *Streptococcus pyogenes* strain SF370. Virology **302:**245-58.

Cantalupo, P. G., B. Calgua, G. Zhao, A. Hundesa, A. D. Wier, J. P. Katz, M. Grabe, R. W. Hendrix, R. Girones, D. Wang, and J. M. Pipas. 2011. Raw sewage harbors diverse viral populations. MBio **2** e00180-11.

Carneiro, B. A., J. Fujii, G. A. Brito, C. Alcantara, R. B. Oria, A. A. Lima, T. Obrig, and R. L. Guerrant. 2006. Caspase and bid involvement in *Clostridium difficile* toxin A-induced apoptosis and modulation of toxin A effects by glutamine and alanyl-glutamine *in vivo* and *in vitro*. Infection and Immunity **74:**81-7.

Carter, G. P., J. I. Rood, and D. Lyras. 2010. The role of toxin A and toxin B in *Clostridium difficile*-associated disease: Past and present perspectives. Gut Microbes **1:**58-64.

Chae, S., L. Eckmann, Y. Miyamoto, C. Pothoulakis, M. Karin, and M. F. Kagnoff. 2006. Epithelial cell I kappa B-kinase beta has an important protective role in *Clostridium difficile* toxin A-induced mucosal injury. J Immunol **177:**1214-20.

Chassard, C., K. P. Scott, P. Marquet, J. C. Martin, C. Del'homme, M. Dapoigny, H. J. Flint, and A. Bernalier-Donadille. 2008. Assessment of metabolic diversity within the intestinal microbiota from healthy humans using combined molecular and cultural approaches. FEMS Microbiol Ecol **66:**496-504.

Chen, H. J., J. C. Tsai, W. C. Hung, S. P. Tseng, P. R. Hsueh, and L. J. Teng. 2011. Identification of fusB-mediated fusidic acid resistance islands in *Staphylococcus epidermidis* isolates. Antimicrob Agents Chemother **55:**5842-9.

Chen, X., E. G. Kokkotou, N. Mustafa, K. R. Bhaskar, S. Sougioultzis, M. O'Brien, C. Pothoulakis, and C. P. Kelly. 2006. *Saccharomyces boulardii* inhibits ERK1/2 mitogen-activated protein kinase activation both *in vitro* and *in vivo* and protects against *Clostridium difficile* toxin A-induced enteritis. J Biol Chem **281:**24449-54.

Chibani-Chennoufi, S., A. Bruttin, M. L. Dillmann, and H. Brussow. 2004. Phage-host interaction: an ecological perspective. J Bacteriol **186:**3677-86.

Cislo, M., M. Dabrowski, B. Weber-Dabrowska, and A. Woyton. 1987. Bacteriophage treatment of suppurative skin infections. Arch Immunol Ther Exp. **2:**175–183.

Citron, D. M., C. V. Merriam, K. L. Tyrrell, Y. A. Warren, H. Fernandez, and E. J. Goldstein. 2003. *In vitro* activities of ramoplanin, teicoplanin, vancomycin, linezolid, bacitracin, and four other antimicrobials against intestinal anaerobic bacteria. Antimicrob Agents Chemother **47:**2334-8.

Citron, D. M., K. L. Tyrrell, C. V. Merriam, and E. J. Goldstein. 2012. Comparative *in vitro* activities of LFF571 against *Clostridium difficile* and 630 other intestinal strains of aerobic and anaerobic bacteria. Antimicrob Agents Chemother **56:**2493-503.

Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res **37:**D141-5.

Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. Farrow. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol **44:**812-26.

Cook, M. T., G. Tzortzis, D. Charalampopoulos, and V. V. Khutoryanskiy. 2012. Microencapsulation of probiotics for gastrointestinal delivery. J Control Release **162:**56-67.

Cornely, O. A. 2012. Current and emerging management options for *Clostridium difficile* infection: what is the role of fidaxomicin? Clinical Microbiology and Infection **18**:28-35

Cottrell, G. S., S. Amadesi, S. Pikios, E. Camerer, J. A. Willardsen, B. R. Murphy, G. H. Caughey, P. J. Wolters, S. R. Coughlin, A. Peterson, W. Knecht, C. Pothoulakis, N. W. Bunnett, and E. F. Grady. 2007. Protease-activated receptor 2, dipeptidyl peptidase I, and proteases mediate *Clostridium difficile* toxin A enteritis. Gastroenterology **132:**2422-37.

Coulondre, C., and J. H. Miller. 1977. Genetic studies of the lac repressor. IV. Mutagenic specificity in the lacI gene of *Escherichia coli.* J Mol Biol **117:**577-606.

Cowles, P. B. 1934. A Bacteriophage for *Cl. tetani*. J Bacteriol **27:**163-4.

Cruz, M. P. 2012. Fidaxomicin (Dificid), a novel oral macrocyclic antibacterial agent for the treatment of *Clostridium difficile*-associated diarrhea in adults. P T **37:**278-81.

Cummings, J. H., M. J. Hill, T. Jivraj, H. Houston, W. J. Branch, and D. J. A. Jenkins. 1979. Effect of Meat Protein and Dietary Fiber on Colonic Function and Metabolism. Changes in Bowel Habit, Bile-Acid Excretion, and Calcium-Absorption. American Journal of Clinical Nutrition **32:**2086-2093.

d' Herelle, F. 1917. Sur un microbe invisible antagoniste des bacilles dysenterique. Academy of Science **165:**373-5.

Davies, A. H., A. K. Roberts, C. C. Shone, and K. R. Acharya. 2011. Super toxins from a super bug: structure and function of *Clostridium difficile* toxins. Biochem J **436:**517-26.

Dawson, M. J., and R. W. Scott. 2012. New horizons for host defense peptides and lantibiotics. Curr Opin Pharmacol. **12**: 545-50

de Lalla, F., G. Privitera, E. Rinaldi, G. Ortisi, D. Santoro, and G. Rizzardini. 1989. Treatment of *Clostridium difficile*-associated disease with teicoplanin. Antimicrob Agents Chemother **33:**1125-7.

Delzenne, N. M., A. M. Neyrinck, and P. D. Cani. 2011. Modulation of the gut microbiota by nutrients with prebiotic properties: consequences for host health in the context of obesity and metabolic syndrome. Microb Cell Fact **10 Suppl 1:**S10.

Dineen, S. S., A. C. Villapakkam, J. T. Nordman, and A. L. Sonenshein. 2007. Repression of *Clostridium difficile* toxin gene expression by CodY. Mol Microbiol **66:**206-19.

Dixon, B. 2004. New dawn for phage therapy. Lancet Infect Dis **4:**186.

Do, A. N., S. K. Fridkin, A. Yechouron, S. N. Banerjee, G. E. Killgore, A. M. Bourgault, M. Jolivet, and W. R. Jarvis. 1998. Risk factors for early recurrent *Clostridium difficile*-associated diarrhea. Clinical Infectious Diseases **26:**954-9.

Dobson, A., F. Crispie, M. C. Rea, O. O'Sullivan, P. G. Casey, P. G. Lawlor, P. D. Cotter, P. Ross, G. E. Gardiner, and C. Hill. 2011. Fate and efficacy of lacticin 3147-producing *Lactococcus lactis* in the mammalian gastrointestinal tract. Fems Microbiology Ecology **76:**602-614.

Dolman, C. E., and E. Chang. 1972. Bacteriophages of *Clostridium botulinum*. Can J Microbiol **18:**67-76.

Domenech, O., G. Francius, P. M. Tulkens, F. Van Bambeke, Y. Dufrene, and M. P. Mingeot-Leclercq. 2009. Interactions of oritavancin, a new lipoglycopeptide derived from vancomycin, with phospholipid bilayers: Effect on membrane permeability and nanoscale lipid membrane organization. Biochim Biophys Acta **1788:**1832-40.

Domingo, E., E. Martinez-Salas, F. Sobrino, J. C. de la Torre, A. Portela, J. Ortin, C. Lopez-Galindez, P. Perez-Brena, N. Villanueva, R. Najera, and et al. 1985. The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance-a review. Gene **40:**1-8.

Drudy, D., E. Calabi, L. Kyne, S. Sougioultzis, E. Kelly, N. Fairweather, and C. P. Kelly. 2004. Human antibody response to surface layer proteins in *Clostridium difficile* infection. FEMS Immunol Med Microbiol **41:**237-42.

Drudy, D., N. Harnedy, S. Fanning, M. Hannan, and L. Kyne. 2007. Emergence and control of fluoroquinolone-resistant, toxin A-negative, toxin B-positive *Clostridium difficile*. Infect Control Hosp Epidemiol **28:**932-40.

Dubberke, E. R., and M. A. Olsen. 2012. Burden of *Clostridium difficile* on the healthcare system. Clinical Infectious Diseases **55 Suppl 2:**S88-92.

Dubberke, E. R., K. A. Reske, Y. Yan, M. A. Olsen, L. C. McDonald, and V. J. Fraser. 2007. *Clostridium difficile*-associated disease in a setting of endemicity: Identification of novel risk factors. Clinical Infectious Diseases **45:**1543-1549.

Dudley, M. N., J. C. McLaughlin, G. Carrington, J. Frick, C. H. Nightingale, and R. Quintiliani. 1986. Oral bacitracin vs vancomycin therapy for *Clostridium difficile*-induced diarrhea. A randomized double-blind trial. Arch Intern Med **146:**1101-4.

Dupuy, B., R. Govind, A. Antunes, and S. Matamouros. 2008. *Clostridium difficile* toxin synthesis is negatively regulated by TcdC. J Med Microbiol **57:**685-9.

Durmaz, E., S. M. Madsen, H. Israelsen, and T. R. Klaenhammer. 2002. *Lactococcus lactis* lytic bacteriophages of the P335 group are inhibited by overexpression of a truncated CI repressor. J Bacteriol **184:**6532-44.

Dvoskin, S., W. C. Xu, N. C. Brown, I. B. Yanachkov, M. Yanachkova, and G. E. Wright. 2012. A novel agent effective against *Clostridium difficile* infection. Antimicrob Agents Chemother **56:**1624-6.

Eaton, T., J. O. Falkinham, and C. F. Vonreyn. 1995. Recovery of *Mycobacterium avium* from Cigarettes. Journal of Clinical Microbiology **33:**2757-2758.

Egerer, M., T. Giesemann, T. Jank, K. J. F. Satchell, and K. Aktories. 2007. Auto-catalytic cleavage of *Clostridium difficile* toxins a and B depends on cysteine protease activity. Journal of Biological Chemistry **282:**25314-25321.

Eidhin, D. B. N., J. B. O'Brien, M. S. McCabe, V. Athie-Morales, and D. P. Kelleher. 2008. Active immunization of hamsters against *Clostridium difficile* infection using surface-layer protein. FEMS Immunology and Medical Microbiology **52:**207-218.

Eiseman, B., W. Silen, G. S. Bascom, and A. J. Kauvar. 1958. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. Surgery **44:**854-9.

Eklund, M. W., F. T. Poysky, S. M. Reed, and C. A. Smith. 1971. Bacteriophage and the toxigenicity of *Clostridium botulinum* type C. Science **172:**480-2.

Elmer, G. W., C. M. Surawicz, and L. V. McFarland. 1996. Biotherapeutic agents. A neglected modality for the treatment and prevention of selected intestinal and vaginal infections. JAMA **275:**870-6.

Erskine, J. M. 1973. Characteristics of *Erwinia amylovora* bacteriophage and its possible role in the epidemology of fire blight. Can J Microbiol **19:**837-45.

Erume, J., P. Wijemanne, E. M. Berberov, S. D. Kachman, D. J. Oestmann, D. H. Francis, and R. A. Moxley. 2012. Inverse relationship between heat stable enterotoxin-b induced fluid accumulation and adherence of F4ac-positive enterotoxigenic *Escherichia coli* in ligated jejunal loops of F4ab/ac fimbria receptor-positive swine. Vet Microbiol. 161:315-24.

Fallingborg, J. 1999. Intraluminal pH of the human gastrointestinal tract. Dan Med Bull **46:**183-96.

Faust, G., D. Langelier, H. Haddad, and D. Menard. 2002. Treatment of recurrent pseudomembranous colitis (RPMC) with stool transplantation: report of 6 casesm Canadian Journal of Gastroenterology **16:**43A.

Fekety, R. 1997. Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. American College of Gastroenterology, Practice Parameters Committee. American Journal of Gastroenterology **92:**739-50.

Fenton, S., D. Stephenson, and C. Weder. 1974. Pseudomembranous colitis associated with antibiotic therapy - an emerging entity. Can Med Assoc J **111:**1110-1, 1114.

Fernie, D. S., R. O. Thomson, I. Batty, and P. D. Walker. 1983. Active and passive immunization to protect against antibiotic associated caecitis in hamsters. Dev Biol Stand **53:**325-32.

Ferreira, C. L., L. Grzeskowiak, M. C. Collado, and S. Salminen. 2011. *In vitro* evaluation of *Lactobacillus gasseri* strains of infant origin on adhesion and aggregation of specific pathogens. J Food Prot **74:**1482-7.

Flotterod, O., and G. Hopen. 1991. [Refractory *Clostridium difficile* infection. Untraditional treatment of antibiotic-induced colitis]. Tidsskr Nor Laegeforen **111:**1364-5.

Fogg, P. C., S. M. Gossage, D. L. Smith, J. R. Saunders, A. J. McCarthy, and H. E. Allison. 2007. Identification of multiple integration sites for Stx-phage Φ24B in the *Escherichia coli* genome, description of a novel integrase and evidence for a functional anti-repressor. Microbiology **153:**4098-110.

Fooks, L. J., and G. R. Gibson. 2003. Mixed culture fermentation studies on the effects of synbiotics on the human intestinal pathogens *Campylobacter jejuni* and *Escherichia coli*. Anaerobe **9:**231-242.

Fortier, L. C., and S. Moineau. 2007. Morphological and genetic diversity of temperate phages in *Clostridium difficile*. Appl Environ Microbiol **73:**7358-66.

Franks, A. H., H. J. Harmsen, G. C. Raangs, G. J. Jansen, F. Schut, and G. W. Welling. 1998. Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-

targeted oligonucleotide probes. Applied and Environmental Microbiology **64:**3336-3345.

Freeman, J., S. D. Baines, D. Jabes, and M. H. Wilcox. 2005. Comparison of the efficacy of ramoplanin and vancomycin in both *in vitro* and *in vivo* models of clindamycin-induced *Clostridium difficile* infection. J Antimicrob Chemother **56:**717-25.

Freeman, J., S. D. Baines, K. Saxton, and M. H. Wilcox. 2007. Effect of metronidazole on growth and toxin production by epidemic *Clostridium difficile* PCR ribotypes 001 and 027 in a human gut model. J Antimicrob Chemother **60:**83-91.

Freeman, J., F. J. O'Neill, and M. H. Wilcox. 2003. Effects of cefotaxime and desacetylcefotaxime upon *Clostridium difficile* proliferation and toxin production in a triple-stage chemostat model of the human gut. Journal of Antimicrobial Chemotherapy **52:**96-102.

Frobisher, M., and J. Brown. 1927. Transmissible toxicogenicity of *Streptococci.* Bull. John Hopkins Hospital **41:**167-173.

Furuse, K., T. Sakurai, A. Hirashima, M. Katsuki, A. Ando, and I. Watanabe. 1978. Distribution of ribonucleic acid coliphages in south and east Asia. Appl Environ Microbiol **35:**995-1002.

Garborg, K., B. Waagsbo, A. Stallemo, J. Matre, and A. Sundoy. 2010. Results of faecal donor instillation therapy for recurrent *Clostridium difficile*-associated diarrhoea. Scand J Infect Dis **42:**857-61.

García, P., C. Madera, B. Martínez, and A. Rodríguez. 2007. Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages. International Dairy Journal **17:**1232-1239.

Garcia, P., B. Martinez, J. M. Obeso, R. Lavigne, R. Lurz, and A. Rodriguez. 2009. Functional genomic analysis of two *Staphylococcus aureus* phages isolated from the dairy environment. Appl Environ Microbiol **75:**7663-73.

Gardiner, D. F., T. Rosenberg, J. Zaharatos, D. Franco, and D. D. Ho. 2009. A DNA vaccine targeting the receptor-binding domain of *Clostridium difficile* toxin A. Vaccine **27:**3598-604.

Gardiner, G. E., M. C. Rea, B. O'Riordan, P. O'Connor, S. M. Morgan, P. G. Lawlor, P. B. Lynch, M. Cronin, R. P. Ross, and C. Hill. 2007. Fate of the two-component lantibiotic lacticin 3147 in the gastrointestinal tract. Appl Environ Microbiol **73:**7103-9.

Geissler, K. 2007. Unusual placement of a nasogastric tube. Radiol Technol **78:**253.

Genth, H., S. C. Dreger, J. Huelsenbeck, and I. Just. 2008. *Clostridium difficile* toxins: more than mere inhibitors of Rho proteins. Int J Biochem Cell Biol **40:**592-7.

George, R. H., J. M. Symonds, F. Dimock, J. D. Brown, Y. Arabi, N. Shinagawa, M. R. Keighley, J. Alexander-Williams, and D. W. Burdon. 1978. Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. Br Med J **1:**695.

Gerding, D. N., S. Johnson, L. R. Peterson, M. E. Mulligan, and J. Silva, Jr. 1995. *Clostridium difficile*-associated diarrhea and colitis. Infect Control Hosp Epidemiol **16:**459-77.

Geric, B., R. J. Carman, M. Rupnik, C. W. Genheimer, S. P. Sambol, D. M. Lyerly, D. N. Gerding, and S. Johnson. 2006. Binary toxin-producing, large clostridial toxin-negative *Clostridium difficile* strains are enterotoxic but do not cause disease in hamsters. J Infect Dis **193:**1143-50.

Geric, B., S. Johnson, D. N. Gerding, M. Grabnar, and M. Rupnik. 2003. Frequency of binary toxin genes among *Clostridium difficile* strains that do not produce large clostridial toxins. J Clin Microbiol **41:**5227-32.

Gersemann, M., S. Becker, I. Kubler, M. Koslowski, G. Wang, K. R. Herrlinger, J. Griger, P. Fritz, K. Fellermann, M. Schwab, J. Wehkamp, and E. F. Stange. 2009. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. Differentiation **77:**84-94.

Giannasca, P. J., Z. X. Zhang, W. D. Lei, J. A. Boden, M. A. Giel, T. P. Monath, and W. D. Thomas, Jr. 1999. Serum antitoxin antibodies mediate systemic and mucosal protection from *Clostridium difficile* disease in hamsters. Infect Immun **67:**527-38.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr **125:**1401-12.

Gibson, G. R., and X. Wang. 1994. Bifidogenic properties of different types of fructo-oligosaccharides. Food Microbiology **11:**491-498.

Gill, C. I., and I. R. Rowland. 2002. Diet and cancer: assessing the risk. Br J Nutr **88 Suppl 1:**S73-87.

Gill, J. J., and P. Hyman. 2010. Phage choice, isolation, and preparation for phage therapy. Current Pharmaceutical Biotechnology. **11:**2-14.

Goh, S., B. J. Chang, and T. V. Riley. 2005 a. Effect of phage infection on toxin production by *Clostridium difficile*. J Med Microbiol **54:**129-35.

Goh, S., P. F. Ong, K. P. Song, T. V. Riley, and B. J. Chang. 2007. The complete genome sequence of Clostridium difficile phage phiC2 and

comparisons to phiCD119 and inducible prophages of CD630. Microbiology **153:**676-85.

Goh, S., T. V. Riley, and B. J. Chang. 2005 b. Isolation and characterization of temperate bacteriophages of *Clostridium difficile.* Appl Environ Microbiol **71:**1079-83.

Goldstein, E. J., D. M. Citron, P. Sears, F. Babakhani, S. P. Sambol, and D. N. Gerding. 2011. Comparative susceptibilities to fidaxomicin (OPT-80) of isolates collected at baseline, recurrence, and failure from patients in two phase III trials of fidaxomicin against *Clostridium difficile* infection. Antimicrob Agents Chemother **55:**5194-9.

Gorbach, S. L., T. W. Chang, and B. Goldin. 1987. Successful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG. Lancet **2:**1519.

Gorski, A., and B. Weber-Dabrowska. 2005. The potential role of endogenous bacteriophages in controlling invading pathogens. Cell Mol Life Sci **62:**511-9.

Goulding, D., H. Thompson, J. Emerson, N. F. Fairweather, G. Dougan, and G. R. Douce. 2009. Distinctive profiles of Infection and Pathology in hamsters infected with *Clostridium difficile* strains 630 and B1. Infection and Immunity **77:**5478-5485.

Govind, R., J. A. Fralick, and R. D. Rolfe. 2006. Genomic organization and molecular characterization of *Clostridium difficile* bacteriophage ΦCD119. J Bacteriol **188:**2568-77.

Govind, R., J. A. Fralick, and R. D. Rolfe. 2011. *In vivo* lysogenization of a *Clostridium difficile* bacteriophage ΦCD119. Anaerobe **17:**125-129.

Govind, R., G. Vediyappan, R. D. Rolfe, B. Dupuy, and J. A. Fralick. 2009. Bacteriophage-mediated toxin gene regulation in *Clostridium difficile*. J Virol **83:**12037-45.

Grant, R. B., and H. P. Riemann. 1976. Temperate phages of *Clostridium perfringens* type C1. Can J Microbiol **22:**603-10.

Greenberg, R. N., T. C. Marbury, G. Foglia, and M. Warny. 2012. Phase I dose finding studies of an adjuvanted *Clostridium difficile* toxoid vaccine. Vaccine **30:**2245-9.

Grehan, M. J., T. J. Borody, S. M. Leis, J. Campbell, H. Mitchell, and A. Wettstein. 2010. Durable alteration of the colonic microbiota by the administration of donor fecal flora. J Clin Gastroenterol **44:**551-61.

Grinblat, J., A. Weiss, B. Grosman, D. Dicker, and Y. Beloosesky. 2004. Diarrhea in elderly patients due to *Clostridium difficile* associated with *Salmonella* and *Shigella* infection. Arch Gerontol Geriatr **39:**277-82.

Gustafsson, A., S. Lund-Tonnesen, A. Berstad, T. Midtvedt, and E. Norin. 1998. Faecal short-chain fatty acids in patients with antibiotic-associated diarrhoea, before and after faecal enema treatment. Scand J Gastroenterol **33:**721-7.

Guzman, C., J. Jofre, A. R. Blanch, and F. Lucena. 2007. Development of a feasible method to extract somatic coliphages from sludge, soil and treated biowaste. J Virol Methods **144:**41-8.

Hagens, S., and U. Blasi. 2003. Genetically modified filamentous phage as bactericidal agents: a pilot study. Letters in Applied Microbiology **37:**318-23.

Hagens, S., A. Habel, U. von Ahsen, A. von Gabain, and U. Blasi. 2004. Therapy of experimental *Pseudomonas* infections with a nonreplicating genetically modified phage. Antimicrob Agents Chemother **48:**3817-22.

Hall, I. C., and E. O'Toole. 1935. Intestinal flora in new-born infants; with a description of a new pathogenic anaerobe, *Bacillus difficilis*. American Journal of Diseases of Children **49:**390-402.

Halvorson, S. A., A. S. Cedfeldt, and A. J. Hunter. 2011. Fulminant, non-antibiotic associated *Clostridium difficile* colitis following *Salmonella* gastroenteritis. J Gen Intern Med **26:**95-7.

Hamm, E. E., D. E. Voth, and J. D. Ballard. 2006. Identification of *Clostridium difficile* toxin B cardiotoxicity using a zebrafish embryo model of intoxication. Proc Natl Acad Sci U S A **103:**14176-81.

Hammer, Ø., D. A. T. Harper, and P. D. Ryan. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica **4**:9pp.

Hanlon, G. W. 2007. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. Int J Antimicrob Agents **30:**118-28.

Harper, D. R., J. Anderson, and M. C. Enright. 2011. Phage therapy: delivering on the promise. Ther Deliv **2:**935-47.

Havelaar, A. H., and W. M. Hogeboom. 1983. Factors affecting the enumeration of coliphages in sewage and sewage-polluted waters. Antonie Van Leeuwenhoek **49:**387-97.

Hawkins, C., D. Harper, D. Burch, E. Anggard, and J. Soothill. 2010. Topical treatment of *Pseudomonas aeruginosa* otitis of dogs with a bacteriophage mixture: a before/after clinical trial. Vet Microbiol **146:**309-13.

He, D., S. Sougioultzis, S. Hagen, J. Liu, S. Keates, A. C. Keates, C. Pothoulakis, and J. T. Lamont. 2002. *Clostridium difficile* toxin A triggers

human colonocyte IL-8 release via mitochondrial oxygen radical generation. Gastroenterology **122:**1048-57.

Health Protection Agency. 2008. Nucleic acid extraction by the Boom method. National Standard Method VSOP 20 Issue 1. http://www.hpa-standardmethods.org.uk/pdf_virology.asp.

Hendrix, R. W. 2002. Bacteriophages: evolution of the majority. Theor Popul Biol **61:**471-80.

Hidalgo, M., M. J. Oruna-Concha, S. Kolida, G. E. Walton, S. Kallithraka, J. P. Spencer, and S. de Pascual-Teresa. 2012. Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. J Agric Food Chem **60:**3882-90.

Hing, T. C., S. Ho, D. Q. Shih, R. Ichikawa, M. Cheng, J. Chen, X. Chen, I. Law, R. Najarian, C. P. Kelly, R. L. Gallo, S. R. Targan, C. Pothoulakis, and H. W. Koon. 2012. The antimicrobial peptide cathelicidin modulates *Clostridium difficile*-associated colitis and toxin A-mediated enteritis in mice. Gut. doi:10.1136/gutjrn-2012-302180.

Hinkson, P. L., C. Dinardo, D. DeCiero, J. D. Klinger, and R. H. Barker, Jr. 2008. Tolevamer, an anionic polymer, neutralizes toxins produced by the BI/027 strains of *Clostridium difficile*. Antimicrob Agents Chemother **52:**2190-5.

Hopkins, M. J., and G. T. Macfarlane. 2002. Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection. J Med Microbiol **51:**448-54.

Horgan, M., O. O'Sullivan, A. Coffey, G. F. Fitzgerald, D. van Sinderen, O. McAuliffe, and R. P. Ross. 2010. Genome analysis of the *Clostridium difficile* phage ΦCD6356, a temperate phage of the Siphoviridae family. Gene **462:**34-43.

Horton, K. M., F. M. Corl, and E. K. Fishman. 2000. CT evaluation of the colon: inflammatory disease. Radiographics **20:**399-418.

Hua, J., R. W. Scott, and G. Diamond. 2010. Activity of antimicrobial peptide mimetics in the oral cavity: II. Activity against periopathogenic biofilms and anti-inflammatory activity. Mol Oral Microbiol **25:**426-32.

Huang, I. H., M. Waters, R. R. Grau, and M. R. Sarker. 2004. Disruption of the gene (*Spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. FEMS Microbiol Lett **233:**233-40.

Huelsenbeck, J., S. Dreger, R. Gerhard, H. Barth, I. Just, and H. Genth. 2007. Difference in the cytotoxic effects of toxin B from *Clostridium difficile*

strain VPI 10463 and toxin B from variant *Clostridium difficile* strain 1470. Infect Immun **75:**801-9.

Hurst, C. J., and C. P. Gerba. 1979. Development of a quantitative method for the detection of enteroviruses in soil. Appl Environ Microbiol **37:**626-32.

Hurst, C. J., C. P. Gerba, and I. Cech. 1980. Effects of environmental variables and soil characteristics on virus survival in soil. Appl Environ Microbiol **40:**1067-79.

Hutchinson, R., A. R. Ahmed, and D. Menzies. 2008. A case of intramural oesophageal dissection secondary to nasogastric tube insertion. Ann R Coll Surg Engl **90:**W4-7.

Hyman, P., and S. T. Abedon. 2010. Bacteriophage host range and bacterial resistance. Adv Appl Microbiol **70:**217-48.

Iizuka, M., H. Itou, S. Konno, J. Chihara, M. Tobita, H. Oyamada, I. Toyoshima, K. Sasaki, A. Sato, Y. Horie, and S. Watanabe. 2004. Elemental diet modulates the growth of *Clostridium difficile* in the gut flora. Aliment Pharmacol Ther **20 Suppl 1:**151-7.

Imamovic, L., E. Balleste, J. Jofre, and M. Muniesa. 2010. Quantification of Shiga toxin-converting bacteriophages in wastewater and in fecal samples by real-time quantitative PCR. Appl Environ Microbiol **76:**5693-701.

Ioseliani, G. D., G. D. Meladze, N. S. Chkhetiia, M. G. Mebuke, and N. I. Kiknadze. 1980. Use of bacteriophage and antibiotics for prevention of acute postoperative empyema in chronic suppurative lung diseases. Grudn Khir. **6:**63–67.

Ishida, Y., T. Maegawa, T. Kondo, A. Kimura, Y. Iwakura, S. Nakamura, and N. Mukaida. 2004. Essential involvement of IFN-gamma in *Clostridium difficile* toxin A-induced enteritis. J Immunol **172:**3018-25.

Ishihara, T., T. Kadoya, N. Endo, and S. Yamamoto. 2006. Optimization of elution salt concentration in stepwise elution of protein chromatography using linear gradient elution data. Reducing residual protein A by cation-exchange chromatography in monoclonal antibody purification. J Chromatogr A **1114:**97-101.

Jalan, K. N., W. Sircus, W. I. Card, C. W. Falconer, C. B. Bruce, G. P. Crean, J. P. McManus, W. P. Small, and A. N. Smith. 1969. An experience of ulcerative colitis. Toxic dilation in 55 cases. Gastroenterology **57:**68-82.

Janvilisri, T., J. Scaria, A. D. Thompson, A. Nicholson, B. M. Limbago, L. G. Arroyo, J. G. Songer, Y. T. Grohn, and Y. F. Chang. 2009. Microarray identification of *Clostridium difficile* core components and divergent regions associated with host origin. J Bacteriol **191:**3881-91.

Jarchum, I., M. Liu, L. Lipuma, and E. G. Pamer. 2011. Toll-like receptor 5 stimulation protects mice from acute *Clostridium difficile* colitis. Infection and Immunity **79:**1498-503.

Jawhara, S., K. Habib, F. Maggiotto, G. Pignede, P. Vandekerckove, E. Maes, L. Dubuquoy, T. Fontaine, Y. Guerardel, and D. Poulain. 2012. Modulation of intestinal inflammation by yeasts and cell wall extracts: strain dependence and unexpected anti-inflammatory role of glucan fractions. PLoS One **7:**e40648.

Jefferson, K. K., M. F. Smith, Jr., and D. A. Bobak. 1999. Roles of intracellular calcium and NF-kappa B in the *Clostridium difficile* toxin A-induced up-regulation and secretion of IL-8 from human monocytes. J Immunol **163:**5183-91.

Jeffrey, N. G., S. A. Hirota, O. Gross, Y. Li, A. Ulke-Lemee, M. S. Potentier, L. P. Schenck, A. Vilaysane, M. E. Seamone, H. Feng, G. D. Armstrong, J. Tschopp, J. A. Macdonald, D. A. Muruve, and P. L. Beck. 2010. *Clostridium difficile* toxin-induced inflammation and intestinal injury are mediated by the inflammasome. Gastroenterology **139:**542-52, 552 e1-3.

Jia, H., W. J. Satumba, G. L. Bidwell, 3rd, and M. C. Mossing. 2005. Slow assembly and disassembly of lambda Cro repressor dimers. J Mol Biol **350:**919-29.

Johnson, A. P. 2010. New antibiotics for selective treatment of gastrointestinal infection caused by *Clostridium difficile*. Expert Opin Ther Pat **20:**1389-99.

Johnson, S., S. R. Homann, K. M. Bettin, J. N. Quick, C. R. Clabots, L. R. Peterson, and D. N. Gerding. 1992. Treatment of asymptomatic *Clostridium difficile* carriers (fecal excretors) with vancomycin or metronidazole. A randomized, placebo-controlled trial. Ann Intern Med **117:**297-302.

Johnson, S., C. Schriever, M. Galang, C. P. Kelly, and D. N. Gerding. 2007. Interruption of recurrent *Clostridium difficile*-associated diarrhea episodes by serial therapy with vancomycin and rifaximin. Clinical Infectious Diseases **44:**846-8.

Johnson, S., C. Schriever, U. Patel, T. Patel, D. W. Hecht, and D. N. Gerding. 2009. Rifaximin Redux: treatment of recurrent *Clostridium difficile* infections with rifaximin immediately post-vancomycin treatment. Anaerobe **15:**290-1.

Joint Formulary Committee. 2012. British National Formulary. London: BMJ Group and Pharmaceutical Press **63**.

Jordan, S. R., and C. O. Pabo. 1988. Structure of the lambda complex at 2.5 A resolution: details of the repressor-operator interactions. Science **242:**893-9.

Jorup-Ronstrom, C., A. Hakanson, A. K. Persson, T. Midtvedt, and E. Norin. 2006. [Feces culture successful therapy in *Clostridium difficile* diarrhea]Lakartidningen **103:**3603–3605.

Jump, R. L., Y. Li, M. J. Pultz, G. Kypriotakis, and C. J. Donskey. 2011. Tigecycline exhibits inhibitory activity against *Clostridium difficile* in the colon of mice and does not promote growth or toxin production. Antimicrob Agents Chemother **55:**546-9.

Jump, R. L., M. J. Pultz, and C. J. Donskey. 2007. Vegetative *Clostridium difficile* survives in room air on moist surfaces and in gastric contents with reduced acidity: a potential mechanism to explain the association between proton pump inhibitors and *C. difficile*-associated diarrhea? Antimicrob Agents Chemother **51:**2883-7.

Kamboj, M., P. Khosa, A. Kaltsas, N. E. Babady, C. Son, and K. A. Sepkowitz. 2011. Relapse versus reinfection: surveillance of *Clostridium difficile* infection. Clinical Infectious Diseases **53:**1003-6.

Kang, J. K., J. S. Hwang, H. J. Nam, K. J. Ahn, H. Seok, S. K. Kim, E. Y. Yun, C. Pothoulakis, J. T. Lamont, and H. Kim. 2011. The insect peptide coprisin prevents *Clostridium difficile*-mediated acute inflammation and mucosal damage through selective antimicrobial activity. Antimicrob Agents Chemother **55:**4850-7.

Katchar, K., C. P. Taylor, S. Tummala, X. Chen, J. Sheikh, and C. P. Kelly. 2007. Association between IgG2 and IgG3 subclass responses to toxin A and recurrent *Clostridium difficile*-associated disease. Clin Gastroenterol Hepatol **5:**707-13.

Katzenelson, E., B. Fattal, and T. Hostovesky. 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl Environ Microbiol **32:**638-9.

Kaur, S., C. Vaishnavi, K. K. Prasad, P. Ray, and R. Kochhar. 2011. Effect of *Lactobacillus acidophilus* & epidermal growth factor on experimentally induced *Clostridium difficile* infection. Indian J Med Res **133:**434-41.

Kaushik, N., M. Pietraszewski, J. J. Holst, and S. J. O'Keefe. 2005. Enteral feeding without pancreatic stimulation. Pancreas **31:**353-9.

Kelly, C. P., S. Becker, J. K. Linevsky, M. A. Joshi, J. C. O'Keane, B. F. Dickey, J. T. LaMont, and C. Pothoulakis. 1994. Neutrophil recruitment in *Clostridium difficile* toxin A enteritis in the rabbit. J Clin Invest **93:**1257-65.

Kelly, C. P., and L. Kyne. 2011. The host immune response to *Clostridium difficile*. J Med Microbiol **60:**1070-9.

Kelly, C. P., C. Pothoulakis, and J. T. LaMont. 1994. *Clostridium difficile* colitis. N Engl J Med **330:**257-62.

Kelly, C. P., C. Pothoulakis, F. Vavva, I. Castagliuolo, E. F. Bostwick, J. C. O'Keane, S. Keates, and J. T. LaMont. 1996. Anti-*Clostridium difficile* bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of *C. difficile* toxins. Antimicrob Agents Chemother **40:**373-9.

Kelly, C. R., L. de Leon, and N. Jasutkar. 2012. Fecal microbiota transplantation for relapsing *Clostridium difficile* infection in 26 patients: methodology and results. J Clin Gastroenterol **46:**145-9.

Ketley, J. M., T. J. Mitchell, D. C. Candy, D. W. Burdon, and J. Stephen. 1987. The effects of *Clostridium difficile* crude toxins and toxin A on ileal and colonic loops in immune and non-immune rabbits. J Med Microbiol **24:**41-52.

Khoruts, A., J. Dicksved, J. K. Jansson, and M. J. Sadowsky. 2010. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent *Clostridium difficile*-associated diarrhea. J Clin Gastroenterol **44:**354-60.

Kibbe, W. A. 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res **35:**W43-6.

Kim, H., S. H. Rhee, E. Kokkotou, X. Na, T. Savidge, M. P. Moyer, C. Pothoulakis, and J. T. LaMont. 2005. *Clostridium difficile* toxin A regulates inducible cyclooxygenase-2 and prostaglandin E2 synthesis in colonocytes via reactive oxygen species and activation of p38 MAPK. J Biol Chem **280:**21237-45.

Kim, H. J., A. Roux, and A. L. Sonenshein. 2002. Direct and indirect roles of CcpA in regulation of *Bacillus subtilis* Krebs cycle genes. Mol Microbiol **45:**179-90.

Kim, J. W., K. L. Lee, J. B. Jeong, B. G. Kim, S. Shin, J. S. Kim, H. C. Jung, and I. S. Song. 2010. Proton pump inhibitors as a risk factor for recurrence of *Clostridium-difficile*-associated diarrhea. World J Gastroenterol **16:**3573-7.

Kim, K., L. K. Pickering, H. L. DuPont, N. Sullivan, and T. Wilkins. 1984. *In vitro* and *in vivo* neutralizing activity of human colostrum and milk against purified toxins A and B of *Clostridium difficile*. J Infect Dis **150:**57-62.

Kim, S. J., K. T. Rim, H. Y. Kim, and J. S. Yang. 2010. Mutagenicity of octane and tetrasodium pyrophosphate in bacterial reverse mutation (Ames) test. J Toxicol Sci **35:**555-62.

King, A. M. Q., M. J. Adams, E. B. Carstens, and E. J. Lefkowitz (ed.). 2012. Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego.

Kink, J. A., and J. A. Williams. 1998. Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. Infect Immun **66:**2018-25.

Knoop, F. C. 1979. Clindamycin-associated enterocolitis in guinea pigs: evidence for a bacterial toxin. Infection and Immunity **23:**31-3.

Kobayashi, T., T. Yagi, M. Murakami, M. Jinbo, S. Saito, T. Takahashi, T. Yamada, H. Kunichika, and H. Gohra. 2011. Staged surgical repair for extensive cardiovascular damage by syphilis. Ann Thorac Surg **92:**1503-6.

Kokkotou, E., A. C. Moss, A. Michos, D. Espinoza, J. W. Cloud, N. Mustafa, M. O'Brien, C. Pothoulakis, and C. P. Kelly. 2008. Comparative efficacies of rifaximin and vancomycin for treatment of *Clostridium difficile*-associated diarrhea and prevention of disease recurrence in hamsters. Antimicrob Agents Chemother **52:**1121-6.

Koo, H. L., N. J. Ajami, Z. D. Jiang, H. L. Dupont, R. L. Atmar, D. Lewis, P. Byers, P. Abraham, R. A. Quijano, D. M. Musher, and E. J. Young. 2009. A nosocomial outbreak of norovirus infection masquerading as *Clostridium difficile* infection. Clinical Infectious Diseases **48:**e75-7.

Koon, H. W., D. Q. Shih, J. Chen, K. Bakirtzi, T. C. Hing, I. Law, S. Ho, R. Ichikawa, D. Zhao, H. Xu, R. Gallo, P. Dempsey, G. Cheng, S. R. Targan, and C. Pothoulakis. 2011. Cathelicidin signaling via the Toll-like receptor protects against colitis in mice. Gastroenterology **141:**1852-63 e1-3.

Kopp, E. D., and S. Seregard. 2004. Epiphora as a side effect of topical mitomycin C. British Journal of Ophthalmology **88:**1422-1424.

Krivan, H. C., and T. D. Wilkins. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect Immun **55:**1873-7.

Kroyer, J. M., and D. H. Dean. 1979. Isolation and rapid screening of deletion mutants of temperate *Bacillus subtilis* molecular cloning vehicle ρ14. Current Microbiology **3:**45-50.

Kucharewicz-Krukowska, A., and S. Slopek. 1987. Immunogenic effect of bacteriophage in patients subjected to phage therapy. Arch Immunol Ther Exp (Warsz) **35:**553-61.

Kuehne, S. A., S. T. Cartman, J. T. Heap, M. L. Kelly, A. Cockayne, and N. P. Minton. 2010. The role of toxin A and toxin B in *Clostridium difficile* infection. Nature **467:**711-3.

Kumar, M., T. Mathur, T. K. Barman, G. Ramkumar, A. Bhati, G. Shukla, V. Kalia, M. Pandya, V. S. Raj, D. J. Upadhyay, C. Vaishnavi, A.

Chakrabarti, B. Das, and P. K. Bhatnagar. 2012. *In vitro* and *in vivo* Activity of a Novel Ketolide RBx 14255 against *Clostridium difficile*. Antimicrob Agents Chemother. 56:5986-9.

Kurtz, C. B., E. P. Cannon, A. Brezzani, M. Pitruzzello, C. Dinardo, E. Rinard, D. W. Acheson, R. Fitzpatrick, P. Kelly, K. Shackett, A. T. Papoulis, P. J. Goddard, R. H. Barker, Jr., G. P. Palace, and J. D. Klinger. 2001. GT160-246, a toxin binding polymer for treatment of *Clostridium difficile* colitis. Antimicrob Agents Chemother **45:**2340-7.

Kutter, E., and A. Sulakvelidze. 2005. Bacteriophages: Biology and Applications. CRC Press, Boca Raton.

Kyne, L., M. B. Hamel, R. Polavaram, and C. N. P. Kelly. 2002. Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. Clinical Infectious Diseases **34:**346-353.

Kyne, L., M. Warny, A. Qamar, and C. P. Kelly. 2001. Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. Lancet **357:**189-93.

Kyne, L., M. Warny, A. Qamar, and C. P. Kelly. 2000. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. N Engl J Med **342:**390-7.

Ladero, V., P. Garcia, V. Bascaran, M. Herrero, M. A. Alvarez, and J. E. Suarez. 1998. Identification of the repressor-encoding gene of the *Lactobacillus* bacteriophage A2. J Bacteriol **180:**3474-6.

Lahdes-Vasama, T. T., R. Sihvonen, and T. Iber. 2009. Perforation of the upper and lower segments of atretic esophagus (type C) secondary to nasogastric tube insertion. Pediatr Surg Int **25:**537-8.

Lai, Y., A. E. Villaruz, M. Li, D. J. Cha, D. E. Sturdevant, and M. Otto. 2007. The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in *Staphylococci*. Mol Microbiol **63:**497-506.

LaMarche, M. J., J. A. Leeds, A. Amaral, J. T. Brewer, S. M. Bushell, G. Deng, J. M. Dewhurst, J. Ding, J. Dzink-Fox, G. Gamber, A. Jain, K. Lee, L. Lee, T. Lister, D. McKenney, S. Mullin, C. Osborne, D. Palestrant, M. A. Patane, E. M. Rann, M. Sachdeva, J. Shao, S. Tiamfook, A. Trzasko, L. Whitehead, A. Yifru, D. Yu, W. Yan, and Q. Zhu. 2012. Discovery of LFF571: an investigational agent for *Clostridium difficile* infection. J Med Chem **55:**2376-87.

Lamont, I., H. Richardson, D. R. Carter, and J. B. Egan. 1993. Genes for the establishment and maintenance of lysogeny by the temperate coliphage 186. J Bacteriol **175:**5286-8.

Lancaster, J. W., and S. J. Matthews. 2012. Fidaxomicin: The Newest Addition to the Armamentarium Against *Clostridium difficile* Infections. Clinical Therapeutics **34:**1-13.

Larson, K. C., P. P. Belliveau, and L. M. Spooner. 2011. Tigecycline for the treatment of severe *Clostridium difficile* infection. Ann Pharmacother **45:**1005-10.

Laverty, G., S. P. Gorman, and B. F. Gilmore. 2011. The potential of antimicrobial peptides as biocides. Int J Mol Sci **12:**6566-96.

Lawley, T. D., S. Clare, A. W. Walker, M. D. Stares, T. R. Connor, C. Raisen, D. Goulding, R. Rad, F. Schreiber, C. Brandt, L. J. Deakin, D. J. Pickard, S. H. Duncan, H. J. Flint, T. G. Clark, J. Parkhill, and G. Dougan. 2012. Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. Plos Pathogens **8:**e1002995.

Leav, B. A., B. Blair, M. Leney, M. Knauber, C. Reilly, I. Lowy, D. N. Gerding, C. P. Kelly, K. Katchar, R. Baxter, D. Ambrosino, and D. Molrine. 2010. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). Vaccine **28:**965-9.

Leblebicioglu, H., M. Sunbul, S. Esen, and C. Eroglu. 2003. Jarisch-Herxheimer reaction in Leptospirosis. Eur J Clin Microbiol Infect Dis **22:**639; author reply 640.

Lee, B. Y., M. J. Popovich, Y. Tian, R. R. Bailey, P. J. Ufberg, A. E. Wiringa, and R. R. Muder. 2010. The potential value of *Clostridium difficile* vaccine: an economic computer simulation model. Vaccine **28:**5245-53.

Lee, N. Y., Y. T. Huang, P. R. Hsueh, and W. C. Ko. 2010. *Clostridium difficile* bacteremia, Taiwan. Emerg Infect Dis **16:**1204-10.

Levisohn, R. 1970. Phenotypic reversion by hydroxylamine. A new group of suppressible phage T4 rII mutants. Genetics **64:**1-9.

Lewis, S., S. Burmeister, and J. Brazier. 2005. Effect of the prebiotic oligofructose on relapse of *Clostridium difficile*-associated diarrhea: a randomized, controlled study. Clin Gastroenterol Hepatol **3:**442-8.

Lewis, S. J., S. Franco, G. Young, and S. J. O'Keefe. 1996. Altered bowel function and duodenal bacterial overgrowth in patients treated with omeprazole. Aliment Pharmacol Ther **10:**557-61.

Lewis, S. J., L. F. Potts, and R. E. Barry. 1998. The lack of therapeutic effect of *Saccharomyces boulardii* in the prevention of antibiotic-related diarrhoea in elderly patients. J Infect **36:**171-4.

Li, J., L. N. Wang, and H. Y. Zheng. 2012. Jarisch-Herxheimer reaction among syphilis patients in China. J Eur Acad Dermatol Venereol. doi 10.1111/j.1468-3083.2012.04544.x

Libby, D. B., and G. Bearman. 2009. Bacteremia due to *Clostridium difficile*-review of the literature. International Journal of Infectious Diseases **13:**e305-9.

Libby, J. M., B. S. Jortner, and T. D. Wilkins. 1982. Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect Immun **36:**822-9.

Lindemann, B. F., C. Klug, and A. Schwienhorst. 2002. Evolution of bacteriophage in continuous culture: a model system to test antiviral gene therapies for the emergence of phage escape mutants. J Virol **76:**5784-92.

Litvinova, A. M., V. M. Chtetsova, and I. G. Kavtreva. 1978. Evaluation of efficacy of the use of *E. coli-Proteus* bacteriophage in intestinal dysbacteriosis in premature infants. Vopr Okhr Materin Det. **9:**42–44.

Liu, Y., Q. Zhang, C. Fang, S. Zhu, Y. Tang, and S. Huang. 2005. Effect of glutathione on UV induction of prophage lambda. Arch Microbiol **183:**444-9.

Lo, J. O., V. Wu, D. Reh, S. Nadig, and M. K. Wax. 2008. Diagnosis and management of a misplaced nasogastric tube into the pulmonary pleura. Arch Otolaryngol Head Neck Surg **134:**547-50.

Loc Carrillo, C. L., R. J. Atterbury, A. El-Shibiny, P. L. Connerton, E. Dillon, A. Scott, and I. F. Connerton. 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. App. Env. Micro. **71:**6554-6563.

Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell. 2000. Molecular Cell Biology, 4th ed. W. H. Freeman, New York.

Loo, V. G., A. M. Bourgault, L. Poirier, F. Lamothe, S. Michaud, N. Turgeon, B. Toye, A. Beaudoin, E. H. Frost, R. Gilca, P. Brassard, N. Dendukuri, C. Beliveau, M. Oughton, I. Brukner, and A. Dascal. 2011. Host and pathogen factors for *Clostridium difficile* infection and colonization. N Engl J Med **365:**1693-703.

Lopman, B., H. Vennema, E. Kohli, P. Pothier, A. Sanchez, A. Negredo, J. Buesa, E. Schreier, M. Reacher, D. Brown, J. Gray, M. Iturriza, C. Gallimore, B. Bottiger, K. O. Hedlund, M. Torven, C. H. von Bonsdorff, L. Maunula, M. Poljsak-Prijatelj, J. Zimsek, G. Reuter, G. Szucs, B. Melegh, L. Svennson, Y. van Duijnhoven, and M. Koopmans. 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet **363:**682-8.

Lorca, G. L., Y. J. Chung, R. D. Barabote, W. Weyler, C. H. Schilling, and M. H. Saier, Jr. 2005. Catabolite repression and activation in *Bacillus subtilis*: dependency on CcpA, HPr, and HprK. J Bacteriol **187:**7826-39.

Louie, T., M. Gerson, D. Grimard, S. Johnson, A. Poirier, K. Weiss, J. Peppe, J. Donovan, and D. Davidson. 2007. Results of a phase III trial comparing tolevamer, vancomycin and metronidazole in patients with *Clostridium difficile*-associated diarrhoea (CDAD). Poster K-425a. Presented at the 47th Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology., Washington, DC.

Louie, T., M. Miller, C. Donskey, K. Mullane, and E. J. Goldstein. 2009a. Clinical outcomes, safety, and pharmacokinetics of OPT-80 in a phase 2 trial with patients with *Clostridium difficile* infection. Antimicrob Agents Chemother **53:**223-8.

Louie, T. J., J. Emery, W. Krulicki, B. Byrne, and M. Mah. 2009b. OPT-80 eliminates *Clostridium difficile* and is sparing of bacteroides species during treatment of *C. difficile* infection. Antimicrob Agents Chemother **53:**261-3.

Louie, T. J., M. A. Miller, K. M. Mullane, K. Weiss, A. Lentnek, Y. Golan, S. Gorbach, P. Sears, and Y. K. Shue. 2011. Fidaxomicin versus vancomycin for *Clostridium difficile* infection. N Engl J Med **364:**422-31.

Louie, T. J., J. Peppe, C. K. Watt, D. Johnson, R. Mohammed, G. Dow, K. Weiss, S. Simon, J. F. John, Jr., G. Garber, S. Chasan-Taber, and D. M. Davidson. 2006. Tolevamer, a novel nonantibiotic polymer, compared with vancomycin in the treatment of mild to moderately severe *Clostridium difficile*-associated diarrhea. Clinical Infectious Diseases **43:**411-20.

Lowe, B. R., J. G. Fox, and J. G. Bartlett. 1980. *Clostridium difficile*-associated cecitis in guinea pigs exposed to penicillin. Am J Vet Res **41:**1277-9.

Lowy, I., D. C. Molrine, B. A. Leav, B. M. Blair, R. Baxter, D. N. Gerding, G. Nichol, W. D. Thomas, Jr., M. Leney, S. Sloan, C. A. Hay, and D. M. Ambrosino. 2010. Treatment with monoclonal antibodies against *Clostridium difficile* toxins. N Engl J Med **362:**197-205.

Luna-Herrera, J., M. V. Reddy, and P. R. Gangadharam. 1995. *In vitro* activity of the benzoxazinorifamycin KRM-1648 against drug-susceptible and multidrug-resistant tubercle bacilli. Antimicrob Agents Chemother **39:**440-4.

Lund-Tonnesen, S., A. Berstad, A. Schreiner, and T. Midtvedt. 1998. [*Clostridium difficile*-associated diarrhea treated with homologous feces]. Tidsskr Nor Laegeforen **118:**1027-30.

Lyerly, D. M., E. F. Bostwick, S. B. Binion, and T. D. Wilkins. 1991. Passive immunization of hamsters against disease caused by *Clostridium*

difficile by use of bovine immunoglobulin G concentrate. Infect Immun **59:**2215-8.

Lyerly, D. M., K. E. Saum, D. K. Macdonald, and T. D. Wilkins. 1985. Effects of *Clostridium-difficile* toxins given intragastrically to animals. Infection and Immunity **47:**349-352.

Lyras, D., J. R. O'Connor, P. M. Howarth, S. P. Sambol, G. P. Carter, T. Phumoonna, R. Poon, V. Adams, G. Vedantam, S. Johnson, D. N. Gerding, and J. I. Rood. 2009. Toxin B is essential for virulence of *Clostridium difficile*. Nature **458:**1176-1181.

MacConnachie, A. A., R. Fox, D. R. Kennedy, and R. A. Seaton. 2009. Faecal transplant for recurrent *Clostridium difficile*-associated diarrhoea: a UK case series. QJM **102:**781-4.

Macfarlane, G. T., and S. Macfarlane. 2007. Models for intestinal fermentation: association between food components, delivery systems, bioavailability and functional interactions in the gut. Current Opinion in Biotechnology **18:**156-162.

Macfarlane, G. T., S. Macfarlane, and G. R. Gibson. 1998. Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon. Microb Ecol **35:**180-7.

Mahoney, D. E., P. D. Bell, and K. B. Easterbrook. 1985. 2 Bacteriophages of *Clostridium difficile*. Journal of Clinical Microbiology **21:**251-254.

Mandalari, G., C. Nueno-Palop, G. Bisignano, M. S. Wickham, and A. Narbad. 2008. Potential prebiotic properties of almond (*Amygdalus communis* L.) seeds. Appl Environ Microbiol **74:**4264-70.

Mandalari, G., C. Nueno Palop, K. Tuohy, G. R. Gibson, R. N. Bennett, K. W. Waldron, G. Bisignano, A. Narbad, and C. B. Faulds. 2007. *In vitro* evaluation of the prebiotic activity of a pectic oligosaccharide-rich extract enzymatically derived from bergamot peel. Appl Microbiol Biotechnol **73:**1173-9.

Mandell, G. L., J. E. Bennett, and R. Dolin. 2010. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 7th edition ed, vol. 1. Churchill Livingstone, London.

Mani, N., and B. Dupuy. 2001. Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. Proc Natl Acad Sci U S A **98:**5844-9.

Martin-Pelaez, S., G. R. Gibson, S. M. Martin-Orue, A. Klinder, R. A. Rastall, R. M. La Ragione, M. J. Woodward, and A. Costabile. 2008. *In vitro* fermentation of carbohydrates by porcine faecal inocula and their

influence on *Salmonella* Typhimurium growth in batch culture systems. FEMS Microbiol Ecol **66:**608-19.

Mascio, C. T., L. I. Mortin, K. T. Howland, A. D. Van Praagh, S. Zhang, A. Arya, C. L. Chuong, C. Kang, T. Li, and J. A. Silverman. 2012. *In vitro* and *in vivo* characterization of CB-183,315, a novel lipopeptide antibiotic for the treatment of *Clostridium difficile*. Antimicrob Agents Chemother. 56:5023-30.

Masker, W. E., L. A. Dodson, and M. Maupin. 1985. Mutagenesis of bacteriophage T7 and T7 DNA by alkylation damage. J Virol **56:**644-6.

Mathur, T., M. Kumar, T. K. Barman, G. R. Kumar, V. Kalia, S. Singhal, V. S. Raj, D. J. Upadhyay, B. Das, and P. K. Bhatnagar. 2011. Activity of RBx 11760, a novel biaryl oxazolidinone, against *Clostridium difficile*. J Antimicrob Chemother **66:**1087-95.

Matsuzaki, S., M. Yasuda, H. Nishikawa, M. Kuroda, T. Ujihara, T. Shuin, Y. Shen, Z. Jin, S. Fujimoto, M. D. Nasimuzzaman, H. Wakiguchi, S. Sugihara, T. Sugiura, S. Koda, A. Muraoka, and S. Imai. 2003. Experimental protection of mice against lethal *Staphylococcus aureus* infection by novel bacteriophage phi MR11. J Infect Dis **187:**613-24.

Mattila, E., V. J. Anttila, M. Broas, H. Marttila, P. Poukka, K. Kuusisto, L. Pusa, K. Sammalkorpi, J. Dabek, O. P. Koivurova, M. Vahatalo, V. Moilanen, and T. Widenius. 2008. A randomized, double-blind study comparing *Clostridium difficile* immune whey and metronidazole for recurrent *Clostridium difficile*-associated diarrhoea: efficacy and safety data of a prematurely interrupted trial. Scand J Infect Dis **40:**702-8.

May, T., R. I. Mackie, G. C. Fahey, Jr., J. C. Cremin, and K. A. Garleb. 1994. Effect of fiber source on short-chain fatty acid production and on the growth and toxin production by *Clostridium difficile*. Scand J Gastroenterol **29:**916-22.

Mayer, M. J., V. Garefalaki, R. Spoerl, A. Narbad, and R. Meijers. 2011. Structure-based modification of a *Clostridium difficile*-targeting endolysin affects activity and host range. J Bacteriol **193:**5477-86.

Mayer, M. J., A. Narbad, and M. J. Gasson. 2008. Molecular characterization of a *Clostridium difficile* bacteriophage and its cloned biologically active endolysin. J Bacteriol **190:**6734-40.

McDonald, L. C., G. E. Killgore, A. Thompson, R. C. Owens, Jr., S. V. Kazakova, S. P. Sambol, S. Johnson, and D. N. Gerding. 2005. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N Engl J Med **353:**2433-41.

McFarland, L. V., G. W. Elmer, and C. M. Surawicz. 2002. Breaking the cycle: Treatment strategies for 163 cases of recurrent *Clostridium difficile* disease. American Journal of Gastroenterology **97:**1769-1775.

McLaughlin, J. R., H. C. Wong, Y. E. Ting, J. N. Van Arsdell, and S. Chang. 1986. Control of lysogeny and immunity of *Bacillus subtilis* temperate bacteriophage SP beta by its d gene. J Bacteriol **167:**952-9.

Meader, E., M. J. Mayer, M. J. Gasson, D. Steverding, S. R. Carding, and A. Narbad. 2010. Bacteriophage treatment significantly reduces viable *Clostridium difficile* and prevents toxin production in an *in vitro* model system. Anaerobe **16:**549-54.

Meimandipour, A., M. Shuhaimi, M. Hair-Bejo, K. Azhar, B. M. Kabeir, B. Rasti, and A. M. Yazid. 2009. *In vitro* fermentation of broiler cecal content: the role of *Lactobacilli* and pH value on the composition of microbiota and end products fermentation. Lett. App. Microbiol. **49:**415-420.

Mellow, M. H., and A. Kanatzar. 2011. Colonoscopic fecal bacteriotherapy in the treatment of recurrent *Clostridium difficile* infection-results and follow-up. J Okla State Med Assoc **104:**89-91.

Metheny, N. A., R. E. Clouse, Y. H. Chang, B. J. Stewart, D. A. Oliver, and M. H. Kollef. 2006. Tracheobronchial aspiration of gastric contents in critically ill tube-fed patients: frequency, outcomes, and risk factors. Critical Care Medicine **34:**1007-15.

Miliutina, L. N., and N. V. Vorotyntseva. 1993. Current strategy and tactics of etiotropic therapy of acute intestinal infections in children. Antibiot Khimioter. **1:**46–53.

Mitchell, T. J., J. M. Ketley, D. W. Burdon, D. C. A. Candy, and J. Stephen. 1987. Biological mode of action of *Clostridium difficile* toxin-A - a novel enterotoxin. J. Med. Microbiol. **23:**211-219.

Mitchell, T. J., J. M. Ketley, S. C. Haslam, J. Stephen, D. W. Burdon, D. C. A. Candy, and R. Daniel. 1986. Effect of Toxin-A and Toxin-B of *Clostridium difficile* on Rabbit Ileum and Colon. Gut **27:**78-85.

Modi, N., N. Gulati, K. Solomon, T. Monaghan, A. Robins, H. F. Sewell, and Y. R. Mahida. 2011. Differential binding and internalization of *Clostridium difficile* toxin A by human peripheral blood monocytes, neutrophils and lymphocytes. Scand J Immunol **74:**264-71.

Moir-Blais, T. R., F. J. Grundy, and T. M. Henkin. 2001. Transcriptional activation of the *Bacillus subtilis* ackA promoter requires sequences upstream of the CcpA binding site. J Bacteriol **183:**2389-93.

Monot, M., C. Boursaux-Eude, M. Thibonnier, D. Vallenet, I. Moszer, C. Medigue, I. Martin-Verstraete, and B. Dupuy. 2011. Reannotation of the genome sequence of *Clostridium difficile* strain 630. J Med Microbiol **60:**1193-9.

Morales, C. A., B. B. Oakley, J. K. Garrish, G. R. Siragusa, M. B. Ard, and B. S. Seal. 2012. Complete genome sequence of the podoviral bacteriophage ΦCP24R, which is virulent for *Clostridium perfringens*. Arch Virol **157:**769-72.

Morello, E., E. Saussereau, D. Maura, M. Huerre, L. Touqui, and L. Debarbieux. 2011. Pulmonary bacteriophage therapy on *Pseudomonas aeruginosa* cystic fibrosis strains: first steps towards treatment and prevention. PLoS One **6:**e16963.

Moreno, M. S., B. L. Schneider, R. R. Maile, W. Weyler, and M. H. Saier, Jr. 2001. Catabolite repression mediated by the CcpA protein in *Bacillus subtilis*: novel modes of regulation revealed by whole-genome analyses. Mol Microbiol **39:**1366-81.

Mortensen, P. B., and M. R. Clausen. 1996. Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease. Scand J Gastroenterol Suppl **216:**132-48.

Mullane, K. M., M. A. Miller, K. Weiss, A. Lentnek, Y. Golan, P. S. Sears, Y. K. Shue, T. J. Louie, and S. L. Gorbach. 2011. Efficacy of fidaxomicin versus vancomycin as therapy for *Clostridium difficile* infection in individuals taking concomitant antibiotics for other concurrent infections. Clinical Infectious Diseases **53:**440-7.

Muller, M. 1983. Mode of action of metronidazole on anaerobic bacteria and protozoa. Surgery **93:**165-71.

Musher, D. M., S. Aslam, N. Logan, S. Nallacheru, I. Bhaila, F. Borchert, and R. J. Hamill. 2005. Relatively poor outcome after treatment of *Clostridium difficile* colitis with metronidazole. Clinical Infectious Diseases **40:**1586-90.

Musher, D. M., N. Logan, A. M. Bressler, D. P. Johnson, and J. F. Rossignol. 2009. Nitazoxanide versus vancomycin in *Clostridium difficile* infection: a randomized, double-blind study. Clinical Infectious Diseases **48:**e41-6.

Musher, D. M., N. Logan, R. Hamill, H. L. Dupont, A. Lentnek, A. K. Gupta, and J. F. Rossignol. 2006 a. Nitazoxanide in the treatment of *Clostridium difficile* associated disease. Gastroenterology **130:**A316-A316.

Musher, D. M., N. Logan, R. J. Hamill, H. L. DuPont, A. Lentnek, A. Gupta, and J. F. Rossignol. 2006 b. Nitazoxanide for the treatment of *Clostridium difficile* colitis. Clinical Infectious Diseases **43:**421-427.

Musher, D. M., N. Logan, V. Mehendiratta, N. A. Melgarejo, S. Garud, and R. J. Hamill. 2007. *Clostridium difficile* colitis that fails conventional metronidazole therapy: response to nitazoxanide. J Antimicrob Chemother **59:**705-10.

Mutters, R., C. Nonnenmacher, C. Susin, U. Albrecht, R. Kropatsch, and S. Schumacher. 2009. Quantitative detection of *Clostridium difficile* in hospital environmental samples by real-time polymerase chain reaction. J Hosp Infect **71:**43-8.

Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol **59:**695-700.

Nagy, E., and J. Foldes. 1991. Electron microscopic investigation of lysogeny of *Clostridium difficile* strains isolated from antibiotic-associated diarrhea cases and from healthy carriers. APMIS **99:**321-6.

Nale, J. Y., J. Shan, P. T. Hickenbotham, W. N. Fawley, M. H. Wilcox, and M. R. Clokie. 2012. Diverse temperate bacteriophage carriage in *Clostridium difficile* 027 strains. PLoS One **7:**e37263.

Nieuwdorp, M., E. Van Nood, P. Speelman, H. A. Van Heukelem, J. M. Jansen, C. E. Visser, E. J. Kuijper, J. F. W. M. Bartelsman, and J. J. Keller. 2008. Treatment of recurrent *Clostridium difficile*-associated diarrhoea with a suspension of donor faeces. Ned. Tijdschr Geneeskd. **152:**1927-1932.

Nieves, B. M., F. Gil, and F. J. Castillo. 1981. Growth inhibition activity and bacteriophage and bacteriocin-like particles associated with different species of *Clostridium*. Can J Microbiol **27:**216-25.

Nottrott, S., J. Schoentaube, H. Genth, I. Just, and R. Gerhard. 2007. *Clostridium difficile* toxin A-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. Apoptosis **12:**1443-53.

Novak-Weekley, S. M., and M. H. Hollingsworth. 2008. Comparison of the premier toxin A and B assay and the TOX A/B II assay for diagnosis of *Clostridium difficile* infection. Clinical and Vaccine Immunology **15:**575-578.

Nuenen, M. H. M. C. v., P. D. Meyer, and K. Venema. 2003. The effect of various inulins and *Clostridium difficile* on the metabolic activity of the human colonic microbiota *in vitro*. Microbial Ecology in Health and Disease. **15:**137-144.

O'Brien, J. A., B. J. Lahue, J. J. Caro, and D. M. Davidson. 2007. The emerging infectious challenge of *Clostridium difficile*-associated disease in Massachusetts hospitals: Clinical and economic consequences. Infection Control and Hospital Epidemiology **28:**1219-1227.

O'Connor, J. R., M. A. Galang, S. P. Sambol, D. W. Hecht, G. Vedantam, D. N. Gerding, and S. Johnson. 2008. Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*. Antimicrob Agents Chemother **52:**2813-7.

O'Keefe, S. J. 2010. Tube feeding, the microbiota, and *Clostridium difficile* infection. World J Gastroenterol **16:**139-42.

O'May, G. A., N. Reynolds, and G. T. Macfarlane. 2005 a. Effect of pH on an *in vitro* model of gastric microbiota in enteral nutrition patients. Appl Environ Microbiol **71:**4777-83.

O'May, G. A., N. Reynolds, A. R. Smith, A. Kennedy, and G. T. Macfarlane. 2005 b. Effect of pH and antibiotics on microbial overgrowth in the stomachs and duodena of patients undergoing percutaneous endoscopic gastrostomy feeding. J Clin Microbiol **43:**3059-65.

Olano-Martin, E., K. C. Mountzouris, G. R. Gibson, and R. A. Rastall. 2000. *In vitro* fermentability of dextran, oligodextran and maltodextrin by human gut bacteria. Br J Nutr **83:**247-55.

Oliveira, A., R. Sereno, A. Nicolau, and J. Azeredo. 2009. The influence of the mode of administration in the dissemination of three coliphages in chickens. Poult Sci **88:**728-33.

Ostle, A. G., and J. G. Holt. 1979. Elution and inactivation of bacteriophages on soil and cation-exchange resin. Appl Environ Microbiol **38:**59-65.

Otwinowski, Z., R. W. Schevitz, R. G. Zhang, C. L. Lawson, A. Joachimiak, R. Q. Marmorstein, B. F. Luisi, and P. B. Sigler. 1988. Crystal structure of trp repressor/operator complex at atomic resolution. Nature **335:**321-9.

Ouwehand, A. C., E. Isolauri, P. V. Kirjavainen, and S. J. Salminen. 1999. Adhesion of four *Bifidobacterium* strains to human intestinal mucus from subjects in different age groups. FEMS Microbiol Lett **172:**61-4.

Papatheodorou, P., C. Zamboglou, S. Genisyuerek, G. Guttenberg, and K. Aktories. 2010. Clostridial glucosylating toxins enter cells via clathrin-mediated endocytosis. PLoS One **5:**e10673.

Paredes-Sabja, D., C. Bond, R. J. Carman, P. Setlow, and M. R. Sarker. 2008. Germination of spores of *Clostridium difficile* strains, including isolates from a hospital outbreak of *Clostridium difficile*-associated disease (CDAD). Microbiology **154:**2241-50.

Parfitt, T. 2005. Georgia: an unlikely stronghold for bacteriophage therapy. Lancet **365:**2166-7.

Parkes, G. C., J. D. Sanderson, and K. Whelan. 2009. The mechanisms and efficacy of probiotics in the prevention of *Clostridium difficile*-associated diarrhoea. Lancet Infect Dis **9:**237-44.

Paterson, D. L., J. Iredell, and M. Whitby. 1994. Putting back the bugs: bacterial treatment relieves chronic diarrhoea. Med J Aust **160:**232-3.

Paul, V. D., S. Sundarrajan, S. S. Rajagopalan, S. Hariharan, N. Kempashanaiah, S. Padmanabhan, B. Sriram, and J. Ramachandran. 2011. Lysis-deficient phages as novel therapeutic agents for controlling bacterial infection. BMC Microbiology **11**.

Paumgartner, G., and U. Beuers. 2002. Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. Hepatology **36:**525-31.

Pawlowski, S. W., G. Calabrese, G. L. Kolling, J. Platts-Mills, R. Freire, C. AlcantaraWarren, B. Liu, R. B. Sartor, and R. L. Guerrant. 2010. Murine model of *Clostridium difficile* infection with aged gnotobiotic C57BL/6 mice and a BI/NAP1 strain. J Infect Dis **202:**1708-12.

Payment, P., S. Fortin, and M. Trudel. 1984. Ferric chloride flocculation for nonflocculating beef extract preparations. Appl Environ Microbiol **47:**591-2.

Pechine, S., A. Gleizes, C. Janoir, R. Gorges-Kergot, M. C. Barc, M. Delmee, and A. Collignon. 2005 a. Immunological properties of surface proteins of *Clostridium difficile*. J Med Microbiol **54:**193-6.

Pechine, S., C. Janoir, and A. Collignon. 2005 b. Variability of *Clostridium difficile* surface proteins and specific serum antibody response in patients with *Clostridium difficile*-associated disease. J Clin Microbiol **43:**5018-25.

Pedersen, M., M. Ligowska, and K. Hammer. 2010. Characterization of the CI repressor protein encoded by the temperate lactococcal phage TP901-1. J Bacteriol **192:**2102-10.

Pelaez, T., E. Cercenado, L. Alcala, M. Marin, A. Martin-Lopez, J. Martinez-Alarcon, P. Catalan, M. Sanchez-Somolinos, and E. Bouza. 2008. Metronidazole resistance in *Clostridium difficile* is heterogeneous. J. Clin. Microbiol. **46:**3028-3032.

Pepin, J., L. Valiquette, M. E. Alary, P. Villemure, A. Pelletier, K. Forget, K. Pepin, and D. Chouinard. 2004. *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ **171:**466-72.

Perelle, S., M. Gibert, P. Bourlioux, G. Corthier, and M. R. Popoff. 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect Immun **65:**1402-7.

Persky, S. E., and L. J. Brandt. 2000. Treatment of recurrent *Clostridium difficile*-associated diarrhea by administration of donated stool directly through a colonoscope. American Journal of Gastroenterology **95:**3283-3285.

Petrof, E. O., M. J. Ciancio, and E. B. Chang. 2004. Role and regulation of intestinal epithelial heat shock proteins in health and disease. Chin J Dig Dis **5:**45-50.

Petrof, E. O., G. B. Gloor, S. J. Vanner, S. J. Weese, D. Carter, M. C. Daigneault, E. M. Brown, K. Schroeter, and E. Allen-Vercoe. 2013. Stool substitute transplant therapy for the eradication of *Clostridium difficile* infection: 'RePOOPulating' the gut. Microbiome **1**e.

Petry, N., I. Egli, C. Chassard, C. Lacroix, and R. Hurrell. 2012. Inulin modifies the Bifidobacteria population, fecal lactate concentration, and fecal pH but does not influence iron absorption in women with low iron status. Am J Clin Nutr **96:**325-31.

Possemiers, S., J. Van Camp, S. Bolca, and W. Verstraete. 2005. Characterization of the bactericidal effect of dietary sphingosine and its activity under intestinal conditions. Int J Food Microbiol **105:**59-70.

Post, K. W., B. H. Jost, and J. G. Songer. 2002. Evaluation of a test for *Clostridium difficile* toxins A and B for the diagnosis of neonatal swine enteritis. Journal of Veterinary Diagnostic Investigation **14:**258-259.

Prakash, S., L. Rodes, M. Coussa-Charley, and C. Tomaro-Duchesneau. 2011. Gut microbiota: next frontier in understanding human health and development of biotherapeutics. Biologics **5:**71-86.

Qamar, A., S. Aboudola, M. Warny, P. Michetti, C. Pothoulakis, J. T. LaMont, and C. P. Kelly. 2001. *Saccharomyces boulardii* stimulates intestinal immunoglobulin A immune response to *Clostridium difficile* toxin A in mice. Infect Immun **69:**2762-5.

Rac, M. W., L. G. Greer, and G. D. Wendel, Jr. 2010. Jarisch-Herxheimer reaction triggered by group B Streptococcus intrapartum antibiotic prophylaxis. Obstet Gynecol **116 Suppl 2:**552-6.

Radostis, O. G. C., D. Blood, and K. R. Hinhcliff. 2000. Reconstitution of ruminal microflora, 9th ed. W.B. Saunders Company, Ltd.

Rambler, M., and L. Margulis. 1979. An ultraviolet light induced bacteriophage in *Beneckea gazogenes*. Orig Life **9:**235-40.

Ramesh, V., J. A. Fralick, and R. D. Rolfe. 1999. Prevention of *Clostridium difficile*-induced ileocecitis with bacteriophage. Anaerobe **5:**69-78.

Raya, R. R., P. Varey, R. A. Oot, M. R. Dyen, T. R. Callaway, T. S. Edrington, E. M. Kutter, and A. D. Brabban. 2006. Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce *Escherichia coli* O157:H7 levels in sheep. Appl Environ Microbiol **72:**6405-10.

Rea, M. C., E. Clayton, P. M. O'Connor, F. Shanahan, B. Kiely, R. P. Ross, and C. Hill. 2007. Antimicrobial activity of lacticin 3,147 against clinical *Clostridium difficile* strains. J Med Microbiol **56:**940-6.

Rea, M. C., A. Dobson, O. O'Sullivan, F. Crispie, F. Fouhy, P. D. Cotter, F. Shanahan, B. Kiely, C. Hill, and R. P. Ross. 2011. Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. Proc Natl Acad Sci U S A **108 Suppl 1:**4639-44.

Rehman, A., F. A. Heinsen, M. Koenen, K. Venema, H. Knecht, S. Hellmig, S. Schreiber, and S. J. Ott. 2012. Effects of probiotics and antibiotics on the intestinal homeostasis in a computer controlled model of the large intestine. BMC Microbiol **12:**47.

Reineke, J., S. Tenzer, M. Rupnik, A. Koschinski, O. Hasselmayer, A. Schrattenholz, H. Schild, and C. von Eichel-Streiber. 2007. Autocatalytic cleavage of *Clostridium difficile* toxin B. Nature **446:**415-9.

Riegler, M., R. Sedivy, C. Pothoulakis, G. Hamilton, J. Zacherl, G. Bischof, E. Cosentini, W. Feil, R. Schiessel, J. T. LaMont, and et al. 1995. *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium *in vitro*. J Clin Invest **95:**2004-11.

Rodriguez-Palacios, A., H. R. Staempfli, T. Duffield, and J. S. Weese. 2007. *Clostridium difficile* in retail ground meat, Canada. Emerg Infect Dis **13:**485-7.

Rodriguez-Palacios, A., H. R. Stampfli, T. Duffield, A. S. Peregrine, L. A. Trotz-Williams, L. G. Arroyo, J. S. Brazier, and J. S. Weese. 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg Infect Dis **12:**1730-6.

Rogers, M. A., M. T. Greene, S. Saint, C. E. Chenoweth, P. N. Malani, I. Trivedi, and D. M. Aronoff. 2012. Higher rates of *Clostridium difficile* infection among smokers. PLoS One **7:**e42091.

Rohlke, F., C. M. Surawicz, and N. Stollman. 2010. Fecal flora reconstitution for recurrent *Clostridium difficile* infection: results and methodology. J Clin Gastroenterol **44:**567-70.

Rolfe, R. D., and J. P. Iaconis. 1983. Intestinal colonization of infant hamsters with *Clostridium difficile*. Infect Immun **42:**480-6.

Roseman, D., and R. L. Richardson. 1969. Isolation of bacteriophage for *Clostridium tetani*. J Virol **3:**350.

Rupnik, M., M. H. Wilcox, and D. N. Gerding. 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **7:**526-36.

Russell, G., J. Kaplan, M. Ferraro, and I. C. Michelow. 2010. Fecal bacteriotherapy for relapsing *Clostridium difficile* infection in a child: a proposed treatment protocol. Pediatrics **126:**e239-42.

Ryan, E. M., S. P. Gorman, R. F. Donnelly, and B. F. Gilmore. 2011. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. J Pharm Pharmacol **63:**1253-64.

Ryan, E. T., J. R. Butterton, R. N. Smith, P. A. Carroll, T. I. Crean, and S. B. Calderwood. 1997. Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. Infect Immun **65:**2941-9.

Ryan, J., C. Murphy, C. Twomey, R. Paul Ross, M. C. Rea, J. MacSharry, B. Sheil, and F. Shanahan. 2010. Asymptomatic carriage of *Clostridium difficile* in an Irish continuing care institution for the elderly: prevalence and characteristics. Ir J Med Sci **179:**245-50.

Saksena, S., and R. K. Tandon. 1997. Ursodeoxycholic acid in the treatment of liver diseases. Postgrad Med J **73:**75-80.

Salminen, S., C. Bouley, M. C. Boutron-Ruault, J. H. Cummings, A. Franck, G. R. Gibson, E. Isolauri, M. C. Moreau, M. Roberfroid, and I. Rowland. 1998. Functional food science and gastrointestinal physiology and function. Br J Nutr **80 Suppl 1:**S147-71.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, Second ed. Cold Spring Harbor Laboratory Press.

Sambrook, J., and D. W. Russell (ed.). 2006. The Condensed Protocols From Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, New York.

Santos, R. L., M. Raffatellu, C. L. Bevins, L. G. Adams, C. Tukel, R. M. Tsolis, and A. J. Baumler. 2009. Life in the inflamed intestine, *Salmonella* style. Trends Microbiol **17:**498-506.

Santos, S. B., C. M. Carvalho, S. Sillankorva, A. Nicolau, E. C. Ferreira, and J. Azeredo. 2009. The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. BMC Microbiol **9:**148.

Sapkota, A. R., S. Berger, and T. M. Vogel. 2010. Human Pathogens Abundant in the Bacterial Metagenome of Cigarettes. Environmental Health Perspectives **118:**351-356.

Saulnier, D. M., G. R. Gibson, and S. Kolida. 2008. *In vitro* effects of selected synbiotics on the human faecal microbiota composition. FEMS Microbiol Ecol **66:**516-27.

Savidge, T. C., W. H. Pan, P. Newman, M. O'Brien, P. M. Anton, and C. Pothoulakis. 2003. *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. Gastroenterology **125:**413-20.

Saxton, K., S. D. Baines, J. Freeman, R. O'Connor, and M. H. Wilcox. 2009. Effects of exposure of *Clostridium difficile* PCR ribotypes 027 and 001 to fluoroquinolones in a human gut model. Antimicrob Agents Chemother **53:**412-20.

Scaria, J., L. Ponnala, T. Janvilisri, W. Yan, L. A. Mueller, and Y. F. Chang. 2010. Analysis of ultra low genome conservation in *Clostridium difficile*. PLoS One **5:**e15147.

Schallehn, G. 1985. Clostridium sordellii bacteriophage active on *Clostridium difficile*. Lett. App. Microbiol. **1:**71-74.

Schiffrin, E. J., F. Rochat, H. Link-Amster, J. M. Aeschlimann, and A. Donnet-Hughes. 1995. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci **78:**491-7.

Scholl, D., S. Adhya, and C. R. Merril. 2002. Bacteriophage SP6 is closely related to phages K1-5, K5, and K1E but encodes a tail protein very similar to that of the distantly related P22. J Bacteriol **184:**2833-6.

Scholl, D., S. Rogers, S. Adhya, and C. R. Merril. 2001. Bacteriophage K1-5 encodes two different tail fiber proteins, allowing it to infect and replicate on both K1 and K5 strains of *Escherichia coli*. J Virol **75:**2509-15.

Schwan, A., S. Sjolin, U. Trottestam, and B. Aronsson. 1984. Relapsing *Clostridium difficile* enterocolitis cured by rectal infusion of normal faeces. Scand J Infect Dis **16:**211-5.

Scott, J., P. Thompson-Mayberry, S. Lahmamsi, C. J. King, and W. M. McShan. 2008. Phage-associated mutator phenotype in group A *Streptococcus*. J Bacteriol **190:**6290-301.

Seal, D., S. P. Borriello, F. Barclay, A. Welch, M. Piper, and M. Bonnycastle. 1987. Treatment of relapsing *Clostridium difficile* diarrhoea by administration of a non-toxigenic strain. Eur J Clin Microbiol **6:**51-3.

Sebaihia, M., B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, N. R. Thomson, A. P. Roberts, A. M. Cerdeno-Tarraga, H. Wang, M. T. Holden, A. Wright, C. Churcher, M. A. Quail, S. Baker, N. Bason, K. Brooks, T. Chillingworth, A. Cronin, P. Davis, L. Dowd, A. Fraser, T. Feltwell, Z. Hance, S. Holroyd, K. Jagels, S. Moule, K. Mungall, C. Price, E. Rabbinowitsch, S. Sharp, M. Simmonds, K. Stevens, L. Unwin, S. Whithead, B. Dupuy, G. Dougan, B. Barrell, and J. Parkhill. 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nature Genetics **38:**779-86.

Sebald, M., and M. Popovitch. 1967. [Study of the bacteriophages of *Clostridium histolyticum* strain P]. Ann Inst Pasteur (Paris) **113:**781-9.

Seeley, N. D., and S. B. Primrose. 1982. The isolation of bacteriophages from the environment. J Appl Bacteriol **53:**1-17.

Sekulovic, O., M. Meessen-Pinard, and L. C. Fortier. 2011. Prophage-stimulated toxin production in *Clostridium difficile* NAP1/027 lysogens. J Bacteriol **193:**2726-34.

Sell, T. L., D. R. Schaberg, and F. R. Fekety. 1983. Bacteriophage and bacteriocin typing scheme for *Clostridium difficile*. J Clin Microbiol **17:**1148-52.

Serra-Moreno, R., S. Acosta, J. P. Hernalsteens, J. Jofre, and M. Muniesa. 2006. Use of the lambda Red recombinase system to produce recombinant prophages carrying antibiotic resistance genes. BMC Mol Biol **7:**31.

Serra-Moreno, R., J. Jofre, and M. Muniesa. 2008. The CI repressors of Shiga toxin-converting prophages are involved in coinfection of *Escherichia coli* strains, which causes a down regulation in the production of Shiga toxin 2. J. Bacteriol. **190:**4722-4735.

Serwer, P., S. J. Hayes, J. A. Thomas, and S. C. Hardies. 2007. Propagating the missing bacteriophages: a large bacteriophage in a new class. Virol J **4:**21.

Serwer, P., S. J. Hayes, S. Zaman, K. Lieman, M. Rolando, and S. C. Hardies. 2004. Improved isolation of undersampled bacteriophages: finding of distant terminase genes. Virology **329:**412-24.

Seth, A. K., M. R. Geringer, K. T. Nguyen, S. P. Agnew, Z. Dumanian, R. D. Galiano, K. P. Leung, T. A. Mustoe, and S. J. Hong. 2013. Bacteriophage therapy for *Staphylococcus aureus* biofilm-infected wounds: a new approach to chronic wound care. Plast Reconstr Surg **131:**225-34.

Sethi, A. K., W. N. Al-Nassir, M. M. Nerandzic, and C. J. Donskey. 2009. Skin and environmental contamination with vancomycin-resistant *Enterococci* in patients receiving oral metronidazole or oral vancomycin treatment for *Clostridium difficile*-associated disease. Infect Control Hosp Epidemiol **30:**13-7.

Shahani, L., and J. Koirala. 2012. Intravenous immunoglobulin in treatment of *Clostridium difficile* colitis. BMJ Case Rep **2012**.

Shan, J., K. V. Patel, P. T. Hickenbotham, J. Y. Nale, K. R. Hargreaves, and M. R. Clokie. 2012. Prophage carriage and diversity within clinically relevant strains of *Clostridium difficile*. Appl Environ Microbiol **78:**6027-34.

Shields, P. A., and S. R. Farrah. 1986. Concentration of viruses in beef extract by flocculation with ammonium sulfate. Appl Environ Microbiol **51:**211-3.

Shimamura, T., S. Nakamura, M. Hayase, and S. Nishida. 1974. Mitomycin-induced lysis of *Clostridium sordellii*. J Med Microbiol **7:**277-83.

Siebenga, J. J., H. Vennema, E. Duizer, and M. P. Koopmans. 2007. Gastroenteritis caused by norovirus GGII.4, The Netherlands, 1994-2005. Emerg Infect Dis **13:**144-6.

Siitonen, S., H. Vapaatalo, S. Salminen, A. Gordin, M. Saxelin, R. Wikberg, and A. L. Kirkkola. 1990. Effect of *Lactobacillus* GG yoghurt in prevention of antibiotic associated diarrhoea. Ann Med **22:**57-9.

Silverman, M. S., I. Davis, and D. R. Pillai. 2010. Success of self-administered home fecal transplantation for chronic *Clostridium difficile* infection. Clin Gastroenterol Hepatol **8:**471-3.

Small, J. D. 1968. Fatal enterocolitis in hamsters given lincomycin hydrochloride. Lab Anim Care **18:**411-20.

Sokol, H., B. Pigneur, L. Watterlot, O. Lakhdari, L. G. Bermudez-Humaran, J. J. Gratadoux, S. Blugeon, C. Bridonneau, J. P. Furet, G. Corthier, C. Grangette, N. Vasquez, P. Pochart, G. Trugnan, G. Thomas, H. M. Blottiere, J. Dore, P. Marteau, P. Seksik, and P. Langella. 2008. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A **105:**16731-6.

Song, X. Y., J. G. Bartlett, K. Speck, A. Naegeli, K. Carroll, and T. M. Perl. 2008. Rising economic impact of *Clostridium difficile* - associated disease in adult hospitalized patient population. Infection Control and Hospital Epidemiology **29:**823-828.

Songer, J. G. 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. Anim Health Res Rev **5:**321-6.

Songer, J. G., R. Jones, M. A. Anderson, A. J. Barbara, K. W. Post, and H. T. Trinh. 2007. Prevention of porcine *Clostridium difficile*-associated disease by competitive exclusion with nontoxigenic organisms. Vet Microbiol **124:**358-61.

Sorg, J. A., and A. L. Sonenshein. 2008. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. J Bacteriol **190:**2505-12.

Spigaglia, P., and P. Mastrantonio. 2002. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol **40:**3470-5.

Stabler, R. A., M. He, L. Dawson, M. Martin, E. Valiente, C. Corton, T. D. Lawley, M. Sebaihia, M. A. Quail, G. Rose, D. N. Gerding, M. Gibert, M. R. Popoff, J. Parkhill, G. Dougan, and B. W. Wren. 2009. Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. Genome Biol **10:**R102.

Starr, J. M., H. Martin, J. McCoubrey, G. Gibson, and I. R. Poxton. 2003. Risk factors for *Clostridium difficile* colonisation and toxin production. Age and Ageing **32:**657-660.

Steele, J., H. Feng, N. Parry, and S. Tzipori. 2010. Piglet models of acute or chronic *Clostridium difficile* illness. J Infect Dis **201:**428-34.

Stewart, M. L., D. A. Timm, and J. L. Slavin. 2008. Fructooligosaccharides exhibit more rapid fermentation than long-chain inulin in an *in vitro* fermentation system. Nutr Res **28:**329-34.

Stringer, S. C., M. D. Webb, S. M. George, C. Pin, and M. W. Peck. 2005. Heterogeneity of times required for germination and outgrowth from single spores of nonproteolytic *Clostridium botulinum*. Appl Environ Microbiol **71:**4998-5003.

Stubbe, H., J. Berdoz, J. P. Kraehenbuhl, and B. Corthesy. 2000. Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. J Immunol **164:**1952-60.

Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff. 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol Lett **186:**307-12.

Sujatha, S., and I. Praharaj. 2012. Glycopeptide resistance in gram-positive cocci: a review. Interdiscip Perspect Infect Dis **2012:**781679.

Sulakvelidze, A., Z. Alavidze, and J. G. Morris, Jr. 2001. Bacteriophage therapy. Antimicrob Agents Chemother **45:**649-59.

Sullivan, A., and C. E. Nord. 2002. The place of probiotics in human intestinal infections. Int J Antimicrob Agents **20:**313-9.

Sullivan, N. M., S. Pellett, and T. D. Wilkins. 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect Immun **35:**1032-40.

Sun, X. M., H. Y. Wang, Y. R. Zhang, K. Chen, B. Davis, and H. P. Feng. 2011. Mouse relapse model of *Clostridium difficile* infection. Infection and Immunity **79:**2856-2864.

Tan, K. S., B. Y. Wee, and K. P. Song. 2001. Evidence for holin function of *tcdE* gene in the pathogenicity of *Clostridium difficile*. J Med Microbiol **50:**613-9.

Tedesco, F. J. 1982. Treatment of recurrent antibiotic-associated pseudomembranous colitis. American Journal of Gastroenterology **77:**220-1.

Terada, A., H. Hara, M. Kataoka, and T. Mitsuoka. 1992. Effect of Lactulose on the Composition and Metabolic Activity of the Human Faecal Flora Microbial Ecology in Health and Disease **5:**43-50.

Thapa, D., R. Losa, B. Zweifel, and R. J. Wallace. 2012. Sensitivity of pathogenic and commensal bacteria from the human colon to essential oils. Microbiology.**158**:2870-7

Thygesen, J. B., H. Glerup, and B. Tarp. 2012. *Saccharomyces boulardii* fungemia caused by treatment with a probioticum. BMJ Case Rep **2012**. doi: 10.1136/bcr.06.2011.4412

Tomoda, K., K. Kubo, T. Asahara, A. Andoh, K. Nomoto, Y. Nishii, Y. Yamamoto, M. Yoshikawa, and H. Kimura. 2011. Cigarette smoke decreases organic acids levels and population of *Bifidobacterium* in the caecum of rats. J Toxicol Sci **36:**261-6.

Trun, N., and J. Trempy. 2003. Fundamental Bacterial Genetics. Blackwell Science Ltd., Oxford.

Trzasko, A., J. A. Leeds, J. Praestgaard, M. J. Lamarche, and D. McKenney. 2012. Efficacy of LFF571 in a hamster model of *Clostridium difficile* infection. Antimicrob Agents Chemother **56:**4459-62.

Tvede, M., and J. Rask-Madsen. 1989. Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. Lancet **1:**1156-60.

Underwood, S., S. Guan, V. Vijayasubhash, S. D. Baines, L. Graham, R. J. Lewis, M. H. Wilcox, and K. Stephenson. 2009. Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. J Bacteriol **191:**7296-305.

van der Vijver, J. C., M. M. van Es-Boon, and M. F. Michel. 1975. Induction of mutation in *Staphylococcus aureus* by ethylmethane sulphonate. J Med Microbiol **8:**265-77.

Varga, J., V. L. Stirewalt, and S. B. Melville. 2004. The CcpA protein is necessary for efficient sporulation and enterotoxin gene (cpe) regulation in *Clostridium perfringens*. J Bacteriol **186:**5221-9.

Verdu, E., F. Viani, D. Armstrong, R. Fraser, H. H. Siegrist, B. Pignatelli, J. P. Idstrom, C. Cederberg, A. L. Blum, and M. Fried. 1994. Effect of

omeprazole on intragastric bacterial counts, nitrates, nitrites, and N-nitroso compounds. Gut **35:**455-60.

Vieira, A., Y. J. Silva, A. Cunha, N. C. Gomes, H. W. Ackermann, and A. Almeida. 2012. Phage therapy to control multidrug-resistant *Pseudomonas aeruginosa* skin infections: *in vitro* and *ex vivo* experiments. Eur J Clin Microbiol Infect Dis **31:**3241-9.

Villano, S. A, T. W., Seiberling M, Gerding DN, Gomez A, Monnot-Chase E. 2010. Phase I evaluation of an oral suspension of VP 20621, spores of a non-toxigenic *C. difficile* strain (NTCD) in healthy older subjects pretreated with oral vancomycin. Abstract number F1-2127b presented at Proceedings of the 50th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, Massachusetts, USA.

Voth, D. E., and J. D. Ballard. 2005. *Clostridium difficile* toxins: Mechanism of action and role in disease. Clinical Microbiology Reviews **18:**247-63.

Ward, P. B., and G. P. Young. 1997. Dynamics of *Clostridium difficile* infection. Control using diet. Adv Exp Med Biol **412:**63-75.

Warny, M., J. Pepin, A. Fang, G. Killgore, A. Thompson, J. Brazier, E. Frost, and L. C. McDonald. 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet **366:**1079-84.

Warren, C. A., G. M. Calabrese, Y. S. Li, S. W. Pawlowski, R. A. Figler, J. Rieger, P. B. Ernst, J. Linden, and R. L. Guerrant. 2012. Effects of adenosine A(2A) receptor activation and alanyl-glutamine in *Clostridium difficile* toxin-induced ileitis in rabbits and cecitis in mice. BMC Infectious Diseases **12**.doi: 10.1186/1471-2334-12-13.

Watanakunakorn, C. 1984. Mode of action and *in vitro* activity of vancomycin. J Antimicrob Chemother **14 Suppl D:**7-18.

Waters, E. H., J. P. Orr, E. G. Clark, and C. M. Schaufele. 1998. Typhlocolitis caused by *Clostridium difficile* in suckling piglets. J Vet Diagn Invest **10:**104-8.

Wenisch, C., B. Parschalk, M. Hasenhundl, A. M. Hirschl, and W. Graninger. 1996. Comparison of vancomycin, teicoplanin, metronidazole, and fusidic acid for the treatment of *Clostridium difficile*-associated diarrhea. Clinical Infectious Diseases **22:**813-8.

Wetzler, M., and A. E. Barron. 2011. Progress in the *de novo* design of structured peptoid protein mimics. Biopolymers **96:**556-60.

Wilcox, M. H., W. N. Fawley, and P. Parnell. 2000. Value of lysozyme agar incorporation and alkaline thioglycollate exposure for the environmental recovery of *Clostridium difficile*. J Hosp Infect **44:**65-9.

Wilcox, M. H., L. Mooney, R. Bendall, C. D. Settle, and W. N. Fawley. 2008. A case-control study of community-associated *Clostridium difficile* infection. Journal of Antimicrobial Chemotherapy **62:**388-396.

Williamson, K. E., M. Radosevich, and K. E. Wommack. 2005. Abundance and diversity of viruses in six Delaware soils. Appl Environ Microbiol **71:**3119-25.

Williamson, K. E., K. E. Wommack, and M. Radosevich. 2003. Sampling natural viral communities from soil for culture-independent analyses. Appl Environ Microbiol **69:**6628-33.

Wilson, K. H., and J. N. Sheagren. 1983. Antagonism of toxigenic *Clostridium difficile* by nontoxigenic *C. difficile*. J Infect Dis **147:**733-6.

Wilson, K. H., J. N. Sheagren, and R. Freter. 1985. Population dynamics of ingested *Clostridium difficile* in the gastrointestinal tract of the Syrian hamster. J Infect Dis **151:**355-61.

World Health Organisation. 2011. WHO Expert Committee on Biological Standardization.

Wren, M. W., M. Sivapalan, R. Kinson, and N. R. Shetty. 2009. Laboratory diagnosis of *Clostridium difficile* infection. An evaluation of tests for faecal toxin, glutamate dehydrogenase, lactoferrin and toxigenic culture in the diagnostic laboratory. Br J Biomed Sci **66:**1-5.

Wullt, M., and I. Odenholt. 2004. A double-blind randomized controlled trial of fusidic acid and metronidazole for treatment of an initial episode of *Clostridium difficile*-associated diarrhoea. J Antimicrob Chemother **54:**211-6.

Yadav, M., M. C. Huang, and E. J. Goetzl. 2011. VPAC1 (vasoactive intestinal peptide (VIP) receptor type 1) G protein-coupled receptor mediation of VIP enhancement of murine experimental colitis. Cell Immunol **267:**124-32.

Yaeger, M., N. Funk, and L. Hoffman. 2002. A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. J Vet Diagn Invest **14:**281-7.

Yan, F., and D. B. Polk. 2002. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem **277:**50959-65.

Yang, C. J., N. Y. Lee, Y. H. Lin, H. C. Lee, W. C. Ko, C. H. Liao, C. H. Wu, C. Y. Hsieh, P. Y. Wu, W. C. Liu, Y. C. Chang, and C. C. Hung. 2010. Jarisch-Herxheimer reaction after penicillin therapy among patients with syphilis in the era of the hiv infection epidemic: incidence and risk factors. Clinical Infectious Diseases **51:**976-9.

Yoon, S. S., and L. J. Brandt. 2010. Treatment of refractory/recurrent *C. difficile*-associated disease by donated stool transplanted via colonoscopy: a case series of 12 patients. J Clin Gastroenterol **44:**562-6.

Yoshida, M., A. Masuda, T. T. Kuo, K. Kobayashi, S. M. Claypool, T. Takagawa, H. Kutsumi, T. Azuma, W. I. Lencer, and R. S. Blumberg. 2006. IgG transport across mucosal barriers by neonatal Fc receptor for IgG and mucosal immunity. Springer Seminars in Immunopathology **28:**397–403.

You, D. M., M. A. Franzos, and R. P. Holman. 2008. Successful treatment of fulminant *Clostridium difficile* infection with fecal bacteriotherapy. Ann Intern Med **148:**632-3.

Zablotny, R., and D. G. Fraenkel. 1967. Glucose and gluconate metabolism in a mutant of *Escherichia coli* lacking gluconate-6-phosphate dehydrase. J Bacteriol **93:**1579-81.

Zausig, Y. A., B. M. Graf, and R. Gust. 2008. Occurrence of a pneumothorax secondary to malpositioned nasogastric tube: a case report. Minerva Anestesiologica **74:**735-738.

Zedtwitz-Liebenstein, K., C. Wenisch, S. Patruta, B. Parschalk, F. Daxbock, and W. Graninger. 2002. Omeprazole treatment diminishes intra- and extracellular neutrophil reactive oxygen production and bactericidal activity. Critical Care Medicine **30:**1118-1122.

Zeiser, J. J., J. Klodmann, H. P. Braun, R. Gerhard, I. Just, and A. Pich. 2011. Effects of *Clostridium difficile* Toxin A on the proteome of colonocytes studied by differential 2D electrophoresis. J Proteomics **75:**469-79.

Zhang, J., and T. L. Madden. 1997. PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation. Genome Res **7:**649-56.

Zhang, Z., S. Schwartz, L. Wagner, and W. Miller. 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol **7:**203-14.

Zihler, A., M. Gagnon, C. Chassard, C. P. Braegger, and C. Lacroix. 2010. Combination of *in vitro* intestinal continuous fermentation and intestinal cell models to investigate *Salmonella* and probiotics interactions in infected gut microbiota. Journal of Biotechnology **150:**S316-S317.

Appendices

Appendix 1: Table of bacterial strains used throughout the thesis.

Genus and species	Strain	Source	Sensitivity to ΦCD27
Clostridium difficile	NCTC 11204	1	S
Clostridium difficile	NCTC 11205	1	S
Clostridium difficile	NCTC 11206	1	R
Clostridium difficile	NCTC 11207	1	S
Clostridium difficile	NCTC 11208	1	R
Clostridium difficile	NCTC 11209	1	S
Clostridium difficile	NCTC 11223	1	R
Clostridium difficile	NCTC 12726	1	R
Clostridium difficile	NCTC 12727	1	R
Clostridium difficile	NCTC 12728	1	R
Clostridium difficile	NCTC 12729	1	R
Clostridium difficile	NCTC 12730	1	R
Clostridium difficile	NCTC 12731	1	R
Clostridium difficile	NCTC 12732	1	R
Clostridium difficile	NCTC 12733	1	R
Clostridium difficile	NCTC 12734	1	R
Clostridium difficile	NCTC 11382	1	R
Clostridium difficile	R23 521	2	R
Clostridium difficile	R23 524	2	R
Clostridium difficile	R23 613*	2	R
Clostridium difficile	R23 614	2	R
Clostridium difficile	R23 621	2	R
Clostridium difficile	R23 635	2	R
Clostridium difficile	R23 639	2	R
Clostridium difficile	R23 642	2	R
Clostridium difficile	R23 720	2	R
Clostridium difficile	R23 727	2	R
Clostridium difficile	R23 732*	2	R
Clostridium difficile	R23 737	2	R
Clostridium difficile	G83/03	2	R
Clostridium difficile	DSMZ 12056	3	R
Clostridium difficile	DSMZ 12057	3	R
Clostridium difficile	NNUH-1	4	R
Clostridium difficile	NNUH-2	4	R
Clostridium difficile	NNUH-3	4	R
Clostridium difficile	NNUH-4	4	R
Clostridium difficile	NNUH-5	4	R
Clostridium difficile	NNUH-7	4	R
Clostridium difficile	NNUH-8	4	R
Clostridium difficile	PIG-ENV	5	R
Clostridium sporogenes	BL81-04	6	R
Clostridium sporogenes	BL84-18	6	R

Clostridium sporogenes	BL84-17	6	R
Clostridium sporogenes	BL02-01	6	R
Clostridium sordellii	ATCC 13356	7	R
Clostridium sordellii	NCTC 13356	1	R
Clostridium bifermentans	C22-10	6	R
Escherichia coli	K12	8	R
Micrococcus luteus	FI10640	8	R

Table A1: Bacterial strains used throughout the thesis. Sources 1) National Collection of Type Cultures, Central Public Health Laboratory, London, U.K; 2) kindly provided by Dr. Jonathan Brazier, Anaerobe Reference Unit, Dept. of Medical Microbiology and PHLS, University Hospital of Wales, Cardiff (*ribotype 027); 3), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), GmbH Inhoffenstrasse 7 B, 38124 Braunschweig, Germany; 4) clinical faecal samples; 5) environmental isolates; 6) kindly provided by Dr. Clare Aldus; 7) American Type Culture Collection, Virginia, U.S.A. and 8) kindly provided by Dr. Diane Hatziioanou. S, shows sensitivity to infection by bacteriophage ΦCD27; R, insensitive to ΦCD27.

Appendix 2: Local and Regional Ethics Committee (LREC) letter

Norfolk NHS
Primary Care Trust

Department of Community Paediatrics
Norfolk & Norwich University Hospital NHS Trust
Colney Lane
Norwich
NR4 7UY

Tel: (01603) 287624
Fax: (01603) 287584

Ref: RFR/BLN/

20 November 07

Professor Mike Gasson
Institute of Food Research
Norwich Research Park
NORWICH
NR4 7UA

Dear Professor Gasson

Thank you for your letter of 14 November about your research on Clostridium difficile. You asked whether ethics and governance approval is necessary for the taking of faecal specimens from the microbiology laboratory. You have supplied me with quite a lot of information but I think the answer depends on some further information which is not in your letter. This of course will have to have the approval of the head of the microbiology laboratory, who ought I think probably to be part of the research team. They obviously need to be happy to release specimens and to confirm that there are no financial implications.

Secondly, I am not sure whether you are taking any clinical information at all. If you are (whether or not this is patient identifiable) then you will need ethical and governance approval. However, if you are simply collecting specimens of stool with no additional information, then I think that this would not require ethical or governance approval. I am afraid that even age, sex and basic demographic details do constitute clinical information and if you were including this then it would be necessary to seek ethical approval.

Finally, the head of the laboratory will need to confirm the safety arrangements for their staff and your staff and the general public about transporting faecal specimens. Again, this is a fairly straightforward issue but it does just need to be agreed, confirmed and signed off.

I am filing a copy of this letter and your letter in the R&D department and will take some further advice about whether the guidance I have suggested above is indeed appropriate.

I hope this letter is helpful.

Yours Sincerely

Dr Richard Reading
Consultant Community Paediatrician

Appendix 3: Colon model results continued; The effect of phage therapy on the growth of *Bifidobacterium spp*., total anaerobes, Gram-positive cocci and total *Clostridium spp*.

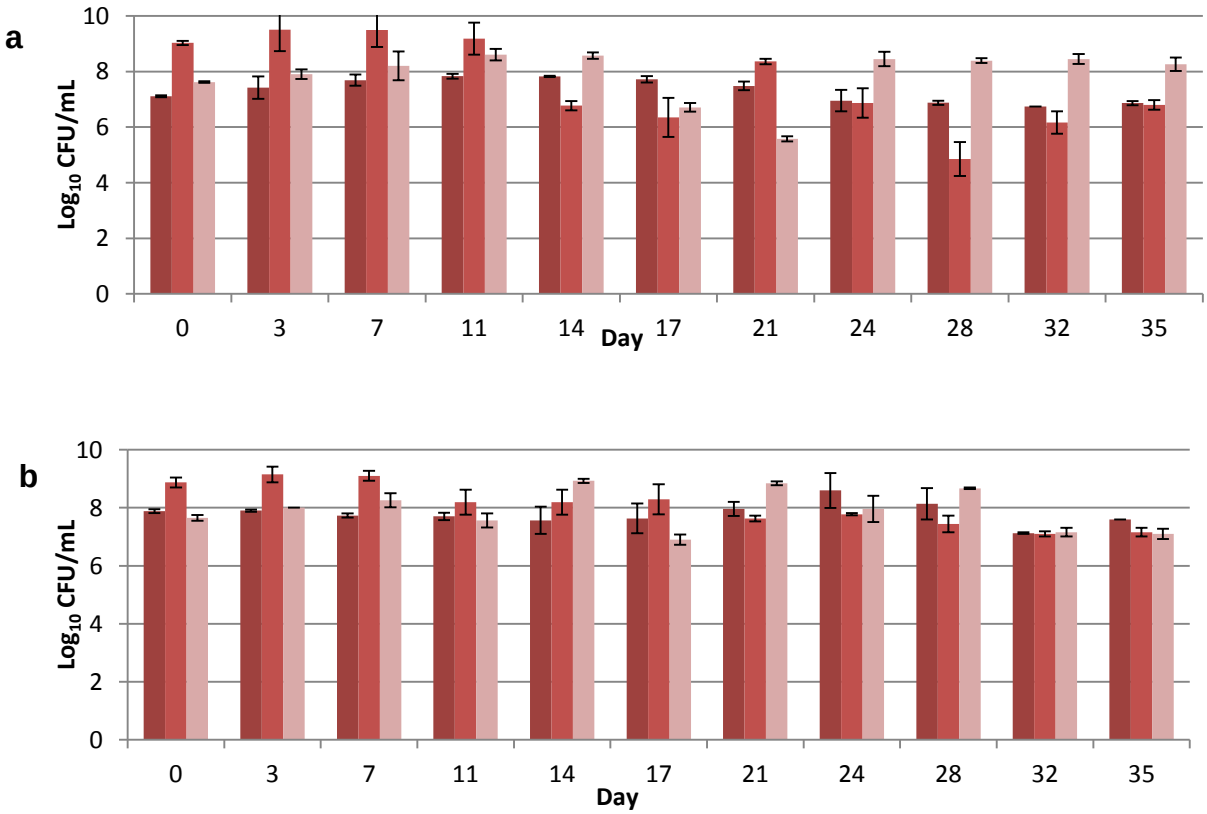


Figure A.1. *Bifidobacterium spp*. recovered from the (a) control and the (b) phage-treated ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

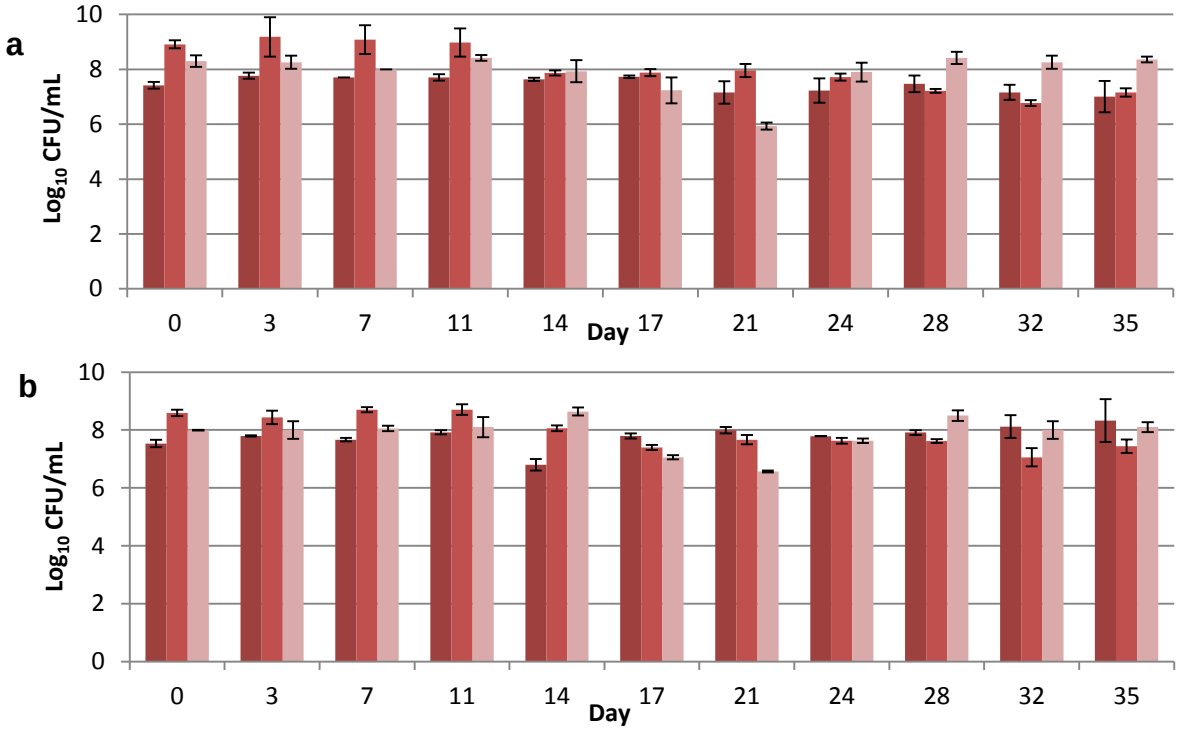


Figure A.2. *Bifidobacterium spp*. recovered from the (a) control and the (b) phage-treated transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

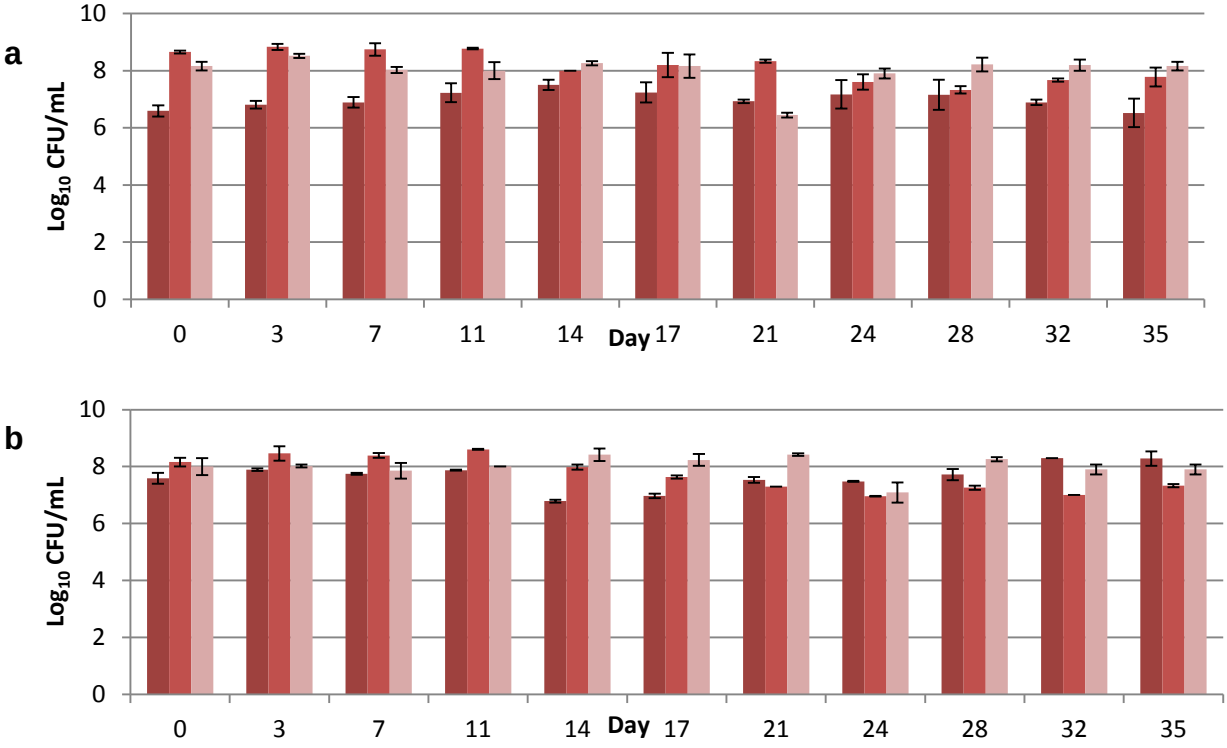


Figure A.3. *Bifidobacterium spp*. recovered from the (a) control and the (b) phage-treated descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

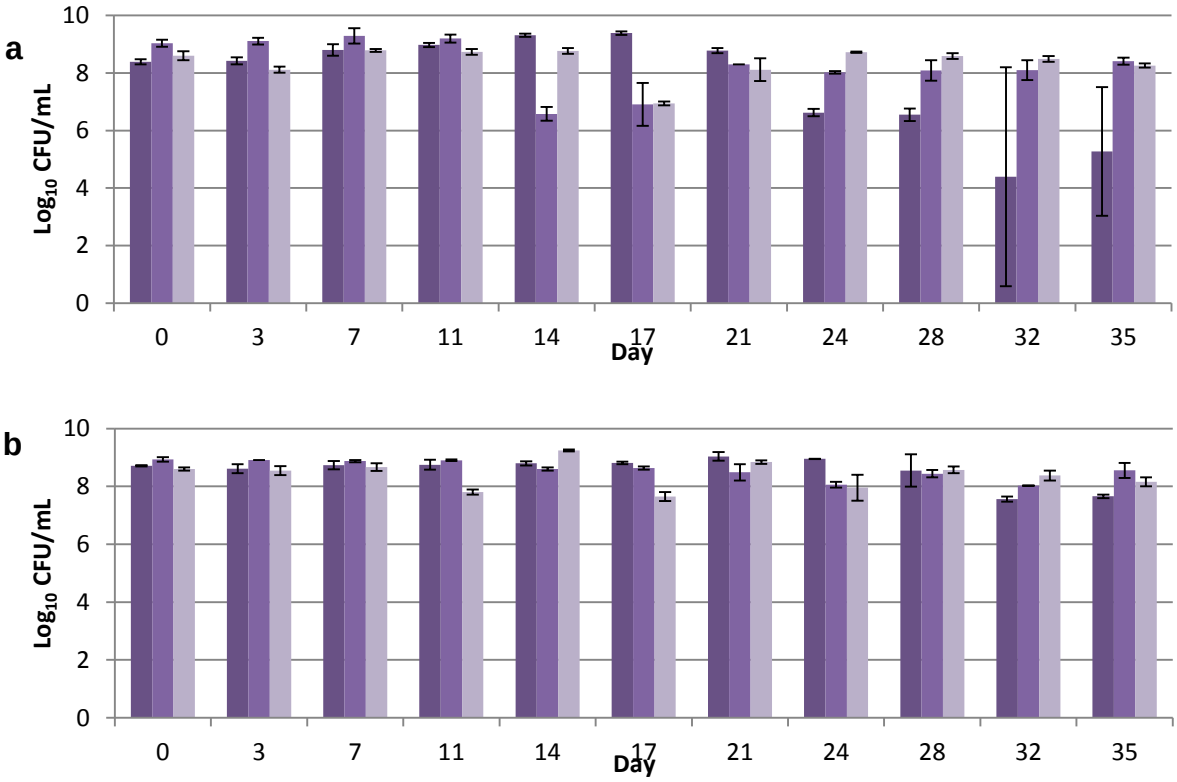


Figure A.4. Total anaerobes recovered from the (a) control and the (b) phage-treated ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

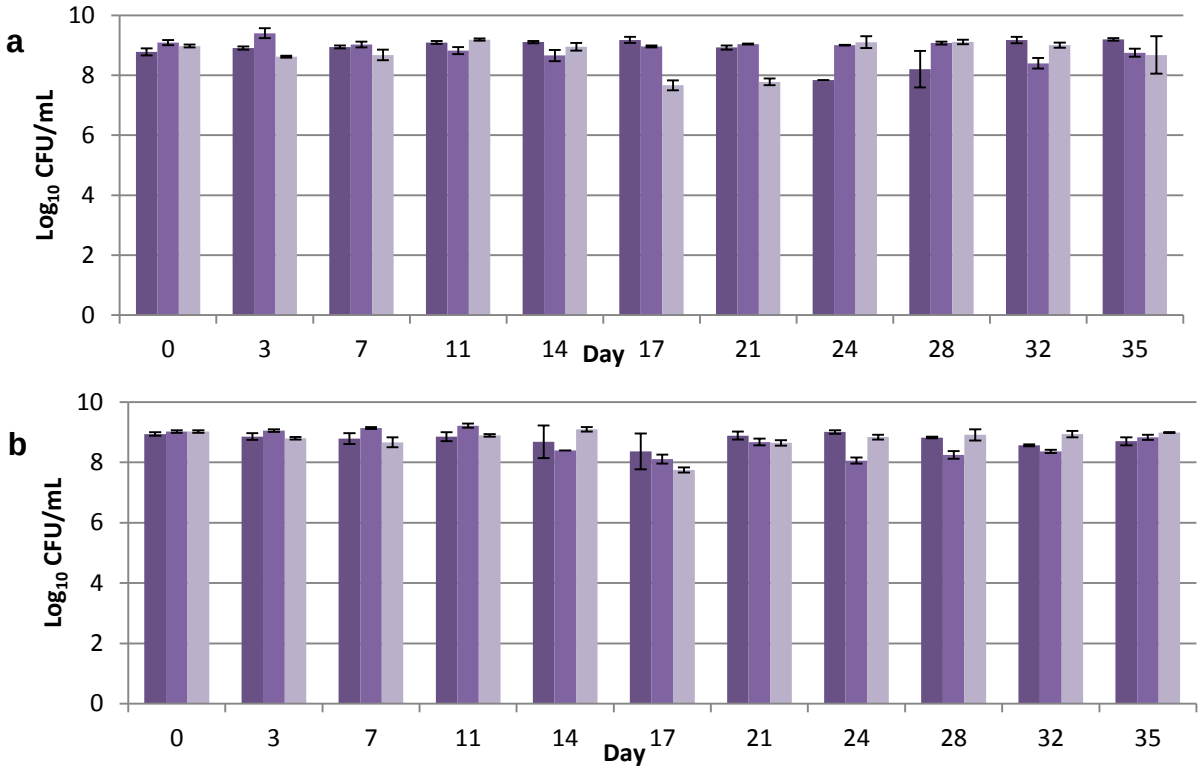


Figure A.5. Total anaerobes recovered from the (a) control and the (b) phage-treated transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

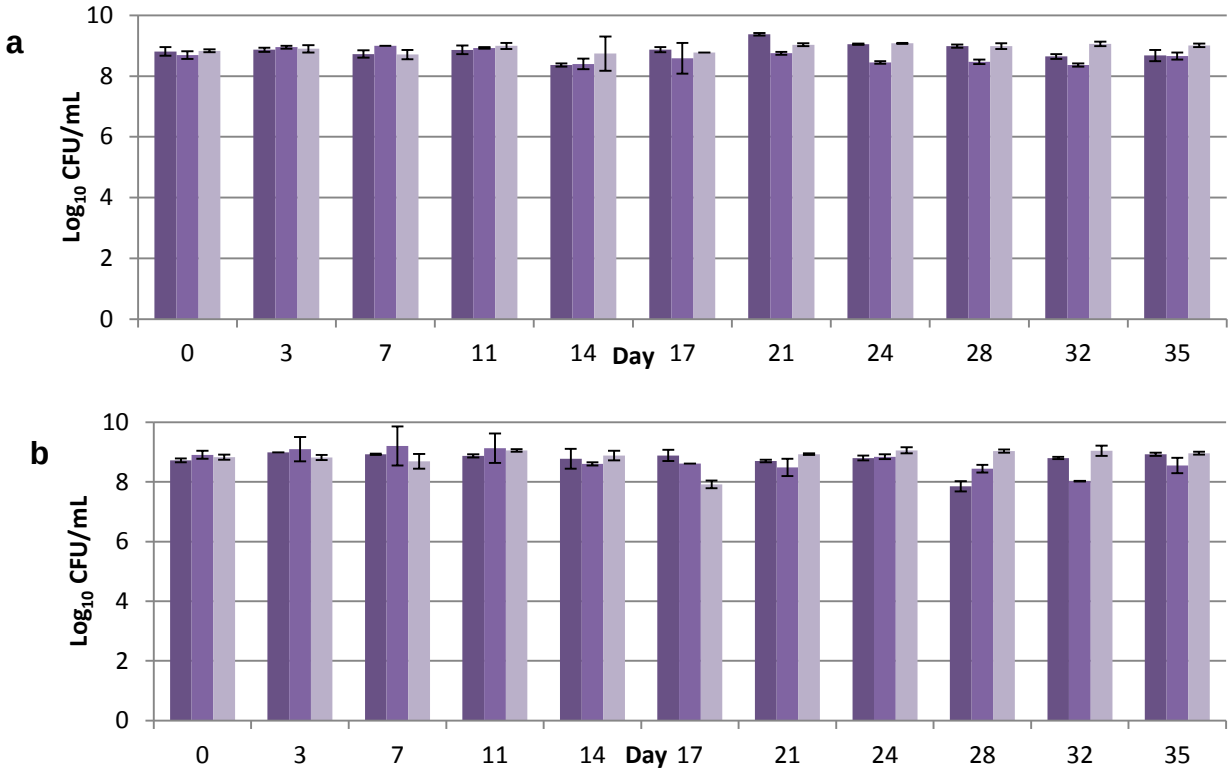


Figure A.6. Total anaerobes recovered from the (a) control and the (b) phage-treated descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

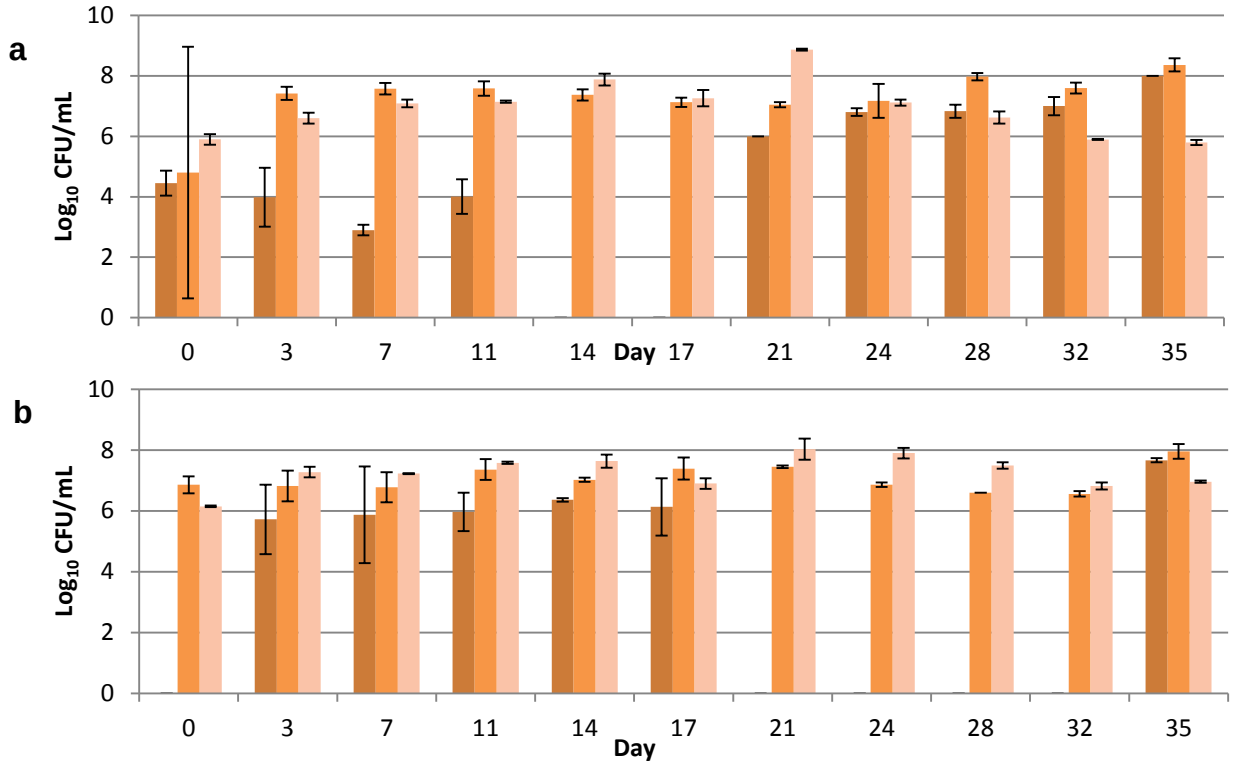


Figure A.7. Gram positive cocci recovered from the (a) control and the (b) phage-treated ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

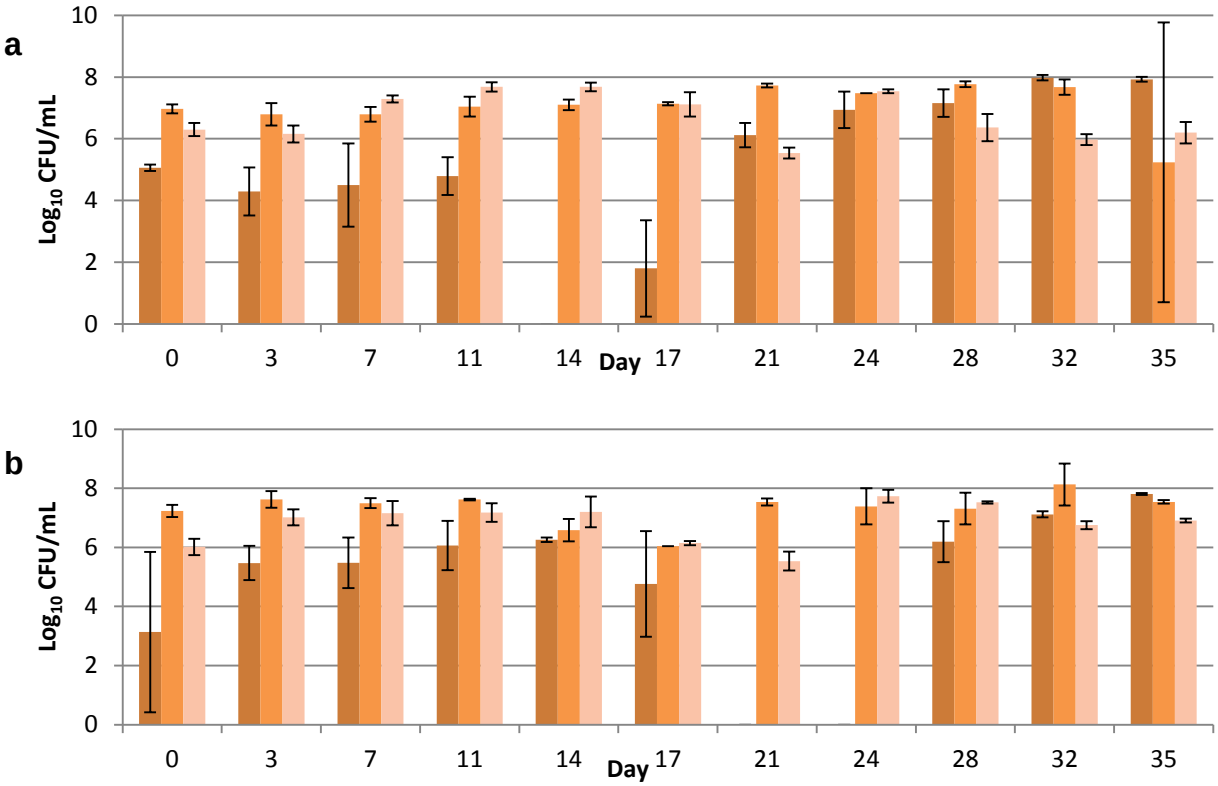


Figure A.8. Gram positive cocci recovered from the (a) control and the (b) phage-treated transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

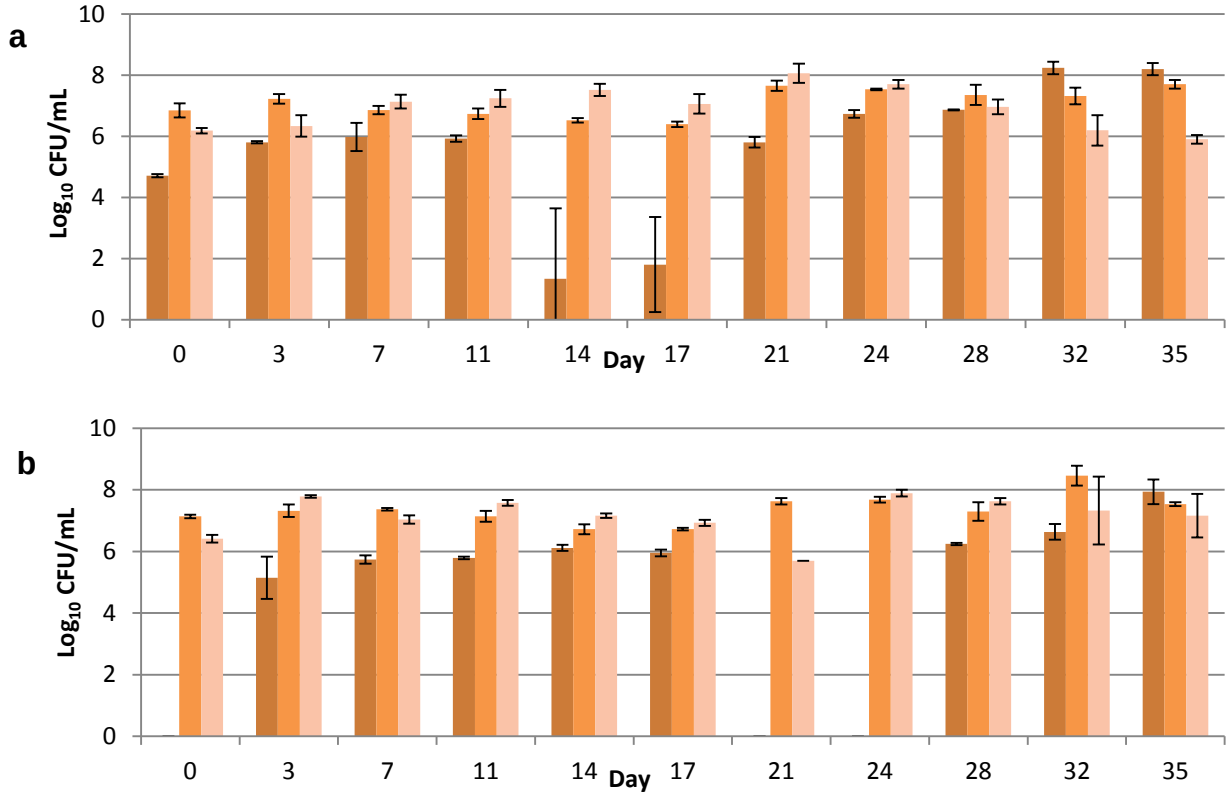


Figure A.9. Gram positive cocci recovered from the (a) control and the (b) phage-treated descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

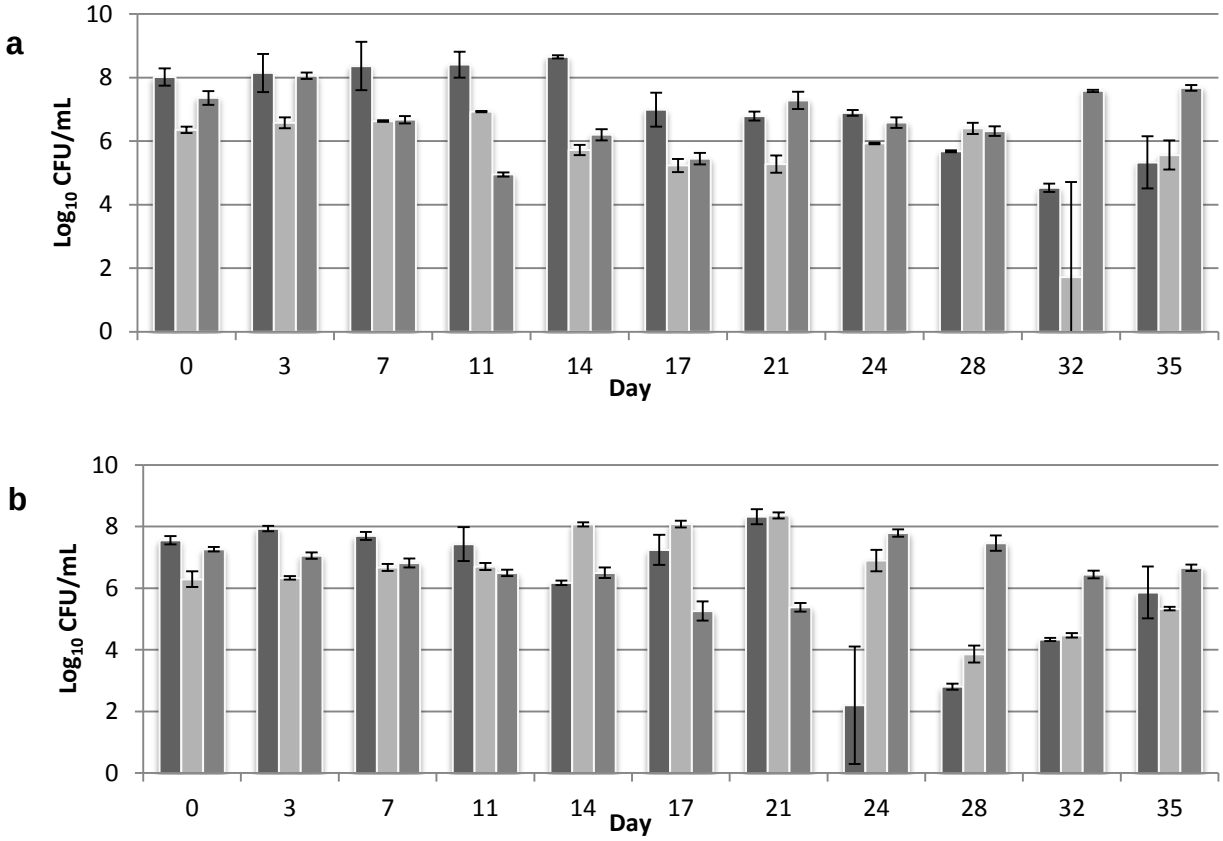


Figure A.10. Total *Clostridium spp*. recovered from the (a) control and the (b) phage-treated ascending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

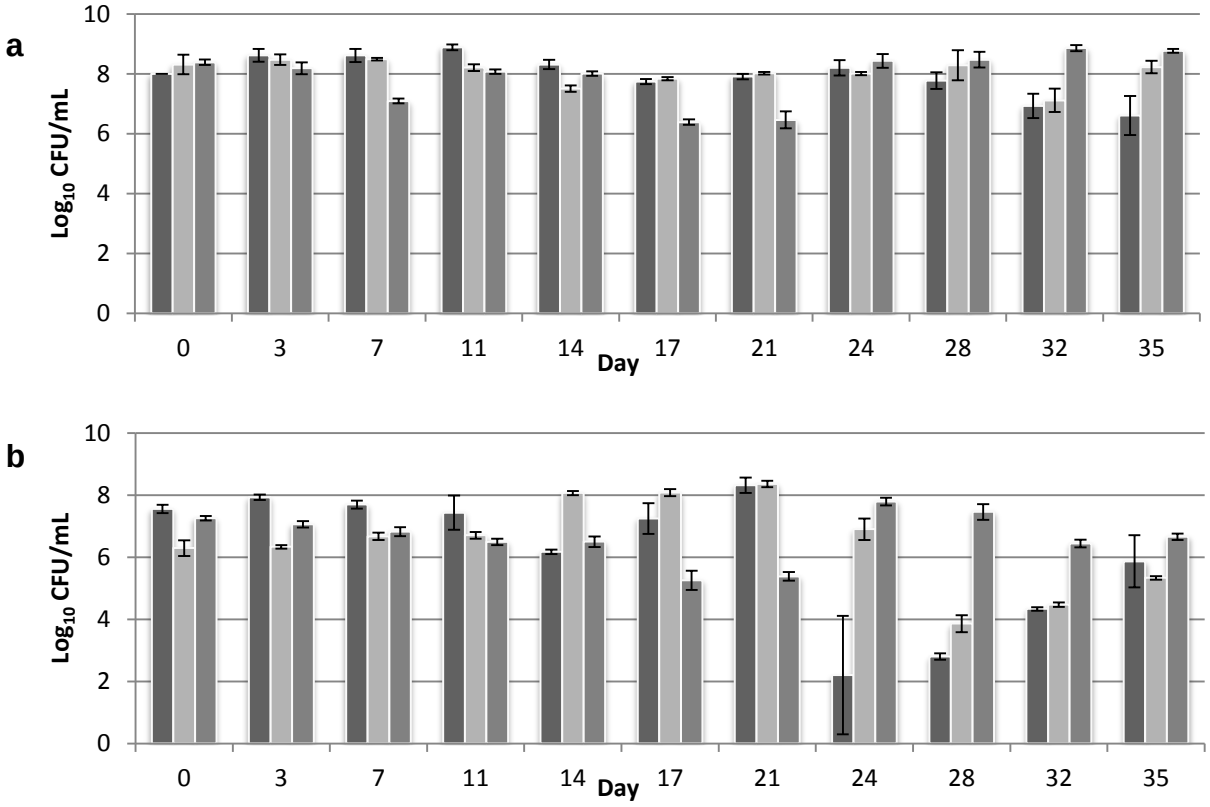


Figure A.11. Total *Clostridium spp*. recovered from the (a) control and the (b) phage-treated transverse sections of the colon model. = replicate 1 = replicate 2 and = replicate 3

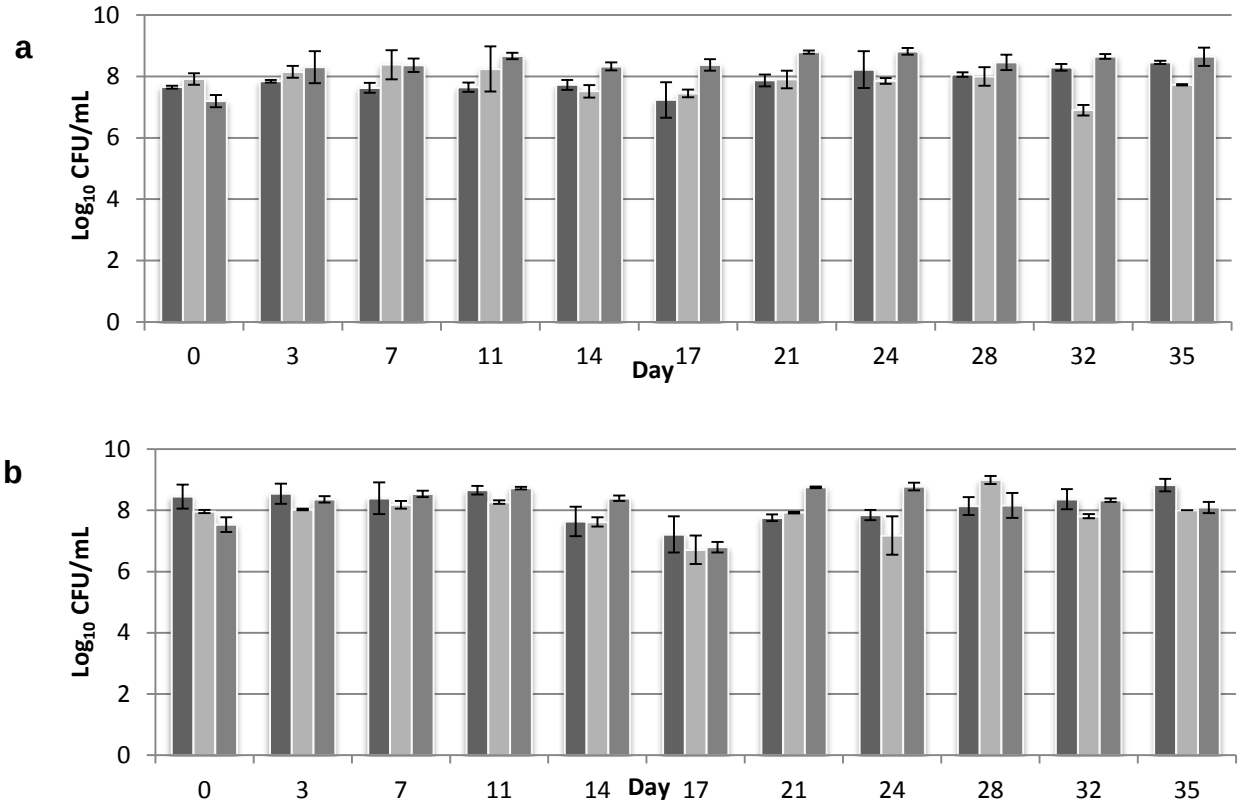


Figure A.12. Total *Clostridium spp*. recovered from the (a) control and the (b) phage-treated descending sections of the colon model. = replicate 1 = replicate 2 and = replicate 3