DEVELOPMENT OF A ROBUST CELL CULTURE MODEL TO INVESTIGATE THE THERAPEUTIC POTENTIAL OF *CLOSTRIDIUM DIFFICILE* BACTERIOPHAGES

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ABSTRACT

Development of a robust cell culture model to investigate the therapeutic potential of C*lostridium difficile* bacteriophages

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Clostridium difficile infection is usually associated with long stays in hospitals and extended antibiotic use. Current treatment of the infection is with further use of broad spectrum antibiotics. The bacterium is becoming more resistant to antibiotics, therefore alternative treatments are being sought. One of which is bacteriophage therapy.

There have been numerous *in vitro* and animal based studies designed to observe the efficiency of phages against *C. difficile*, however none of these have considered the potential interactions both may have with the gastrointestinal epithelial cell layer.

This study aimed to develop and optimise a cell culture model which could be used to investigate the interactions and dynamics of bacteriophages and *C. difficile* within a natural setting designed to mimic the gastrointestinal tract.

Results showed that *C. difficile* levels dropped further after phage treatment in the presence of cells compared to with bacteriophages alone. Phages and *C. difficile* were enumerated with varying levels of mucus and with different treatment regimens. Interestingly, the interactions changed in the presence of mucus. Bacteriophages were generally more active against planktonic *C. difficile* rather than against bacteria that had attached to a mucus layer. Moreover phages were able to attach to the epithelial cells in the presence of mucus. The mucin levels of HT29-MTX-E12 were measured by indirect ELISA. Despite extensive optimisation of the indirect ELISA the mucin levels were unable to be quantified.

The results of this study not only demonstrates the successful use of the cell culture model but also highlights the potential of bacteriophage therapy against *C. difficile* infection.

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I would like to dedicate this thesis to the memory of my late father Rama and my former boss Paul van Empel.

Publications

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Abbreviations

BHI……Brain heart infusion

CDC……...Centre for disease control and prevention

CDI……*Clostridium difficile* infection

CFU……...Colony forming units

dH_2O……..Distilled water

DMEM…...Dulbecco's modified Eagle's medium

DMSO……Dimethyl sulfoxide

DNA……...Deoxyribonucleic Acid

dPBS……...Dulbecco's phosphate buffered saline

dsDNA……Double stranded DNA

dsRNA……Double stranded RNA

EDTA……..Ethelynediaminetetraacetic acid

ELISA…….Enzyme linked immunosorbent assay

FA broth…..Fastidious anaerobic broth

FBS……..Foetal bovine serum

FDA…….Food and drug administration

FMT…… Faecal microbiota transplantation

GI tract…….Gastrointestinal tract

MOI……..Multiplicity of infection

MRSA……..Methicillin resistant *Staphylococcus aureus*

OD……...Optical density

PFU……..Plaque forming units

PTS……..Proline threonine serine

RNA……Ribonucleic acid

RT qPCR.....Real time quantitative polymerase chain reaction

SEM……Standard error of mean

ssDNA…….Single stranded DNA

ssRNA…….Single stranded RNA

TcdA……...Toxin A

TcdB……Toxin B

TcdC……Negative regulator for toxin production

TcdE……Holin

TcdR……Positive regulator for toxin production

TEM……Transmission electron microscopy

WHO……World Health Organisation

Chapter 1. INTRODUCTION

C. difficile infection (CDI) is typically a hospital acquired infection which results from use of broad spectrum antibiotics. Current treatment of the infection means further antibiotic use which *C. difficile* is rapidly developing resistance to. Additionally there are more cases of recurrent CDI recorded, therefore alternative treatments are being sought with bacteriophages being one of the potential replacement treatments. In order for the treatment to be developed the efficiency of bacteriophages against *C. difficile* needs to be assessed within as natural a setting as possible. Therefore a model using human colon epithelial cells has been developed.

To date, there have been numerous studies where cell lines have been used to assess the interactions of human and animal cells with bacteria. There have also been many studies where cell lines have been used to determine the interactions between bacteriophages and cells. However there is yet to be a successful model that can simultaneously assess all three together. The information that can be gathered from the development of such a model featuring all three entities would be particularly valuable for studying the potential for bacteriophages to be used to treat CDI.

For this PhD project, it was proposed that a suitable cell culture model mimicking the conditions of the gastrointestinal environment (including the anaerobic environment and presence of mucus) could be devised to assess the therapeutic potential of bacteriophages against *C. difficile* infection.

1.1. Introduction to *C. difficile*

Clostridium difficile is derived from the Greek word “Kloster” meaning spindle. It was first isolated from stools of healthy new born babies in 1935 and named *Bacillus difficilis* due to its difficulty in being isolated and its requirement of strict anaerobic conditions for successful growth (Goudarzi et al. 2014). Its name was changed to *Clostridium difficile* (*C. difficile*) in the 1970s (Kuipers & Surawicz 2008).

C. difficile is a bacillus shaped bacteria that grows in strictly anaerobic conditions (Zucca et al. 2013). The bacteria have the ability to form dormant spores when exposed to conditions that cause stress (Viswanathan et al. 2010). Examples of conditions that cause stress are the presence of oxygen and exposure to non-optimal temperature. The spores formed by *C. difficile* are resistant to many extreme conditions such as heat, drying and chemical agents such as disinfectants and the acidity of the stomach (Kuijper et al. 2006).

C. difficile is part of the normal intestinal microbiota and has shown to have been cultured from stools of approximately 80 % of healthy new born babies and infants and 1-3 % healthy adults (Kuijper et al. 2006). The percentage of adults with *C. difficile* in their stools significantly rises to 20-50 % amongst patients who have had surgery or experienced long stays in hospital (Kuijper et al. 2006).

1.1.1. *C. difficile* infection

C. difficile is responsible for causing *C. difficile* infection (CDI) - a severe toxin mediated intestinal disease. The infection is the most common problem in hospitals and nursing homes, particularly where the patients frequently receive antibiotic treatment (Rupnik et al. 2009). The global health threat of multidrug resistance has continued to rise and it has been predicted that it will cause as many as 10 million deaths worldwide by 2050 (O'Neill 2014). The individuals most at risk to CDI are the elderly (over 65 years) and the very young - those who are under one year of age (Kuijper et al. 2006). The risk factor is increased by the age of patients and other underlying factors such as prolonged duration of hospital stay, antimicrobial therapy and ongoing conditions such as inflammatory bowel diseases and immunodeficiency disorders (Goudarzi et al. 2014).

CDI can be classed as endogenous or exogenous (Denève et al. 2009). Endogenous infections originate from strains carried by the patient and exogenous infections occur by exposure to the environment where infection can be spread by contaminated healthcare workers from hospital sources or from contaminated environments (Goudarzi et al. 2014). It is spread via the faecal-oral route and can be acquired by the ingestion of *C. difficile* spores which remain dormant in the environment. The small intestine has an anaerobic environment which is suitable for the spores to germinate to the active

vegetative state and subsequent colonisation takes place in the large intestine. The patient may acquire the *C. difficile* bacteria during hospitalisation but may not be susceptible to the infection until they are exposed to various antimicrobial agents. In 1974 a clear link was established between pseudomembranous colitis (one of the most serious symptoms of CDI) and the use of the antibiotic clindamycin (Tedesco et al. 1976). The use of various antimicrobial agents was found to disrupt the commensal bacteria located in the colon, allowing for the proliferation of *C. difficile* and subsequent toxin production causing the symptoms of the infection to manifest (Goudarzi et al. 2014)

Figure 1.1 shows how CDI can be spread within a hospital setting and illustrates that *C. difficile* colonisation does not always equate to symptomatic CDI (Rupnik et al. 2009).

The symptoms of CDI can range from asymptomatic to more severe manifestations. Asymptomatic individuals carry *C. difficile* and shed it in their stools (without diarrhoea) and facilitate the spread of spores within the environment (figure 1.1), albeit at lower concentrations than patients with visible symptoms (Rupnik et al. 2009). Studies have shown that the frequency of the natural presence of *C. difficile* increases from 3 % in healthy adults to 20-30 % in hospitalised patients and rises even further to 50 % in patients who have had long hospital stays (Badger et al. 2012). *C. difficile* associated disease has been linked with hospital stays that have been extended by 4 to 14 days (McFarland et al. 2007).

The more severe manifestations of the infection include abdominal cramps, varying levels of diarrhoea, fever, dehydration and an increased white blood cell count. At its most severe, CDI can be life threatening as inflammation and dilation of the colon (colitis) can lead to perforation (Goudarzi et al. 2014).

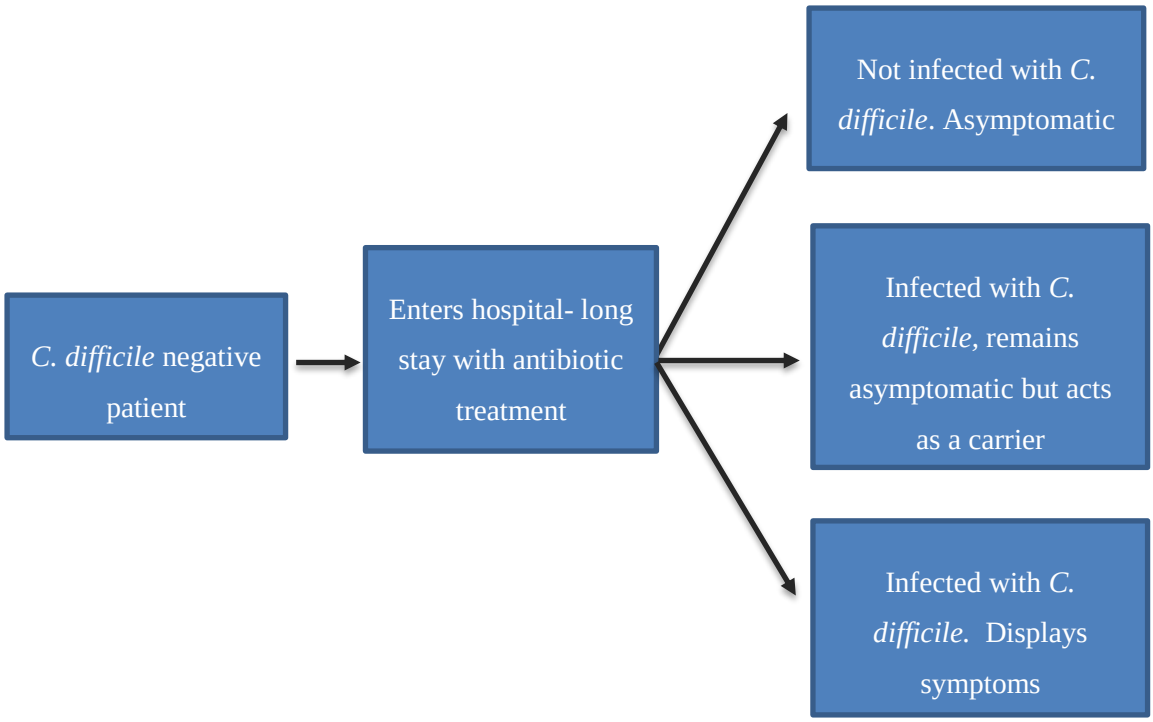

Figure 1.1: Schematic of how *C. difficile* infection spreads within a hospital environment. Adapted from Rupnik et al 2009.

1.1.2. CDI prevalence

C. difficile has now been recognised as the leading cause of hospital associated infectious diarrhoea amongst adults within developed countries. It was once seen as a "mildly troublesome" antibiotic associated diarrhoea in elderly patients who had been hospitalised for longer periods of time. However, in more recent times it has progressively been seen as a major public health problem (Bouza 2012). The severity of the problem can be seen in the paper by Poxton et al who in 2006 reported that in some hospitals, the incidence of CDI exceeded that of methicillin-resistant *Staphylococcus aureus* (MRSA) (Poxton 2006). Awareness of *C. difficile* rose, coinciding with an increased severity of the disease and an increase in the number of patients being infected, particularly those who would not have previously considered to be at risk. Hospitalisations due to *C. difficile* within the US increased from 25,200 to 110,600 between 1998 and 2009 but plateaued between 2008 and 2009 (Burke & Lamont 2014). The UK also showed a visible rise in CDI with 1100 cases being

diagnosed in 1990 and a peak of 55,635 being reached in 2006 (Cartman et al. 2010). There was a 28 % increase in deaths associated with CDI observed between 2006 and 2007 (Cartman et al. 2010). In 2015, there were 19,269 reported cases of CDI with 488 fatalities recorded (Nale et al. 2018). The increase in recorded cases may be primarily due to the reporting of all incidences being made compulsory in the UK from 2004. The increased life expectancy of the general population would also put more people at risk (Burke & Lamont 2014). Based on hospitalised patients in the USA, it is estimated that CDI occurs in approximately 700,000 patients a year with approximately 30,000 deaths but these figures do not account for long term care, nursing homes or outpatient cases (Lucado et al. 2012). Lower hygiene standards combined with overcrowding in hospitals, increased antibiotic resistance and emergence of hypervirulent strains have all been reported to have played significant roles in the increased incidence rate (Cartman et al. 2010). An increase in community acquired *C. difficile* associated disease in low risk populations was also reported (Burke & Lamont 2014).

The hypervirulent strains of *C. difficile* ribotype 027 have been identified as key contributors to the rapid increase in new cases of CDI in North America (Bouza 2012). This epidemic ribotype was particularly associated with more complications including perforation of the colon, higher death rate and a poor response to the antibiotic treatment of choice – metronidazole (Pépin et al. 2004). *C. difficile* 027 was studied extensively and it was found that the emergence of the strain also correlated with resistance to antibiotic fluoroquinolones (Le Monnier et al. 2014). However, it has been confirmed that there are many other strains responsible for the increased rates of CDI e.g. ribotype 078 is known to produce disease of a similar severity to 027 and is prevalent in Europe(Mullish & Williams 2018).

Epidemiological data on incidence and prevalence rates are heavily reliant on the accuracy of the initial diagnosis of CDI. It has been revealed that a significant percentage of CDI cases are missed due to clinicians failing to request tests for *C. difficile* toxins when a patient is suffering from unexplained bouts of diarrhoea (Bouza 2012). Furthermore, some laboratories use diagnostic tests with very low sensitivity or those which are not applied appropriately (Bouza 2012; Mullish & Williams 2018) e.g stand-alone enzyme immunoassays for toxin detection (Barbut et al. 2003). A

combination of the failure to request for appropriate tests along with the choice of diagnosis methods used may account for the majority of the under-reporting of CDI in Europe. There have been improvements in testing methods and the use of nucleic acid amplification tests which detect the genes that encode *C. difficile* toxins is the preferential diagnosis method(Carroll 2011; Mullish & Williams 2018). The improvement in testing has addressed deficiencies in the diagnosis of CDI and has led to better epidemiological reporting.

1.1.3. CDI virulence and pathogenesis

Although much has been reported on *C. difficile* pathogenesis with respect to toxin production, little is known about the interactions between *C. difficile* and the mammalian gut. The first and most important steps of the pathogenic process are the implantation, growth and subsequent colonisation of *C. difficile* in the gut lumen (Le Monnier et al. 2014; Denève et al. 2009). The contamination by *C. difficile* spores results in subsequent adhesion and colonisation of vegetative *C. difficile* in the small intestine. The spores germinate to the vegetative form of *C. difficile* due to the favourable conditions within the gut lumen namely the anaerobic environment, temperature (~37 °C) and disturbance of the intestinal microbiota and barrier function caused by the use of antibiotics (Le Monnier et al. 2014). There are many virulence factors that may contribute key roles in adherence and intestinal colonisation. The surface proteins play a key role in facilitating colonisation. Adhesins have been identified within many *C. difficile* proteins including the S layer proteins located within the 66 kDa cell wall protein Cwp66 and the flagella components FliC (flagellin) and FliD (flagellar cap protein) (Denève et al. 2009). In addition to the adhesins produced, all strains of *C. difficile* produce hydrolytic enzymes as well as the cysteine protease Cwp84 which is thought to be involved in spreading the infection due to its ability to degrade the mucosal extracellular matrix proteins (Le Monnier et al. 2014).

The second phase of pathogenesis is characterised by the production and release of toxins A and B – the major, high molecular weight virulence factors of *C. difficile* belonging to a group of large clostridial toxins. Toxins A and B are the principal toxins responsible for the symptoms experienced by CDI patients (Rupnik et al. 2009). The toxins are also the primary markers for diagnosis of CDI and can be detected in stools of

patients by antibody based and cytotoxicity assays (Voth & Ballard 2005). Toxin A is an enterotoxin which is encoded by the gene *tcdA* whilst *tcdB* encodes the cytotoxin Toxin B. These two toxins have been found to be cytotoxic i.e they are toxic to living cells (Gerding et al. 2014). Both genes are located on a pathogenicity locus (PaLoc) containing three additional accessory genes *tcdC*, *tcdR* and *tcdE* (see figure 1.2). TcdC is a negative regulator of toxin synthesis (Dupuy et al. 2008), whilst TcdR is a transcription initiation factor activator critical for expression of toxin genes (Carter et al. 2012); together they modulate the toxin gene expression (Viswanathan et al. 2010). The gene *tcdE* encodes a putative holin (Denève et al. 2009) which is involved in the release of progeny phage from infected bacterial cells; therefore, although not confirmed, TcdE may play a key role in toxin release (Carter et al. 2012).

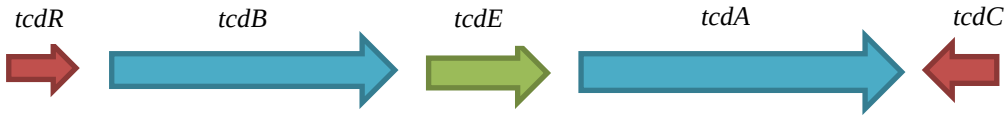

Figure 1.2: Structure of the pathogenicity locus (PaLoc) of *C. difficile*.

The toxin genes are in blue, regulatory genes are in red and tcdE is in green. Diagram adapted from Carter et al 2012.

A third, binary toxin known as CDT is produced by approximately 20 % of *C. difficile* strains some of which are hypervirulent (Viswanathan et al. 2010; Le Monnier et al. 2014) Clinical studies have shown that CDT- producing strains could be associated with more severe clinical presentations of CDI and high mortality (Barbut et al. 2007). Additionally it has been observed that the hypervirulent strains, namely from ribotype 027, produce higher levels of TcdA and TcdB along with CDT (Denève et al. 2009).

The binary toxin has both a binding component and an enzymatic component encoded by *cdtA* and *cdtB*. CDT also contributes to cytoskeleton disorganisation (Denève et al. 2009). Collectively they play a role in regulation of toxin production and release of toxin from the cell (Voth & Ballard 2005).

1.1.4. Current treatment of CDI

As previously mentioned, CDI results from broad-spectrum antibiotics depleting the normal gut flora creating a suitable environment for *C. difficile* to proliferate in the colon(Ivarsson et al. 2015). Resistance to widely used antibiotics such as clindamycin and moxifloxacin is one of the root causes of CDI(Ivarsson et al. 2015; Tickler et al. 2014).

Metronidazole and vancomycin have been the treatment for CDI for the last 30 years. Between 1977 and 1980, most doctors prescribed 125 – 500 mg oral vancomycin for 7-14 days (Fekety et al. 1989). In general, 90 % of patients with CDI were successfully treated (Aslam et al. 2005). In 1982, metronidazole was effectively used to treat 13 *C. difficile* infected patients over 7-10 days. The efficacy of metronidazole and vancomycin were compared in a trial and shown to be equally effective (Teasley et al. 1983).

Metronidazole is a nitroimidazole prodrug (a compound that remains inactive until administered) which is taken up and reduced by bacterial cells. It binds to DNA causing it to lose its helical structure, inhibiting nucleic acid synthesis (McFarland 2011). Metronidazole is typically the first choice for treatment of milder bouts of CDI as it is 10 times cheaper than vancomycin. Vancomycin is a glycopeptide which works by inhibiting the synthesis of peptidoglycans necessary for keeping the cell wall in Gram positive bacteria intact. The antibiotic has been Food and Drug Administration (FDA) approved for treatment of CDI but it is now only used to treat severe infection or given to patients who remain infected after using metronidazole. The reduced usage of vancomycin is due to the emergence of vancomycin-resistant bacteria(Ivarsson et al. 2015). Vancomycin has minimal side effects for the patient as it is not absorbed. In contrast, metronidazole is absorbed and therefore is associated with side effects such as headaches, nausea and vomiting. Additionally, minimal quantities of the drug reaches the site of infection as the majority of it is absorbed by the time it reaches the colon (Louie et al. 2011).

The main disadvantage of metronidazole and vancomycin is that they are both broad-spectrum antibiotics thus causing significant damage to the endogenous gut microbiota.

The reduced protection of the microbiota barrier results in patients being exposed to a higher risk of re-infection(Ivarsson et al. 2015). Moreover, emerging strains of *C. difficile* are less susceptible to both metronidazole and vancomycin. Treatment failure rates with metronidazole have risen from < 16 % before 2003 to > 35 % since 2004 and vancomycin treatment failures are rising at a similar rate (McFarland 2011). There has been a reduction in the success rate of antimicrobial therapy with patients failing to respond to treatment and others relapsing after treatment has been stopped (Aslam et al. 2005).

In 2011, fidaxomicin was given approval by the FDA for treatment of CDI. Fidaxomicin is a macrolide that works by inhibiting RNA polymerase therefore inhibiting growth (Wilcox 2016). Unlike metronidazole and vancomycin, fidaxomicin has a narrow spectrum of activity against *C. difficile* and does not affect many species of the natural microbiota of the gut including *Bifidobacterium* and *Bacteroides*(Ivarsson et al. 2015). Reducing the damage to the microbiota is fundamental in protecting the patients from disruptive, dangerous and also costly recurrences of infection. Phase III clinical trial data was used to compare the recurrence of infection between fidaxomicin and vancomycin. Results showed that there was only 13.3 % recurrence of infection after treatment with fidaxomicin compared to 24 % with vancomycin (Louie et al. 2011). In addition, fidaxomicin is poorly absorbed by the intestine and much like vancomycin has minimal side effects.

Despite the advantageous results of fidaxomicin compared with metronidazole and vancomycin, the use of fidaxomicin has remained limited because of the high cost of the treatment. A 10 day course of fidaxomicin in tablet form costs $ 2800 in comparison to vancomycin treatment at $ 680 and the significantly lower cost of metronidazole at $ 22 (Ivarsson et al. 2015). Frequent use of fidaxomycin is not cost effective (Ivarsson et al. 2015) and for it to be deemed as such the drug would need to be priced at less than half of its current price(Ivarsson et al. 2015).

In order to eliminate *C. difficile* from the colon, administration of antibiotics should be ceased and the colon microbiota allowed to recover spontaneously; however, this process could take a minimum of 12 weeks during which time patients may suffer from an infection recurrence (Leffler and Lamont 2015). Many treatment avenues for CDI

have been investigated including several antibiotics, absorbents, anti-diarrhoeal medication, competitive probiotic strains, anti-inflammatory medications, immune system enhancers and the focus of this thesis, bacteriophages. These various treatments have been designed to target different stages within the pathogenic pathway of CDI, particularly the key virulence factors of *C. difficile* (McFarland 2011). It has been accepted that removing *C. difficile* completely from the environment is not a feasible option due to its spore forming ability and the fact that it is commonly found in both wild and domestic animals (McFarland 2011). Most antibiotics are designed to target the growth of the vegetative *C. difficile* cells.

C. difficile is becoming significantly more resistant to several antibiotics and so it is important that new alternative, non-antibiotic approaches are identified in order to avoid future cross-resistance (Zucca et al. 2013). In addition, new antibiotics will need a narrow spectrum of activity in order to avoid the disruption of the patient's intestinal microbiota, which can otherwise lead to CDI recurrence. They should be active against multiple targets to slow down the rapid emergence of resistance and should ideally be as effective but less costly than fidaxomicin (Zucca et al. 2013).

Phase III clinical trials for the treatment of CDI have been carried on two antibiotics; the first, named cadazolid inhibits protein synthesis and weakly inhibits DNA synthesis and the second known as surotomycin induces cytoplasmic membrane depolorisation(Locher et al. 2014; Mascio et al. 2012; Knight-Connoni et al. 2016). Although they possess very different mechanisms of action they have both shown similarities to fidaxomicin in that they both display strong bactericidal activity against *C. difficile*, are minimally absorbed and therefore can successfully reach and maintain therapeutic concentrations at the site of action needed in the colon and they are inactive against key components of the gut flora (Chilton et al. 2014). Additionally, surotomycin has shown statistically lower recurrence rates than with vancomycin (Knight-Connoni et al. 2016). Cadazolid has been deemed safe and well tolerated; however, results from clinical trials have shown that it remains inferior to vancomycin, therefore further commercial development of the drug for treatment of CDI is unlikely (Gerding et al. 2019).

There are a number of treatments which do not involve the use of antibiotics that are being studied in order to determine the success levels in treating CDI. Recently a new treatment of CDI known as faecal microbiota transplantation (FMT) has been developed. FMT restores the diversity of the gut microbiota and reverses the imbalance of microbes that often leads to the proliferation of *C. difficile* and the subsequent onset of CDI. A blended and filtered faecal suspension is prepared from a fresh donor stool and is administered to the patient via either the upper or lower gastrointestinal (GI) tract (McCune et al. 2014). The procedure rapidly restores the diversity of the intestinal microbiota, preventing the proliferation of *C. difficile* and has shown remarkably high success rates for the treatment of recurrent CDI (over 90 %). There has also been growing interest in the use of FMT for the treatment of primary CDI in its most severe form. To date, very few studies have documented the success rate of FMT and much more work is needed to determine the efficacy and safety of the treatment for primary CDI (Leffler & Lamont 2015). Significant hurdles still exist affecting its widespread use, namely safe and standardised large scale production and formulation are required. Additionally, continuous research is being carried out in order to define the key bacteria within a healthy stool which can be used as a treatment that may be administered orally (McCune et al. 2014).

Another protective measure against CDI currently being examined is the potential for vaccination. The vaccine would act against the toxins of *C. difficile* and should be a relatively inexpensive preventative measure. Initial phase I studies have shown strong antitoxin responses in healthy volunteers immunised with toxoids of TcdA and TcdB (Kotloff et al. 2001). Phase I trials were completed to test the immunogenicity, safety and efficacy of the vaccination. The toxoid vaccine proved to be 'safe and tolerable' compared to the placebo. Additionally, antibodies against both toxins were detected. This has now moved onto phase III trials, one of which involves 15,000 study participants and is being evaluated over a period of three years (Leffler and Lamont 2015).

Although there are many promising treatments for CDI, the focus for this study will be the efficiency of phage therapy against *C. difficile* when in the presence of colon epithelial cells.

1.2. Bacteriophages

Bacteriophages, or phages are viruses that only infect bacteria. They are 50 times smaller than bacteria and are the most abundant biological entity (approximately 10^{31}) on Earth (O'Flaherty et al. 2009). Bacteriophages are ubiquitous but mainly found in soil, water and in several food products (Ly-Chatain 2014). Data has shown that phages play crucial roles in regulating bacterial populations and are responsible for the death of approximately 20-40 % of all marine surface bacteria every 24 hrs (Wittebole et al. 2014).

1.2.1. History of bacteriophage research

In 1910 French-Canadian Felix d'Herelle observed round zones of clearance (areas of no bacterial growth) in cultures spread on agar plates whilst carrying out studies on locust control (Golkar et al. 2014). He concluded that the areas of lysis were caused by viral parasites. Four years later, Frederick Twort, a British bacteriologist, proposed that the zones of clearance could be due to viruses, however this was a theory that Twort was unable to pursue(Twort 1915; Twort 1925). The name bacteriophage ("bacterium eater") was coined in 1916 by Felix d'Herelle who demonstrated their safety by ingesting them himself (D'Herelle 1931). D'Herelle also first tested phages in human patients in 1917. Patients included a 12 year old boy suffering with severe dysentery (D'Herelle 1931). A single treatment was enough to ease the patient's symptoms and he went on to make a full recovery (Golkar et al. 2014). Although phage therapy showed great promise, early work produced some conflicting findings. Failures of treatment with bacteriophages that occurred in later years were attributed to many factors including a lack of understanding of phage biology, poor experimental techniques, poor quality of phage preparations and a lack of understanding of the underlying causes of the ailment being treated (O'Flaherty et al. 2009). The commercialisation of antibiotics during the 1940's led to a decline in the progression of phage therapy, particularly in Western countries (O'Flaherty et al. 2009). Bacteriophage therapy will be discussed in more detail in section 1.3.

1.2.2. Structure of bacteriophages

Bacteriophages are classified based on their morphology and nucleic acid. Most known phages have double stranded DNA (dsDNA), whilst some have RNA (either single stranded or double stranded) or single stranded DNA (ssDNA). Phages can be grouped as filamentous or isosahedral without tails. 96 % of known bacteriophages are in the order *Caudavirales*, have tails and may possess a lipid-containing envelope or contain lipids in the particle shell (Ackermann 2009). They have dsDNA, a capsid and tail and are approximately 24-400 nm in length. The *Caudavirales* can be further divided into three families - *Siphoviridae* with long non-contractile tails (61 %), *Myoviridae* with contractile tails consisting of a sheath and a central tube (25 % of tailed bacteriophages), and *Podoviridae* with short tails (14 %). The structure of the phages in all three families is similar. The capsid is attached to the tail through a connector that plays many fundamental roles during the infection cycle including packaging dsDNA into the capsid and participating in the release of phage DNA into the host bacterium. The phage DNA is delivered to the host bacterial cell through the tail which has fibres at its end so it is able to bind to its specific bacterial receptor (Ackermann 2009). The structure of a typical bacteriophage is shown in figure 1.3.

The remaining bacteriophages are pleomorphic and filamentous bacteriophages. These are further classified into ten small families and make up approximately 4 % of the bacteriophage population (Ackermann 2009).

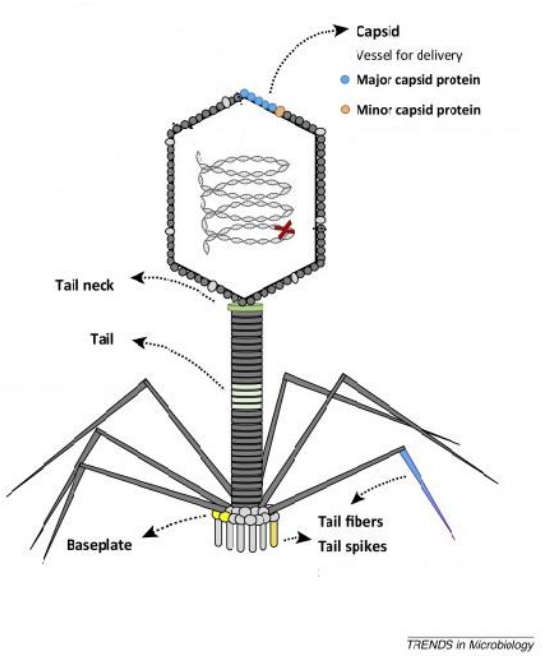

Figure 1.3: Structure of a bacteriophage. (Adapted from Nobrega et al. 2015)

1.2.3. Bacteriophage life cycle

Bacteriophages are characterised into two main groups according to type of infection- lytic or lysogenic. The phage first binds to specific receptors on the host bacterial cell surface and injects its DNA into the bacterium. Following binding, bacteriophages either undergo the lytic cycle or the lysogenic cycle. Figure 1.4 shows a diagram of the phage life cycles (Gill & Hyman 2010). During the lytic cycle, the release of the phage DNA causes the protein machinery within the host bacterium to switch enabling new phages to be produced. The process is mediated by two key components, holin which produces pores within the cytoplasmic membrane and endolysin resulting in the production of hydrolytic enzymes which in turn cleaves the bacterial cell wall. This combination of events depletes the resources of the host cell making it weak, causing the cell to lyse (Schmelcher & Loessner 2014). New phages are then released into the extracellular space.

The lysogenic cycle, on the other hand involves the integration of the phage DNA into the host cell genome - known as a prophage (Davies et al. 2016). The prophage is replicated along with the host bacterial genome resulting in the new bacteria inheriting

the viral DNA. This process can continue for many generations with no consequences for the bacterial host. The lysogenic cycle is thought to be a survival tactic during periods or stress and resource limitation (Stewart & Levin 1984). The cycle can eventually return back to the lytic cycle where phages are released; however, this process is spontaneous (Davies et al. 2016). Bacteriophages that follow the lysogenic cycle are not suitable for phage therapy due to their ability to remain in the cycle for several generations without affecting the bacterial host (Orlova 2012). In some cases, the prophage may encode genes for virulence factors which can be horizontally transferred from one bacterium to another by transduction (O'Flaherty et al. 2009) further benefitting the bacterial host. Therefore, phage therapy mainly relies on the use of lytic bacteriophages.

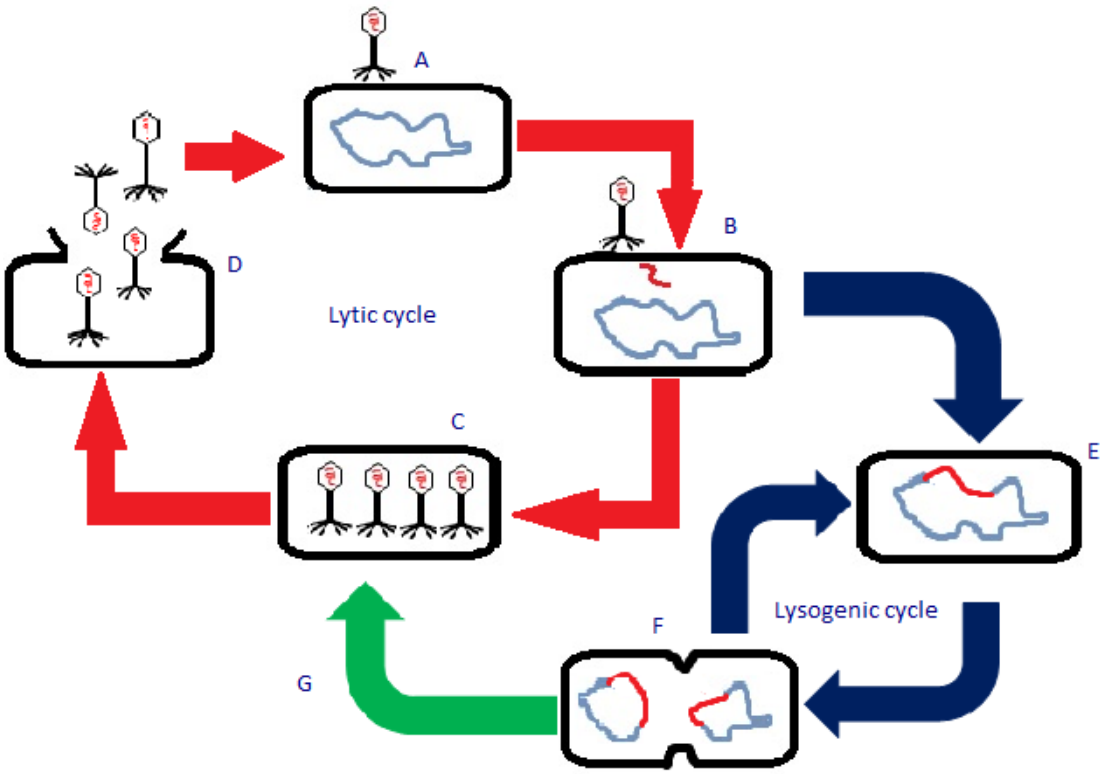

Figure 1.4: Summary of bacteriophage life cycle.

The bacteriophage attaches to the bacterium (A), and injects its DNA (B). The cycle splits into the lytic cycle, where phage DNA is replicated and new bacteriophages are assembled and released (C and D), or the lysogenic cycle where the phage DNA integrates into the bacterial chromosome and is passed on when the cell divides (E and F). The lysogenic cycle can spontaneously return to the lytic cycle at any time (G).

There is a stage between the lytic and lysogenic cycle known as pseudolysogeny. It is possible that pseudolysogeny was observed when bacteriophages were first discovered by Twort and D'Herelle, (Delbruck 1946)The phenomenon has been defined as a stage of stalled development displayed by phages (Los & Wegrzyn 2012), but on the whole, pseudolysogeny still remains poorly described and there is yet to be a commonly accepted definition (Los & Wegrzyn 2012; Mirzaei & Maurice 2017). The phage does not partake in either the lytic or the lysogenic cycles as it does not immediately initiate a lytic cycle nor does it integrate its genome into the host. Instead, it remains as a circular DNA structure within the cytoplasm which replicates in synchrony with host replication (Los & Wegrzyn 2012). This phenomenon is thought to occur during conditions that may be unsuitable for efficient phage production e.g. when the host bacterium is nutrient deprived and undergoing starvation. Pseudolysogeny can be terminated with the initiation of lysogenisation or lytic growth when the conditions for the bacterium improves (Los & Wegrzyn 2012). The role of pseudolysogeny in phage-host interactions has been underestimated and has only been explored more recently(Los & Wegrzyn 2012; Cenens et al. 2013)..

Most bacteriophages tend to be infectious only to the bacteria that carry its complementary receptor which in turn determines the lytic phage host range. The host specificity varies amongst phages, many of which are strain specific whereas other phages have shown that they are capable of infection across a broader range of bacterial species (Koskella & Meaden 2013).

1.3. Bacteriophage Therapy

As mentioned in section 1.1.4, one of the potential alternative treatments of CDI is bacteriophage therapy- the practice of using phages to treat bacterial infections. Phage therapy has been around for almost a century. Frederick Twort observed "eaten edges of colonies of *Staphylococcus."* He managed to filter cultures grown from these colonies and spot them on lawns of different *Staphylococcus* strains where he observed zones of clear lysis (Twort 1915). A couple of years later Felix d'Herelle conceived the idea of using phages therapeutically and demonstrated their use for treating pathogens

such as *Shigella dysentriae* in 1919(D'Herelle 1931). Originally, phage therapy was the subject of much controversy due to poor documentation and a variable success rate. Many mistakes were made during the early trials due to poor understanding of bacteriophages and basic purification techniques being used along with poor storage protocols causing low phage titres and contamination from bacteria. Further logistical and technical obstacles in the development of phage therapy e.g. the delivery of phages to the site of infection led to research being predominantly abandoned for the more favourable research field of antibiotic development (Lin et al. 2017). However, research continued in parts of Eastern Europe where clinical phage therapy is now used extensively to treat antibiotic resistant infections. Although the development of antibiotics and their subsequent use revolutionised healthcare and improved quality of life, antibiotic resistant genes encoding for bacterial resistance to common antibiotics continue to pose a serious threat to current treatment of the most common diseases. To cause further concern, these genes are now more abundant in the environment (Zhang et al. 2009) causing organisations such as the Centre for Disease Control (CDC) and World Health Organisation (WHO) to declare antibiotic resistance a global threat. The CDC have estimated antibiotic resistant infections cause two million illnesses and at least 23,000 deaths a year in USA alone (Centers for Disease Control and Prevention (CDC) 2013; Laxminarayan et al. 2013). The UK government 2016 Review on Antimicrobial Resistance stated that an estimated 700,000 people globally die each year from antibiotic resistant infections with the death toll predicted to increase to 10 million by 2050 (UK Government review 2016)

Phage therapy is widely used in Eastern European countries such as Russia, Poland and Georgia where phage preparations are readily available as over the counter products at pharmacies (Zucca et al. 2013). Bacteriophages have been used therapeutically in Poland and Georgia against infections caused by a wide variety of organisms such as *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, *Enterobacter*, *Proteus*, *Serratia*, *Acinetobacter* and *Pseudomonas.* The phage treatments are given as multiphage preparations or are integrated into wound dressings and have been proven successful in 85 % of cases where antibiotic treatment previously failed (O'Flaherty et al. 2009). Bacteriophages are also being used as measures to control disease in animal populations, notably in livestock (Wall et al. 2010). The use of phage therapy in

Western countries was quickly abandoned in the 1940s as it was poorly understood and antibiotics were becoming readily available (Zucca et al. 2013). However, the more recent concerns of antibiotic resistance has resulted in phage therapy research being considered as a high priority (Zucca et al. 2013).

There are many advantages that phage therapy holds over antibiotics. A major advantage is that bacteriophages are host specific meaning they only infect a few strains of a bacterial species. In contrast, antibiotics are not target specific and can cause adverse effects to normal bacterial flora in addition to the target bacteria (Loc-Carrillo & Abedon 2011). Phage therapy would be particularly beneficial for infections such as CDI which occurs as a result of adverse effects from the normally used broad spectrum antibiotics on the gut microbiota. As well as this, the narrow host range also reduces the risk of phage associated resistance. Bacteria can develop resistance to bacteriophages by different mechanisms including; spontaneous mutations, restriction modification systems and adaptive immunity via CRISPR-Cas (Oechslin 2018). Spontaneous mutations whereby the bacterium loses its phage receptor by a mutation in a gene is the main mechanism which drives phage resistance and phage-bacterial coevolution (Oechslin 2018). However, the resistance to bacteriophages does not cause a problem for phage use or phage therapy as the rate at which bacteria develop resistance to phages is ten-fold lower than to antibiotics (Ly-Chatain 2014). Additionally, when bacterial resistance to a bacteriophage does occur, a new phage can quickly be isolated to target and destroy the new strain - a process which is much faster than the development of antibiotics (Ly-Chatain 2014). The specificity of phages can also act as a disadvantage particularly when it comes to treating infections that may be caused by more than one strain of bacterium. However, this can be counteracted by using a combination of bacteriophages known as a phage cocktail whereby several phages are prepared together resulting in them being active against a range of pathogens (Lin et al. 2017). The success of phage cocktails depends on having prior knowledge of which pathogens need to be treated for each infection. Designing a phage cocktail is thought to be more complicated than designing an antibiotic treatment regime due to the huge diversity of environmental bacteriophages. When designing the cocktail, the phage life cycle has to be taken on board as lysogenic phages are very common particularly in the gut microbiota (Minot et al. 2011). Although lysogenic bacteriophages have the potential to

complicate treatment due to their ability to horizontally transfer antibiotic resistance genes, it has been reported that such a cocktail containing only lysogenic phages was able to eliminate a skin wound infection in a mouse model. The phage cocktail was successful as one of the bacteriophages was a growth inhibitor which targeted the capsulated *A. baumanii* (the cause of the infection) resulting in the loss of the capsule which is the key virulence factor of this particular bacterium. Once this was removed, the bacterium was able to be lysed by the four remaining bacteriophages that made up the phage cocktail (Regeimbal et al. 2016).

Phage therapy takes advantage of the bacteriophage's ability to replicate, meaning only a single dose may be required compared to the multiple doses of antibiotics that are frequently used (Loc-Carrillo & Abedon 2011). They also act fast and can be used as an alternative for patients who are allergic to antibiotics. As the action of phages does not interfere with antibiotics it is feasible to use the two side by side in combination treatments (Zucca et al. 2013).

Currently there are no phage therapy products that have been approved for human use within the EU or United States as the full scope of phage therapy is yet to be fully realised. However, in the food industry there have been many phage preparations that have been approved by the FDA which are being used for the biocontrol of pathogens particularly for bacteria such as *Salmonella* spp, *E.coli* and *Campylobacter* spp found in meat, fruits, vegetables and dairy products (Lin et al. 2017).

1.4. Phage therapy models

There are several successful models for phage therapy described in the literature which clearly establish a paradigm for phage treatment of infections. These include treatments for infections caused by common bacteria such as *Pseudomonas, E.coli, Salmonella, Staphylococcus* and *Streptococcus* (McVay et al. 2007; Raya et al. 2006; Wall et al. 2010). The phage therapy models for the pathogens listed have mainly been *in vivo* based. A study carried out on pigs showed that bacteriophages could be used to reduce the amount of *Salmonella* in pigs (Wall et al. 2010). Another example of an animal

model was the use of mice to show how phage therapy could be applied to burn wounds that had been infected with *P. aeruginosa.* Phage therapy successfully reduced the mortality levels of mice that had been infected with *P. aeruginosa* (McVay et al. 2007).

Bacteriophages have also been used within the food industry as decontamination agents and as further alternatives to antibiotics (Nannapaneni & Soni 2015). The models used for the development of phage therapy in this industry have varied between *in vivo* or *in vitro* models and have been tried amongst several genera of bacteria (Brüssow 2012; Nale et al. 2018).

1.4.1. Bacteriophage models against *C. difficile*

There have been limited studies on phage therapy of CDI despite there being numerous bacteriophages that have previously been characterised (Nale et al. 2016). A possible reason could be the high proportion of lysogenic phages compared to lytic phages that have been isolated.

There are two key studies that demonstrate the action of lytic phages on *C. difficile* within a phage therapy model. The studies made use of single phages as a treatment option. Ramesh et al used a hamster model and showed that 14 out of 18 phage treated hamsters survived *C. difficile* challenge whereas those without phages died within 96 hours (Ramesh et al. 1999). Meader et al used an *in vitro* batch fermentation model which mimicked the anaerobic conditions of the human colon and showed that prophylactic phage therapy resulted in negative toxin results along with a significant decrease in the number of viable *C. difficile* cells (Meader et al. 2010). The conclusions of both studies indicated that bacteriophages were a suitable option for treatment and prevention of CDI. More recently, Nale et al progressed the idea by combining single phages together in order to form a treatment cocktail for the treatment of the infection (Nale et al. 2015). The phage cocktail was tested both *in vitro* and *in vivo* in a hamster model. In addition, Nale et al used the wax moth larvae *Galleria mellonella* as a model to show the efficiency of treatment of CDI using phages prophylactically and through remedial treatments. It was determined that both treatment regimens resulted in prolonged survival of the larvae confirming that bacteriophages were an efficacious treatment against CDI (Nale et al. 2016).

Although the use of *G. mellonella* provides a valuable alternative to other more costly and ethically problematic models, the main limitation of the model is that the organism differs significantly from humans. Despite offering a good insight into phage therapy of infections caused by *C. difficile*, the model is lacking in several elements which prevents the model from being directly correlated with the human counterpart. Similarly, the study by Ramesh et al was carried out in hamsters – an organism which displays many features that resemble the physiology and metabolism in humans (Ramesh et al. 1999). Hamsters have the added advantage of presenting similar symptoms of CDI to what would be seen in humans. However, this type of model was not able to take into account the complex nature of the human colon, particularly the mucus layer that lines the colon epithelial cells.

Likewise, the *in vitro* model developed by Meader et al successfully replicated certain conditions of the human colon e.g. the anaerobic nature of the colon (Meader et al. 2010). Meader's group also took into account the idea of the gut microbiota playing an active role in the proliferation of CDI. However it is a technically difficult experiment to run and once again, the idea of the epithelial mucosal layer was omitted from the study.

1.4.2. The use of cell lines in potential models for phage therapy

The development of an *in vitro* model using cell lines to study the dynamics between phages and bacteria could be particularly beneficial. Cell lines are straightforward and cost effective to maintain, and cell based models are reproducible and can be easily replicated which is advantageous for statistical purposes. An additional advantage for the use of cell lines over an equivalent animal model is they are not as time consuming to run (Shan et al. 2018).

Human cell lines are frequently used as preclinical models to assess responses to drugs particularly in drug screening and toxicity studies (Shan et al. 2018). The use of *in vitro* human cell lines has numerous advantages. They are a renewable resource and tend to be well controlled systems that can be easily manipulated where many phenotypes can be measured simultaneously e.g. cytotoxicity, growth rate and changes in gene expression (Niu & Wang 2015).

Cell lines have been widely used to show how bacteria are able to attach to the cell surface and proliferate (Gagnon et al. 2013; Naz et al. 2013). The adherence of bacteria to the cells is a crucial first step in the initiation of an infection and there is a large amount of literature that covers this subject. Of particular focus has been the adherence of bacteria to epithelial cells predominantly lung and intestinal cells. There are numerous examples of studies, many of which are not relevant to this project, but a couple worth mentioning include, the attempt to identify bacterial adhesins responsible for the adherence of *P. aeruginosa* to lung epithelial cells (Azghani et al. 2002) and the study of the interactions of probiotic bacteria *Lactobacillus* and *Bifidobacterium* strains with the intestinal epithelial cells (Candela et al. 2008).

Many cell line studies have used the concept of co-culture in order to create a more realistic *in vitro* structure of the intestinal cell layer structure. A co-culture is where more than one cell line is cultured together and has generally been applied to studies assessing the permeability of the intestinal cells. Once again, there are numerous examples of this but a noteworthy example is a study where the rates of diffusion and drug absorption were compared between Caco-2/HT29-MTX co-cultured cells and a single culture of Caco-2 cells (Hilgendorf et al. 2000). An additional study assessed the permeability of the intestine using the same co-culture (Beduneau et al. 2014). Caco-2 cells have the ability to form polorised monolayers in culture which are consistent to those found in the enterocytes within the small intestine (Gagnon et al. 2013). The colon epithelial cell HT29 is a colon cancer cell line, and HT29-MTX is its mucus producing derivative (Martinez-Maqueda et al. 2015). Triple co-cultures have also been used to create a model of the respiratory tract where epithelial cells, dendritic cells and macrophages were cultured together to simulate the human airway barrier (Blom et al. 2016).

Several studies have been previously carried out to determine the interactions between intestinal epithelial cells and *C. difficile.* The research has mainly been focussed on how the bacteria adhere to the intestinal epithelial cells (Drudy et al. 2001). One such study investigated the adhesive factors that played a role in *C. difficile* adherence to both Caco-2 and HT29-MTX cells (Eveillard et al. 1993). Cell lines have also been used to determine what happens next in order for the infection to manifest particularly

in relation to the cytotoxic effect on the cells that results from the toxin production of *C. difficile* (Valdes et al. 2015).

Recently cell culture has been applied more frequently to study the interactions between cells and bacteriophages to explore the concept of phage therapy. Examples include the use of A549 lung epithelial cells to measure the efficiency of phage therapy against *Acinetobacter baumanni* (Shen et al. 2012) and using a cystic fibrosis bronchial epithelial cell line to demonstrate that bacteriophages have the ability to kill *P. aeruginosa* (Alemayehu et al. 2012). More recently the intestinal epithelial cells HT29 and Caco-2 cells were used to investigate the immune responses that result from *E. coli* bacteriophages (Mirzaei et al. 2017). Despite all of the examples mentioned, to date there has been no research published about a cell culture model that can be used to assess phage therapy against *C. difficile* infection.

The determination of an appropriate cell line for use within a study is a factor that needs to be given a lot of thought. An example of a study where intestinal cell lines were evaluated for use was one by Gagnon et al, who compared Caco-2, HT29 and HT29-MTX cells to investigate the adherence and invasion of *Salmonella* (Gagnon et al. 2013). The data from this particular study indicated that the HT29-MTX model was the most suitable for the study of pathogen and cell interactions (Gagnon et al. 2013). There are studies that have been carried out using Caco-2 cells to research *C. difficile* pathogenesis; however, for the purpose of this project HT29 and later HT29-MTX-E12 cells were selected. The colon epithelial cell lines were considered to be the most relevant for this project as CDI and its potential phage therapy are the focus and *C. difficile* colonises the large intestine.

A primary focus of my PhD project was the observation of the interactions between phages and *C. difficile* and how these changed in the presence of epithelial cells and a mucus layer such as the one present in the gastrointestinal tract (described in section 1.5.2).

1.5. The gastrointestinal tract

1.5.1. Overview of intestinal epithelial barrier

Within the human body, all of its surfaces and cavities are lined by layers of epithelial cells which are connected by junctions serving three main purposes; the maintenance of tissue integrity by adhesion, the formation of a selective barrier, and signalling enabling communication to control cell behaviour and tissue function (Citi 2018). The intestinal epithelial layer is the largest and most important of these selective barriers against the external environment (Groschwitz & Hogan 2009). The cell monolayer is made up of 20 billion adjoining cells (Capaldo et al. 2017) which forms the boundary between tissues of the intestine and the external environment (Stadnyk 2009). The gastrointestinal (GI) tract itself has an approximate surface area of 32 m^2 (Luissint et al. 2016).

The structure of the intestinal barrier consists of a monolayer of columnar epithelial cells so called as they are rectangular-like in shape and often resemble a column (see figure 1.5). These cells are separated from the contents of the lumen by mucus which will be described further in section 1.5.2. Within the small intestine the epithelium is folded to form villi and crypts; although in the large intestine there are only crypts present (Luissint et al. 2016).

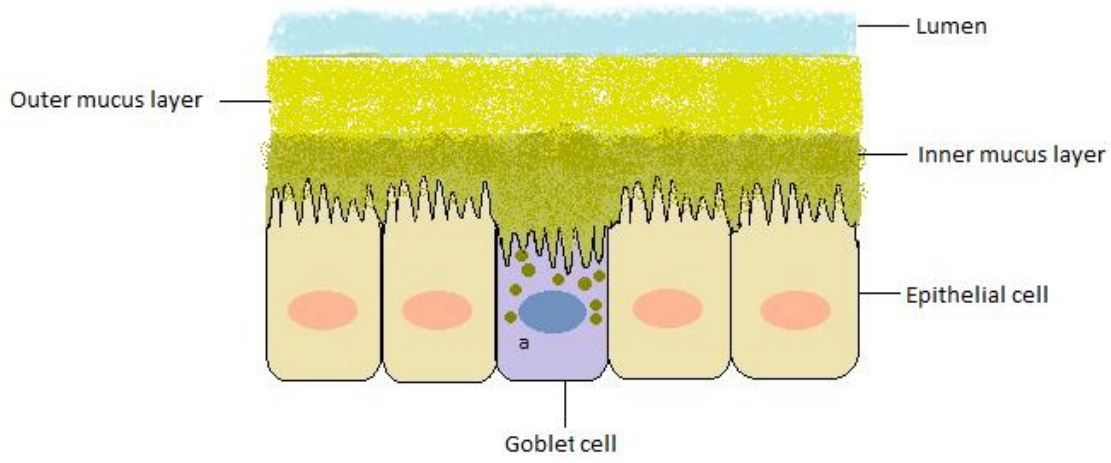

Figure 1.5: Structure of the GI tract. Adapted from Johansson et al 2016

The epithelial and goblet cells with the mucus layers. The section marked "a" shows the secretion of mucus from the goblet cells.

Like all efficient epithelial cell layers, the intestinal wall functions as a selectively permeable barrier. Ingested food must be digested, absorbed and processed for excretion. The intestinal barrier has the ability to allow essential dietary nutrients, electrolytes and water to be transported from the intestinal lumen into the main circulation of the body. It does this whilst simultaneously providing a barrier against the commensal microorganisms needed to ferment the undigested food material and the more harmful entities such as toxins, microorganisms and foreign antigens (Groschwitz & Hogan 2009). Therefore it acts as both a barrier and a filter. The essential nutrients can be taken up by the cells of two different pathways, the transepithelial pathway where transport occurs directly through the cells and the paracellular pathway where transport occurs in the spaces between the epithelial cells (Turner 2009). Breaking or even slightly damaging the epithelial barrier can cause serious problems linked with infection and inflammation (Citi 2018). It has also been noted that physical stress (potentially caused by illness and infection) can play a huge role in the function of the GI tract leading to changes in gut permeability as well as alterations in ion, fluid and mucus secretion and absorption. Usually the commensal microorganisms do not cause disease but when the mucosal surface is damaged, the commensal microorganisms can become opportunistic pathogens (McGuckin et al. 2011).

1.5.2. Mucus layer

As mentioned previously in section 1.5.1 the GI tract has a large surface area (32 m^2), all of which is exposed to the contents of the intestines. It is a major entry point for potential pathogens and for this reason an efficient defence system is crucial for protection. The defence system is complex and made up of several levels; however, it is the mucus layer that provides the physical protection. Mucus can be found in several places within the human body including both the upper and lower respiratory tracts but for the purpose of this thesis the mucus located within the digestive system will be the key focal point.

The intestinal mucus layer is found overlying the epithelium (see figure 1.5) and provides the first line of defence against physical and chemical injury that could potentially be caused by ingested food, microbes and microbial products (Kim & Ho 2010). Mucus production occurs in the goblet cells where it is secreted and forms part

of the innate immune system (Hansson 2012). The mucus layer contributes significantly to an already spectacular system where digestion of food can occur without the organs digesting themselves and the high concentration of hydrochloric acid (HCl) produced in the stomach can be tolerated. Additionally, the GI tract harbours more bacteria than it does host cells, yet despite rapid multiplication, the bacteria are seldom allowed to take over (Johansson et al. 2013). The human commensal flora or microbiota are present within the GI tract in their billions and are primarily dominated by the phyla Firmicutes and Bacteroidetes (50 % and 30 % respectively). For the relevance of this thesis *C. difficile* is part of the Firmicutes phylum.

The stomach and the colon have two layers of mucus whereas the small intestine only has one layer. The inner layer of mucus found in the colon is firmly attached to the epithelium and can range in thickness between 50-200 µm. (Hansson 2012). The inner layer has a compact and layered appearance and has been found to be almost completely bacteria free. It has been hypothesised that a major function of mucus is its ability to limit bacterial contact with the epithelium and aid in transporting the bacteria elsewhere. The mucus may also act as a filter as the bacteria may be too big to pass through (Johansson et al. 2010; Johansson et al. 2011; Johansson et al. 2008). However, there is limited knowledge on what mechanisms the pathogenic bacteria use to evade the protection system. There is an estimated number of 10^{13} – 10^{14} commensal bacteria located within the adult intestines. The high commensal bacteria count is thought to be because the intestines contain many of the nutrients they require. The mucus layer is a direct source of carbohydrates for the bacteria as the mucus glycan is the carbon source (Sicard et al. 2017). Additionally, the mucus layer contains numerous attachment sites and the body temperature is optimal within the intestines (Johansson et al. 2010). As mentioned previously, the human host has a number of mechanisms to make sure the host is not taken over by the bacteria whether commensal or pathogenic. The mechanisms include the adaptive immune system where secretory IgA is produced and secreted and the innate immune system where components including lysozymes and antibacterial peptides are released. The mucus layer is part of the innate immune system due to its ability to prevent pathogens from reaching and persisting on the intestinal epithelial surfaces (Sicard et al. 2017). The intestinal immune system appears to be most active within the small intestine as there is more exposure to the bacteria.

Passage through the small intestine is fast (approximately 3-5 hours) and therefore there is a limited time frame for the bacteria to be able to replicate and increase in number. On the other hand, the transit through the colon is much slower (30-40 hours) and so the bacteria can stay here for a longer time (Johansson et al. 2010). In the colon, the high numbers of commensal bacteria have an interdependent relationship with the host whereby in normal conditions the bacteria are tolerated by the host but still pose a serious threat.

The mucus layer is made up of mucins, non-specific antimicrobial molecules such as antimicrobial peptides (secreted antibodies that target specific microbial antigens) and other intestinal proteins (Sicard et al. 2017). A major component of mucus are the secreted mucins which are large, highly glycosylated proteins made up of more than 80 % carbohydrate. The mucin proteins are referred to as MUC followed by a number which identifies the MUC protein more specifically. The mucin domain glycans bind a lot of water due to its hygroscopic and hydrophilic properties generating most of the typical gel-like properties that mucus typically has (Johansson et al. 2013). The gel-like features of mucus are a big disadvantage when it comes to its study as it tends to collapse if it is not well hydrated and can be lost if formaldehyde is used to fix the tissue. An additional problem is that normal mucus is made up of 98 % water and therefore can be close to invisible under the microscope (Hansson 2012).

Mucins have domains known as PTS domains which give them specific properties which have been summarised in table 1.1. The PTS domains are so called as they are structures found within the glycoproteins and are made up of the amino acids proline, threonine and serine (Sicard et al. 2017). As seen in table 1.1, there are two main types of mucin; transmembrane and gel-forming (Johansson et al. 2013). Transmembrane mucins have a domain that enables anchoring to the cell membrane which occurs on the apical side of the epithelial cells, (the side of the cell that is exposed to the lumen and the contents of the intestine). The second mucin type- the gel forming mucins have the key function of building the mucus. It is this mucus that is designed to protect and lubricate the GI tract (Johansson et al. 2013).

Table 1.1 Mucins located in the GI tract (Table adapted from Johansson et al 2013)

Mucin (and type)	**Cell type from which it is expressed**	**Function**
MUC1 (transmembrane)	Epithelial cells	Signalling, protection
MUC2 (gel-forming)	Goblet cells Paneth cells	Protection, lubrication, entrapment
MUC3 (transmembrane)	Enterocytes	Apical surface protection
MUC4 (transmembrane)	Epithelial cells Goblet cells	Signalling, protection
MUC5AC (gel-forming)	Mucous cells	Protection, lubrication, entrapment
MUC5B (gel-forming)	Mucous cells Goblet cells	Protection, lubrication, entrapment
MUC6 (gel-forming)	Mucous cells	Protection, lubrication, entrapment
MUC7 (gel-forming)	Mucous cells	Protection
MUC12 (transmembrane)	Enterocytes	Apical surface protection
MUC13 (transmembrane)	Enterocytes	Apical surface protection
MUC16 (transmembrane)	Epithelial cells	Apical surface protection
MUC17 (transmembrane)	Enterocytes	Apical surface protection

The mucus located throughout the digestive system varies with different mucin proteins being more prominent in each part of the digestive tract. In the mouth, MUC5B and MUC7 are produced by the salivary glands and act as a form of lubrication for the passage of ingested food through the oesophagus. The stomach has a two layered mucus system with an inner mucus which is attached and an outer unattached loose mucus layer (Atuma et al. 2001). The mucus system becomes a single layered system in the small intestine where a single type of surface mucin (MUC2) is produced. MUC2 is an unattached, discontinuous mucus which is less well defined. Here, the mucus is secreted at the top of the crypts and moves upwards between the villi. The colon, the location of CDI has a two layered mucus system like the stomach. Here the inner layer is dense and firmly attached to the epithelium (Atuma et al. 2001). It forms a matrix like complex (Ashida et al. 2012) whereas the outer layer is loose and unattached and exposed to bacterial proteolytic activity. Similarly to the small intestine, MUC2 is the mucin that is prominent within the colon, where the mucus is almost exclusively MUC2 (Naughton et al. 2014). Therefore it can be assumed that MUC2 behaves differently in both the small and large intestines (Johansson et al. 2013). In 2008, Johansson et al fixed and stained MUC2 in colon tissue and confirmed a stratified appearance in the inner layer. It was also proven that the outer loose layer was more disorganised in appearance. There was further confirmation that bacteria were only present in the outer loose mucus layer allowing the conclusion to be made that bacteria from the colon did not usually reach the epithelial cells (Johansson et al. 2008).

Proteomic studies of the intestinal mucus layer have revealed almost identical protein profiles and further proved that the main component is the gel forming mucin MUC2. Other proteins, particularly cellular proteins have also been identified as the cells are continuously shed into the lumen and trapped within the mucus (Probert & Gibson 2002).

MUC2 is one of the four human gel forming mucins expressed in a site specific fashion throughout the human body and is expressed by the goblet cells. As previously mentioned in this section, the mucus layer of the colon is largely composed of the mucin MUC2. It helps remove the gut contents and intruding microbes. However, it also contains various digestive enzymes, antimicrobial peptides, and immunoglobulins

which are secreted from the epithelial cells and Paneth cells which help prevent bacteria from penetrating the inner mucus layer (Ashida et al 2012). Mucin is usually secreted at a constant rate but can vary in response to bacterial infection or a change in the conditions within the lumen. Mucin secretion within the goblet cells is regulated by several factors including microbial products, inflammatory mediators, hormones, signalling mediators, growth factors and infectious bacteria (Ashida et al 2012). The protein core of MUC2 is made up of 5179 amino acids and has typical mucin domains which gives the mucin an extended conformation and the ability to bind water to a high capacity (Johansson et al. 2010). MUC2 is stored in the goblet cell mucin granulae. When the granulae are released, MUC2 mucin expands in volume. Johansson et al observed how the released mucins spread out and organised a sheet underneath the inner mucus layer. An additional observation is the MUC2 found in the inner layer and secretory vesicles is insoluble; however, the MUC2 in the outer loose mucus layer is readily soluble (Johansson et al. 2010). It has been hypothesised that this is due to the proteolytic cleavages that take place in the MUC2 of the outer mucus layer. The cleavages do not disrupt MUC2 but instead allow the mucus to expand four times in volume. The expansion of MUC2 also increases the capacity of the mucin glycans to bind water (Johansson et al. 2010). As previously stated, the commensal bacteria live and thrive in the outer loose mucus layer of the colon which may be because the MUC2 mucin network expands in volume allowing the bacteria to penetrate into the mucin network.

1.5.3. Bacteria interactions with mucus in gut

As mentioned in section 1.5.1 the main functions of the intestinal epithelium are the absorption of nutrients and retention of water and electrolytes whilst forming an efficient barrier against microbes and pathogens. There are multiple defence mechanisms within the GI tract that are used to provide efficient protection from pathogens including the microbiota naturally occurring within the lumen, a high epithelial cell turnover, the integrity of the epithelial membrane and the mucus layer itself (Ashida et al 2012). The interactions with the mucus layer are important for colonisation of the gut commensals and some of the pathogens, particularly those that have evolved to adhere to mucus and exploit it (Juge 2012). However, the pathogens

themselves have numerous strategies where they can bypass the intestinal epithelial defence mechanisms and cause infection. The bacteria must evade the intestinal defences in order to penetrate the mucus layer resulting in colonisation. Some bacteria are able to produce proteases and glycosidases which are able to degrade the mucins creating holes within the mucus barrier allowing the bacteria to pass through. Examples of bacteria that have this mucin degrading function are *Bifidobacterium bifidum, Bacteroides fragilis* and *Ruminococcus spp* (Sicard et al. 2017). Another mechanism that bacteria have been found to use is induce down regulation of the expression of mucin genes (Naughton et al. 2014). Some pathogens use mucus components to modulate the expression of virulence genes and adapt to the host environment. The mucus layer has a protective function and acts as a lubricant for the transit of luminal contents along the digestive tract. There is a state of equilibrium between the growth of the bacteria in the mucus gel and the shedding of the mucus and the epithelial cells into the lumen. The mucus is constantly shed into the lumen therefore, the gut epithelial cells have a turnover time of 3-6 days. The persistence of a microorganism in this environment depends on its ability to replicate at a faster rate than it is shed into the lumen and expelled from the body (Probert & Gibson 2002). The thickness of the mucus layer varies in the gut but the colon is covered by the thickest mucus layer, it is also the location where there is the highest density of microorganisms.

Bacterial populations associated with mucus in the colon are extremely difficult to study due to the invasive methods needed for sample collection. Much has been learnt about human faecal flora as samples are readily accessible; however few data are available on bacteria associated with the colonic mucus. Additionally, the small volume of samples that have been taken are often from diseased individuals and it is therefore unlikely that an accurate depiction of bacteria and mucus within a healthy human could be formed (Probert & Gibson 2002). Studies of bacterial interactions with their hosts have traditionally relied heavily on the use of cell lines and cell culture techniques. Although studies to date have given a remarkable insight into the interactions between the two, it is recognised that there are several limitations with using cell lines, particularly intestinal cell lines. Although cell lines are commonly used, they often do not accurately reflect conditions that are encountered in the gut (Naughton et al. 2013) e.g. in reality there are hundreds of bacterial species all interacting with each other to form a

diverse and complex ecosytem. *In vitro* adhesion assays present structures that are highly likely to be involved with *in vivo* adhesion, but *in vivo* studies are still needed for confirmation. One of the main reasons both *in vitro* and *in vivo* systems are needed is due to the lack of suitability of a unique model system that is able to predict the adhesion ability in every bacterial strain (Laparra & Sanz 2009). Despite the limitations, there have been several key observations of bacteria and host interactions that have been made through the use of intestinal cell lines.

Naughton et al, used cell lines that secreted mucins forming an adherent mucus layer for their study of host interactions with *Campylobacter jejuni* and *Helicobacter pylori* (Naughton et al. 2013). Although the two bacterial species are closely related they displayed two very different mechanisms of interaction with mucus and mucins. *C. jejuni* is an intestinal pathogen whereas *H. pylori* infects the stomach and therefore the first observation made was that the bacteria bound to different mucins. *C. jejuni* logically bound more effectively to the mucins found in chickens. Although *C. jejuni* was able to bind to mucins from other animals i.e. bovine, equine and porcine, stronger interactions were observed with the mucins from the chicken. *H. pylori* bound to a completely different subset of mucins with the greatest affinity being observed with porcine mucin, although the binding was less significant than that observed between *C. jejuni* and chicken mucin. In order to provide further evidence for their observations Naughton et al used the HT29-MTX cell line. The cell line has two sub-types, the first of which (HT29-MTX) secretes mucins into the culture supernatant and the second, HT29-MTX-E12 forms an adherent mucus layer (Naughton et al. 2013). Bacterial colonisation with the mucus producing cells was compared with its non-mucus producing counterpart (HT29). Both *C. jejuni* and *H. pylori* displayed the most efficient colonisation with the adherent mucus layer. A key difference was that *H. pylori* required the presence of an adherent mucus layer for colonisation as demonstrated by a significantly reduced level of colonisation with the secreted mucus cell line HT29-MTX. Also, *H. pylori* was unable to colonise the non-mucus producing HT29. The study confirmed the role that mucus and mucins can play in the colonisation of bacteria leading to subsequent infection (Naughton et al. 2013).

More specifically, in the colonisation process of *C. difficile*, it is highly likely that the bacteria encounter a layer of mucus first. As mentioned in section 1.1.3, toxins A and B are two of the virulence factors of CDI. Other potential accessory virulence factors include the capsule, fimbriae, hydrolytic enzymes. The toxins and accessory virulence factors could potentially be involved in mucus degradation and penetration and combine their functions with adhesins which may play a role in mediating adherence to the mucosal layer (Tasteyre et al. 2001). With this in mind, the properties of adhesion of two flagellar proteins, FliC and FliD to cecal mouse mucus were examined. *In vitro* results suggested that both FliC and FliD proteins were implicated in the attachment of *C. difficile* to the mucus layer of the intestine. Additionally, the flagellated strains appeared to have a better capacity in associating with the cecal wall *in vivo* compared to the non-flagellated *C. difficile* which had a 10 fold lower rate of association (Tasteyre et al. 2001).

1.5.4. Mucus and bacteriophage interactions

The gut microbiota feature a wide range of microorganisms including bacteria, bacteriophages, viruses and fungi. Phages make up a huge proportion of the gut microbiota with approximately 10^{15} located in the human gut alone (Dalmasso et al. 2014). They are also found in large numbers in the respiratory tract, the mouth, urine and serum of both animals and humans (Łusiak-Szelachowska et al. 2017).

Over the past 15 years, there has been a growing interest in the human gut microbiota and its effects on health and disease in humans. The number of publications on the human microbiome and its implications on health and disease increased significantly between 2013 and 2017 where 12,900 articles were published, 80 % of the overall publications of the last 40 years(Cani 2018). However, there have only been a limited number of studies of the gut virome. Most gut phages are members of Caudovirales or single stranded Microviridae. A typically active virome within a healthy human gut consists of anything between 35 and 2800 different types of viruses distributed throughout the gut, and generally increasing in number from the proximal region to the distal region (Łusiak-Szelachowska et al. 2017). The phage virome was shown to be highly dynamic during infancy compared to the much more stable and resilient adult stage. There was a rich and diverse phage community with the majority being

Caudovirales and dsDNA viruses in samples taken from infants. Lim et al noted that over the first two years of life there was a gradual decrease in species richness which also coincided with the infant bacterial microbiome becoming more similar to that found within adults (Lim et al. 2016).

The abundance of bacteriophages within the human gut can alter according to circumstances, for example phages increased in patients with intestinal diseases. This is likely to occur because of the induction of prophages from bacteria that have been stressed (Łusiak-Szelachowska et al. 2017). There has also been a recent observation by Davies et al that some antibiotics, primarily fluoroquinolones may induce the production of prophages. Particularly relevant to this project is that it was reported that treatment with fluoroquinolones increased the production of *C. difficile* phages in human faeces (Davies et al 2016).

It has also been confirmed that viruses are able to migrate across mucosal barriers into the blood and local tissues which could potentially influence the immune system. Phages have been detected in blood samples from both healthy and immunosuppressed patients; however, it is more likely that the movement of bacteriophages from the GI tract into the bloodstream is more likely to occur in diseased individuals as many illnesses can affect the GI tract making the epithelial barrier more permeable to microorganisms (Łusiak-Szelachowska et al. 2017).

It has been suggested that phages play an important role in human immunity and the defence of the mucosal barrier against bacteria. Reports have stated that the phage to bacteria ratio within the mucosa is high and furthermore is much higher in mucosal surfaces than in the surrounding environment. The phages found in these environments can either be ‘free,’ and aid their host bacterial strain by killing related competing strains, or they may develop a conditional symbiotic relationship with their host via the process of lysogeny. Here, the prophages integrate into the host and are able to express genes that enable the fitness or virulence of the host to be increased giving added protection to the host from lysis by related phages (Barr et al. 2013).

Barr et al showed that phages were able to adhere to mucus via their capsid proteins. They confirmed this using three methods (Barr et al. 2013). Epifluorescence

microscopy was used to show the phage concentration in the mucus layer was much higher than the surrounding environment. Cell culture was used to assess phage to mucus adherence using T84 colon epithelial cells and a non-mucus cell line A589 for comparison purposes. Finally, a modified top agar assay was used to prove that the phages significantly adhered more to a mucin coated agar surface (Barr et al. 2013).

An interesting question arose from this work which was also addressed by the same group. Did the number of adherent bacteriophages found on the mucosal surface also reduce microbial colonisation? In order to address this point, Barr et al assessed bacterial attachment on T84 and A589 epithelial cells with and without a pre-treatment of T4 phage. Results showed that phage pre-treatment of the mucus producing tissue culture cells significantly decreased bacterial attachment, whereas pre-treatment of non-mucus producing cells had a much lesser effect on bacterial attachment (Barr et al. 2013). Another observation was that after phage replication, bacterial attachment reduced further compared to when no phage replication occurred within the mucus. This confirmed that pre-treatment of a mucus surface with bacteriophages reduced adherence of a pathogen and further protection was provided by continued bacteriophage replication within the mucus (Barr et al. 2013). Overall the mucus cells incubated with phage showed a 3.6 fold reduction in A589 cell death caused by the bacteria compared to the non-mucus cell line.

At this point, Barr et al developed the BAM model (bacteriophage adherence to mucus). The model demonstrated that adherent phages were able to protect the underlying epithelial cells from bacterial infection and the adherence of phages to the mucosal surface led to a non-host derived antimicrobial defence. However, the BAM model had its limitations. The main limitation was the simplicity of the model. Within an *in vivo* state, the mucus layer would contain several bacteria and a huge variety of bacteriophages. The interactions between the two would be very complex compared to within the BAM model where only one lytic phage and one host bacteria was studied. Therefore, in reality there is most likely going to be a much lower probability of specific phage and bacteria host pairs encountering each other. The mucins will also be secreted continuously by the epithelium meaning that there is also a rapid and ongoing turnover of both bacteria and phage populations within the mucus layer (Barr et al. 2013).

Similar limitations frequent the study of mucus and phage interactions within an *in vitro* setting as in reality the mucosal environment is a complicated environment to replicate. This is because the environment changes according to whether it is healthy or diseased. At the most basic level, the mucus volume, thickness and consistency will change with illness. In addition to this, and thus making it more complex, the gut microbiota will also change significantly which is likely to play a huge role in how bacteriophages interact with the mucus layer.

1.6. Aims of study

The initial optimisation of the epithelial cell model was already initiated as part of a Masters project (Vukusic 2014-University of Leicester). It was confirmed that HT29 and HeLa cell lines were able to survive incubation in anaerobic conditions for up to 10 hours; which was necessary to provide a suitable environment for observations of the interactions between *C. difficile* and phagesFor the purposes of this PhD project the model was further optimised and refined to make the model more streamlined and suitable for its purpose. The aims of the project were as follows:

1. Further development, optimisation and refinement of the HT29 cell model designed to assess the therapeutic potential of *C. difficile* phages.

The growth conditions for *C. difficile* in cell culture medium were optimised. Also, different *C. difficile* strains and phage combinations were trialled to ascertain the most suitable pairing to be used for the model. Finally, CDI treatment scenarios were carried out in the presence of HT29 cells in order to establish the working parameters of the cell culture model.

2. Further development of the model with use of mucus producing epithelial cells

The additional parameter of the mucus layer was introduced. The mucus layer features heavily within the GI tract and is likely to have a significant impact on how phages interact with *C. difficile*. Different treatment regimens were explored and the impact of

varying levels of mucus on the interactions between phages and bacteria were also assessed. The final section of the chapter demonstrated the efficacy of phages once they had bound to the mucus layer of the epithelial cells.

3. Mucin quantification

An indirect ELISA was developed and optimised in order to confirm that the mucus levels were changing as expected within the model. Optimisation involved determining the working concentration for each of the primary antibodies for each MUC protein to be quantified along with the corresponding working concentration for the secondary antibody.

A key aim of this PhD thesis was to delve into the novel concept of studying phages and *C. difficile* on an *in vitro* basis. The presence of colon epithelial cells and a mucus layer would allow for CDI and phage therapy scenarios to be closely mimicked.

Chapter 2. General Methods

2.1. *C. difficile* growth and maintenance

2.1.1. Bacterial strains

For the development and optimisation of the cell culture model an appropriate strain of *C. difficile* had to be selected. *C. difficile* ribotypes 002, 005, 014/020, 027 and 078 were selected for optimising the model. The five ribotypes selected are all clinically relevant ribotypes and were isolated from faecal samples and ribotyped by Dr Krusha Patel (Patel 2012). Faecal specimens were collected from Glenfield General Hospital, Leicester Royal Infirmary, Leicester General Hospital and healthcare centres within the University Hospitals of Leicester, UK NHS Trust between 2008 and 2010 (Patel 2012). *C. difficile* ribotypes were isolated from the samples and cryogenically preserved for long term storage at the laboratory of Professor Martha Clokie (University of Leicester, UK).

R027, in particular has been identified as hypervirulent and one of the main ribotypes responsible for the marked increase in CDI recorded from 2004 (Public Health England 2015). The majority of the experiments throughout this project were carried out using R027 strain AIU. The remaining four ribotypes used within the project have also been identified as prevalent in the UK (Burke & Lamont 2014; Public Health England 2015). R076 (strain T6) was used for propagation of bacteriophages CDHM3 and CDHM6 as described in section 2.2.3.

All recipes for the media required can be found in the appendix.

2.1.2. Bacterial growth

C. difficile strains were cultured on brain heart infusion (BHI) agar plates supplemented with 7 % defibrinated horse blood (hereafter referred to as blood agar plates) and incubated at 37 °C anaerobically in a Don Whiteley chamber for 24-48 hours. Fastidious anaerobic broth (FA broth) was pre-incubated in the Don Whiteley chamber for a minimum of 16-18 hours before one colony of *C. difficile* from the blood agar

plate was used to inoculate 7 mL of the broth. The FA broth culture was incubated overnight.

2.1.3. Cryogenic preservation of *C. difficile*

C. difficile strains were cultured on blood agar plates and incubated as described previously and used to inoculate 7 mL FA broth which was subsequently incubated overnight. Eppendorf tubes containing 1 mL of the overnight culture were centrifuged for 15 minutes at 4000 x g at room temperature. The supernatant was discarded and the pellets resuspended with glycerol solution from the Protect Bacterial Preservers Vial (Technical Service Consultants Ltd, Heywood, UK). The resuspended mixture was transferred back into the vial and immediately moved to -80 °C for long term storage.

2.1.4. Determination of viable *C. difficile*

The viable count of *C. difficile* was determined by calculating the number of colony forming units per mL (CFU/mL) as per the Miles and Misra method (Miles et al. 1938). Ten fold serial dilutions were prepared in BHI medium (for growth curves) or PBS (for epithelial cell model study) and10 µL of each dilution was spotted onto BHI agar plates. The plates were allowed to dry and incubated overnight anaerobically at 37 °C. After incubation, the colonies were counted and the CFU/mL calculated using the following calculation:

CFU/mL = (number of colonies / volume of bacteria plated (mL)) x dilution factor

2.2. Bacteriophages

The bacteriophage that was primarily used throughout the project was CDHS1 which was previously shown to infect the widespread epidemic strain of *C. difficile* R027 in liquid culture (Shan et al. 2018). CDHS1 propagates on *C. difficile* R027 strain AIU. The other phages used for the study were CDHM3 and CDHM6 which propagate on R076 (strain T6) (Hargreaves et al. 2015; Shan et al. 2012; Nale et al. 2012).

2.2.1. Propagation of bacteriophages

With the exception of CDHS1, all phages were propagated by liquid culture. For CDHS1, the plaque assay method was applied as propagation by liquid culture did not result in a high enough titre.

2.2.1.1. Plaque assay

An overnight FA broth culture of *C. difficile* AIU was grown as previously described in section 2.1.2 and 450 µL was added to a 1:1 ratio (7 mL in total) of BHI soft overlay (0.5 %) agar and salt solution (0.4 M magnesium chloride and 0.1 M calcium chloride). All recipes can be found in the appendix. A volume of 250 µL CDHS1 was added to the solution which was mixed and poured onto BHI 1 % agar plates. The agar was allowed to set, incubated overnight anaerobically at 37 °C and checked for complete lysis. The soft agar overlay was collated from the plate and mixed with an equal volume of BHI broth. The mixture was placed on a rocking platform for 30 mins before incubation at 4 °C overnight. Following incubation, the agar-BHI broth mixture was centrifuged at 4000 x g for 15 mins. The supernatant was collected and filtered through a 0.22 µM filter and stored at 4 °C for subsequent spot testing to determine the phage titre. The phage lysate can be kept at 4 °C for a period of up to 2 months with fortnightly spot tests to ensure the titre has not dropped.

2.2.1.2. Liquid propagation of bacteriophages

An overnight FA broth culture of *C. difficile* T6 was prepared as previously described and 500 µL was added to 50 mL of pre-incubated BHI broth and incubated until bacterial growth was in exponential phase indicated by a measurement of $O.D_{550}$ of 0.2. A volume of 100 µL of phage (either CDHM3 or CDHM6) was added to the BHI broth and incubated overnight. Following incubation, the culture was centrifuged at 4000 x g for 15 mins at room temperature. The supernatant was collected and filtered as previously described in section 2.2.1.1. The phage lysate was stored at 4°C.

2.2.2. Determination of bacteriophage titres

The phage titre or number of plaque forming units/mL (PFU/mL) is determined by the spot test. The soft agar, salt solution and FA broth culture were mixed together and poured onto the BHI agar plate as previously described (section 2.2.1.1). 10 fold serial dilutions of the phage were prepared in cold BHI broth (PBS for epithelial cell model) and 10 µL of the diluted phage was spotted onto the BHI agar plate. The plates were incubated overnight as previously described. The plaques were counted and PFU/mL was calculated using the following equation:

PFU/mL = (number of plaques/volume of bacteriophage plated) x dilution factor

2.2.3. Preparation of bacteriophages for experiments

As mentioned previously, the bacteriophages are stored in BHI broth at 4 °C. The presence of the nutrient rich BHI medium compromises the epithelial cells. Therefore, before the experiments can take place, the volume of phage lysate required for the experiment must be transferred to cell culture maintenance medium (see appendix). The phage lysate is washed three times in maintenance medium by centrifugation at 21,000 x g for 30 mins at 4 °C. The final supernatant is discarded and the pellet is resuspended in maintenance medium. The suspension can be stored at 4 °C for a maximum of one week but preferably should be used within 24 hours.

2.3. Cell culture

The growth medium used for growth and passaging of all cell lines was Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (Sigma D5546) supplemented with 10 % (v/v) Foetal Bovine Serum (FBS) (Gibco 10082147), 2mM L-glutamine (Sigma G7513) and 1 mL 100 µg/mL penicillin and 100 U/mL streptomycin (Sigma P4333). Similarly, the maintenance medium for use in the epithelial model in the presence of *C. difficile* and for the washing of the phage lysate was made as described previously, but without antibiotics and with a reduced concentrations of FBS (2 %

(v/v)). All medium and reagents were pre-warmed in a water bath set at 37 °C for ~1 hour before any cell culture work was carried out.

2.3.1. Cell lines

All cell lines were provided by the European Collection of Cell Cultures (HT29 ECACC 91072201, HT29-MTX-E12 ECACC 12040401, HeLa ECACC 93021013) PCR was carried out on a 6 monthly basis to ensure the cell lines remained *Mycoplasma* free. PCR was carried out by Dr Ravinder Chana of University of Leicester.

2.3.2. Culture and maintenance of cell lines

All three cell lines used throughout this PhD project were maintained using the traditional cell culture methods as described in sections 2.3.2-2.3.4. Epithelial cells typically prefer an optimal environment at 36 °C with 5 % CO_2 for incubation (Helgason & Miller 2005).

Cryopreserved cells were revived as follows; a vial was removed from liquid nitrogen storage and rapidly thawed using a water bath set at 37 °C. The contents of the vial were added to 9 mL growth medium and centrifuged for 5 minutes at 200 x g to remove as much of the cryoprotectant as possible. The supernatant was discarded and the pellet was resuspended in 1 mL growth medium. The resuspended pellet was added to 10 mL growth medium in a small tissue culture flask with vented caps (Nunc 136196) (25 cm^2) and incubated for 24-48 hours at 36 °C and 5 % CO_2 until ~70- 80 % confluence was observed.

Once 70- 80 % confluence level was reached, the cells were maintained and scaled up for experimental use as and when required. The growth medium was discarded from the flask. The cells were gently rinsed twice with ~10 mL Dulbecco's phosphate buffered saline (dPBS). After rinsing, the dPBS was discarded and 1 mL of trypsin-EDTA (Sigma) was added to the flask to remove the adhered cells from the plastic surface of the flask. The cells were incubated for ~5 minutes at 36 °C and 5 % CO_2 to allow the trypsin-EDTA to detach the cells from the flask. 10 mL of growth medium was added to the flask and the cells were aspirated to prevent clumping. 1 mL of the cell suspension was added to 10 mL growth medium in a new 25 cm^2 flask and the

remainder was added to a medium (75 cm^2) flask (Nunc 178905) containing 20 mL growth medium to scale up the cell production. The flasks were incubated at 36 °C and 5 % CO_2 until the cells were confluent as previously described. The cells were continuously passaged on a routine basis to ensure the cells never exceeded 70 % level of confluence. For experimental purposes the cells were scaled up to a medium flask; however, for maintenance purposes the cells were either cryopreserved or maintained for a short period in small flasks.

2.3.3. Cryopreservation of cells

Cryopreservation of cells was carried out routinely to maintain a frozen stock of cells. A 75 cm^2 flask with 70- 80 % confluent cells was harvested as described in section 2.3.2 and resuspended in 5-6 mL of growth medium. The cell suspension was centrifuged at 200 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 2.5 mL of FBS and 0.5 mL dimethyl sulfoxide (DMSO) to make a final concentration of 90 % FBS and 10 % DMSO. The suspension was aliquoted into three cryogenic vials each containing 1 mL. The vials were frozen in a controlled freezing container at -80 °C overnight and transferred to liquid nitrogen for long term storage.

2.3.4. Preparation of cells for microtitre plates

The 75 cm^2 cell culture flasks containing 70-80 % confluent cells were used for the preparation of 24 well micro titre plates (Corning, CoStar CLS3527-100EA) for experimental purposes. The cells passaged from one 75 cm^2 flask is sufficient to seed three 24 well plates. The cells were processed as previously described in section 2.3.2. A cell count was performed using a Fuchs-Rosenthal haemocytometer and the cells were adjusted to 7 x 10^4 cells/mL in maintenance medium (DMEM with 2 % (v/v) FBS and 200 mM L- glutamine). Maintenance medium containing a lower percentage of FBS was used for all experiments as initial optimisation carried out in the Masters project demonstrated that a higher concentration of FBS gave false positive results by reducing the CFU/mL count of *C. difficile* (Vukusic 2014). The cell suspension was used to seed 9-12 wells of a 24 well plate so each well contained 70,000 cells (1 mL per well). The plates were incubated at 36 °C and 5 % CO_2 for 2 days until they reached confluence.

2.4. Statistical analysis

GraphPad Prism 7 was used for all statistical analysis which included standard error of mean (SEM) and calculations of P values by t-test.

Chapter 3. Optimisation of HT29 cell culture model

3.1. Introduction

Understanding the interactions that take place between bacteriophages and their corresponding hosts are a necessity for the development of phage therapy as a potential alternative treatment to antibiotics. To date many studies have been carried out to observe phage dynamics with *C. difficile;* however, they have mainly been *in vivo* models, namely hamster models and more recently *Galleria mellonella,* or within *in vitro* broth based cultures or small scale fermentations (Meader et al. 2010; Meader et al. 2013; Ramesh et al. 1999; Nale et al. 2016). These studies have provided valuable insights into how phages may interact with *C. difficile* within its natural settings, however, the methods used within these studies do not take into account all of the conditions or the environment associated with a human host. Of key importance to such dynamics are the epithelial cells located within the GI tract. The cells that line the intestines make up the epithelial cell barrier and therefore encounter all that passes through the GI tract, including bacteria, (whether part of the microbiota or pathogens) and phages. The studies carried out to date have omitted the presence of epithelial cells from their models and therefore lack a key parameter required to assess the potential application of phages against *C. difficile* in a near natural therapeutic setting within a patient. The paper by Shan et al from Prof Clokie's lab has described how a cell culture model was developed within this PhD project in order to mimic some of the conditions that would be expected to be observed within the GI tract where CDI would manifest (Shan et al. 2018).

The study introduced the concept of observing *C. difficile* and phage interactions in the presence of a cell line. It also confirmed that the cell line used for the experiments was able to withstand anaerobic conditions for the duration of the experiment which will be discussed further in section 3.4.1.

The aim of the first results chapter of this thesis was to optimise and refine the model that was first implemented as part of a Masters project (Vukusic 2014) Optimisation included confirmation of the selection of the *C. difficile* strain and assessment of its

growth and stability in cell culture medium along with confirmation of suitable phage selection. It was necessary to ensure that varying levels of adsorption and infectivity were considered to further understand if these differences contributed to the dynamics observed between phages and bacteria and how they act with HT29 cells. Furthermore, two cell lines (the colon epithelial cell line HT29 and the cervical cancer cell line HeLa) were used within this chapter to ascertain that select interactions were specific to cell type and not a general observation. HT29 cells are a human colon adenocarcinoma cell line which was isolated from a primary tumour in 1964 (Martinez-Maqueda et al. 2015). The cells traditionally remain undifferentiated and grow in a multilayer. They are predictably used for the study of colon cancers but have also been used for studies involving the digestion of food due to the expression of characteristics otherwise observed in mature intestinal cells (Martinez-Maqueda et al. 2015). They are also used for studies investigating the intestinal immune response to bacterial infections, and the survival, adhesion and invasion of microorganisms. HT29 cells were chosen for this study as they are a colon epithelial cell line which closely represent the cell types that are observed within the large intestine. HeLa cells originated from a tissue biopsy from patient Henrietta Lacks who died in 1951 of an aggressive form of cervical cancer. These cells are the oldest and most commonly used human cell line (Lucey et al. 2009). As mentioned previously, HeLa cells are a cervical cancer cell line and therefore the use of them for the study of *C. difficile* – a gut pathogen and its corresponding phages, may be deemed unusual. However, HeLa cells were required in order to assess the specificity of the phage adsorption to human cells to elucidate whether the origin of the cells affected adsorption activity.

As mentioned previously, the work described in this chapter involved putting the developed model to use and trialling treatment scenarios. The growth conditions for *C. difficile* in cell culture medium were optimised. Additionally, different *C. difficile* strains and phage combinations were trialled to ascertain the most suitable pairing to be used for the model. Finally, CDI treatment scenarios were carried out in the presence of HT29 cells in order to establish the working parameters of the cell culture model.

3.2. Methods

3.2.1. Optimisation of model

3.2.1.1. Growth curves

Growth curves were used to compare the growing rate of *C. difficile* in both brain heart infusion (BHI) broth (standard growth medium for *C. difficile*) and maintenance medium. All media to be used was pre-incubated in the anaerobic chamber overnight. Approximately 1 mL of an overnight culture of *C. difficile* was used to inoculate 15 mL of pre-incubated BHI broth and 15 mL of pre-incubated maintenance medium (details of the recipes can be found in the appendix). The OD_{550} was taken on an hourly basis in order to confirm that efficient growth of *C. difficile* was occurring, a CFU/mL count was carried out in parallel and the data was plotted. CFU/mL counts were determined using the method described in section 2.1.4.

3.2.1.2. Determination of a suitable method for assessing attachment of *C. difficile* and phages to cells

In order to assess the level of attachment of bacteria and phages to the cells, the mechanism by which the cells were removed from the plastic of the 24 well plates had to be optimised. Both physical and chemical methods were compared. It was necessary for an efficient method to be devised as the mechanism should successfully remove the cells from the plate without compromising the *C. difficile* CFU/mL count. Four methods were tested, three using chemical reagents – 0.06 % Triton X-100, PBS/EDTA and trypsin/EDTA (Sigma) and one using the physical method of scraping using a cell scraper.

HT29 cell monolayers were prepared in 24 well plates as previously described (section 2.3.4). *C. difficile* was grown to exponential phase, washed and transferred to maintenance medium as described in section 3.2.3. 0.1 mL of *C. difficile* was added to the cells and the plate was incubated for four hours at 37 °C in anaerobic conditions. Following incubation, the maintenance medium was removed from all wells and replaced with either 0.06 % Triton X-100, PBS/EDTA or 0.25 mL of trypsin/EDTA. PBS was added to the wells to be processed with the cell scraper. The plate was

incubated aerobically for 10 minutes at 37 °C. For the wells containing trypsin/EDTA, 0.25 mL growth medium was added to the cells in order to neutralise the action of the trypsin and stop the reaction. The wells containing the reagents were aspirated vigorously in order to detach the cells from the plastic of the plate. The remaining wells were processed using the cell scraper. Following the detachment of the cells and bacteria from the base of the wells a CFU/mL count was carried out in order to enumerate the *C. difficile* levels.

3.2.1.3. Interactions of five ribotypes of *C. difficile* with CDHS1 and HT29 cells

Cell culture plates (24 well) were seeded with HT29 cells as described in section 2.3.4 and incubated overnight until a monolayer was formed. Five strains of *C. difficile* were chosen from selected ribotypes as described in section 3.4.3.1 and were grown and prepared as previously described as was the CDHS1 phage lysate. All plates were inoculated with 0.1 mL of its allocated strain of *C. difficile* and 0.1 mL of phages to attain a multiplicity of infection (MOI) of 10. Samples were taken at 4 and 8 hours post inoculation and the planktonic (non-attached *C. difficile* in the supernatant) and attached *C. difficile* levels were enumerated.

3.2.1.4. Interactions between *C. difficile* strain AIU with non-specific phages and HT29 cells

HT29 cells in 24 well plates, *C. difficile* strain AIU and phage lysates of CDHM3, CDHM6 and CDHS1 were prepared as previously described in section 2.3.4. All plates were inoculated with 0.1 mL of *C. difficile* AIU and 0.1 mL of its allocated bacteriophage so a MOI of 10 was attained. Samples were taken at 4 hours post inoculation and planktonic and attached levels of *C. difficile* were enumerated.

3.2.2. Bacteriophages with Hela and HT29 cells

Cell culture plates (24 well) were seeded with both HeLa and HT29 cells and allowed to grow overnight to form a monolayer. Three phage lysates containing CDHM3, CDH6 and CDHS1 were prepared as described in section 2.2.1. The reasons behind the selection of these bacteriophages are addressed in section 3.4.4. The bacteria and phages (0.1 mL of each) were used to inoculate wells containing HT29 and HeLa cells

so a MOI of 10 was attained. Samples were taken at 0, 4 and 8 hours and the phages were enumerated as per the method described in section 2.2.2.

3.2.3. *C. difficile* and bacteriophages on HT29 and HeLa cells

Cell culture plates (1 plate per time point) were seeded with either HeLa or HT29 cells and allowed to grow overnight to form a monolayer. *C. difficile* was grown as described in section 2.1.2 and 1 mL of overnight fastidious anaerobic (FA) broth culture was used to inoculate 15 mL pre-incubated BHI broth. The culture was monitored until it reached an OD_{550} of 0.2-0.3 indicating exponential growth. The culture was washed twice in maintenance medium by centrifugation at 4200 x g for 15 minutes at room temperature and resuspended in an appropriate volume to give a concentration of approximately 10^8 CFU/mL. The wash was necessary to eliminate toxin and BHI carryover into the human cells. The phage lysate was prepared as previously described.

Before the cell monolayer was inoculated, the maintenance medium was removed and replaced with fresh pre-warmed medium (1 mL per well). A MOI of 10 was attained by adding 0.1 mL of *C. difficile* culture to each well of the plate and 0.1 mL of phages to half of the wells in order to represent CDI with phage treatment. The plates were incubated at 37 °C in the anaerobic chamber. At each time point, a plate was removed from the anaerobic chamber and an aliquot of the supernatant was taken immediately to measure free (non-attached) *C. difficile* and phages in the wells. The maintenance medium was discarded and the wells were washed twice with PBS followed by the addition of PBS/EDTA (chosen after comparison of methods described in section 3.2.1.2) for measurement of attached *C. difficile* to the cells. The plate was incubated at 37 °C for 10 minutes to allow the cells to detach from the plate. The contents of the well were aspirated thoroughly to ensure the cells had fully detached and the samples were taken for immediate enumeration. The samples collected for the enumeration of phages were centrifuged at 21,000 x g for 5 minutes. The supernatant was collected and the pellet was discarded. All samples were serially diluted 10 fold in PBS and plated out using the Miles and Misra method as previously described in section 2.1.4. The double overlay agar method was used to determine the phage titre (section 2.2.2).

3.2.4. Prophylactic experiment

This experiment was designed to mimic a prophylactic phage treatment i.e. the treatment was administered prior to the start or detection of the infection. The experiment was set up as described in section 3.2.1.2 using HT29 cells with one alteration. The bacteriophages (0.1 mL) were added to the cells and were incubated anaerobically at 37 °C for either one or two hours. After incubation, 0.1 mL *C. difficile* was added to each well which marked time 0. Samples were taken at 0, 4 and 8 hours post addition of *C. difficile* and bacteria and phages were enumerated.

3.3. Results

3.3.1. Optimisation of model

3.3.1.1. Growth curve

In order for growth conditions in cell culture medium to be established, two different cultures of *C. difficile* strain AIU (R027) were compared. A colony of *C. difficile* was used to inoculate FA and BHI broth. The cultures were allowed to grow overnight before being used to inoculate 15 mL of BHI broth and 15 mL of maintenance medium. The OD_{550} and CFU/mL results can be seen in figures 3.1 and 3.2.

Figure 3.1 shows the growth curve determined by OD_{550} results. The BHI broth overnight culture into maintenance medium was the least successful as the OD_{550} levels did not increase. The FA broth overnight culture into cell culture medium showed growth albeit at a very slow rate with an OD_{550} of 0.268 finally being reached at 9 hours post inoculation (see figure 3.1). There was a further increase in growth observed by the 10 hour time point where the OD_{550} increased to 0.317. After 24 hours the OD_{550} had not increased implying that stationary phase had been reached. This is in complete contrast to what was observed in BHI broth. An OD_{550} of ~0.2 was reached after 2 hours incubation after inoculation from the FA broth overnight culture and 3 hours from the BHI broth overnight culture. The FA broth culture into BHI broth reached

stationary phase as early as 5 hours whereas the BHI broth inoculated with the BHI overnight culture reached stationary phase after 10 hours.

The CFU/mL results (see figure 3.2) confirmed that the *C. difficile* count was highest when grown in BHI broth, particularly when the overnight culture of FA broth was used. However, the results suggested that the bacteria were also able to grow in maintenance medium albeit with a resulting CFU/mL count much lower than that observed in BHI broth (figure 3.2). The CFU/mL results indicated that the stationary phase was reached 3 hours after inoculation, which also corresponded with the OD_{550} results. The OD_{550} reading showed no growth when the BHI broth overnight culture was used to inoculate the cell culture medium; however, CFU/mL results showed that after 24 hours a small level of growth was observed.

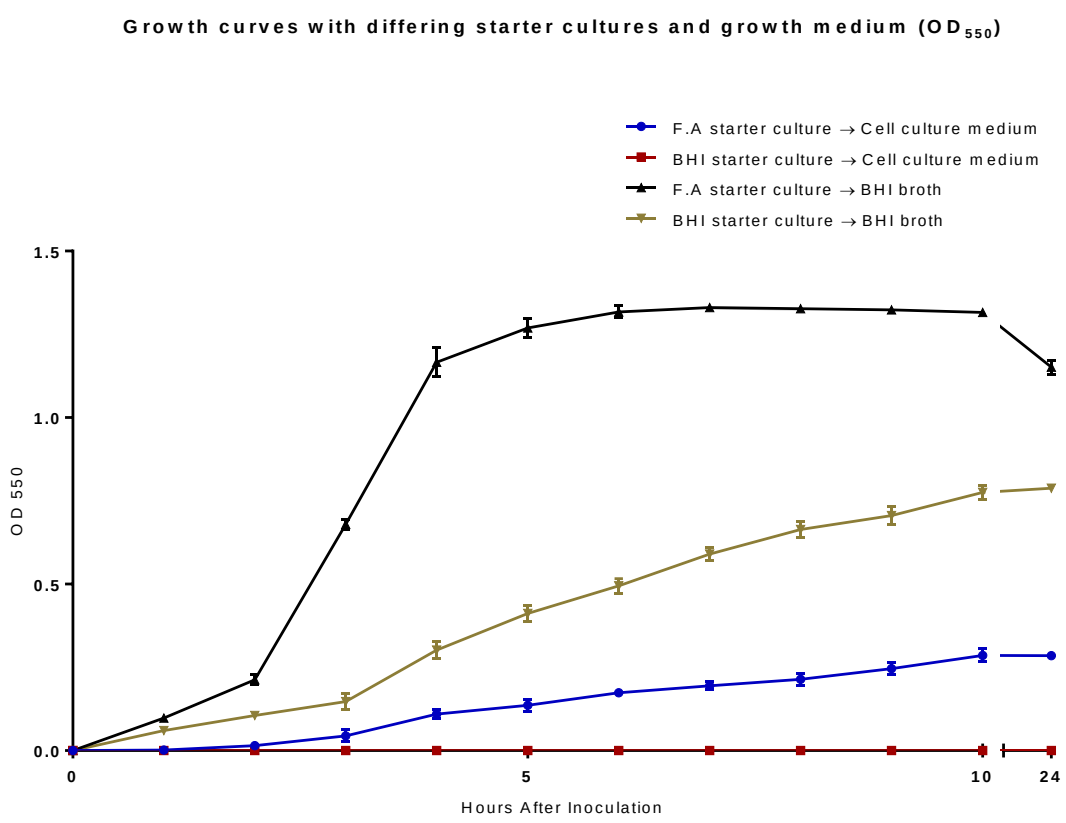

Figure 3.1: Growth curves showing the OD_{550} readings of *C. difficile* strain AIU from starter cultures containing fastidious anaerobic broth or BHI broth inoculated into cell culture maintenance medium and BHI broth.

Overnight cultures from FA broth and BHI broth were used to inoculate maintenance medium and BHI broth. OD_{550} readings were taken hourly for 10 hours and growth curves were plotted. For each growth condition tested, averages ± SEM (error bars) of OD_{550} were calculated from three biological replicates, each with three technical repeats.

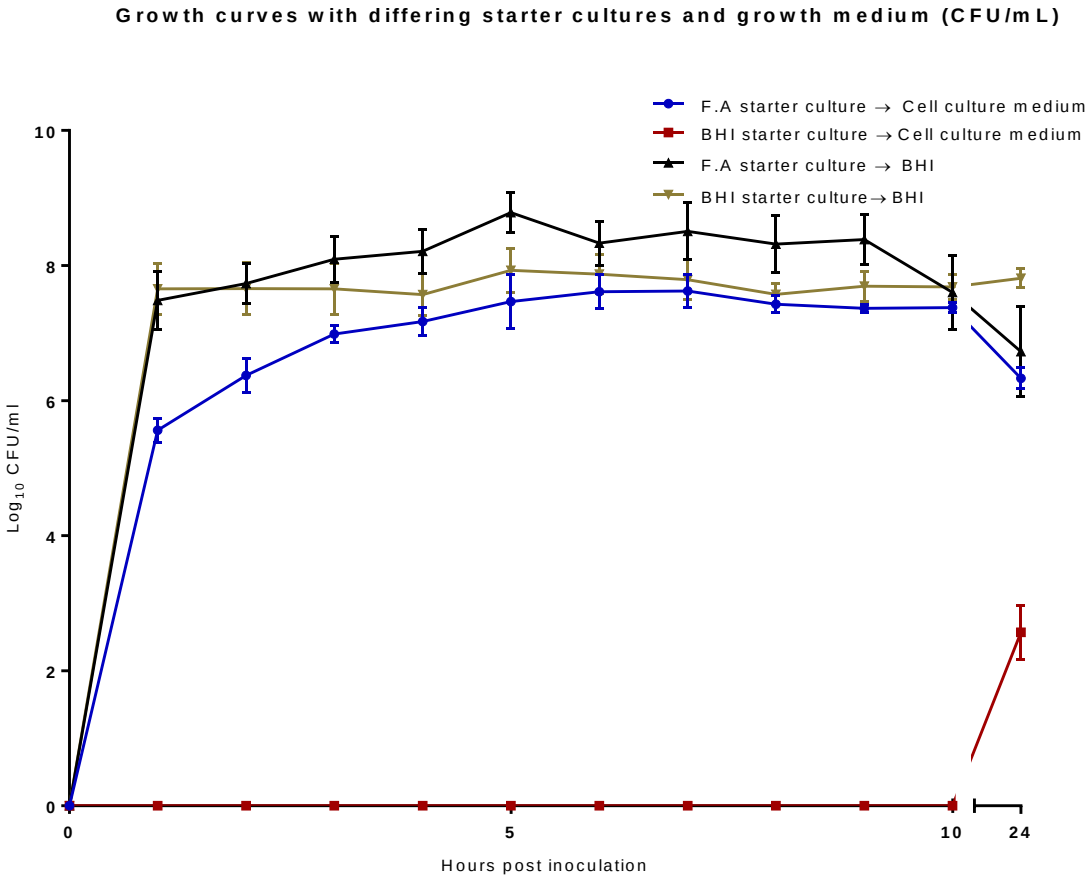

Figure 3.2: Growth curves showing the CFU/mL results of *C. difficile* strain AIU from starter cultures containing fastidious anaerobic broth or BHI broth inoculated into cell culture maintenance medium and BHI broth.

Overnight cultures from FA broth and BHI broth were used to inoculate maintenance medium and BHI broth. Samples were taken hourly for 10 hours, serially diluted (10 fold) and spotted using the Miles and Misra technique. Growth curves were plotted with the results. For each growth condition tested, averages ± SEM (error bars) of CFU/mL were calculated from three biological replicates, each with three technical repeats.

3.3.1.2. Determination of method for attached *C. difficile* enumeration

The method by which the epithelial cells and attached *C. difficile* were removed from the 24 well plate had to be determined in order to prevent false results for the bacterial count. It was essential to use a method that did not affect the CFU/mL count. Four methods were compared, three using chemical reagents and one using the cell scraper. Results are shown in figure 3.3. The use of 0.06 % Triton X-100 resulted in complete

C. difficile death with no bacteria being able to survive incubation with the reagent. PBS/EDTA and trypsin/EDTA displayed similar results to each other with a minimal log reduction in CFU/mL when compared to the control ($p \leq 0.05$). The scraping of the cells resulted in ~1 log reduction ($p \leq 0.01$). *C. difficile* in PBS without cells and planktonic levels of *C. difficile* in the supernatant were included as controls.

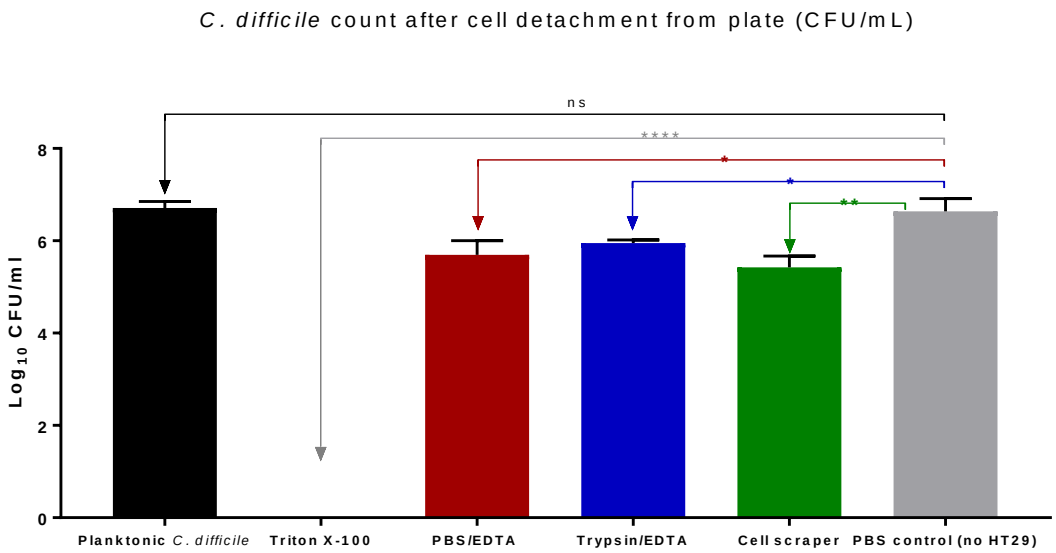

Figure 3.3: Graph showing the results of four different methods of epithelial cell detachment from the 24 well cell culture plate.

Comparison of methods of detachment of epithelial cells with attached bacteria from the cell culture plates. Maximum *C. difficile* recovery was required, therefore each method of epithelial cell and attached bacteria removal was directly compared with the PBS control containing bacteria only (no epithelial monolayer present). For each method, averages ± SEM (error bars) of CFU/mL were calculated from three biological replicates, each with three technical repeats. Mean values were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$, two stars to $p \leq 0.01$ and four stars to $p \leq 0.0001$. A non-statistically significant difference has been denoted with "ns".

3.3.1.3. Interactions of five ribotypes of *C. difficile* with CDHS1 and HT29 cells

Figure 3.4 shows the results of planktonic and attached *C. difficile* CFU/mL counts of different ribotypes with the bacteriophage CDHS1 in the presence of HT29 cells. At the 4 hour time point the attached levels of AIN (ribotype 005) showed a difference of ~0.5 log between phage treated and non-treated cells although this result was not statistically significant. AIN was the only strain that showed a reduction at 4 hours apart from AIU (ribotype 027) which was used as a control. No reductions in planktonic *C. difficile*

levels were observed in all other strains tested. The results at 8 hours post inoculation varied further. None of the strains showed a reduction in attached *C. difficile* levels; however, there was a reduction of ~1 log in planktonic levels of ATJ (ribotype 014/020) when CDHS1 was present (not statistically significant). There was also a reduction of planktonic levels of AIN (ribotype 005) at 8 hours ($p \leq 0.001$).

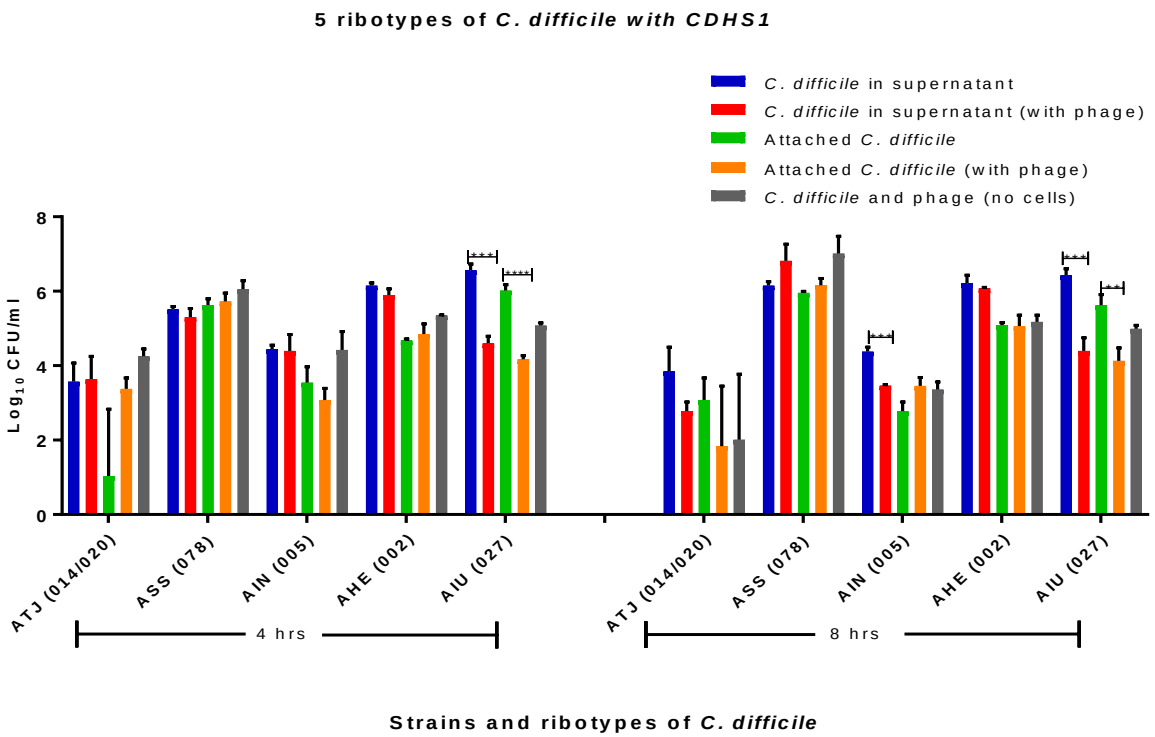

Figure 3.4: Comparison of 5 different *C. difficile* ribotypes with CDHS1 and HT29 cells.

The efficiency of the bacteriophage CDHS1 against five different *C. difficile* ribotypes in the presence of HT29 cells was compared. Ribotype 027 (strain AIU) was used a positive control. For each ribotype, averages ± SEM (error bars) of CFU/mL were calculated from three biological replicates, each with three technical repeats. Corresponding conditions were compared i.e. mean values of phage treated and non-treated *C. difficile* in supernatant were compared to each other. Likewise, mean values of phage treated and non-treated attached *C. difficile* were compared to each other. The mean values were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$, three stars to $p \leq 0.001$ and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

3.3.2. Interactions of non-specific bacteriophages with *C. difficile* strain AIU

Figure 3.5 is a graph illustrating how AIU responds to the phages CDHM3 and CDHM6 and HT29 cells. CDHS1 is included as a positive control. It is evident that in the presence of HT29 cells and both non-specific phages, there is no reduction in attached or planktonic levels of AIU. This is in contrast to the control which showed ~1.5 log reduction for both planktonic and attached *C. difficile* ($p \leq 0.01$ and $p \leq 0.0001$ respectively).

3.3.3. Comparison of interactions of three different bacteriophages with HeLa and HT29 cells

CDHM3, CDHM6 and CDHS1 were added to both HeLa and HT29 cells and enumerated over an 8 hour time course in order to assess what effect the presence of the two different cell lines had on the phage titre. Figure 3.6 shows the results of the three phages on HT29 cells and figure 3.7 shows the results of the three phages on HeLa cells. Out of the three phages, CDHS1 was the only phage that showed a reduction in titre in the presence of both HT29 and HeLa cells. When the phage titres were compared in the presence and absence of the cells, both showed differences. In HT29 cells, ~1 log difference was observed when CDHS1 was measured with the cells compared to without ($p \leq 0.01$ and $p \leq 0.001$) at 4 hours and 8 hours post inoculation respectively. A similar observation was observed with HeLa cells. The titres of CDHM3 and CDHM6 were not affected by the presence of the cells and remained constant throughout the time course for both cell lines.

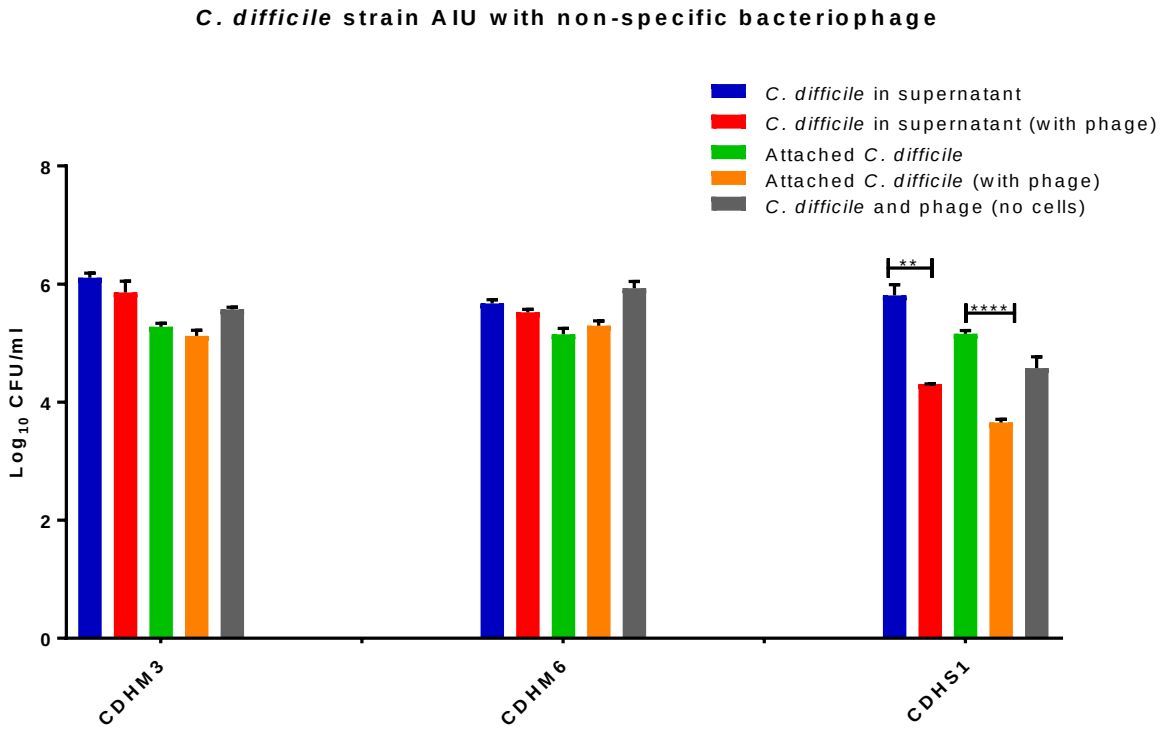

Figure 3.5: Comparison of the activity of phages CDHM3, CDHM6 and CDHS1 against AIU with HT29 cells.

The action of bacteriophages CDHM3, CDHM6 and CDHS1 against AIU with HT29 cells were compared, with CDHS1 acting as a positive control. For each phage, averages ± SEM (error bars) of CFU/mL were calculated from three biological replicates, each with three technical repeats. Corresponding conditions were compared i.e. mean values of phage treated and non-treated *C. difficile* in supernatant were compared to each other. Likewise, mean values of phage treated and non-treated attached *C. difficile* were compared to each other. The mean values were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

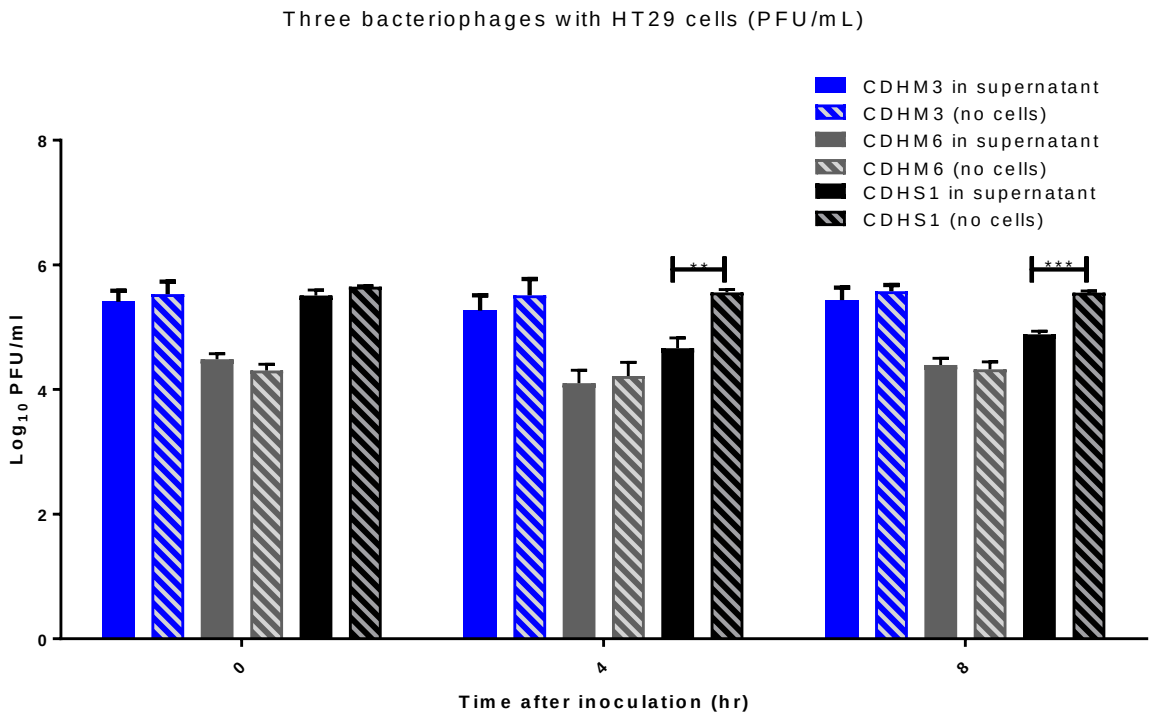

Figure 3.6: Observation of the dynamics of phages CDHM3, CDHM6 and CDHS1 with HT29 cells.

Bacteriophages CDHM3, CDHM6 and CDHS1 were added to HT29 cells and the PFU/mL count was enumerated for phages in the supernatant with and without cells. For each phage, averages ± SEM (error bars) of PFU/mL were calculated from three biological replicates, each with three technical repeats. Corresponding conditions were compared for each phage tested i.e. mean values of phages in the supernatant in the presence of HT29 cells compared to number of phages without HT29 cells. The mean values were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$and three stars to $p \leq 0.001$. Bars that are not marked with asterisks are not statistically significant.

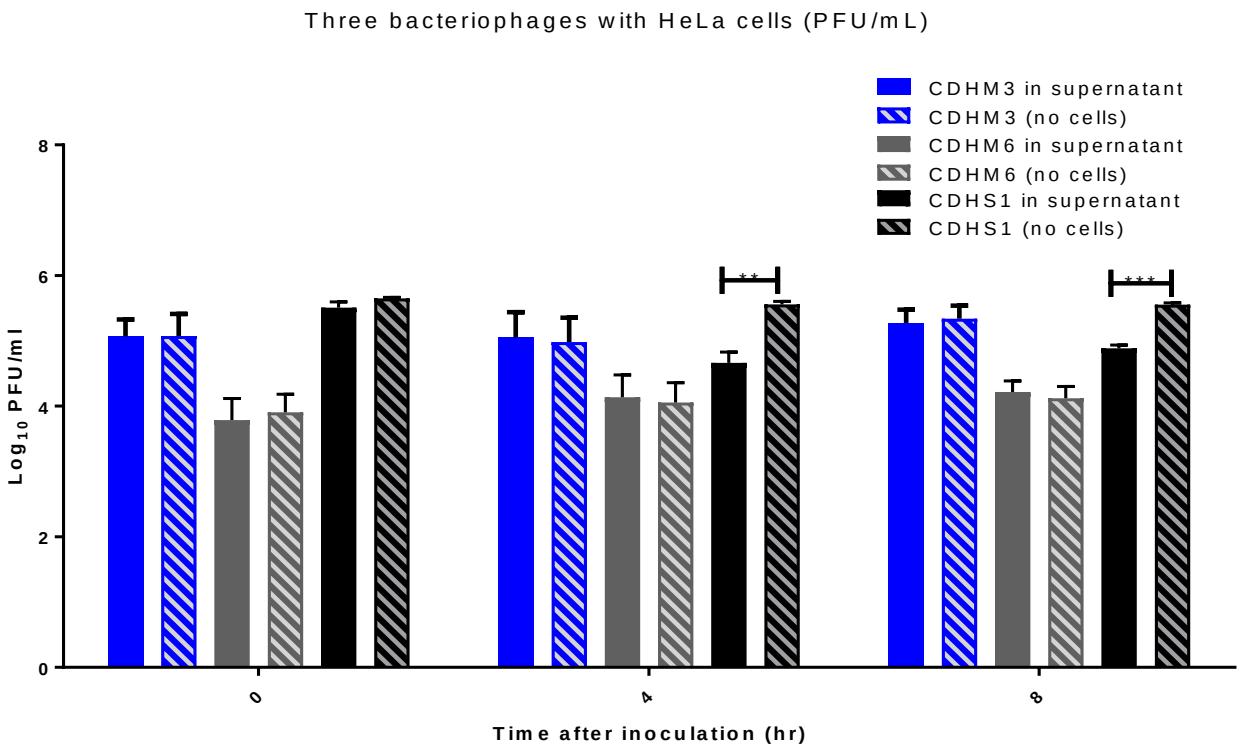

Figure 3.7: Observation of the dynamics of phages CDHM3, CDHM6 and CDHS1 with HeLa cells.

Bacteriophages CDHM3, CDHM6 and CDHS1 were added to HeLa cells and the PFU/mL count was enumerated for phages in the supernatant with and without cells. For each phage, averages ± SEM (error bars) of PFU/mL were calculated from three biological replicates, each with three technical repeats. Corresponding conditions were compared for each phage tested i.e. mean values of phages in the supernatant in the presence of HeLa cells compared to number of phages without HeLa cells. The mean values were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$and three stars to $p \leq 0.001$. Bars that are not marked with asterisks are not statistically significant.

3.3.4. Bacteriophage and *C. difficile* dynamics with HT29 and HeLa Cells

Figures 3.8 and 3.9 show the CFU/mL count of attached or planktonic *C. difficile* when bacteria and phages are added to either HT29 or HeLa cells simultaneously.

C. difficile was able to attach to both cell lines. This can be seen in both figures 3.8 and 3.9 where the CFU/mL of attached *C. difficile* increased after inoculation at time 0. However, *C. difficile* attached to HT29 cells faster than HeLa cells. Maximum bacterial attachment to HT29 cells took place 4 hours after inoculation. Attachment was slower with HeLa cells where there was a gradual increase over the time course with maximum attachment observed at 8 hours post inoculation. The differences observed implies that cell specificity influences how *C. difficile* interacts with the cells.

When phages were present, the attached *C. difficile* to HT29 count reduced by ~2 logs ($p \leq 0.0001$) at 4 hours post inoculation. There was ~1.5 log reduction at 8 hours ($p \leq 0.01$). A reduction of 1.75 logs was observed at 4 hours with HeLa cells ($p \leq 0.01$) which remained constant at 8 hours ($p \leq 0.01$). These differences could indicate that the choice of cells may also play a role in phage killing efficiency.

Similarly, the CFU/mL results of the planktonic *C. difficile* showed that bacteriophages were able to reduce *C. difficile* levels by approximately two logs with HT29 ($p \leq 0.001$) and HeLa ($p \leq 0.01$, $p \leq 0.001$) at 4 and 8 hours post inoculation.

It was clear that the presence of the HT29 cells increased the log reduction further as the *C. difficile* CFU/mL count was lower when the HT29 cells were present compared to the no cell control. In contrast, although a significant log reduction was observed in the presence of the HeLa cells, this reduction further increased when the HeLa cells were not present. This was evident for both attached and planktonic *C. difficile.*

Bacteriophages were unable to attach to either cell line. However, the phage count within the supernatant stayed constant with HT29 cells but dropped by ~0.25- 0.5 logs with HeLa cells. As seen in figure 3.8, a further observation was that the titre of phage CDHS1 was higher in the presence of HT29 cells when directly compared with the non-cell equivalent. The phage titre did not rise from the initial titre at time 0; however, the titre of the non-cell control steadily decreased over the 8 hour time course. This was in

contrast to the phage titre observed with the HeLa cells. The titre dropped in the presence of the cells by the 4 hour time point but rose again by 8 hours, although, the CDHS1 titre without the cells remained constant over the time course.

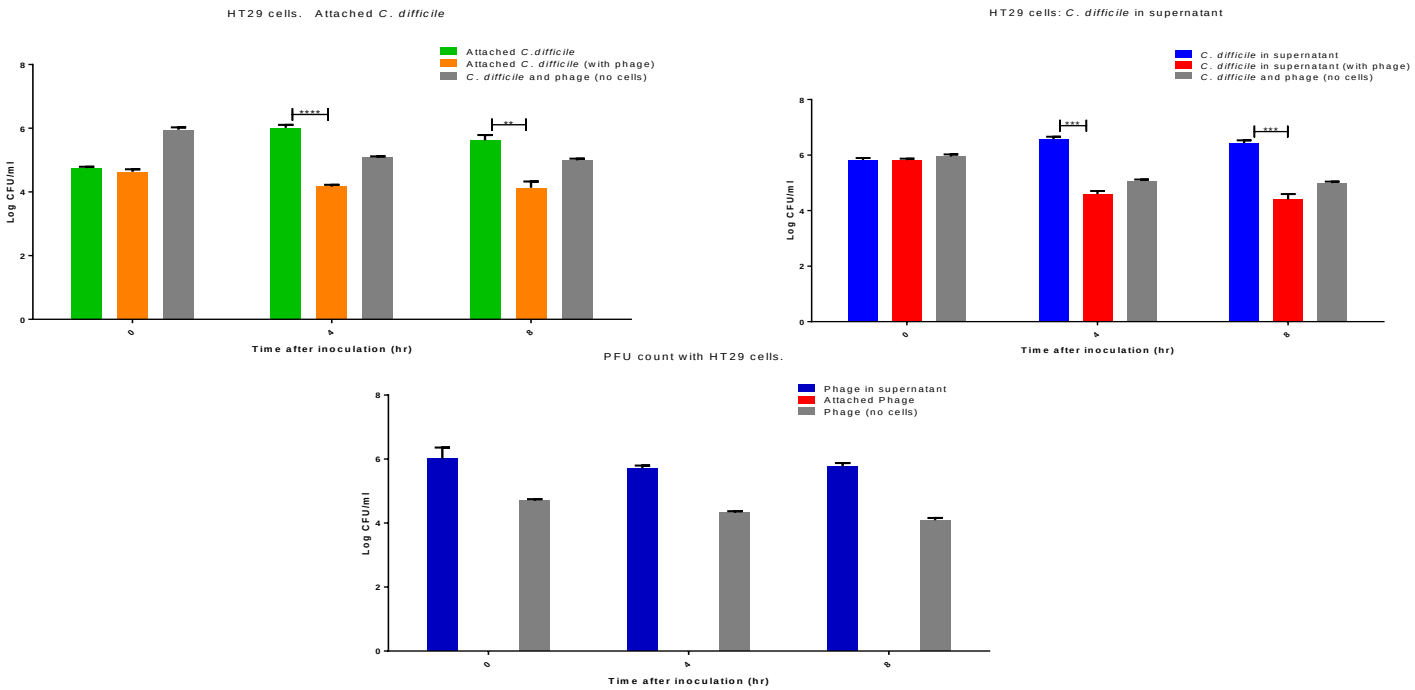

Figure 3.8: Graph showing AIU and CDHS1 with HT29 cells.

C. difficile AIU strain and CDHS1 phages were added to HT29 cells and both were enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached and planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU and PFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria (CFU/mL) were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$, three stars to $p \leq 0.001$ and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

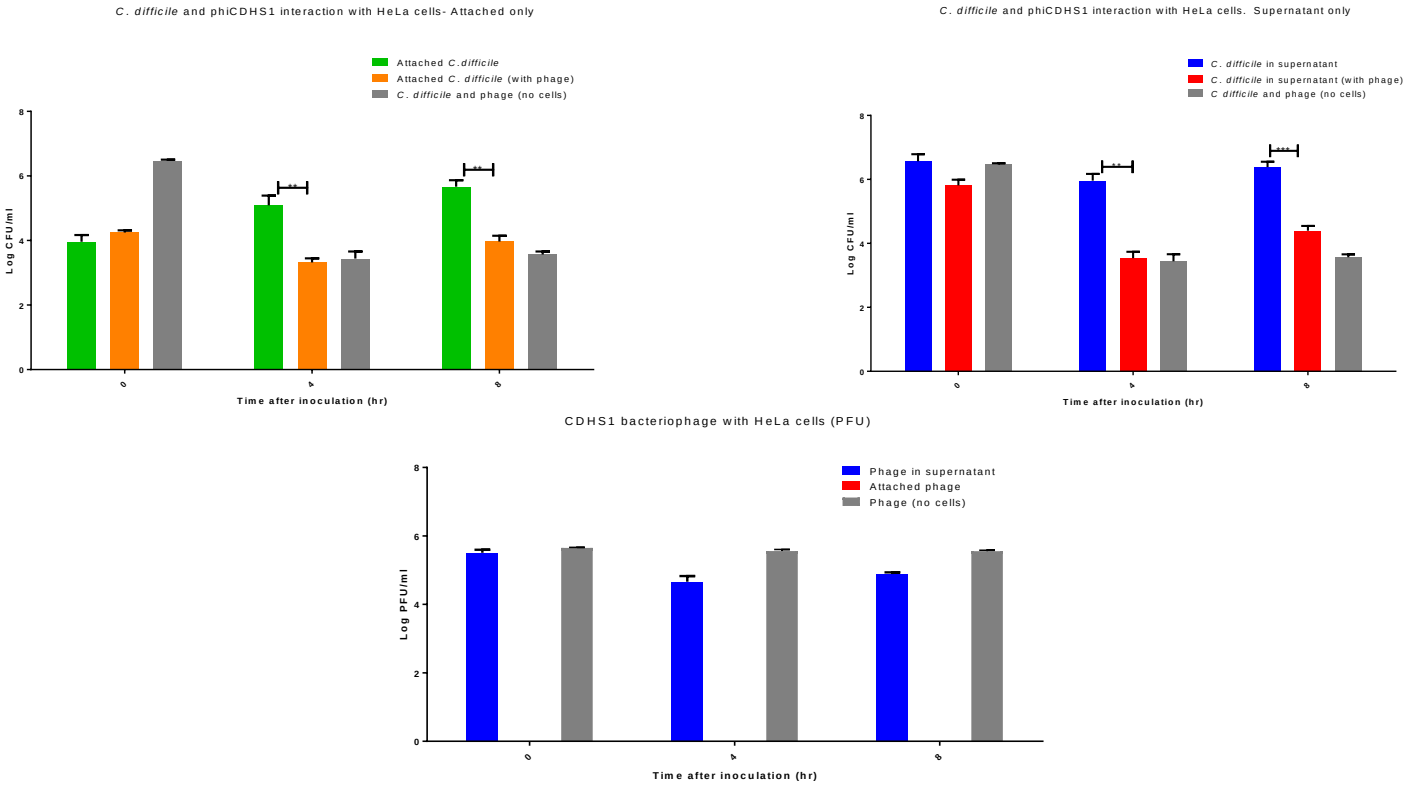

Figure 3.9: Graph showing AIU and CDHS1 with HeLa cells.

C. difficile AIU strain and CDHS1 phages were added to Hela cells and both were enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached and planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU and PFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria (CFU/mL) were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$ and three stars to $p \leq 0.001$. Bars that are not marked with asterisks are not statistically significant.

3.3.5. Prophylactic bacteriophage treatment

Figures 3.10 and 3.11 show the results of 1 and 2 hour phage pre-treatment regimens against *C. difficile* on HT29 cells. The 1 hour treatment regime showed a two log reduction in attached *C. difficile* ($p \leq 0.001$) compared to its non-phage equivalent at the 4 hour time point. By the 8 hour time point, only a one log reduction was observed ($p \leq 0.05$). This was in contrast to the results obtained with the 2 hour prophylactic treatment, where 0.75-1 log reduction in attached *C. difficile* was observed at both 4 and 8 hours post inoculation. Similarly, with the 1 hour prophylactic treatment, the planktonic levels of *C. difficile* displayed a two ($p \leq 0.01$) and one log ($p \leq 0.01$) reduction at 4 and 8 hours respectively whilst the 2 hour prophylactic treatment resulted in a 0.75 log reduction ($p \leq 0.05$) at 4 hours and ~0.5 log reduction (ns) at 8 hours post inoculation.

For both treatment regimens, there was no evidence of phage attachment to the HT29 cells, as no plaques were visible in the PFU/mL reading. The phage titre remained relatively consistent throughout the time course and for both treatment regimens, the titre was higher in the presence of cells compared to the no cell control.

The prophylactic treatment displayed reduced log reductions and significance levels compared to the results attained when phages and *C. difficile* were added to HT29 cells simultaneously (figure 3.8).

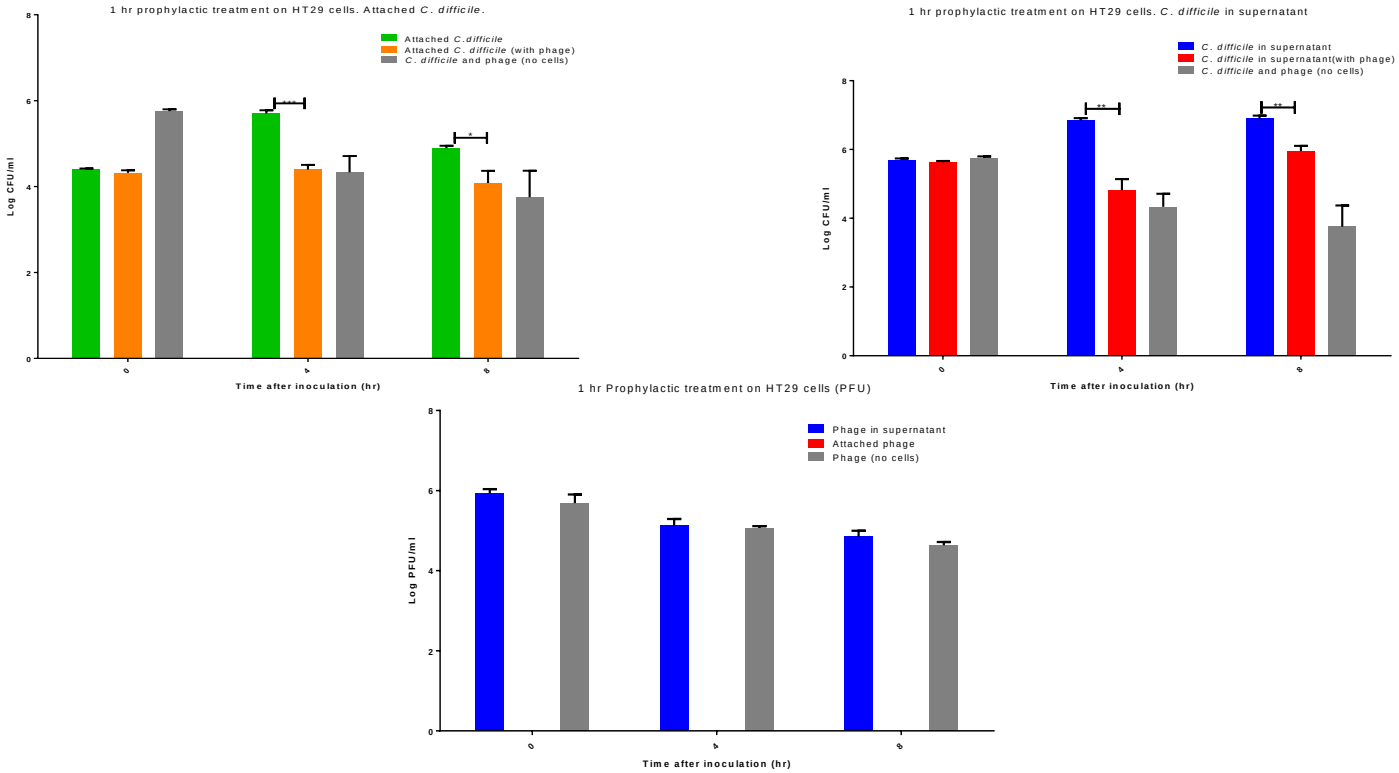

Figure 3.10: Graph showing 1 hour prophylactic treatment of AIU with CDHS1.

HT29 cells were treated prophylactically with CDHS1 bacteriophages before addition of *C. difficile* strain AIU 1 hour later.Both *C. difficile* and phages were enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached and planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU and PFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria (CFU/mL) were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$, two stars to $p \leq 0.01$ and three stars to $p \leq 0.001$. Bars that are not marked with asterisks are not statistically significant.

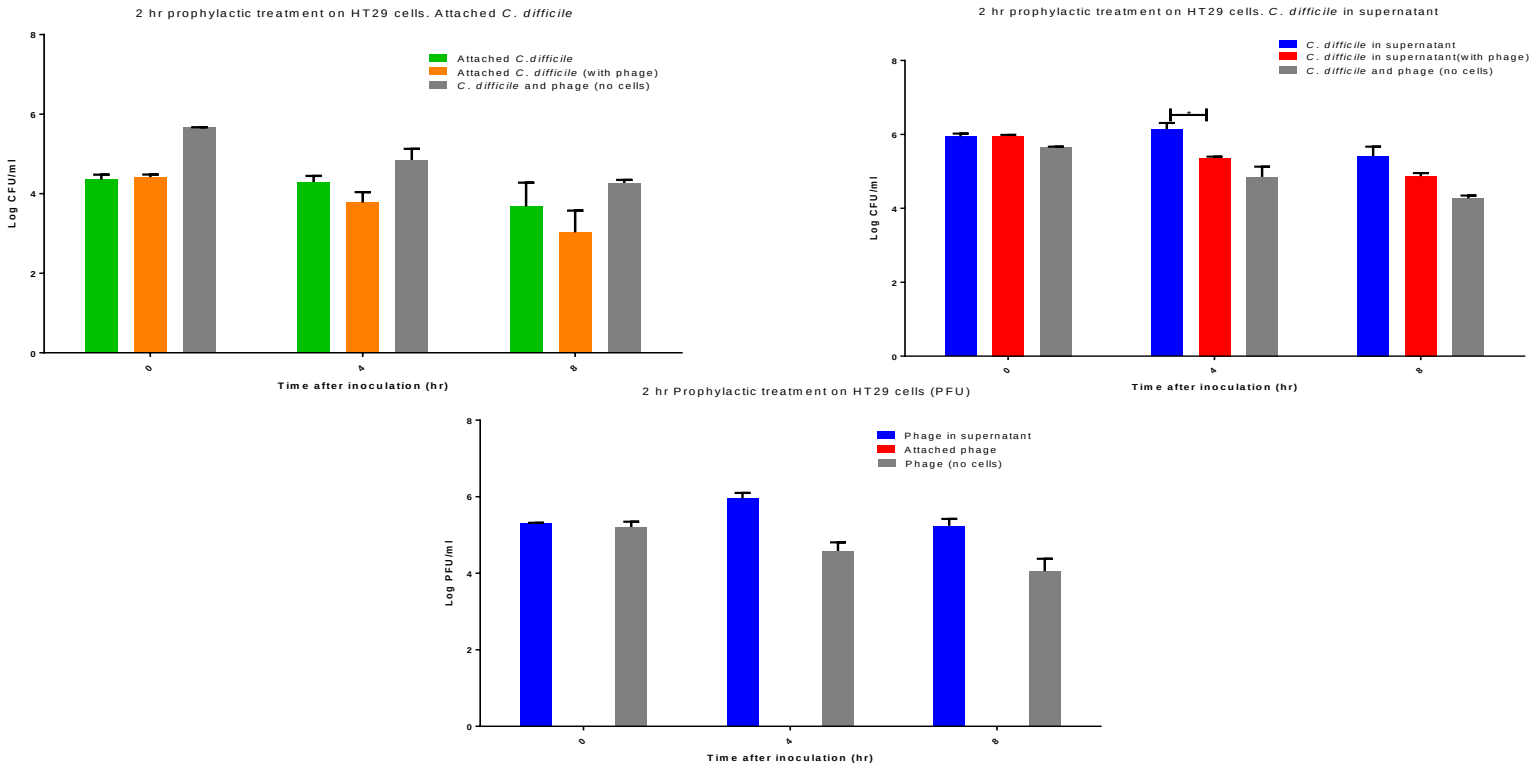

Figure 3.11: Graph showing 2 hour prophylactic treatment of AIU with CDHS1.

HT29 cells were treated prophylactically with CDHS1 bacteriophages before addition of *C. difficile* strain AIU 2 hours later. Both *C. difficile* and phages were enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached and planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU and PFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria (CFU/mL) were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$. Bars that are not marked with asterisks are not statistically significant.

3.4. Discussion

3.4.1. Summary of parameters previously established in Masters project

The Masters project that preceded this PhD project confirmed that both HT29 and HeLa cells were able to survive in anaerobic conditions for the 8-10 hour time frame of the experiment (Vukusic 2014). Epithelial cells typically prefer an environment at 36 °C with 5 % CO_2; however, this environment was not suitable for *C. difficile* or the bacteriophages (Helgason & Miller 2005). It was therefore necessary to ensure that all cell lines to be used in this project were able to survive for the duration of the experiment in a strict anaerobic environment. The viability of the cells was confirmed by measuring the amount of cytoplasmic lactate dehydrogenase (LDH) that was released into the medium (Shan et al. 2018) as LDH would only be released if the cell membrane had been damaged. The LDH value observed was less than 2 % of the maximum release within the 8 hour period for both HT29 and HeLa cells which was the same for both aerobic and anaerobic conditions (Vukusic 2014; Shan et al. 2018). Therefore it was confirmed that the cells were viable in the anaerobic environment for the required duration (Shan et al. 2018). The LDH value along with the use of microscopy was sufficient to confirm the integrity of the cell monolayer along with its viability.

3.4.2. Growth curves

It was necessary to minimise carryover of BHI medium into the cell monolayer. The presence of an excess quantity of BHI medium could subsequently compromise the viability of the cells and the integrity of the epithelial cell monolayer; therefore, it was necessary to devise a method by which *C. difficile* could reach exponential phase and determine whether its titre could be maintained when grown in maintenance medium. Consequently, growth curves were carried out in order to compare growth of *C. difficile* in its standard medium (FA broth and BHI broth) vs growth in maintenance medium which would be used for the study. The results are described in section 3.3.1.1. A general observation was that the growth in maintenance medium was inconsistent. For some replicates growth occurred but was significantly slower than that observed in the BHI medium, but in others no growth was observed at all. The inconsistency is likely to be because even though the cell culture medium is extremely rich it is lacking in the

specific nutrients required for *C. difficile* (Edwards et al. 2013; Nyenje et al. 2013) to reach an exponential rate of growth.

The OD_{550} results showed that *C. difficile* was unable to reach the exponential phase of growth unless the overnight culture was in FA broth and inoculated into BHI broth. In contrast, CFU/mL results indicated that there was a clear exponential phase for FA broth starter cultures inoculated into both BHI broth and cell culture medium. However, the titre of *C. difficile* was ~ 2 logs lower in cell culture medium than in BHI. It was important for the OD_{550} to be consistent and the corresponding CFU/mL results to be as high as possible to maintain the multiplicity of infection (MOI) throughout the experiment. The FA broth starter culture into BHI broth is the typical method in which *C. difficile* is grown for experimental use (Alfa & Sepehri 2013); however the use of BHI broth is detrimental to the epithelial cell monolayer (Shan et al. 2018). Therefore, in order to ensure the bacteria reached the necessary exponential growth for the study, *C. difficile* was allowed to grow in BHI medium until an OD_{550} of 0.2-0.3 was reached. At this point the culture was washed twice in maintenance medium by centrifugation at 4000 x g for 15 minutes at room temperature and the culture was used to inoculate the cells for the experiments. The CFU/mL of *C. difficile* was determined immediately to ensure that the bacterial count remained constant without dropping in value after the washing process. The procedure was implemented into the study for the remainder of the experiments to ensure *C. difficile* was grown to an exponential phase with minimal BHI broth carry over to the epithelial cells.

3.4.3. Determination of optimal method for cell detachment

It was necessary to establish a means by which attached *C. difficile* could be enumerated without the CFU/mL count being compromised thus leading to false results. Four methods of cell detachment were compared and contrasted for use within the cell model using both chemical reagents and physical means for removal of the epithelial cells and attached bacteria. The detergent Triton X-100albeit at a low concentration of 0.06 % was immediately ruled out due to the extreme effect it had on *C. difficile* viability. No bacteria could be recovered after 0.06 % Triton X-100 was added to the cells. Triton X-100 is a detergent that has the ability to dissolve lipids in the cell membrane, thus lysing cells (Holloway 1973). The cell membrane of *C. difficile* contains lipids (Kirk et al. 2017) and therefore it is likely that Triton X-100 lysed the bacteria despite being at a

low concentration. The cell scraping method allowed for a good level of *C. difficile* recovery from the cells, although, a one log reduction was observed. In addition, it was not an efficient process as it was time consuming due to each well having to be scraped individually. The results from Trypsin/EDTA and PBS/EDTA were comparable; however, PBS/EDTA was chosen to prevent the introduction of an additional reagent (trypsin). A further disadvantage for the trypsin/EDTA method is the need for the reaction to be stopped with growth medium which contains FBS (McAtter & Davies 2002). A higher concentration of FBS is required to inactivate the trypsin, the presence of which could subsequently compromise the *C. difficile* result. With the use of PBS/EDTA, once added to the cells and incubated, the samples can be diluted and plated immediately.

3.4.4. Interactions of five ribotypes of *C. difficile* with CDHS1 and HT29 cells

Four strains of *C. difficile* were selected to represent four ribotypes (002, 005, 014/020 and 078) that are all clinically relevant within UK (Burke & Lamont 2014). A further important justification behind the choice of the strains was they were carefully selected according to their relationship with the bacteriophage CDHS1. Strain AHE (ribotype 002) was chosen as CDHS1 is able to infect it with minimal adsorption (Thanki et al. 2018). CDHS1 is unable to infect the remaining three strains AIN (005), ATJ (014/020) and ASS (078), however it is able to adsorb 12 %, 17 % and 13 % respectively (Thanki et al. 2018). . The strain AIU from ribotype 027 is the propagation host of CDHS1 and so this phage is known to bind and infect this particular strain of *C. difficile* (Nale et al. 2012). Therefore, AIU and phage CDHS1 were used as a control combination for the experiment. As described in section 3.3.1.3, results showed that CDHS1 was able to reduce attached levels of AIN at 4 hours and planktonic levels of ATJ at 8 hours. This was a particularly interesting observation as CDHS1 was able to bind to the two strains but not able to infect them. Surprisingly, the CFU/mL count of AHE did not reduce in the presence of the CDHS1 despite the phage's ability to infect the strain. These results indicated that it was not necessary for bacteriophages to infect and bind to its bacterial host for a reduction in CFU/mL count to be observed. This widens the scope for selecting bacteriophages for therapeutic purposes, particularly for use in combination. Bacteriophage cocktails will be discussed further in section 6.3.1.1.

3.4.5. Interactions of non-specific bacteriophages with *C. difficile* strain AIU and HT29 cells

An experiment was designed to assess if non-specific phages were able to prevent *C. difficile* strain AIU from attaching to the HT29 monolayer. The 4 hour time point was selected as optimal attachment of *C. difficile* AIU strain to HT29 cells took place at this time and was therefore sufficient for comparisons to be made between bacteriophages tested. The phages were selected based on their morphology and adsorption rates to AIU. As CDHS1 is a siphovirus, two myoviruses were selected for comparison, CDHM3 and CDHM6 (Nale et al. 2015). In addition, the two selected phages showed a similar adsorption rate to AIU as CDHS1, although they are not able to infect the bacteria (Thanki 2016). Despite the similar adsorption rates between the three bacteriophages, neither of the myoviruses were able to reduce the attachment of AIU to HT29 cells. This indicated that the phage's ability to infect the bacteria was an important factor, but as mentioned in section 3.4.3, multiple bacteriophages with combinations of efficient infectivity and adsorption could be put to use in phage cocktails.

The results from the data sets discussed in sections 3.4.4 and 3.4.5 served multiple purposes in regards to the cell culture model and justified the use of CDHS1 and its *C. difficile* host for use in the model. The bacteria and phage combination not only resulted in efficient bacterial clearance within a broth culture but continued to work effectively together in the presence of cells. It also confirmed that for use in the epithelial cell model, bacteriophages ideally needed to be able to both adsorb and infect the bacteria for efficient clearing of bacteria and being able to do one or the other was not sufficient.

3.4.6. Comparison of interactions of three different bacteriophages with HeLa and HT29 cells

As mentioned previously, the titre of CDHS1 was the only one of the three bacteriophages tested that dropped in the presence of the epithelial cells. At this point it is possible to presume that this drop in titre is due to the phages attaching to the both cell lines. This assumption is feasible as the titres of CDHM3 and CDHM6 remained

constant whether the epithelial cells were present or not indicating that neither the cell culture medium nor the presence of the human cells were detrimental to the phage titre. However, at this stage it cannot be confirmed that the bacteriophages have attached to the epithelial cells as only samples from the supernatant were taken and therefore the attached phages were not enumerated.

3.4.7. *C. difficile* AIU and phage CDHS1 interactions with HT29 and HeLa cells

The comparison of interactions between *C. difficile* and the bacteriophage CDHS1 were carried out with HT29 and HeLa cell lines in order to determine whether the presence of a certain cell type further affected the dynamics indicating a level of specificity. As mentioned previously, HT29 cells are colon epithelial cells and HeLa cells are derived from cervical cancer cells; therefore the likelihood of this impacting the interactions between *C. difficile* and bacteriophages is high. When comparing the results described in section 3.3.4, it was clear that the cell type did not affect the overall trend. The bacteriophages were still able to lyse and kill the bacteria regardless of whether HT29 or HeLa cells were present as significant log reductions were observed for both. However, the presence of the cervical cell line did impair the phage activity against *C. difficile* as the bacteriophages were able to further reduce the CFU/mL count of *C. difficile* in the wells that were kept cell free. This confirmed that phages demonstrate a further level of specificity and were able to infect the gut bacteria more efficiently in the presence of location specific HT29 colon epithelial cells. Additionally, the attachment of bacteria to the cells was more efficient in HT29 cells than HeLa cells. This correlates with *C. difficile* being a gut bacteria that is prominent within the large intestine. The virulence factors (including the S layer and cell wall proteins, the fimbriae and flagella) that aid in colonisation and adherence of *C. difficile* to the GI cells and tissue are most likely to display specificity to the cell type (Awad et al. 2015).

The results showed that the phage titre in the supernatant remained constant in the presence of the HT29 cells but reduced without the cells. This was in contrast to the HeLa cells where the phage titre was lower in the presence of HeLa cells but remained constant without the cells. This further indicated a level of cell and phage specificity. It is possible that there are specific interactions that take place between CDHS1 and HT29 cells which do not take place with HeLa cells, causing the phage titre to drop in the presence of cervical cells compared to colon cells. There are surprisingly very limited

studies on the specific interactions between phages and epithelial cells. Kolotukha and Maliuta stated there was a "lack of bacteriophage or cell specificity" in terms of phage and cell interactions (Kolotukha & Maliuta 1979) when studying the bacteriophage lambda with HeLa, RH and Chinese hamster cells; however, it is not feasible to draw such a conclusion when limited cell lines have been tested. More recent research by Nguyen et al has demonstrated that bacteriophages have the ability to cross epithelial cell layers by means of transcytosis (Nguyen et al. 2017). The research group tested the efficiency by which the T4 bacteriophage could cross MDCK cells and the human cells A549 (lung), T84 and Caco-2 (gut), Huh7 (liver) and hBMec (brain) (Nguyen et al. 2017). However, the group did not compare the rates at which the process occurred and whether this varied between cell types, therefore, it was not possible to conclude whether there was a level of specificity displayed. To date there have been no phage receptors that have been reported or identified on mammalian cells (Shan et al. 2018), as most studies have focussed on the receptors on the bacterial hosts. Therefore, further research is required to determine if there are bacteriophage receptors present on human epithelial cells.

3.4.8. Prophylactic treatment

The 1 hour bacteriophage treatment on HT29 cells was far more effective than the 2 hour treatment, particularly on attached levels of *C. difficile*. Therefore all subsequent experiments in this project using the prophylactic treatment regimens were implemented with the 1 hour treatment plan.

As mentioned in section 3.3.5, the prophylactic treatment on HT29 cells appeared to be less effective in terms of reducing the *C. difficile* count compared to when phages and bacteria were added to HT29 cells simultaneously. It could be argued that bacteriophages and *C. difficile* are unlikely to encounter epithelial cells simultaneously within the intestine. However, as Minot et al stated, the GI tract is one of the largest reservoirs of phages in a human (Minot et al. 2013) and so the potential is there for high numbers of vegetative *C. difficile* and bacteriophages to be present in the gut simultaneously. On the other hand, the intestines contain a huge diversity of bacteriophages and therefore the specific phages that infect *C. difficile*, may only be present in smaller numbers.

3.5. Summary

The experiments in this chapter were implemented in order to further develop and optimise a cell culture model that mimicked the gut allowing for the study of phage and bacteria interactions. The growth conditions for *C. difficile* and the optimal method for detachment of the epithelial cell monolayer from the 24 well tissue culture plate were determined. Following this, further preliminary experiments were carried out to ensure a suitable phage-bacterial host combination were selected for use within the model. The optimised model was put to use and prophylactic treatment schedules were simulated.

Although the model was successfully optimised and put to use in this chapter, there are still many limitations at this point. The first and foremost is the lack of mucus in the current model. As stated in section 1.5.2, mucus is a prominent feature of the GI tract and plays an integral role in protecting the gut from invading pathogens. This concept will be addressed in the next chapter of this thesis where the mucus-producing epithelial cell line HT29-MTX-E12 has been incorporated into the model.

Chapter 4. Further development of epithelial cell model with use of HT29-MTX-E12 cells

4.1. Introduction

The previous chapter of this thesis introduced the development and optimisation of a HT29 epithelial cell model that could be used to elucidate the interactions that take place between *C. difficile* and bacteriophages. Until now the model has provided a rather simplistic view of the GI tract. The aim of this chapter was to recreate more accurate conditions that are observed within the GI tract with the introduction of a mucus producing cell line (mentioned below). The more accurate conditions were subsequently applied to the treatment regimens and the efficiency of phage treatment in varying mucus quantities was explored.

The epithelial cells of the GI tract are lined and protected with a layer of mucus which is part of the defence mechanism of the human digestive system. The development and optimisation of the HT29 cell culture model described in chapter 3 was carried out with non-mucus producing cell lines and the presence of mucus was not considered. In order to develop a more realistic model, it is therefore necessary to use an epithelial cell line which has the ability to produce mucus. The presence of mucus means that conditions within the GI tract can be mimicked and the efficiency of phage activity against *C. difficile* can be assessed within a more accurate environment.

The mucus within the GI tract acts as a physical barrier against food, the microbiota and pathogens (Johansson et al. 2013). The presence of a mucus layer needs to be taken into account when assessing the efficiency of phage therapy because the mucus layer has the potential to influence the way in which bacteriophages interact with bacteria (Mirzaei & Maurice 2017; Barr et al. 2013). It is possible that phages may adhere to the mucus layer which could have positive or negative effects on their ability to infect bacteria. If phages bound via their heads and their tails remained accessible they would be able to adhere to bacteria and cause lysis. The mucus layer could allow the bacteriophages to stay in this orientation for longer periods of time, resulting in a higher level of phage-bacteria interaction. In contrast, if the phages bound tail first their main receptors would be inaccessible leaving them unable to bind to the bacterial cells. In some

circumstances bacterial cells may even increase if the conditions are suitable. Another potential scenario is that bacterial cells may get embedded within the mucus layer deeming them inaccessible to the bacteriophages (Barr et al. 2013). Either way, the mucus layer has the potential to affect the therapeutic activity of phages against pathogens located within the large intestine and it is of paramount importance that more work is carried out in order to understand this further.

The experiments in chapter 3 were designed to demonstrate how a colon epithelial cell model could be used to observe the interactions between human cells, *C. difficile* and phages. This chapter concentrates on the development and refinement of the HT29 cell culture model. To further refine the model, the presence of mucus was considered for the cell culture model, therefore, from this point onwards HT29-MTX-E12 cells were used throughout. HT29-MTX-E12 cells are a derivative of the HT29 cells used in chapter 3 which have been adapted for growth in methrotrexate. They are able to produce mucus, thus making the cell model conditions more true to those observed in the GI tract. Methotrexate is traditionally used as an anti-cancer reagent and can be found in many chemotherapeutic agents. It acts by interfering with the cells' metabolic pathway by acting as an inhibitor of dihydrofolate reductase (DHFR), an enzyme which plays an essential role in the synthesis of DNA and some amino acids (Rajagopalan et al. 2002). HT29-MTX-E12 cells were a result of its parental cell line HT29 adapting to a medium containing 10^{-6} M of methotrexate which occurred over 6 months - the length of time required for HT29 cells to acquire the morphology and mucin producing characteristics of the goblet cells (Meader et al. 2010).

It was imperative that the subclone HT29-MTX-E12 was used as it forms an adherent mucus layer whereas HT29-MTX secretes mucins into the culture supernatant (Naughton et al. 2014). The presence of an adherent mucus layer was necessary in order to provide an environment closer to that of the GI tract so that the dynamics between phages and *C. difficile* could be further understood.

Another key concept introduced within this chapter is the concept of the mucus production of HT29-MTX-E12 cells increasing over a 21 day period. Lesuffleur et al stated that the cells were able to continue producing mucus over a period of time. The number of cells producing mucus increased each day until its maximum was reached at 21 days (Lesuffleur et al. 1993). An experiment was devised for this PhD project

linking with the results by Lesuffleur et al to determine the extent by which increased mucus production affected phage activity against *C. difficile*. The prophylactic and remedial treatment regimens were also taken into account for these experiments (see sections 4.3.2.2 and 4.3.2.3).

Another idea developed and tested in this chapter is the efficiency of bacteriophages that have bound to the epithelial cells. The phages were allowed to bind to the cells in the presence of increasing levels of mucus and interactions with *C. difficile* were observed.

4.2. Methods

4.2.1. Experimental Set up

As HT29-MTX-E12 cells grew at the same rate as HT29 cells, the cell culture flasks were maintained and plates seeded with 7 x 10^4 cells per ml as described in section 2.3.4. The remainder of the experimental set up remained unchanged and was carried out as described in section 3.2.3. The method for the prophylactic treatment experiments also remained the same (section 3.2.4). All experiments carried out using HT29-MTX-E12 cells were completed using bacteriophage CDHS1 with its host *C. difficile* strain AIU (ribotype 027).

4.2.2. Bacteriophage and *C. difficile* interactions with HT29-MTX-E12 cells

HT29-MTX-E12 cells were cultured and maintained as previously described in section 2.3.2. For the mucus production study, the experiments were carried out on days 0, 7, 14 and 21 post seeding of the 24 well microtitre plates. The plates were seeded as described in section 2.3.4 and the day 0 plates were used the following day. For the remaining time points the cells were incubated in the plates until their designated day of use. The maintenance medium was changed on alternate days until the cells were ready for use.

The use of bacteriophages as a remedial treatment was also taken into account, in addition to the general observations with phages and *C. difficile* being added to cells simultaneously and the prophylactic treatment option. Here, *C. difficile* was incubated

with HT29-MTX-E12 cells anaerobically at 37 °C for 1 hour, representing the start of a CDI. Following the incubation period, the bacteriophages were added, simulating treatment of the infection. *C. difficile* and phage enumeration was carried out as previously described.

4.2.3. Assessment of bound phage activity

This experiment was designed to assess the activity of phages that had bound to the epithelial cell monolayer. This was done by adding a wash step before the addition of *C. difficile* and therefore only phages that had attached to the cells (or entered the mucus layer) would be able to infect the bacteria.

Bacteriophages were added to the cells and the two were incubated together anaerobically for 1 hour at 37 °C. Prior to the addition of *C. difficile*, the wells were washed twice with dPBS to remove any unbound phages. The *C. difficile* and phage enumeration was completed as previously described.

4.3. Results

4.3.1. Replication of HT29 experiments with HT29-MTX-E12 cells

In order to ascertain that the mucus producing HT29-MTX-E12 cells were suitable for use it was necessary to repeat the experiments originally carried out on the non-mucus producing HT29 cells. These experiments were designed to be a direct replication of the experiments completed with HT29 cells during the optimisation process. This was to ensure that no adverse reactions caused by the change in cells occurred and that any future observations were most likely due to the presence of the mucus rather than for any other reason.

4.3.1.1. Bacteriophage and *C. difficile* added to HT29-MTX-E12 cells together

This experiment was repeated as previously described in section 3.3.4 for the non-mucus producing HT29 cells. The results were comparable to those attained from the experiments with HT29 cells. A reduction in planktonic CFU/mL of ~2 logs ($p \leq 0.0001$) at 4 and 8 hours post inoculation with phage treatment was observed.

Similarly, a two log reduction was observed at the 4 hour time point in the attached *C. difficile* count with phage treatment but this dropped to a 1.5 log reduction ($p \leq 0.001$) at 8 hours (figure 4.1).

For the attached *C. difficile* count, both the mucus producing and non-mucus producing cells showed a reduction in CFU/mL of two logs ($p \leq 0.0001$) at 4 hours implying that phage activity against attached *C. difficile* numbers was not affected by the presence of mucus. At the 8 hour time point, there was a smaller reduction between the phage treated and non- treated samples. A reduction of ~1.25 logs ($p \leq 0.01$) was observed, implying that phages were less active at 8 hours in the non-mucus producing cells. The trend was not repeated for the planktonic *C. difficile* count (figure 4.1). A bigger reduction of two logs was observed between phage treated and non-treated mucus producing cells ($p \leq 0.0001$) compared to the 1.5 log reduction in non-mucus producing cells ($p \leq 0.001$). These reductions remained consistent at the 8 hour time point. Therefore, the presence of mucus may affect the activity of bacteriophages on planktonic *C. difficile* levels but length of time may not be a contributing factor.

As observed with HT29 cells, no attached phages were detected in the mucus producing cells (figure 3.8) suggesting that the mucus does not cause the bacteriophages to visibly attach to the cells. The phage levels in the supernatant remained relatively consistent over the time course – a trend that was also observed with HT29 cells.

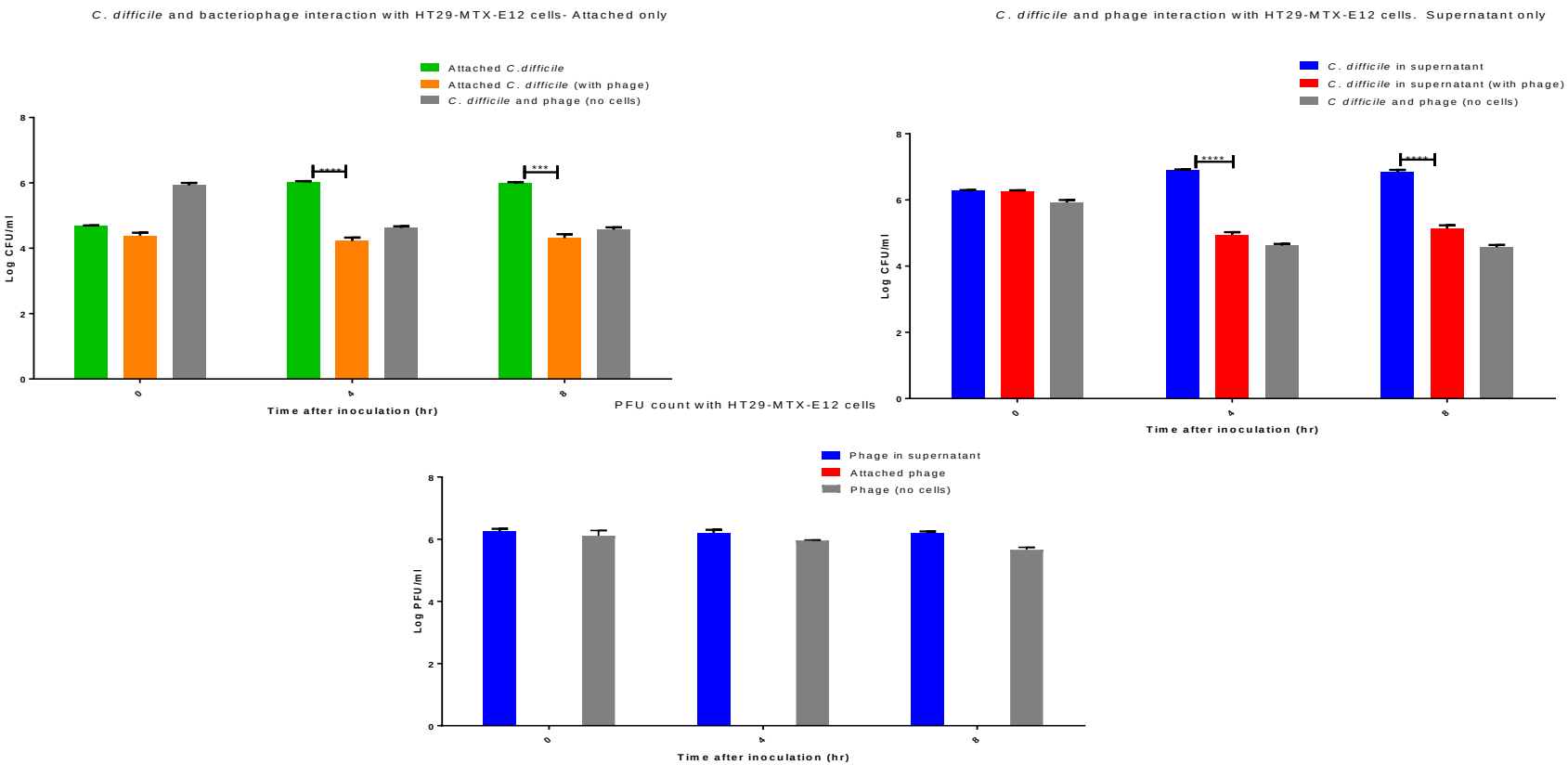

Figure 4.1: Bacteriophages and *C. difficile* added to HT29-MTX-E12 cells simultaneously.

HT29-MTX-E12 cells and *C. difficile* were treated with CDHS1 bacteriophages. Both *C. difficile* and phages were enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells where significant differences are observed on both planktonic and attached *C. difficile* with phage treatment. The efficacy of phages on attached and planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU and PFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria (CFU/mL) were used to statistically analyse the results using T-tests, with three stars being assigned to $p \leq 0.001$ and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

4.3.1.2. Prophylactic treatment of *C. difficile* infection using bacteriophages

Phages were applied to HT29-MTX-E12 cells prophylactically as described for HT29 cells in section 3.2.4. A 1 hour and 2 hour prophylactic treatment was applied to the cells prior to the addition of *C. difficile.* The results can be seen in figures 4.2 and 4.3.

For the 1 hour prophylactic treatment, ~1.5 log reductions were observed at the 4 hour time point in both attached and planktonic *C. difficile* (($p \leq 0.001$) and ($p \leq 0.0001$) respectively figure 4.2). There was no difference between phage treated and non-treated cells at 8 hours for both attached and planktonic *C. difficile* and the CFU/mL count between phage treated and non-treated cells remained more or less unchanged. The extra hour of prophylactic treatment did not make a difference to the results. The 2 hour prophylactic treatment resulted in ~1.5 log reduction at 4 hours for both attached and planktonic *C. difficile* (figure 4.3). The only difference observed was at 8 hours where a one log reduction ($p \leq 0.05$) in CFU/mL was observed between the phage treated and non- treated planktonic *C. difficile.*

The PFU/mL results showed no visible attachment of phages to the cells and a consistent number remained present within the supernatant throughout the time course. The results were similar to what was observed with HT29 cells.

Comparisons were made between results attained from HT29-MTX-E12 cells and its non-mucus counterpart. At this point, it was unclear whether the presence of mucus contributed to the efficiency of phages against both attached and planktonic *C. difficile.*

The results from the 1 hour prophylactic treatment experiment with HT29-MTX-E12 cells at 4 hours were comparable with its HT29 counterpart. However, at the 8 hour time point, no reduction was observed on the mucus cells but a reduction of one log was observed on HT29 cells for attached and planktonic *C. difficile.*

The results from the 2 hour prophylactic treatment on HT29 cells showed there was no reduction of attached *C. difficile* and only one log reduction for planktonic *C. difficile* at 4 hours. There was also no observed reduction of attached or planktonic bacteria at 8 hours (see figure 3.11). In contrast, there was ~1.5 log reduction observed at 4 hours for both attached and planktonic *C. difficile* and one log reduction observed at 8 hours in the mucus producing cells (figure 4.3).

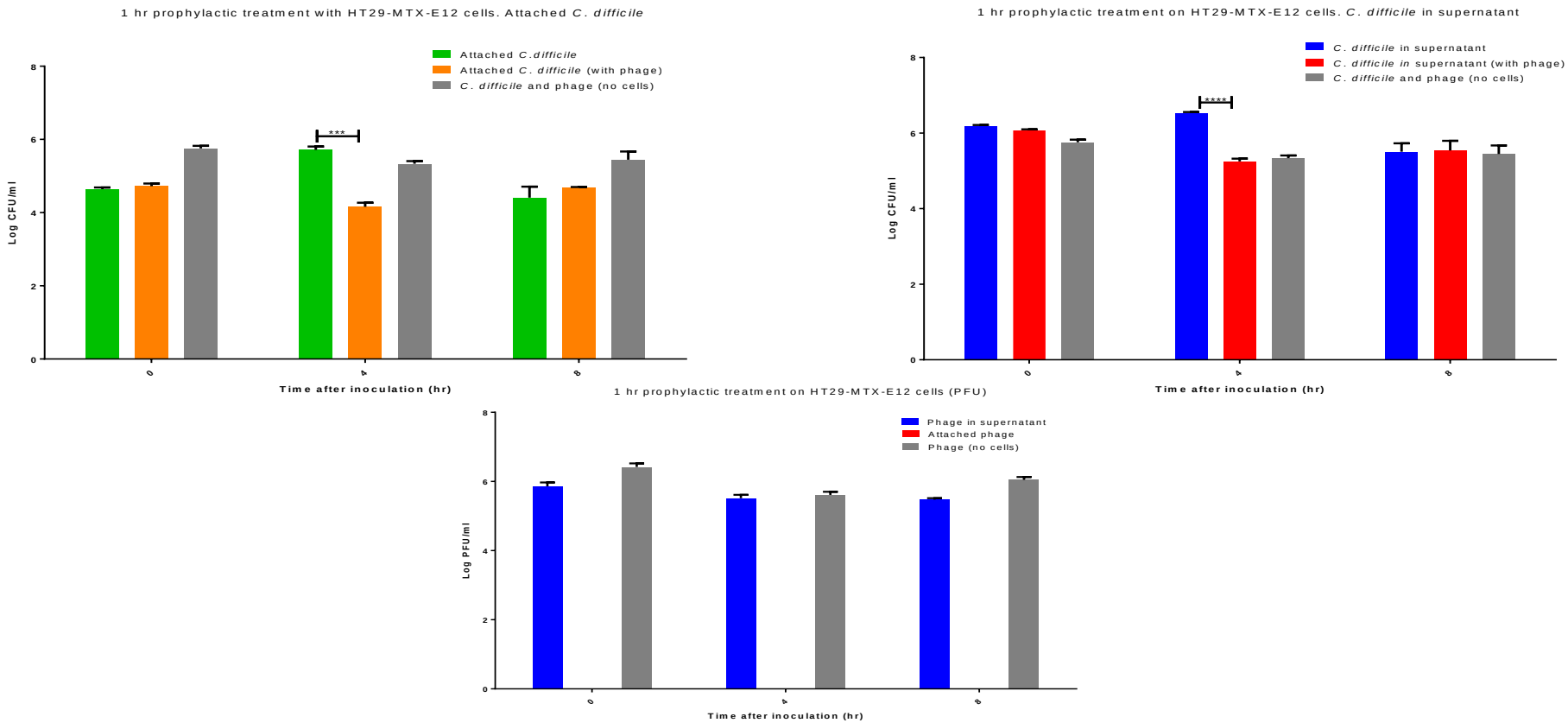

Figure 4.2: 1 hour prophylactic treatment of HT29-MTX-E12 cells

HT29-MTX-E12 cells and *C. difficile* were treated prophylactically for 1 hour with CDHS1 bacteriophages. Both *C. difficile* and phages were enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached and planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU and PFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria (CFU/mL) were used to statistically analyse the results using T-tests, with three stars being assigned to $p \leq 0.001$ and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

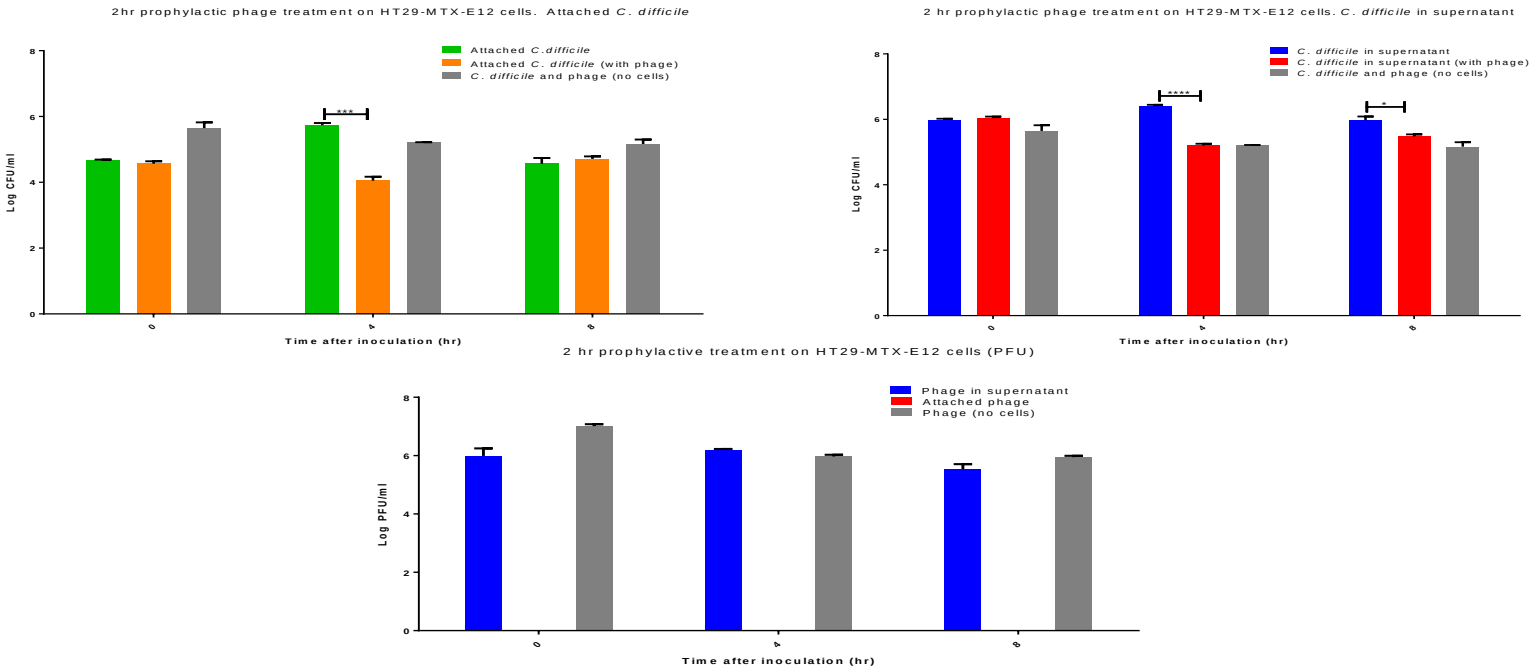

Figure 4.3: 2 hour prophylactic treatment of HT29-MTX-E12 cells

HT29-MTX-E12 cells and *C. difficile* were treated prophylactically for 2 hours with CDHS1 bacteriophages. Both *C. difficile* and phages were enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached and planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU and PFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria (CFU/mL) were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$, three stars to $p \leq 0.001$ and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

4.3.2. Observation of phage CDHS1 and *C. difficile* (AIU) dynamics with increasing mucus production

In these experiments I determined how phages interacted with *C. difficile* over 21 days as mucus levels increased. The mucus layer has the potential to influence the way in which bacteriophages act against the *C. difficile* and therefore needs to be considered. Each experiment was carried out on four separate days over a 21 day period in order to take the varying mucus levels into account.

4.3.2.1. Simultaneous addition of bacteriophage and *C. difficile*

For this particular experiment both *C. difficile* and bacteriophages were added to the cells simultaneously. The methods and time points were kept the same as previous experiments. Each experiment was carried out on days 0, 7, 14 and 21 indicating the length of time the cell monolayer was maintained for (described in section 4.2.2) so the effects of the varying mucus levels could be assessed.

Figure 4.4 shows the levels of attached *C. difficile* with and without phage treatment. The 4 hour post inoculation time point at day 0 had the largest log reduction of ~ 2 logs and was significantly different as demonstrated by the t-test ($p \leq 0.0001$). The subsequent experimental days (7, 14 and 21) showed a far smaller log reduction (0.5 log reduction for days 7 and 14 ($p \leq 0.01$)) and 1 log reduction for day 21 ($p \leq 0.001$)). Day 0 was the only experimental day where there was a visible log reduction of ~1 log ($p \leq 0.01$) in CFU/mL between phage treated and non-treated cells at 8 hours post inoculation. The other three experimental days showed no reduction and no statistically significant results at 8 hours for attached *C. difficile*.

Figure 4.5 shows the effect of varying mucus levels on phage activity on planktonic *C. difficile.* As previously noted with the attached *C. difficile* data, the biggest log reduction was seen on day 0 which was also the case for the planktonic *C. difficile.* There was a 2 log reduction between phage treated and non- treated wells at both 4 hours and 8 hours post inoculation ($p \leq 0.0001$). As the mucus levels increased over experimental days 7, 14 and 21, a few differences were noted. At 4 hours post inoculation, the log reduction reduced to ~1.5 logs at day 7 and 1 log at day 14 ($p \leq 0.0001$); however, at day 21 the log reduction diminished even further to ~0.5 log ($p \leq 0.001$). This trend was echoed at 8 hours post inoculation where there was ~1 log

reduction on day 7 ($p \leq 0.01$), ~0.5 log reduction on day 14 and no log reduction on day 21.

The PFU/mL results also showed key differences over the four experimental days (figure 4.6). The most obvious difference was the detection of attached phages. Attached phages were not detected until days 14 and 21. The PFU/mL increased by ~0.5 log between the 4 and 8 hour time point on days 14 and 21. Attached phages were not detected when *C. difficile* was not present, indicating that a key factor of the attachment process of bacteriophages to epithelial cells is the presence of the bacteria. It appears that *C. difficile* is required for the attachment of phages to the epithelial cells regardless of the quantity of mucus present. The phage titre in the supernatant remained consistent over the time course irrespective of the presence of *C. difficile* and did not show noticeable variation despite there being increasing amounts of mucus.

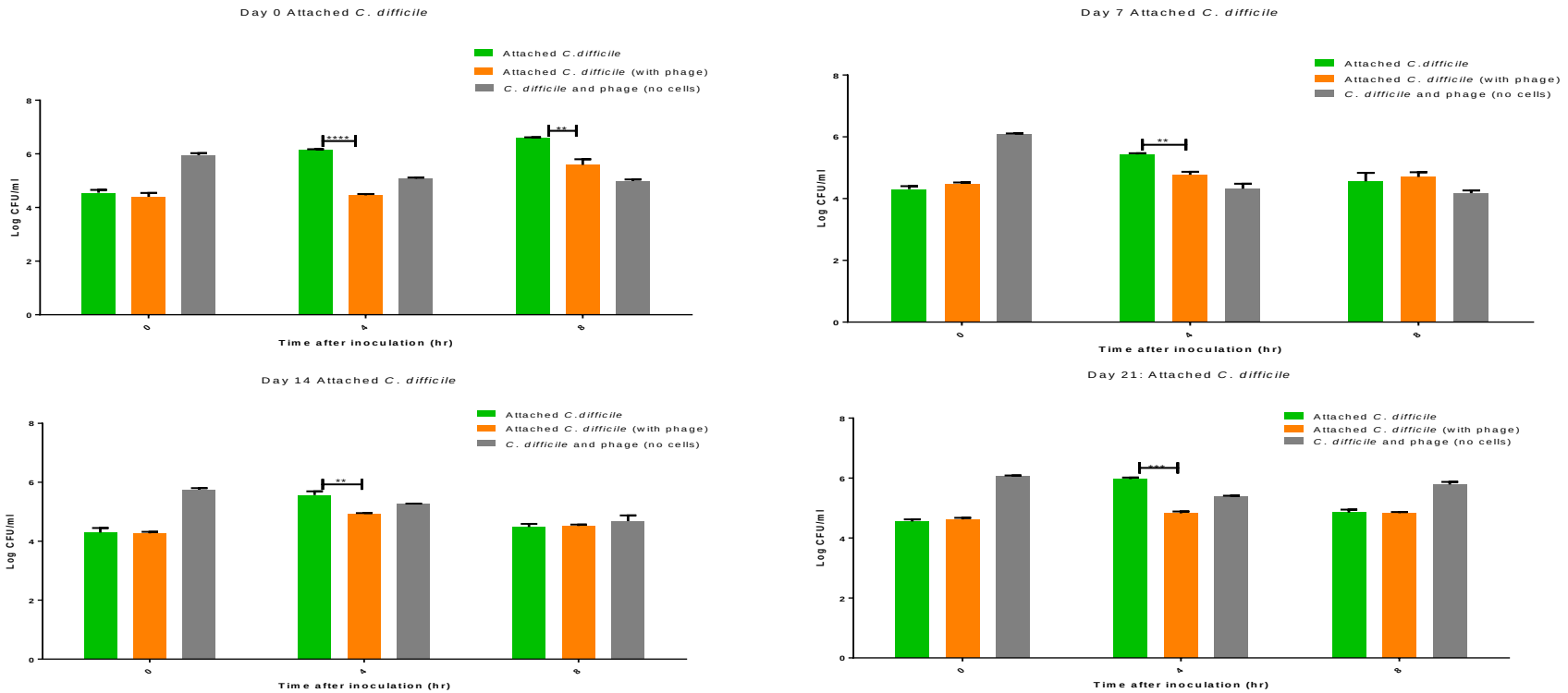

Figure 4.4: Attached levels of *C. difficile* with HT29-MTX-E12 cells observed over 21 days

The graphs show the CFU/mL counts of attached *C. difficile* on days 0, 7, 14 and 21. .HT29-MTX-E12 cells and *C. difficile* were treated with CDHS1 bacteriophages and comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$, three stars to $p \leq 0.001$ and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

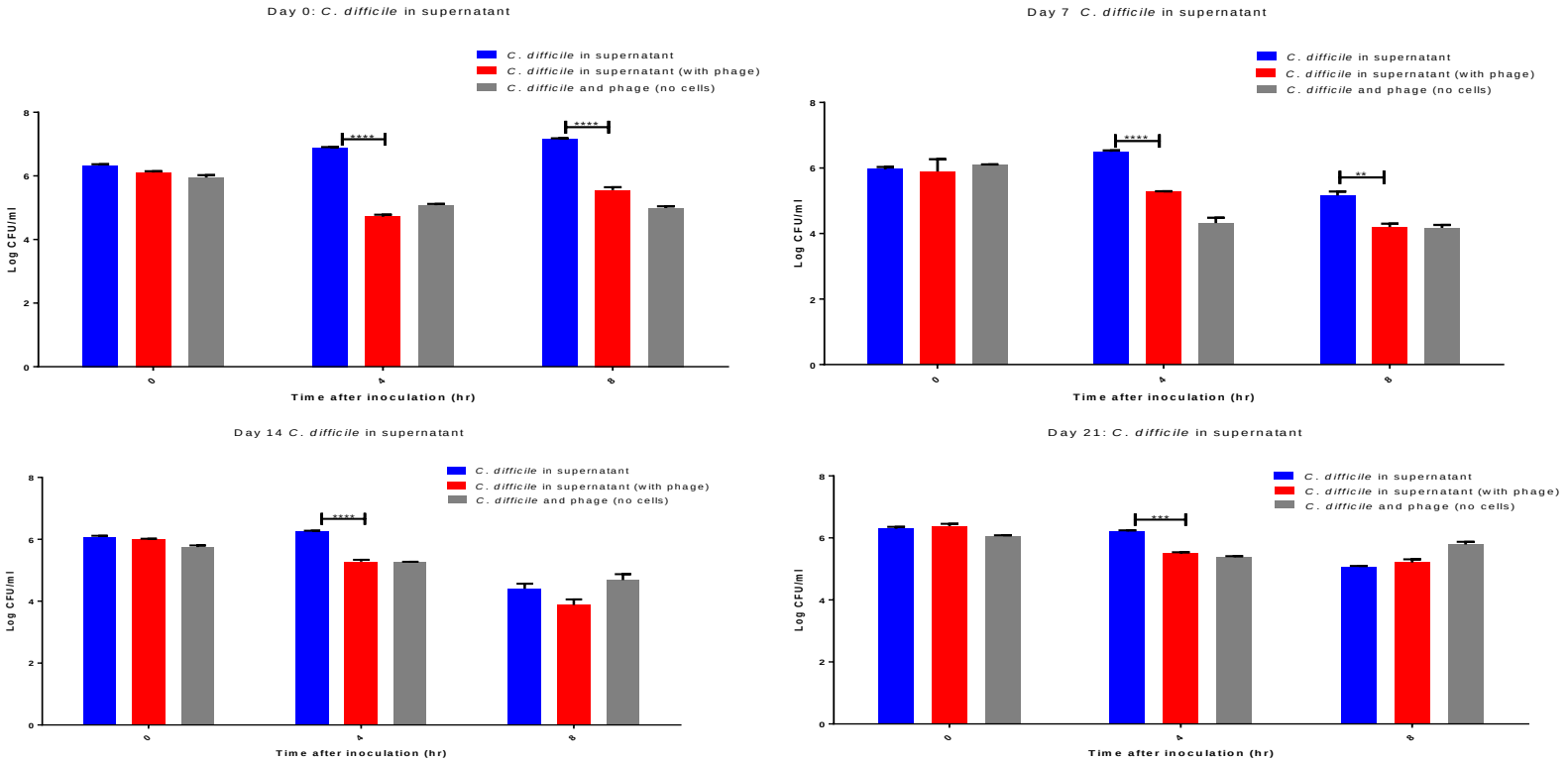

Figure 4.5: Planktonic levels of *C. difficile* with HT29-MTX-E12 cells observed over 21 days

The graphs show the CFU/mL counts of planktonic *C. difficile* on days 0, 7, 14 and 21. HT29-MTX-E12 cells and *C. difficile* were treated with CDHS1 bacteriophages. *C. difficile* was enumerated at T=0, 4 and 8 hours. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU /mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests, with two stars being assigned to $p \leq 0.01$, three stars to $p \leq 0.001$ and four stars to $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

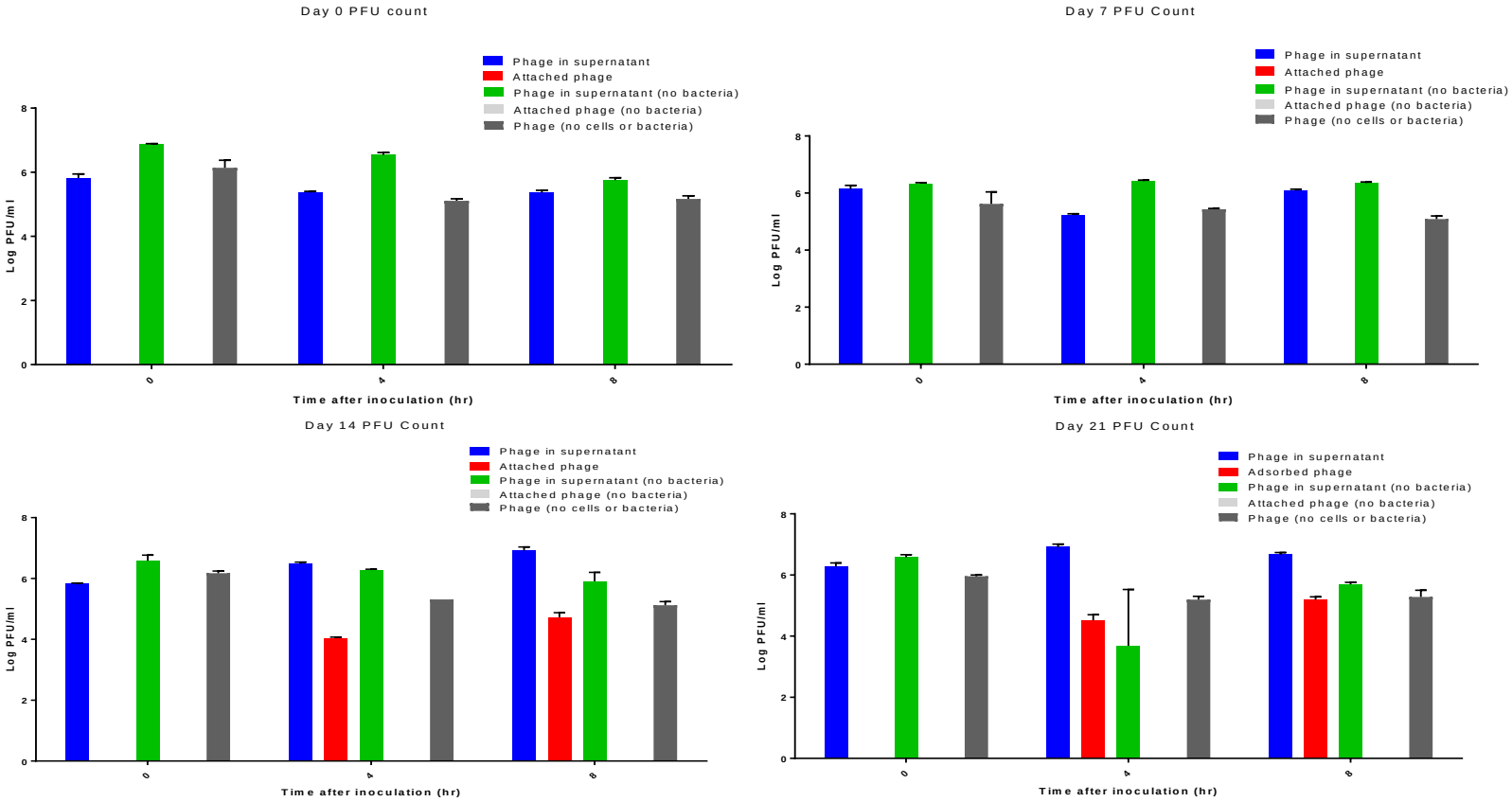

Figure 4.6: Bacteriophage counts with HT29-MTX-E12 cells observed over 21 days.

The graphs show the PFU/mL counts of bacteriophages on days 0, 7, 14 and 21. Averages ± SEM (error bars) of PFU/mL were calculated from three biological replicates, each with three technical repeats.

4.3.2.2. Bacteriophage prophylactic treatment

This experiment was designed to replicate a prophylactic treatment scenario against CDI. Bacteriophages were added to the epithelial cell layer and incubated 1 hour before the addition of *C. difficile* which simulated the start of the infection. The impact of the mucus levels was taken into account for the observations of this experiment.

The figures are separated into attached and planktonic CFU/mL values of *C. difficile*. Figure 4.7 shows the attached levels of *C. difficile.* For all experimental days (excluding day 7), the 4 hour post inoculation time point remained the optimum time for attachment of *C. difficile* to the cells. However, the differences in the levels of attachment between phage treated and non-treated appeared to be far less than what was observed in figure 4.4 where phages and *C. difficile* were added simultaneously. On day 0, there was a reduction of ~1 log ($p \leq 0.05$) between phage treated and non-treated cells which reduced to 0.5 log at 8 hours ($p \leq 0.05$). Day 7 revealed some unexpected results. Approximately ~0.5 log difference ($p > 0.05$) was observed between phage treated and non-treated wells at 4 hours post inoculation. Similarly, at 8 hours, a minimal reduction was observed. At day 14 a similar trend to day 0 was noted with ~1 log ($p \leq 0.01$) and 0.5 log ($p \leq 0.05$) reductions being recorded at 4 and 8 hours post inoculation respectively. At day 21 where the maximum quantity of mucus was expected, there was a 1 log reduction ($p \leq 0.001$) of attached *C. difficile* at 4 hours but no reduction observed at 8 hours.

Figure 4.8 shows the effect of the 1 hour prophylactic treatment on planktonic levels of *C. difficile.* On day 0 there was a reduction of ~ 1 log at 4 and 8 hours ($p \leq 0.01$ and $p \leq 0.05$). At the 4 hour time point of day 7 there was ~1 log reduction ($p \leq 0.001$). There was a small reduction of ~0.25-0.5 log at 8 hours ($p > 0.05$). On days 14 and 21, similar log reductions were observed at 4 hours with a 1 log reduction ($p \leq 0.001$) observed on both experimental days. At the 8 hour time point there was a reduction of ~1 log on day 14 and a much smaller reduction of ~0.25 log on day 21 ($p \leq 0.01$) .

The PFU/mL results (see figure 4.9) were similar to those observed for the experiment described in section 4.3.2.1. There were no visibly attached phages detected until days 14 and 21. As seen previously, there were also no attached bacteriophages detected in the absence of *C. difficile*.

The results of the 1 hour prophylactic treatment have revealed that the phages are able to act more efficiently against *C. difficile* whilst it is still in the supernatant. This indicates that once the bacteria has attached to the cells, there is a form of protection against the bacteriophages, reducing the rate at which the phages are able to infect and kill *C. difficile*. It is unclear at this stage whether the protection observed is from the mucus layer or from the act of attachment. It is also not possible to conclude whether increasing mucus levels have an impact on phage activity against attached or planktonic *C. difficile.*

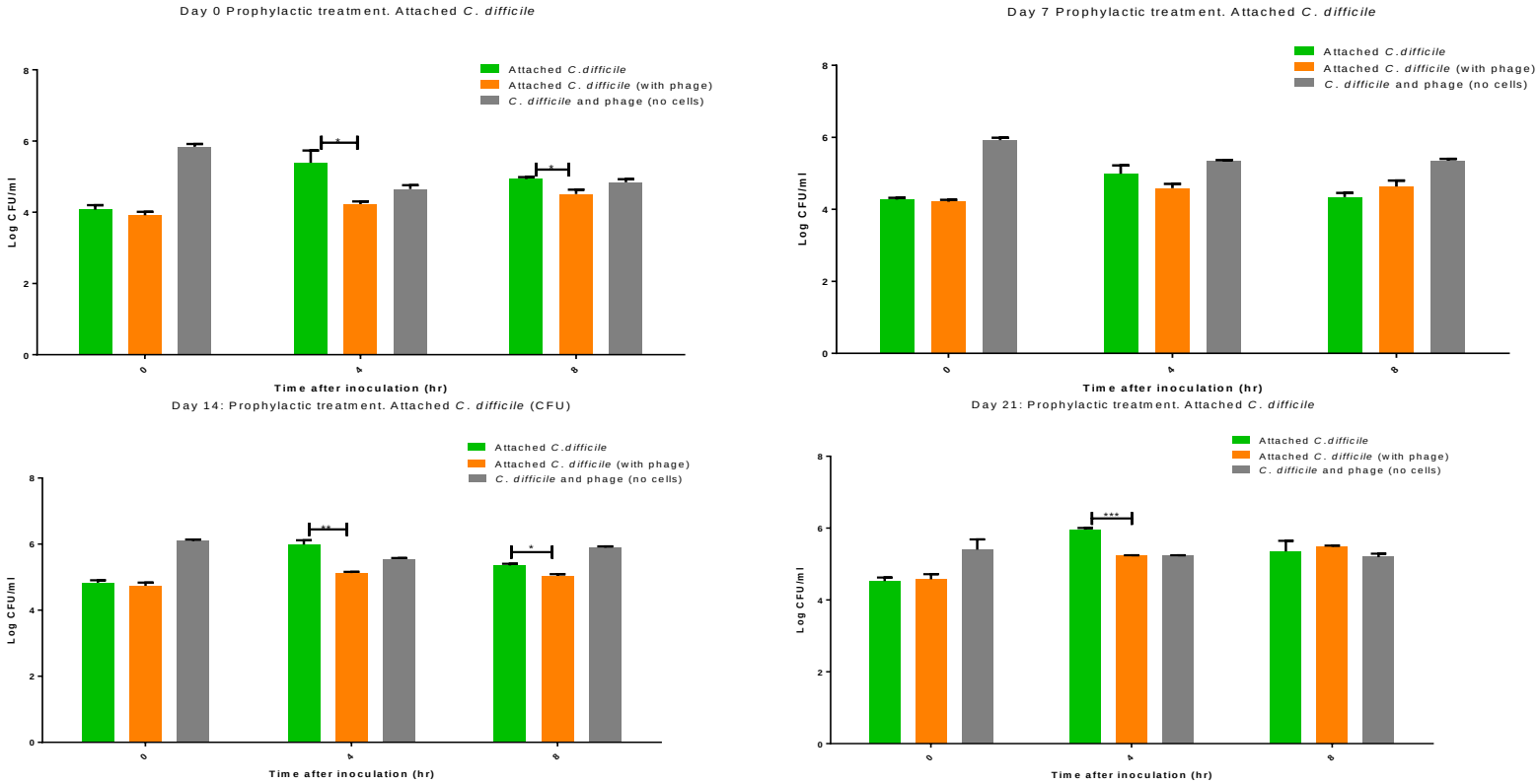

Figure 4.7: Attached levels of *C. difficile* over 21 days after a 1 hour bacteriophage prophylactic treatment

The graphs show the CFU/mL counts of attached *C. difficile* on days 0, 7, 14 and 21. HT29-MTX-E12 cells were treated with bacteriophages for one hour prior to the addition of *C. difficile*. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$, two stars to $p \leq 0.01$ and three stars to $p \leq 0.001$. Bars that are not marked with asterisks are not statistically significant.

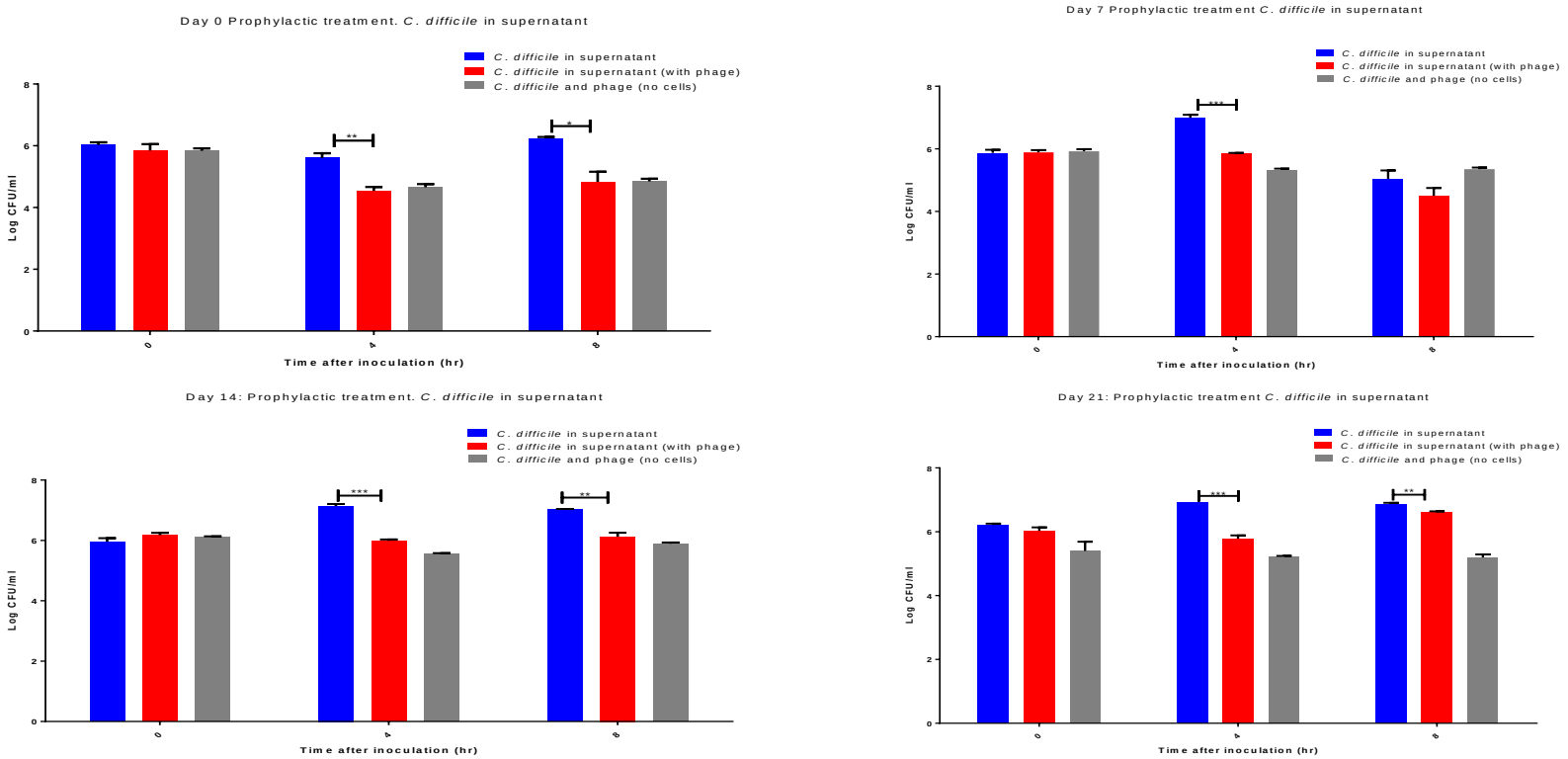

Figure 4.8: Planktonic levels of *C. difficile* over 21 days after a 1 hour bacteriophage prophylactic treatment

The graphs show the CFU/mL counts of planktonic C. difficile on days 0, 7, 14 and 21. HT29-MTX-E12 cells were treated with bacteriophages for one hour prior to the addition of C. difficile. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on planktonic C. difficile was assessed by measurement of CFU/mL. For each of the CFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$, two stars to $p \leq 0.01$ and three stars to $p \leq 0.001$. Bars that are not marked with asterisks are not statistically significant.

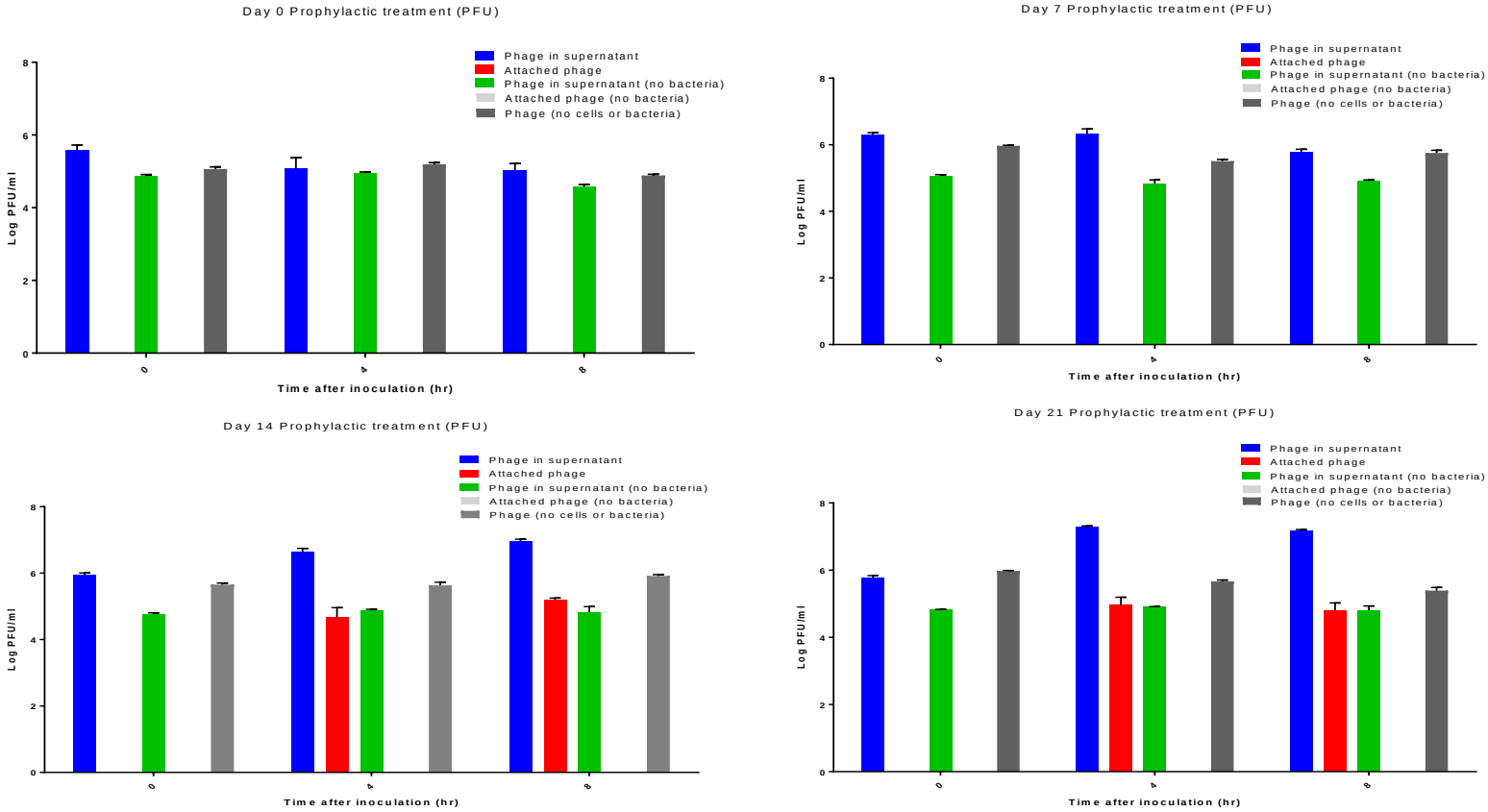

Figure 4.9: Bacteriophage counts over 21 days after a 1 hour bacteriophage prophylactic treatment

The graphs show the PFU/mL counts of bacteriophages on days 0, 7, 14 and 21 after a 1 hour prophylactic treatment with phages. Averages ± SEM (error bars) of PFU/mL were calculated from three biological replicates, each with three technical repeats

4.3.2.3. Bacteriophage remedial treatment

The remedial treatment experiment was designed to replicate an infection and treatment scenario. The infection was allowed to manifest first by inoculating the epithelial cell layer with *C. difficile* and incubating them together for 1 hour prior to the addition of phages. Once again, the difference in mucus levels was an additional parameter and so the experiments were repeated four times over a 21 day period.

The difference in log reduction of attached *C. difficile* between phage treated and non-treated wells for experimental days 0 and 7 were consistent (see figure 4.10). On both days, ~1 log reduction was observed at both 4 and 8 hours post addition of phages. The results from day 14 showed the biggest reduction of ~2 logs which occurred at both 4 and 8 hours ($p \leq 0.01$ and $p \leq 0.05$ respectively). At day 21 there was a 0.25-0.5 log reduction at 4 hours (ns) however, the reduction between phage treated and non- treated cells increased to ~1.5 log at 8 hours ($p \leq 0.01$).

Figure 4.11 shows the effect of remedial treatment with phages against planktonic *C. difficile*. There was a 2 log and 1.5 log reduction between phage treated and non-treated cells at 4 and 8 hours respectively ($p \leq 0.05$) on day 0. At day 7 there was a 1 log reduction at 4 hours which increased to 1.5 logs at 8 hours ($p \leq 0.05$). Day 14 saw 1.5 log reductions for both time points . Day 21 exhibited reductions of 1.5 and 2 logs at 4 and 8 hours respectively ($p \leq 0.01$).

The PFU/mL results (figure 4.12) showed a key difference between the results from this set of experiments and those from the prophylactic experiments and the experimental set where phages and *C. difficile* were added to the cells simultaneously. Attached phages were detected on days 0 and 7 in contrast to the experiments described in sections 4.3.2.1 and 4.3.2.2 where attached phages were only detectable on days 14 and 21 (figures 4.6 and 4.9). It is probable that the attached PFU/mL count on days 0, 7 and 14 at the 0 hour time point were anomalies as it is highly unlikely that phages would be able to attach that quickly after being added to the epithelial cells with *C. difficile*. The presence of larger error bar for those readings (see figure 4.12) confirms that the values are likely to be anomalies. Once again there were no attached phages detected when *C. difficile* was absent, further implying that the attachment of bacteriophages to the cells is somewhat dependent to the presence of *C. difficile*.

The efficiency of phages was more consistent in the remedial experiments regardless of whether *C. difficile* was attached or in the supernatant. It appeared that in a scenario where CDI was allowed to manifest first, the phages were able to act as efficiently against both attached and planktonic bacteria.

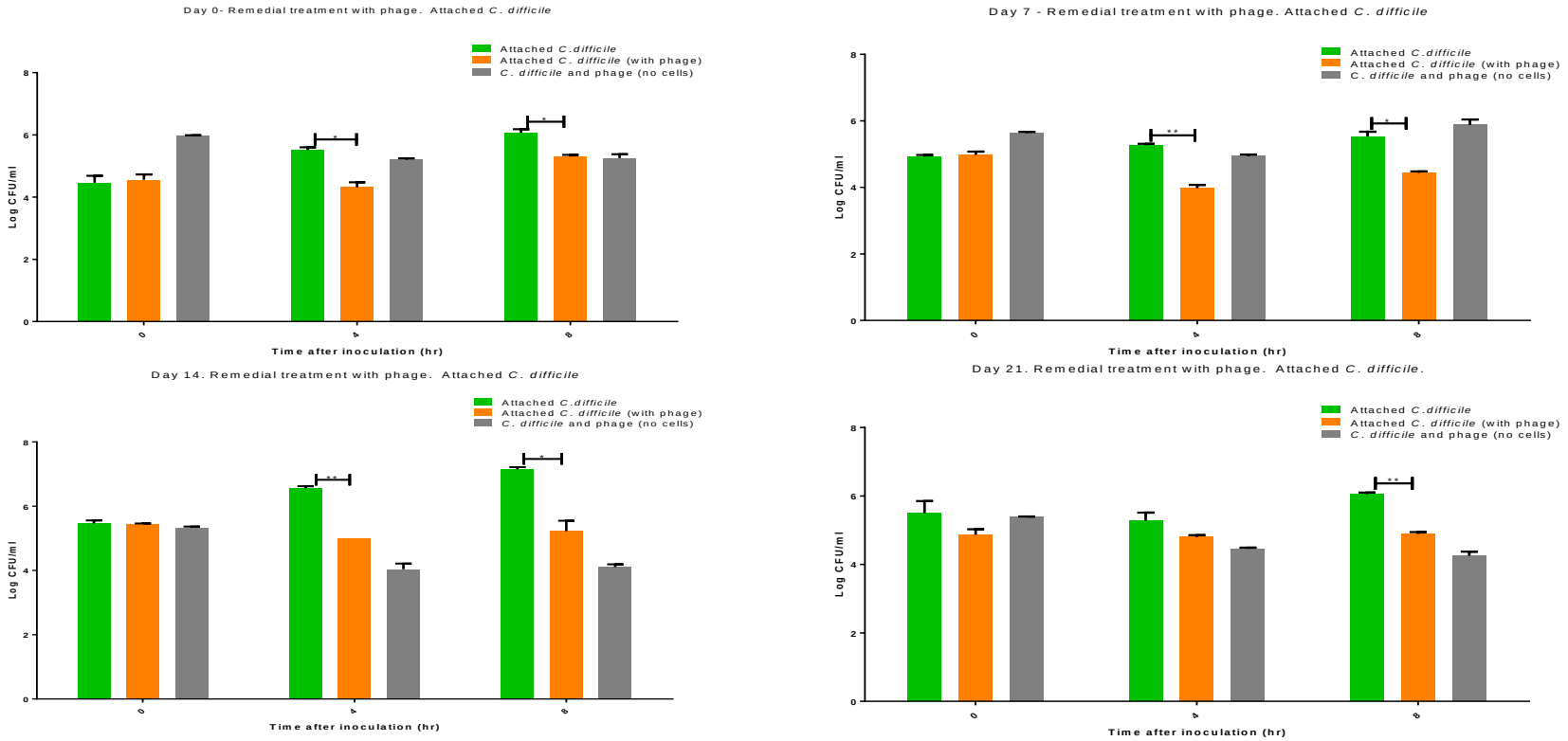

Figure 4.10: Attached levels of *C. difficile* over 21 days after remedial treatment with bacteriophages 1 hour after infection with *C. difficile*

The graphs show the CFU/mL counts of attached *C. difficile* on days 0, 7, 14 and 21. HT29-MTX-E12 cells were treated with bacteriophages one hour after the addition of *C. difficile*. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$ and two stars to $p \leq 0.01$. Bars that are not marked with asterisks are not statistically significant.

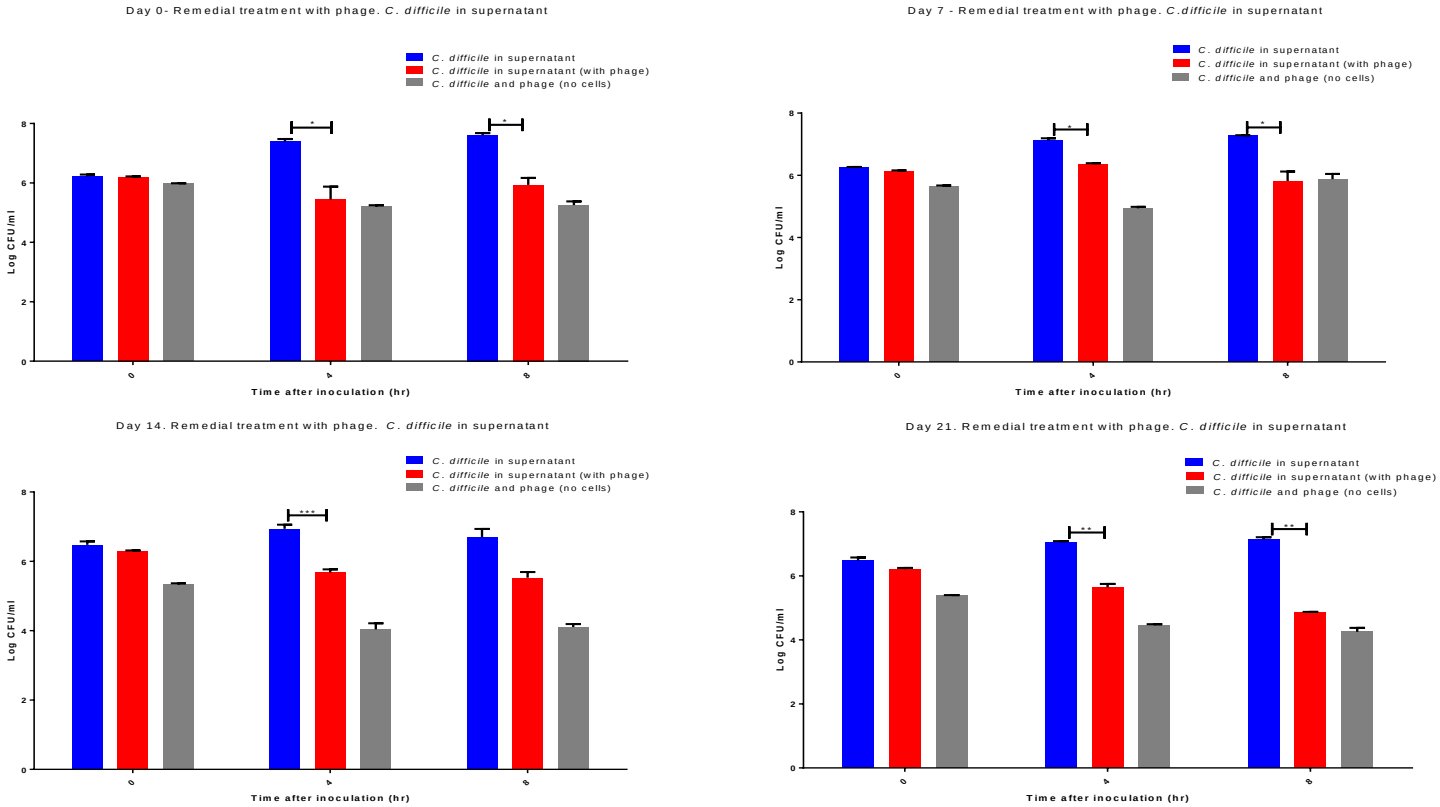

Figure 4.11: Planktonic levels of *C. difficile* over 21 days after remedial treatment with bacteriophages 1 hour after infection with *C. difficile*

The graphs show the CFU/mL counts of planktonic *C. difficile* on days 0, 7, 14 and 21. HT29-MTX-E12 cells were treated with bacteriophages one hour after the addition of *C. difficile.* Comparisons were made between phage treated and non-treated wells. The efficacy of phages on planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests, with one star being assigned to $p \leq 0.05$, two stars to $p \leq 0.01$ and three stars to $p \leq 0.001$. Bars that are not marked with asterisks are not statistically significant.

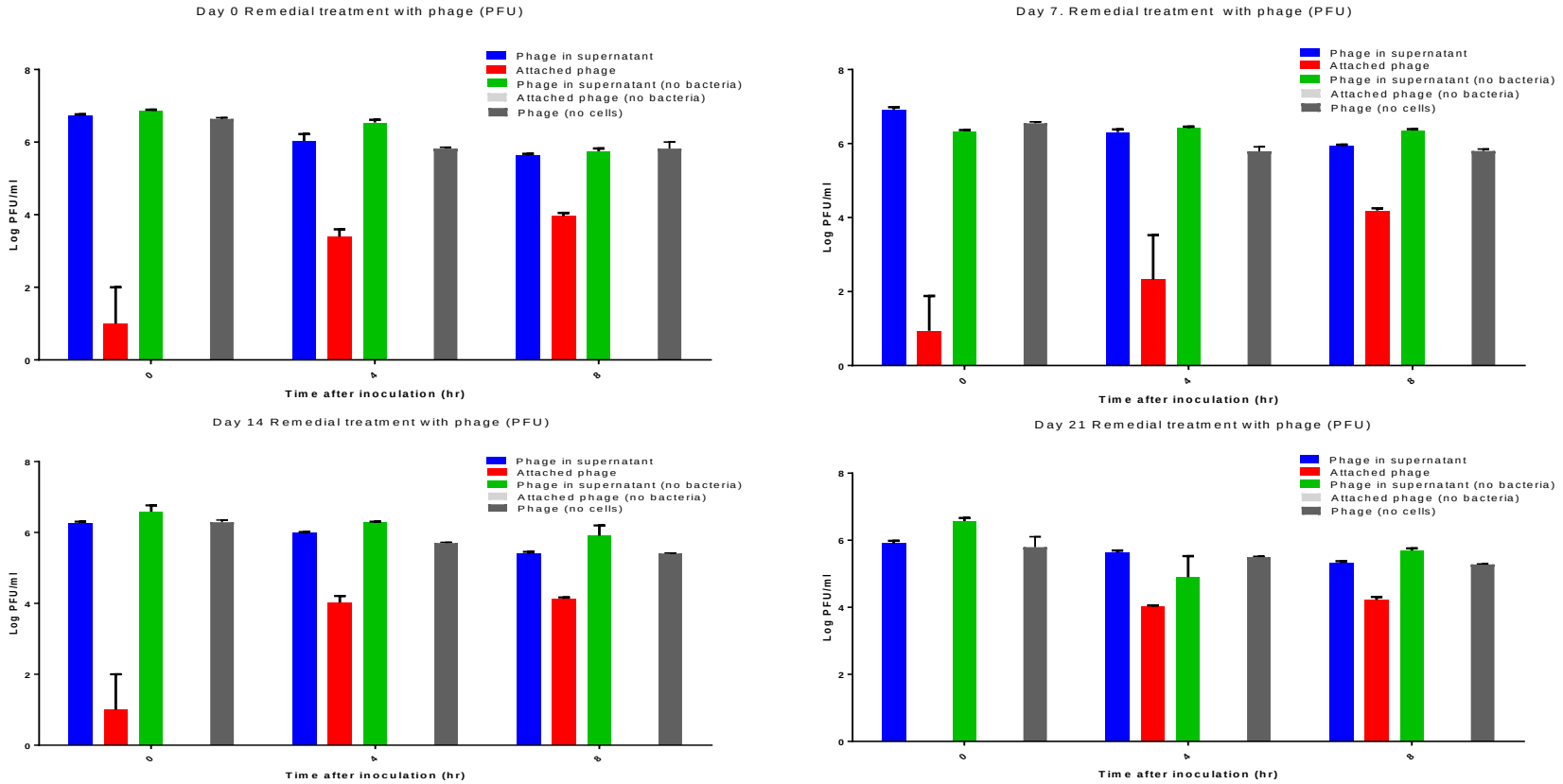

Figure 4.12: Bacteriophage counts over 21 days after remedial treatment with phages 1 hour after infection with *C. difficile*

The graphs show the PFU/mL counts of bacteriophages on days 0, 7, 14 and 21 after a remedial treatment 1 hour after infection with *C. difficile*. Averages ± SEM (error bars) of PFU/mL were calculated from three biological replicates, each with three technical repeats

4.3.3. Bound bacteriophage activity

An experiment was designed to assess phage activity once they had bound to the epithelial cell and mucus layer. The bacteriophages were allowed to bind to HT29-MTX-E12 cells for 1 hour, the unbound phages were washed off and *C. difficile* was added. This was repeated on days 0, 7, 14 and 21 over the same 21 day period as previous mucus relating experiments described in this chapter.

Figures 4.13, 4.14 and 4.15 show the results from this set of experiments. The most interesting observation was that the bound phages had no effect on the CFU/mL count of attached *C. difficile* (figure 4.13). There was no difference observed between phage treated and non-treated wells on all four experimental days. However, this was not the case for the planktonic *C. difficile* (figure 4.14). Reductions of 1 log ($p \leq 0.001$) and 0.5 log ($p \leq 0.05$) were observed at 4 hours post inoculation on days 0 and 7 respectively. At 8 hours post inoculation, approximately 0.75 log reduction of planktonic *C. difficile* was observed on days 0 and 7 ($p \leq 0.01$) and a log reduction was observed on days 14 and 21 ($p \leq 0.0001$ and $p \leq 0.001$ respectively).

A somewhat unusual result was observed with the PFU/mL results (figure 4.15). Despite the unbound phages being washed off before the addition of *C. difficile*, there was still a high count of bacteriophages in the supernatant. It was expected for the count to be much lower; however, it is possible that the phages are not irreversibly attached to the mucus layer and are therefore able to detach and re-enter the supernatant. As seen in the previous experiments, phages only visibly attach to the cells in the latter stages where more mucus is being produced, in this case only on days 14 and 21. An additional observation was that at day 21 it took 8 hours for attached bacteriophages to be detected however at day 14 attached phages were detected as early as 4 hours.

A possible reason for such a marked difference between the activity of bound phages on attached *C. difficile* compared to planktonic *C. difficile* is that the bacteriophages may only be able to infect the bacterial cells before they have attached to the epithelial monolayer themselves, i.e. whilst they are still in the supernatant. Once attached, it is possible that the bacteria may embed themselves within the mucus layer causing them to be inaccessible to the phage receptors. Another consideration to take into account is the orientation by which the phages have bound to the epithelial cells. If they have

bound to the epithelial cells tail side down, their receptors are not accessible for successful attachment to bacteria. However if they bind head side down, theoretically their tails should be sticking up and the bacteria should be able to successfully attach to the tails.

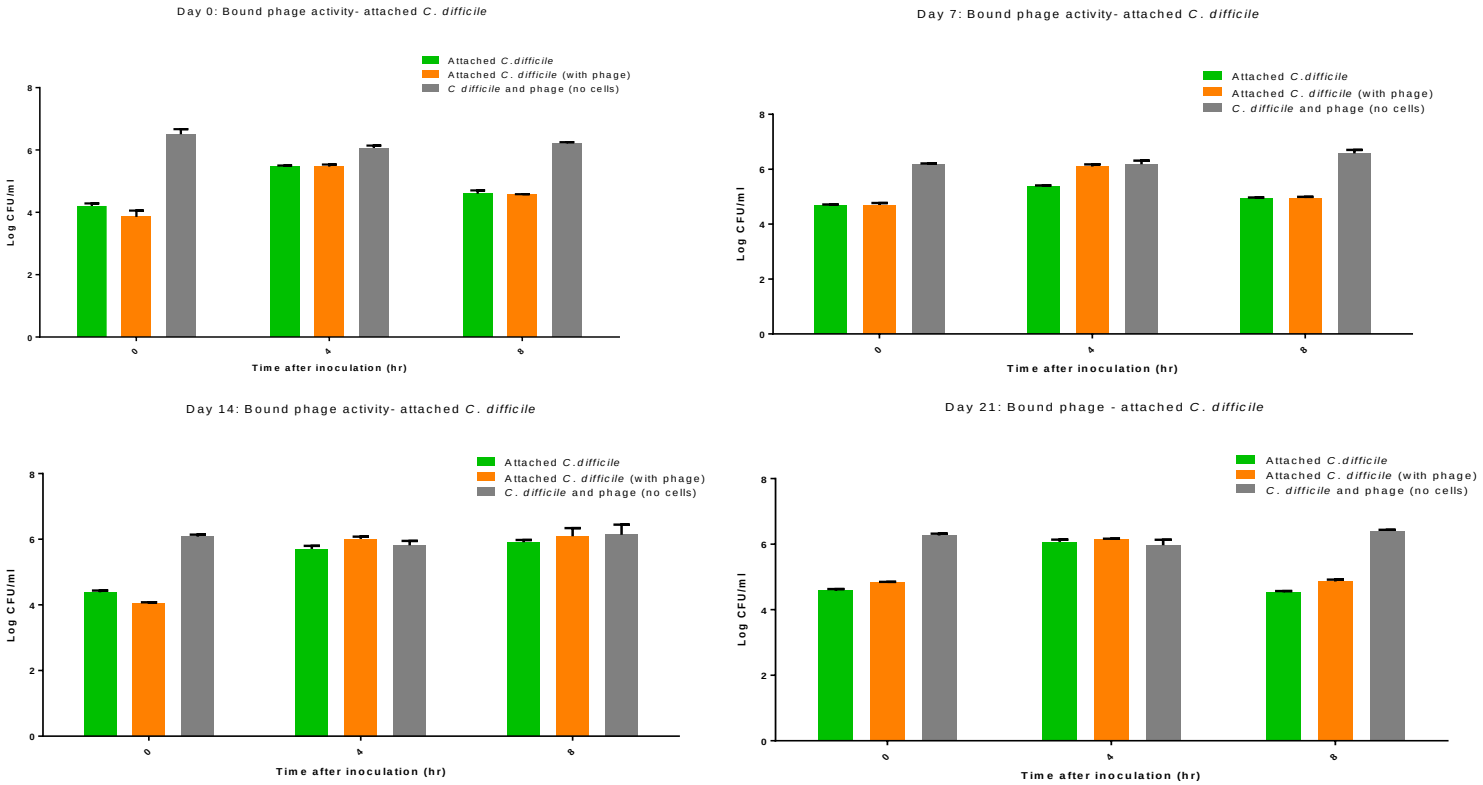

Figure 4.13: Attached CFU/mL count of *C. difficile* over 21 days after treatment with phages bound to HT29-MTX-E12

HT29-MTX-E12 cells were treated with phages for one hour. The epithelial cell monolayer was washed prior to the addition of *C. difficile* thus allowing only bound phages to treat the *C. difficile.* Comparisons were made between phage treated and non-treated wells. The efficacy of phages on attached *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests. Bars that are not marked with asterisks are not statistically significant.

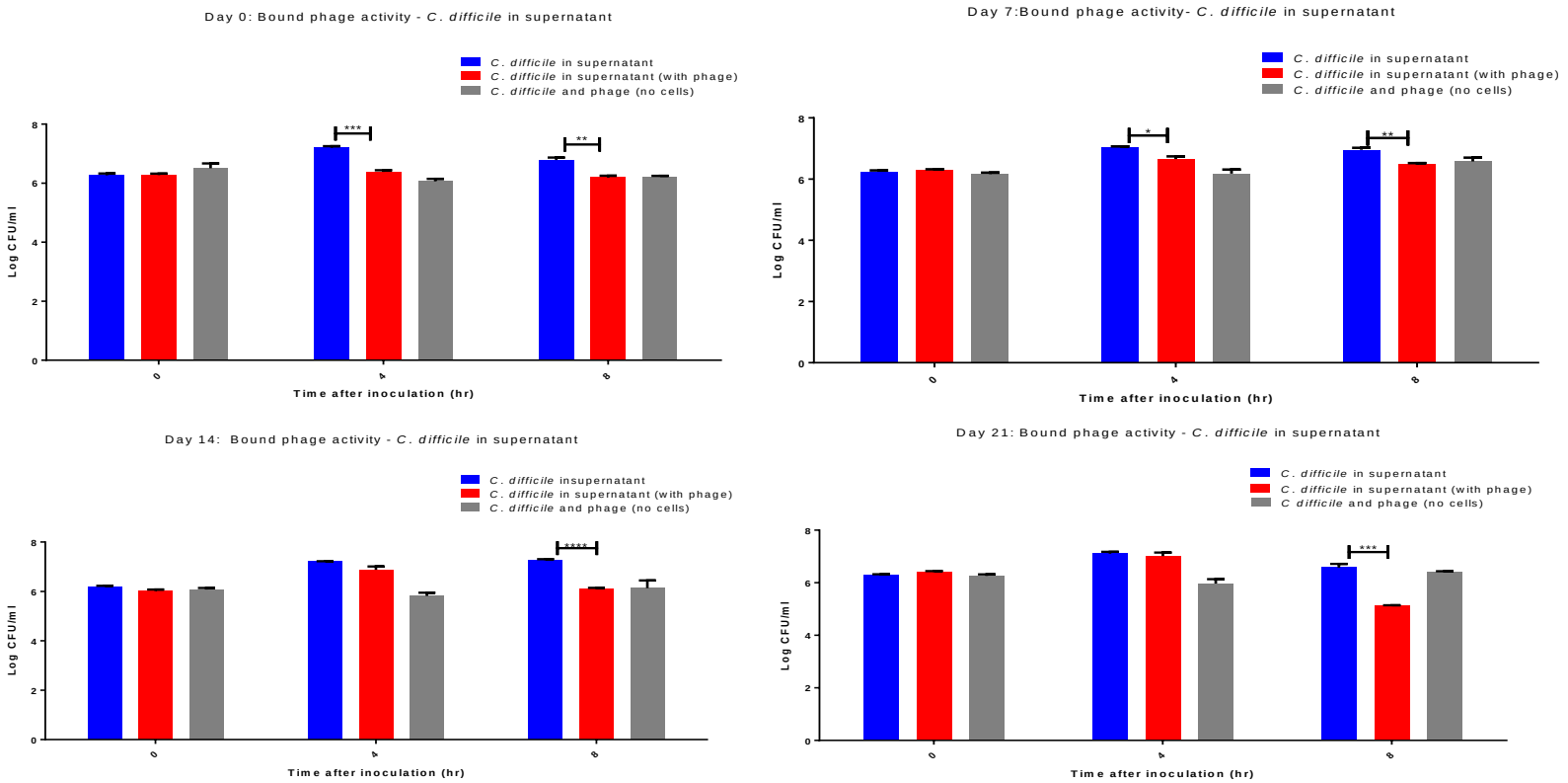

Figure 4.14: Planktonic CFU/mL count of *C. difficile* over 21 days after treatment with phages bound to HT29-MTX-E12

HT29-MTX-E12 cells were treated with phages for one hour. The epithelial cell monolayer was washed prior to the addition of *C. difficile* thus allowing only bound phages to treat the *C. difficile*. Comparisons were made between phage treated and non-treated wells. The efficacy of phages on planktonic *C. difficile* was assessed by measurement of CFU/mL. For each of the CFU/mL counts, averages ± SEM (error bars) were calculated from three biological replicates, each with three technical repeats. The mean values of bacteria were used to statistically analyse the results using T-tests with one star being assigned to $p \leq 0.05$, two stars to $p \leq 0.01$, three stars to $p \leq 0.001$ and $p \leq 0.0001$. Bars that are not marked with asterisks are not statistically significant.

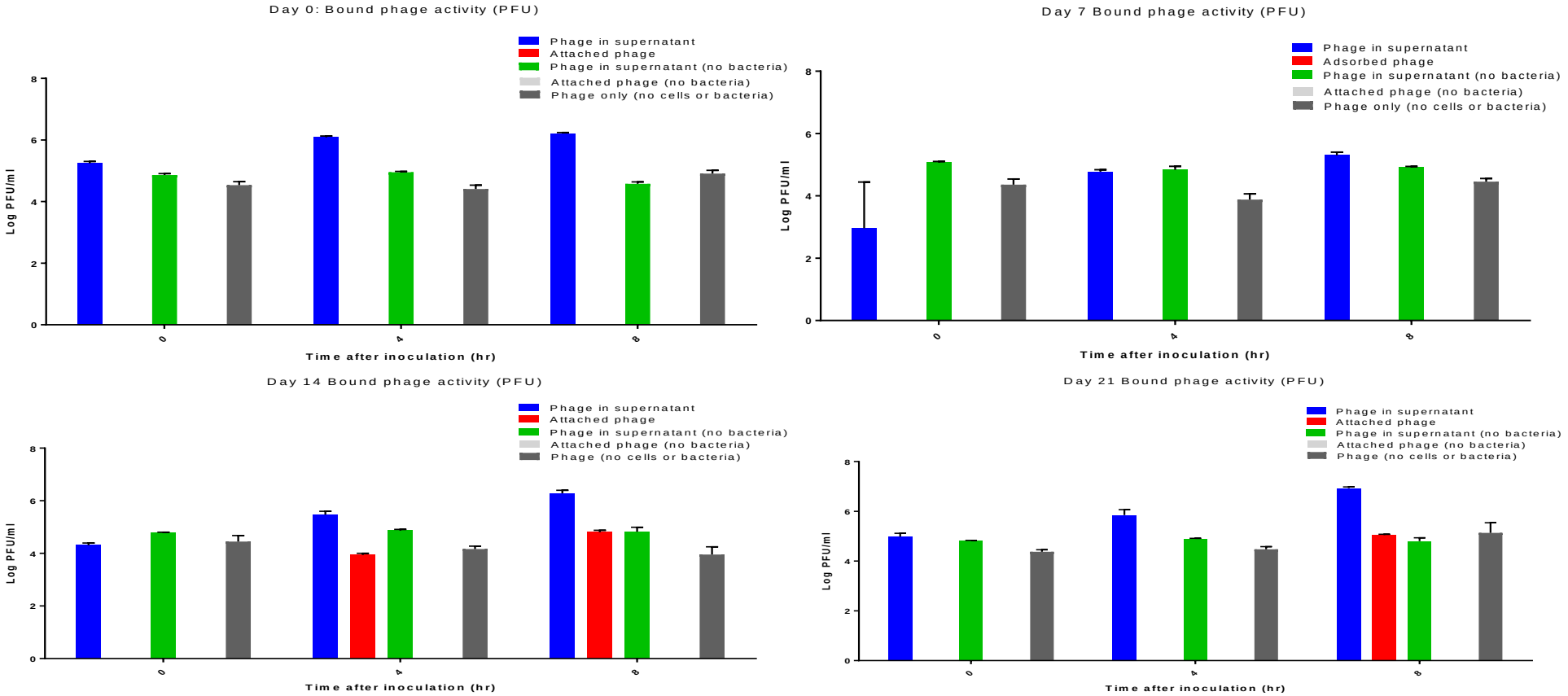

Figure 4.15: Bacteriophage counts over 21 days after treatment of *C. difficile* with phages bound to HT29-MTX-E12

The graphs show the PFU/mL counts of bacteriophages on days 0, 7, 14 and 21 after the cells were treated with bacteriophages for 1 hour before washing; allowing only bound bacteriophages to treat the *C. difficile*. Averages ± SEM (error bars) of PFU/mL were calculated from three biological replicates, each with three technical repeats.

4.4. Discussion

The experiments within this chapter were focussed on improving the previously developed cell culture model designed to elucidate the dynamics between phages and *C. difficile* within as realistic a setting as possible. The GI tract is lined with a mucus layer which plays a key role in defending against potential pathogens (Johansson et al. 2014). It is certain that both bacteria and phages come into contact with the mucus in the GI tract (Barr et al. 2015); therefore it is of paramount importance that the presence of mucus is taken into consideration for the model.

HT29-MTX-E12 cells were used for all experiments within this chapter which is the same cell line that was used for the optimisation of the model (HT29) with the one key difference that the cells have been differentiated to secrete mucus (Gagnon et al. 2013). This chapter investigated the concept of varying levels of mucus which may not only alter the interactions between phages and *C. difficile* but also impact the infection and killing efficiency of the bacteriophages themselves. It also explored the different phage treatment regimens that could be applied. The idea that phages were able to bind to the epithelial cell layer causing a direct impact on the way it interacts with *C. difficile* was also considered.

It was clear that the presence of mucus changed the dynamics between the phages, bacteria and the epithelial cells in certain scenarios. The possible reasons why the varying mucus levels affected the dynamics in only some scenarios will be discussed in this section.

4.4.1. Replication of HT29 experiments with use of HT29-MTX-E12 cells

The results attained from the experiments using the mucus producing HT29-MTX-E12 cells were first compared to parallel experiments carried out on HT29 cells in section 3.3.4. At this stage it was unclear whether the presence of mucus contributed to the efficiency of phage activity against *C. difficile.* There were no obvious differences between the HT29 and HT29-MTX-E12 experiments when phages and *C. difficile* were added to the cells simultaneously. The lack of

difference indicates that the presence of mucus does not have an effect on the phages' ability to infect and kill planktonic or attached *C. difficile*. An alternate explanation is that the layer of mucus at this point is small (Lesuffleur et al. 1990) and its quantity is not enough to have a significant impact on the phage activity.

A similar observation was made from the prophylactic set of experiments. The duration of phage treatment (either 1 or 2 hours) did not make a significant difference to the results. A 1.5-2 log reduction was observed in both the attached and planktonic *C. difficile* data sets. There was no evidence in the form of PFU/mL counts to show that bacteriophages were able to attach to the epithelial cell monolayer. The 1 hour prophylactic treatment with phages was sufficient for there to be effective infection and killing of *C. difficile.* The 2 hour incubation gave the same end result and was therefore no more efficient than the 1 hour treatment. Therefore the 1 hour treatment was the chosen duration used for all subsequent prophylactic related experiments.

It is likely that the layer of mucus produced in HT29-MTX-E12 cells at this stage was too small to show a marked difference in results between those observed with the HT29 cells. The HT29-MTX-E12 cells were cultured overnight for these experiments and therefore it is likely that only a minimal amount of mucus was present (Lesuffleur et al. 1993) which could explain the lack of difference in the results observed using HT29 cells versus HT29-MTX-E12 cells.

4.4.2. Observation of phage and *C. difficile* dynamics with increasing mucus production

Throughout this set of experiments the varying levels of mucus were taken into account whilst further assessing the interactions between phages and *C. difficile*. The experiments were split into three separate groups according to when the phages and *C. difficile* were added to the cells. In the first group *C. difficile* and bacteriophages were added to the cells at the same time, the second was designed to demonstrate a 1 hour prophylactic treatment and the final group was to depict a remedial form of treatment.

A general observation for all three groups was that the phages were slightly more active against planktonic *C. difficile* particularly in the prophylactic set of experiments and when *C. difficile* and phages were added simultaneously. For the remedial treatment experiments the outcome was more or less the same for both planktonic and attached *C. difficile*. It appeared that whilst the bacteria remained planktonic and unattached in the supernatant, they were far more accessible to the phages in all three treatment scenarios. An additional observation was that the 4 hour time point was key to the findings of these experiments. As with HT29 cells, maximum attachment occurred by this time. Furthermore, phage activity against both the attached and planktonic *C. difficile* was optimal at 4 hours post inoculation. The main exception was during the remedial treatment at day 21, when the presence of maximum levels of mucus meant that optimal phage activity took place at the 8 hour time point.

It is clear to see that the mucus levels do have an impact on the dynamics observed between bacteriophages and *C. difficile*; however, it is not as obvious of an impact as expected. The data from this project strongly corroborate with an observation made by Barr et al who stated that phages used the mucus layer to their advantage by trapping themselves within it enabling them to attack invading bacteria before they reached the tissue (Barr et al. 2013). His observation coincides with the data from this chapter which showed that phages were more efficient at killing *C. difficile* in the supernatant compared to when it had attached to the cell monolayer. Much more work on the impact of the mucus levels on the action of bacteriophages needs to be completed. This will be described in the future work (chapter 6).

The finding that was the most apparent in these experiments was the presence of attached phages. No visible plaques for attached phages were present when the model was first optimised with HT29 cells nor when the experiments were first replicated on HT29-MTX-E12 cells. However, when the cells were cultured up to 21 days attached PFU/mL counts were feasible. Both the prophylactic simulation and the experimental set where *C. difficile* and phages were added to the cells together produced visible plaques showing that phages were able to attach to the

epithelial cells at 14 and 21 days. The remedial treatment indicated that phages could attach to the cells as early as day 0 and day 7. What was more interesting was the phages were unable to bind to the cell monolayer when *C. difficile* was omitted in the phage and cell only control. This strongly suggests that the presence of the bacteria plays a key role in the attachment of the phages to the epithelial cell and mucus layer. Why this is the case is yet to be determined. There is a possibility that the presence of bacteria may stimulate certain receptors in the phages (Gill & Hyman 2010; Górski et al. 2017) with the intention of making it easier for phages to attach to their bacterial hosts but instead they are attaching to the cells.

4.4.3. Bound bacteriophage activity

Results from these experiments showed that after the phages had bound to the epithelial cells they were unable to infect and kill attached *C. difficile*. The graphs in figure 4.13 showed there was no difference in the attached CFU/mL count between phage treated and non-treated cells. The same trend was seen over the four experimental days despite the levels of mucus increasing over this period. In contrast, the graphs representing the planktonic CFU/mL count showed a statistically significant difference between phage treated and non-treated cells at both time points on days 0 and 7 and at 8 hours post inoculation on days 14 and 21. This observation strongly indicates that once the phages have bound to the cells they are able to infect and kill planktonic *C. difficile* (Barr et al. 2013), however, once the bacteria have attached to the cells they are no longer susceptible to phages.

A possible reason behind the observed difference is that *C. difficile* may embed itself within the mucus so that it becomes inaccessible to the phages. In order for the phages to successfully attach to the bacteria it is necessary for the phage receptors to have access to their corresponding binding sites on the bacteria (Tsonos et al. 2014; Górski et al. 2017). If the mucus layer has covered the bacteria, binding cannot take place, thus the attached bacteria are protected from the phages. Another idea to take into account is the orientation by which the phages have bound to the epithelial cells. If they have bound to the epithelial cells

tail side first then the receptors are trapped within the mucus and not accessible for successful attachment to the bacteria (Barr et al. 2013); however if they bound head side down, theoretically their tails should be sticking up and the planktonic *C. difficile* should be able to successfully attach to the tails. Determining the orientation of the phages is something to consider for the future work of this study and will be discussed more in section 6.3.2.

4.5. Summary

In summary, this chapter has further enhanced the cell culture model described in chapter 3 of this thesis. The mucus producing epithelial cells (HT29-MTX-E12) were implemented into the model and the experiments carried out in this chapter were designed to investigate how the presence of mucus affected the interactions between phages and *C. difficile.* The final results chapter of the thesis explores mucin quantification in order to confirm the presence and increasing quantity of mucus described to further consolidate the findings of this chapter.

Chapter 5. Mucin quantification by Indirect ELISA

5.1. Introduction

The previous chapter introduced the rationale behind including the presence of mucus in the mammalian cell model developed within the work described in this thesis. The majority of the experiments described in the chapter focussed on the impact of mucus on phage and *C. difficile* interactions and the ability of HT29-MTX-E12 cells to increase its secretion of mucus over a 21 day period. To measure the mucus quantitatively it was necessary to determine the mucin levels within the model. As literature suggests, the gel-forming nature of mucins and its extreme size makes the quantification of mucin notoriously difficult (Harrop et al. 2012). Additionally, the hygroscopic and hydrophilic properties of the mucin domain glycans which cause an increase in their ability to bind large quantities of water generating most of the typical gel-like properties of mucus, are also a disadvantage when it comes to its study (Johansson et al. 2013). Mucus tends to collapse if it is not well hydrated and can be lost if formaldehyde is used to fix the tissue. An additional problem is that normal mucus is made up of 98 % water and therefore is difficult to visualise under the microscope (Hansson 2012).

An established method for mucin quantification is the indirect ELISA. Its use was successfully demonstrated in a study by Barnett et al who investigated the effects of caprine milk oligosaccharides on intestinal barrier function and the mucus secreting cells (Barnett et al. 2016). In Barnett's study a co-culture combining Caco-2 and mucus secreting HT29-MTX was used to recreate the intestinal tract. The co-culture was used to determine if mucin concentrations were affected by the presence of caprine milk, which is thought to be similar to human milk (Barnett et al. 2016). The indirect ELISA was implemented to document the findings in terms of changes in mucin concentration, therefore the mucin protein abundance in response to a caprine milk oligosaccharide enriched fraction was established (Barnett et al. 2016).

Lesuffleur et al demonstrated that the expression of each MUC protein in HT29-MTX cells varied over a specific time period (Lesuffleur et al. 1993). There are many mucin proteins within the GI tract which could be quantified as a measure of mucus levels, (some of which have been listed in table 1.1) but for the purposes of this project the selection was narrowed down to three proteins MUC2, MUC3 and MUC5AC. The three proteins were selected according to when they were predominantly expressed by the cells. MUC2 expression was shown to be low in early cultures but increased gradually over time and therefore could act as a marker for monitoring the gradual increase of mucus over the 21 day time frame. MUC3 protein was chosen because it was detectable from day 7 and increased significantly to its peak at day 14. Finally MUC5AC was selected as it is the main protein expressed in HT29-MTX-E12 cells (Barnett et al. 2016) and should also show a dramatic increase in protein levels between days 7 and 14 (Lesuffleur et al. 1993).

Before the indirect ELISA could be implemented, it first had to be optimised to ensure that all parameters for the assay were accurately defined. As the quantities of three different MUC proteins were to be determined, primary antibodies against each individual protein were required and optimisation was carried out accordingly.

The aim of the optimisation process was to increase the ratio of sample signal to background reading. This is generally achieved by simultaneously reducing the background whilst increasing the sample signal thus avoiding false positives. These specific measures would ensure that any observed reading would be solely due to the presence of the mucin protein and not any background noise.

5.2. Methods

5.2.1. Cell culture sample preparation

The cell lysate and spent medium from HT29-MTX-E12 cells were both required to quantify the mucus levels. Samples were taken at days 0, 7, 14 and 21 over the

same three week period that corresponded with previous experiments in section 4.3.2. The cells were cultured, maintained and used to seed the microtitre plates as previously described in sections 2.3.2 and 2.3.4. To process the spent medium samples, 1 mL of cell culture supernatant was removed per well of the 24 well tissue culture plate (on experimental days 0, 7, 14 and 21) to which 250 µL protein inhibitor solution (1 tablet (Sigma 8830) dissolved in 10.5 mL PBS) was added. The samples were centrifuged at 4000 x g for 5 minutes at 4 °C. The pellet was discarded and the supernatant was removed and stored at -80 °C until required.

After the spent medium was removed from the well as described at the beginning of this section, 1 mL of protein inhibitor solution with 1% Triton X-100 was added per well to process the cell lysate. The plate was incubated at 37 °C for 10 mins to allow the cells to lyse. Following incubation, the lysate was collected and centrifuged at 4000 x g for 5 minutes at 4 °C. The supernatant was removed and stored at -80 °C until required.

5.2.2. Checkerboard method

The checkerboard method - so named due to the layout of the 96 well plate and the resulting concentrations in each well, was used to determine the working concentration of the primary antibody (MUC2 and MUC5 supplied by Sigma, product codes SAB1412430 and WH0004586M7 respectively. MUC3 supplied by ThermoFisher MA1-35702). A schematic of the layout is shown in figure 5.1. The figure shows the plate layout and the different concentrations in each well after all the dilutions have been carried out.

The antigens used to coat the plates were provided by 2B Scientific (MUC2 and MUC3) and My BioSource (MUC5AC). Recombinant MUC2 - product code RPA705Hu01, Recombinant MUC3 - product code RPB031Hu01 and Recombinant MUC5AC - product code MBS2544888.

The method is as follows. The antigen (positive control of each MUC protein, details given above) was serially diluted two-fold in coating buffer (see appendix)

across the plate and was incubated overnight at 4 °C. Following overnight incubation, the contents of the well were discarded and 150 µL blocking buffer (see appendix) was added to all the wells. The plate was incubated at room temperature for 1 hour and washed three times using 0.1 % PBS-Tween (see appendix). With each wash, the plate was allowed to stand for 5 minutes before the liquid was discarded and blotted thoroughly to ensure minimal liquid carryover. The primary antibody was diluted two fold down the plate in 1 % BSA and incubated for 1 hour at room temperature. The plates were washed once with 0.1 % PBS-Tween and three times with PBS. The secondary antibody (SouthernBiotech product code – 6170-05) was diluted in 1 % (w/v) BSA (see appendix) and added to the plate (50 µL per well) followed by 1 hour incubation at room temperature. After a final wash with 0.05 % PBS-Tween (see appendix) followed by three washes with PBS, 100 µL of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma T0440) was added per well to the whole plate. The plate was incubated at room temperature for 30 minutes in the dark. The final step was the addition of the stop solution (0.16 M sulfuric acid- see appendix) to the plate (50 µL per well) before the absorbance was read in the plate reader at 450nm.

All ELISA reagents required and made within the laboratory are listed in the appendix. Working concentrations of primary and secondary antibodies were determined through optimisation. The coating buffer could be made in advance and stored at 4 °C up to one month. All other reagents needed to be made fresh.

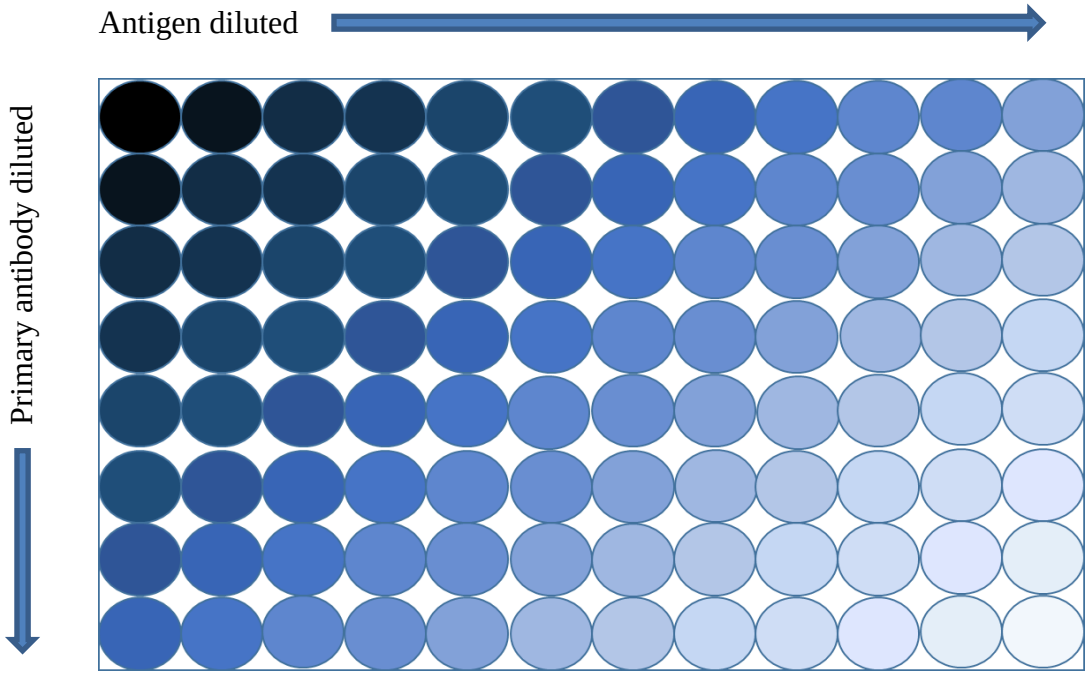

Figure 5.1: Schematic showing the layout of the checkerboard system

The antigen was added to the first column of the plate and diluted along the rows, whilst the primary antibody is put in the top row and diluted down the plate. The change in colour gradient indicates the changes in concentration.

5.2.3. Indirect ELISA method

The cell samples processed and stored at -80 °C as described in section 5.2.1 were used to coat a medium binding ELISA plate. The spent medium and cell lysate samples were added to the first column of the 96 well plate in duplicate and serially diluted two-fold across the plate in coating buffer. The corresponding positive control was also included on the plate and diluted in the same way. The final column in the plate was left sample-free in order to assess the background reading. The plate was incubated overnight at 4°C. The same method as described in section 5.2.2 was used. Briefly, the plates were washed, blocked and washed again as previously described. The corresponding primary antibody was diluted to the concentration pre-determined by the checkerboard method and 50 µL was added to each well of the plate. The plate was incubated at room temperature for an hour. After a further washing step the secondary antibody was added to the plate at the concentration determined in the optimisation steps

described in section 5.3.1.2 and incubated for an hour at room temperature. The plate was washed a final time before the addition of TMB substrate and incubation for 30 mins at room temperature in the dark after which the reaction was stopped with H_2SO_4 and read at 450 nm.

5.3. Results

5.3.1. Optimisation of Indirect ELISA

The first parameter to be determined was the blocking buffer. Barnett et al used 3 % bovine serum albumin (BSA)., An alternate blocking buffer that is traditionally used for ELISAs is skimmed milk buffer where skimmed milk powder is dissolved in PBS (see appendix) for use in the blocking step (Xiao & Isaacs 2012); therefore the two were compared.

The second parameter to determine was the optimal dilution factor of the secondary antibody. Initially, an empirical value of 1:2500 was chosen according to the manufacturer's recommended dilution range of 1:2000 - 1:8000 for ELISAs.

The determination of the working concentration of the secondary antibody was a pre-requisite for the subsequent optimisation of the coating antigen and primary antibody concentrations, which was carried out by using the checkerboard method described in section 5.2.2.

5.3.1.1. Blocking buffer

A 96 well plate was set out as seen in figure 5.2. It was decided that the plate would not be coated in order to assess the interactions of the buffers, antibodies and substrates alone thus determining the level of background resulting from the reagents and antibodies themselves. The plate was divided into two halves. The top half of the plate was blocked with 3 % BSA (labelled A) and the bottom half

of the plate was blocked with 5 % skimmed milk (labelled B). The plate was further divided four times (labelled 1-4) making eight segments on the plate. The complete ELISA method as described in section 5.2.3, was carried out in segments 1A and 1B. In the remaining segments the reagents were separated in order to identify if one may give a higher reading. In segments 2A and 2B the primary antibody was added, followed by the substrate. Segments 3A and 3B were used to test the secondary antibody followed by the substrate and segments 4A and 4B tested the substrate only without the addition of the antibodies. The results are shown in table 5.1. A higher reading was observed in the full ELISA columns (1A and 1B) and the secondary antibody columns (3A and 3B). However, it was clear that the blocking buffer did not have an adverse effect on the signal observed as all of the readings were similar throughout when comparing rows A and B. However, it evident that the signal attained was background, potentially caused by the secondary antibody, therefore, it was of paramount importance to determine the optimal working concentration of secondary antibody for the assay.

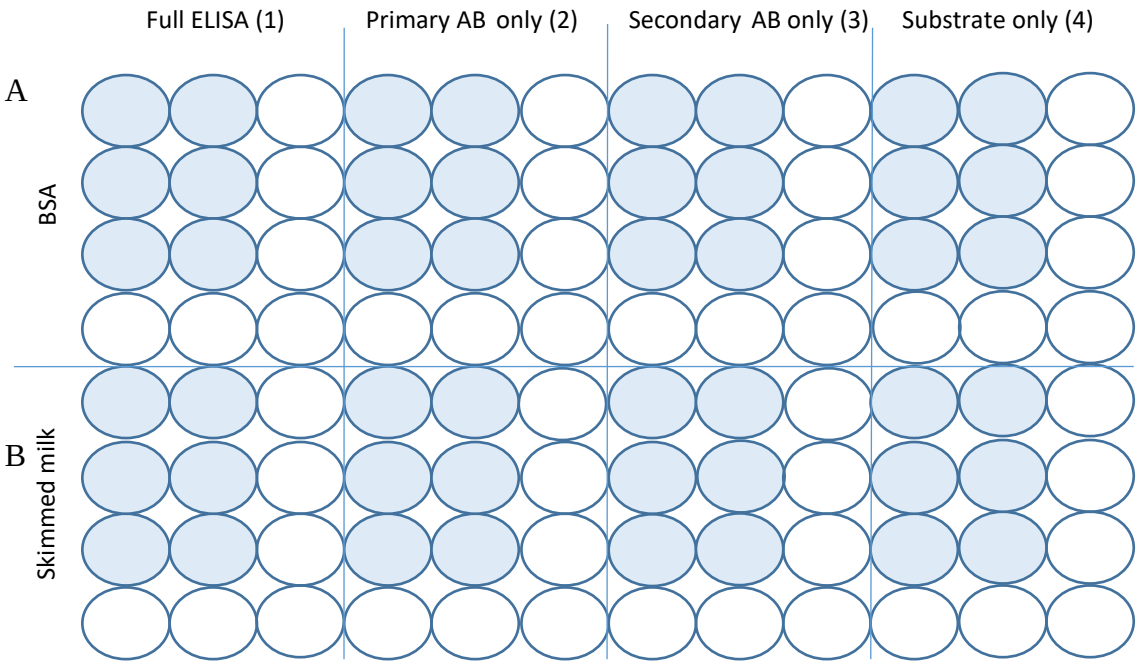

Figure 5.2: The plate layout used for the optimisation of the indirect ELISA.

The upper and lower halves are labelled A and B respectively. The 12 columns are divided into four parts and are thus labelled 1-4. The wells in blue represent the wells used for the optimisation process, the wells in white remained unused.

5.3.1.2. Concentration of Secondary Antibody

As mentioned previously, the secondary antibody appeared to be responsible for a higher background signal. No signal was observed when the primary antibody and substrate were used by themselves in segments 2A, 2B, 4A and 4B, and there was an obvious signal when a full ELISA was carried out in segments 1A and 1B and when only the secondary antibody was used (3A and 3B). Presumably, the higher signal was generated by the dilution of the working stock of the secondary antibody. Therefore, the experiment was repeated with further dilution of the secondary antibody to 1:4000. Results from this experiment can be seen in table 5.2.

Table 5.1: Results of indirect ELISA optimisation with the primary antibody MUC2. Two potential blocking buffers (BSA and Skimmed milk) were compared. Secondary antibody diluted 1:2500.

MUC2 Secondary AB 1:2500	**Full ELISA (1)**			**MUC2 Primary AB only (2)**			**Secondary AB only (3)**			**Substrate only (4)**	
BSA (A)	0.41	0.61		0.04	0.04		0.43	0.53		0.04	0.04
	0.53	0.42		0.04	0.04		0.49	0.61		0.04	0.04
	0.55	0.52		0.04	0.04		0.47	0.49		0.04	0.04
SKIMMED MILK (B)	0.45	0.53		0.04	0.03		0.55	0.51		0.04	0.04
	0.54	0.39		0.04	0.03		0.49	0.46		0.04	0.03
	0.55	0.50		0.04	0.04		0.50	0.39		0.04	0.04

Table 5.2: Results of indirect ELISA optimisation using the MUC2 primary antibody. Blocking buffers BSA and skimmed milk were compared. The secondary antibody dilution was further diluted to 1:4000

MUC2 AB 1:4000	Full ELISA (1)			MUC2 Primary AB only (2)			Secondary AB only (3)			Substrate only (4)	
BSA (A)	0.17	0.17		0.04	0.04		0.23	0.19		0.04	0.04
	0.18	0.17		0.04	0.04		0.17	0.13		0.04	0.04
	0.22	0.16		0.03	0.03		0.11	0.12		0.04	0.04
SKIMMED MILK (B)	0.17	0.26		0.04	0.04		0.18	0.10		0.04	0.04
	0.16	0.29		0.04	0.03		0.15	0.15		0.04	0.04
	0.21	0.20		0.04	0.04		0.17	0.20		0.04	0.04

Table 5.2 shows the background reading was successfully reduced after the secondary antibody was further diluted as the signals observed in segments 1A, 1B, 3A and 3B were all lower compared to what was seen in table 5.1. There was also further confirmation that there was no difference between the two blocking buffers for MUC2 as both resulted in similar end readings.

To ensure the blocking buffer and the secondary antibody did not affect the assay further, the process was repeated for both MUC3 and MUC5AC. This would give additional confirmation that the same blocking buffers and the same concentration of secondary antibody could be used throughout for all proteins. The results for these experiments can be seen in table 5.3. The primary antibodies were tested by themselves and a full ELISA was also completed with the MUC3 and MUC5AC

primary antibodies to ensure the 1:4000 dilution of the secondary antibody did not provide an excess background reading.

Table 5.3: Results of indirect ELISA optimisation using MUC3 and MUC5AC primary antibodies and comparing two potential blocking buffers. The secondary antibody was diluted to 1:4000.

	Full ELISA MUC3			MUC3 Primary AB only			Full ELISA MUC5AC			MUC5AC Primary AB only	
BSA	0.08	0.10		0.04	0.04		0.08	0.09		0.04	0.04
	0.08	0.10		0.03	0.03		0.08	0.07		0.04	0.04
	0.09	0.08		0.03	0.03		0.08	0.10		0.04	0.03
SKIMMED MILK	0.08	0.08		0.04	0.03		0.08	0.07		0.04	0.04
	0.10	0.08		0.04	0.04		0.07	0.07		0.03	0.03

It was decided that 3 % BSA would be the chosen blocking buffer for the remaining assays. This was to maintain consistency as 1 % BSA was the manufacturer's recommended diluent for the antibodies.

5.3.1.3. Determination of primary antibody concentration by checkerboard method.

The three primary antibodies were reconstituted in PBS and recommended working stock concentrations of 1 µg/mL were prepared according to the manufacturer's instructions. In order to ensure a full range of dilutions were covered the starting concentration of each primary antibody was 5 µg/mL.

The first set of results in tables 5.4 and 5.5 shows each MUC protein with a starting concentration of 1000 ng/mL diluted two-fold to 0.98 ng/mL and primary antibody concentrations starting at 2500 ng/mL to 19.53 ng/mL. Tables 5.4A and B for MUC2 and MUC3 respectively showed a constant signal throughout. All results were similar to the background signal output making it impossible to identify whether there was a signal due to the recombinant protein coat or background. Table 5.5C shows the checkerboard for MUC5AC. Here, a positive signal was generated which subsequently reduced as a result of the decreasing concentrations of the antigen in the wells (highlighted in green).

As there was no difference between sample signals and background levels for MUC2 and MUC3, the standard protein concentration range was increased 10 fold to 10,000 ng/mL in an attempt to differentiate between the sample and background signals. Therefore the concentration of standard proteins tested started at 10,000 ng/mL and went down to 9.77 ng/mL. The results for the two assays for MUC2 and MUC3 are shown in table 5.6A and B respectively. Once again the results for MUC3 showed no signal despite the higher concentration of standard protein used. A signal was attained for MUC2 protein as highlighted in green in table 5.6A, however the signal to background ratio still remained low. The maximum signal obtained was not as high as the signal for MUC5AC. Therefore it was necessary to improve the signal and to try and obtain a signal for MUC3. As the maximum concentration for the standard protein was now being used, the maximum concentration of the primary antibody was increased from 2500 ng/mL to 10,000 ng/mL.

Table 5.4: Results of MUC2 (A) and MUC3 (B) checkerboard indirect ELISA

A		STANDARD RECOMBINANT PROTEIN MUC2 ng/mL											
		1000	500	250	125	62.5	31.25	15.63	7.81	3.91	1.95	0.98	BLANK
Primary Antibody ng/mL	2500	0.08	0.08	0.07	0.07	0.08	0.09	0.07	0.08	0.27	0.08	0.09	0.12
	1250	0.06	0.08	0.07	0.07	0.06	0.07	0.07	0.08	0.08	0.06	0.07	0.17
	625	0.07	0.08	0.07	0.07	0.07	0.07	0.07	0.08	0.07	0.06	0.09	0.13
	312.5	0.06	0.07	0.07	0.07	0.06	0.07	0.06	0.06	0.07	0.07	0.07	0.12
	156.25	0.06	0.07	0.08	0.07	0.07	0.06	0.06	0.06	0.06	0.07	0.09	0.12
	78.13	0.07	0.06	0.06	0.07	0.07	0.07	0.06	0.07	0.07	0.07	0.08	0.12
	39.06	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.08	0.11
	19.53	0.07	0.07	0.06	0.06	0.07	0.08	0.07	0.07	0.07	0.07	0.09	0.13

B		STANDARD RECOMBINANT PROTEIN MUC3 ng/mL											
		1000	500	250	125	62.5	31.25	15.63	7.81	3.91	1.95	0.98	BLANK
Primary Antibody ng/mL	2500	0.09	0.09	0.09	0.08	0.08	0.10	0.08	0.08	0.09	0.09	0.08	0.21
	1250	0.08	0.10	0.08	0.08	0.10	0.08	0.08	0.08	0.08	0.08	0.10	0.16
	625	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.09	0.08	0.08	0.15
	312.5	0.07	0.09	0.08	0.07	0.08	0.09	0.07	0.07	0.08	0.08	0.09	0.13
	156.25	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.07	0.08	0.08	0.10	0.14
	78.13	0.08	0.09	0.08	0.07	0.07	0.07	0.07	0.08	0.07	0.08	0.10	0.12
	39.06	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.08	0.07	0.08	0.12
	19.53	0.08	0.09	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.13

Table 5.5: Results of MUC5AC (C) checkerboard indirect ELISA

C		STANDARD RECOMBINANT PROTEIN MUC5AC ng/mL											
		1000	**500**	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**BLANK**
Primary Antibody ng/mL	**2500**	1.09	0.75	0.58	0.45	0.34	0.24	0.18	0.16	0.13	0.12	0.12	0.13
	1250	1.03	0.68	0.52	0.43	0.34	0.23	0.16	0.15	0.12	0.11	0.11	0.11
	625	1.04	0.69	0.54	0.42	0.31	0.22	0.15	0.13	0.11	0.10	0.12	0.12
	312.5	1.01	0.69	0.54	0.41	0.27	0.20	0.15	0.12	0.10	0.10	0.11	0.13
	156.25	0.94	0.67	0.51	0.41	0.31	0.21	0.14	0.12	0.10	0.09	0.09	0.11
	78.13	0.87	0.62	0.48	0.38	0.28	0.18	0.12	0.12	0.10	0.09	0.09	0.13
	39.06	0.73	0.50	0.37	0.31	0.23	0.15	0.12	0.12	0.09	0.09	0.10	0.11
	19.53	0.62	0.43	0.36	0.27	0.20	0.17	0.12	0.10	0.13	0.10	0.09	0.11

Table 5.6: Results of MUC2 and MUC3 checkerboard indirect ELISA, with starting concentration of standard protein at 10,000ng/mL

	A	Standard Recombinant protein MUC2 ng/mL											
		10,000	5000	2500	1250	625	312.5	156.25	78.13	39.06	19.53	9.77	Blank
Primary Antibody ng/mL	2500	0.54	0.28	0.34	0.20	0.17	0.15	0.14	0.12	0.12	0.15	0.12	0.16
	1250	0.60	0.30	0.33	0.21	0.15	0.16	0.16	0.11	0.14	0.14	0.16	0.13
	625	0.52	0.30	0.28	0.19	0.17	0.14	0.16	0.15	0.15	0.15	0.13	0.13
	312.5	0.46	0.24	0.23	0.16	0.13	0.13	0.18	0.11	0.12	0.16	0.12	0.11
	156.25	0.46	0.24	0.18	0.12	0.12	0.12	0.17	0.12	0.16	0.12	0.15	0.14
	78.13	0.38	0.19	0.16	0.12	0.11	0.10	0.12	0.12	0.13	0.14	0.14	0.14
	39.06	0.38	0.16	0.11	0.13	0.12	0.13	0.12	0.12	0.13	0.14	0.13	0.14
	19.53	0.37	0.17	0.11	0.12	0.12	0.12	0.11	0.11	0.13	0.18	0.12	0.14

	B	Standard Recombinant protein MUC3 ng/mL											
		10,000	5000	2500	1250	625	312.5	156.25	78.13	39.06	19.53	9.77	Blank
Primary Antibody ng/mL	2500	0.10	0.10	0.11	0.11	0.09	0.13	0.10	0.11	0.14	0.10	0.12	0.14
	1250	0.12	0.10	0.10	0.09	0.10	0.10	0.11	0.11	0.11	0.11	0.11	0.14
	625	0.13	0.10	0.10	0.11	0.12	0.13	0.15	0.13	0.11	0.11	0.14	0.16
	312.5	0.13	0.10	0.09	0.10	0.11	0.09	0.11	0.10	0.12	0.11	0.12	0.13
	156.25	0.12	0.10	0.09	0.10	0.09	0.11	0.10	0.10	0.10	0.11	0.12	0.15
	78.13	0.11	0.09	0.09	0.10	0.09	0.18	0.09	0.11	0.11	0.12	0.12	0.15
	39.06	0.11	0.09	0.09	0.11	0.11	0.12	0.11	0.10	0.12	0.12	0.15	0.14
	19.53	0.12	0.11	0.10	0.12	0.10	0.11	0.10	0.11	0.09	0.12	0.15	0.13

Tables 5.7A and B show the results of the MUC2 and MUC3 checkerboards respectively with increased concentrations of the primary antibodies. Starting with a higher primary antibody concentration was successful for the MUC2 protein (highlighted in green) as a signal from the samples was visible. Ideally the signal should reduce steadily, (as seen with MUC5AC in table 5.5) across the columns due to the two fold dilutions of the standard protein, however, for MUC2 there was a large drop observed between 10,000 ng/mL and 5000 ng/mL. As shown in table 5.7B no positive signal was observed for MUC3 as the readings generated from the samples were indistinguishable from the background values.

The decision was made that due to time constraints MUC3 should be eliminated from the proteins to be quantified and the focus should remain on MUC2 and MUC5AC. The justification behind this decision will be discussed further in section 5.4. The working concentration of primary antibody for subsequent ELISAs was determined from the checkerboard results in table 5.5C and 5.6A; 312.5 ng/mL MUC5AC and 5000 ng/mL for MUC2 respectively.

Table 5.7: Indirect ELISA checkerboard for MUC2 (A) and MUC3 (B) with higher concentrations of primary antibody

	A	MUC2 Standard Recombinant Protein ng/mL											
		20,000	10000	5000	2500	1250	625	312.5	156.25	78.13	39.06	19.53	Blank
Primary antibody ng/mL	10000	1.25	1.07	0.38	0.34	0.24	0.16	0.14	0.14	0.13	0.13	0.11	0.10
	5000	1.04	0.89	0.34	0.29	0.19	0.18	0.11	0.10	0.12	0.10	0.11	0.15
	2500	0.83	0.75	0.24	0.22	0.16	0.12	0.10	0.11	0.11	0.10	0.10	0.12
	1250	0.71	0.55	0.21	0.17	0.14	0.11	0.11	0.11	0.09	0.09	0.09	0.11
	625	0.66	0.46	0.19	0.16	0.12	0.11	0.10	0.09	0.10	0.10	0.10	0.11
	312.5	0.44	0.25	0.13	0.12	0.10	0.09	0.10	0.09	0.09	0.10	0.10	0.10
	156.25	0.44	0.47	0.25	0.15	0.14	0.10	0.11	0.08	0.10	0.11	0.11	0.11
	78.125	0.57	0.30	0.17	0.13	0.12	0.10	0.10	0.10	0.11	0.10	0.10	0.10

	B	MUC3 Standard Recombinant Protein ng/mL											
		20,000	10000	5000	2500	1250	625	312.5	156.25	78.13	39.06	19.53	Blank
Primary antibody ng/mL	10000	0.13	0.07	0.09	0.08	0.08	0.10	0.08	0.08	0.09	0.09	0.08	0.21
	5000	0.08	0.07	0.08	0.08	0.07	0.08	0.08	0.08	0.08	0.07	0.10	0.16
	2500	0.11	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.09	0.08	0.08	0.15
	1250	0.09	0.08	0.08	0.07	0.08	0.09	0.07	0.07	0.08	0.08	0.09	0.13
	625	0.08	0.08	0.08	0.08	0.08	0.08	0.09	0.07	0.08	0.06	0.10	0.14
	312.5	0.10	0.09	0.09	0.07	0.08	0.07	0.08	0.08	0.07	0.08	0.10	0.12
	156.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.12
	78.125	0.12	0.09	0.08	0.08	0.08	0.08	0.09	0.08	0.08	0.08	0.08	0.13

5.3.2. Implementation of Indirect ELISA

The working stock concentrations of primary and secondary antibodies for MUC2 and MUC5AC were determined in sections 5.3.1.2 and 5.3.1.3 respectively. These concentrations were implemented into the indirect ELISA for the quantification of mucin in the cell lysate and spent medium samples taken at days 0, 7, 14 and 21. Each plate had a positive and negative control in addition to the test samples. The samples were plated in duplicate. The cell lysate results are highlighted in grey.

The results are shown in tables 5.8 and 5.9 for MUC2 and tables 5.10 and 5.11 for MUC5AC. The absorbance levels of both the cell lysate and spent medium were of the same value as the blank wells and the negative controls. The assay was unable to quantify the MUC2 levels within both the cell lysate and the spent medium for all four test days. The positive control confirmed that the assay worked to a certain extent due to an obvious reduction in OD reading output corresponding to the changes in concentration across the plate, however, this did not extend to the test samples.

This was also the case for MUC5AC. The positive control confirmed the assay itself was steadfast (tables 5.10 and 5.11), but once again, the absorbance readings from the test samples were too close to those of the blank wells and the negative control. The first absorbance readings of the cell lysate results in tables 5.10F and 5.11G appeared to be slightly higher than the blank readings. However, the result to background ratio was not large enough for this to be confirmed as a positive reading and therefore could not be used to quantify the MUC5AC levels.

Table 5.8: Results of indirect ELISA quantifying MUC2 proteins in cell culture samples from days 0 (A) and 7 (B)

A	Protein concentration ng/mL											
Day 0	**20,000**	**10,000**	**5000**	**2500**	**1250**	**625**	**312.50**	**156.25**	**78.13**	**39.06**	**19.53**	**BLANK**
Protein Std 1	1.08	0.85	0.44	0.36	0.29	0.21	0.17	0.15	0.16	0.14	0.11	0.11
Protein Std 2	1.10	0.89	0.50	0.34	0.30	0.25	0.18	0.17	0.19	0.13	0.10	0.15
Cell lysate 1	0.12	0.11	0.10	0.12	0.13	0.11	0.16	0.14	0.13	0.12	0.11	0.13
Cell lysate 2	0.11	0.12	0.14	0.13	0.11	0.15	0.11	0.10	0.10	0.11	0.12	0.12
Spent medium 1	0.11	0.09	0.10	0.09	0.10	0.09	0.10	0.11	0.10	0.12	0.10	0.11
Spent medium 2	0.10	0.10	0.09	0.10	0.11	0.11	0.09	0.08	0.09	0.10	0.09	0.10
Neg control 1	0.11	0.12	0.10	0.11	0.12	0.11	0.10	0.11	0.11	0.10	0.10	0.11
Neg control 2	0.12	0.12	0.11	0.12	0.11	0.11	0.12	0.10	0.10	0.11	0.10	0.12

B	Protein concentration ng/mL											
Day 7	**20,000**	**10,000**	**5000**	**2500**	**1250**	**625**	**312.50**	**156.25**	**78.13**	**39.06**	**19.53**	**BLANK**
Protein Std 1	1.07	0.79	0.34	0.31	0.27	0.19	0.15	0.14	0.13	0.12	0.12	0.18
Protein Std 2	1.11	0.81	0.40	0.31	0.26	0.21	0.19	0.18	0.14	0.12	0.12	0.17
Cell lysate 1	0.18	0.14	0.11	0.15	0.13	0.14	0.15	0.13	0.12	0.14	0.11	0.16
Cell lysate 2	0.16	0.14	0.14	0.15	0.14	0.13	0.14	0.16	0.12	0.13	0.11	0.13
Spent medium 1	0.12	0.11	0.11	0.10	0.09	0.10	0.09	0.11	0.08	0.10	0.10	0.14
Spent medium 2	0.10	0.09	0.09	0.10	0.08	0.11	0.09	0.09	0.09	0.10	0.09	0.10
Neg control 1	0.12	0.11	0.12	0.10	0.10	0.09	0.11	0.12	0.10	0.10	0.11	0.11
Neg control 2	0.12	0.11	0.14	0.11	0.12	0.11	0.11	0.10	0.10	0.11	0.11	0.13

Table 5.9: Results of indirect ELISA quantifying MUC2 proteins in cell culture samples from days 14 (C) and 21 (D)

C	Protein concentration ng/mL											
Day 14	**20,000**	**10,000**	**5000**	**2500**	**1250**	**625**	**312.50**	**156.25**	**78.13**	**39.06**	**19.53**	**BLANK**
Protein Std 1	1.04	0.89	0.38	0.34	0.24	0.16	0.14	0.14	0.13	0.13	0.11	0.10
Protein Std 2	1.06	0.84	0.40	0.38	0.31	0.21	0.14	0.15	0.13	0.12	0.11	0.11
Cell lysate 1	0.11	0.14	0.10	0.12	0.12	0.15	0.12	0.10	0.09	0.12	0.10	0.12
Cell lysate 2	0.11	0.12	0.12	0.13	0.12	0.12	0.13	0.10	0.11	0.12	0.11	0.10
Spent medium 1	0.09	0.10	0.07	0.07	0.08	0.07	0.09	0.07	0.09	0.09	0.10	0.12
Spent medium 2	0.09	0.08	0.08	0.09	0.07	0.10	0.08	0.08	0.08	0.08	0.10	0.10
Neg control 1	0.10	0.11	0.12	0.10	0.12	0.10	0.14	0.12	0.10	0.11	0.11	0.12
Neg control 2	0.13	0.12	0.10	0.12	0.11	0.10	0.12	0.13	0.13	0.13	0.10	0.12

D	Protein concentration ng/mL											
Day 21	**20,000**	**10,000**	**5000**	**2500**	**1250**	**625**	**312.50**	**156.25**	**78.13**	**39.06**	**19.53**	**BLANK**
Protein Std 1	0.99	0.65	0.45	0.38	0.34	0.24	0.20	0.17	0.16	0.13	0.14	0.15
Protein Std 2	1.00	0.77	0.34	0.36	0.30	0.28	0.24	0.19	0.16	0.12	0.12	0.19
Cell lysate 1	0.14	0.13	0.14	0.12	0.13	0.11	0.11	0.10	0.10	0.10	0.10	0.12
Cell lysate 2	0.12	0.11	0.11	0.13	0.10	0.12	0.10	0.12	0.10	0.11	0.11	0.13
Spent medium 1	0.10	0.09	0.08	0.10	0.09	0.09	0.08	0.10	0.11	0.11	0.10	0.11
Spent medium 2	0.09	0.10	0.10	0.09	0.11	0.10	0.09	0.08	0.09	0.10	0.11	0.12
Neg control 1	0.12	0.10	0.11	0.09	0.11	0.11	0.09	0.09	0.10	0.10	0.12	0.11
Neg control 2	0.13	0.10	0.12	0.11	0.10	0.11	0.10	0.10	0.11	0.09	0.10	0.13

Table 5.10: Results of indirect ELISA quantifying MUC5AC proteins in cell culture samples from days 0 (E) and 7 (F)

E	Protein concentration ng/mL											
Day 0	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**BLANK**
Protein Std 1	0.97	0.95	0.75	0.66	0.44	0.31	0.26	0.17	0.16	0.12	0.11	0.12
Protein Std 2	1.00	0.97	0.87	0.71	0.53	0.44	0.37	0.19	0.16	0.12	0.12	0.19
Cell lysate 1	0.15	0.15	0.12	0.11	0.12	0.12	0.13	0.11	0.18	0.09	0.11	0.10
Cell lysate 2	0.20	0.19	0.21	0.15	0.13	0.11	0.11	0.10	0.11	0.10	0.12	0.14
Spent medium 1	0.10	0.09	0.08	0.08	0.09	0.08	0.08	0.10	0.12	0.09	0.09	0.09
Spent medium 2	0.14	0.12	0.11	0.14	0.14	0.13	0.16	0.15	0.14	0.15	0.18	0.18
Neg control 1	0.12	0.11	0.10	0.09	0.10	0.10	0.11	0.10	0.12	0.14	0.11	0.13
Neg control 2	0.14	0.10	0.13	0.12	0.11	0.11	0.09	0.12	0.11	0.13	0.10	0.12

F	Protein concentration ng/mL											
Day 7	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**BLANK**
Protein Std 1	1.22	1.02	0.85	0.78	0.55	0.38	0.24	0.19	0.13	0.12	0.12	0.20
Protein Std 2	1.17	1.06	0.87	0.71	0.49	0.36	0.22	0.17	0.16	0.12	0.12	0.19
Cell lysate 1	0.21	0.18	0.20	0.16	0.17	0.17	0.23	0.16	0.17	0.15	0.22	0.18
Cell lysate 2	0.22	0.23	0.19	0.16	0.22	0.18	0.19	0.17	0.16	0.16	0.18	0.14
Spent medium 1	0.12	0.10	0.11	0.11	0.10	0.11	0.12	0.15	0.13	0.14	0.17	0.16
Spent medium 2	0.13	0.12	0.10	0.13	0.14	0.12	0.14	0.13	0.11	0.12	0.14	0.18
Neg control 1	0.13	0.11	0.11	0.09	0.10	0.12	0.11	0.13	0.12	0.10	0.11	0.15
Neg control 2	0.16	0.11	0.14	0.12	0.13	0.11	0.10	0.14	0.13	0.12	0.11	0.17

Table 5.11: Results of indirect ELISA quantifying MUC5AC proteins in cell culture samples from days 14 (G) and 21 (H)

G	Protein concentration ng/mL											
Day 14	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**BLANK**
Protein Std 1	1.14	1.06	0.82	0.74	0.56	0.36	0.22	0.15	0.16	0.14	0.12	0.17
Protein Std 2	1.06	1.00	0.91	0.79	0.68	0.45	0.36	0.23	0.16	0.15	0.12	0.19
Cell lysate 1	0.22	0.24	0.20	0.16	0.17	0.18	0.16	0.17	0.16	0.15	0.18	0.14
Cell lysate 2	0.20	0.22	0.18	0.15	0.13	0.15	0.11	0.12	0.13	0.10	0.13	0.14
Spent medium 1	0.13	0.08	0.10	0.08	0.11	0.10	0.11	0.10	0.10	0.12	0.09	0.09
Spent medium 2	0.13	0.11	0.10	0.14	0.13	0.15	0.16	0.13	0.12	0.11	0.14	0.16
Neg control 1	0.13	0.11	0.09	0.08	0.10	0.11	0.10	0.13	0.12	0.10	0.11	0.14
Neg control 2	0.14	0.10	0.11	0.12	0.10	0.11	0.09	0.13	0.11	0.13	0.10	0.12

H	Protein concentration ng/mL											
Day 21	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**BLANK**
Protein Std 1	0.97	0.95	0.75	0.66	0.44	0.31	0.26	0.17	0.16	0.12	0.11	0.12
Protein Std 2	1.00	0.97	0.87	0.71	0.53	0.44	0.37	0.19	0.16	0.12	0.12	0.19
Cell lysate 1	0.16	0.13	0.14	0.11	0.13	0.12	0.11	0.09	0.10	0.09	0.10	0.12
Cell lysate 2	0.11	0.10	0.11	0.09	0.10	0.12	0.08	0.13	0.10	0.11	0.11	0.11
Spent medium 1	0.10	0.08	0.08	0.10	0.10	0.09	0.09	0.10	0.12	0.10	0.11	0.11
Spent medium 2	0.11	0.12	0.11	0.10	0.11	0.12	0.10	0.11	0.12	0.10	0.09	0.13
Neg control 1	0.11	0.10	0.09	0.10	0.09	0.11	0.10	0.10	0.11	0.10	0.11	0.12
Neg control 2	0.13	0.11	0.13	0.11	0.12	0.10	0.11	0.11	0.10	0.11	0.10	0.11

5.4. Discussion

When it became clear that the optimised ELISA was not a successful tool to use to quantify the three selected MUC proteins I focussed on MUC2 and MUC5AC. These proteins were selected because the mucus located within the intestinal tract is almost exclusively MUC2 (Naughton et al. 2014). MUC2 is also directly affected by the presence of CDI. Studies have shown that patients suffering from CDI showed a marked decrease in MUC2 production which coincides with the deficient mucus barrier which is caused by the infection (Engevik et al. 2014). MUC2 is therefore a significant mucin protein to focus on in terms of research of treatment of *C. difficile.* As previously mentioned MUC5AC is the main mucin protein produced by HT29-MTX-E12 cells (Martinez-Maqueda et al. 2015). Significant increases in protein levels should be observed particularly between days 7 and 14 thus providing a plausible explanation for continuing to quantify this protein.

Despite thorough optimisation of the assay for MUC2 and MUC5AC, the indirect ELISA was unable to detect an increase in mucin production for any of the MUC proteins tested. This does not match previous findings (Barnett et al. 2016; Lesuffleur et al. 1993). Barnett et al were able to quantify mucin proteins of a Caco-2/HT29-MTX co-culture and Lesuffleur stated that the mucus secreted from HT29-MTX increased in quantity over a period of up to 21 days and stabilised afterwards. The use of the positive control confirmed that the ELISA was a working assay and therefore the limitation was from the test samples rather than the assay itself. It could be that the level of the MUC proteins within the samples were beyond the detection range of the assay. This is most certainly the case for MUC2. In order to attain a suitable OD signal from the recombinant protein, the working concentrations had to be 10 fold higher than those used for MUC5AC indicating that the ELISA was only able to detect higher levels of the MUC2 protein. For the MUC5AC assays, it is possible that there was a level of MUC5AC detectable by the ELISA within the cell lysate at days 7 and 14; however, due to the low signal to background reading ratio this could not be confirmed.

In order to successfully quantify the MUC proteins in the cell culture samples, a more sensitive ELISA could be implemented. One such ELISA is the sandwich ELISA. This type of ELISA is considered to be 2-5 times more sensitive than the indirect ELISA

(Shah & Maghsoudlou 2016). For the sandwich ELISA technique, the plate is coated with a known concentration of antibody which is selected to capture the desired antigen. The non-specific binding sites are blocked and the sample containing the antigen of interest is applied to the plate. A specific primary antibody is added to "sandwich" the antigen. Finally, the enzyme-linked secondary antibody, followed by the substrate are added to the plate and a plate reader is used to measure the OD output (Wiederschain & Crowther 2009). A key advantage of the sandwich ELISA compared to the indirect ELISA include: minimal sample purification is needed as the sandwich ELISA is highly sensitive and specific to the antigen of interest. However, it is more expensive and time consuming than the indirect ELISA. Additionally, the primary and secondary antibodies must be "matched pairs" unlike the indirect ELISA which is more flexible (Shah & Maghsoudlou 2016).

Purification and concentration of the proteins could also be considered in the future. Protein purification can be carried out by fast protein liquid chromatography (FPLC) or high protein liquid chromatography (HPLC) and if required, further concentration of the proteins can be carried out by dialysis or ultra-filtration (Schömig et al. 2016). These techniques could ensure the samples fall within the detection range of the assay however both (particularly protein concentration) could be technically difficult due to the small sample volumes from cell culture.

There are additional explanations of why the assay was unable to detect the presence of MUC2 and MUC5AC from HT29-MTX-E12 cells. The choice of cell line could have had an impact. Barnett et al and Lesuffleur et al both used HT29-MTX cells for their studies (Barnett et al. 2016; Lesuffleur et al. 1993). The HT29-MTX cell line releases the mucus into the supernatant, whereas the HT29-MTX-E12 cells used in this project form an adherent mucus layer (Naughton et al. 2013). The cell line could have a direct effect on how the mucus proteins are detected as they may be readily quantifiable when present within the supernatant but not as an adherent layer. It is also possible that some of the mucus that was adhered to the cell monolayer may have been lost when the sample was processed, particularly when the cells were lysed with Triton X-100 (described in section 5.2.1) affecting the readings taken from the cell lysate sample. As well as this, the secretion rate of the mucus and the specificity of the ELISA would need

to be considered, although it is unlikely that the specificity of the ELISA was an issue in this study due to the successful standard curves produced by the positive controls.

5.5. Summary

An indirect ELISA was optimised with the aim of quantifying the increasing mucus levels of HT29-MTX-E12 cells over a 21 day period. The assay worked well for the positive controls but was unable to quantify the selected MUC proteins successfully. The reasons behind this have been discussed in section 5.4. The exact reason of why the mucin proteins could not be quantified by ELISA could not be established within the scope of this project. Alternative methods of quantifying the mucin levels will be discussed in section 6.3.3.

Chapter 6. Conclusions and Future work

This section will summarise the conclusions from the three results chapters of this thesis along with the limitations of the study. Future work that could be carried out to further advance the work within the PhD project will also be considered.

6.1. Key findings of this study

CDI remains a consistent problem to patients, particularly the elderly, who are facing antibiotic treatment and longer stays in hospitals. Like most infections, CDI is treated with antibiotics; however, a sharp increase in antibiotic resistant strains is posing a threat to these more common treatment methods (Lin et al. 2017). The antibiotic resistance era has led to alternative treatments being sought, one of which is bacteriophage therapy. In order for bacteriophage treatment of CDI to be explored, the safety and efficacy have to be established. Numerous models have been developed in order to assess the efficiency of the potential treatment of CDI with bacteriophages. Most models have been *in vivo* based, primarily in hamsters with a few laboratory batch fermentation models also being used (Ramesh et al. 1999; Meader et al. 2010). More recently *Galleria mellonella* has been introduced as an *in vivo* model along with *in vitro* broth based models (Nale et al. 2018; Nale et al. 2016). The aim of this PhD project was to develop and optimise a cell culture model using colon epithelial cell lines to observe the dynamics between phages and *C. difficile* and assess the therapeutic potential of *C. difficile* bacteriophages.

The idea of the cell culture model was first developed as a Masters project. During the Masters project it was shown that HT29 cell lines were able to survive in the anaerobic conditions required by *C. difficile* for the 8-10 hours duration of the experiments. Within the scope of this PhD project, the model was further developed, refined and optimised.

The optimisation process has been described in chapter 2. Here, the key methods used throughout the project were established, including growth of *C. difficile* for use in cell culture medium and enumeration of planktonic and attached *C. difficile.* As well as optimisation, the chapter also compared how phages and bacteria behaved in the

presence of two differing cell lines (HT29 and HeLa cells- colon and cervical cancer cell lines respectively). Results indicated that there was a level of specificity demonstrated by both the bacteriophages and the bacterium. Firstly, *C. difficile* took longer to attach to HeLa cells compared to HT29 cells. Another observation confirming specificity was the phages' ability to reduce *C. difficile* levels further in the absence of HeLa cells compared to with cells. In contrast, the presence of HT29 cells increased the killing efficiency of the bacteriophages. The specificity demonstrated by both *C. difficile* and the bacteriophages is likely to be due to the cell type and the location from which they had been isolated. *C. difficile* is a bacterium known to reside and proliferate within the gut and therefore specificity towards colon HT29 cells is likely.

The model was further developed in chapter 3 with the presence of mucus taken into account. The mucus layer is a prominent feature of the gastrointestinal tract and plays an important defensive role as a physical barrier against pathogens. For this reason it was of paramount importance that the presence of mucus was considered when the cell culture model was developed. The chapter also observed the interactions between bacteriophages and *C. difficile* in potential treatment regimens (prophylactic and remedial) with varying mucus levels. Additionally the efficiency of bound phages were investigated.

The presence of mucus altered the dynamics observed between bacteriophages and *C. difficile.* Firstly, the phages were able to attach to the epithelial cell layer as the mucus levels increased. A further observation was that bacteriophages were unable to attach to the cells without *C. difficile* being present. Bacteriophages that had bound to the epithelial cells and those given prophylactically were far more effective against planktonic *C. difficile* compared to attached bacteria. Once *C. difficile* had attached to the cells and mucus they appeared to be protected from the phages. This was also observed by Barr et al who noted that bacteriophages took advantage of a mucus layer by embedding themselves and attacking bacteria before they reached the epithelial cell layer (Barr et al. 2013). Once the bacteria attached to the mucus it is highly likely that the receptors of both phages and bacteria are less accessible due to their separate interactions with the mucus layer. The remedial treatment was consistently effective against both planktonic and attached bacteria.

The final results chapter discussed the optimisation of an indirect ELISA developed to quantify the mucus layer on HT29-MTX-E12 cells. Quantification of mucin levels was unsuccessful using the ELISA method. The assay was optimised successfully using two positive controls (MUC2, the prevalent mucus protein of the colon and MUC5AC, the main protein produced by HT29-MTX-E12 cells). However, when the samples were measured a positive signal was not obtained. If feasible, further optimisation of the assay could be repeated in order to reduce the background readings to increase the signal to background ratio. It is more likely that the samples had mucin levels that were beyond the scope of detection by indirect ELISA and therefore an alternative method of quantification needs to be considered. This is discussed in section 6.3.3.

6.2. Limitations of study

The main limitation of the cell culture model was that it remained static throughout the experimental time frame and did not take into account the movement that naturally occurs within the digestive system. In reality, the mucus is continuously moving in the gastrointestinal tract and therefore a constant mucus layer is not maintained at any given time (Johansson et al. 2013). The movement of the mucus layer is an important factor. As it moves, it takes along attached bacteria and bacteriophages which would further impact the dynamics between them. The small intestine is designed to limit bacterial exposure and so by the process of flushing there is an intense motor activity where the mucus and attached bacteria are moved along into the colon (Johansson et al. 2013). In contrast, the large intestine is designed to harbour commensal bacteria however, movement still occurs in this region. Unlike the small intestine which uses the aforementioned flushing mechanism, the movement within the colon is in the form of vigorous peristaltic waves which are generated from the enteric nervous system. The waves are powerful and are able to carry the mucus with it. In addition, the inner layer of mucus is constantly renewed by the goblet cells and has a turnover time of approximately 1 hour (Johansson et al. 2013). It is important to note that the mucus layer also changes in the presence of *C. difficile* and its infection. The levels of MUC2 reduce in the presence of CDI which corroborates with the fact that there is a reduced protective barrier function (Engevik et al. 2014).

A second limitation of this PhD project is that *C. difficile* spores were not taken into account. All experiments were carried out using vegetative *C. difficile* cells, the bacterium's active, infection causing state, as bacteriophages are not able to infect *C. difficile* in its spore form. However, CDI is usually spread by the ingestion of spores via the faecal-oral route, particularly in a hospital setting and therefore should be taken into account within an experimental model. It would be interesting to investigate how *C. difficile* spores interacted with the mucus layer and how efficiently they were able to germinate in those conditions. The observation of spores would be technically difficult within the cell culture model developed for this PhD project due to the time constraints that result from culturing epithelial cells in an anaerobic environment. In the cell culture model developed for this project both the HT29 and HT29-MTX-E12 cells were able to remain viable with an intact monolayer for 8-10 hours -the maximum time needed for the experiment. If *C. difficile* spores were going to be used, the germination time would have to be added on to the experimental time. The extra time required could lead to erroneous results due to the epithelial cell monolayer being compromised due to the extended duration spent in anaerobic conditions. Likewise, if the spores were put in an environment that was not suited to them then they would not be able to germinate into the vegetative cells. Therefore a new model would have to be devised in order to take into account the *C. difficile* spores and the use of cell culture.

6.3. Future work

6.3.1. Cell model development

There are many ways in which the cell model could be further improved in order to create a more effective setting for investigating the therapeutic potential of bacteriophages against CDI. A natural progression to the model would be to keep the experimental set up as it is, but work with more bacteriophages and introduce phage cocktails as a treatment option.

The cell culture model itself could be further improved firstly by using a colon cell line which does not derive from cancer e.g. CCD-18co. Cell lines, particularly carcinoma

cell lines are easy to culture and maintain and many of which have been in culture for decades. This means that the cell lines are now well adapted to their medium and may have altered morphology and may even differ genetically from their tissue of origin (Pan et al. 2008). Primary cells are isolated directly from the tissues and will therefore maintain their normal cell morphology along with the many markers and functions that are seen *in vivo* (Pan et al. 2008). Disadvantages of primary cells include their finite lifespan and limited expansion capacity, their sensitivity and their need for additional nutrients. However, the establishment of a primary cell model will further refine the work carried out in this thesis.

Another method for improving the model is with the use of co-culture. The gastrointestinal tract is a difficult environment to replicate *in vitro*, but it is possible that the use of co-culture featuring two or more cell lines or cell types would be more accurate. Another way the model could be improved is with the use of 3D cell culture which would be beneficial for further determination of the interactions between phages and bacteria with the cell monolayer.

6.3.1.1. Bacteriophage cocktails

Throughout this project the bacteriophage CDHS1 was used with its accompanying host *C. difficile* strain AIU (ribotype 027). Many studies have been published to show the efficiency of bacteriophage cocktails on a wide range of bacteria including *P. aeruginosa, E. coli and Salmonella spp* (McVay et al. 2007; Wall et al. 2010). Studies using *C. difficile* phage cocktails made up of two to four bacteriophages have also been successful. To date these have been applied *in vitro* in laboratory scale studies including batch fermentations (Meader et al. 2013) and *in vivo* in a hamster and a *G. mellonella* model (Nale et al. 2016; Nale et al. 2018), but have yet to be carried out on epithelial cells from the gastrointestinal tract. For this reason, it would be beneficial to try different phage cocktail combinations within the cell culture model developed within this thesis. It would be interesting to see if data attained from applying the phage cocktails to a cell culture model correlated with what was observed in previous studies. In order for this to take place it is likely that a different *C. difficile* ribotype should be used so multiple bacteriophages could be made use of. It may also be possible to assess

combination treatment using both antibiotics and phages and applying both to an epithelial layer infected with *C. difficile.*

6.3.1.2. Co-culture of cells

The co-culture of cells involves the combination of two or more cell lines. The use of co-culture in a model such as the one developed in this study would give a more accurate depiction of the structure of the cells located within the gastrointestinal tract. There are a wide range of cell types that feature within the GI tract, the aforementioned epithelial cells, enterocytes, goblet cells and Paneth cells to name a few (see table 1.1). Primary cells as mentioned previously can also be considered. A co-culture featuring at least two of these cell types would improve the current model by creating a more complex cell layer that the bacteria and phages could interact with. It would be interesting to consider whether a cell layer made up of multiple cell types could alter how *C. difficile* adheres to the cells and whether the phage killing efficiency would be affected. An additional parameter that could be investigated is whether the mucin production from the various cell types changes the dynamics even further. Co-cultures could be applied using cells that secrete mucus in combination with the cells that form an adherent mucus layer.

6.3.1.3. 3D cell culture

A natural progression from a cell culture or co-culture model is 3D cell culture. The technique is frequently used to demonstrate how bacteria interact with epithelial cells. Ordinary cell culture on a two dimensional plane has been recently deemed far too simple and overlooks certain parameters including the communication between adjacent cells (Haycock 2011). Additionally 2D cell cultures tend to use one individual cell line. The co-culture to a certain extent solves this by introducing another cell type but further enhancement could be made using 3D cell culture. With the advent of 3D cell culture models, the opportunity is there to take a step closer to recreate the natural conditions as seen *in vivo*. They offer biologically superior structures which can be used to study complex interactions that are otherwise not possible with 2D cell culture (Ravi et al. 2015).

For the 3D structure to be achieved, a scaffold is usually required and this can be made from many biomaterials. A typical 3D cell culture application which is of particular relevance to the model developed in this thesis would be the use of a Transwell® insert. The epithelial cell line could be grown on this membrane and be used to demonstrate whether bacteria and/or phages are able to pass through the cells and membrane to the area located below or whether they get embedded in the monolayer. This technique can also be applied to the mucus cells. HT29-MTX-E12 cells can be grown on the membrane to form a monolayer in order to determine whether both bacteriophages and *C. difficile* are able to pass through the membrane or if they get trapped within the mucus. The process can be repeated over a time course with varying levels of mucus as has been done for this PhD project. It would be expected that the phages would be able to pass through the membrane without the monolayer being present due to their small size, however it would be interesting to note whether they were able to pass through the cell monolayer and whether the presence of mucus either inhibits them completely or slows them down.

6.3.2. Microscopy

Microscopy could be applied in multiple ways to gather more data on how bacteriophages interact with the mucus layer. Transmission electron microscopy (TEM), is usually the chosen method to attain clear images of phages. However, this method is usually applied when bacteriophages have been isolated and are the only entity within a sample. In this particular case TEM would not be possible as not only is it technically difficult to prepare slides with a cell monolayer for analysis, the added complication of phages and bacteria would cause an additional challenge. Ordinarily the cell monolayer would have to be prepared on a coverslip. Alternatively, the sample would have to be embedded in resin so that sections could be taken in order for the cell monolayer to be observed by cross section. This method is more straightforward in non-mucus producing cells, however when the added variable of mucus is included the method becomes far more complicated.

The use of microscopy for a visual representation of bacteriophages within the cell culture model would be far more challenging and therefore unfortunately beyond the scope of the PhD project. In order for phages to be located within the mucus and cell

monolayer, it is most likely that a staining process would need to be utilised; whereby a stain would be applied so each variable (the cell monolayer, the mucus, the phages and the bacteria) could be visible. This would involve a great deal of optimisation to ensure that the stains for each element did not interact with each other.

Barr et al previously made use of epifluorescence microscopy in order to assess the adherence of phages and bacteria to mucus. Here, the mucus was extracted directly from various mucosal surfaces and the number of bacteriophages were enumerated using SYBR gold staining and epifluorescence microscopy (Barr et al. 2013). Although this method could be applied to the cell culture model, it would be more suited as a method of enumeration as demonstrated by Barr et al. A further enumeration method is not required for the cell culture model devised in this project as the CFU/mL and PFU/mL counts appeared to be accurate and provided sufficient data. Furthermore, epifluorescence microscopy is not a suitable method to identify the exact location of phages within the cell and mucus layer, nor is it a suitable method to determine the orientation of which the bacteriophages have bound to the mucus. The location and orientation of the phages are two of the main unknowns which if studied further, would provide a deeper insight into the interactions between phages, the infecting bacteria and the mucus layer.

6.3.3. Mucin quantification

The challenging aspects of quantifying mucus by indirect ELISA have already been discussed in sections 5.1 and 5.4. The idea of using a more sensitive sandwich ELISA has also been considered in section 5.4. This section will consider the alternative methods for mucin quantification which could be applied.

An alternative method for mucin quantification is real time qPCR. Here, the mucin mRNA expression levels can be measured and compared. The use of a probe would be more suitable compared to the intercalating dye due to its higher specificity (Ye et al. 2012). A further justification for the use of a probe, is it is unclear how abundant the MUC proteins are within the cells, particularly as the ELISA initially failed to detect them. The use of a dye such as SYBR green could result in the primers binding to the

wrong target or the formation of primer dimers and therefore a probe would be more appropriate.

For a simpler and subjective method of quantification, a staining method using Alcian blue, Periodic acid-Schiff (Pas) stain or a combination of the two could be applied. The Alcian blue stain tends to target the more acidic mucins whereas the Pas stain targets the neutral mucins (Lamacchia et al. 2018). Using a combination of the two would enable both acidic and neutral mucins to be identified. These staining methods would show the presence or absence of mucus stained in one colour whilst the epithelial cells would stain in another colour. Light microscopy can be used to observe the changing levels of mucus on each experimental day. The staining method would successfully provide a visual representation of the changing mucus levels which could be suitable for the purposes of a cell culture model such as the one developed for this PhD thesis. However limitations will arise if the difference in mucus levels are small and not clearly visible by means of staining and microscopy. Therefore a more accurate method where the mucus could be quantified would be far more beneficial.

6.4. Final conclusion

The data from this PhD thesis suggests that a cell culture model can be utilised to study the interactions between bacteriophages and *C. difficile*. Furthermore, the model has been refined to take into account the anaerobic conditions and presence of mucus within the gastrointestinal tract. The use of the model successfully showed cell specificity displayed by both phages and *C. difficile*. The model was also used to mimic potential bacteriophage treatment regimens with changing mucus levels in order to recreate various scenarios that could take place in a real life setting. Although the model is in its preliminary stages and currently has its limitations, it has the potential to be improved to play a key role in establishing in detail phage - *C. difficile* dynamics within an epithelial cell environment.

Chapter 7. Appendix – Media, buffers and solutions

Media

All media were autoclaved at 121 °C for 15 minutes unless stated otherwise.

Blood Agar (7 %)

Brain Heart Infusion (BHI) powder……14.8 g

Bacteriological Agar No 1……...4 g

Distilled water…….....365 mL

Autoclave and allow to cool to approximately 50 °C.

Add 35 mL of defibrinated horse blood. Mix thoroughly by inversion. Dispense into plates as required. Store at 4 °C for a maximum of two weeks.

BHI Agar (1 %)

BHI Powder……..……14.8 g

Bacteriological Agar No 1……...4 g

Distilled water…….400 mL

Autoclave, allow to cool to approximately 50 °C and dispense into plates as required. Store at 4 °C for a maximum of two weeks.

BHI Broth

BHI Powder……..14.8 g

Distilled water……...400 mL

Autoclave. Store at room temperature for a maximum of one month.

Fastidious Anaerobic Broth

Fastidious anaerobic broth powder……2.39 g

Distilled water…….80 mL

Autoclave. Allow to cool to approximately 50 °C. Store at 4 °C a maximum of one month if unopened, two weeks if opened. Can be stored in approximately 7 mL aliquots at 37 °C in the anaerobic chamber for up to one week.

Double strength BHI agar (overlay)

BHI powder…….14.8 g

Bacteriological Agar No 1……2 g

Distilled water……200 mL

Autoclave. Store at 55 °C for a maximum of two weeks.

Cell culture growth medium

Dulbecco's Modified Eagle's Medium……200 mL

Supplemented with:

Foetal Bovine Serum (FBS).……...20 mL

L-glutamine (200 mM)…….2 mL

Penicillin/Streptomycin…….1mL

L-glutamine was filter sterilised prior to addition to the medium. Store at 4 °C for a maximum of one month.

Cell culture maintenance medium

Dulbecco's Modified Eagle's Medium……200 mL

Supplemented with:

Foetal Bovine Serum (FBS)…….....4 mL

L-glutamine (200 mM)…….2 mL

L-glutamine was filter sterilised prior to addition to the medium. Store at 4 °C for a maximum of one month.

<u>Buffers and Solutions</u>

Double strength salt solution

Calcium chloride dihydrate 99 % ($CaCl_2$)…….0.6 g

Magnesium chloride hexahydrate ($MgCl_2$)……....32.48 g

Distilled water……up to 200 mL

Dissolve the salts in 150 mL first, then make up to 200 mL. Autoclave. Store at 55 °C for up to one month.

Dulbecco's Phosphate Buffered Saline (dPBS)

Dulbecco's Phosphate Buffered Saline (dPBS)...…….1 tablet

Distilled water……...100 mL

Autoclave. Store at room temperature for up to six months if unopened. Once opened, discard after one month.

ELISA reagents

Coating buffer

Sodium carbonate (NA_2CO_3)……..0.61 g

Sodium hydrocarbonate ($NaHCO_3$)……..1.2 g

Distilled water…….200 mL

Store at 4 °C for a maximum of three months.

Blocking buffer (3 % Bovine serum albumin fraction V)

To be made fresh each ELISA run:

Bovine serum albumin fraction V (3 % BSA)……. 0.3 g

PBS…….10 mL

Quantity scaled up or down as required.

Blocking buffer (5 % skimmed milk)

To be made fresh each ELISA run:

Skimmed milk powder …….0.5 g

PBS……...10 mL

Quantity scaled up or down as required.

Diluent for dilutions of primary and secondary antibodies (1 % BSA)

BSA fraction V…….0.1 g

PBS……...10 mL

Quantity scaled up or down as required.

PBS-Tween (0.1 %) Washing buffer

Tween 20…….....0.4 mL

PBS……..up to 400 mL

PBS-Tween (0.05 %) Washing buffer

Tween 20…….....0.2 mL

PBS……..up to 400 mL

Stop solution- 0.16 M Sulfuric Acid

1 M Sulfuric acid (H_2SO_4)……3 mL

Distilled water……...197 mL

Chapter 8. Bibliography

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