THE STUDY OF *CLOSTRIDIOIDES DIFFICILE* SPORE GERMINATION FOR THE

DEVELOPMENT OF A PRO-GERMINATION SPORICIDAL STRATEGY FOR THE ELIMINATION

OF *C. DIFFICILE* SPORES

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The study of *Clostridioides difficile* spore germination for the development of a pro-germination sporicidal strategy for the elimination of *C. difficile* spores

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Thesis Abstract

The highly resistant spores of *C. difficile* are key to the transmission of *C. difficile* infection and are capable of withstanding adverse environmental conditions including the antimicrobial activity of traditional biocides but reactivate through germination in response to specific environmental germinants and co-germinants. The research presented in this thesis utilised an optical density germination assay to explore the induction of *C. difficile* spore germination by germinants and co-germinants to further understand the regulation of *C. difficile* spore germination and utilise this knowledge in a pro-spore germination strategy to eliminate *C. difficile* spores from the environment. The bile salt, taurocholate was an efficient germinant, while cholate, glycocholate and deoxycholate exhibited divergent germination activity with spores of *C. difficile* NCTC 11204 and *C. difficile* reference strains representing ribotypes 001, 002 and 015. Only glycine and calcium had the capacity as co-germinant to induce germination of *C. difficile* spores but combining glycine or calcium with selected amino acids reduced or completely inhibited the germination response of *C. difficile* spores. A novel bile salt-derived compound, C109, developed to have dual germination and antimicrobial functionality with *C. difficile* spores successfully initiated germination and also demonstrated antimicrobial activity against *C. difficile* vegetative cells. To develop a *C. difficile* sporicidal smart surface a series of polymeric formulations incorporating C109 and additional novel compounds C114 and C119 were evaluated. Germination of *C. difficile* spores was successfully induced but polymer composition and processing conditions were found to influence germination activity. Evaluation of the antimicrobial activity of polymer formulations incorporating C109 demonstrated a statistically significant reduction in the recovery of viable *C. difficile* spores. The dual germination and antimicrobial activity of the polymer formulation incorporating C109 was therefore successfully demonstrated, providing proof of concept for a sporicidal polymeric biomaterial targeting *C. difficile* spores, deserving of further development and investigation.

Keywords: *Clostridioides difficile*, *C. difficile* spores, *C. difficile* germination, pro-spore germination elimination strategy

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Abbreviations

ARHAI - Advisory Committee on Antimicrobial Prescribing, Resistance and Healthcare Associated Infection

ASE - Accelerated solvent extraction

ANOVA - Analysis of variance

BKC - Benzalkonium chloride

BHI - Brain heart infusion

BHIS - Brain heart infusion media supplement with 0.1% (w/v) L-cysteine and 0.5% (w/v) yeast extract

CAMPs - Cationic antimicrobial peptides

CWP - Cell wall protein

CWPs - Cell wall proteins

CDI - *Clostridioides difficile* infection

CDT - *Clostridium difficile* transferase

CDRN - *Clostridium difficile* Ribotyping Network

CryoSIM - Cryo-structured illumination microscopy

Cryo-SXT - Cryogenic soft X-ray tomography

DPA - Dipicolinic acid

EIA - Enzyme immunoassay

EIAs - Enzyme immunoassays

ESCMID - European Society of Clinical Microbiology and Infectious Diseases

FMT - Faecal microbiota transplantation

H2RAs - Histamine type-2 receptor antagonists

HAIs - Healthcare associated infections

HDPE - High density polyethylene

HPV - Hydrogen peroxide vapour

Ig - Immunoglobulin

IL - Interleukin

MAL - Muramic-δ-lactam

MAPK - Mitogen-activated protein kinase

MSCRAMMs - Microbial surface components recognising adhesive matrix molecules

NSAIDs - Non-steroidal anti-inflammatory drugs

NF-kB - Nuclear factor Kappa B

OD_{570} - Optical density at 570 nm

PBS - Phosphate Buffered Saline

PPIs - Proton pump inhibitors

SASPs - Small acid-soluble proteins

SDW - Sterile distilled water

SEM - Scanning electron microscopy

SHDPE - Stabilised high-density polyethylene

SLP - Surface layer protein

SLPs - Surface layer proteins

STC - Sodium taurocholate

T101 - Trigonox 101

TEM - Transmission electron microscopy

TLR - Toll-like receptor

TNFα - Tumour necrosis factor-alpha

Tris-HCL - Tris(hydroxymethyl)aminomethane hydrochloride

UHDPE - Unstabilised high density polyethylene

UV - Ultraviolet

VRE - Vancomycin-resistant *Enterococcus*

Chapter 1

1.0 Introduction.

1.1 *Clostridioides difficile.*

Clostridioides difficile is a Gram-positive, anaerobic, cytotoxin-producing, spore-forming bacillus, which is motile due to peritrichous flagella (Lawson *et al.*, 2016; Bongaerts and Lyerly, 1994; Purcell *et al.*, 2012). Isolated from the stool of healthy new-borns, it was originally named *Bacillus difficile*, to reflect the difficulty in isolating and culturing the anaerobic bacterium (Hall and O'Toole, 1935; Kelly and LaMont, 2008). It was identified as the causative agent of antibiotic associated colitis in 1978, where the relationship between the production of cytotoxin and disease was demonstrated (Bartlett *et al.*, 1978; Lance George *et al.*, 1978).

The vegetative cells of *C. difficile* are 0.5-1.9 μm wide, 3.0-16.0 μm in length and grow optimally under anaerobic conditions at 30-37°C but are unable to survive in aerobic environments (Lawson *et al.*, 2016; Jump *et al.*, 2007). The aerotolerant metabolically dormant spores produced by *C. difficile* however are highly resistant to standard disinfectant procedures, allowing them to persist in the environment and are therefore responsible for the transmission of *C. difficile* infection (CDI) (Deakin *et al.*, 2012; Vedantam *et al.*, 2012). The ovoid spores of *C. difficile* which vary in size, 0.5-0.7 μm in width and 1-1.5 μm in length, can be visualised microscopically by utilisation of the endospore stain developed by Schaeffer and Fulton in 1933 (Lawson *et al.*, 2016; Snelling *et al.*, 2010; Schaeffer and Fulton, 1933). *C. difficile* can be cultured using a variety of selective media but the addition of the bile salt, sodium taurocholate to culture media is required to germinate spores (Hink *et al.*, 2013; Lister *et al.*, 2014; Wilson, 1983). Colonies of *C. difficile* vary in size but have a characteristic 'ground glass' morphology on non-selective media (Curry, 2010). A distinctive

phenotypic 'barnyard' odour is associated with cultures of *C. difficile* due to production of specific volatile organic compounds (Curry, 2010; Probert *et al.*, 2004).

Until relatively recently, *C. difficile* belonged to the genus *Clostridium* which also includes the clinically relevant but genetically distinct pathogens *Clostridium botulinum*, *Clostridium tetani* and *Clostridium perfringens* (Bahl and Dürre, 2001; Yutin and Galperin, 2013). A proposed reclassification of the genus *Clostridium* by Collins *et al*. (1994) and then by Lawson and Rainey (2016) to restrict the genus to those closely related to the type species *Clostridium butyricum* had consequences for the inclusion of *C. difficile* (Collins *et al.*, 1994; Lawson and Rainey, 2016).

Kingdom: Bacteria

Phylum: Firmicutes

Class: Clostridia

Order: Clostridiales

Family: Clostridiaceae

Genus: *Clostridioides*

Species: *difficile*

Figure 1.1 Taxonomy and classification of *C. difficile* (Oren and Rupnik, 2018, Sandhu and McBride, 2018).

A 16S rRNA gene sequence analysis of *C. difficile* demonstrated its lack of genetic similarity with other *Clostridia* leading to proposals for its accommodation within the new genus of *Peptoclostridium* and its reclassification as *Clostridioides difficile* (figure 1.1) (Lawson *et al.*, 2016; Yutin and Galperin, 2013). While the genus *Peptoclostridium* has not been widely adopted, both *Clostridioides difficile* and *Clostridium difficile* (abbreviated *C. difficile*) are now accepted as valid in the nomenclature (Oren and Rupnik, 2018; Sandhu and McBride, 2018). A more recent phylogenetic study, based on the conserved genes of *Clostridium* species was in agreement with the 16S rRNA based taxonomy of Collins *et al.* (1994) and Yutin and Galperin (2013) and the consequent reclassification of *C. difficile* (Yu *et al.*, 2019).

1.2 *C. difficile* infection (CDI).

CDI is a major cause of infective diarrhoea in healthcare environments and the community, accounting for 20-30% of antibiotic associated diarrhoea and is a substantial cause of morbidity and mortality (Balsells *et al.*, 2019; NICE, 2015). The development of CDI is driven by 3 key events; the alteration of the normal gut microbiota, colonisation of the colon by *C. difficile* and finally the proliferation of the organism and production of its toxins which are responsible for the symptoms and clinical presentation of CDI through mucosal damage and inflammation (Poutanen and Simor, 2004; Kelly *et al.*, 1994).

1.3 Incidence of *C. difficile* infection.

In the early 1990's, CDI was poorly understood, transmission between hospital patients was recognised but it was unclear if *C. difficile* was a component of the normal flora or transient coloniser (McFarland *et al.*, 1989; Bartlett, 1994). CDI was considered a self-limiting disease following the withdrawal of antimicrobial therapy, and not the threat to public health recognised today (Curry, 2010; Depestel and Aronoff, 2013). Over the past 30 years CDI has emerged as a significant infectious disease that is associated with acquisition in clinical environments and the community (Depestel and Aronoff, 2013). An increase in cases of CDI in the early 2000's was attributed to the emergence of the PCR ribotype 027, causing significant outbreaks in the USA and across Europe (Kuijper *et al.*, 2006). Strains belonging to this ribotype were responsible for extensive outbreaks of *C. difficile* in Quebec, Canada and were linked with increased morbidity and mortality, resulting in the term of 'hypervirulent' associated with ribotype 027 (Cookson, 2007; Yakob *et al.*, 2015). In the UK *C. difficile* received national publicity following outbreaks at two UK hospitals. During the period between April 2003 and March 2006, a series of minor and major outbreaks occurred at Stoke Mandeville Hospital, Buckinghamshire Hospital NHS Trust, resulting in 498 cases of CDI and 172 deaths associated with *C. difficile* (The Healthcare Commission, 2006).

Similarly, during outbreaks that occurred at Maidstone and Tunbridge Wells NHS Trust between October 2005 and September 2006, more than 500 patients developed CDI, leading to 60 deaths where *C. difficile* was considered to be the contributing factor (The Healthcare Commission, 2007). The 027 hypervirulent ribotype was identified in patient samples during the outbreaks periods at both NHS trusts and was implicated as factor in the increased severity of disease and proportion of deaths (The Healthcare Commission, 2006; 2007). The reports investigating the outbreaks at Buckinghamshire Hospital NHS Trust and Maidstone and Tunbridge Wells NHS Trust, implicated a failure to isolate patients and inadequate infection control, including infrequent handwashing by staff, leading to an increased risk of transmission of CDI (The Healthcare Commission, 2006; 2007).

In England, the number of deaths, where *C. difficile* was stated on the death certificate, peaked at 7,916, in 2007 (Office for National Statistics, 2013). Rates of CDI in the UK have declined dramatically since the introduction of a multi-component care bundle to prevent healthcare associated infections (HAI), but it remains a major contributor to the burden of HAI in the USA, causing almost half a million infections and 29,000 deaths in the USA in one year (Department of Health, 2007a; Balsells *et al.*, 2019; Lessa *et al.*, 2015). *C. difficile* was the most frequently reported healthcare-associated pathogen in a multistate prevalence survey of HAI in the USA (Magill *et al.*, 2014). In 2013 and again in 2019, the Centre for Disease control and Prevention (CDC) declared *C. difficile* an urgent threat to public health. In the USA in 2017, 223,900 cases of *C. difficile* were recorded with at least 12,800 deaths (Centers for Disease Control and Prevention, 2019; 2013)*.*

CDI was considered to be principally a nosocomial infection, with hospitalised patients being at high risk, until the emergence of community-acquired infections (Hensgens *et al.*, 2012b). It is likely that the extent of community-acquired CDI was underestimated and underdiagnosed due to a lack of understanding of CDI outside of a hospital setting (Gupta and Khanna, 2014). CDI is defined as community-acquired if the onset of symptom occurs in the community or within 48-hours of admission to a hospital, with no hospitalization in the past 12 weeks (Kuijper *et al.*, 2006; McDonald

et al., 2007). Hospital-acquired or healthcare facility-associated CDI is defined by the onset of symptom more than 48-hours after admission or less than 4 weeks after discharge from a healthcare facility (Kuijper *et al.*, 2006; McDonald *et al.*, 2007). Rates of CDI have continued to decline in England since 2007, undoubtably due the implementation of multicomponent control measures to reduce CDI. The most recently available data for England reveals a total of 12,275 cases of *C. difficile* infection as reported by NHS trusts between 1 April 2018 and 31 March 2019. However the proportion of community acquired CDI cases is of increasing concern, with community associated, community-onset CDI causing 3,467 cases in England in 2018/19 (Public Health England, 2019a; Department of Health and Health Protection Agency, 2008).

1.4 Risk factors of *C. difficile* infection.

CDI is classically precipitated by exposure to broad spectrum antibiotics which disrupt the normal intestinal microbiota, the most common risk factor to development of CDI (Poutanen and Simor, 2004; Bignardi, 1998; Theriot *et al.*, 2016). Dysbiosis of the normal gut microbiota by broad spectrum antibiotics is strongly associated with proliferation and colonisation of *C. difficile* (Vedantam *et al.*, 2012). Any factor that alters the commensal gut microbiota, which normally prevents establishment of CDI by a phenomenon known as

**Table 1.1 Antibiotic classes and their association with *C. difficile* infection[*].
Adapted from Leffler and Lamont (2015).**

Antibiotic Class	Association with CDI
Clindamycin	Very common
Ampicillin	Very common
Amoxicillin	Very common
Cephalosporins	Very common
Fluoroquinolones	Very common
Other Penicillins	Common
Sulphonamides	Common
Trimethoprim	Common
Trimethoprim-sulfamethoxazole	Common
Macrolides	Common
Aminoglycosides	Uncommon
Bacitracin	Uncommon
Metronidazole	Uncommon
Teicoplanin	Uncommon
Rifampin	Uncommon
Chloramphenicol	Uncommon
Tetracyclines	Uncommon
Carbapenems	Uncommon
Daptomycin	Uncommon
Tigecycline	Uncommon

[*]Specific antibiotics are listed if their association with *C. difficile* differs from that of other antibiotics in their class.

colonisation resistance, increases the risk of CDI by facilitating the colonisation of *C. difficile* present in the environment (Viswanathan *et al.*, 2010; Poutanen and Simor, 2004). Patients with *C. difficile* demonstrate a reduction in the diversity of colonic commensal bacteria; *Bacteroides*, *Prevotella*, and *Bifidobacteria*, leading to the loss of colonisation resistance (Rea *et al.*, 2012; Kelly, 2012).

Patients on antimicrobial therapy have been found to be on average at 6-fold higher risk of developing CDI compared to individuals not on antimicrobial therapy (Bignardi, 1998). Almost all classes of antibiotics have been linked with CDI (table 1.1), (Leffler and Lamont, 2015; Teng *et al.*, 2019). Systematic review and meta-analysis has identified carbapenems, second, third and fourth generation cephalosporins, trimethoprim/sulphonamides, fluoroquinolones and penicillin combinations to be most strongly associated with hospital-associated CDI (Slimings and Riley, 2021; Slimings and Riley, 2014; NICE, 2015). Antibiotics most strongly association with community acquired-CDI are clindamycin, fluoroquinolones, cephalosporins, monobactams and carbapenems (Brown *et al.*, 2013; Deshpande *et al.*, 2013). In the month following the termination of antibiotic therapy, patients are at a 7-10 fold increased risk of developing CDI and remain at an increased risk up to 3 months after the antibiotic usage (Hensgens *et al.*, 2012a).

Susceptibility to CDI increases with age, with the majority of cases occurring in patients over the age of 65 years (Viswanathan *et al.*, 2010; Bignardi, 1998; Loo *et al.*, 2005). The incidence of CDI per 100, 000 people increases 3-fold in people aged 45-64 years and 13-fold in those aged >65 years compared to younger adults aged 18–44 years (Lessa *et al.*, 2015). When considering those over 65 years, increasing age is associated with progressively increasing risk of CDI, until patient age reaches the late 80's where CDI risk remains elevated (Olsen *et al.*, 2018). In England, CDI rates were highest among patients ≥ 85 years of age in the financial year 2018/19 (Public Health England, 2019a). The overall health status, including infections, healthcare utilization, acute conditions and frailty indicators of patients over 65 years are the most important determinants of the risk of CDI in the elderly (Olsen *et al.*, 2018). The high rate of CDI in the elderly is also attributed to a failure of

the immune system to mount an effective response due an inability of the normal gut microbiota to rapidly and fully recover after antibiotic treatment for the infection (Crogan and Evans, 2007).

The association between CDI and clinical environments has long been established (Eze *et al.*, 2017). A prolonged stay in hospital is strongly associated with exposure to *C. difficile*, increasing the likelihood of exposure and colonisation from strains present in hospitalised asymptomatic and symptomatic patients (Bignardi, 1998; Asha *et al.*, 2006; Clabots *et al.*, 1992). Multiple admissions to hospital and admission to intensive care units have also been determined to increase the risk of CDI (Palmore *et al.*, 2005; Bignardi, 1998). A study by Asha *et al.* (2006) equated each additional day of hospital a stay to an ~2%-increased risk of infection. When considering the duration of hospital admission as a risk factor, it is often unclear if this is a contributing risk factor or a consequence of CDI (Bignardi, 1998). The transmission of *C. difficile* and its persistence in hospital settings is thought to be exacerbated by re-admission of previously hospitalized and asymptomatic patients (Clabots *et al.*, 1992). An increased risk of CDI in clinical environments also extends to long-term care and nursing facilities where patients are elderly and have underlying disease (Bauer *et al.*, 2011; Simor *et al.*, 2002). The risk associated with clinical environments is not relevant for community-acquired CDI, the traditional risk factors already discussed are not typically associated as cases of community-acquired CDI are seen in individuals not considered to be at risk, including those who have not received recent antibiotic therapy and healthy younger adults (Gupta and Khanna, 2014; Wilcox *et al.*, 2008; Khanna *et al.*, 2012).

Patient comorbidities are an additional risk factor for hospital-acquired CDI, community acquired CDI and complicated CDI (Bauer *et al.*, 2011; Furuya-Kanamori *et al.*, 2015; Abou Chakra *et al.*, 2014). A study that examined the association between comorbidities and the risk of community acquired CDI, identified inflammatory bowel disease as the strongest risk factor for CDI, followed by renal failure and hematologic cancers (Furuya-Kanamori *et al.*, 2015). The evidence to understand the link between CDI and comorbidities is complex and requires further examination.

Patients with multiple comorbidities may also be more likely to be admitted to hospital, receiving antibiotic therapy and to be of advanced age.

An increased risk of CDI has also been associated with several non-antibiotic drugs. The use of proton pump inhibitors (PPIs) and histamine type-2 receptor antagonists (H2RAs) in the treatment of acid-related upper gastrointestinal disorders was assessed as a risk factor of CDI by systematic review. This study identified an increased risk of taking antisecretory therapy in those infected with *C. difficile*, with a greater association of PPI use compared with H2RA use (Leonard *et al.*, 2007). A more recent meta-analysis of studies showed a significant association between the use of PPIs and the risk of CDI (Trifan *et al.*, 2017). Treatment with PPIs inhibits the production of gastric acid and significantly alters the microbial community in the gut, which is believed to promote the proliferation of *C. difficile* (Amir *et al.*, 2014; Trifan *et al.*, 2017).

Meta-analysis to understand the association between the use of non-steroidal anti-inflammatory drugs (NSAIDs) and both community-based and healthcare-associated CDI demonstrated a significant increase in the risk of CDI in patients exposed to NSAIDs (Permpalung *et al.*, 2016). In a study in the UK, a risk of CDI was associated with the use of diclofenac, but not with other NSAIDs (Suissa *et al.*, 2012). In *C. difficile*-infected mice, a non-steroidal anti-inflammatory drug caused prostaglandin deregulation and disruption to the intestinal barrier which was also paralleled by dysbiosis of the intestinal microbiota and an altered inflammatory and immune response (Maseda *et al.*, 2019).

Cytotoxic chemotherapeutic agents, particularly methotrexate, have similarly been associated with the development of CDI in the absence of antibiotics (Anand and Glatt, 1993). Chemotherapy agents disrupt the gut microbiota and cause damage to epithelial cells, promoting infection with *C. difficile* (Neemann and Freifeld, 2017).

1.5 Transmission of *C. difficile* infection.

Spore formation by *C. difficile* is crucial for survival and dissemination in the environment. The infectious agent of CDI, the dormant aerotolerant and highly resistant spore facilitates transmission (Deakin *et al.*, 2012; Paredes-Sabja *et al.*, 2014; Vedantam *et al.*, 2012). A major reservoir of *C. difficile* in clinical environments are patients with CDI who shed spores, contaminating the environment and transmitting CDI to vulnerable patients (McFarland *et al.*, 1989; Samore *et al.*, 1996; Crobach *et al.*, 2018). Symptomatic CDI patients as well as asymptomatic carriers of *C. difficile* contribute to the contamination of the healthcare environment and the transmission of CDI, with the shedding of spores continuing even after the cessation of symptoms and the completion of antibiotic therapy (Sethi *et al.*, 2010; Riggs *et al.*, 2007). *C. difficile* is introduced to clinical environments through the admission of colonised patients. The reported rates of colonisation upon admission varies significantly between studies, with one study reporting of up 55% of patients testing positive for *C. difficile* upon admission to hospital (Clabots *et al.*, 1992; McFarland *et al.*, 1989; Johnson *et al.*, 1990). The variation between studies is multifactorial but is additionally determined by the methodology used to identify colonised patients. In a more recent large Canadian study only 4% of patients tested positive for *C. difficile* upon admission (Loo *et al.*, 2011).

The contaminated healthcare environment is a significant reservoir of *C. difficile* (Ellis Mulligan *et al.*, 1979; Kim *et al.*, 1981; Kaatz *et al.*, 1988). An early study investigating the contamination of hospital environments by *C. difficile*, determined patients with CDI excreted $1 \times 10^4\text{-}10^7$ of *C. difficile* per gram of faeces (Ellis Mulligan *et al.*, 1979). A higher level of contamination is consequently associated with rooms occupied by patients with CDI, but contaminating spores are also found in non-CDI patient rooms (Dubberke *et al.*, 2007). *C. difficile* has been isolated from the floors of the hospital wards, buzzers, bed rails, bedsheets and other high contact surfaces (Samore *et al.*, 1996). This contamination is likely to originate from CDI patients who previously occupied rooms or transmitted from the hands of healthcare workers who transfer *C. difficile* spores from

the environment or from direct contact with CDI patients (Dubberke *et al.*, 2007; Samore *et al.*, 1996; Bobulsky *et al.*, 2008).

The presence of CDI in the community suggests there are alternative sources of *C. difficile* and modes of transmission (Gupta and Khanna, 2014). A study which utilised whole genome sequencing to compare single-nucleotide variants of *C. difficile* isolates from healthcare and community settings, demonstrated that 45% of all isolates were genetically distinct from all previously tested isolates, suggesting that CDI was transmitted from asymptomatic individuals or environmental sources (Eyre *et al.*, 2013a). Otten *et al.* (2010) proposed four routes of transmission of CDI in the community: person-to-person, environment-to-person, animal-to-person and foodborne consumption. The transmission from symptomatic and asymptomatic individuals through close contact and environmental contamination is certainly relevant in community as well as clinical settings but individuals may be at risk from other modes of transmission. *C. difficile* has been associated with a variety of animals, including household pets and those reared for food production (Kachrimanidou *et al.*, 2019). A study of the prevalence of *C. difficile* in pet cats and dogs demonstrated 11% were colonised at the time of admission to the veterinary hospital (Clooten *et al.*, 2008). The distribution of *C. difficile* in farm animals and humans employed in the farm industry was examined in a study conducted in the Netherlands. Animals and humans were found to be colonised with identical isolates of *C. difficile*, suggesting interspecies transmission (Knetsch *et al.*, 2014). The evidence of animals as vectors of *C. difficile* suggests that CDI could be considered a zoonotic disease (Kachrimanidou *et al.*, 2019). Individual may also be exposed to *C. difficile* from the consumption of contaminated food (Otten *et al.*, 2010). Several studies, summarised by Hensgens *et al.* (2012b); Warriner *et al.* (2017) have demonstrated the isolation of *C. difficile* from foods; including processed meat, fish and seafood, processed and raw vegetables. *C. difficile* has also been isolated from seawater and freshwater samples and, concerningly from treated water including tap water (Al Saif and Brazier, 1996). The origins of food and water as sources of *C. difficile* are likely to be from animal agriculture, the soil and compost used to grow produce and the wide

distribution of *C. difficile* throughout the environment (Warriner *et al.*, 2017; Al Saif and Brazier, 1996). *C. difficile* has also been found in the soil of rural parks, household gardens and playgrounds (Al Saif and Brazier, 1996; Båverud *et al.*, 2003). There is limited knowledge on the prevalence of *C. difficile* spores in indoor domestic environments, a small study of the contamination of household environments by *C. difficile* identified 32% of the samples of household items were positive for *C. difficile,* with all households within the study having a least one positive sample of *C. difficile* (Warriner *et al.*, 2017; Alam *et al.*, 2014).

1.6 *C. difficile* acquisition and colonisation.

Acquisition begins with the ingestion of *C. difficile* via the faecal–oral route (Gerding *et al.*, 2008; Kelly *et al.*, 1994). Ingested vegetative cells of *C. difficile* are predominantly eliminated by the gastric acid of the stomach whereas the spores are highly resistant to the acid environment and pass into the small intestine which facilitates germination (Poutanen and Simor, 2004; Jump *et al.*, 2007). Prior dysbiosis of the gut microbiota and loss of colonisation resistance, typically associated with antibiotic usage provides the opportunity for outgrowing vegetative *C. difficile* to colonise the host (Vedantam *et al.*, 2012; Freeman and Wilcox, 1999). While the exact mechanism of colonisation resistance has not been fully determined, it is proposed to be a combination of host defences resulting from the stimulation of the immune system by health-associated commensal microbiota and direct competition with these microbes for the ecological niche within the gut (Lawley and Walker, 2013). A reduction of the diversity of the commensal microbiota following the administration of broad-spectrum antibiotic therapy in turn alters the metabolome of the gut (Rea *et al.*, 2012; Theriot *et al.*, 2016). This is caused by the depletion of commensal microbes which bio-transform primary bile salts, resulting in a shift in the intestinal environment that favours the proliferation of *C. difficile* (Theriot *et al.*, 2016; Winston and Theriot, 2016; Sorg and Sonenshein, 2008).

C. difficile colonisation does not always result in disease. Ingestion can lead to asymptomatic carriage (figure 1.2) which appears to be beneficial in providing protection from the risk of symptomatic CDI in the future (Poutanen and Simor, 2004; Shim *et al.*, 1998). Asymptomatic carriage of *C. difficile* differs between different age groups (Schäffler and Breitrück, 2018). New-borns and infants are commonly and transiently colonised asymptomatically with *C. difficile (Bolton et al., 1984).* A study by Jangi and Lamont (2010) determined 37% of neonates were colonised with *C. difficile* but other research suggests that this could be up to 60%–70% (Barbut and Petit, 2001; Bolton *et al.*, 1984). Several factors are believed to prevent disease in the very young, including an absence of *C. difficile* toxin-binding receptors or downstream processes in the immature gut mucosa (Jangi and Lamont, 2010; Eglow *et al.*, 1992). The rate of *C. difficile* colonisation peaks within the first month of life, then declines significantly over the first year (Jangi and Lamont, 2010). There is also variation in the rate of reported asymptomatic carriage of *C. difficile* in adults (Schäffler and Breitrück, 2018). Studies which have examined the rate of *C. difficile* symptomatic colonisation amongst adult patients upon admission to hospital, summarised by Crobach *et al.* (2018) suggest that colonisation rates ranges from 4-15%, with the majority reporting rates of under 10%. Similar levels of colonisation are also reflected in healthy adults from the general population (Ozaki *et al.*, 2004). Colonisation of healthy individuals is likely transient. In a study of the colonisation of healthy adults, unique isolates were more frequently isolated from participants throughout the testing period than continuous colonisation by the same *C. difficile* isolate (Ozaki *et al.*, 2004). The prevalence of *C. difficile* colonisation amongst the elderly in long-term care facilities is believed to be higher than that of the general population although there is considerable variation between the

reported rates in different types of care facility and the country where the study was conducted (Rodriguez *et al.*, 2014). The rate of asymptomatic colonisation is thought to be between 4-20% of elderly residents of long-term care facilities, although much higher rates have been described (Eyre *et al.*, 2013b; Simor *et al.*, 2002; Arvand *et al.*, 2012). Arvand *et al.* (2012) reported the colonization by toxigenic *C. difficile* isolates was ten-times higher in nursing home residents than in the general population outside long-term care facilities. In a study by Riggs *et al.* (2007) during an outbreak of *C. difficile,* 51% of elderly residents at a long-term care facility were asymptomatic carriers of toxigenic *C. difficile* and were associated with significant levels of skin and environmental contamination. Residents identified as asymptomatic carriers were monitored up to 3 months after the initial study, where 83% were found to be persistently colonised.

Progression to an infection with *C. difficile* is influenced by the commensal gut microbiota and host immune response (Crobach *et al.*, 2018; Sun and Hirota, 2015). CDI patients have a reduced diversity of the gut microbiota compared to healthy controls (Antharam *et al.*, 2013; Rea *et al.*, 2012). Members of the Clostridia class in the *Ruminococcaceae* and *Lachnospiraceae* families were
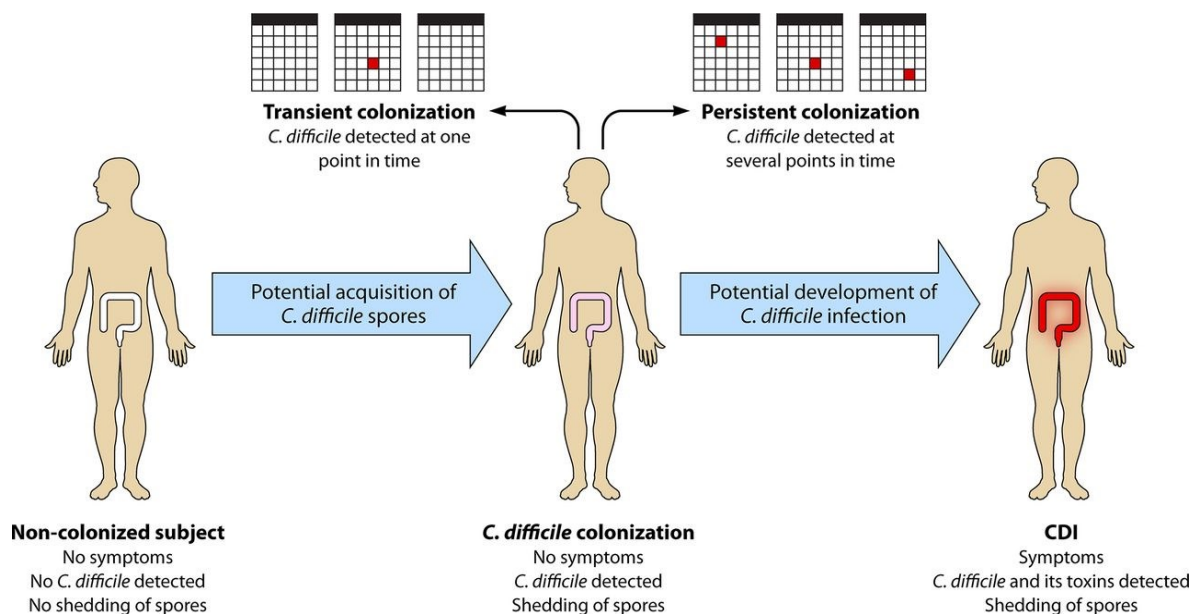
Figure 1.2 *C. difficile* asymptomatic colonisation and *C. difficile* infection (Crobach *et al*., 2018).

found to be significantly depleted or absent in CDI patients (Antharam *et al.*, 2013). The normally abundant and essential anaerobic bacteria produce short-chain fatty acids (SCFAs) including butyrate which is a major energy source for colonic mucosal epithelial cells (Antharam *et al.*, 2013; Wong *et al.*, 2006; Cook and Sellin, 1998). Butyrate also has a protective effect on the health of the colonic mucosa, promoting cell renewal, including differentiation, proliferation and apoptosis (Wong *et al.*, 2006; Cook and Sellin, 1998). The disruption of the gut microbiota and depletion of beneficial butyrogenic bacteria may lead to epithelial dysfunction, altering the susceptibility to infection by *C. difficile* by compromising host defences (Antharam *et al.*, 2013). Concomitantly the depletion of members of the *Ruminococcaceae* and *Lachnospiraceae* families prevents the biotransformation of primary bile acids to secondary bile acids by 7-dehydroxylases produced by members of the commensal gut microbiota, leading to increased levels of primary bile acids which are important determinants of *C. difficile* germination (Theriot *et al.*, 2016; Ridlon *et al.*, 2006; Sorg and Sonenshein, 2008; 2010). In antibiotic-treated mice, loss of members of the *Ruminococcaceae* and *Lachnospiraceae* families correlated with a significant reduction of secondary bile acids (Theriot *et al.*, 2016).

The host's first line of defence from *C. difficile* is the physical barrier of the intestinal epithelia, upon colonisation this can be overcome by the cytotoxic effect of the toxins, causing a disruption to epithelial tight junctions (Péchiné and Collignon, 2016). The toxins are also responsible for the activation of the inflammatory signalling cascades of the innate immune system, mediated by intestinal epithelial and immune cells including macrophages, monocytes and mast cells (Péchiné and Collignon, 2016; Sun and Hirota, 2015). The proceeding inflammatory response determines the progression and severity of CDI (Poutanen and Simor, 2004; Kelly and Kyne, 2011). An adaptive immune response is also induced by the toxins along with non-toxin antigens providing protective memory which can curtail disease severity (Péchiné and Collignon, 2016; Sun and Hirota, 2015). Detectable serum IgG and IgA antibodies against *C. difficile* toxins are found in 60% of adults and older children (Viscidi *et al.*, 1983). It is thought that this is the result of antibody production in

infancy and stimulation via environmental exposure to *C. difficile* throughout adult life (Viscidi *et al.*, 1983; Kelly and Kyne, 2011).

1.7 Recurrent *C. difficile* infection.

Despite antibiotic treatment, recurrent CDI occurs in approximately 25% of cases, although reported rates of recurrence vary considerably (4->50%) (Bakken *et al.*, 2013; Debast *et al.*, 2014; Enoch *et al.*, 2018; Johnson, 2009a). Recurrent CDI is not a result of treatment failure but is defined as an episode of CDI occurring within 8 weeks of resolution of symptoms and completion of treatment of a previous episode of CDI (Johnson, 2009b; McDonald *et al.*, 2007; Kuijper *et al.*, 2006). The majority of recurrent CDI occurs within the first month of completing antibiotic therapy to treat the primary episode of CDI (Kelly, 2012). Recurrence occurs as a result of either relapse due to persistent colonisation of the same strain of C. *difficile* or reinfection due to the acquisition of a new strain of C. *difficile* (Johnson, 2009a; Wilcox *et al.*, 1998). Recurrent CDI due to relapse occurs in a shorter time period following the primary episode, the average duration between recurrent
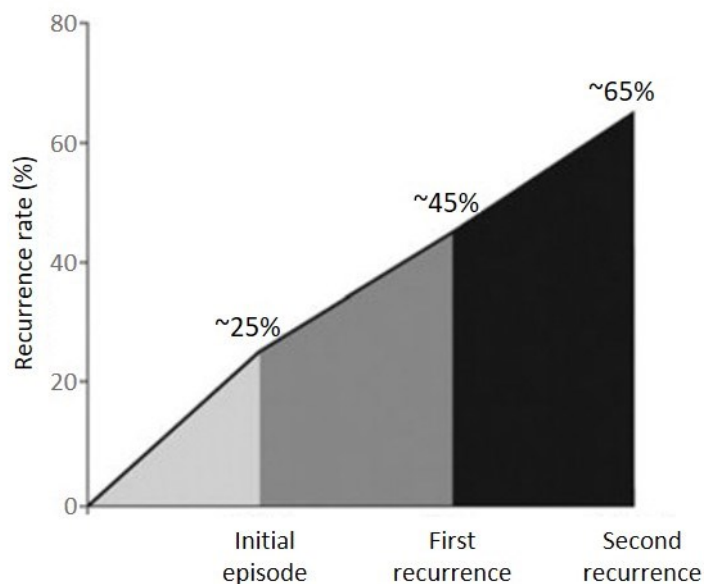
Figure 1.3 The recurrence of *C. difficile* infection following the initial episode and the first and second recurrence. Adapted from Kelly (2012).

episodes was determined to be 14.5 days for relapse and 42.5 days for reinfection in a study by Johnson *et al.* (1989). Barbut *et al.* (2000) determined recurrence was more frequently caused by relapse than reinfection, but only marginally with 48% of the observed recurrences due to re-infection. Patients who have experienced a recurrent episode of CDI are at higher risk of experiencing further episodes of CDI which can occur over the course of weeks, months or even years, severely impacting quality of life (figure 1.3) (Kelly, 2012; Johnson, 2009a). Repeated cycles of antibiotic therapy to treat recurrent CDI cause continued disruption to the gut microbiota, extending the risk of recurrence (Kelly, 2012). This prolonged disruption of the commensal microbial community is a proposed mechanism of recurrent CDI; patients with recurrent infection demonstrate a decrease in the diversity of the gut microbiome suggesting the restoration of colonisation resistance is inhibited (Chang *et al.*, 2008). Recurrence is also associated with low antibody titres of immunoglobulin M (IgM) and immunoglobulin G (IgG) anti-toxin A and elevated levels of interleukin-8 (IL-8) as a result of a polymorphism in the promoter gene, leading to altered neutrophil recruitment and inflammatory response (Kyne *et al.*, 2001; Garey *et al.*, 2010; Abou Chakra *et al.*, 2014).

1.8 *C. difficile* infection pathogenicity.

In addition to the well characterised cytotoxins (toxin A and toxin B), *C. difficile* produces several virulence factors that contribute to colonisation and pathogenesis including adhesion, tissue invasion, regulation of bacterial growth and metabolism, and modulation of the host immune response (Vedantam *et al.*, 2012).

1.8.1 *C. difficile* non-toxin virulence factors.

1.8.1.1 Adherence to host cells.

The ability to adhere and colonise host cells are important virulence factors that contribute significantly to disease (Merrigan *et al.*, 2013). The flagellum and type IV pilus, usually associated with swimming chemotaxis and gliding motility respectively are involved in *C. difficile* colonisation and adherence (Tasteyre *et al.*, 2001a; Varga *et al.*, 2006). The flagellin filament (FliC) and flagellin cap protein (FliD) encoded by *fliC* and *fliD* respectively adhere to the layer of mucus that coats the intestinal epithelium while FliD also has a role in initial adhesion to host cells (Tasteyre *et al.*, 2000; Tasteyre *et al.*, 2001b). *C. difficile* flagellin induces the up-regulation of pro-inflammatory gene expression and pro-inflammatory cytokine synthesis in host cells: FliC activates the nuclear factor Kappa B (NF-kB) and mitogen-activated protein kinase (MAPK) signalling pathway via Toll-like receptor 5 (TLR5) promoting the production of Interleukin 8 (IL-8) (Batah *et al.*, 2016; Yoshino *et al.*, 2013). *C. difficile* flagellum gene expression is also connected to the regulation of toxin genes, the disruption of early-stage flagellum genes (associated with the flagellum motor machinery) was associated with decreased expression of the *C. difficile* pathogenicity locus (PaLoc), including the toxin genes (*tcdB* and *tcdA*) and regulator (*tcdR*) (Aubry *et al.*, 2012). Conversely *fliC* and *fliD* mutants studied by Baban *et al.* (2013) demonstrated increased expression of *tcdA*. The reciprocal relationship reported between flagellum gene expression and toxin production seems to be derived

from the laboratory strains used to study the link between flagellum genes and pathogenesis and are attributed to uncharacterised mechanisms of global regulation of *C. difficile* virulence factors (Stevenson *et al.*, 2015). The expression of flagellum and toxins may have a synergistic effect; the absence of flagella in toxin-producing *C. difficile* strains reduced the degree of mucosal inflammation in a CDI mouse model (Batah *et al.*, 2017). The production of flagella by *C. difficile* may provide an advantage, they may not be essential for colonisation and adherence (Stevenson *et al.*, 2015). Not all *C. difficile* strains produce flagella but genome analysis demonstrates *C. difficile* encode putative Type IV pilus genes (Borriello *et al.*, 1988; Melville and Craig, 2013). The type IV pili promote adherence to the host epithelium and are important for persistence in the host, this is proposed to enable the formation of microcolonies that can establish surface-associated biofilm formation (McKee *et al.*, 2018; Maldarelli *et al.*, 2016). As part of the unique regulation of gene expression during biofilm formation, flagellin *fliC* is decreased while the major pilin encoded by *pilA1* demonstrates enhanced expression (Maldarelli *et al.*, 2016).

C. difficile cell-wall proteins (CWPs) and other adhesins are implicated as important colonisation factors, allowing *C. difficile* to adhere and proliferate. The predominant cell-wall protein (CWP) of *C. difficile* involved in adherence to intestinal epithelia cells are the surface layer proteins (SLPs) (Calabi *et al.*, 2002; Merrigan *et al.*, 2013). *C. difficile* expresses two distinct SLPs, a low molecular weight surface layer protein (SLP) of 32-38 kDa and a high molecular weight SLP of 42-48 kDa (Calabi *et al.*, 2001). Both are encoded by the *slpA* gene and together form a complex of a regularly arranged lattice on the external surface of the bacterium (Calabi *et al.*, 2001; Calabi *et al.*, 2002; Fagan *et al.*, 2009). The CWP Cwp84 cleaves SlpA to produce the high molecular weight SLP and the low molecular weight SLP. The protease activity of Cwp84 also extends to the degradation of fibronectin, laminin, and vitronectin which may aid host invasion (Janoir *et al.*, 2007). SLP binding is mediated almost exclusively by the high-MW subunit which is highly conserved. The low molecular weight SLP displays sequence variation between strains but how this is involvement in host interaction is not clear (Calabi *et al.*, 2002; Calabi *et al.*, 2001; Mori and Takahashi, 2018).
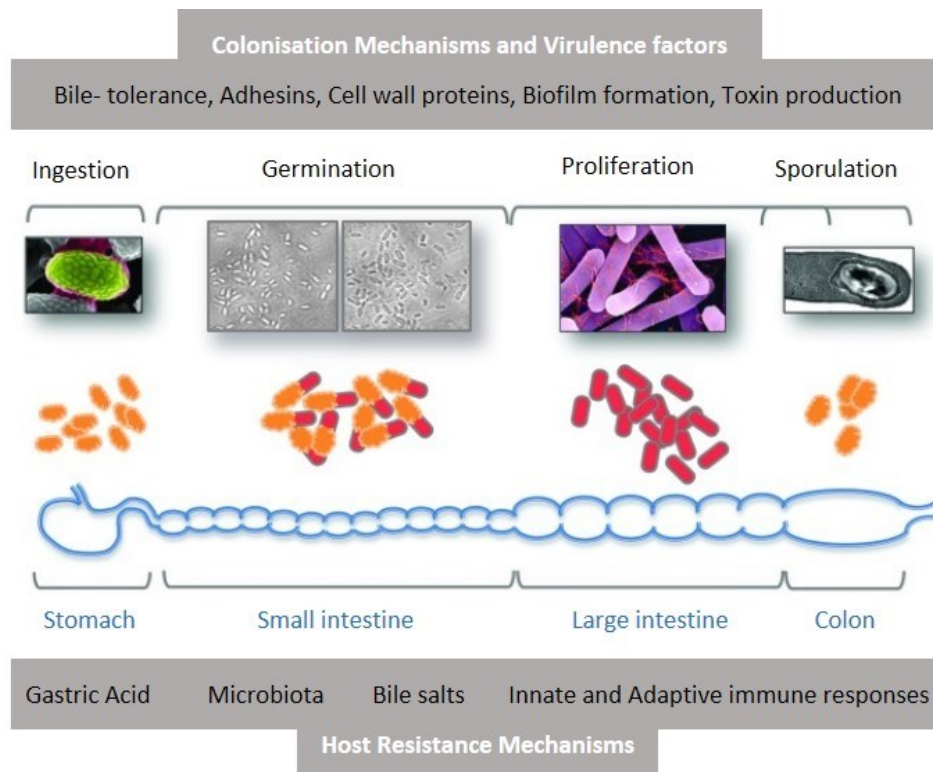
Figure 1.4 Schematic of *C. difficile* infection. Adapted from Vedantam *et al.* (2012). Spores (orange), vegetative cells (red) and key events of *C. difficile* pathogenesis are depicted along with the bacterial and host factors that influence or modulate disease.

Clinical strains of *C. difficile* display diverse variations in surface protein profiles of SlpA with distinct adherence capabilities to epithelia cells in culture (Merrigan *et al.*, 2013). SLPs also have a role in activating the innate immune system through Toll-like receptor-4 (TLR-4). The subsequent production of inflammatory cytokines, initiated by downstream signalling of NF-kB leads to immune cell activation (Ryan *et al.*, 2011). SLPs from hypervirulent strains have been demonstrated to generate a more potent inflammatory response, inducing higher levels of cytokines and chemokines production by macrophages while inhibiting phagocytosis (Lynch *et al.*, 2017).

Several other CWPs contribute to *C. difficile* cell adherence and colonization including Cwp66. Cwp66 was the first adhesin identified in *C. difficile* as a surface-associated heat shock protein with adhesive properties (Waligora *et al.*, 2001). CwpV promotes aggregation of *C. difficile,* this is

mediated through the antigenically distinct repetitive C-terminal domain which displays high variablity between strains, suggesting a role in evasion of the host immune system (Reynolds *et al.*, 2011).

C. difficile toxin mediated the disruption of cell tight junctions results in the exposure of the underlying extracellular matrix (Pothoulakis, 2000). *C. difficile* bacterial matrix binding proteins known as microbial surface components recognising adhesive matrix molecules (MSCRAMMs) recognize ligands displayed on the extracellular matrix (Arato *et al.*, 2019). The MSCRAMMs fibronectin binding protein (FbpA) and collagen-binding protein (CbpA) are involved in *C. difficile* intestinal adhesion, contributing to the interaction of *C. difficile* and the host mucosa (Barketi-Klai *et al.*, 2011; Tulli *et al.*, 2013). A protein more recently attributed to *C. difficile* MSCRAMMs CD2831, is a collagen binding protein that may also promote biofilm formation and has a suggested role in evading the host immune system by inhibiting the complement cascade (Arato *et al.*, 2019). Other proteins reported to contribute to *C. difficile* adherence include the surface associated lipoprotein CD0873 and heat shock protein GroEL (Kovacs-Simon *et al.*, 2014; Hennequin *et al.*, 2001).

1.8.1.2 Biofilm formation.

C. difficile biofilm formation has been demonstrated *in vitro* and via *in vivo* animal models but the role of biofilms in CDI is still being understood. It is suspected to be an important virulence factor for disease and persistence (Vuotto *et al.*, 2018; Donelli *et al.*, 2012; Soavelomandroso *et al.*, 2017). The sessile microbial community of a biofilm facilitates the survival in hostile environments and resistance to killing by antibiotics (Hall-Stoodley *et al.*, 2004; Đapa *et al.*, 2013). Under laboratory conditions, *C. difficile* forms characteristic structurally complex biofilms composed of a matrix of bacterial cells, proteins, polysaccharide and extracellular DNA. Similarly to other pathogenic bacterial species the ability to form biofilms varies between *C. difficile* strains (Đapa *et al.*, 2013). As might be predicted, disruption of the quorum-sensing regulator *luxS* has a dramatic effect on

the ability of *C. difficile* to form biofilms. A *luxS* mutant was incapable of forming bacterial monolayer, an important step in the establishment of biofilm formation. Mutants of the master regulator of sporulation, Spo0A, and germination regulator, SleC demonstrated defects in biofilm formation, but the ability to form a biofilm was not eliminated (Đapa *et al.*, 2013).

As previously indicated, *C. difficile* adhesins are also implicated in biofilm formation, Cwp84 is suspected to be involved the modulation between biofilm and planktonic growth states (Đapa *et al.*, 2013; Pantaléon *et al.*, 2015). A *cwp84* mutant constructed by Pantalean *et al.* (2015) displayed a restricted growth rate but enhanced biofilm formation, with a denser biofilm than the parental strain (Pantaléon *et al.*, 2015). The modulation of biofilm formation by Cwp84 may be linked with the inherent formation of the surface layer through the cleavage of SlpA or cleavage of yet unidentified proteins (Pantaléon *et al.*, 2015). As previously described, *C. difficile* flagellum and type IV pili positively influence the formation of biofilms. A *fliC* mutant reduced mature biofilm accumulation while a non-piliated mutant was significantly deficient in early biofilm formation (Đapa *et al.*, 2013; Maldarelli *et al.*, 2016).

Bile salts are also implicated in the biofilm form of growth. The primary bile salt chenodeoxycholate, and the bile acid conjugates, glycochenodeoxycholate and taurochenodeoxycholate, significantly induced biofilm formation in *C. difficile* (Dubois *et al.*, 2019). Biofilm formation induced by a sub-lethal concentration of secondary bile salts deoxycholate may also have a role in asymptomatic persistence of *C. difficile* in the host (Dubois *et al.*, 2019).

1.8.1.3 Evasion of host defences.

As already discussed, the modulation or evasion of the immune system are important virulence factors in the pathogenesis of CDI and several mechanisms are employed by *C. difficile* to overcome host defensins (Vedantam *et al.*, 2012). Host-produced cationic antimicrobial peptides (CAMPs)

induce the *C. difficile dlt* operon, resulting in the modification of the cell wall by addition of D-alanine esters to teichoic acids, providing a mechanism of resistance to CAMPs (McBride and Sonenshein, 2011a). Similarly sub-inhibitory concentrations of lysozyme activate transcription factors involved in extracellular stress, resulting in the modification of *C. difficile* peptidoglycan providing resistance to lysozyme (Ho *et al.*, 2014). McBride and Sonenshein (2011b) also outlined another mechanism employed by *C. difficile* to adapt to the presence of CAMPs. Exposure to CAMPs caused the induction of the *cprABC* operon which encodes an ABC transporter system, suggesting that resistance to CAMPs may also be accomplished through the export of these peptides by an ABC transporter (McBride and Sonenshein, 2011b).

1.8.1.4 Formation of spores.

The ability to produce spores, the major vector of CDI transmission, is also considered a virulence factor of *C. difficile* (Deakin *et al.*, 2012). Spores excreted by colonised individuals ensure long-term survival and dissemination beyond the host environment (McFarland *et al.*, 1989; Samore *et al.*, 1996). Transmission within clinical settings and dispersal in the community have given rise to the description of *C. difficile* spores as ubiquitous in the environment (Gerding *et al.*, 2008; Bauer and Kuijper, 2015; Freeman *et al.*, 2010). Spores are metabolically dormant and intrinsically resistant to adverse environmental conditions, ensuring reactivation occurs only in response to defined signals associated with the host (Deakin *et al.*, 2012; Driks, 2003; Gil *et al.*, 2017; Setlow, 2003). The important resistant properties of the spore, sporulation and spore germination is discussed further in successive sections.

1.8.2 *C. difficile* toxin virulence factors.

1.8.2.1 Toxins A and B.

The most significant virulence factors of *C. difficile,* toxins TcdA (Toxin A), TcdB (Toxin B) and, in some strains, the binary toxin, are responsible for the clinical presentation of CDI (Bongaerts and Lyerly, 1994; Voth and Ballard, 2005). Toxin-producing strains and non-toxigenic *C. difficile* strains are both capable of colonising the host, but only toxigenic strains are associated with disease (Vedantam *et al.*, 2012). TcdA and TcdB are encoded within a 19.6 Kb genetic island in the chromosome of *C. difficile* known as the pathogenicity locus (PaLoc) and are expressed during late logarithmic and stationary phases of growth in response to environmental stimuli (Hammond and Johnson, 1995; Davies *et al.*, 2011; Hundsberger *et al.*, 1997). The PaLoc encodes the toxins TcdA and TcdB, along with TdR, TcdC and TcdE which regulate toxin production and secretion (Hundsberger *et al.*, 1997; Hammond and Johnson, 1995; Voth and Ballard, 2005). The RNA polymerase sigma factor *tcdR*, positively regulates toxin expression along with its own expression while *tcdC* is thought to negatively regulate the transcription of *tcdA* and *tcdB* (Mani and Dupuy, 2001; Hundsberger *et al.*, 1997). Recent analysis of TcdC suggest it is associated with the bacterial cell surface and may exert an alternative mechanism of regulation (Oliveira Paiva *et al.*, 2020). The role of TcdE is thought to be the export of TcdA and TcdB from the bacterial cell (Govind and Dupuy, 2012).

There has been conflicting evidence of the individual roles of toxin A and B in disease. Previous studies have suggested a synergistic action of the toxins, whereby toxin A causes the majority of pathology and toxin B is unable to initiate disease unless the epithelium has first been damaged by the effects of toxin A (Lyerly *et al.*, 1985; Bongaerts and Lyerly, 1994). Lyras *et al.* (2009) demonstrated toxin B was essential for virulence. Disruption to *tcdB* resulted in an attenuated virulence phenotype, while disruption of *tcdA* produced results similar to the wildtype strain. Following the study by Lyras *et al*. in 2009, Kuehne *et al.* (2010) established that production of toxin

A or B in isolation was able to induce a disease state in the hamster infection model, attenuation occurred only when both toxins were inactivated (Kuehne *et al.*, 2010). The discrepancy between the studies does not seem to be due to the construction methodology or the animal model utilised but the inherent variability of toxin activity, where toxins have differing substrate specificity and host cell selectivity (Janoir, 2016; Kuehne *et al.*, 2010).

The glycosyltransferase toxins TcdA and TcdB share 68% sequence similarity and are shown to be structurally similar by electron microscopy (Davies *et al.*, 2011; Pruitt *et al.*, 2010). Both toxins enter the cell through receptor mediated endocytosis and execute cytotoxicity by transferring glycosyl residues to Rho GTPases, Rac and Cdc42 proteins (Davies *et al.*, 2011; Voth and Ballard, 2005; Jank *et al.*, 2007). The Rho family GTPases interact with a range of effector molecules to regulate several essential cellular functions including epithelial barrier permeability and cell to cell contact, immune cell migration, phagocytosis, cytokine production and immune cell signalling (Chandrasekaran and Lacy, 2017; Jank *et al.*, 2007). Toxin mediated glycosylation of Rho GTPases results in actin depolymerisation and rearrangement of the cytoskeleton, disruption of tight junctions and cell rounding which results in the loss of the epithelial cell barrier along with vital downstream signalling pathways and ultimately caspase-3 and caspase-9 mediated apoptosis (figure 1.5) (Nusrat *et al.*, 2001; Voth and Ballard, 2005; Jank *et al.*, 2007; Davies *et al.*, 2011). The increase in vascular permeability due to the opening tight junctions causes fluid accumulation and haemorrhage (Pothoulakis, 2000; Poxton *et al.*, 2001).

The toxins also stimulate epithelial cells and resident mucosal immune cells to produce multiple proinflammatory cytokines and chemokines including tumour necrosis factor-alpha (TNFα) and interleukins IL-1 and IL-8, leading to the activation of neutrophils through the activation of the NF-κB and TLR4 and TLR5 signalling pathways and the mitogen-activated protein kinase (MAPK) pathways (Pothoulakis, 2000; Poxton *et al.*, 2001; Madan and Petri, 2012; Chandrasekaran and Lacy, 2017). The loss of the epithelial cell barrier causes an acute activation of innate immune response
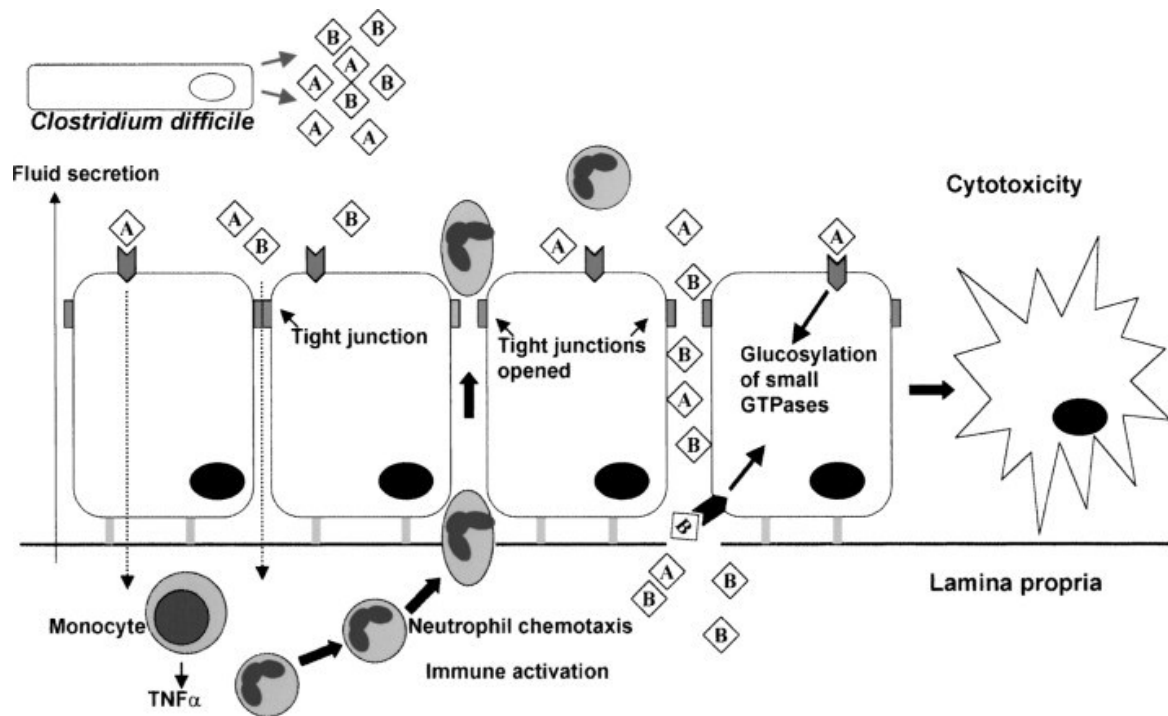
Figure 1.5 Action of the *C. difficile* toxins A and B on the intestinal epithelium (Poxton *et al.*, 2001). Originally adapted from Thelestam and Chaves-Olarte (2000).

inducing further inflammatory cytokine production by lymphocytes and mast cells and the migration the accumulation of neutrophils (Voth and Ballard, 2005; Shen, 2012; Madan and Petri, 2012). Collectively, the severe intestinal damage and activation of the inflammasome caused by TcdA and TcdB increases intestinal permeability and fluid accumulation which manifests as diarrhoea, the hallmark symptom of CDI (Carter *et al.*, 2012; Chandrasekaran and Lacy, 2017).

Finally, the inflammatory response to toxins is believe to supress the proliferation of other microbes while *C. difficile* upregulates expression of genes that are involved in utilisation of amino acids for growth, *C. difficile* thereby reduces competition for resources and generates more resources to promote its own proliferation (Fletcher *et al.*, 2021).

1.8.2.2 Binary toxin.

Some *C. difficile* clinical isolates produce the *Clostridium difficile* transferase (CDT), also known as the binary toxin which has ribosyl-transferase activity, causing cytoskeleton reorganisation, inactivation of cell signalling pathways and cell death (Barth *et al.*, 2004; Awad *et al.*, 2014). The binary toxin is encoded by two genes *cdtA* and *ctd*B encoded on a 6.2 Kb chromosomal region known as Cdt locus or CdtLoc, along with response regulator gene *cdtR* which positively regulates binary toxin expression (Carter *et al.*, 2007). The binary toxin protein components, the enzymatically active CdtA and the binding component CtdB are secreted separately and associate on the surface of host cell (Perelle *et al.*, 1997; Barth *et al.*, 2004). Binary toxin ADP-ribosylates actin, inducing the depolymerization of the actin cytoskeleton causing the redistribution of microtubules and formation of protrusions at the surface of intestinal epithelial cells, aiding the adherence of *C. difficile* to the surface of epithelia cells (Gonçalves *et al.*, 2004; Schwan *et al.*, 2009). A mutant strain producing only the binary toxin caused disease in an animal model, but the symptoms caused are not consistent with that of CDI (Kuehne *et al.*, 2014). The production of binary toxin by *C. difficile* is associated with severe patient outcomes of CDI, suggesting that it may potentiate the toxicity of TcdA and TcdB and lead to more severe disease (Gerding *et al.*, 2014; Kuehne *et al.*, 2014).

1.9 Symptoms of *C. difficile* infection.

The clinical presentation of CDI ranges from asymptomatic carriage, mild diarrhoea to life threatening pseudomembranous colitis (Table 1.2) (Kelly et al., 1994; Poutanen and Simor, 2004). The onset of diarrhoea caused by CDI typically occurs 4–9 days after antibiotic therapy but up to a third of patients develop diarrhoea after the cessation of antibiotics therapy which can be up to weeks later (Kelly and LaMont, 1998). Patients with *C. difficile* colitis in the absence of pseudomembranes experience watery diarrhoea, lower abdominal pain and systemic symptoms such nausea, dehydration and a low-grade fever (Kelly et al., 1994; Poutanen and Simor, 2004). More acute colitis presents as profuse debilitating diarrhoea along with systematic manifestation including fever, severe abdominal pain and distention (Kelly et al., 1994). The most severe manifestation of CDI is pseudomembranous colitis, which is characterised by formation of microulcerations of the colonic mucosa, which are covered by an inflammatory pseudomembrane (Figure 1.6) (Kelly and LaMont, 1998). The adherent plaques of the pseudomembranes cover large areas of the colon which undergoes pronounced thickening (Kelly and LaMont, 1998; Kelly et al., 1994). The intense inflammatory response, tissue damage and neutrophil recruitment are thought to promote the formation of pseudomembranes observed in severe cases of CDI (Voth and Ballard, 2005; Sun and Hirota, 2015).

Table 1.2 Spectrum of CDI clinical presentation. Adapted from Leffler and Lamont (2015).

Severity	Clinical Manifestations
Asymptomatic carrier	No signs or symptoms
Mild	Mild diarrhoea (3 to 5 unformed bowel movements per day), afebrile status, mild abdominal discomfort or tenderness.
Moderate	Moderate non-bloody diarrhoea, moderate abdominal discomfort or tenderness, nausea with occasional vomiting, dehydration, white-cell count >15 $x10^9$/L, blood urea nitrogen or creatinine levels above normal range
Severe	Severe or bloody diarrhoea, pseudomembranous colitis, severe abdominal pain, vomiting, ileus, temperature >38.9°C, white-cell count >20 $x10^9$/L, albumin level <0.0025 g/L, acute kidney injury.
Complicated	Toxic megacolon, peritonitis, respiratory distress, and hemodynamic instability

Fulminant colitis and pseudomembranous colitis caused by CDI can lead to rare but serious and life-threatening complications including prolonged ileus, perforation of the bowel, toxic megacolon, peritonitis, sepsis, multiple organ dysfunction syndrome and death (Kelly and LaMont, 1998; Rubin *et al.*, 1995; Dobson *et al.*, 2003; Cone and Wetzel, 1982). Fulminant colitis accounts for the most serious complications of CDI, patients are acutely unwell presenting with lethargy, fever, and severe abdominal pain. Diarrhoea is prominent with the exception of patients who develop an ileus (Kelly and LaMont, 1998; Kelly *et al.*, 1994)
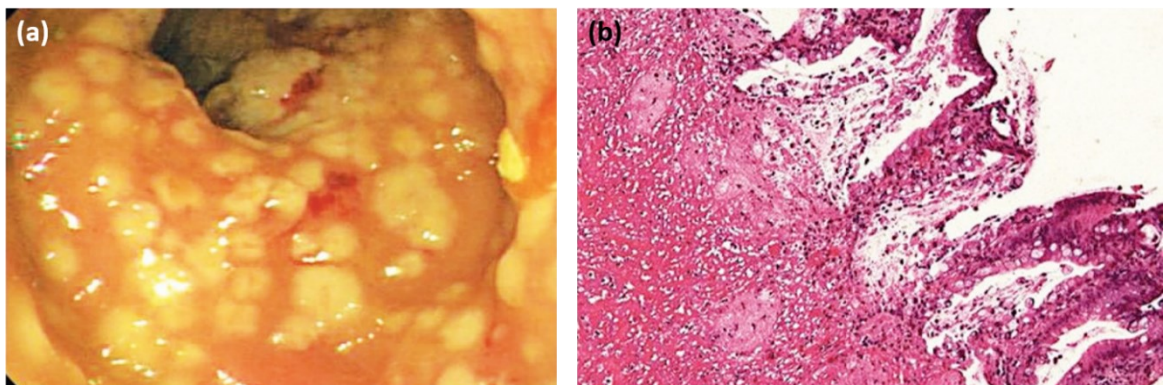
Figure 1.6 Pseudomembranous colitis. Colonoscopy imaging of multiple discrete pseudomembranous lesions (a) Hematoxylin and eosin stained biopsy of pseudomembranous lesion (b) Chen and Shih (2011).

1.10 *C. difficile* sporulation and spore structure.

The formation of spores by *C. difficile* is critical for survival in an aerobic atmosphere, resistance to extreme environmental conditions and the dissemination and persistence of CDI (Paredes-Sabja *et al.*, 2014; Deakin *et al.*, 2012). The sporulation signalling pathway of *C. difficile* is based on the knowledge of other spore formers and members of the *Clostridia* (Al-Hinai *et al.*, 2015). The initiation of *C. difficile* sporulation is under the control of the master regulator Spo0A and is thought to be triggered by environmental signals associated with the host, nutrient depletion and quorum sensing (Steiner *et al.*, 2011; Wetzel and McBride, 2020; Edwards and McBride, 2014; Higgins and Dworkin, 2012; Paredes-Sabja *et al.*, 2014). Pro-sporulation signals result in histidine kinase phosphorylation of the master transcription regulator, Spo0A, and subsequent activation of sigma factors σ^F, σ^E, σ^G, and σ^K that control the transcription of genes required for spore formation (Paredes-Sabja *et al.*, 2014; Fimlaid *et al.*, 2013). *C. difficile* sporulation is indirectly repressed by the regulator CodY in response to nutrient acquisition (Nawrocki *et al.*, 2016). While these sigma factors are conserved across spore-forming bacteria, their activation and regulation in *C. difficile* occurs in a divergent pathway to *Bacillus* and other *Clostridium* species (Fimlaid *et al.*, 2013). The induction of the sporulation transcription factors coincides with morphological changes and communication between the mother cell and the forespore facilitates the formation of the mature oval spore structure that develops sub-terminally in the mother cell (Saujet *et al.*, 2014; Lawson *et al.*, 2016).

The conserved protective bacterial spore structure has constituents and features that are not associated with the vegetative cell which contribute to its resistance and durability (Figure 1.7) (Driks, 2003; Setlow, 2006). At the centre of the spore is the dehydrated spore core containing DNA, tRNA, ribosomes and enzymes essential for the return to metabolic activity during germination (Setlow, 2006). The low water content is the primary factor in spore enzymatic dormancy and resistance to wet heat, making up just 25-55% spore core wet weight, depending on the species
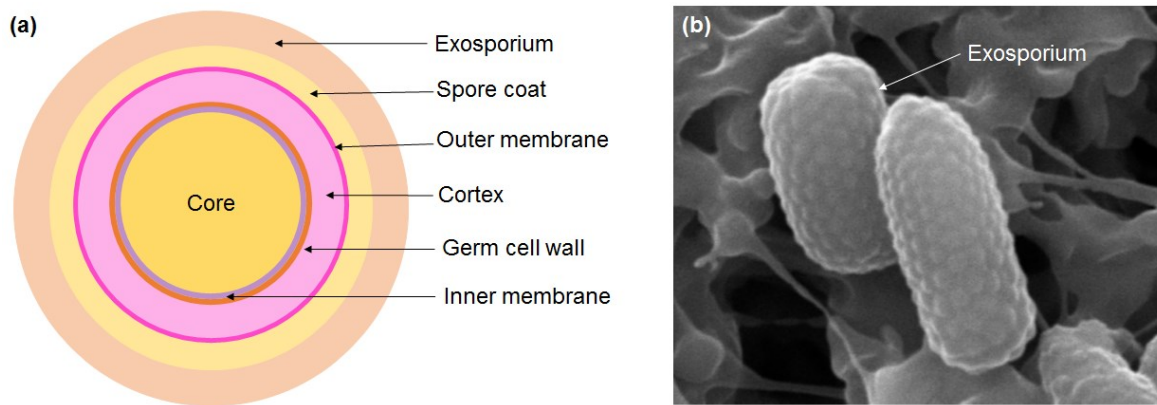
Figure 1.7 *C. difficile* spore structure. (a) Schematic cross-sectional representation of spore layer structure (not to scale). (b) Scanning electron microscopy image of the *C. difficile* strain NCTC 11204 spore surface showing the exosporium layer.

(Gerhardt and Marquis, 1989). The core is saturated with dipicolinic acid (DPA) chelated 1:1 with the divalent cation Ca^{2+} as Ca-DPA contributes to the reduced core water content (Setlow, 2007; Setlow, 2006). DNA within the spore core is bound to protective small acid-soluble proteins (SASPs), which alter the properties of DNA. The SASPs are found exclusively in the spore core and are responsible for spore resistance to desiccation, damage by heat and ultraviolet (UV) radiation (Setlow, 2014; Setlow, 2006). Recently the role of the SASPs, SspA and SspB produced by *C. difficile* were investigated; SspA was demonstrated as the major contributor of resistance to ultraviolet (UV) radiation while deletion of *sspA* along with *sspB* resulted in defective sporulation (Nerber and Sorg, 2021).

The spore core is surrounded by the inner membrane which acts as a permeability barrier and contributes to spore resistance by preventing the entry of DNA damaging compounds (Nicholson *et al.*, 2000). The inner membrane is further surrounded by the peptidoglycan-containing germ cell wall which becomes the outgrowing cell wall upon germination (Paredes-Sabja *et al.*, 2014; Setlow, 2006). Next the large cortex layer is composed of spore-specific modified cross-linked peptidoglycan containing muramic-δ-lactam (MAL) which is important for recognition and selective hydrolysis by cortex lytic enzymes during germination (Popham, 2002; Paredes-Sabja *et al.*, 2014).

The presence of the cortex is essential for spore dormancy and resistance to heat and ethanol (Popham, 2002). The cortex is enclosed by the outer membrane, which, in contrast to the surrounding spore coat, is not believed to be a significant permeability barrier or to have a role in spore resistance (Nicholson *et al.*, 2000). The proteins of the spore coat protect against attack from enzymes, chemicals and biocides (Setlow, 2014). The morphogenetic protein, CotL is essential for the correct assembly of the spore coat, cortex and exosporium, a *cotL* knockout resulted in morphological defects and reduction of the abundance of several coat and exosporium proteins (Alves Feliciano *et al.*, 2019). The multi-layered protein exosporium which demonstrates variability in its morphology between and within strains of *C. difficile*, is not present in all spore formers. The morphogenetic proteins CdeC and CdeM are involved in the recruitment assembly of *C. difficile* exosporium which is demonstrated also to influence *C. difficile* pathogenesis (Pizarro-Guajardo *et al.*, 2016; Calderón-Romero *et al.*, 2018). CdeC specifically contributes to spore resistance: spores lacking the *C. difficile* exosporium cysteine-rich protein (CdeC) demonstrate reduced resistance to lysozyme, ethanol and heat treatment (Barra-Carrasco *et al.*, 2013).

1.11 *C. difficile* spore germination.

To colonise the host, spores of *C. difficile* reactivate in the presence of specific environmental signals (germinants) associated with the small intestine, leading to the return to full metabolic activity and vegetative cell growth, a process known as germination (Gil *et al.*, 2017; Viswanathan *et al.*, 2010; Setlow, 2003). Although it has long been recognised that the addition of the primary bile salt, taurocholate, to culture media enhances the recovery of *C. difficile* spores in the laboratory, Sorg *et al.* (2008) demonstrated that the bile salt, cholate, along with conjugated bile salts glycocholate and taurocholate, induce the germination of *C. difficile* spores (Wilson, 1983; Sorg and Sonenshein, 2008). Exposure to the primary bile acids and secondary bile salts during passage through the gastrointestinal tract is the crucial signal that regulates the germination. Primary bile acids, cholic acid and chenodeoxycholic acid, which are secreted into the small intestine to assist the digestion of lipids acid are conjugated with taurine or glycine to produce primary bile salts derivatives (figure 1.8) (Ridlon *et al.*, 2006). Although active transport of the primary bile salts ensures the majority are recycled back to the liver, a low concentration enters the large intestine and is deconjugated by bacterial species of the intestinal microbiota to generate cholate and chenodeoxycholate (Ridlon *et al.*, 2006; Thomas *et al.*, 2001). In the colon there is disproportionate passive reabsorption of chenodeoxycholate leading to a higher ratio of remaining cholate (Mekhjian *et al.*, 1979). In healthy individuals 7α-dehyroxylation of the primary bile salts by selective microbiota species generates secondary bile aids deoxycholate and lithocholate (figure 1.8 and 1.9). However, in the absence of these microbiota species, for example following their elimination by broad spectrum antibiotics such as the beta-lactam antibiotics, conversion to secondary bile acids is reduced, resulting in an increased concentration of the *C. difficile* germinants (Ridlon *et al.*, 2006; Theriot *et al.*, 2016). Correspondingly, the presence of secondary bile salts is associated with colonisation resistance, by inhibition of *C. difficile* germination and outgrowth (Theriot *et al.*, 2016). Responding only in the presence of cholate-derived germinants ensures *C. difficile* spores germinate specifically in the ileum, preventing germination until spores receive the germination-positive

signals that are indicative of the small intestine environment (Koenigsknecht *et al.*, 2015; Kochan *et al.*, 2018b). Sorg and Sonenshein (2009) also established that the primary bile acid, chenodeoxycholate, inhibits taurocholate-mediated germination in a competitive manner. The higher affinity of *C. difficile* for chenodeoxycholate, thereby supresses germination until the concentration of germinant overcomes the inhibitory action of chenodeoxycholate (Sorg and
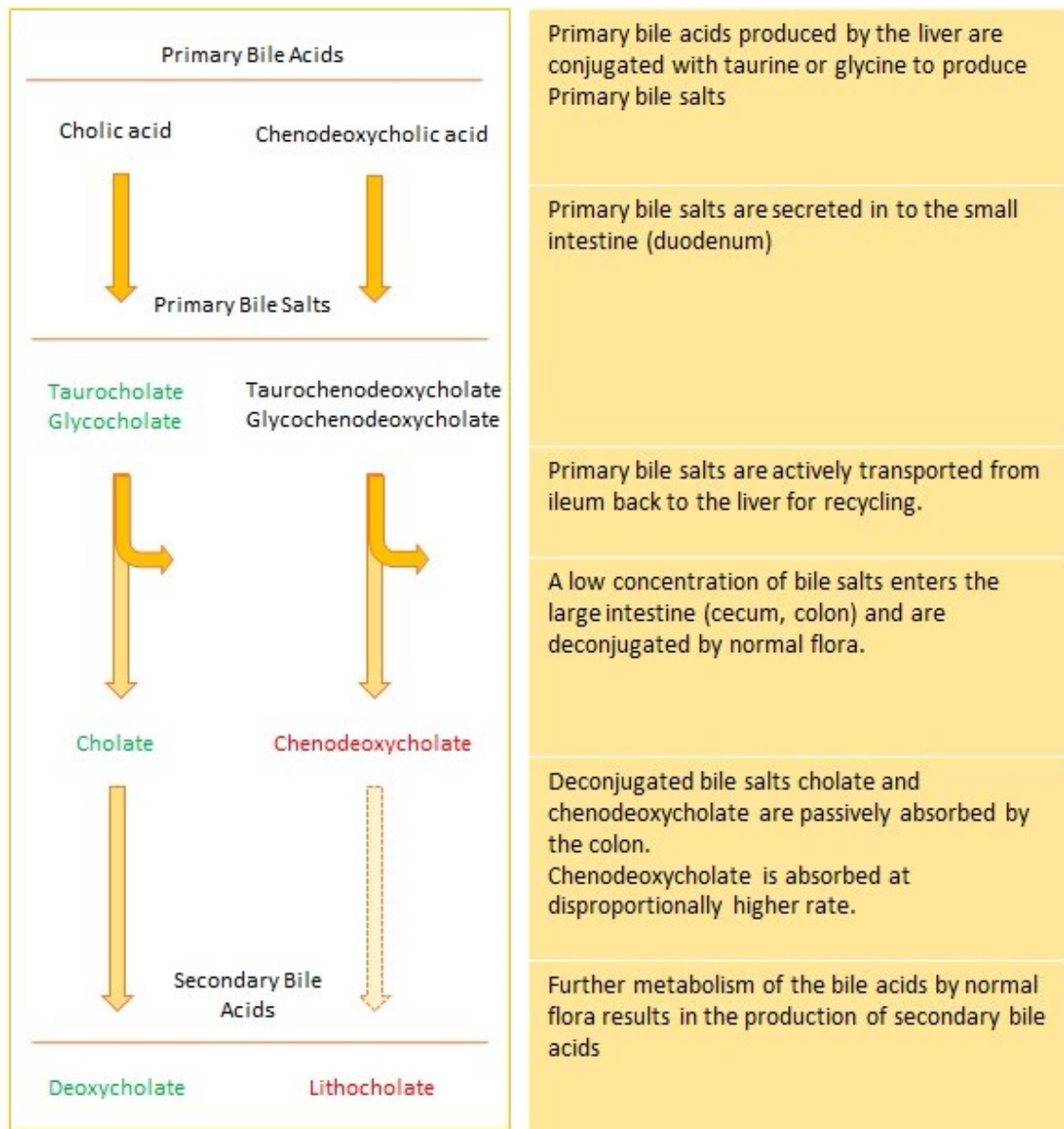
Figure 1.8 Modification of primary bile acids during transit through the small and large Intestines. Green text indicates a bile salt or bile acid that has *C. difficile* spore germination activity; red text indicates an inhibitor (Sorg and Sonenshein, 2008, 2009, 2010).

Sonenshein, 2008; 2010; 2009). The secondary bile salt, lithocholate, is also an inhibitor of germination, whereas deoxycholate is a weak spore germinant but inhibits growth of *C. difficile* (Sorg and Sonenshein, 2010; 2008; Thanissery *et al.*, 2017). With the exception of structural alterations of conjugated bile salt derivatives, bile salt germinants and inhibitors of *C. difficile* are structurally similar, and the presence or absence of the 12α-hydroxyl group appears to determine the promotion or suppression of germination (figure 1.9) (Sorg and Sonenshein, 2010). Variation in germinant specificity however has been recognised across clinically relevant strains of *C. difficile* (Heeg *et al.*, 2012). Heeg *et al.* (2012) demonstrated variation in the rate and extent of germination in response to taurocholate; furthermore, taurocholate-mediated germination was not inhibited by chenodeoxycholate in some clinical isolates. Disparity in the germination response to bile salt
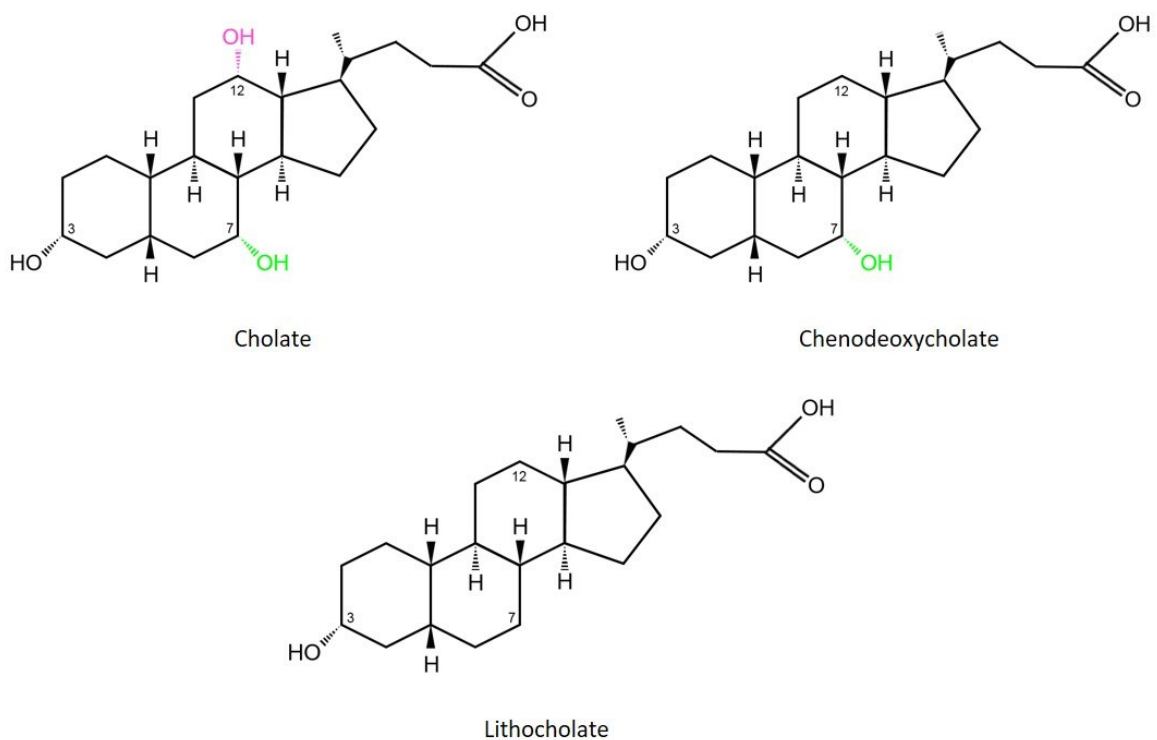
Figure 1.9 Structure of the primary bile salts cholate and chenodeoxycholate, and the secondary bile acid lithocholate. The 7α-hydroxyl group (highlighted in green) is dehydroxylated during conversion of primary bile salts to secondary bile acids. The 12α-hydroxyl group (highlighted in pink) is a structural determinant that differentiates between germinants and inhibitors of *C. difficile* germination.

germinants and inhibitors was also demonstrated across a broad range of *C. difficile* ribotypes (Thanissery *et al.*, 2017). A selected mutation of the identified bile salt receptor CspC in the *C. difficile* UK1 strain background modified germinant specificity: chenodeoxycholate induced germination of *C. difficile* spores instead of having inhibitory action in an optical density-based germination assay (Francis *et al.*, 2013). By contrast, through assessment of germination on solid media (pre-treated with potential germinants), Rohlfing *et al.* (2019) recently demonstrated that the selected CspC mutation ($cspC_{G457R}$) in strain 630Δ*erm* resulted in heightened sensitivity to germinants and co-germinants, but did not induce germination in response to chenodeoxycholate.

C. difficile germination does not occur in the presence of a single germinant, a co-germinant is usually required. Sorg and Sonenshein (2008) identified the amino acid glycine as a *C. difficile* spore co-germinant and the component of complex media that provides the additional signal required for germination. Other amino acids have also been established as co-germinants, but their activity varies considerably; although glycine has the greatest co-germination activity, with L-alanine, taurine and L-glutamine displaying further activity. Although this is strain-dependant, amino acids with branched side chains typically demonstrate the least co-germinant activity (Wheeldon *et al.*, 2011; Kochan *et al.*, 2018b; Sorg and Sonenshein, 2008; Shrestha and Sorg, 2017). Histidine was found to enhance taurocholate- and glycine-mediated germination significantly whereas a specific combination of 5 amino acids (valine, arginine, aspartic acid, histidine and glycine) in addition to taurocholate demonstrated similar germination activity to that of taurocholate and complex media. The combination of the amino acid co-germinants is believed to be an additive effect rather than synergistic effect (Wheeldon *et al.*, 2008a; Wheeldon *et al.*, 2011; Kochan *et al.*, 2018b). The activity of amino-acid co-germinants is highly temperature dependant; lysine and serine, not previously considered as co-germinants and D-forms of amino acids including D-alanine and D-serine, demonstrate co-germinant activity at the physiologically relevant temperature of 37°C (Shrestha *et al.*, 2017; Shrestha and Sorg, 2017). The role of glycine as a *C. difficile* co-germinant is thought to

be linked to its requirement for optimal growth through its incorporation into peptidoglycan of the vegetative cell wall (Bhattacharjee *et al.*, 2016b; Peltier *et al.*, 2011).

More recently, *C. difficile* spore germination was demonstrated in response to taurocholate and calcium (Kochan *et al.*, 2017). Calcium functions as a co-germinant, inducing germination in conjunction with the bile salt germinant taurocholate, and is also presumed to be involved in the transmission of the germination signal by activating cortex degradation during taurocholate- and glycine-derived germination, through an unidentified calcium-dependant enzyme (Kochan *et al.*, 2017). Germination in response to calcium as a co-germinant can be induced by either exogenous (derived from the host) or endogenous calcium released during rehydration of the spore core as Ca-DPA (Kochan *et al.*, 2017). Calcium demonstrates synergistic activity with amino acid co-germinants, and enhances the rate of germination in the presence of taurocholate and low concentrations of amino acid (Kochan *et al.*, 2018b). Crucially, this synergistic activity together with amino-acid co-germinants is able to overcome the inhibitory activity of the chenodeoxycholate (Kochan *et al.*, 2018b).

1.12 Regulation of *C. difficile* spore germination.

Universally across spore-forming bacteria, the binding of germinants and in some cases co-germinants to their appropriate receptors results in the transformation of a dormant spore into a metabolically active vegetative cell. The process of spore germination has largely been determined from the study of germination in the model organism *Bacillus subtilis* and other *Clostridia* (Setlow, 2003). Although there is commonality between some *Bacillus* and *Clostridia* species, the *C. difficile* spore germination regulation pathway is unique amongst spore-formers (Bhattacharjee *et al.*, 2016b; Setlow, 2003). Typically the *Ger* receptors are responsible for the detection of germinants and are highly conserved across *Bacillus* and *Clostridioides* species; however, distinctively, *C. difficile* does not possess Ger-type receptors but instead senses germinants and co-germinants via

the Csp subtilisin-like serine proteases of the *CspBAC* locus (Francis *et al.*, 2013; Shrestha *et al.*, 2019; Bhattacharjee *et al.*, 2016b). The products of this locus, CspB, CspA and CspC are key regulators of germination signal transduction, and interactions between the Csp proteins during spore formation are likely to be important for effective germination. Specific to *C. difficile,* gerG controls the incorporation of CspA, CspB and CspC in the mature spore (figure 1.10a) and is required for *C. difficile* spores to complete germination (Donnelly *et al.*, 2017). In the absence of functional GerG, the decreased levels of CspA, CspB and CspC alter the detection of germinants and inhibit downstream cortex hydrolysis (Donnelly *et al.*, 2017). The catalytic triad of the Csps proteases, consisting of aspartate, histidine, and serine residues, is functional in only CspB (Adams *et al.*, 2013; Francis *et al.*, 2013). Evolutionary alteration of the catalytic residues of CspA and CspC eliminates their ability to process the cortex lytic enzyme to its active form and transduce the germination signal (Francis *et al.*, 2013). Restoration of the catalytic triad residues of CspC and CspA does not restore protease activity and results in varied phenotypic consequences. In the case of CspC, the restoration of specific catalytic residues reduced the stability and levels of CspC in sporulating cells and caused defective germination while the restoration of residues of CspA did not affect the levels of CspBA or alter germination activity (Donnelly *et al.*, 2020).
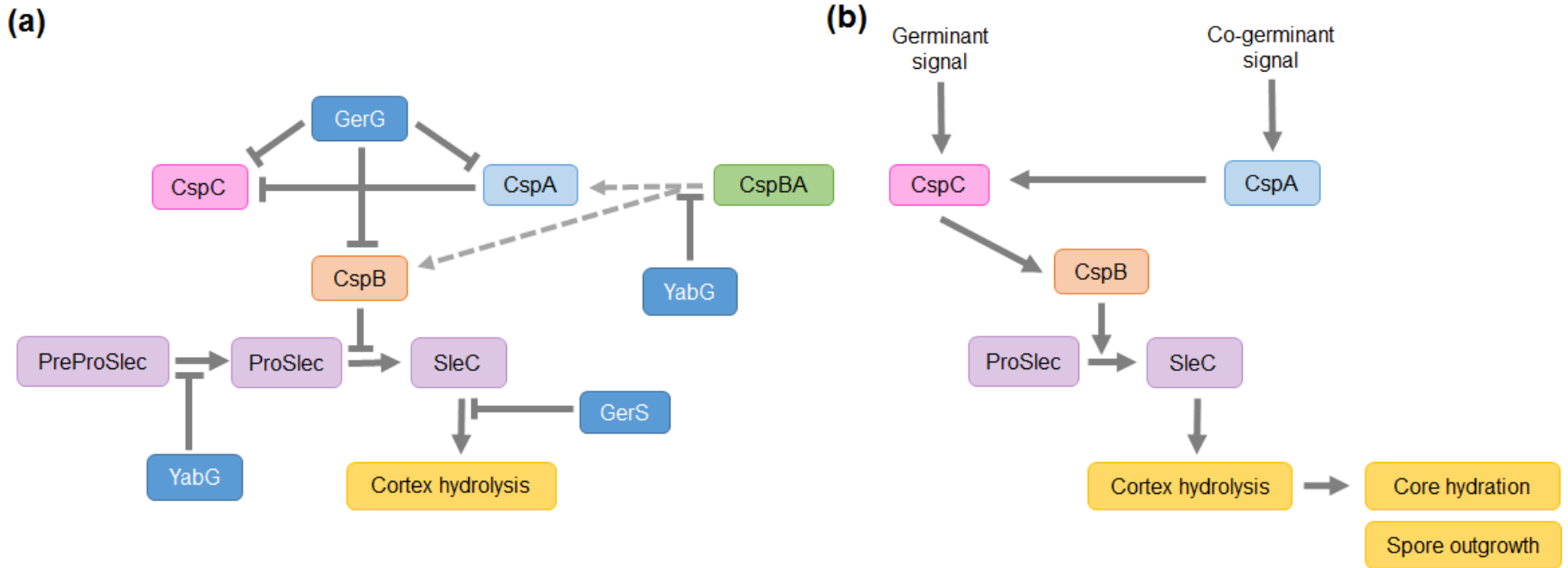
Figure 1.10 A model of *C. difficile* germination regulation pathways. (a) The role of key regulators of *C. difficile* germination, (b) and the sequence of the *C. difficile* germination signal cascade (Francis *et al.*, 2013, Shrestha *et al.*, 2019, Donnelly *et al.*, 2017, Adams *et al.*, 2013, Bhattacharjee *et al.*, 2016, Francis *et al.*, 2015, Francis and Sorg, 2016, Burns *et al.*, 2010, Diaz *et al.*, 2018, Fimlaid *et al.*, 2015).

CspC has been identified as the proposed bile salt germinant receptor and is responsible for the transmission of the germination signal downstream to CspB, although the mechanism by which this occurs has yet to be fully determined (Francis *et al.*, 2013; Bhattacharjee *et al.*, 2016a). However, a greater abundance of CspC protein in mature spores of clinically relevant *C. difficile* strains did not correlate with an increased germination rate, suggesting CspC has a more complex role in *C. difficile* germination signal transduction and may inhibit downstream processes (Bhattacharjee *et al.*, 2016a). Research by Rohlfing *et al.* (2019) demonstrated that mutation of specific residues of CspC resulted in increased sensitivity to the germinant taurocholate and even negated the need for taurocholate to induce germination at low frequency. CspA and CspB are encoded by *cspBA* as a fusion protein and undergo interdomain cleavage during spore formation (Adams *et al.*, 2013; Francis *et al.*, 2013). CspBA is believed to stabilise CspC through sporulation and spore maturation, and thus regulates the level of CspC incorporated into mature spores (Kevorkian *et al.*, 2016). Deletion of *cspA* results in mature spores that are germination-defective and contain reduced level of CspC protein (Kevorkian *et al.*, 2016). Recently the role of CspA in *C. difficile* spore germination has been expanded through research by Shrestha *et al.* (2019) that implicates CspA in the recognition of amino acid and calcium co-germinants. Site directed deletions in *cspA* eliminated the requirement for co-germinants during taurocholate-mediated germination, although these mutants still responded to glycine as a co-germinant (Shrestha *et al.*, 2019). The interdomain processing of CspBA to CspB and CspA is dependent upon the protease YabG (figure 1.10a) during sporulation (Kevorkian *et al.*, 2016). Cleavage of CspBA regulates appropriate germination: the mutation of YabG protease produces spores that are able to germinate in response to the germinant taurocholate alone. By contrast to the CspA mutant discussed previously, the YabG mutant no longer requires or responds to amino acid co-germinants (Shrestha *et al.*, 2019).

CspB, the only subtilisin-like serine protease produced by *C. difficile* that has catalytic activity is controlled by CspC through a putative protein-protein interaction (figure 1.10b) and is required for cortex hydrolase activation (Adams *et al.*, 2013; Francis *et al.*, 2015; Donnelly *et al.*, 2017).

Activation of CspB by CspC initiates the cleavage of the N-terminal region of pro-SleC to produce the active cortex lytic enzyme, SleC (Francis and Sorg, 2016; Adams *et al.*, 2013). Upon activation, SleC degrades the specialised peptidoglycan of the cortex, releasing stored Ca-DPA and rehydrating the spore core via a mechanosensing mechanism (Francis and Sorg, 2016; Burns *et al.*, 2010). Eliminating the catalytic activity of CspB has a significant impact on *C. difficile* spore germination, and inhibits processing of the active protease SleC, but does not eliminate germination completely, suggesting other enzymes are also involved in cortex hydrolysis (Adams *et al.*, 2013). YabG also regulates of the cortex lytic enzyme and processes preproSlec to proSleC; elimination of YabG prevents incorporation of proSleC in the mature spore (Kevorkian *et al.*, 2016; Shrestha *et al.*, 2019).

GerS regulates germination through its modification of the peptidoglycan of the cortex and thus regulates the activity of SleC (Diaz *et al.*, 2018). Germination deficient GerS mutants are unable to degrade the cortex and complete germination because these cortex-specific modifications are required for the recognition by SleC (Diaz *et al.*, 2018; Fimlaid *et al.*, 2015). Furthermore the inactivation of GerS results in the inability to cause disease in the *C. difficile* hamster infection model (Fimlaid *et al.*, 2015).

By contrast to other spore-forming bacteria, cortex degradation by cortex lytic enzymes occurs before the release of Ca-DPA during *C. difficile* germination (Francis *et al.*, 2015). Hydrolysis of cortex peptidoglycan relieves osmotic constraints inside the core, and this is detected by the mechanosensing protein, SpoVAC, that facilitates the release of Ca-DPA (Francis and Sorg, 2016).

1.13 *C. difficile* spore germination models.

Considering the understanding of *C. difficile* germination discussed above, models predicting the transduction of the germination signal have been hypothesised. Kochan *et al*. (2018) proposes a model whereby bile salt germinants acts as a 'chemical key' when binding to its 'lock', CspC (Kochan *et al.*, 2018a; Kochan *et al.*, 2017). Activation of CspC by germinants facilitates the transport of co-germinants including calcium and amino acids through the outer membrane via an unidentified mechanism. CspB is activated by binding to calcium, which is either exogenous or released from the spore core when amino acids bind to the co-germinant receptor. CspB processes proSleC to its active form SleC, which degrades the peptidoglycan of the cortex, leading to core rehydration, DPA release and spore outgrowth (Kochan *et al.*, 2017).

In the alternative 'germinosome' model, based on the findings of Adams *et al.* (2013); Bhattacharjee *et al.* (2016); Fimlaid *et al.* (2015); Francis *et al.* (2013) and informed by the observed co-localization of germinant receptors at the inner membrane of *Bacillus subtilis* spores, the products of the *cspBAC* operon together with proSleC are co-located in the cortex component of the outer-membrane as a 'germinosome complex' (Adams *et al.*, 2013; Francis *et al.*, 2013; Fimlaid *et al.*, 2015; Bhattacharjee *et al.*, 2016b; Kochan *et al.*, 2018a; Griffiths *et al.*, 2011). Their proximity is hypothesised to facilitate direct interaction such that when taurocholate binds to the germinant receptor CspC, CspB is subsequently activated. The activation of CspB, together with simultaneous interaction of calcium and/or amino acid co-germinants with the germinosome, facilitates CspB-mediated processing of proSlec to SleC and ultimately cortex degradation (Kochan *et al.*, 2018a). Integrating the recent findings by Shresha *et al*. (2019) that identify CspA as the proposed amino acid co-germinant receptor within the germinosome complex model, the proximity and physical interaction of CspC and CspA with CspB is hypothesised to inhibit its protease activity, thereby regulating germination (figure 1.11). Upon binding of germinants and co-germinants to their

receptors, CspC and CspA dissociate from CspB, allowing the processing of proSleC to SleC (Shrestha *et al.*, 2019)

Recent characterisation of the crystal structure of CspC by Rohlfing *et al.* (2019) was unable to determine whether bile salt germinants interact directly or indirectly with CspC. Mutational binding studies of CspC identified mutants that had increased sensitivity to the germinant taurocholate but also had increased sensitivity to amino acid co-germinants (Rohlfing *et al.*, 2019). Although CspC plays a key role in germination signal transduction, it may not be to bind directly to bile salts germinants. The authors propose that it may interact with the unidentified bile salt germinant receptor and the co-germinant receptor to transduce the germination signal (Rohlfing *et al.*, 2019). CspC activation may induce a conformational change that permits its interaction with CspB, resulting in induction of protease activity and subsequent interaction of CspA with CspB of the germinosome complex (Rohlfing *et al.*, 2019; Shrestha *et al.*, 2019). The authors also discuss an alternative role for CspC in the transport of germinants to their receptors located in the cortex by altering the permeability of the spore outer membrane (Shrestha *et al.*, 2019). This explanation of the role of CspC in germination regulation has similarities to the 'lock and key' model but also incorporates the co-location of the germinosome complex.
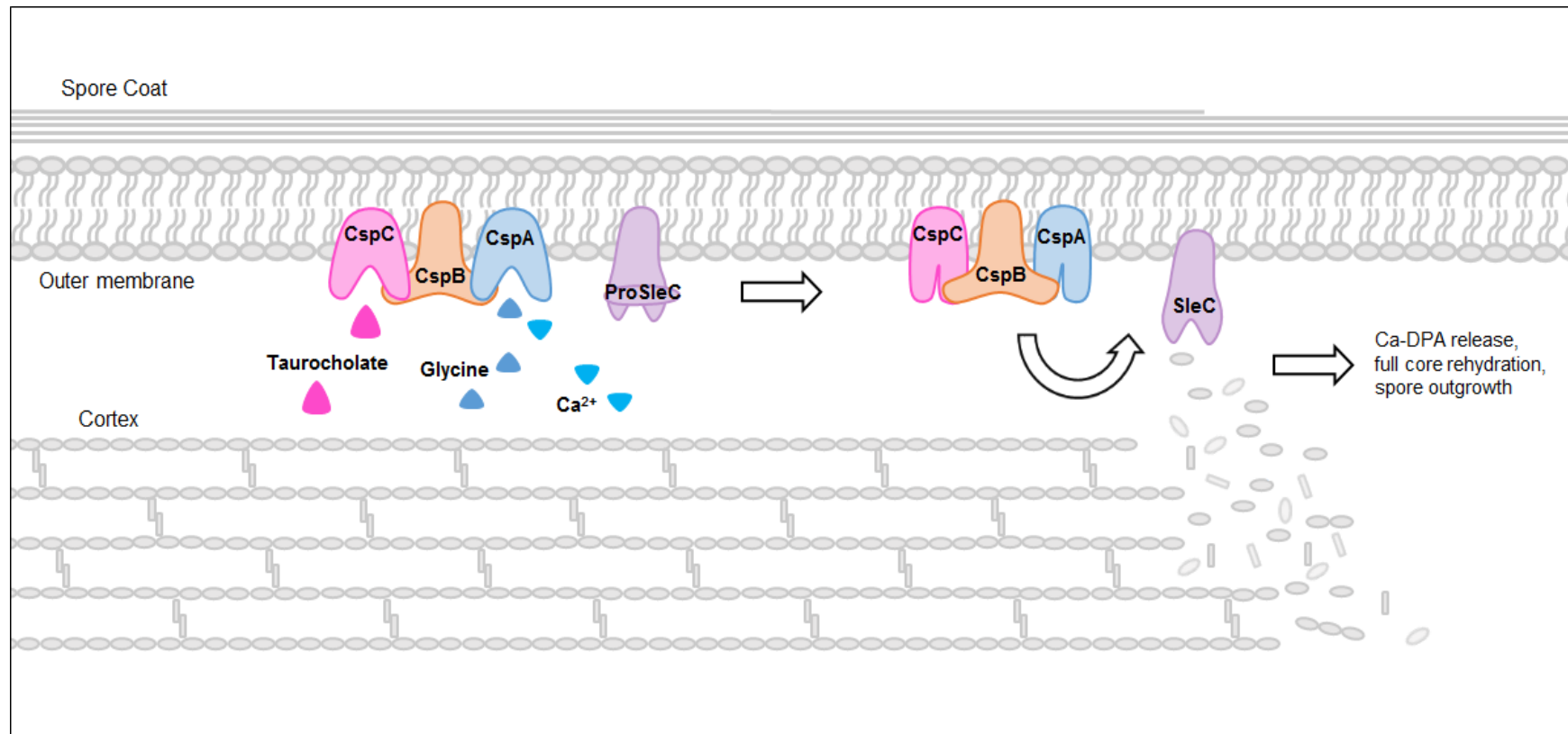
Figure 1.11 An updated germinosome complex model demonstrating germinant and co-germinant receptor activation and downstream signalling leading to germination of *C. difficile* spores. The model incorporates the recent identification of CspA as the cogerminant receptor and its interaction with CspB, which together with CspC activation results in processing of proSlec to SleC, subsequent cortex degradation, calcium dipicolinic acid (Ca-DPA) release, core rehydration, and spore outgrowth. Model modified from Kochan *et al*. (2018) and Shrestha *et al*. (2019).

1.14 Laboratory diagnosis of *C. difficile* infection.

Because it is not possible to clinically distinguish CDI from other causes of healthcare-associated diarrhoea, laboratory diagnosis is essential. There is substantial debate in the literature on the most appropriate methodology to diagnose CDI with specificity, sensitivity, cost, time required for results and the severity of patient symptoms, all considerations. An effective diagnostic approach should determine if patients require treatment and isolation to reduce transmission in a timely manner, preventing the progression of severe CDI and complications while also avoiding unnecessary treatment or withdrawal of antibiotic therapy for those patients who do not have CDI (Gerding *et al.*, 2016). If a patient has diarrhoea (Bristol Stool Chart types 5-7) that is not clearly attributable to an underlying condition or therapy then it is necessary to determine if this is due to CDI (Lewis and Heaton, 1997; Department of Health, 2012). Diarrhoeal samples should be tested for *C. difficile* from hospital patients aged >2 years, all community patients aged >65 years, and from community patients aged <65 years, wherever clinically indicated. The department of health advisory committee on antimicrobial prescribing, resistance and healthcare associated infection (ARHAI) advise a two stage testing approach for the diagnosis of CDI which consists of a lateral flow glutamate dehydrogenase enzyme immunoassay (EIA) test or a PCR test to screen stool samples, followed by a sensitive toxin EIA test or a cytotoxin assay (Department of Health, 2012). The enzyme, glutamate dehydrogenase is produced by both toxigenic and non-toxigenic strains of *C. difficile* and its detection by EIA is highly sensitive as a diagnostic marker for *C. difficile* but is unable to distinguish between colonisation or CDI (Swindells *et al.*, 2010; Shetty *et al.*, 2011). The alternative first stage of real-time PCR testing can be used to detect the presence of toxin genes including *tcdA, tcdB* or *tcdC* directly from patient stools (Crobach *et al.*, 2009; de Jong *et al.*, 2012). For the second stage test, commercially available rapid and highly specific enzyme immunoassays (EIAs) for *C. difficile* toxin detection in patient stool include tests for toxin A only or toxins A and B in combination (Bartlett and Gerding, 2008; Yolken *et al.*, 1981). Anti-toxin antibodies capture the antigen if present in the stool supernatant which are then bound by a second set of anti-toxin

antibodies, sandwiching the antigen. The addition of anti-second antibody conjugated to a chromogenic substrate visualises the reaction as a colour change, indicating the presence of *C. difficile* toxins (Lall *et al.*, 2017). The cytotoxin assay requires the inoculation of stool filtrate onto a monolayer of a cell culture which is subsequently observed for a toxin B induced cell rounding, to confirm the cytopathic effect is specific to *C. difficile* toxin, neutralization with antiserum is completed. This technique requires specialist expertise and laboratory facilities with results taking 24-48 hours (Crobach *et al.*, 2009; Chang *et al.*, 1978). The culture of *C. difficile* is not routine but is recommended in outbreaks alongside toxin detection to facilitate strain type monitoring and surveillance (Department of Health, 2012; Public Health England, 2018).

1.15 *C. difficile* ribotyping.

Epidemiological studies of *C. difficile* utilise several typing techniques including pulsed-field gel electrophoresis, restriction enzyme analysis, PCR-ribotyping, toxinotyping, arbitrary-primed PCR and random amplification of polymorphic DNA. Consequently *C. difficile* isolates are often referred to by multiple typing designations (eg:NAP1/BI/027) (Fawley *et al.*, 2015). In England, the standardized ribotyping by the *Clostridium difficile* Ribotyping Network (CDRN) provides enhanced surveillance of *C. difficile* strains monitoring and identifying the emergence of clinically important *C. difficile* ribotypes (Wilcox *et al.*, 2012). PCR-ribotyping, a DNA-typing technique comprises the extraction of DNA from *C. difficile* cultures, PCR amplification of the 16S-23S rRNA gene intergenic spacer region with specific primers and capillary gel electrophoresis analysis of the PCR fragments. This is a standardised, rapid, discriminatory and cost-effective methodology for the epidemiological monitoring of *C. difficile* (Fawley *et al.*, 2015).

Over 650 unique *C. difficile* ribotypes have been identified, with the number continuing to expand (Fawley *et al.*, 2015). Specific ribotypes are prevalent in different geographical regions, with the predominant ribotypes in Europe including 027, 001/072, 014/020, 140, 002, 010, 078, 018, 015 and 176 (Bauer *et al.*, 2011; Davies *et al.*, 2016). Ribotypes 002, 014, 015, 005, 023 and 078 are the most prevalent in England (Public Health England, 2019b). There has been a decreasing presence of *C. difficile* ribotypes 027, 001 and 106 in England and Northern Ireland between 2008 and 2018 with a compensatory increase in the other ribotypes (Public Health England, 2019b). A two-year analysis of *C. difficile* ribotypes in west London hospitals found ribotypes 002 and 015 were the most frequently identified while ribotype 022 was associated with increased mortality and ribotype 005 was most commonly associated with recurrent *C. difficile* when compared to other ribotypes (Herbert *et al.*, 2019).

Ribotype 027, also known as North American pulsed-field gel electrophoresis type 1, restriction endonuclease analysis type B1 (NAP1/B1/027), is the most commonly isolated ribotype in European

countries (Loo *et al.*, 2005; McDonald *et al.*, 2005; Davies *et al.*, 2016). Described as hypervirulent, *C. difficile* ribotype 027 is responsible for significant outbreaks that have occurred in the USA and Europe (Kuijper *et al.*, 2006; Cookson, 2007; Fatima and Aziz, 2019). Ribotype 027 strains produce binary toxin (Carter 2007) which enhances the toxicity of TcdA and TcdB and may be an important virulent factor of 027 ribotype strains (Carter *et al.*, 2007; Gerding *et al.*, 2014). Ribotype 027 is also associated with increased expression of toxins A and B; a mutation in the TcdC gene, the negative regulator of *tcdA* and *tcdB* was found to cause hyper-expression of toxins A and B, approximately 16 times and 23 times higher respectively than control strains (Warny *et al.*, 2005). Ribotype 027 has been associated with increased disease severity and mortality but this has been contradicted where patient outcomes were conversely not significantly worse as a result ribotype 027 when compared to CDI caused by other strains (See *et al.*, 2014; Walk *et al.*, 2012). Phylogenetic analysis of *C. difficile* ribotype 027 demonstrates two epidemic lineages with distinct patterns of global spread (He *et al.*, 2013). Despite evidence of other enhanced virulence factors that potentially contribute to the hypervirulence of ribotype 027, the acquisition of several mobile genetic elements which convey resistance to a number of antibiotic classes is believed to be the driving force in the evolution and persistence of *C. difficile* ribotype 027 in healthcare settings (He *et al.*, 2013).

1.16 Treatment of *C. difficile* infection.

Upon diagnosis of CDI, isolation of the patient and implementation of appropriate infection control measures is mandatory to prevent transmission within the hospital (Debast *et al.*, 2014; Public Health England, 2013; McDonald *et al.*, 2018). Immediate treatment measures to mitigate CDI include adequate replacement of fluid and electrolytes and the review of any controllable risk factors including discontinuing the use of antibiotic therapy, PPIs and anti-motility medications (Debast *et al.*, 2014). Any inciting antibiotic agent(s) should be discontinued as soon as possible to allow the normal intestinal flora to be re-established (McDonald *et al.*, 2018; Public Health England, 2013). Continuation of the antibiotic therapy is associated with treatment failure and recurrent CDI (Modena *et al.*, 2006; Mullane *et al.*, 2011)

1.16.1 Antibiotic treatment of CDI.

Appropriate antibiotic treatment of CDI is based on the severity of disease (Gerding *et al.*, 2016). Metronidazole and vancomycin have been the main antibiotic agents used in the treatment of CDI but there are now more antibiotics treatment options (McDonald *et al.*, 2018). Public Health England recommends that patients with mild or moderate CDI should receive oral metronidazole (dose: 400–500 mg 3 times a day for 10–14 days), diarrhoea should resolve in 1-2 weeks (Public Health England, 2013). The clinical practice guideline for CDI produced by Infectious Diseases Society of America and Society for Healthcare Epidemiology of America alternatively recommends vancomycin or fidaxomicin over metronidazole for an initial episode of CDI (McDonald *et al.*, 2018).

1.16.1.1 Metronidazole.

Oral metronidazole is demonstrated as effective treatment for CDI and has the advantage of low cost (Nelson *et al.*, 2017). Metronidazole is activated by reduction when inside the target anaerobic bacterial cell where it inhibits protein synthesis by interacting with DNA causing strand breakage and the loss of helical DNA structure (Edwards, 1993). Treatment of CDI with metronidazole is not appropriate for patients at high risk of severe or life-threatening disease such as those of advanced age or comorbidities (Shen and Surawicz, 2008; Debast *et al.*, 2014). Data suggest that amongst patients aged 65 years and under with mild CDI, treatment with metronidazole has similar clinical outcomes to vancomycin and is therefore an appropriate treatment for younger patients with an initial mild episode of CDI (Appaneal *et al.*, 2019). There is evidence of the emergence of reduced susceptibility of some strains of *C. difficile* to metronidazole and a rise in clinical treatment failure associated with ribotype 027, although mechanisms remain unclear (Baines *et al.*, 2008; Pépin *et al.*, 2007). Recently *C. difficile* resistance to metronidazole has been attributed to the acquisition of an internationally disseminated plasmid, pCD-METRO which is maintained in the absence of selective pressure (Boekhoud *et al.*, 2020).

1.16.1.2 Vancomycin.

For patients with severe CDI, or those where metronidazole treatment has failed, Public Health England recommends oral vancomycin (dose: 125 mg 4 times a day for 10–14 days) due to the superiority of vancomycin in the treatment of severe CDI compared to metronidazole (Public Health England, 2013; Zar *et al.*, 2007). The clinical cure rate of oral vancomycin has been shown to be superior to metronidazole in randomized, placebo-controlled trials (Zar *et al.*, 2007). Vancomycin is bactericidal, inhibiting the polymerization of peptidoglycan in the bacterial cell wall by binding to the acyl-D-ala-D-ala portion of the growing peptidoglycan cell wall (Nagarajan, 1991). It is poorly absorbed from the gastrointestinal tract leading to high luminal drug levels which results in rapid

suppression of *C. difficile* (Debast *et al.*, 2014; Al-Nassir *et al.*, 2008). For complicated CDI, vancomycin administered orally together with intravenously administered metronidazole is the regimen of choice (McDonald *et al.*, 2018). This has been demonstrated to improve mortality in critically ill patients (Rokas *et al.*, 2015). Life-threatening disease can be treated by vancomycin given via a nasogastric tube and/or by rectal installation (Public Health England, 2013; Apisarnthanarak *et al.*, 2002). Treatment of CDI with vancomycin has incorrectly been associated with an increased risk of colonization with vancomycin-resistant *Enterococcus* (VRE), however the frequency of VRE contamination was found to be similar between patients treated with metronidazole or vancomycin (Sethi *et al.*, 2009).

1.16.1.3 Fidaxomicin and treatment of recurrent CDI.

Oral fidaxomicin was introduced as a new option for the treatment of CDI in Europe in 2012 (Johnson and Wilcox, 2012). Fidaxomicin is a macrocyclic antibiotic that prevents bacterial RNA synthesis by binding and inhibiting the action of DNA-dependent RNA polymerase, it has a narrow spectrum of activity against Gram-positive bacteria which includes *C. difficile* (Johnson and Wilcox, 2012). Its lack of activity against Gram-negative bacilli is an important advantage, promoting the maintenance of colonization resistance (Johnson and Wilcox, 2012). Fidaxomicin is recommended for patients with multiple co-morbidities who are receiving concomitant antibiotics or those with recurrent CDI, whether mild, moderate or severe, because of their increased risk of further recurrences (Public Health England, 2013). Fidaxomicin was significantly more effective than vancomycin in achieving clinical cure in the presence of concomitant antibiotic therapy and in preventing recurrence regardless of concomitant antibiotic use (Mullane *et al.*, 2011). Two phase 3 randomised controlled trials demonstrated fidaxomicin was non-inferior to vancomycin in the initial clinical cure of CDI, but was superior in reducing recurrence (Crook *et al.*, 2012).

Recurrent CDI occurs in approximately 25% of patients and is associated with a diminished quality of life due to repeated episodes of diarrhoea (Kelly, 2012; Johnson, 2009b). For patients with a first recurrence of CDI, treatment with oral vancomycin or fidaxomicin is recommended (McDonald 2018). In patients with a first recurrence of CDI, fidaxomicin demonstrated similar efficacy to vancomycin, it was found to be superior in preventing a second recurrence compared to the standard course of vancomycin (Cornely 2012). The superior efficacy may be linked to suppression of spores in the faeces of patients treated with fidaxomicin which also leads to a reduction in environmental contamination (Davies 2020). The European society of clinical microbiology and infectious diseases (ESCMID) recommend multiple recurrent episodes of CDI should be treated with fidaxomicin, if unresponsive to repeated antibiotic treatment, faecal microbiota transplantation (FMT) in combination with oral antibiotic treatment is strongly recommended (Debast *et al.*, 2014).

1.16.1.4 Alternative and future antibiotic therapy for the treatment of CDI.

Rifaximin, a non-absorbed rifamycin antibiotic that remains largely in the gastrointestinal tract has good antimicrobial activity against *C. difficile*, is well tolerated by patients with minimal adverse effects (Public Health England, 2013; Descombe *et al.*, 1994). It has been demonstrated as a viable alternative for the treatment of CDI by Systematic review (Ng *et al.*, 2019). Rifaximin could also be utilised in conjunction with other therapies, being efficacious in reducing the rate of recurrence, however resistance to rifaximin is a significant issue that could limited its practicality for the treatment of CDI (Ng *et al.*, 2019).

Nitazoxanide and its main metabolite, tizoxanide are antimicrobial against *C. difficile* and have been shown to be as effective at as metronidazole in the treatment of CDI but could be more beneficial as an effective alternative for the treatment of recurrent CDI (Dubreuil *et al.*, 1996; Musher *et al.*, 2006).

Ridinilazole is a potential future antimicrobial agent for the treatment of CDI, it demonstrates bactericidal activity against *C. difficile* and was found to suppress the release of IL-8 from cell lines exposed to *C. difficile* while also reducing the production of toxin A and B (Bassères *et al.*, 2016). It has a narrow-spectrum of activity, demonstrating limited activity against the commensal of microbes of the gut helping to preserve the gut microbiota (Cho *et al.*, 2019). Two clinical trials are currently in progress to evaluate ridinilazole for the treatment for CDI in comparison to vancomycin (ClinicalTrials.gov, 2018a; c).

Teicoplanin may be an effective antibiotic to treat CDI as an alternative to vancomycin but the quality of the evidence is currently very low (Nelson *et al.*, 2017). The novel antimicrobials; tedizolid, dalbavancin, and ceftobiprole have been evaluated for activity against *C. difficile* with dalbavancin and tedizolid demonstrating potential as therapeutic agents for the treatment of CDI (Binyamin *et al.*, 2018).

Other antibiotics that have clinically relevant activity against *C. difficile* are nitazoxanide, ramoplanin and tigecycline, however due to limited data, high cost, resistance and reports of unfavourable adverse-events, these are not current routinely used for the treatment of CDI (Leffler and Lamont, 2015).

1.16.1.5 Antibiotic resistance.

While resistance to antibiotics has not limited treatment options for CDI, as is the case of many other clinically relevant pathogens, antibiotics have influenced the epidemiology of CDI and the emergence of hypervirulent strains. The increased use of fluoroquinolones was correlated with the acquisition of mutations in DNA gyrase (*gyrA*) in two independent lineages of *C. difficile* ribotype 027 (Centers for Disease Control and Prevention, 2019; He *et al.*, 2013). *C. difficile* is unsusceptible to many antibiotics commonly used in the treatment of bacterial infections and are known risk

factors of CDI (Spigaglia, 2016; Peng *et al.*, 2017). Moreover, the formation of dormant spores by *C. difficile* are intrinsically resistant to antibiotics (Gil *et al.*, 2017; Driks, 2003).

Horizontal gene transfer is a key mechanism driving *C. difficile* evolution, mobile genetic elements are responsible for the acquisition of an extensive array of genes involved in antimicrobial resistance, virulence, host interaction and the production of surface structures by *C. difficile* (Sebaihia *et al.*, 2006). *C. difficile* resistance to metronidazole and vancomycin is low, although there is evidence of reduced susceptibility of *C. difficile* to these antibiotics across European surveillance; 0.11% and 0.87% of the strains investigated were resistant to metronidazole and vancomycin respectively (Spigaglia, 2016; Freeman *et al.*, 2015). Adler *et al.* (2015) described a *C. difficile* ribotype 027 strain with reduced susceptibility to metronidazole and vancomycin that had disseminated across Israel to become the dominant strain. *C. difficile* fidaxomicin resistance is extremely rare, no fidaxomicin isolates were found to be resistant or intermediately resistant during European surveillance, as reported by Freeman *et al.* (2015). Rifampicin resistant clinical isolates of *C. difficile* and corresponding resistance to rifaximin have been reported with resistance rates appearing to be on the rise (Spigaglia, 2016; Eitel *et al.*, 2015). Rifampicin resistance has been demonstrated across multiple *C. difficile* ribotypes (Freeman *et al.*, 2015).

1.16.2 Non-antibiotic treatment of CDI.

Several non-antibiotic therapies for the treatment of CDI have been described and are predominantly utilised when antibiotic treatment failure occurs, or patients have severe complications. Severe or recurrent CDI, warrants intravenous immunoglobulin containing anti-toxin antibodies against *C. difficile* toxins (Department of Health, 2011). The administration of neutralizing human monoclonal antibodies against *C. difficile* toxins A and B significantly reduced the recurrence of CDI in a randomised controlled study, but did not reduce the time to the resolution of diarrhoea or the number of days of hospitalization for the initial episode (Lowy *et al.*, 2010). A case report by Shah *et al.* (2014) described the successful treatment of a patient with comorbidities and severe CDI, who had not responded to antimicrobial therapy with a single dose of intravenous immunoglobulin. No recurrence of CDI occurred following the intravenous immunoglobulin therapy Shah *et al.* (2014).

Bezlotoxumab a monoclonal antibody directed against toxin B produced by *C. difficile*, has been approved as adjunctive therapy for patients who are receiving antibiotic treatment for CDI and who are at high risk for recurrence (Wilcox *et al.*, 2017). A retrospective study of the use of bezlotoxumab in CDI patients in a Finnish hospital demonstrated it was effective in the prevention of recurrent CDI in 73% of patients and may be a suitable alternative treatment to those in need of FMT (Oksi *et al.*, 2019).

Tolevamer a novel *C. difficile* toxin-binding polymer, developed to ameliorate the disease without adversely affecting normal flora, successfully neutralises the toxins produced by multiple strains of *C. difficile* including those produced by epidemic NAP1/BI/027 strains (Hinkson *et al.*, 2008). The outcome of two controlled trials comparing the efficacy of tolevamer with metronidazole and vancomycin to treat CDI found tolevamer was inferior to antibiotic therapy but may be suitable as adjunctive treatment with standard antibiotic treatment (Johnson *et al.*, 2014).

DAV132 is a therapeutic designed to bind excess antibiotics in the colon to prevent dysbiosis of the gut microbiota (Burdet *et al.*, 2017). It is currently undergoing a clinical trial to determine its efficacy of DAV132 in hospitalized patients receiving fluoroquinolones at high risk of CDI (ClinicalTrials.gov, 2018b).

Surgical intervention by means of colectomy is necessary for severely ill CDI patients, such as those with toxic megacolon (McDonald *et al.*, 2018; Public Health England, 2013). The mortality of CDI patients undergoing an emergency colectomy is very high but the surgical intervention for selected patients is lifesaving. A retrospective observational cohort study suggest colectomy reduces mortality in very high-risk patients (Lamontagne *et al.*, 2007). A more recent alternative surgical intervention to colectomy for the treatment of severe CDI is the diverting loop ileostomy which preserves the colon and has been shown to have an improved mortality rate compared to colectomy in a multicentre study (Ferrada *et al.*, 2017).

1.16.3 Faecal microbiota transplantation.

FMT aims to restore the health-associated commensal microbiota in patients who have recurrent CDI by introducing enteric bacteria from the faeces of healthy donors. The subsequent alteration of intestinal environment becomes inhospitable for *C. difficile* and allows colonisation resistance to be restored (NICE, 2014; Tariq *et al.*, 2018). The inhibitory mechanism of action against CDI is thought to include niche competition for nutrients, production of short chain fatty acids and the abundance of primary and secondary bile acids (Lawley and Walker, 2013; Theriot *et al.*, 2016). The exact microbial constituents of the faecal microbiota that provide resistance against *C. difficile* are not known but the phyla Bacteroidetes and Firmicutes are thought to comprise critical components of the material that needs to be transplanted (Antharam *et al.*, 2013). Successful FMT is correlated with the normalisation of the faecal bile acid composition, decreasing primary bile acid

concentrations and increasing secondary bile acids to levels found prior to antibiotic treatment (Weingarden *et al.*, 2016).

FMT is recommended for patients with multiple recurrences of CDI who have failed appropriate antibiotic treatments (McDonald *et al.*, 2018; NICE, 2014). The first randomized double-blind clinical study that compared the outcome of standard antibiotic therapy to FMT was published in 2013 and demonstrated FMT was significantly more effective for the treatment or recurrent CDI than vancomycin (van Nood *et al.*, 2013). A more recent systematic review and meta-analysis of published studies and case series of FMT for the treatment of recurrent CDI determined FMT was highly effective, with clinical resolution of CDI following FMT of 92% across the studies included. FMT was found to be more effective at resolving recurrent CDI than vancomycin therapy (Quraishi *et al.*, 2017). The mode of FMT delivery, dosage and number of FMT infusions influence the efficacy of FMT for the treatment of recurrent CDI; multiple infusions increase the rate of efficacy of FMT while the use of fresh or frozen FMT product does not influence the outcome (Ianiro *et al.*, 2018).

FMT is currently a highly diverse biological product with variation derived from the microbial communities of the donor and final FMT product along with the mode of delivery to the recipient patient (Schäffler and Breitrück, 2018; Tariq *et al.*, 2018). Observational studies have shown FMT is highly successful in the treatment of CDI, but the efficacy described in clinical trials has been variable. A meta -analysis of randomised control trials determined FMT was associated with lower cure rates than seen with observational studies. Variation appears to be derived from the inclusion and exclusion criteria of randomised control trials, the mode of delivery of FMT and the definition of clinical resolution of CDI (Tariq *et al.*, 2018).

The microbial composition and diversity of the donor is a major factor and reliable predictor of the success of FMT (Kump *et al.*, 2018). A recent study by Littmann *et al.* (2021) demonstrated the inflammatory immune status of a recipient can limit the efficacy of FMT. Utilising a *C. difficile* infection model and subsequent effective treatment of FMT in mice, the authors demonstrated

immunodeficient mice exhibited exacerbated intestinal inflammation and impaired resolution of CDI following FMT compared to control animals. Immunodeficient FMT non-responsive mice failed to restore the of intestinal metabolite profile to pe-infection concentrations, including secondary bile acids (Littmann *et al.*, 2021). Weingarden *et al.* (2016) demonstrated bile acids at concentrations found in patients after FMT inhibit germination and vegetative growth of clinical strains of *C. difficile,* supporting the hypothesis that the restoration of bile acid composition following FMT is another important factor in the therapeutic efficacy of FMT.

1.17 Therapies to prevent CDI.

1.17.1 Live biotherapeutic products.

The use of probiotics for the prophylactic treatment of CDI is being studied. The findings of a meta-analysis on the use of probiotics for the prevention of CDI and antibiotic-associated diarrhoea indicate that probiotics given concurrently with antibiotics reduce the risk of CDI (Pattani *et al.*, 2013). The use of a prophylactic probiotic treatment consisting of 3 species of *Lactobacillus*, in parallel to antibiotic therapy at a Quebec community hospital was associated with a reduction in CDI rates and recurrences (Maziade *et al.*, 2015). Existing data suggest that these could be beneficial at preventing CDI but the data set included a higher CDI incidence than might be expected based in the patient population, suggesting a bias toward a benefit of the probiotic (McDonald *et al.*, 2018). The synthetic probiotic lactobacilli, Syn-LAB 2.0 and Syn-LAB 2.1 which display the *C. difficile* adhesin SlpA on their cell-surface, could be another non-antibiotic intervention for CDI. Syn-LAB 2.0 and Syn-LAB 2.1 exhibited a protective effect in animals that were subsequently exposed to *C. difficile* but have not progressed to clinical trials (Vedantam *et al.*, 2018).

Prevention of primary CDI may be possible with probiotics, but it is less clear if probiotics are beneficial to patients who have experienced a previous episode of CDI, the efficacy of probiotics for the prevention of recurrent CDI is believed to be linked to the extent of disruption of the host colonic microbiota (Johnson *et al.*, 2012). Limitations of the available data on the use of probiotics for the prevention of CDI remain, including differences in probiotic formulations studied, duration of probiotic administration, duration of study follow-up, and inclusion of patients (McDonald *et al.*, 2018). There is currently insufficient evidence to recommend administration of probiotics for primary prevention of CDI (McDonald *et al.*, 2018; Debast *et al.*, 2014).

The promotion of colonisation by a non-toxigenic strain of *C. difficile* in order to fill the environmental niche exploited by toxin-producing *C. difficile* has been an approach suggested to prevent CDI recurrence (Kelly and LaMont, 2008; Sambol *et al.*, 2002). Treatment of patients who

had experience a single episode or a single recurrence of CDI, with a non-toxigenic *C. difficile* strain was demonstrated to provide protection from recurrent CDI in a phase 2 clinical trial (Gerding *et al.*, 2015). Although it is possible for non-toxigenic *C. difficile* strains to acquire the PaLoc and antibiotic resistance genes from toxigenic strains through horizontal gene transfer the NTCD-M3 non-toxigenic *C. difficile* strain has been endorsed by the European Medicine Agency (EMA) for advancement to a phase 3 clinical trial (Brouwer *et al.*, 2013; Imwattana *et al.*, 2020; Destiny Pharma PLC, 2022)

1.17.2 Anti-sporulation and anti-germination therapies.

C. difficile sporulation is crucial to the dissemination and transmission of CDI, while germination is an essential step in the colonisation of the host. Therapies that target these events may offer an opportunity to disrupt the chain of infection and prevent CDI in vulnerable patients (Paredes-Sabja *et al.*, 2014; Deakin *et al.*, 2012). Cephamycins have been shown to inhibit sporulation of *C. difficile* by binding to penicillin binding proteins. When administered in combination with standard antibiotic treatment, cephamycin antibiotics were found to prevent recurrent CDI in a mouse model (Srikhanta *et al.*, 2019). While this suggests an antibiotic therapy that specifically targets recurrent CDI, it is not applicable to the prevention of primary CDI and could cause prolonged dysbiosis of the gut microbiota and exacerbate symptoms as cephmycins are known as risk factors of CDI (Wilcox, 2020).

The primary bile salt, chenodeoxycholate and analogues of chenodeoxycholate competitively inhibit *C. difficile* germination in the presence of potent germinant taurocholate (Sorg and Sonenshein, 2010; 2009). Therapeutic use of chenodeoxycholate is not possible as it is dehydroxylated at 7α-position by the colonic flora to lithocholate which is insoluble and has been linked to colorectal carcinogenesis (Sorg and Sonenshein, 2010; Nagengast *et al.*, 1995).

Chenodeoxycholate analogues may also be vulnerable to the active and passive reabsorption of bile salts for recycling by the liver (Ridlon *et al.*, 2006).

A synthesized cholate analogue, the cholic amide meta-sulfonic derivative (CamSA) has been shown to provide protection from CDI in a mouse model challenged with *C. difficile* spores. The authors demonstrated a single dose 50 mg/kg of CamSA given at the time of infection completely protected animals from CDI. A sub-optimal dose of CamSA or administration 24 hours prior to infection with *C. difficile* spores delayed the onset of CDI along with less severe symptoms (Howerton *et al.*, 2013). CamSA alone was insufficient in preventing CDI in the hamster model but instead delayed the onset of disease. When administered alongside low doses of vancomycin the survival rate of animals was improved (Howerton *et al.*, 2018). Building on these findings, a more potent cholic acid amide inhibitor of *C. difficile* germination was developed in part to address the variation in germination inhibitory activity of CamSA in different strains *of C. difficile*. *In vivo* activity of this inhibitor has not yet been reported (Sharma *et al.*, 2018).

Stoltz *et al.* (2017) synthesised a series of chenodeoxycholic acid and ursodeoxycholic acid derivatives with substitutions at the C3, C7 and C24 positions. While several bile acid analogues were identified as inhibitors of taurocholate-induced germination of *C. difficile* spores, candidate 21b which featured an additional sulphate group at the C7-position to overcome enterohepatic re-uptake, was found to be both a potent inhibitor of *C. difficile* germination and poorly permeable in a model of intestinal epithelial absorption, indicated by its remains within the gut lumen (Stoltz *et al.*, 2017).

1.17.3 Vaccines.

Vaccination that target the toxins of *C. difficile* could be an effective strategy for the prevention of CDI and several vaccine candidates have been explored or are currently undergoing clinical trials. A toxoid vaccine, known as Cdiffense, developed Sanofi Pasteur was undergoing a phase 3 clinical trial until it was terminated in 2017. Interim analysis determined it was unlikely to meet its primary objective as it did not effectively prevent CDI in participants (ClinicalTrials.gov, 2013; de Bruyn *et al.*, 2021). A vaccine candidate of recombinant fusion protein of TcdA and TcdB C-terminal domains (VLA84 or IC84) developed by Valneva completed phase 2 trials but has not progressed to phase 3 (ClinicalTrials.gov, 2014).

A genetically modified and formalin-inactivated toxoid vaccine developed by Pfizer was shown to be safe, well tolerated, and immunogenic in healthy adults aged 65–85 years in a phase 3 trial (ClinicalTrials.gov, 2015; Kitchin *et al.*, 2020). This vaccine candidate is also being assessed in a global clinical trial named the Clover trial which has recruited >1700 participants across 23 countries. It aims to determine if the vaccine is effective at preventing CDI in patients aged 50 years and over who are at risk (ClinicalTrials.gov, 2017).

DNA-based vaccines targeting the toxins have also been developed, including a plasmid-based vaccine (pTAB) which encodes toxin A and B receptor binding domains of *C. difficile* which was found to reduced infection severity in hamster and murine CDI models (Fehér *et al.*, 2017; Zhang *et al.*, 2016).

While antitoxin vaccines, target and neutralise toxins in the host they do not prevent colonisation or inhibit the growth of *C. difficile* (Zhu *et al.*, 2018). Vaccines that targeting *C. difficile* surface antigens have been investigated to address this problem (Zhu *et al.*, 2018; Fehér *et al.*, 2017). Targets include the surface layer proteins, Cwp84, flagellum protein (FliD) and surface polysaccharide but data is currently limited to animal models and has not progressed to human subjects (Fehér *et al.*, 2017).

1.18 Strategies to prevent CDI.

1.18.1 Interventions to minimise the incidence and transmission of CDI.

As part of a multi-component care bundle to prevent HAIs, specific policies and procedures were implemented to address the risk posed by *C. difficile*. The purpose was to improve and measure the implementation of key elements of care and reduce risk of CDI by ensuring they are performed every time and for every patient. Five main factors were identified as being necessary to reduce the incidence of CDI: prudent antibiotic prescribing, hand hygiene, environmental decontamination, isolation of CDI patients and personal protective equipment (Department of Health, 2007a). To support compliance, healthcare workers should be well educated of the infection control measures and have an understanding of *C. difficile* contamination of the environment, transmission and disinfection strategies (Vonberg *et al.*, 2008).

All NHS Trusts in England are required to participate in the Department of Health's mandatory CDI reporting system and to report all cases of *C. difficile* toxin positive diarrhoea in patients over 2 years of age (Department of Health and Health Protection Agency, 2008). This requires the testing of samples for all patients aged 65 years and above and for those aged less than 65 years if this is clinically indicated. Diagnostic laboratories are also required to provide information that identifies if specimens are hospital-associated or community-associated (Department of Health and Health Protection Agency, 2008). CDI surveillance is essential to detect increases in incidence, disease severity and identify risk-factors for CDI acquisition and opportunities for intervention (Vonberg *et al.*, 2008)

1.18.1.1 Antibiotic prescribing.

Appropriate prescribing of antibiotics should minimize the frequency and duration of high-risk antibiotic therapy and the number of antibiotic agents prescribed, to reduce CDI risk (McDonald *et al.*, 2018). This encompasses the wider goal of antibiotic stewardship to optimize clinical outcomes while minimizing unintended consequences of antimicrobial use, including toxicity, the selection of pathogenic organisms (such as *C. difficile*), and the emergence of resistance (Dellit *et al.*, 2007). Restrictive antibiotic guidelines for NHS trusts recommend the use narrow-spectrum agents for empirical treatment where appropriate, avoidance of the use of clindamycin and second and third generation cephalosporins, especially in the elderly and to minimise the use of fluoroquinolones, carbapenems and prolonged courses of aminopenicillins (Department of Health and Health Protection Agency, 2008). Antibiotic stewardship programmes ensuring the selection of the most appropriate antibiotic, duration, dose and method of administration for patients have been demonstrated to reduce the incidence of CDI in hospital inpatients (Goff, 2011; Baur *et al.*, 2017)

1.18.1.2 Isolation of patients with CDI.

To prevent transmission and limit *C. difficile* contamination within the healthcare environment, it is strongly recommended that patients with suspected potentially infectious diarrhoea be moved immediately into a single room (Department of Health and Health Protection Agency, 2008). Cohort care for CDI patients should be used if single rooms are not available (Department of Health, 2007a). Screening of patients to identify asymptomatic carriers has been implemented during outbreaks but was not shown to reduce the rate of CDI (Vonberg *et al.*, 2008).

1.18.1.3 Hand hygiene and personal protective equipment.

Patient to patient transfer of *C. difficile* via healthcare workers hands should be considered an important route of transmission of CDI (Jullian-Desayes *et al.*, 2017). Increased levels of *C. difficile* environmental contamination have been correlated with an increase in the isolation of *C. difficile* from the hands of healthcare workers (Samore *et al.*, 1996). All healthcare workers should wash their hands with soap and water before and after contact with patients with suspected or proven CDI. They must use disposable gloves and aprons for any physical contact with such patients, and with the patient's immediate environment and body fluids (Department of Health and Health Protection Agency, 2008). Common antiseptic handwash or antiseptic hand rub preparations are not reliably sporicidal against *Clostridium* species but the mechanical action while washing hands with soap and water aids the physical removal of spores from the surface of contaminated hands (World Health Organisation, 2009). Alcohol-based hand sanitizers should not be used as an alternative to handwashing with soap as they do not reduce the number of viable *C. difficile* spores (Department of Health and Health Protection Agency, 2008; Jabbar *et al.*, 2010). All staff or visitors entering an isolation-room should use disposable gloves and aprons for all contact with the patient and the patient's environment (Department of Health and Health Protection Agency, 2008). Contaminated gloves and aprons should then be removed prior to touching any other surface in order to prevent the contamination and transmission of *C. difficile* (Manian *et al.*, 1996). Along with staff, visitors should wash their hands with soap and water before and after CDI patient contact (Department of Health and Health Protection Agency, 2008).

1.18.1.4 Environmental decontamination.

Clinical environments are contaminated with *C. difficile* spores shed by symptomatic CDI patients and asymptomatic carrier; cleaning is therefore essential to minimise the reservoir of these spores (Samore *et al.*, 1996; Riggs *et al.*, 2007; Sethi *et al.*, 2010). The contamination of patient room

surfaces, and equipment plays an important role in the transmission of *C. difficile* between patients (Weber *et al.*, 2013). Studies investigating *C. difficile* contamination in clinical settings have recovered *C. difficile* from toilets and commodes, sinks, beds and bedrails, call buttons, light switches and floors (McFarland *et al.*, 1989; Verity *et al.*, 2001). Fawley *et al*. (2005) demonstrated a significant correlation between the prevalence of environmental *C. difficile* contamination associated with patients and healthcare workers and CDI incidence rates (Fawley *et al.*, 2005).

Environmental decontamination to prevent CDI includes the implementation of enhanced cleaning in areas with CDI patients. The Department of Health recommends the use of chlorine-based disinfectants or other sporicidal products to reduce environmental contamination with *C. difficile* spores as per local policy (Department of Health, 2007a). Environmental cleaning of rooms or bed spaces of *C. difficile* patients should be carried out at least daily using chlorine-based cleaning agents (at least 1,000 ppm available chlorine), clinical areas should be regularly assessed for cleanliness (Department of Health and Health Protection Agency, 2008).

Terminal cleaning, the deep cleaning of mattresses, bed space, bay or ward area after the discharge, transfer or death of a patient with CDI should be thorough (Department of Health, 2007a; Department of Health and Health Protection Agency, 2008). All areas should be cleaned using chlorine-containing cleaning agents (at least 1,000 ppm available chlorine) or vaporised hydrogen peroxide (Department of Health and Health Protection Agency, 2008). Routine environmental screening for *C. difficile* is not recommended but can be used to evaluate cleaning standards (Department of Health and Health Protection Agency, 2008; Vonberg *et al.*, 2008).

1.18.2 Disinfectants and biocides.

Contamination of the clinical environment by *C. difficile* spores is an important route of transmission for CDI, the use of sporicidal disinfectants and biocides to eliminate this contamination is widely accepted as a control measure to prevent hospital-acquired CDI (Wilcox *et al.*, 2003; Wullt *et al.*, 2003). Hospital cleaning agents and biocides are highly effective against the vegetative cells of *C. difficile* but their efficacy against the spores is often inadequate due to the chemical resistant properties of spores which allows them to withstand common decontamination methods (Fawley *et al.*, 2007; Edwards *et al.*, 2016). Clinical environments are frequently soiled with organic material that inhibits the sporicidal activity of disinfectants and biocides (Vohra and Poxton, 2011). Detergent based cleaning is necessary to remove this organic debris, such as that excreted alongside *C. difficile* spores in faeces, but this does not eliminate spores in the environment (Wilcox *et al.*, 2003).

Effective sporicidal products are needed to eliminate *C. difficile* contamination that persist in clinical settings as commercially available biocides for use in hospitals to target bacterial spores demonstrate inadequate decontamination in laboratory test conditions (Speight *et al.*, 2011). The laboratory testing methodology used to evaluate the activity of sporicidal agents against *C. difficile* varies amongst studies and often recommends exposure times of disinfectants and biocides far beyond what can reasonably be achieved in a hospital setting (Wilcox *et al.*, 2003; McDonald *et al.*, 2018). Clinical and laboratory strains of *C. difficile* have also been reported to demonstrate considerable variation in susceptibility to sporicidal disinfectants and biocides (Edwards *et al.*, 2016; Kenters *et al.*, 2017). Of concern, enhanced sporulation of *C. difficile* has also been demonstrated in the presence of subinhibitory concentrations and even at the manufacturer's recommended concentrations of hospital cleaning agents (Wilcox and Fawley, 2000; Fawley *et al.*, 2007).

In clinical settings the efficacy of disinfectants and biocides to eliminate *C. difficile* spores is difficult to determine as cleaning practices and frequency differ and sporicidal agents are used alongside

multicomponent infection control measures or as intervention during outbreak settings (McDonald *et al.*, 2018; Schoyer and Hall, 2020).

1.18.2.1 Chlorine-based agents.

Chlorine-releasing disinfectants and biocides including sodium hypochlorite (NaOCl) and sodium dichloroisocyanurate (NaDCC) are the most the effective agents to eliminate *C. difficile spores* and are therefore recommended to reduce environmental contamination (MacLeod-Glover and Sadowski, 2010; Lawley *et al.*, 2010; McDonald *et al.*, 2018; Department of Health and Health Protection Agency, 2008). In a comparison of the efficacy of hospital cleaning agents used at recommended working concentrations, only chlorine-based agents were able to inactivate *C. difficile* spores (Fawley *et al.*, 2007). The concentration of available chlorine, presence of organic debris and exposure time of *C. difficile* spores are important factors that influence the efficacy of chlorine-releasing disinfectants againg *C. difficile* resulting in considerable variation of reported results (Perez *et al.*, 2005; Wheeldon *et al.*, 2008b; Ungurs *et al.*, 2011). The cleaning of surfaces with detergents prior to the use of chlorine-releasing disinfectants is demonstrated to enhance efficacy against *C. difficile* spores compared to the use of a chlorine-releasing disinfectant alone (Ungurs *et al.*, 2011). While hypochlorite and sodium dichloroisocyanurate are biocidal, they are not effective at cleaning surfaces. Formulations that combine chlorine-releasing agents and detergent can overcome this problem (Wilcox *et al.*, 2003)

Perez *et al.* (2005) utilised the quantitative carrier test to determine sporicidal activity of several chlorine releasing products at a range of concentrations. All formulations were able to eliminate *C. difficile* spores to below detectable levels within 30 minutes of exposure, in the absence of any organic debris. Moreover, the majority of chlorine-releasing disinfectants achieved the 6-log reduction of *C. difficile* spores within 20 minutes, however variation in the sensitivity of *C. difficile* was associated with the culture media used to prepare the inoculum for the experiment (Perez *et*

al., 2005). Disparity in the susceptibility to chlorine-releasing disinfectants was also demonstrated across clinical isolates of *C. difficile* by Joshi *et al*. (2017). A 10 minute exposure to a dichloroisocyanurate-based disinfectant at half the recommended concentration (500 ppm) produced the variable reduction of spore viability of between 4 to 6-log of *C. difficile* strains of different ribotypes (Joshi *et al.*, 2017).

Speight *et al.* (2011) examined the *C. difficile* sporicidal activity of 23 commercially available chlorine-releasing disinfectants for use in clinical settings using the standard suspension test. The manufacturer's recommended concentrations were tested at short (1 minute) and long (60 minutes) contact times and under clean and dirty conditions. Only eight of these chlorine-releasing disinfectants achieved the required 3-log reduction of *C. difficile* viability, specified as sporicidal efficacy by the modified suspension test, in 1 minute under dirty conditions. A further 5 chlorine-releasing disinfectants achieved the required 3-log reduction of *C. difficile* in 1 minute under clean conditions only or within 60 minutes in either clean or dirty conditions (Speight *et al.*, 2011).

In an early study during an outbreak, Kaatz *et al.* (1988) demonstrated the introduction of hypochlorite-based disinfection (500 ppm available chlorine) reduced *C. difficile* environmental contamination to 21% of samples compared to the prior rate of 31% of samples. The use of hypochlorite at a higher concentration was even more effective, reducing *C. difficile* environmental contamination by 98% (Kaatz *et al.*, 1988). Substituting a quaternary ammonium disinfectant to a hypochlorite solution was found to significantly decrease CDI rates from 8.6 cases to 3.3 cases per 1000 patient-days. Following the intervention, when the disinfection regime was switched back to the use of quaternary ammonium disinfectant, CDI incidence increased to 8.1 cases per 1000 patient-days, suggesting the reduction of *C. difficile* environmental contamination as a result of hypochlorite disinfection decreased nosocomial transmission of CDI (Mayfield *et al.*, 2000). A hospital-based study examined the efficacy of cleaning with a chlorine-releasing disinfectant or detergent in separate wards in reducing environmental *C. difficile* contamination over 2 years. Over

the duration of the study the level of *C. difficile* environmental contamination detected was similar on both wards but there was a significant reduction in the incidence of CDI rates from 8.9 to 5.3 cases per 100 admissions (Wilcox *et al.*, 2003).

There are several disadvantages to the use chlorine-based products in a clinical setting, including the risk of respiratory exposure to healthcare workers completing cleaning regimes, corrosion of metal surfaces and equipment and an undesirable odour (Wilcox and Fawley, 2000; Speight *et al.*, 2011). While there are some effective alternatives, worryingly sub-inhibitory concentration of non-chlorine based disinfectants have been found to enhance *C. difficile* sporulation (Wilcox and Fawley, 2000).

1.18.2.2 Hydrogen peroxide.

Hydrogen peroxide is an oxidising agent, producing hydroxyl radicals which react with biomolecules causing the damage of DNA, proteins and the disruption of cell membranes, which upon biodegradation converts to non-toxic oxygen and water (Linley *et al.*, 2012). Hydrogen peroxide is sporicidal, rapidly eliminating *C. difficile* spores on contact. A 1 minute exposure of 1% hydrogen peroxide resulted in the immediate inactivation of 75% of the spores while 10% hydrogen peroxide caused the inactivation of >99% of the spores in the same time period (Lawley *et al.*, 2010). In an evaluation of hard surface disinfectants measured via the quantitative carrier test, the hydrogen peroxide (7%) releasing biocide, Virox STF achieved a 6-log reduction of *C. difficile* spores within 10 minutes (Perez *et al.*, 2005).

Automated room decontamination using hydrogen peroxide vapour (HPV) offers a no-touch disinfection method that has been shown to significantly reduce CDI rates in hospital setting (Manian *et al.*, 2013; Boyce *et al.*, 2008). In a study investigating the terminal disinfection of CDI patient rooms, the delivery of 35% HPV, forming microscopic condensation of hydrogen peroxide on clinical surfaces, replaced the normal use of a chlorine-based disinfectant resulting in a 60%

reduction in CDI rates. During the study period terminal disinfection was implemented by cleaning with a non-sporicidal disinfectant followed by decontamination with HPV. The CDI rate was demonstrated to decrease from 1.0 case per 1000 patient-days before HPV usage, to 0.4 cases per 1000 patient-days in the first 24 months of HPV usage, suggesting the elimination of *C. difficile* spores from the environment reduced exposure to vulnerable patients (McCord *et al.*, 2016). The use of hydrogen peroxide dry-mist disinfection was found to be significantly more effective than 0.5% sodium hypochlorite solution at eradicating *C. difficile* spores in a study conducted in French hospitals to evaluate terminal disinfection of *C. difficile* in patient rooms. After decontamination with hydrogen peroxide dry-mist only, 2% of environmental samples from patient rooms were positive for *C. difficile* compared to 12% in hypochlorite-treated rooms (Barbut *et al.*, 2009). Following the increase in CDI rates at a 500-bed hospital in the USA, multicomponent control measures were implemented alongside the introduction of HPV decontamination to decrease environmental contamination of *C. difficile*. HPV decontamination resulted in a reduction of *C. difficile* contamination to below a detectable level of the sites sampled. The implementation of HPV along with control measures resulted in a reduction in CDI incidence to 1.28 cases per 1,000 patient-days from 2.28 cases per 1,000 patient-days prior to intervention (Boyce *et al.*, 2008). Detergent-based cleaning of surfaces is still required prior to HPV decontamination. Rooms must also be sealed during the decontamination process which typically takes 3-4 hours (Boyce *et al.*, 2008; Manian *et al.*, 2013). Hydrogen peroxide levels must return to 1 ppm before it is safe for healthcare staff to enter (Boyce *et al.*, 2008).

1.18.2.3 Other sporicidal disinfectants.

While several chemicals have sporistatic activity only a limited number are sporicidal and suitable for inclusion in disinfectant or biocide formulations for use in clinical settings (Russell, 1990). Wullt *et al*. (2003) investigated the sporicidal activity of glutaraldehyde, peracetic acid, acidified nitrite

and isopropanol against *C. difficile*. Peracetic acid (1.6%) demonstrated rapid sporicidal activity achieving a 4-log reduction within 5 minutes exposure. The same reduction in viability of *C. difficile* required 30 minutes exposure to glutaraldehyde (2%) and acidified nitrite. 70% isopropanol displayed no measurable sporicidal activity after 30 minutes (Wullt *et al.*, 2003). The sporicidal activity of peracetic acid has also been demonstrated in the laboratory, achieving a >3 log reduction of *C. difficile* spores in 10 to 30 minutes of exposure, depending upon the reporting study (Block, 2004; Wheeldon *et al.*, 2008b). In a hospital setting the introduction of peracetic acid-based disinfectant for daily and terminal decontamination was associated with an increase in the incidence of CDI case. Subsequent evaluation of the peracetic acid-based disinfectant determined the concentration of peracetic acid was significantly below expected levels which did not have sufficient sporicidal activity against *C. difficile* to adequately eliminate environmental contamination (Cadnum *et al.*, 2017).

Glutaraldehyde-based disinfectants are routinely used in the sterilisation of endoscopes. Their sporicidal activity against *C. difficile* has been demonstrated via standardised testing but efficacy is dependent upon the specific disinfectant formulation, with concentration below 2% glutaraldehyde inhibiting the sporicidal decontamination activity (Cowan *et al.*, 1993; Rutala *et al.*, 1993). Glutaraldehyde-based disinfectants are irritants and sensitisers, requiring precautions for their routine use in hospital settings (Cowan *et al.*, 1993).

1.18.3 Ultraviolet-light decontamination.

Germicidal UV-light for disinfection and decontamination involves the emission of shortwave (100-280 nm) UV radiation to inactivate microorganisms by targeting DNA, causing the formation of pyrimidine dimers which inhibits DNA replication (Reed, 2010; Rastogi *et al.*, 2010). Germicidal UV-light technology has been demonstrated as a promising decontamination method for the eliminated of *C. difficile* spores on surfaces in a hospital environment (Boyce *et al.*, 2011; Nerandzic

et al., 2010). Boyce *et al.* (2011) utilised a modified version of the quantitative carrier test to evaluate the efficacy of mobile UV-light technology for the decontamination of *C. difficile*. On average a 2-log reduction of spores inoculated onto metal discs and placed in various position around the room was achieved, including those out of the line of exposure of the device. Similarly, Nerandzic *et al.* (2010) who inoculated *C. difficile* spores directly on the surface of laboratory work benches achieved a 2-3-log reduction in the recovery of *C. difficile* amongst the multiple laboratory strains tested, following the use of a UV-light device. When the same mobile UV-light device was utilised in patient rooms that previously housed CDI patients and had not been terminally cleaned, of the 9 sites that were previously positive for *C. difficile* only one remained positive after germicidal UV-light decontamination (Nerandzic *et al.*, 2010). UV-light decontamination is a no-touch disinfectant technology intended to enhance manual environmental cleaning by decontaminating surfaces inadequately cleaned by manual practices, particularly terminal decontamination of patient rooms. UV-light devices require placement in multiple positions to achieve the delivery of UV-light to surfaces throughout the room (Miller *et al.*, 2015). The efficacy of germicidal UV-light decontamination is inhibited by the presence of debris and applicable to only hard surfaces in clinical setting, indicating manual cleaning regimes are necessary for optimal results (Nerandzic *et al.*, 2010). UV-light devices are remotely operated and cannot be used when rooms are occupied or during rapid turnover of patient rooms (Nerandzic *et al.*, 2010; Levin *et al.*, 2013). The decontamination process time to prepare and deploy the UV-light devices per patient room varies, approximately 45 minutes is required to complete UV-light decontamination that specifically targets the elimination of bacterial spores, but is not reported to add significantly more time to cleaning regimes (Levin *et al.*, 2013; Nerandzic *et al.*, 2010; Pegues *et al.*, 2017).

In a hospital setting, Miller *et al.* (2015) demonstrated a 56.9% reduction in CDI rates in the period where the UV-light disinfection was introduced, but this was in combination with multidisciplinary team intervention to address the incidence of *C. difficile* and so the reduction in CDI rate cannot be attributed to the efficacy of UV-light for decontamination exclusively. In a multicentre randomised

assessment of enhanced terminal room disinfection strategies the utilisation of UV-light terminal decontamination did not significantly change the incidence of CDI, but it should be noted that the comparator was the cleaning of rooms with bleach based disinfectant and rooms decontaminated with UV-light were also cleaned with bleach based disinfectant prior to the UV-light treatment (Anderson *et al.*, 2017). Levin *et al.* (2013) also used UV-light as part of terminal disinfection as an adjunct to decontamination with a chlorine-based disinfectant. CDI attributed deaths and colectomies were decreased, and the rate of CDI was reduced by 53% during the study period where UV-light was used part of terminal disinfection, compared to the previous year where chlorine-based disinfectant was also used (Levin *et al.*, 2013).

1.18.4 Pro spore-germination elimination strategy.

A strategy to eliminate *C. difficile* spores from the environment termed 'germinate to exterminate' involves the exposure of *C. difficile* spores to a germinant, triggering the irreversible process of germination and loss of the spore's resistant properties (Wheeldon, 2008; Wheeldon *et al.*, 2010; Setlow, 2003). Exploiting the germination process to produce bacterial cells that are sensitive to traditional elimination methods was first suggested by Stuy (1956). Wheeldon *et al.* (2008a) utilised a germinant solution of 1% sodium taurocholate in thioglycolate medium in combination with the antimicrobial properties of copper to enhance the killing of *C. difficile* spores. Similarly, Nerandzic and Donskey (2010) demonstrated that *C. difficile* spores exposed to a germinant solution were eliminated by UV-C radiation or heat. Furthermore this study was completed on the surfaces within a hospital environment, demonstrating the potential application in clinical settings (Nerandzic and Donskey, 2010). Later, the same authors combined the germinant solution with a disinfectant containing a quaternary ammonium compound (Nerandzic and Donskey, 2016). Sensitising *C. difficile* spores with the dual germinant and biocide solution significantly reduced the number of spores recovered from hospital surfaces (Nerandzic and Donskey, 2016). This strategy has also been

applied to eliminate spores on the surface of skin. An *ex vivo* porcine skin model was utilised to demonstrate the enhanced elimination of *C. difficile* spores by exposure to germinants and co-germinants in an ethanol solution, comparable to that of an alcohol-based hand sanitizer (Nerandzic and Donskey, 2017). These data suggest that activation of germination of spores and elimination of the sensitive outgrowing vegetative cells could provide an effective strategy to reduce the contamination of *C. difficile* spores on the surfaces of clinical environments.

1.18.5 Antimicrobial surfaces.

An alternative to disinfectants in clinical environments is the use of antimicrobial smart surfaces or antimicrobial polymeric materials that have the ability to inhibit or kill microorganisms (Kamaruzzaman *et al.*, 2019). Antimicrobial polymeric materials can incorporate a wide variety of antimicrobial agents including biocides and antimicrobial peptides which are typically covalently bonded to a polymer matrix, producing reusable, chemically stable and long-term antimicrobial activity (Kamaruzzaman *et al.*, 2019; Xue *et al.*, 2015). This targeted approach has been demonstrated to be effective against a large number of clinically relevant microorganism (Muñoz-Bonilla and Fernández-García, 2012; Kamaruzzaman *et al.*, 2019). Antimicrobial polymers with activity against *C. difficile* have been developed that incorporate molecules that mimic host defence peptides. These polymers demonstrated inhibitory action against *C. difficile* vegetative cells and successfully prevented spore outgrowth but did not eliminate the spores themselves or their ability to germinate and so do not offer a solution to the transmission of CDI in hospital environments (Liu *et al.*, 2014).

1.19 Aims and objectives.

A greater understanding of the environmental signals that initiate *C. difficile* germination is of importance to prevent CDI in vulnerable patients. The aim of the research presented in this thesis was to study the germinants and co-germinants of *C. difficile* spores to understand the mechanism of germination regulation and provide insight for the development of disinfectant strategies that exploit the inappropriate triggering of germination to eliminate *C. difficile* spores that contaminate the clinical environment. The aims of this research were achieved by completing the following objectives:

- To utilise an establish methodology to study the initiation of *C. difficile* spore germination.

- To determine the germination activity of bile salts to initiate the germination *C. difficile* spores and investigate the environmental factors that influence spore germination.

- To determine the co-germinant activity of amino acids and calcium to initiate *C. difficile* spore germination.

- To investigate the germination response of *C. difficile* spores exposed to glycine or calcium in combination or with other amino acid co-germinants.

- To study the morphology and structure of germinating *C. difficile* spores utilising scanning and transmission electron microscopy and cryogenic soft X-ray tomography.

- To investigate the germination and antimicrobial activity of lithocholate derived novel compound C109.

- To evaluate the germination and antimicrobial activity of polymeric biomaterial formulations incorporating bile salt-derived novel compounds to target C. *difficile.*

2.0 Measurement of *Clostridioides difficile* spore germination.

2.1 Introduction.

The germination of bacterial spores is initiated by the presence of germinants which activate germination receptors and a signal cascade leading to the physiological transformation of the dormant spore into a metabolically active vegetative cell (Setlow, 2003). In *C. difficile* the germination signal cascade is transduced by Csps proteins and the conversion of the cortex lytic enzyme SleC to its active form, causing the degradation of specialised peptidoglycan of the cortex (Adams *et al.*, 2013; Francis and Sorg, 2016; Burns *et al.*, 2010). The removal of the osmotic constraints imposed by the cortex is detected by SpoVAC a mechanosensing protein, triggering the release of DPA and Ca^{2+} from the spore core and its rehydration (Francis and Sorg, 2016; Francis *et al.*, 2015; Kochan *et al.*, 2018b). The sequence of events during *C. difficile* spore germination contrasts to that of the well-studied spore former *B. subtilis* where DPA release and partial rehydration of the spore core occurs before the degradation of the cortex peptidoglycan (Vepachedu and Setlow, 2007; Setlow, 2003). The release of H^+, monovalent cations, degradation of the SASPs and full rehydration of the *C. difficile* spore core facilitates enzymatic and metabolic activity and expansion of the germ cell wall, completing the transition to an outgrowing vegetative cell (Setlow, 2003).

During the past 2 decades, a greater understanding of the germinants and co-germinants responsible for initiating germination of *C. difficile* has been established. In 2008, Sorg and Sonenshein demonstrated *C. difficile* germination is triggered by bile salt germinants and the amino acid co-germinant glycine. The primary bile salt taurocholate was identified as the most effective germinant, with cholate, along with the conjugated bile salt glycocholate, also able to induce the

germination of *C. difficile* spores. Importantly, bile salt-induced germination was found to only occur in complex media or in the presence of the co-germinant glycine (Sorg and Sonenshein, 2008). The co-germinant activity of more amino acids was identified but their ability to enhance germination varies. Glycine has the greatest co-germination activity, with L-alanine, taurine and L-glutamine demonstrating less activity (Sorg and Sonenshein, 2008; Kochan *et al.*, 2018b; Wheeldon *et al.*, 2011). Most recently Ca^{2+} was identified as a *C. difficile* co-germinant and was found to enhance the gemination activity of taurocholate and low concentrations of amino acid co-germinants (Kochan *et al.*, 2018b).

While specific bile salts act as *C. difficile* germinants, others are known to inhibit germination in a competitive manner. The secondary bile salt chenodeoxycholate and bile acid lithocholate inhibit taurocholate mediated germination (Sorg and Sonenshein, 2009; 2010). In the host, the balance of bile salts in the colon is modified by specific microbiota species which 7α-dehyroxylate primary bile salts to produce secondary bile acids, an environment that prohibits germination of *C. difficile* spores. When the intestinal microbiota is disrupted, the production of secondary bile acids is reduced, giving rise to an environment with an increased concentration of *C. difficile* germinants (Ridlon *et al.*, 2006; Theriot *et al.*, 2016; Sorg and Sonenshein, 2008).

In addition to the balance of germinants and inhibitors of germination, environmental factors regulate germination. *C. difficile* spores germinate optimally at 37°C but germination also occurs at lower temperatures (Wheeldon *et al.*, 2008a). An environmental pH of ~6.5-7.5 is required for optimum germination while acidic and basic conditions reduce the rate or inhibit germination of *C. difficile* spores (Wheeldon *et al.*, 2008a; Paredes-Sabja *et al.*, 2008; Kochan *et al.*, 2018b). However, the activation of germination does not require an anaerobic atmosphere or nutrient-rich media necessary for spore outgrowth (Wheeldon *et al.*, 2008a; Sorg and Sonenshein, 2008). The interaction of spore germinants with their receptors and the specific environmental conditions discussed, ensure *C. difficile* spores germinate only in the host (Gil *et al.*, 2017).

The transformational changes which results in the end of spore dormancy also cause the loss of the spore's resistance properties (Setlow, 2003). DNA inside the core is no longer protected by the reduced water content previously derived through the high percentage of Ca-DPA and the binding of DNA to SASPs which contribute to the resistance to desiccation, heat and UV radiation (Setlow, 2014; Setlow, 2006). Protection from DNA damaging compounds is no longer provided by the restrictive permeability barriers of the outer membranes and the loss of the specialised cortex peptidoglycan reduces the resistance to heat and ethanol (Nicholson *et al.*, 2000; Popham, 2002). Protection against enzymatic damage, chemicals and biocides is decreased as the vegetative cell emerges from the proteinaceous spore coat (Setlow, 2014).

The initial stages of germination, where the resistant properties of the spore are lost but before restoration of full metabolic activity or outgrowth, occurs within a short time frame of just minutes (Francis and Sorg, 2016; Burns *et al.*, 2010; Kochan *et al.*, 2018b; Carr *et al.*, 2010; Wheeldon *et al.*, 2008a). This rapid change is a biological characteristic of germination and can be monitored or measured using a number of experimental techniques (Setlow, 2003; Hindle and Hall, 1999; Vary and Halvorson, 1965; Levinson and Hyatt, 1966). Occurring during the early stages of germination, the release of Ca-DPA and other monovalent cations from the *C. difficile* spore core is a property of the germinating spore that can be used to monitor germination (Francis *et al.*, 2015; Francis and Sorg, 2016; Burns *et al.*, 2010; Hindle and Hall, 1999). Spore Ca-DPA release can be quantified through the utilisation of terbium chloride, which forms a highly fluorescent complex with DPA. Germination can therefore be measured experimentally in a terbium DPA assay as an increase in fluorescence (Hindle and Hall, 1999). This methodology has been further developed as a colorimetric assay, with the addition of pyrocatechol violet, which also forms a complex with terbium and is displaced by DPA, resulting in a colour change from blue to yellow (Clear *et al.*, 2013).

An established technique to analyse the kinetics of germination is spectrophotometry, monitoring the optical density of purified spore suspensions in the visible light range (Powell, 1950; Vary and Halvorson, 1965; Cabrera-Martinez *et al.*, 2003). Germinating spores can be studied by monitoring the characteristic drop in optical density at 600 nm, representing the decrease in refractive index of spores as the spores swell and rehydrate during germination (Moir and Smith, 1990; Tehri *et al.*, 2018). This methodology was successfully utilised with purified *C. difficile* spores to characterise germination in response to bile salt germinants and amino acid co-germinants (Sorg and Sonenshein, 2008; Heeg *et al.*, 2012). The decrease in refractive index of germinating spores can also be studied via phase contrast microscopy, the change in refraction is observed as a transition from phase bright to phase dark spores (Tehri *et al.*, 2018).

The low water content of the spore's core provides protection from heat damage (Gerhardt and Marquis, 1989; Setlow, 2006; Setlow, 2007). The consequent rehydration of the spore during germination leads to the loss of resistance to heat, a property which can be used to assess germination (Levinson and Hyatt, 1966). Utilising this methodology, spore suspensions are exposed to control and germination test conditions before a high temperature heat-challenge and rapid cooling. Ungerminated and therefore heat resistant spores are then enumerated by inducing germination on media containing characterised germinants to determine survival (Levinson and Hyatt, 1966). This method is therefore reliant on subsequently germinating the spores that have remained ungerminated during the germination test conditions. Similarly, germination can be indirectly measured through the loss of resistance to toxic chemicals (Levinson and Hyatt, 1966). The hydrolysis of the spore cortex and the breakup of proteinaceous spore coat and exosporium during germination result in an increase in susceptibility to biocides and other chemicals, including ethanol (Popham, 2002; Barra-Carrasco *et al.*, 2013; Setlow, 2014). Loss of resistance to heat and ethanol of *C. difficile* spores was successfully employed by Wheeldon *et al.* (2008a) to study germination of *C. difficile* in response to bile salt germinants and at optimum pH conditions respectively.

The aim of the research presented in this chapter was to establish the methodology for analysis of *C. difficile* spore germination spectrophotometrically, through the use of the germination optical density assay. This permitted study of the initial germination response of *C. difficile* to bile salts and the examination of other environmental factors that may influence or regulate germination. Knowledge gained from these studies can be used to inform and build upon the pro-germination elimination strategy, 'germinate to exterminate' to reduce environmental contamination of *C. difficile* the source of transmission of CDI (Wheeldon *et al.*, 2010; Kaatz *et al.*, 1988; Otten *et al.*, 2010).

2.2 Methods.

2.2.1 Bacterial strains and growth conditions.

C. difficile NCTC 11204 (ribotype 001) was sourced from Jon Brazier (HPA Anaerobic Reference Laboratory, Cardiff). *C. difficile* reference ribotype strains 001, 002 and 015 were sourced from Andrew Sails (Newcastle HPA Laboratory, Newcastle upon Tyne). Frozen stocks of *C. difficile* were stored at -80˚C on Microbank™ beads (Pro Lab Diagnostics, Canada) and resuscitated by inoculating a bead onto Wilkins-Chalgren agar (Oxoid, Basingstoke, UK) supplemented with 0.1% (w/v) sodium taurocholate hydrate (Alfa Aesar, UK) and incubating anaerobically (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK) at 37°C for 48 hours.

2.2.2 Preparation of spore suspensions of *C. difficile*.

Spore suspensions of *C. difficile* were prepared using a modified version of the methodology described by Heeg *et al.* (2012). Frozen stocks of *C. difficile* NCTC 11204 and reference ribotype strains 001, 002 and 015 stored at -80°C were used to inoculate Wilkins-Chalgren agar (Oxoid, Basingstoke, UK) supplemented with 0.1% (w/v) sodium taurocholate and incubated anaerobically (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK) at 37°C for 48 hours.

Under anaerobic conditions, a single colony of the resuscitated *C. difficile* strain was used to inoculate 10 ml of pre-reduced brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) which was incubated anaerobically at 37°C for 4 hours. Aliquots of 100 µl of the *C. difficile* culture were inoculated and spread on the surface of BHI agar supplement with 0.1% (w/v) L-cysteine and 0.5% (w/v) yeast extract (BHIS). The inoculated BHIS agar plates were incubated anaerobically at 37°C for 5-7 days. The presence of a high concentration of spores was confirmed by staining with 5% (w/v) malachite green solution and counterstaining with 0.5% (v/v) safranin as per the Schaeffer-Fulton endospore staining technique (Schaeffer and Fulton, 1933). Spores were harvested from the

surface of the BHIS agar plates using a sterile cotton swab moistened with sterile distilled water (SDW) and suspended in SDW. The crude spore suspension was incubated at 4°C overnight.

The spore suspension was washed by resuspending the spores in ice-cold sterile phosphate buffered saline (PBS) and 0.1% (v/v) Tween 80 and centrifuging at 8800g for 10 minutes at 4°C. Each time the supernatant was discarded carefully ensuring the lower layer of the pellet was not disturbed. This was repeated 5 times. A further 5 washes were completed by resuspending the spores in ice-cold sterile PBS and 0.01% (v/v) Tween 80 and centrifuging at 8800g for 10 minutes at 4°C. A minimum of a final 5 washes were completed in 20 ml ice-cold SDW and centrifuging at 4700g for 15 minutes at 4°C. A pure spore suspension was confirmed by the Schaeffer-Fulton staining technique, characterised by an absence of vegetative cells and cell debris (Schaeffer and Fulton, 1933). Spore suspensions that were identified to contain residual vegetative cells or cell debris were treated with further washes with 20 ml ice-cold SDW and centrifuging at 4700g for 15 minutes at 4°C and re-examined using the Schaeffer-Fulton staining technique. Pure spore suspensions were checked for clumping by phase-contrast microscopy and to confirm the transition from phase bright to phase dark when initiated by a germinant.

2.2.3 Determination of germination of *C. difficile* spores by measurement of optical density.

The methodology of the measurement of germination by the optical density assay was based on that described by Sorg and Sonenshein (2008) and Heeg *et al.* (2012). The optical density at 570 nm (OD_{570}) of the purified *C. difficile* spore suspensions was determined using a spectrophotometer (Jenway, UK) and adjusted to an OD_{570} of 3.5 in preparation for the optical density assay. Aliquots of 20 μl of the spore suspension or SDW were loaded into the wells of a clear flat bottom 96-well plate followed by 180 μl of appropriate media, achieving a final OD_{570} of the spore suspension of 0.35. The OD_{570} was measured immediately (time zero) using the ELx808 Absorbance plate reader

(BioTek, USA) and every minute thereafter for 60 minutes during incubation at room temperature. A 3 second medium shake was included prior to each OD_{570} measurement to prevent the adhesion of spores to the wells of the 96-well plate. The relative ratios of the OD_{570} at the various time points to the OD_{570} at time zero were plotted against time. To investigate the germination activity of bile salts, BHIS broth was supplemented with the bile salts; sodium taurocholate, sodium glycocholate, sodium cholate and sodium chenodeoxycholate. To determine the influence of pH and the presence of butyrate (Alfa Aesar, Heysham, UK) on germination, the pH of the BHIS broth supplemented to achieve a final concentration of 6.9 mM sodium taurocholate was adjusted to pH 4, 5, 6, 7, 8 and pH 9 or was supplemented with butyrate to achieve a final concentration 5, 10, 15, 20, 25 and 30 mM. To achieve the required pH of BHIS broth, the media was adjusted with 1.0 M hydrochloric acid or 1.0 M sodium hydroxide.

2.2.4 Statistical analysis.

Spore germination optical density data presented consists of the mean of a minimum of 6 replicates and the standard deviation of these experiments. A one-way analysis of variance (ANOVA) was used to determine the germination activity of sodium taurocholate, the influence of pH and the presence of butyrate with *C. difficile* NCTC 11204. A two-way ANOVA was used to determine the germination activity of bile salts: sodium glycocholate, sodium cholate and sodium chenodeoxycholate and sodium taurocholate with *C. difficile* reference ribotypes 001, 002 and 015. The *post-hoc* Bonferroni's multiple comparisons test was used to statistically analyse the germination activity of various test conditions. Both statistical tests were completed using the GraphPad Prism software (GraphPad, USA).

2.3 Results.

2.3.1 Preparation of purified *C. difficile* spore suspensions.

In order to investigate the germination activity of bile salt and environmental factors that influence germination the optical density germination assay methodology was selected. The production of *C. difficile* spore suspension with the absence of vegetative cells and cell debris is therefore necessary for the measurement of *C. difficile* germination spectrophotometrically. To ensure the *C. difficile* spore suspensions were suitable for the germination optical density assay, the Schaeffer and Fulton
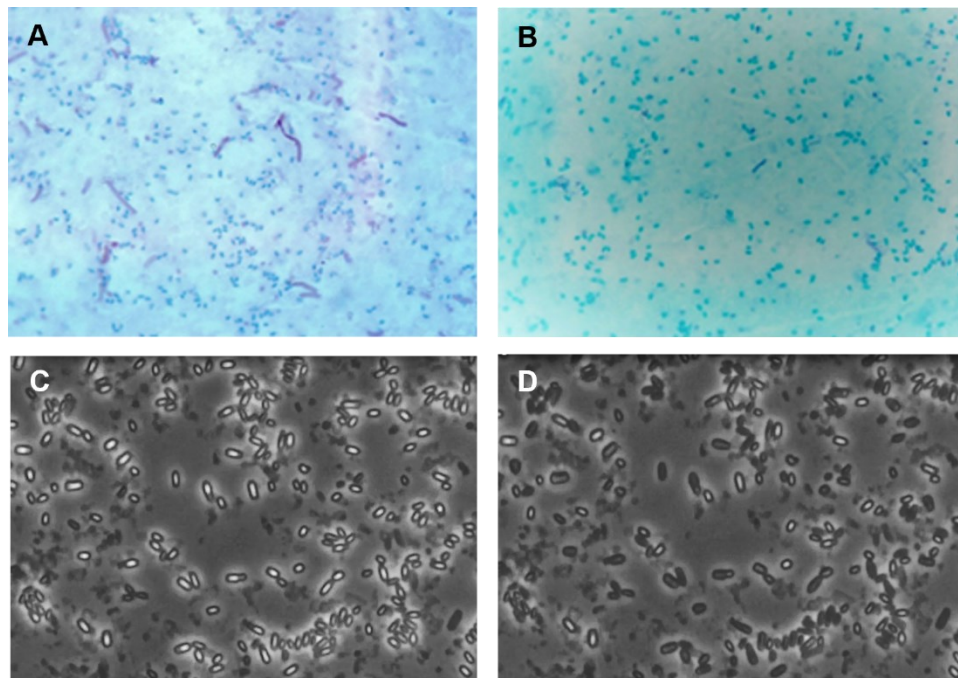
Figure 2.1 Visualisation of *C. difficile* spore suspensions. *C. difficile* strain NCTC 11204 spore suspension stained using the Schaeffer and Fulton (1933) endospore staining technique viewed under x100 objective prior to (A) and post purification (B). Spores were stained with 5% (w/v) Malachite green solution, vegetative cells are counterstained with 0.5% (v/v) Safranin solution. Phase contrast microscopy viewed under x100 objective of purified *C. difficile* spore suspension prior to (C) and 10 minutes post exposure to germinant taurocholate (D). Images are representative of routine endospore staining and phase contrast microscopy.

(1933) endospore staining technique was utilised to identify the presence of vegetative cells post the purification of spore suspensions. Only spore suspensions of ≥99% purity were accepted for use in the optical density assay (figure 2. 1B). Spore suspensions were also observed under phase contrast microscopy. *C. difficile* spores demonstrated the phase bright appearance and were observed to transition to phase dark upon exposure to characterised germinants and co-germinants (figure 2.1C and D).

2.3.2 Determination of *C. difficile* spore germination by measurement of optical density.

The germination activity of the bile salt taurocholate was investigated utilising the optical density germination assay. Exposure of *C. difficile* spores to taurocholate at a concentration of 6.9 mM and 0.1 % (w/v), equivalent to 1.86 mM, in BHIS resulted in an expected decrease in relative optical density at 570 nm over the time course of the assay, demonstrating spore germination. The results (figure 2.2) demonstrate an initial rapid decrease in relative OD_{570} response to both tested concentrations of taurocholate, during the first 5 minutes of the assay. The rate of the decrease in relative OD_{570} declines between 5 and 10 minutes, with a further gradual reduction over the remainder of the assay. Taurocholate at 6.9 mM caused a 32% decrease of relative OD_{570} from 1.0 at time point zero to 0.68 at 60 minutes. A decrease in relative OD_{570} from 1.0 to 0.72 at 60 minutes was also demonstrated by 0.1 % (w/v) taurocholate, a 28% decrease. The achieved decrease in relative OD_{570} after 60 minutes, caused by 6.9 mM and 0.1 % (w/v) taurocholate was determined to be statistically significant ($P < 0.001$).

The negative control, where *C. difficile* spores were exposed to BHIS only demonstrated a small reduction in relative OD_{570} over the time course of the assay from 1.0 to 0.95, a 5% reduction. This was not determined to be a statistically significant decrease. To ensure any observed decrease in optical density was the result of the change in refractive index of germination spores, controls of BHIS, BHIS with 6.9 mM and 0.1 % (w/v) taurocholate in the absence of spores were included in the
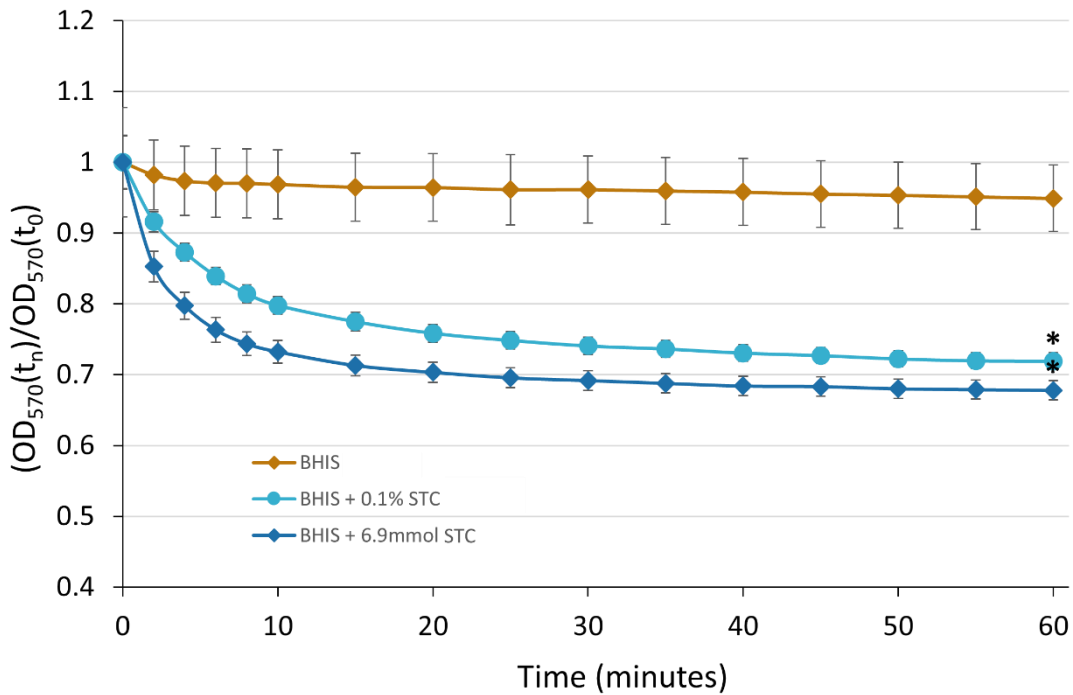
Figure 2.2 The activity of primary bile salt taurocholate to induce *C. difficile* spore germination measured via optical density. Purified *C. difficile* NCTC 11204 spores were mixed with BHIS broth only: orange diamonds, in BHIS with 0.1% (w/v) sodium taurocholate (STC): light blue circles, BHIS with 6.9 mM STC: dark blue diamonds. Error bars represent standard deviation. Statistically significant reductions of the relative optical density values at 60 minutes compared to time point zero are indicated by an asterisk (*P* < 0.0001).

optical density. No change in relative OD_{570} in the absence of spores was observed over +/- 2.2% (data not shown on the graph).

2.3.3 The effect of pH on the germination of *C. difficile* spores by measurement of optical density.

Utilising the optical density germination assay, the effect of pH on taurocholate-induced *C. difficile* germination was investigated. The exposure of *C. difficile* spores to taurocholate in BHIS at pH 5, 7, 8 and 9 failed to initiate germination, with minimal changes to the relative OD_{570} observed. These

were not found to be statistically significant (figure 2.3). Taurocholate-induced *C. difficile* germination occurred only in BHIS at pH 6 and in unmodified BHIS, with a pH of 6.6 as demonstrated by the decrease in relative OD_{570} during the time course of the assay. *C. difficile* germination in both BHIS at pH 6 and in unmodified BHIS test conditions was demonstrated by the characteristic initial rapid decrease in relative OD_{570} followed by a reduction in the rate of decrease. A steeper decline in relative OD_{570} during the initial 20 minutes of the assay, and therefore a faster rate of *C. difficile* germination is observed for the unmodified BHIS at pH 6.6 when compared to taurocholate-induced germination in BHIS at pH 6. At completion of the assay (60 minutes), both BHIS at pH 6 and unmodified BHIS test conditions demonstrated a statistically significant decrease in relative OD_{570} compared to the start of the assay ($P < 0.0001$), a reduction of 30% and 31% respectively. The final relative OD_{570} values for *C. difficile* spores germinated with BHIS at pH 6 and unmodified BHIS were comparable and not determined to be statistically significantly different from each other.

As previously, to ensure that any observed decrease in optical density was the result of the change in refractive index of germination spores, controls of BHIS with 6.9 mM at the various pH values but in the absence of *C. difficile* spores were included. Small changes in the relative OD_{570} germination during the time course of the assay were observed but these the were not over +/-4.4% (data not shown on the graph). *C. difficile* spores exposed to BHIS at the various pH values but in the absence of the germinant taurocholate, demonstrated observable changes in the relative OD_{570} germination during the time course of the assay, particularly BHIS at pH 7, 8 and 9. To investigate this further, spores were examined under phase contrast microscopy and were determined to be phase bright, indicating the spores remained ungerminated under these conditions.
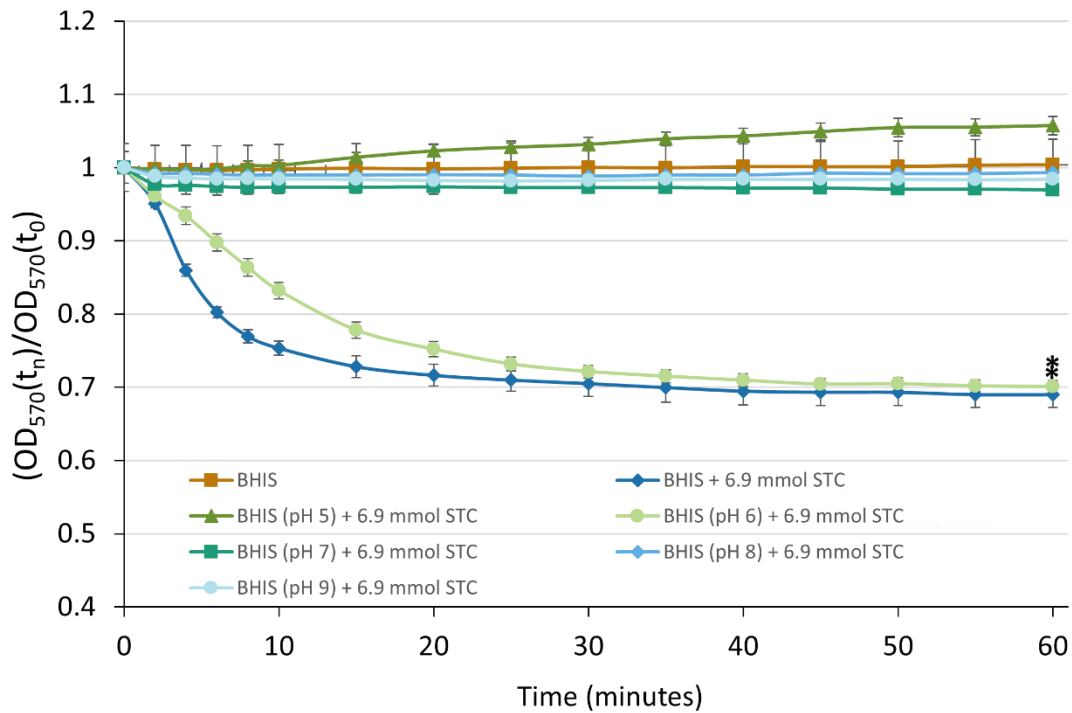
Figure 2.3 The effect of pH on taurocholate-induced germination of *C. difficile* spores. Purified *C. difficile* NCTC 11204 spores were mixed with unmodified BHIS broth only: orange squares, unmodified BHIS with 6.9 mM STC: dark blue diamonds, BHIS with 6.9 mM STC at pH 5: dark green triangles, BHIS with 6.9 mM STC at pH 6: light green circles, BHIS with 6.9 mM STC at pH 7: dark green squares, BHIS with 6.9 mM STC at pH 8: light blue diamonds, BHIS with 6.9 mM STC at pH 9: light blue circles. Error bars represent standard deviation. Statistically significant reductions of the relative optical density values at 60 minutes compared to time point zero are indicated by an asterisk ($P < 0.0001$).

2.3.4 The effect of butyrate on the germination of *C. difficile* spores by measurement of optical density.

A biologically relevant range of concentrations of the short-chain fatty acid butyrate was selected to investigate its influence on taurocholate-induced *C. difficile* spore germination. *C. difficile* spores exposed to taurocholate in the presence of all tested concentrations of butyrate demonstrated the

characteristic drop in relative OD_{570} indicating *C. difficile* spore germination, however there was variation in the extent of the change relative OD_{570} in the presence of the different concentration of butyrate (figure 2.4). The presence of butyrate at 5 mM and 10 mM, both resulted in a change in relative OD_{570} from 1.0 at time point zero to 0.76 at 60 minutes, a 24% decrease ($P < 0.0001$). The presence of 15, 20 and 25 nmol butyrate was demonstrated to cause a decrease in relative OD_{570} from 1.0 at the start of the assay to 0.79, 0.75 and 0.75 at 60 minutes respectively, a 22%, 25% and 25% reduction ($P < 0.0001$). The highest concentration of butyrate tested, 30 mM resulted in the smallest decrease in relative OD_{570} from 1.0 to 0.83 at 60 minutes, a 17% reduction ($P < 0.0001$).

The positive control, *C. difficile* spores exposed to taurocholate in the absence of butyrate, showed a 28% decrease of relative OD_{570} from 1.0 at time point zero to 0.71 at 60 minutes. The concentrations of butyrate tested were not found to result in a statistically different final relative OD_{570} from the positive control. The final relative OD_{570} values for *C. difficile* spores germinated in the presence of each concentration of butyrate were compared but were not found to be statistically significantly different from each other.

Negative controls, where *C. difficile* spores were exposed to BHIS and the various concentrations of butyrate in the absence of taurocholate demonstrated a small reduction in relative OD_{570} over the time course of the assay, this was not beyond a 2% reduction. Controls of BHIS, the various concentrations of butyrate and taurocholate in the absence of spores were included to ensure the observed decrease in optical density was the result of the change in refractive index of germinating spores. No change in relative OD_{570} was observed over +/- 2.3% during the assay.
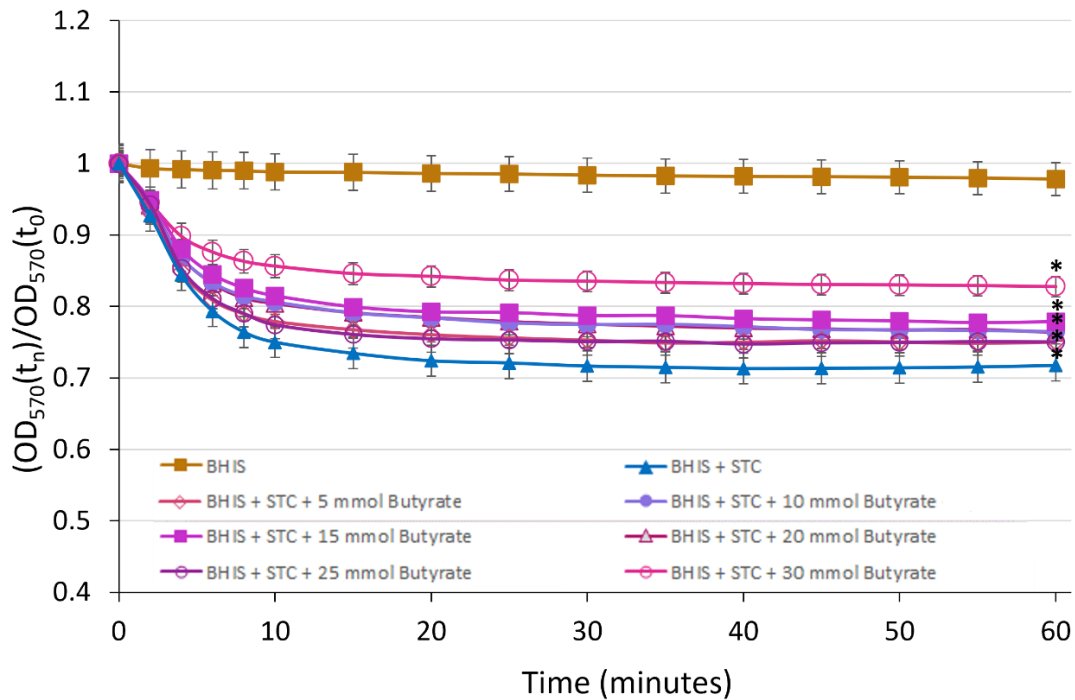
Figure 2.4 The effect of butyrate on taurocholate-induced germination of *C. difficile* spores.

Purified *C. difficile* NCTC 11204 spores were mixed with BHIS broth only: orange squares, BHIS with 6.9 mM STC: blue triangles, BHIS with 6.9 mM STC and 5 mM butyrate: pink diamonds, BHIS with 6.9 mM STC and 10 mM butyrate: purple circles, BHIS with 6.9 mM STC and 15 mM butyrate: purple squares, BHIS with 6.9 mM STC and 20 mM butyrate: open pink triangles, BHIS with 6.9 mM STC and 25 mM butyrate: open purple circles, BHIS with 6.9 mM STC and 30 mM butyrate: open pink circles. Error bars represent standard deviation. Statistically significant reductions of the relative optical density values at 60 minutes compared to time point zero are indicated by an asterisk (*P* < 0.001).

2.3.5 The germination response of *C. difficile* spores of strain NCTC 11204 to bile salts.

The germination response of *C. difficile* NCTC 11204 spores to a range of bile salts was investigated utilising the optical density assay. The exposure of *C. difficile* spores to 6.9 mM cholate caused a gradual decrease in relative OD_{570} over the course of the assay but this reduction was minimal, with a decrease from 1.0 at time point zero to 0.97 at 60 minutes, a 3.2% decrease (figure 2.5A). *C. difficile* spores exposed to 6.9 mM glycocholate did not cause a noticeable drop in relative OD_{570} over the course of the assay. The relative OD_{570} at 60 minutes was 0.99, a 0.1% decrease (figure 2.5B). Spores of *C. difficile* exposed to 6.9 mM deoxycholate caused a gradual decrease in relative OD_{570} over the course of the assay but similarly to cholate this reduction was minimal. A gradual decrease in relative OD_{570} from 1.0 at time point zero to 0.95 at 60 minutes, a 5% decrease was observed in response to 6.9 mM deoxycholate (figure 2.6A). The observed changes in relative optical density caused by cholate, glycocholate and deoxycholate were not found to be statistically significant, indicating they did not initiate *C. difficile* spore germination.

A positive control of *C. difficile* spores exposed to taurocholate where the germination activity of cholate, glycocholate and deoxycholate were investigated, caused a relative OD_{570} from 1.0 at time point zero to 0.7, 0.68 and 0.68 at 60 minutes, a decrease of 30, 32 and 32%, respectively. Negative controls, where *C. difficile* spores were exposed to BHIS demonstrated small changes in relative OD_{570} over the time course of the assays, equally to a maximum of +/- 3.5% change at 60 minutes. Controls of BHIS, BHIS with cholate, glycocholate, deoxycholate and taurocholate in the absence of spores were included in the optical density assays. No change in relative OD_{570} was observed over +/- 1.5%.

The activity of chenodeoxycholate to induce *C. difficile* spore germination along with the taurocholate-induced germination inhibitory action of chenodeoxycholate was also investigated. *C. difficile* spores exposed to 6.9 mM chenodeoxycholate only did not cause a significant change in relative OD_{570} over the course of the assay. The presence of chenodeoxycholate resulted in a change

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in relative OD_{570} from 1.0 at time point zero to 1.03 at 60 minutes, a 3% increase (figure 2.6B). The control, where *C. difficile* spores were exposed to taurocholate caused an expected rapid reduction in relative OD_{570}, characteristic of spore germination, causing a decrease from 1.0 at time point zero to 0.67 at 60 minutes, a 33% reduction. The exposure of *C. difficile* spores to 6.9 mM taurocholate in combination with 6.9 mM chenodeoxycholate, did not cause a noticeable change in relative OD_{570} over the course of the assay. The relative OD_{570} of 1.0 at time point zero and 1.007 at 60 minutes was observed, a 0.7% change (figure 2.6B).

Controls of BHIS, BHIS with chenodeoxycholate and or taurocholate in the absence of spores were included in the optical density assay. No change in relative OD_{570} was observed over +/- 1.5%. A negative control, where *C. difficile* spores were exposed to BHIS demonstrate a small change in relative OD_{570} over the time course of the assays, equally to +/- 3.2% change at 60 minutes.
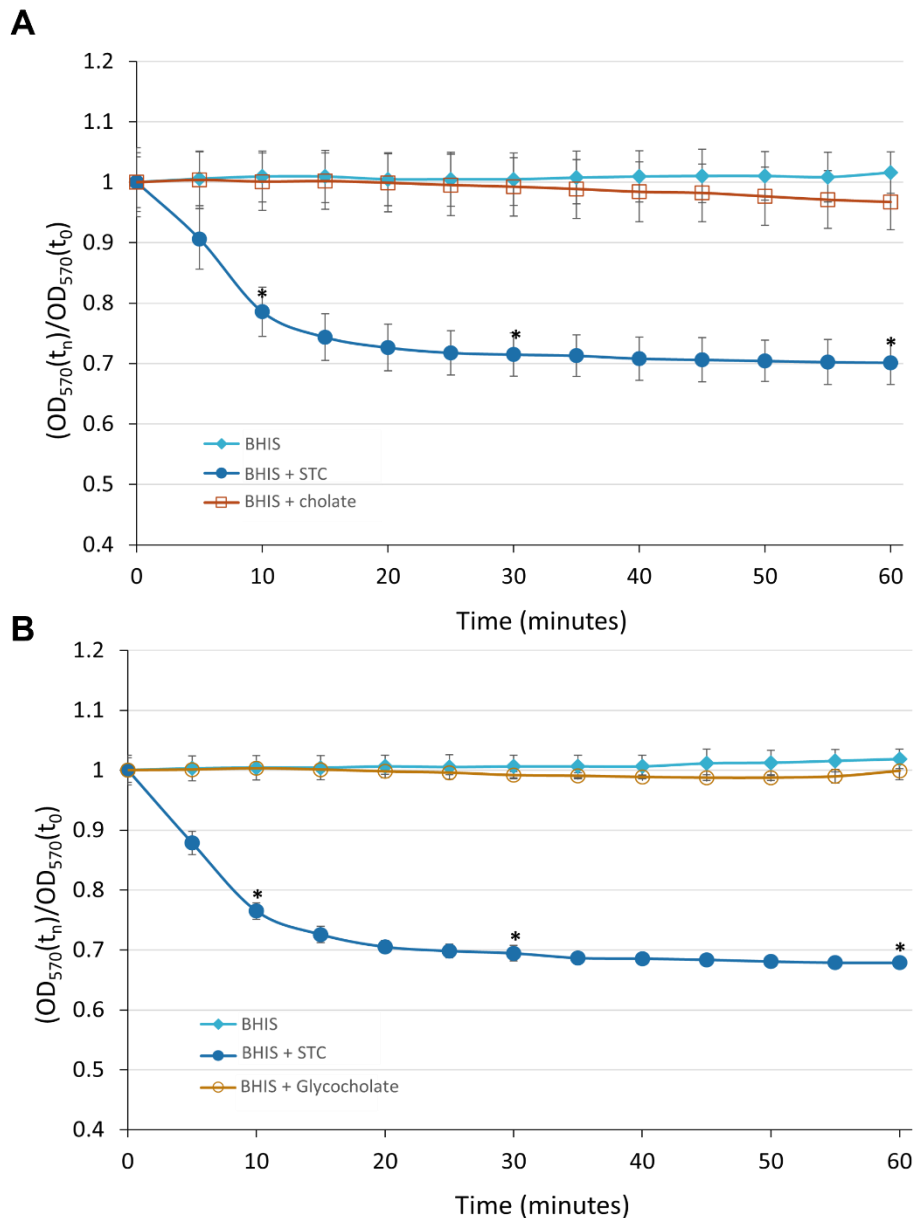
Figure 2.5 The activity of bile salts cholate and glycocholate to induce *C. difficile* spore germination. The activity of cholate (A) and glycocholate (B) to induce *C. difficile* germination was investigated. Purified *C. difficile* NCTC 11204 spores were mixed with BHIS broth only: light blue diamonds, BHIS with 6.9 mM STC: dark blue circles, BHIS with 6.9 mM cholate: open red squares, BHIS with 6.9 mM sodium glycocholate: open orange circles. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values at various time points 10, 30 and 60 minutes compared to time point zero are indicated by an asterisk ($P < 0.001$).
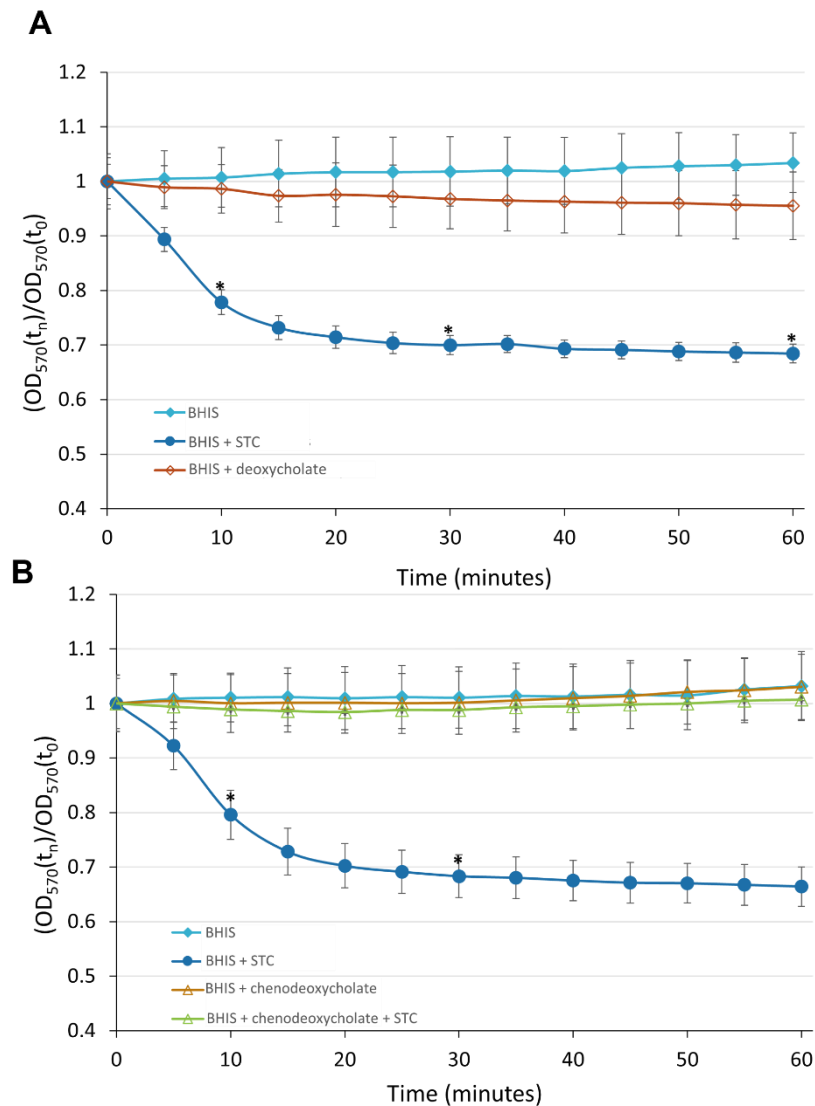
Figure 2.6 The effect of deoxycholate and chenodeoxycholate on *C. difficile* spore germination.

The activity of deoxycholate (A) and chenodeoxycholate (B) to induce *C. difficile* germination and the activity of chenodeoxycholate to inhibit taurocholate-induced germination investigated. Purified *C. difficile* NCTC 11204 spores were mixed with BHIS broth only: light blue diamonds, BHIS with 6.9 mM STC: dark blue circles, BHIS with 6.9 mM sodium deoxycholate: open red diamonds, BHIS with 6.9 mM sodium chenodeoxycholate: open orange triangles, BHIS with 6.9 mM sodium taurocholate and 6.9 mM sodium chenodeoxycholate: open green triangles. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values at various time points 10, 30 and 60 minutes compared to time point zero are indicated by an asterisk ($P < 0.001$).

2.3.6 The germination response of *C. difficile* ribotypes 001, 002 and 015 to bile salts.

The germination response of *C. difficile* spores of reference ribotypes 001, 002 and 015 strains to bile salts taurocholate, cholate, glycocholate and deoxycholate was investigated utilising the optical density assay. Spores of *C. difficile* ribotype 001 demonstrated the characteristic rapid drop in relative optical density, only in the presence of the bile salt taurocholate. Exposure of spores of ribotype reference strain 001 to 6.9 mM taurocholate resulted in a statistically significant decrease in relative OD_{570} from 1.0 at time point zero to 0.76 at 60 minutes ($P < 0.0001$), a 24% decrease (figure 2.7A). Statistically significant reductions in the relative OD_{570} were also observed at 10 ($P < 0.0001$), and 30 minutes ($P < 0.0001$), when compared to time point zero. The exposure of ribotype reference strain 001 to 6.9 mM cholate, glycocholate and deoxycholate resulted in minimal changes to the relative OD_{570} over the time course of the assay, these were not determined to be statistically significant.

Spores of *C. difficile* ribotype 002 demonstrated a divergent response to bile salts, a decrease in relative optical density was observed following exposure to taurocholate, cholate and deoxycholate. The presence of taurocholate resulted in a statistically significant decrease in relative OD_{570} from 1.0 at time point zero to 0.64 at 10 minutes ($P < 0.0001$), 0.62 at 30 minutes ($P < 0.0001$) and 0.62 at 60 minutes ($P < 0.0001$), a reduction of 37% (figure 2.7B). Exposure of spores of the ribotype 002 reference strain to cholate also resulted in a gradual but substantial drop in relative optical density, indicating spore germination. Here the presence of cholate resulted in a statistically significant decrease in relative OD_{570} from 1.0 at time point zero to 0.80 at 30 minutes ($P = 0.0008$) and 0.72 at 60 minutes ($P < 0.0001$), a reduction of 28% (figure 2.7B). A similar response of spores of the ribotype 002 reference strain to deoxycholate was observed. The presence of deoxycholate resulted in a statistically significant decrease in relative OD_{570} from 1.0 at time point zero to 0.77 at 30 minutes ($P < 0.0001$) and 0.74 at 60 minutes ($P < 0.0001$), a reduction of 26% (figure 2.7B). The exposure of ribotype reference strain 002 to 6.9 mM glycocholate resulted in minimal changes to

the relative OD_{570} over the time course of the assay, these were not determined to be statistically significant.

As for the spores of *C. difficile* ribotype 001 reference strain, spores of the ribotype 015 reference strain demonstrated a drop in relative optical density, only in the presence of the bile salt taurocholate. Exposure of spores of the ribotype reference strain to 015 to 6.9 mM taurocholate resulted in a decrease in relative OD_{570} from 1.0 at time point zero to 0.9 at 60 minutes, but this was not determined to be statistically significant (figure 2.7A). The exposure of ribotype reference strain 015 to 6.9 mM cholate, glycocholate and deoxycholate resulted in minimal changes to the relative OD_{570} over the time course of the assay, these were not determined to be statistically significant.

Negative controls, where *C. difficile* spores of ribotype reference strains 001, 002 and 015 were exposed to BHIS only, demonstrated small changes in relative OD_{570} over the time course of the assays, equally to a maximum of +/- 2% change at 60 minutes. Controls of BHIS, BHIS with taurocholate, cholate, glycocholate and deoxycholate in the absence of spores were included in the optical density assays. No change in relative OD_{570} was observed over +/- 6%.
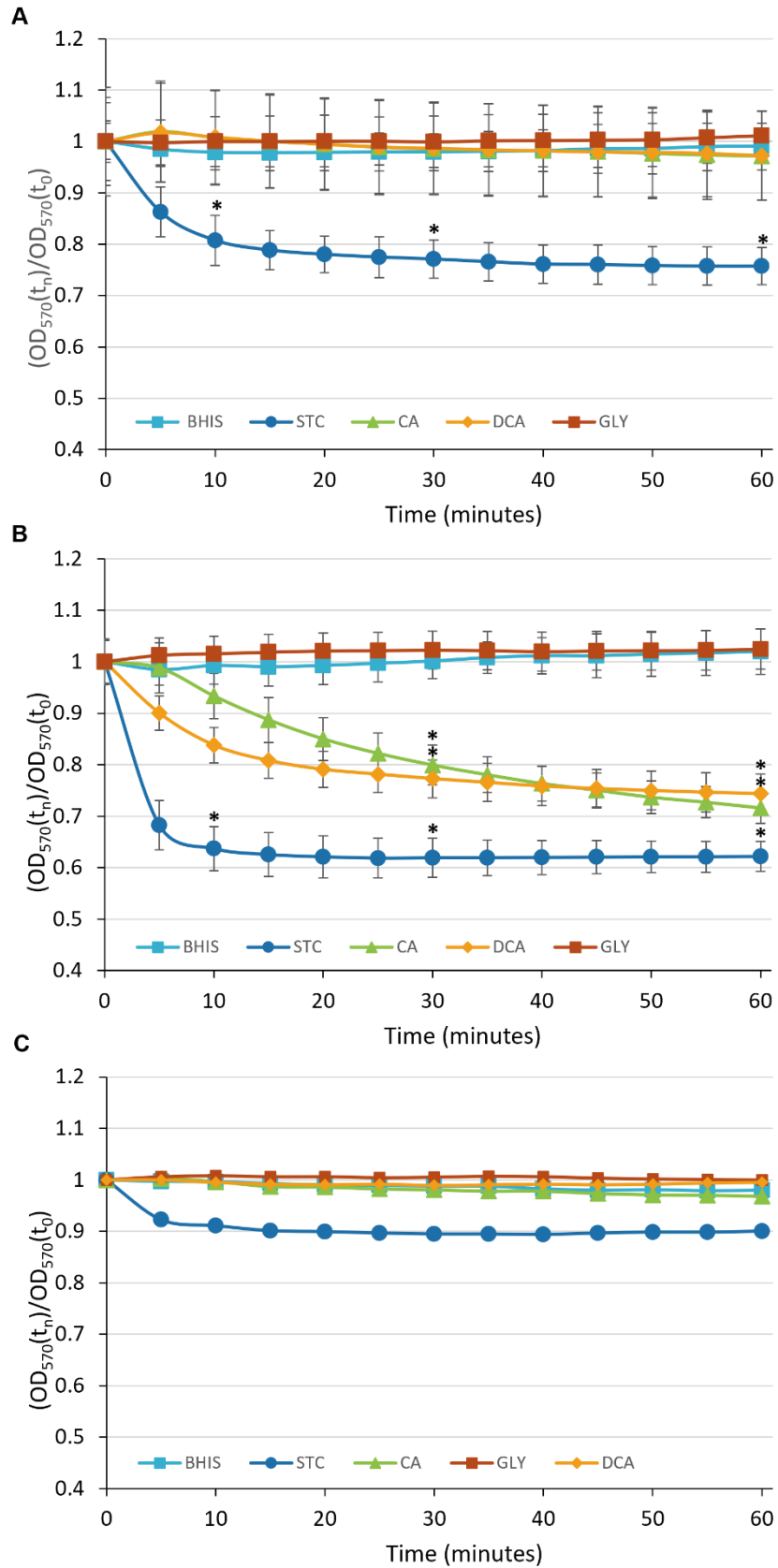
Figure 2.7 The germination response of *C. difficile* ribotypes 001 (A), 002 (B) and 015 (C) to bile salts. *Legend continued on next page.*

Figure 2.7 The germination response of *C. difficile* ribotypes to bile salts. The activity of bile salts taurocholate, cholate, glycocholate and deoxycholate induce germination of *C. difficile* ribotype 001 (A), 002 (B) and 015 (C) was investigated. Purified *C. difficile* spores were mixed with BHIS broth only: light blue squares, BHIS with 6.9 mM STC: dark blue circles, BHIS with 6.9 mM cholate: green triangles, BHIS with 6.9 mM sodium glycocholate: red squares, BHIS with 6.9 mM sodium deoxycholate: yellow diamonds. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values at various time points 10, 30 and 60 minutes compared to time point zero are indicated by an asterisk (P <0.0001).

2.4 Discussion.

In order to investigate the germination response of *C. difficile* spores to compounds that may function as germinants, co-germinants and inhibitors of germination or to study the environmental factors that influence *C. difficile* spore germination, a reliable and sensitive method is required to monitor germination. In this study, the germination optical density assay was selected as it provides a real-time measurement of the initial stages of spore germination, following activation by germinants, where the partial rehydration of the spore core and the release of Ca-DPA occurs (Setlow, 2014; Setlow, 2006). This method facilitates the quantification of the rate of the germination response and the extent of spore *C. difficile* germination without the requirement of the transformation of the spore to a metabolically active vegetative cell as is the case for methods which measure germination via the loss of resistance to heat or ethanol (Moir and Smith, 1990; Levinson and Hyatt, 1966). Utilising the optical density germination assay, this work characterised the germination response of *C. difficile* spores to bile salts, confirming previously identified *C. difficile* germinants and inhibitors. This methodology was also used to demonstrate the influence of environmental pH and the presence of the fatty acid butyrate on taurocholate-induced *C. difficile* germination.

By successfully establishing the use of the germination optical density assay with *C. difficile* spores, the induction of germination of *C. difficile* in response to the known germinant taurocholate in BHIS media was replicated. The exposure of spores of *C. difficile* NCTC 11204 to taurocholate in BHIS broth induced the characteristic rapid reduction in optical density (figure 2.2), a measure of the decreasing refractive index of germinating spores as they rehydrate and swell (Sorg and Sonenshein, 2008; Moir and Smith, 1990). The rapid germination response of *C. difficile* NCTC 11204 to 0.1 % (w/v) taurocholate was in agreement with the data presented by Sorg and Sonenshein (2008) where the germination activity of the bile salt germinant was initially characterised. In addition, the germination response of *C. difficile* to 0.1 % (w/v) taurocholate, the germination

activity of 6.9 mM taurocholate was investigated. This concentration of taurocholate was selected based on a previous study by Wheeldon *et al.* (2008a) and its biological relevance of the maximal duodenal and ileum concentrations (Leverrier *et al.*, 2003; Mallory *et al.*, 1973; Northfield and McColl, 1973). This is a considerably higher concentration than that selected by Sorg and Sonenshein (2008); 0.1% (w/v) sodium taurocholate is equivalent to a concentration of 1.86 mM. Germination of *C. difficile* NCTC 11204 spores in response to 6.9 mM taurocholate was demonstrated to occur more rapidly during the initial phase of the optical density assay and the extent of the germination response was greater, with a larger final reduction in relative optical density, compared to that found with 0.1 % (w/v) taurocholate.

The pH of culture media has previously been demonstrated as an important factor influencing *C. difficile* germination (Wheeldon *et al.*, 2008a; Paredes-Sabja *et al.*, 2008; Kochan *et al.*, 2018b). The investigation of the influence of pH on taurocholate-induced *C. difficile* germination demonstrated significant germination occurred only with a narrow pH range. Germination of *C. difficile* spores induced by taurocholate, was demonstrated with BHIS media at pH 6 and unmodified BHIS at pH 6.6 exclusively (figure 2.3). These findings differ to that of Paredes-Sabja *et al.* (2008) and Wheeldon *et al.* (2008a) who demonstrated the germination of *C. difficile* spores in media over a wider pH range. Paredes-Sabja *et al.* (2008) demonstrated *C. difficile* spore germination occurred between a range of pH 6 and pH 7.5 while Wheeldon *et al.* (2008a) reported germination of *C. difficile* spore between pH 5.5 to 8.5, but the extent of the germination response varied. Acute acidic or basic conditions inhibited *C. difficile* germination, with the most efficient germination being demonstrated between pH 6.5-7.7. However germination in this study was measured via loss of resistance ethanol. Germination of *C. difficile* spores over a broader pH range was also reported by Kochan *et al.* (2018b). *C. difficile* spore germination, measured via the optical density assay, occurred optimally between pH 6.5 and 8.5 (Kochan *et al.*, 2018b). In this investigation the exposure of *C. difficile* spores to the germinant at pH 7 and beyond, failed to induce germination.

The presence of butyrate largely had minimal influence on the germination of *C. difficile*. Germination was demonstrated at all concentrations of butyrate examined although the extent of the germination of *C. difficile* spores appeared to be reduced for some concentrations of butyrate tested. However, the extent of germination was not determined to be significantly altered from the taurocholate-induced germination of *C. difficile* (figure 2.4). *C. difficile* germination in the presence of butyrate occurred at concentrations within and beyond that of the physiologically relevant values of butyrate of 10-20 mM, although the total short chain fatty acid intestinal concentration is thought to be much greater (Kiefer *et al.*, 2006).

The investigation of the germination activity of bile salts cholate, glycocholate and deoxycholate with spores of *C. difficile* NCTC 11204, demonstrated limited germination. Cholate identified as a *C. difficile* germinant did not produce the rapid germination response when monitored via the optical density assay in this investigation or by that of (Sorg and Sonenshein, 2008). Cholate is a weak germinant, where the germination response has been observed over a time scale of hours on solid media (Sorg and Sonenshein, 2008). Equally glycocholate demonstrated no germination activity with spores of *C. difficile* NCTC 11204 when measured via the optical density assay. It has also demonstrated a failure to induce germination over a short time frame in the study by Sorg and Sonenshein (2008) but was also identified as a weak germinant due its ability to induce germination when incorporated into BHIS agar. Deoxycholate did not induce a statistically significant decrease in relative optical density of the *C. difficile* NCTC 11204 spores over the time scale of the assay, but a slight reduction was observed at 60 minutes. This is similar to the germination activity of deoxycholate reported by Bhattacharjee *et al.* (2016a). In contrast to bile salts cholate and glycocholate, deoxycholate has been found to induce germination of *C. difficile* but inhibit its growth. Chenodeoxycholate a known inhibitor of *C. difficile* germination failed to induce the germination of *C. difficile* NCTC 11204 spores and was found to completely inhibit taurocholate-induced germination of *C. difficile* NCTC 11204 spores (figure 2.6) (Sorg and Sonenshein, 2008; 2009). This is in accordance with the demonstrated competitive inhibitory action of

chenodeoxycholate with *C. difficile* germinants, preventing germination (Sorg and Sonenshein, 2009; 2010). Of the bile salts investigated for germination activity, only the well characterised *C. difficile* germinant taurocholate was identified to be a strong initiator of *C. difficile* NCTC 11204 spore germination with the optical density assay used in this work (Sorg and Sonenshein, 2008; Heeg *et al.*, 2012).

To examine the diversity of the germination response of *C. difficile* to bile salts, spores of reference ribotype strains of *C. difficile* representing 001, 002 and 015 were exposed to taurocholate, cholate, glycocholate and deoxycholate. The representative ribotypes were chosen based on their prevalence in Europe and the UK with 002 and 015 being identified as the most frequently isolated ribotype in a study of West London hospitals (Bauer *et al.*, 2011; Public Health England, 2019b; Herbert *et al.*, 2019). Spores of *C. difficile* ribotype 001 reference strain demonstrated a similar germination response to taurocholate, cholate, glycocholate and deoxycholate as *C. difficile* NCTC 11204, also ribotype 001. Cholate, glycocholate and deoxycholate failed to induce germination of ribotype 001 reference strain, while a rapid germination activity was observed with taurocholate (figure 2.7A), although the extent of the germination response was not as pronounced as for *C. difficile* NCTC 11204 (figure 2.2). The germination activity of cholate, glycocholate and deoxycholate was also similar with *C. difficile* ribotype 015 reference strain, with no detectable activation of germination. Exposure of spores of ribotype 015 reference strain to taurocholate-initiated germination but the activity was weaker and to a lower extent than seen with ribotype 001 or NCTC 11204 (figure 2.7C). In contrast, spores of *C. difficile* ribotype 002 reference strain demonstrated a divergent germination response to cholate, glycocholate and deoxycholate compared with ribotype 001 and 015 reference strains and *C. difficile* NCTC 11204. Rapid germination of *C. difficile* ribotype 002 spores was observed in response to taurocholate. Germination of ribotype 002 spores was also initiated by the presence cholate and deoxycholate (figure 2.7B), demonstrating their activity as *C. difficile* germinants. A varied germination response to bile salts germinants across different strains of *C. difficile* has previously been reported in clinical isolates. In the study by Heeg *et al.* (2012) the

activity of taurocholate to induce germination was diverse and notably chenodeoxycholate did not inhibit germination of some *C. difficile* clinical isolates. This work and that of others clearly demonstrate taurocholate as a strong initiator of *C. difficile* germination, causing the activation of germination across the limited number of strains of *C. difficile* used in this investigation (Sorg and Sonenshein, 2008; Heeg *et al.*, 2012). The germination response of the representative ribotypes strains in this work demonstrates the potential for a varied response of clinical isolates of *C. difficile* to characterised *C. difficile* germinants, an important consideration when developing a pro-germination decontamination strategy to eliminate *C. difficile* spores in the environment.

Primary bile acids; cholic acid and chenodeoxycholic acid are conjugated with glycine and taurine prior to release into the small intestine, producing secondary bile salts: glycocholate, taurocholate, glycochenodeoxycholate and taurochenodeoxycholate (Ridlon *et al.*, 2006). While active transport returns the majority of the secondary bile salts back to the liver, low concentrations are deconjugationed by bile salt hydrolases produced by species of the intestinal microbiota, forming cholic acid and chenodeoxycholic acid (Ridlon *et al.*, 2006; Thomas *et al.*, 2001). These primary bile acids are passively reabsorbed however reabsorption of chenodeoxycholate is disproportionate leading to a higher remaining concentration of cholate (Mekhjian *et al.*, 1979). Specific members of the intestinal microbiota, including the *Ruminococcaceae* and *Lachnospiraceae* families, modify cholate and chendeoxycholate through 7α-dehydroxylation to produce the secondary bile acids deoxycholate and lithocholate (Ridlon *et al.*, 2006; Theriot *et al.*, 2016). Disruption of the gut microbiota by broad spectrum antibiotics, a key risk factor for CDI diminishes the members of the intestinal microbiota responsible for the biotransformation of primary bile acids to secondary bile acids by 7α-dehydroxylation, creating an environment that promotes the germination of *C. difficile* spores (Vedantam *et al.*, 2012; Theriot *et al.*, 2016). The modulation of bile acids and salts is an important factor in germination of *C. difficile* spores in the host (Theriot *et al.*, 2016; Ridlon *et al.*, 2006; Sorg and Sonenshein, 2008; 2009).

The specific members of the *Ruminococcaceae* and *Lachnospiraceae* families also produce short-chain fatty acids (SCFAs) including butyrate which is important for healthy functioning of the colonic mucosal (Cook and Sellin, 1998). The possible inhibition of *C. difficile* germination in the presence of butyrate seen in this study is likely due to the influence by the high concentration of butyrate on the pH in the media, creating an acidic environment that impedes germination. The inhibitory activity of high concentrations of butyrate on *C. difficile* proliferation and colonisation resistance has been demonstrated but the inhibitory mechanism of butyrate was not found to be exclusively due to the effect on environmental pH (Rolfe, 1984). The inhibitory activity of butyrate on *C. difficile* has been predicted to disrupt of metabolic pathways and interfere with cellular signalling or enzyme function (Gregory *et al.*, 2021). This may indicate that butyrate acts as another environmental cue that supresses germination when there is an abundance of the *Ruminococcaceae* and *Lachnospiraceae* families within the healthy intestinal microbiota of the host (Antharam *et al.*, 2013).

To successfully examine the germination response of *C. difficile*, preparation of purified spore suspension that are free from vegetative cell debris was critical to a reliable measurement of *C. difficile* germination using the optical density assay. The purification process of the spore suspension however eliminates the biological variation of spores morphotypes, including spores at various stages of sporulation. Similarly, it does not reflect the condition of spores that are excreted by CDI patients into the environment (Samore *et al.*, 1996). Preparation of purified spore suspensions is likely to eliminate the natural heterogeneity of the germination response of spores to germinants, a characteristic of *C. difficile* clinical isolates (Ghosh and Setlow, 2009; Heeg *et al.*, 2012). The methodology of spore preparation likely also influences the abundance of *C. difficile* germinant receptors and proteins involved in the germination signal cascade, as seen in other spore formers (Ramirez-Peralta *et al.*, 2012). The abundance of CspB and CspC was demonstrated to be altered in spores of *C. difficile* prepared in different culture media (Shrestha and Sorg, 2019). The necessary laboratory-based culture and purification of *C. difficile* spores in order to perform the

optical density may result in a germination response that does not accurately reflect the biology of spores that contaminate the clinical environment.

An alternative method to measure the initial transformation of the spore following the triggering of germination, but also requiring the use of pure spore suspensions, is the quantification of spore Ca-DPA release using terbium chloride (Hindle and Hall, 1999). This method has been routinely utilised to study germination of *C. difficile* spores, but the experimental conditions of this methodology have been found to influence the germination of *C. difficile* spores (Shrestha and Sorg, 2019). Terbium was demonstrated to enhance taurocholate-induced germination and is predicted to act as a *C. difficile* co-germinant, in a similar mechanism as calcium (Kochan *et al.*, 2017; Shrestha and Sorg, 2019). The germination enhancing effect of terbium was also found to extend to other metal ions and was associated with the preparation of *C. difficile* spores in peptone-rich culture media (Shrestha and Sorg, 2019).

The initial stages of germination, where the resistant properties of the spore are lost presents an opportunity to eliminate spores using the pro-germination, germinate to exterminate strategy (Wheeldon *et al.*, 2010). During this initial stage of germination, the loss of refractive index of rehydrating spore population can also be measure via a decrease optical density facilitating a sensitive and reproducible method to monitor *C. difficile* germination. This methodology provides an alternative to traditional methods of measuring spore germination which determine the heat or ethanol resistance of spores exposed to germinants and are reliant on subsequently inducing germination and outgrowth of *C. difficile* spores. The germination response of *C. difficile* spores, measured by the optical density assay in this chapter are confirmatory of previously published studies. Sodium taurocholate was demonstrated to be a potent germinant of *C. difficile* NCTC 11204, in addition to other well studied laboratory and clinical strains of *C. difficile* (Sorg and Sonenshein, 2008; Heeg *et al.*, 2012). A varied germination response rate to sodium taurocholate was observed across the representative ribotype clinical strains of *C. difficile.* This divergent

germination response was also seen when the representative ribotype strains of *C. difficile* were exposed to other relevant bile salts. The rate and extent of *C. difficile* NCTC 11204 spore germination was influenced by environmental factors including pH. In conclusion, the germination response of *C. difficile* clinical isolates is anticipated to vary to any bile salt germinant, with environmental factors also influencing the germination response of the spore population. These are important considerations for the development of a *C. difficile* pro-germination spore elimination strategy.

Chapter 3

3.0 Investigation of the co-germinant potential of amino acids and calcium in the germination of *Clostridioides difficile* spores.

3.1 Introduction.

To initiate *C. difficile* spore germination, a bile salt germinant and a co-germinant are required to transduce the germination signal, causing the transformation of the dormant spore (Sorg and Sonenshein, 2008; Kochan *et al.*, 2017). The term co-germinant is distinctive of the description of *C. difficile* germination, despite other Clostridia requiring multiple germinants to trigger efficient germination (Bhattacharjee *et al.*, 2016b). The first co-germinant of *C. difficile* germination identified was glycine, as the component of complex nutrient media that facilitates efficient *C. difficile* spore germination with the germinant taurocholate (Sorg and Sonenshein, 2008). Glycine has subsequently been demonstrated as an effective co-germinant of the spores of multiple laboratory and clinical strains of *C. difficile* (Sorg and Sonenshein, 2008; Howerton *et al.*, 2011; Shrestha and Sorg, 2017; Kochan *et al.*, 2017).

The profile of amino acid co-germinants was expanded by Wheeldon *et al.* (2011) who demonstrated the activity of arginine, aspartic acid, valine, and histidine but only when utilised in combination with glycine to induce spore germination of *C. difficile* laboratory strain NCTC 11204. The efficiency of glycine co-germinant activity was found to be enhanced when combined with arginine, aspartic acid, valine, or histidine. Glycine together with histidine or a mixture of glycine and the four amino acids (arginine, aspartic acid, valine, and histidine) was found to be a particularly effective co-germinant combination to trigger *C. difficile* spore germination. The activity of these co-germinant combinations was also demonstrated as effective with four clinical *C. difficile* strains (Wheeldon *et al.*, 2011). More recently Shrestha and Sorg (2017) conducted a detailed

characterisation of amino acid co-germinants of *C. difficile*; 16 amino acids were identified to act as co-germinant to induce germination of *C. difficile* UK1 spores. Activity was varied and was highly dependent on amino acid concentration and temperature. The co-germinant activity of these amino acids was ranked according to the calculated EC_{50} values, the concentration at which half maximum germination was achieved, revealing alanine, taurine, glutamine, histidine, serine, arginine to have the highest efficacy to induce germination, after glycine. The same approach was taken with *C. difficile* M68 but resulted in a different order of the ranking of amino acids to efficiently induce germination (Shrestha and Sorg, 2017).

The activity of *C. difficile* amino acid co-germinants appears to be highly strain dependant with the exception of glycine, which has demonstrated consistent activity (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2011; Howerton *et al.*, 2011; Shrestha and Sorg, 2017; Kochan *et al.*, 2017). The capacity of other amino acids to function as co-germinants is temperature dependant; amino acids including D- forms of alanine and serine not previously identified as *C. difficile* co-germinants were demonstrated to have activity as co-germinants at physiologically-relevant 37°C (Shrestha *et al.*, 2017; Shrestha and Sorg, 2017). The germination response of some *C. difficile* clinical strains was shown to be highly dependent upon prior heat activation. Spores of some *C. difficile* strains, obtained during a CDI outbreak, also germinated in response to alternative germinants including potassium, phosphate and Ca-DPA (Paredes-Sabja *et al.*, 2008).

C. difficile spore germination can also be initiated by calcium when in combination with taurocholate. Calcium and other cations were identified to stimulate germination along with taurocholate, but the co-germination activity of calcium was highly efficient. Kochan *et al.* (2017) demonstrated rapid initiation of spore germination of 3 *C. difficile* strains following exposure to calcium and taurocholate. In addition to exogenous calcium functioning as a co-germinant to stimulate germination, endogenous calcium was also demonstrated to be essential for glycine and taurocholate-induced *C. difficile* germination. This was suggested to be due to the activation of

calcium-dependant enzymes involved in the initiation of spore germination (Kochan *et al.*, 2017). Analysis of the kinetics of *C. difficile* spore germination in response to taurocholate and calcium or glycine indicates calcium is 10-fold more efficient at triggering germination. Significantly, calcium and glycine act synergistic when in combination to facilitate germination of *C. difficile* spores in the presence of suboptimal concentration of co-germinants (Kochan *et al.*, 2017; Kochan *et al.*, 2018b).

Amino acids and calcium as co-germinants are believed to activate the co-germinant receptor CspA, functioning through the same signalling pathway to induce germination (Kochan *et al.*, 2018b; Shrestha *et al.*, 2019). A study of the role of co-germinants in the regulation of *C. difficile* germination revealed the inactivation of the protease involved in processing of CspA or the deletion of the coding region of CspA generated *C. difficile* spores that did not require a co-germinant to initiate germination (Kevorkian *et al.*, 2016; Shrestha *et al.*, 2019). Current models of the germination signalling pathway suggest that the bile salt germinant signal is required prior to the activation of the co-germinant receptor. In the 'Lock and Key' model the germinant receptor CspC is first activated by the binding of germinants, allowing the influx of calcium and amino acids co-germinants across the outer membrane to reach the co-germinant receptor, CspA (Kochan *et al.*, 2017; Kochan *et al.*, 2018b). The 'germinosome' model suggests the germinant and co-germinant receptors are co-located in a complex along with protease CspB. Due to their proximity, the binding of bile salts to CspC and co-germinants to CspA, releases CspB to transfer the germination signal by activating the cortex lytic enzyme SleC (Kochan *et al.*, 2018b; Shrestha *et al.*, 2019; Francis and Sorg, 2016; Lawler *et al.*, 2020).

The current knowledge of *C. difficile* co-germinants has been utilised in *C. difficile* spore pro-germination elimination strategies. The four amino acids: arginine, aspartic acid, valine and histidine, identified by Wheeldon *et al.* (2011) to work cooperatively as co-germinants with glycine were utilised in a germination solution to investigate the initiation of germination and subsequent susceptibility of germinating spores to biocides (Wheeldon *et al.*, 2011; 2010). Incubation of *C.*

difficile spores with the germination solution for 1 hour resulted in a 3-log reduction in recovery of *C. difficile* (Wheeldon *et al.*, 2010). Similarly, a cocktail of 18 amino acid co-germinants was subsequently included in a 'spore germination solution' along with taurocholate and other components with the purpose of triggering *C. difficile* spore germination (Nerandzic and Donskey, 2010; 2013). The application of the germinant solution to surfaces in a clinical environment followed by UV radiation was demonstrated to enhance the elimination of *C. difficile* spores (Nerandzic and Donskey, 2010). The germinant solution was also demonstrated to increase the sensitivity of *C. difficile* spores to nisin (Nerandzic and Donskey, 2013).

The aim of the research presented in this chapter was to investigate the co-germination activity of amino acids and calcium to induce germination of *C. difficile* strain NCTC 11204. The germination optical density assay was selected to measure the germination response of *C. difficile* to co-germinants during the initial phase of germination. Experiments sought to characterise the co-germinant activity of amino acids as the exclusive co-germinant with germinant sodium taurocholate and to examine the germination response of spores exposed to glycine and calcium with other amino acid co-germinants. This research aims to enhance current knowledge of *C. difficile* spore co-germinants and provide further understanding of the relevant conditions under which co-germinants could be utilised in a spore-germination elimination strategy.

3.2 Methods.

3.2.1 Bacterial strains and growth conditions.

C. difficile NCTC 11204 was sourced from Jon Brazier (HPA Anaerobic Reference Laboratory, Cardiff). Frozen stocks of *C. difficile* were stored at -80˚C on Microbank™ beads (Pro Lab Diagnostics, Canada) and resuscitated by inoculating a bead onto Wilkins-Chalgren agar (Oxoid, UK) supplemented with 0.1% (w/v) sodium taurocholate hydrate (Alfa Aesar, UK) and incubating anaerobically (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK) at 37°C for 48 hours.

3.2.2 Preparation of spore suspensions of *C. difficile*.

Spore suspensions of *C. difficile* NCTC 11204 were prepared using a modified version of the methodology described by Heeg *et al.* (2012) as described on chapter 2, section 2.2.2 of this thesis.

3.2.3 Determination of germination of *C. difficile* spores by measurement of optical density.

The methodology of the measurement of germination by the optical density assay was based on that described by Sorg and Sonenshein (2008) and Heeg *et al.* (2012). The optical density at 570nm (OD_{570}) of the purified *C. difficile* spore suspensions was determined using a spectrophotometer (Jenway, UK) and adjusted to an OD_{570} of 3.5 in preparation for the optical density assay. Aliquots of 20 µl of the spore suspension or SDW were loaded into the wells of a clear flat bottom 96-well plate followed by 180 µl of appropriate germination buffer, achieving a final OD_{570} of the spore suspension of 0.35. The OD_{570} was measured immediately (time zero) using the ELx808 Absorbance plate reader (BioTek, USA) and every minute thereafter for 60 minutes during incubation at room temperature. A 3 second medium shake was included prior to each OD_{570} measurement to prevent

the adhesion of spores to the wells of the 96-well plate. The relative ratios of the OD_{570} at the various time points to the OD_{570} at time zero were plotted against time. To investigate the co-germination activity of amino acids and calcium, a germination buffer of Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.3-7.4 was supplemented with 6.9 mM sodium taurocholate and 0.4% (w/v) of the amino acids: glycine, aspartic acid, alanine, methionine, valine, serine, arginine, histidine, and 50 mM calcium chloride.

3.2.4 Statistical analysis.

Spore germination optical density data presented consists of the mean of a minimum of six replicates and the standard deviation of these experiments. A two-way analysis of variance (ANOVA) was used to determine the co-germination activity of amino acids and calcium on *C. difficile* germination. The *post-hoc* Bonferroni's multiple comparisons test was used to statistically analyse the germination activity of various test conditions. Both statistical tests were completed using the GraphPad Prism software (GraphPad, USA).

3.3 Results.

3.3.1 The efficacy of amino acids as co-germinants of *C. difficile* spore germination.

The optical density germination assay was utilised to investigate the co-germination activity of amino acids in the initiation of *C. difficile* spore germination along with known bile salt germinant taurocholate. Exposure of *C. difficile* spores to 0.4% (w/v) glycine and sodium taurocholate at a concentration of 6.9 mM resulted in a decrease in relative optical density at 570nm over the one hour timeframe of the assay. The results (figure 3.1) demonstrate a moderate decrease in relative OD_{570} during the initial 30 minutes, with a following gradual reduction over the remainder of the assay. Glycine 0.4% (w/v) together with 6.9 mM sodium taurocholate caused a 32.2% decrease of relative OD_{570} from 1.0 at time point zero to 0.68 at 60 minutes. A statistically significant ($P < 0.001$) decrease in relative OD_{570} of *C. difficile* spores exposed to 0.4% (w/v) glycine and sodium taurocholate was observed at 30 and 60 minutes when compared to *C. difficile* spores and 0.4% (w/v) glycine only (in the absence of the bile salt germinant sodium taurocholate) at the equivalent time point.

To ensure any observed decrease in relative optical density was the result of the transition in refractive index of germination spores, controls of the germination buffer Tris-HCl with 6.9 mmol and Tris-HCl with 0.4% (w/v) glycine in the absence of spores were included in the optical density assay. No change in relative OD_{570} was observed over +/-1.3% at the end point of the assay. Negative controls where *C. difficile* spores were exposed to the germination buffer Tris-HCl and sodium taurocholate or Tris-HCl and 0.4% (w/v) glycine resulted in a small change in relative OD_{570} over the time course of the assay from 1.0 to 1.03, a -3.5% change.
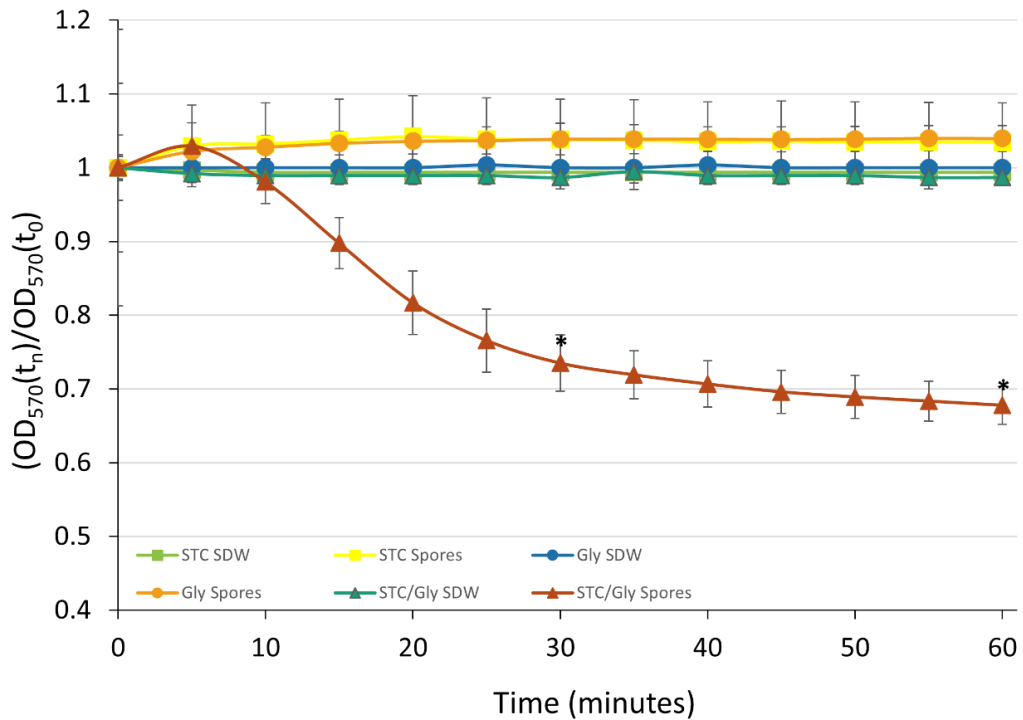
Figure 3.1 The effect of amino acid glycine on *C. difficile* spore germination. The activity of glycine to co-induce *C. difficile* NCTC 11204 spore germination with sodium taurocholate was investigated. Tris-HCl buffer was mixed with SDW and 6.9 mM sodium taurocholate: light green squares, purified *C. difficile* spores and 6.9 mM sodium taurocholate: yellow squares, SDW and 0.4% (w/v) of glycine: dark blue circles, purified *C. difficile* spores and 0.4% glycine: orange circles, SDW, 6.9 mM sodium taurocholate and 0.4% (w/v) of glycine: dark green triangles, purified *C. difficile* spores, 6.9 mM sodium taurocholate and 0.4% (w/v) glycine: red triangles. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values of at points 30 and 60 minutes compared to purified *C. difficile* spores and 0.4%(w/v) glycine only at the equivalent time point are indicated by an asterisk (*P* < 0.001).
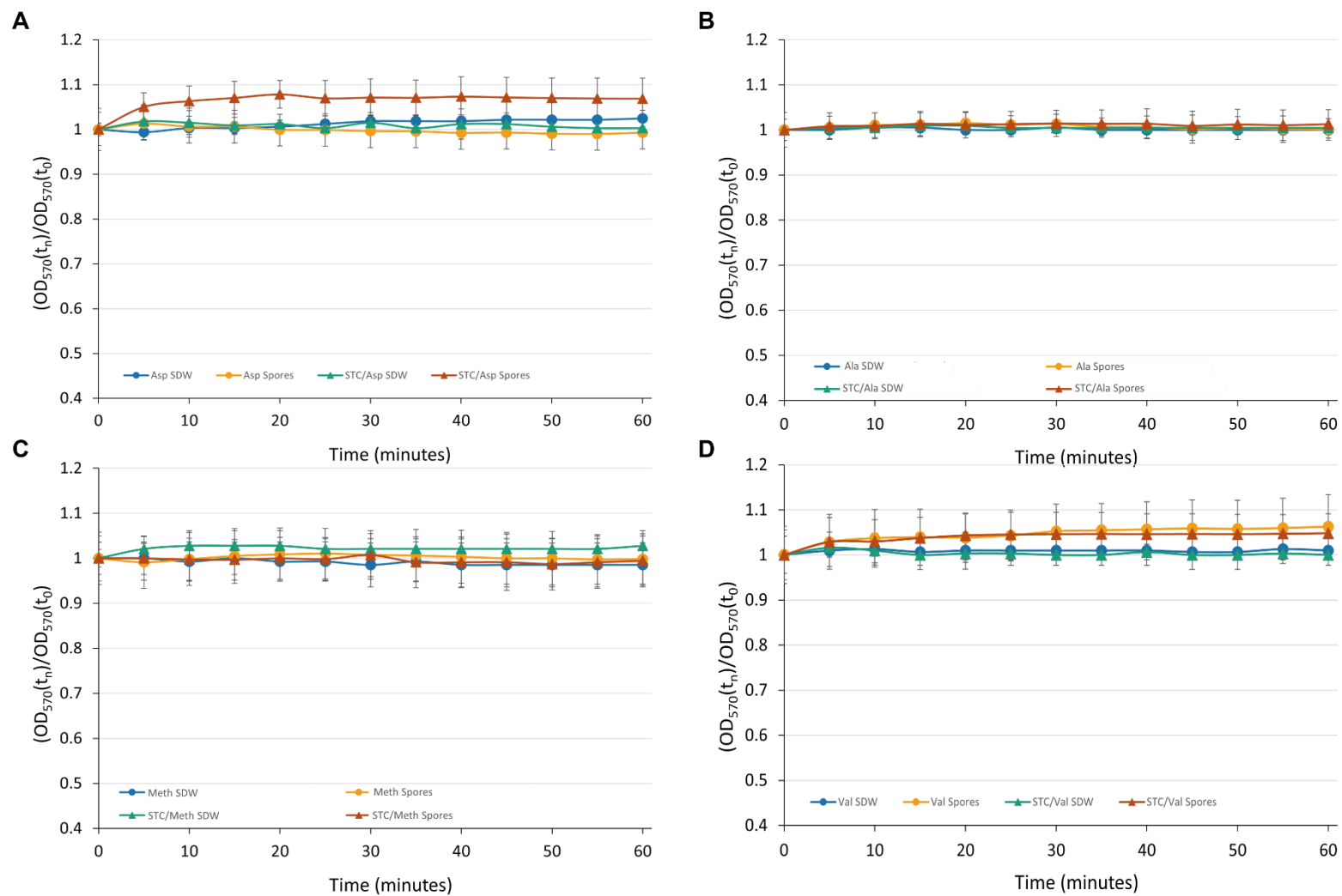
Figure 3.2 The effect of amino acids aspartic acid (A), alanine (B), methionine (C), and valine (D) on *C. difficile* spore germination. *Legend continued on next page.*

Figure 3.2 The effect of amino acids aspartic acid, alanine, methionine, and valine on *C. difficile* NCTC 11204 spore germination. The activity of aspartic acid (A), alanine (B), methionine (C), and valine (D) to co-induce *C. difficile* spore germination with sodium taurocholate was investigated. Tris-HCl buffer was mixed with SDW and 0.4% (w/v) of the specified amino acid: dark blue circles, purified *C. difficile* spores and 0.4% (w/v) of the specified amino acid: orange circles, SDW, 6.9 mM sodium taurocholate and 0.4% (w/v) glycine: dark green triangles, purified *C. difficile* spores, 6.9 mM sodium taurocholate and 0.4% (w/v) glycine: red triangles. Error bars represent standard deviation.

The co-germination activity of amino acids aspartic acid, alanine, methionine, valine, serine, arginine, and histidine in the germination of *C. difficile* spores was also investigated. These amino acids aspartic acid, alanine, methionine, valine, serine, arginine, and histidine failed to initiate *C. difficile* spore germination in combination with 6.9 mM of bile salt germinant taurocholate during the time course of the assay (figure 3.2 and 3.3). No statistically significant decrease in relative OD_{570} of *C. difficile* spores exposed to 0.4% (w/v) of the specified amino acid and sodium taurocholate was observed. As for the optical density assay with 0.4% (w/v) glycine, to ensure any decreases in relative optical density were the results of the transition in refractive index of germination spores, controls of the germination buffer Tris-HCl with 6.9 mmol sodium taurocholate and 0.4% (w/v) of the specified amino acid in the absence of spores were included in the optical density assay. No change in relative OD_{570} was observed over +/-2.2% at the end point for the assays completed with the specified amino acids. For the negative controls where no amino acids were present in combination with sodium taurocholate, *C. difficile* spores were exposed to the germination buffer and 0.4% (w/v) of the specified amino acid, this resulted in a small change in relative OD_{570} at the end point of the assay a maximum of a -4.3% change across the different assays.
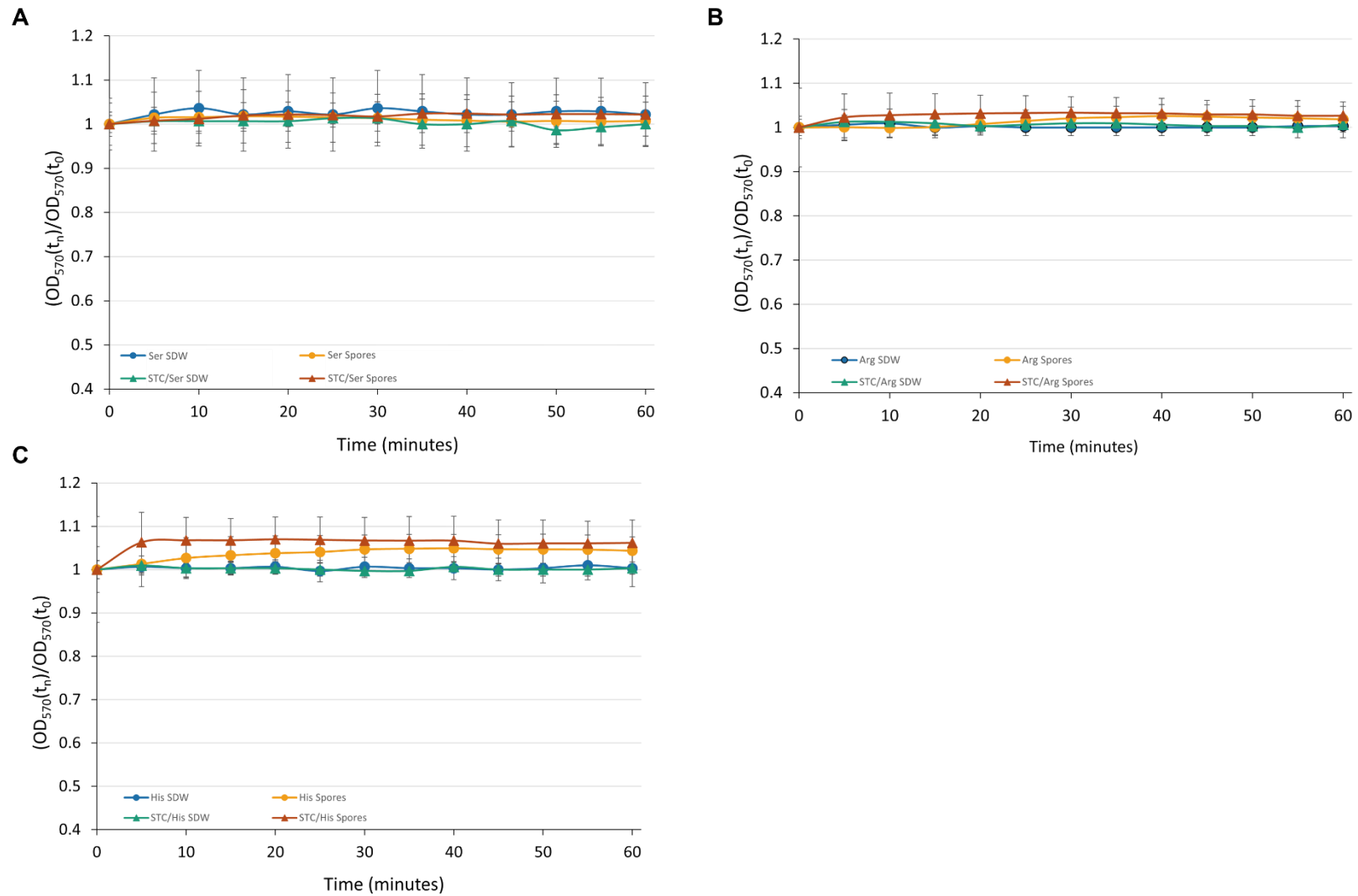
Figure 3.3 The effect of amino acids serine (A), arginine (B), and histidine (C) on *C. difficile* spore germination. *Legend continued on next page.*

Figure 3.3 The effect of amino acids serine, arginine, and histidine on *C. difficile* NCTC 11204 spore germination. The activity of serine (A), arginine (B), and methionine (C), to co-induce *C. difficile* spore germination with sodium taurocholate was investigated. Tris-HCl buffer was mixed with SDW and 0.4% (w/v) of the specified amino acid: dark blue circles, purified *C. difficile* spores and 0.4% of the specified amino acid: orange circles, SDW, 6.9 mM sodium taurocholate and 0.4% glycine: dark green triangles, purified *C. difficile* spores, 6.9 mM sodium taurocholate and 0.4%(w/v) glycine: red triangles. Error bars represent standard deviation.

3.3.2 The efficacy of calcium in combination with amino acids as co-germinants of *C. difficile* NCTC 11204 spore germination.

The optical density assay was also utilised to investigate the co-germination activity of amino acids in combination with calcium in the initiation of *C. difficile* spore germination along with known bile salt germinant taurocholate. As previously demonstrated (figure 3.1), exposure of *C. difficile* spores to 0.4% (w/v) glycine and sodium taurocholate at a concentration of 6.9 mM, resulted in a reduction in relative OD_{570} over the time course of the assay, causing a 34.4% decrease of relative OD_{570} at 60 minutes. The decrease in relative OD_{570} of *C. difficile* spores exposed to 0.4% (w/v) glycine and sodium taurocholate observed at 30 and 60 minutes when compared to *C. difficile* spores and 0.4% (w/v) glycine only (in the absence of the bile salt germinant sodium taurocholate) at the equivalent time point was determined to be statistically significant ($P < 0.001$). A similar germination response of *C. difficile* spores when exposed to 50 mM calcium chloride and 6.9 mM sodium taurocholate was also observed (figure 3.4A). The results demonstrate a moderately rapid rate of reduction in the relative OD_{570} during the initial 30 minutes, with a more gradual reduction over the remainder of the 60 minute assay. Calcium chloride and sodium taurocholate caused a 32.2% decrease of relative OD_{570} from 1.0 at time point zero to 0.70 at 60 minutes. A statistically significant ($P < 0.001$) decrease in relative OD_{570} of *C. difficile* spores exposed to 50 mM calcium chloride and sodium

Figure 3.4 The effect of calcium in combination with amino acids glycine and aspartic acid on *C. difficile* spore germination. The activity of calcium and glycine (A), and calcium and aspartic acid (B) to co-induce *C. difficile* NCTC 11204 spore germination with sodium taurocholate was investigated. Purified *C. difficile* spores and Tris-HCl buffer were mixed with 0.4% of the specified amino acid: dark blue diamonds, 6.9 mM sodium taurocholate and 0.4% (w/v) of the specified amino acid: red circles, 6.9 mM sodium taurocholate and 50 mM calcium chloride: purple squares, 6.9 mM sodium taurocholate, 50 mM calcium chloride and 0.4% of the specified amino acid: light green triangles. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values at time points 10, 30 and 60 minutes compared to purified *C. difficile* spores and 0.4% (w/v) of the specified amino acid only at the equivalent time point are indicated by an asterisk ($P < 0.001$).

taurocholate was observed at 10, 30 and 60 minutes when compared to *C. difficile* spores and 0.4% (w/v) glycine only at the equivalent time point. When *C. difficile* spores were exposed to 0.4% (w/v) glycine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate germination was initiated, causing a rapid decrease in the relative OD_{570} during the initial 10 minutes of the assay followed by a gradual reduction over the remainder of the assay. A reduction in relative OD_{570} from 1.0 at time point zero to 0.68 at 60 minutes was observed. The decrease in relative OD_{570} at 10, 30 and 60 minutes caused by exposure of *C. difficile* spores to 0.4% (w/v) glycine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate when compared to *C. difficile* spores and 0.4% (w/v) glycine only at the equivalent time point was found to be statistically significant ($P <$ 0.001). The extent of the change in relative OD_{570} in response to 0.4% (w/v) glycine, 50 mM calcium chloride and 6.9 mM sodium taurocholate in combination was similar as for the response to 0.4% (w/v) glycine in combination with 6.9 mM sodium taurocholate or 50 mM calcium chloride in combination with 6.9 mM sodium taurocholate.

The exposure of *C. difficile* spores to 0.4% (w/v) aspartic acid and 6.9 mM sodium taurocholate failed to initiation of *C. difficile* spore germination (figure 3.4B) as in previous experiments (figure 3.2A), while *C. difficile* spores exposed to 50 mM calcium chloride and 6.9 mM sodium taurocholate demonstrated a statistically significant *(P < 0.001)* decrease in relative OD_{570} at 10, 30 and 60 minutes when compared to *C. difficile* spores and 0.4% (w/v) aspartic acid only at the equivalent time point. When *C. difficile* spores were exposed to 0.4% (w/v) aspartic acid in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate germination was not initiated, there was no observed decrease in the relative OD_{570} over the time course of the assay (figure 3.4B).

Controls of the germination buffer Tris-HCl with 6.9 mM sodium taurocholate and 0.4% (w/v) glycine, 0.4% (w/v) aspartic acid or 50 mM calcium chloride in the absence of spores were included in the optical density assay to ensure the observed decrease in relative optical density were the results of the transition in refractive index of germination spores. A maximum change of +/-1.4 %
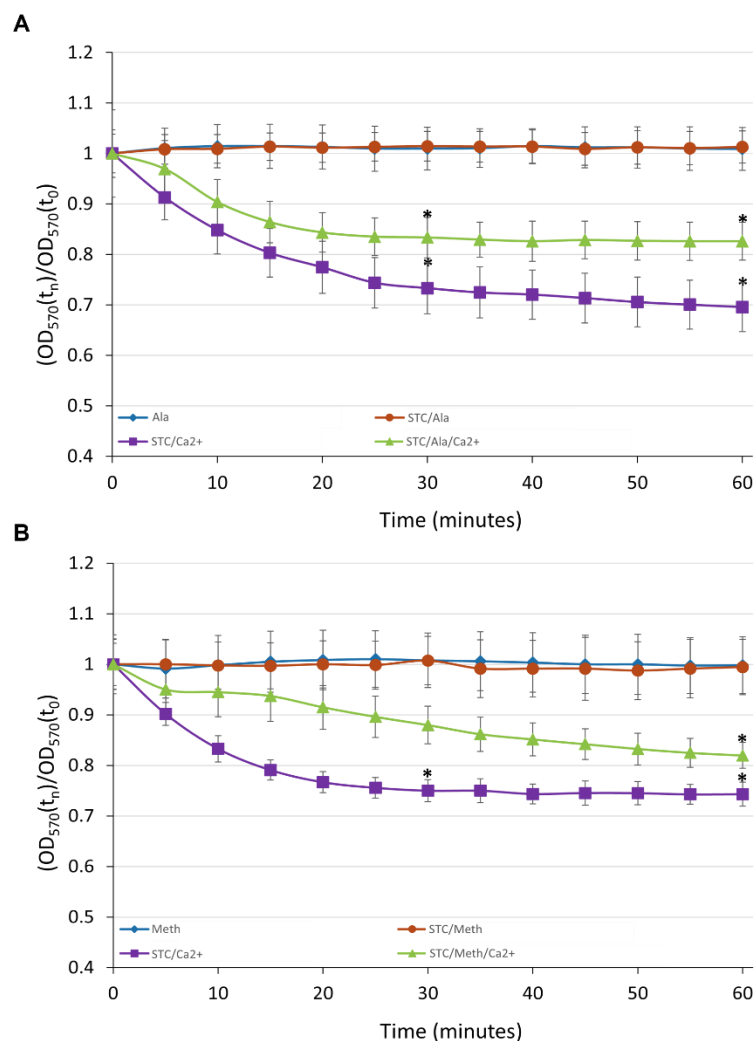
Figure 3.5. The effect of calcium in combination with amino acids alanine and methionine on *C. difficile* **spore germination. The activity of calcium and alanine (A), calcium and methionine (B) to co-induce** *C. difficile* **NCTC 11204 spore germination with sodium taurocholate was investigated. Purified** *C. difficile* **spores and Tris-HCl buffer were mixed with 0.4% (w/v) of the specified amino acid: dark blue diamonds, 6.9 sodium taurocholate and 0.4% (w/v) of the specified amino acid: red circles, 6.9 mM sodium taurocholate and 50 mM calcium chloride: purple squares, 6.9 mM sodium taurocholate, 50 mM calcium chloride and 0.4% (w/v) of the specified amino acid: light green triangles. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values at time points 10, 30 and 60 minutes compared to purified** *C. difficile* **spores and 0.4% (w/v) of the specified amino acid only at the equivalent time point are indicated by an asterisk (*P* < 0.001).**

of the relative OD_{570} was observed at the end point of the assay. Negative controls included in the assays where *C. difficile* spores were exposed to germination buffer Tris-HCl and glycine, aspartic acid or calcium in the absence of sodium taurocholate resulted in a small -2.4% change in relative OD_{570} over the time course of the assay.

As previously established, the exposure of *C. difficile* spores to 0.4% (w/v) alanine, methionine valine, serine, arginine, and histidine with 6.9 mM sodium taurocholate failed to initiation *C. difficile* spore germination while the co-germination activity of calcium in combination with 6.9 mM sodium taurocholate (in the absence of amino acids) was demonstrated as statistically significant ($P < 0.001$) at 30 and 60 minutes for each germination assay (Figures 3.5, 3.6, 3.7). Only when *C. difficile* spores were exposed to 0.4% (w/v) of alanine, methionine, valine, arginine, and histidine and 50 mM calcium chloride in combination with 6.9 mM sodium taurocholate was germination initiated.

C. difficile spores exposed to 0.4% (w/v) of alanine or methionine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate demonstrated a slow decrease in relative OD_{570} over the time course of the assay (figure 3.5). The extent of the germination response, as measured by the relative OD_{570} values at the end point of the assay was reduced compared to that of the response to 50 mM calcium chloride and 6.9 mM sodium taurocholate. A reduction in relative OD_{570} from 1.0 at time point zero to 0.83 and 0.82 at 60 minutes was observed following expose of *C. difficile* spores to alanine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate, and methionine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate, respectively. The decrease in relative OD_{570} at 30 and 60 minutes in response to 0.4% (w/v) alanine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate when compared to that of 0.4% (w/v) alanine only at the equivalent time point was found to be statistically significant ($P = 0.0046$ and P = 0.0073 respectively). The decrease in relative OD_{570} at 60 minutes in response to 0.4% (w/v) methionine in combination with 50 mM calcium chloride and 6.9
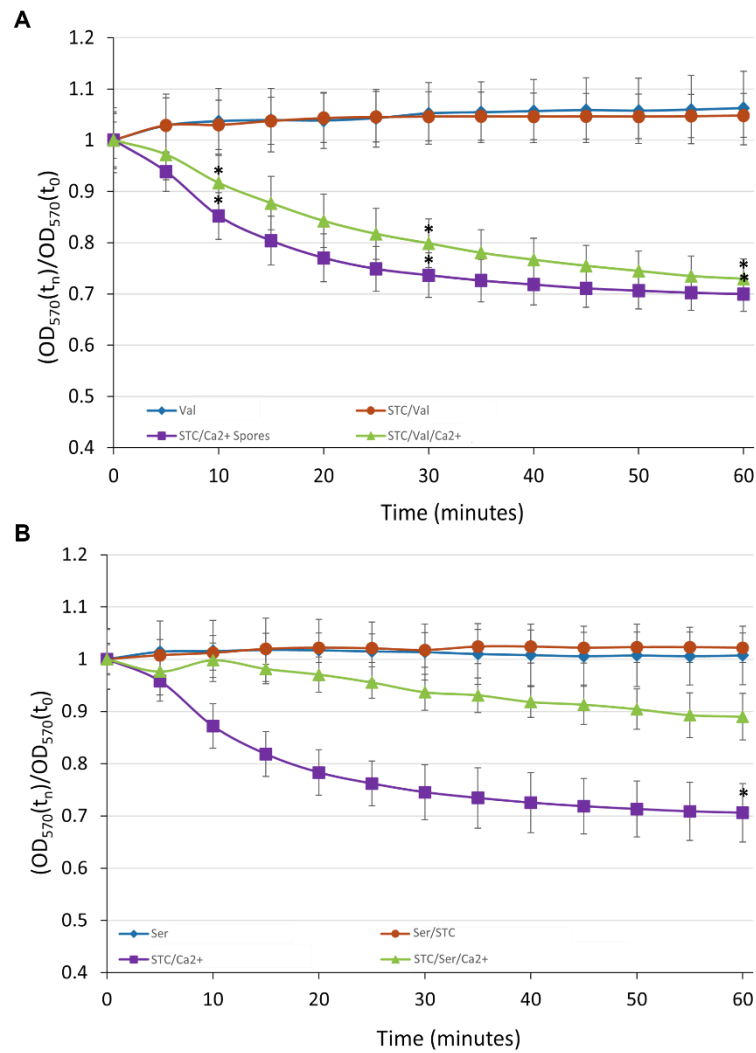
Figure 3.6 The effect of calcium in combination with amino acids valine and serine on *C. difficile* spore germination. The activity of calcium and valine (A), calcium and serine (B) to co-induce *C. difficile* NCTC 11204 spore germination with sodium taurocholate was investigated. Purified *C. difficile* spores and Tris-HCl buffer were mixed with 0.4% (w/v) of the specified amino acid: dark blue diamonds, 6.9 mM sodium taurocholate and 0.4% (w/v) of the specified amino acid: red circles, 6.9 mM sodium taurocholate and 50 mM calcium chloride: purple squares, 6.9 mM sodium taurocholate, 50 mM calcium chloride and 0.4% (w/v) of the specified amino acid: light green triangles. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values at time points 10, 30 and 60 minutes compared to purified *C. difficile* spores and 0.4% (w/v) of the specified amino acid only at the equivalent time point are indicated by an asterisk (*P* < 0.001).

mM sodium taurocholate when compared to that of 0.4% (w/v) methionine only at the equivalent time point was found to be statistically significant ($P < 0.0001$)

Exposure of *C. difficile* spores to valine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate demonstrated a similar pattern of reduction of the relative OD_{570} over the time course of the assay and a similar extent in reduction to that of the response of *C. difficile* spore to 50 mM calcium chloride and 6.9 mM sodium taurocholate (figure 3.6A), a decrease in relative OD_{570} from 1.0 at time point zero to 0.69 at 60 minutes was observed. The decrease in relative OD_{570} at 30 and 60 minutes in response to 0.4% (w/v) valine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate when compared to that of 0.4% (w/v) valine only at the equivalent time point was found to be statistically significant ($P < 0.001$).

C. difficile spores exposed to 0.4% (w/v) serine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate resulted in a slow reduction of the relative OD_{570} over the time course of the assay with a limited extent of the reduction. The decrease in relative OD_{570} at 60 minutes was not determined to be statistically significant (figure 3.6B).

C. difficile spores exposed to 0.4% (w/v) of arginine or histidine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate demonstrated a very similar germination response to that of the response to 50 mM calcium chloride and 6.9 mM sodium taurocholate alone (figure 3.7). The results demonstrate a moderate rate in reduction of the relative OD_{570} during the initial 20 minutes followed by a more gradual reduction over the remainder of the assay. A reduction in relative OD_{570} from 1.0 at time point zero to 0.70 and 0.67 at 60 minutes was observed following expose of *C. difficile* spores to arginine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate, and histidine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate, respectively. The decrease in relative OD_{570} at 30 and 60 minutes in response to 0.4% (w/v) arginine in combination with 50 mM calcium chloride and 6.9 mM sodium taurocholate, and 0.4% (w/v) histidine in combination with 50 mM calcium chloride and 6.9 mM
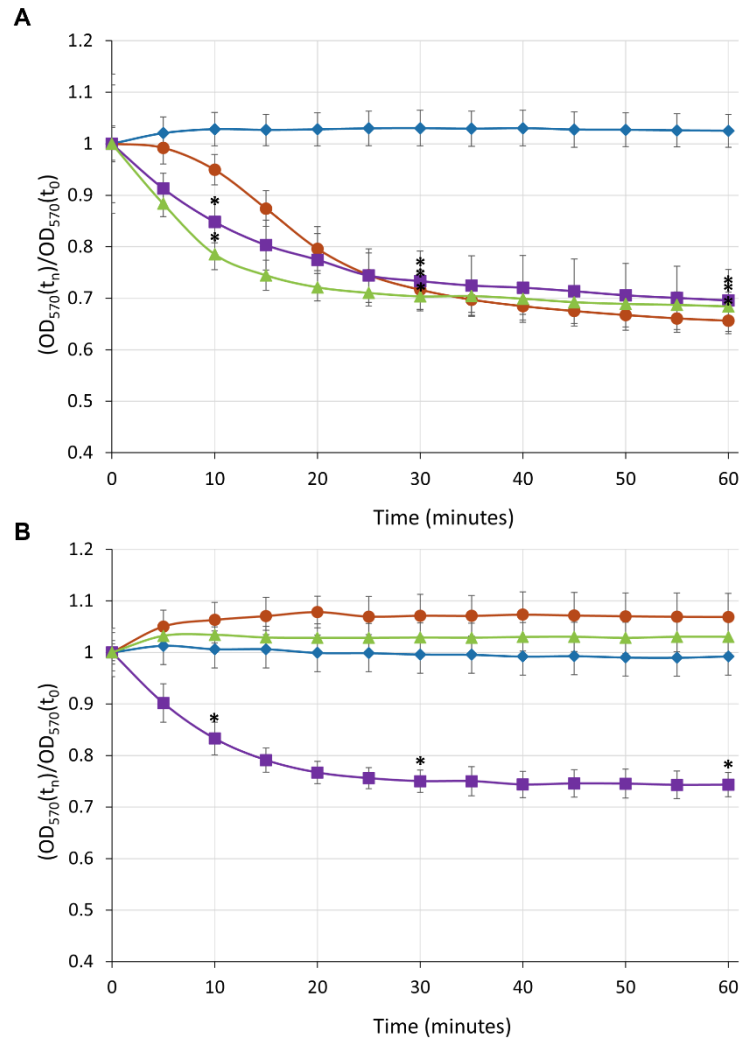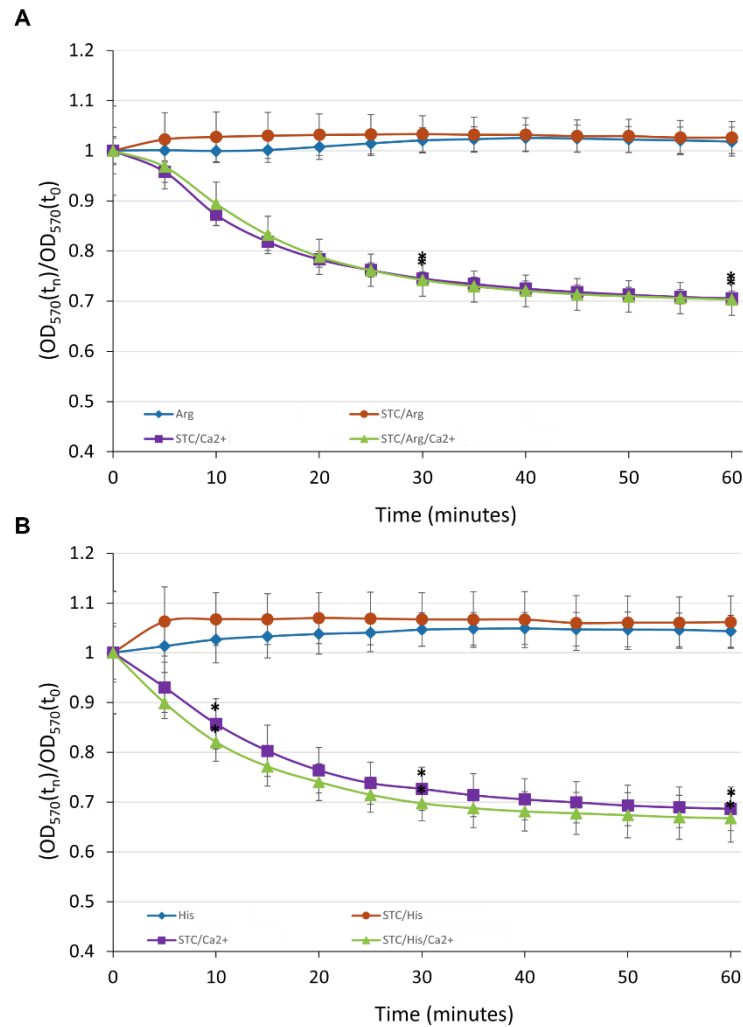
Figure 3.7 The effect of calcium in combination with amino acids arginine and histidine on *C. difficile* spore germination. The activity of calcium and arginine (A), calcium and histidine (B) to co-induce *C. difficile* NCTC 11204 spore germination with sodium taurocholate was investigated. Purified *C. difficile* spores and Tris-HCl buffer were mixed with 0.4% of the specified amino acid: dark blue diamonds, 6.9 mM sodium taurocholate and 0.4% (w/v) of the specified amino acid: red circles, 6.9 mM sodium taurocholate and 50 mM calcium chloride: purple squares, 6.9 mM sodium taurocholate, 50 mM calcium chloride and 0.4% of the specified amino acid: light green triangles. Error bars represent standard deviation. Statistically significant reductions of the optical density ratio values at time points 10, 30 and 60 minutes compared to purified *C. difficile* spores and 0.4% (w/v) of the specified amino acid only at the equivalent time point are indicated by an asterisk ($P < 0.001$).

sodium taurocholate when compared to that of 0.4% (w/v) of the specified amino acid only at the equivalent time point was found to be statistically significant ($P < 0.001$).

Controls of the germination buffer Tris-HCl with 6.9 mM sodium taurocholate and 0.4% (w/v) of the specified amino acid (alanine, methionine, valine, serine, arginine, histidine) or 50 mM calcium chloride in the absence of spores were included in each of the optical density assays to ensure the observed decrease in relative optical density were the results of the transition in refractive index of germination spores. A maximum change of +/-2.2 % of the relative OD_{570} was observed at the end point, across the germination assay conducted. Negative controls included in the assays where *C. difficile* spores were exposed to germination buffer Tris-HCl and alanine, methionine, valine, serine, arginine, histidine, or calcium in the absence of sodium taurocholate resulted in a maximum of a 6.2% change in relative OD_{570} at the end point, across the germination assay conducted.

3.3.3 The efficacy of glycine in combination with other amino acids as co-germinants of *C. difficile* NCTC 11204 spore germination.

The co-germination activity of amino acids in combination with glycine to initiation of *C. difficile* spore germination with known bile salt germinant taurocholate was also investigated. The change in relative OD_{570} at the end point of the assay as a result of exposure of spores to the amino acids combination and sodium taurocholate was compared to that achieve by glycine and sodium taurocholate to determine if the germination response was altered.

C. difficile spores exposed to 0.4% (w/v) valine in combination with 0.4% (w/v) glycine and 6.9 mM sodium taurocholate initiated germination, causing a steady decrease in relative OD_{570} over the time course of the assay (figure 3.8). The rate and extent of spore germination however was limited when compared to that of exposure to 0.4% (w/v) glycine and sodium taurocholate. A reduction in relative OD_{570} from 1.0 at time point zero to 0.78 at 60 minutes, a 21.5% decrease, was observed.
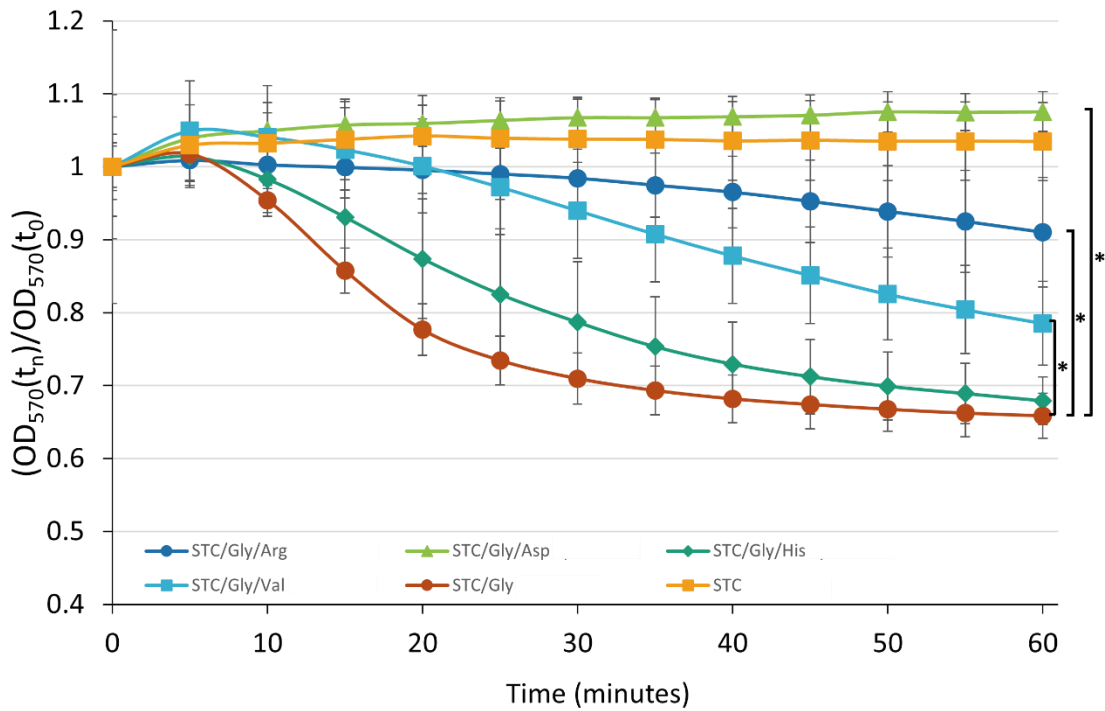
Figure 3.8 The effect of glycine in combination with the amino acids valine, arginine, aspartic acid and histidine on *C. difficile* NCTC 11204 spore germination. The activity of glycine and arginine, glycine and valine, glycine and aspartic acid, glycine and histidine to co-induce *C. difficile* spore germination with sodium taurocholate was investigated. Purified *C. difficile* spores and Tris-HCl buffer were mixed with 6.9 mM sodium taurocholate: orange squares, 0.4% (w/v) glycine: red circles, 0.4% (w/v) glycine and 0.4% (w/v) valine: light blue squares, 0.4% (w/v) glycine and 0.4% (w/v) arginine: dark blue circles, 0.4% (w/v) glycine and 0.4% (w/v) aspartic acid: light green triangles, 0.4% (w/v) glycine and 0.4% (w/v) histidine: dark green diamonds. A statistically significant change in the optical density ratio values at 60 minutes compared to that of purified *C. difficile* spores, 6.9 mM sodium taurocholate and 0.4% glycine at the equivalent time point are indicated by an asterisk ($P < 0.001$).

The relative OD_{570} value at 60 minutes, as a result of exposure of *C. difficile* spore to 0.4% (w/v) valine in combination with 0.4% (w/v) glycine and 6.9 mM sodium taurocholate was found to be

statistically significantly different ($P < 0.001$) from that of 0.4% (w/v) glycine and 6.9 mM sodium taurocholate, which caused a 34.2% decrease in the relative OD_{570} value.

The exposure of *C. difficile* spores to the combination of 0.4% (w/v) arginine and 0.4% (w/v) glycine with 6.9 mM sodium taurocholate caused a reduction in relative OD_{570} over the course of the assay to 0.91 at 60 minutes, a 9% decrease. The relative OD_{570} value achieved by 0.4% (w/v) arginine and 0.4% (w/v) glycine with 6.9 mM sodium taurocholate at 60 minutes was determined to be statistically significantly ($P < 0.001$) from that of 0.4% (w/v) glycine and 6.9 mM sodium taurocholate at the same time point. The exposure of *C. difficile* spores to 0.4% (w/v) aspartic acid in combination with 0.4% (w/v) glycine and 6.9 mM sodium taurocholate failed to initiate *C. difficile* spore germination. Due to the lack of germination response, the relative OD_{570} values of spores exposed to 0.4% (w/v) aspartic acid in combination with 0.4% (w/v) glycine and 6.9 mM sodium taurocholate when compared to 0.4% (w/v) glycine and 6.9 mM sodium taurocholate were found to statistically significantly different ($P < 0.001$) at 60 minutes.

C. difficile spores exposed to 0.4% (w/v) histidine in combination with 0.4% (w/v) glycine and 6.9 mM sodium taurocholate initiated germination, causing a moderately rapid decrease in relative OD_{570} over 60 minutes. The rate and extent of spore germination was similar to that of exposure to 0.4% (w/v) glycine and sodium taurocholate. A reduction in relative OD_{570} from 1.0 at time point zero to 0.68 at 60 minutes, equating to a 32.1% decrease was observed. The relative OD_{570} value achieved by 0.4% (w/v) histidine and 0.4% (w/v) glycine with 6.9 mM sodium taurocholate at 60 minutes was not found to be statistically significantly different from the germination response caused by 0.4% (w/v) glycine and 6.9 mM sodium taurocholate.

Controls of the germination buffer Tris-HCl with 6.9 mM sodium taurocholate, 0.4% (w/v) glycine and 0.4% (w/v) of the specified amino acid (valine, arginine, aspartic acid and histidine) in the absence of spores were included on each of the optical density assay to ensure the observed decrease in relative optical density were the results of the transition in refractive index of

germination spores. A maximum change of +/- 1.2 % of the relative OD_{570} was observed at the end point, across the germination assay conducted. For the negative control *C. difficile* spores were exposed to the germination buffer with Tris-HCl and valine, arginine, aspartic acid and histidine or glycine in the absence of sodium taurocholate which resulted in a maximum of a 6.3% change in relative OD_{570} at the end point, across the germination assay conducted.

3.4 Discussion.

To develop an effective *C. difficile* pro-germination strategy, knowledge of the role of co-germinants in the initiation of *C. difficile* spore germination is an important consideration. Under optimal laboratory conditions the amino acid glycine and calcium have been demonstrated to be potent co-germinants of *C. difficile* but the activity of other amino acids as co-germinants is varied or absent depending on the *C. difficile* strain and experimental conditions utilised in studies (Sorg and Sonenshein, 2008; Howerton *et al.*, 2011; Shrestha and Sorg, 2017; Kochan *et al.*, 2017). While the activity of individual amino acids as co-germinants may vary, combining specific amino acids has been demonstrated to act as an effective co-germinant solution, along with taurocholate to induce *C. difficile* germination (Wheeldon *et al.*, 2010; 2011; Nerandzic and Donskey, 2010; 2013). The role of calcium as a co-germinant to initiate taurocholate-induced *C. difficile* germination was identified more recently (Kochan *et al.*, 2017). Furthermore, the release of endogenous calcium from the Ca-DPA complex during the initial stage of germination, is believed to contribute to the activation of germination to enhance the spore germination response in the presence of low concentration of glycine (Kochan *et al.*, 2017; Kochan *et al.*, 2018b). The dynamics of the interaction of co-germinants glycine and calcium have been studied but understanding of the efficacy of other potential amino acid co-germinants and calcium in combination to induce *C. difficile* spore germination is less clear.

In this chapter the co-germinant activity of selected amino acids was investigated utilising the optical density germination assay. Only glycine and calcium were identified as co-germinants of *C. difficile* NCTC 11204 spore germination. The germination response initiated when selected amino acids were then combined with co-germinants glycine or calcium ranged from no effect to the reduction of *C. difficile* spore germination, thus indicating possible inhibition. No combination of amino acid with co-germinant glycine or calcium was found to enhance *C. difficile* spore germination.

In line with previous studies, the activity of glycine as an amino-acid co-germinant of *C. difficile* spores was confirmed in this study (Sorg and Sonenshein, 2008; Kochan *et al.*, 2017; Shrestha and Sorg, 2017). Utilising the optical density assay, glycine as a co-germinant in combination with bile salt germinant sodium taurocholate caused the rapid initiation of the germination the spores of *C. difficile* NCTC 11204. Amino acids established by Wheeldon *et al.* (2011) to have co-germination activity when in combination with glycine to induce germination of *C. difficile* NCTC 11204 spores, were also investigated using the optical density assay. The amino acids aspartic acid, alanine, methionine, valine, serine, arginine, and histidine, at 0.4% (w/v) failed to demonstrate co-germination activity, in the absence of glycine in this study, in agreement with the findings of (Wheeldon *et al.*, 2011). Calcium was also demonstrated to be a highly effective co-germinant, causing the initiation of *C. difficile* spore germination, confirming the findings reported by (Kochan *et al.*, 2017).

The lack of independent co-germination activity demonstrated by aspartic acid, alanine, methionine, valine, serine, arginine, and histidine is supported Wheeldon's prior work (2011) but is divergent to that reported by Shrestha and Sorg (2017) and for the co-germinant activity of specific amino acids; alanine, methionine, arginine as demonstrated by Howerton *et al.* (2011). Howerton *et al.* (2011) utilised *C. difficile* strain 630 while Shrestha and Sorg (2017) used two *C. difficile* laboratory strains (UK1 and M68) in their studies. *C. difficile* spores of both strains used by Shrestha *et al.* (2017) germinated in response to the above listed amino acids but there was variability in the degree of the germination response achieved with specific amino acids between each of the *C. difficile* strains. An important difference in the experimental approach of Shrestha and Sorg (2017) and Howerton *et al.* (2011) and this study is the temperature at which the germination response to amino acid co-germinants was monitored. Shrestha and Sorg (2017) identified clear differences in activity of amino acids to function as co-germinants at 37°C and 25°C; lysine for instance acted as an effective co-germinant initiate *C. difficile* spore germination at 37°C to but did not at 25°C. Howerton *et al.* (2011) conducted experiments at 30°C in contrast to those in this study and by

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Wheeldon *et al.* (2011) which were undertaken at ambient temperature. The clear influence of temperature on co-germinant activity along with the distinct preference of *C. difficile* strains for specific amino acids co-germinants may explain the differences in amino acid co-germination activity data reported by Shrestha and Sorg (2017) and Howerton *et al.* (2011) and that presented in this study.

When *C. difficile* spores were exposed to co-germinants calcium and glycine in combination with a bile salt germinant, as is predicted to occur *in vivo*, germination was rapidly initiated *in vitro* (Kochan *et al.*, 2017; Shrestha *et al.*, 2019). The germination response of *C. difficile* NCTC 11204 spores when exposed to the combination of the co-germinant calcium and other amino acids previously demonstrated to have no independent co-germinant activity in this study, were also examined. Calcium in combination with either valine, arginine or histidine caused the initiation of spore germination that was highly similar to that of when calcium was the exclusive co-germinant, suggesting there was minimal or no impact on the germination response with the addition of these amino acids. Conversely, the combination of calcium and aspartic acid with germinant sodium taurocholate failed to initiate a germination response, despite the presence of the calcium, known to be a potent co-germinant (Kochan *et al.*, 2018b). When *C. difficile* spores were exposed to amino acids alanine, methionine and serine with calcium and sodium taurocholate, germination was initiated but the rate and extent of the germination response was decreased compared to that of calcium as the exclusive co-germinant, suggesting the co-germinant activity of calcium was inhibited. The combination of calcium and alanine or methionine caused a slow but gradual initiation of germination over the time course of the optical density assay with both amino acids in combination with calcium and taurocholate demonstrating a statistically significant ($P < 0.001$) germination response at the end point of the assay. The combination of calcium and serine also caused a decrease in relative OD_{570} indicating the initiation of germination, but the extent of germination response was not determined to statistically significant after 60 minutes.

Research by Kochan *et al.* (2018b) demonstrated the synergistic activity of calcium and glycine as co-germinants to initiate germination, particularly when glycine was at low concentrations. In this study, calcium and glycine independently or in combination as co-germinants produced a similar germination response, but the use of high concentrations of glycine (0.4% v/w glycine is equivalent to 54 mM) and the experimental approach used here does not allow the identification of synergism. When calcium was combined with valine, arginine, or histidine (and sodium taurocholate) a similar germination response was demonstrated but it is unclear if this is due to the efficient action of calcium as co-germinant or a synergistic interaction. Utilising terbium DPA germination assay, Kochan *et al.* (2018b) also observed this effect, although not with the same profile of amino acids. Calcium was seen to enhance the germination response of alanine, serine and to a limited extent histidine, when at suboptimal concentrations. Importantly these amino acids were not able to act as co-germinants independently, reflecting the observations of this study (Kochan *et al.*, 2018b).

The germination response initiated by the exposure of *C. difficile* spores to alanine or methionine and calcium could be interpreted as the synergistic activity of calcium to enhance germination but alternatively, it could be viewed as an inhibitory effect of the presence of these amino acids on the efficient co-germinant activity of calcium. Furthermore, the germination response exhibited by *C. difficile* spores to the combination of serine and calcium was limited to the point it was not determined to statistically different from the control. Taken together, this suggests that some amino acids may adversely affect the germination response, inhibiting calcium and taurocholate-induced *C. difficile* spore germination.

Combining amino acids with glycine was previously shown to enhance the efficacy of glycine to induce *C. difficile* NCTC 11204 spore germination by Wheeldon *et al.* (2011). However, findings from this study demonstrated the presence of specific amino acids supressed the co-germination activity of glycine and taurocholate to induced germination *C. difficile* NCTC 11204 spores. Histidine in

combination with glycine and sodium taurocholate initiated a germination response that was similar to that of glycine as the exclusive co-germinant, indicating it had minimal or no impact on the germination response, which was not determined to statistically different from that of the germination response to glycine and sodium taurocholate. However, arginine or valine in combination with glycine limited the germination response, causing a statistically significant reduction in the extent of the germination response when compared to glycine and sodium taurocholate. Aspartic acid in combination with glycine abolished the *C. difficile* spore germination response completely. This suggests arginine and valine have a suppressive effect on glycine and taurocholate-induced *C. difficile* spore germination. Furthermore, aspartic acid prohibited the germination of spores completely, indicating it acted as an inhibitor of *C. difficile* NCTC 11204 spore germination. A study of the synergy of glycine with amino acids as co-germinants, via the measurement of Ca-DPA release from germinating spores, revealed glycine at low concentration was not able to act synergistically with amino acids. The authors concluded that when combining amino acids, the germination response would likely be the result of an additive effect (Kochan *et al.*, 2018b). The data presented in this current study indicated the inclusion of specific amino acids was inhibitory to the efficient co-germinant activity of glycine to initiate *C. difficile* germination. Howerton *et al.* (2011) described amino acid that failed to initiate germination as 'weak co-germinants'. The authors also proposed that weak amino acid co-germinants may inhibit the efficient action of strong co-germinants germinants under some circumstances by competing for the co-germinant receptor. Characterisation of the interaction of glycine and other amino acid by Howerton *et al.* (2011) however did not identify a combination of amino acids that inhibited *C. difficile* germination in their research.

Although there are important differences in the experimental approach and reported outcome, evidence of an inhibitory effect is also indicated in the data presented by Wheeldon *et al.* (2011). The activity of glycine independently as a co-germinant initiated the germination of *C. difficile* spores causing an approximate 0.5 log reduction of *C. difficile* NCTC 11204 when challenged to heat

shock at 70°C for 10 minutes. The combination of glutamic acid, glutamine, asparagine, cysteine, or lysine with glycine all resulted in a decrease in the achieved log-reduction and therefore the initiation of *C. difficile* germination (Wheeldon *et al.*, 2011).

With the exception of aspartic acid, the activity of amino acids in combination with glycine to act as co-germinants of *C. difficile* NCTC 11204 confirms the profile of co-germinants identified by Wheeldon *et al.* (2011). The lack of co-germinant activity of aspartic acid with glycine was unexpected when characterising the germination response of the same laboratory strain in this study. This disparity could be due to the differences in experimental methodology and spore preparation. In this study, evidence of spore germination was monitored spectrophotometry by the decrease in refractive index of germination spores while Wheeldon measured germination by utilising the loss of germinated spore's resistant properties to heat. The *C. difficile* spores utilised by Wheeldon *et al.* (2011) underwent a short sporulation period before the crude spore suspension was harvested. This was followed by minimal purification of the suspensions to remove cell debris and other contaminants. In contrast, the utilisation of the optical density germination assay required substantial purification of spores which were derived from a period of sporulation over 5-7 days. This necessary purification process is likely to alter the biological variation amongst spores, producing a homogenous population of spores that germinate more consistently, distinct from those with minimal manipulation. Malyshev and Baillie (2020) demonstrated the morphotype of *C. difficile* spores was highly influenced by the methodology utilised to produce purified suspensions.

The optical density germination assay completed in this study were completed at ambient temperature to reflect the environmental conditions of a clinical setting. Monitoring the germination response of spores of *C. difficile* NCTC 11204 to amino acid co-germinants at 30°C or 37°C as per Howerton *et al.* (2011) and Shrestha and Sorg (2017) may reveal a different profile of amino acids that are able function as co-germinants glycine in the absence or presence of glycine and calcium, however this is not applicable to the administration of a pro spore-germination

elimination strategy which would need to function within the conditions of clinical environment. Equally heat activation of spores in a laboratory environment, was found to enhance the germination response of *C. difficile* spores exposed to a germination solution containing amino acid co-germinants and taurocholate, but this has limited relevance as the biology of heat shocked spores is not consistent with those excreted into the environment by patients (Nerandzic and Donskey, 2013; Samore *et al.*, 1996; Crobach *et al.*, 2018).

The activity of co-germinants as reported in this study are considered in context with the findings of other research but are limited by the use of a single *C. difficile* laboratory strain and the narrow range of amino acids investigated. The strength of conclusions would be improved if the co-germinant activity of a wider selection of amino acids was investigated with multiple laboratory and clinical strains of *C. difficile*. Particularly, the inclusion of *C. difficile* strains utilised by other researchers characterise co-germinant activity of amino acids or calcium.

Collectively, the data presented in this chapter and that reported by other researchers demonstrates the role of co-germinants in the initiation of *C. difficile* germination is complex with clear disparity in the reported activity of co-germinants depending on the selected *C. difficile* strain and the experimental approach taken to monitor germination. Glycine is consistently reported as an efficient amino acid co-germinant across multiple studies but when in combination with specific amino acids in this study, this activity was inhibited. The capacity of amino acids to inhibit co-germinant activity was also seen with calcium. This has import implications when considering the inclusion of co-germinants in a pro-germination strategy to reduce the reservoir of *C. difficile* spores in clinical environments. Including a combination of amino acids co-germinants may prove to be detrimental to spore germination, causing the competitive inhibition of the co-germinant receptor. A strong co-germinant would need to be at a concentration sufficient to overcome any potential inhibitory activity of competing weak amino acids co-germinants. Inclusion of either glycine or calcium as the exclusive co-germinant may overcome this problem but biological contamination in

the environment may also be a source of inhibitory amino acids to limit the efficiency of the initiation of germination to render spores susceptible to antimicrobials.

The research presented in this part of the study further contributes to existing knowledge relating to the co-germination potential of amino acids and calcium in the germination of *C. difficile* spores. In this chapter, the study of amino acids to function as co-germinants during the initial phase of germination revealed an absence of co-germinant activity to initiate *C. difficile* spore germination independently, with the exception of glycine. Calcium was also confirmed as a potent co-germinant of *C. difficile* spore germination. Measurement of the germination response initiated by amino acids in combination with either glycine or calcium identified combinations of co-germinants that were effective at initiating *C. difficile* spore germination and those where specific amino acids inhibited the co-germinant activity of glycine and calcium to initiate germination. Understanding the potential interactions of *C. difficile* spore co-germinants may be a key detail for the development of a successful pro spore-germination elimination strategy to eliminate *C. difficile* spores from the environment.

Chapter 4

4.0 Electron microscopy and cryo-soft X-ray tomography of germinating *Clostridioides difficile* spores.

4.1 Introduction.

The conserved protective structure of *C. difficile* spores ensures their survival and dissemination in the environment to transmit CDI (Driks, 2003; Setlow, 2006; Deakin *et al.*, 2012; Paredes-Sabja *et al.*, 2014). The spore's structure, detailed in figure 1.7, comprises a central dehydrated core preserving the critical enzymes, ribosomes, tRNA and DNA which is saturated with protective small acid-soluble proteins (SASPs). The low water content (25-60% of wet weight) of the core, is essential for dormancy and is maintained by high levels of Ca-DPA (Setlow, 2006; Setlow, 2014; Paredes-Sabja *et al.*, 2014). The spore inner membrane surrounds the spore core, limiting permeability of water and harmful compounds and is itself enclosed by the germ cell wall (Nicholson *et al.*, 2000; Paredes-Sabja *et al.*, 2014; Setlow, 2006). A dense layer of specialised peptidoglycan makes up the large cortex layer providing resistance to heat and is important for dormancy (Popham, 2002; Paredes-Sabja *et al.*, 2014). The cortex is surrounded by the outer membrane, which is not believed to have properties that restrict permeability or contribute to resistance (Nicholson *et al.*, 2000). The proteinaceous multi-layered spore coat protects against large toxic compounds and enzymatic attack (Driks, 2003; Paredes-Sabja *et al.*, 2014). The outermost spore layer, the exposorium has a variable morphology and is not present on all *C. difficile* strains but has been associated with a contribution to spore resistance properties (Pizarro-Guajardo *et al.*, 2016; Barra-Carrasco *et al.*, 2013).

High-resolution microscopy has been used to study the internal structure of *C. difficile* spores but there has been limited examination of the changes that occur upon the initiation of germination

(Paredes-Sabja *et al.*, 2014; Barra-Carrasco *et al.*, 2013; Escobar-Cortés *et al.*, 2013; Joshi *et al.*, 2012; Permpoonpattana *et al.*, 2011). In a study of the morphology and ultrastructure of *C. difficile* spores, transmission electron microscopy (TEM) imaging was used to identify the features of the internal spore structure comprising of the spore core, inner membrane, germ cell wall, cortex, spore coat and exosporium (Rabi *et al.*, 2017). Particularly of interest was the morphology of the exosporium which was observed as an enclosed layer with numerous peaks or bumps protruding from the spore. Scanning electron microscopy (SEM) imaging confirmed the bumpy morphology of the exposorium which covers the entire surface of the spore, and was apparent on spores of all *C. difficile* strains examined in the study (Rabi *et al.*, 2017).

Research has demonstrated the outer structures of *C. difficile* spores are particularly different to those of other spore-forming bacteria. The spore coat is made up of multiple layers or laminations with defined inner and outer coat layers being identified in some TEM images of *C. difficile* spores (Paredes-Sabja *et al.*, 2014; Rabi *et al.*, 2017; Barra-Carrasco *et al.*, 2013; Setlow, 2007). The morphology of the outermost spore layer, the exosporium is distinctive in *C. difficile* spores. A bumpy exosporium phenotype has previously been observed in TEM images of *C. difficile* spores but a thin and smooth morphology or even absence of the exosporium has also been reported. Significantly this variation in morphotype occurs between and also within strains of *C. difficile* (Joshi *et al.*, 2012; Paredes-Sabja *et al.*, 2014; Pizarro-Guajardo *et al.*, 2016). Pizarro-Guajardo *et al.* (2016) reported two distinct phenotypes of the exosporium, a thick morphotype often with protrusions or thin smooth morphotype. Differences in the exosporium phenotype have been shown to be dependent upon the methodology used to generate and purify spore suspensions (Malyshev and Baillie, 2020; Escobar-Cortés *et al.*, 2013). Malyshev and Baillie (2020) demonstrated differing spore preparation and purification techniques resulted in a disparity in the morphology of *C. difficile* spores of the same strain. The inclusion of a sonication step was found to result in a higher proportion of spores with a smooth exosporium morphology. Spore preparation methodology was also found to influence the size and shape of *C. difficile* spores with differences in length and width

of spores reported between the spore production and purification methods (Malyshev and Baillie, 2020).

The research of Kochan *et al.* (2017), Francis *et al.* (2013), Adams *et al.* (2013), Fimlaid *et al.* (2015), Francis and Sorg (2016) and Shrestha *et al.* (2019) has revealed the key regulators of *C. difficile* spore germination, identifying CspC as the bile salt germinant receptor and the requirement of CspA in the recognition of calcium and amino acid co-germinants. While CspC, CspB and CspA are demonstrated as essential for the downstream transmission of the germination signal and transformation of the spore, the exact location of these proteins within the internal structure of the *C. difficile* spore has not been determined. Within the proposed germinosome complex model of the regulation of *C. difficile* germination, CspC, CspB and CspA are predicted to be co-located internally on the spore outer membrane, along with pro-SleC. Germinant and co-germinants must therefore transit the external spore structures of the exposorium and spore coat, crossing the inner membrane to reach their corresponding receptors (Francis *et al.*, 2013; Francis and Sorg, 2016; Adams *et al.*, 2013; Fimlaid *et al.*, 2015; Shrestha *et al.*, 2019; Lawler *et al.*, 2020). The 'Lock and Key' model suggest the germinant receptor CspC and co-germinant receptor CspA are located on the outer membrane and extend into the layers of the spore coat, while CspB is situated internally on the outer membrane. Activation of the germinant receptor facilitates access of co-germinants across the outer membrane and to the internal layers of the spore. A proposed uncharacterised amino acid dependant receptor is located on the spore inner membrane, which facilitates the release of endogenous calcium from the spore core (Kochan *et al.*, 2017; Kochan *et al.*, 2018a).

While TEM imaging has allowed recognition of the internal spore structure, the presence of the inner and outer membrane have not been definitively confirmed within the internal structure of *C. difficile* spores. The fluorescent probe Laurdan (6-dodecanoyl-N,N-dimethyl-2-naphthylamine) can be utilised to detect changes in fluidity of lipid membranes which exists as complex systems combining liquid ordered or disordered states, previously described as gel and liquid phases

(Klymchenko and Kreder, 2014). Distribution of Laurdan within the lipid membrane allows the fluidity of the lipid membrane to be studied: the sensitivity of Laurdan to the polarity of the lipid membrane can be detected by a shift in the Laurdan emission spectrum (Jay and Hamilton, 2017). The peak emission spectra of the liquid ordered (440 nm) and disordered (490 nm) state can be utilised to calculate the generalized polarization function (Parasassi *et al.*, 1990). Laurdan has been used successfully employed to study membrane fluidity of vegetative bacterial cells in response to high pressure and antibiotics (Ulmer *et al.*, 2002; Bessa *et al.*, 2018). Furthermore Hofstetter *et al.* (2012) demonstrated a methodology for Laurdan labelling of spore membranes of *Clostridium* species in order to determine generalized polarization measurements. The generalized polarization values of Laurdan-labelled spores of *Clostridium sporogenes* and *Clostridium beijerinckii* were determined to be high, indicating a liquid ordered state or gel phase of membrane with low fluidity (Hofstetter *et al.*, 2012).

The aim of research presented in this chapter was to utilise high resolution microscopy techniques to study the morphology and structure of *C. difficile* spores following the initiation of germination. Scanning and transmission electron microscopy were selected to investigate changes to the external and internal structures of *C. difficile* spores exposed to bile salt germinants and co-germinants. Novel use of cryogenic soft X-ray tomography (cryo-SXT) and the labelling of *C. difficile* spores with Laurdan sought to examine the internal structural changes that occur during germination and identify the location of the inner and outer spore membrane. Creating a greater understanding of the spore structural changes that occur during the initial phase of *C. difficile* germination and the confirmation of the presence of the inner and outer spore membranes may contributing to clearer understanding of the regulation pathway of *C. difficile* germination.

4.2 Methods.

4.2.1 Bacterial strains and growth conditions.

C. difficile NCTC 11204 was sourced from Jon Brazier (HPA Anaerobic Reference Laboratory, Cardiff). *C. difficile* 630 (NCTC 13307, ribotype 012) was sourced from National Collection of Type Cultures, Public Health England. Frozen stocks of *C. difficile* were stored at -80˚C on Microbank™ beads (Pro Lab Diagnostics, Canada) and resuscitated by inoculating a bead onto Wilkins-Chalgren agar (Oxoid, Basingstoke, UK) supplemented with 0.1% (w/v) sodium taurocholate hydrate (Alfa Aesar, UK) and incubating anaerobically (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK) at 37°C for 48 hours.

4.2.2 Preparation of spore suspensions of *C. difficile*.

Spore suspensions of *C. difficile* NCTC 11204 and *C. difficile* 630 used in electron microscopy and cryo-soft X-ray tomography (cryo-SXT) were prepared using a modified version of the methodology described by Heeg *et al.* (2012) as described in chapter 2, section 2.2.2 of this thesis.

In order to label *C. difficile* spores with Laurdan a single colony of the resuscitated *C. difficile* strains NCTC 11204 and 630 was used to inoculate 10 ml of pre-reduced BHI broth (Oxoid, Basingstoke, UK) and incubated anaerobically at 37°C for 4 hours. Aliquots of 100 µl of the *C. difficile* cultures were then used to inoculate BHIS agar plates pre-treated with 70 mM Laurdan dissolved in 100% ethanol. Briefly, 400 µl of 70 mM Laurdan was spread on the surface of BHIS agar plates (90 mm petri dishes) and allowed to dry in the absence of light. The Laurdan-treated BHIS agar plates were transferred to a light-proof box and pre-reduced in the anaerobic cabinet for 2 hours before inoculation with *C. difficile.* The inoculated Laurdan BHIS agar plates were incubated anaerobically at 37°C for 5-7 days. The presence of a high concentration of spores was confirmed by staining with 5% (w/v) malachite green solution and counterstaining with 0.5% (v/v) safranin as per the

Schaeffer-Fulton endospore staining technique (Schaeffer and Fulton, 1933). Spores were harvested and purified as described in chapter 2, section 2.2.2 of this thesis. Purified spores cultured in the presence of Laurdan were resuspended in SDW and stored at 4°C protected from the dark. The labelling of spores with Laurdan was confirmed by measuring the fluorescence (excitation: 360 nm, emission: 440/490) in a fluorescent plate reader (SpectraMax Gemini EM).

4.2.3 Preparation of *C. difficile* spores for electron microscopy.

Spore suspensions of *C. difficile* NCTC 11204 and *C. difficile* 630 were prepared at an optical density of 3.0 at 570_{nm} equivalent to approximately 1.0×10^8 CFU/ml. Aliquots of 150 µl of spore suspension were transferred to labelled 1.5 ml microcentrifuge tubes containing 1.35 ml BHIS media or BHIS media supplemented with 6.9 mM sodium taurocholate and incubated for 5, 10, 20 and 60 minutes. At the appropriate time period the microcentrifuge tubes containing spores were centrifuged at 17000g for 1 minute to pellet the spores. The supernatant was removed without disturbing the pellet and replaced with 1.35 ml 2.5% glutaraldehyde (v/v), 0.1 M sodium cacodylate buffer at pH 7.3, for 1 hour at ambient temperature to fix the samples. Following fixation, the spore samples were centrifuged for 1 minute at 17000g. The supernatant was removed and replaced with 500 µl 0.1 M sodium cacodylate buffer. The *C. difficile* spore samples were stored at 4°C.

4.2.4 Transmission electron microscopy.

C. difficile spore samples of strains NCTC 11204 and 630 were prepared for TEM by members of the Centre for Electron Microscopy, School of Metallurgy and Materials, University of Birmingham, as described by Rabi *et al.* (2017). Sectioned *C. difficile* spore samples were poststained with 2% (w/v) uranyl acetate and lead citrate to visualise internal structures. Imaging of samples was completed with the Jeol 2100 200kV LaB6 transmission electron microscope at 80-kV.

4.2.5 Scanning electron microscopy.

C. difficile NCTC 11204 spore samples were prepared and imaged by Dr Rachel Sammons, School of Dentistry, College of Medical and Dental Sciences, University of Birmingham, as described by Rabi *et al.* (2017). Following sample dehydration using serial ethanol gradient immersions the *C. difficile* spore samples were gold sputter coated. Imaging of samples was completed with the Zeiss EVO MA-10 scanning electron microscope at working distance of 6.5 mm at 20-kV.

4.2.6 Preparation of *C. difficile* spores for cryo-soft X-ray tomography.

Spore suspensions of unlabelled and Laurdan-labelled *C. difficile* NCTC 11204 and *C. difficile* 630 were prepared at an optical density of 5.0 at 570_{nm}. Aliquots of 150 µl of the spore suspension were transferred to labelled 1.5 ml microcentrifuge tubes containing 1350 µl BHIS media or BHIS media supplemented with 6.9 mM sodium taurocholate under aerobic and anaerobic conditions. Spores were exposed to BHIS media supplemented with 6.9 mM sodium taurocholate for 0.5, 1, 2, 3, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes before centrifugation at 17000g for 1 minute to pellet the spores. The supernatant was removed without disturbing the pellet and replaced with 1350 µl 2.5% (v/v) glutaraldehyde, 0.1 M sodium cacodylate buffer at pH 7.3, for 1 hour at ambient temperature to fix the samples. Following fixation, the spore samples were centrifuged for 1 minute at 17000g. The supernatant was removed and replaced with 500 µl 0.1 M sodium cacodylate buffer. The *C. difficile* spore samples were stored at 4°C.

4.2.7 Cryo-soft X-ray tomography.

Samples of *C. difficile* NCTC 11204 and *C. difficile* 630 spores were prepared and imaged by cryogenic soft X-ray tomography (cryo-SXT) by the members of the Beamline 24 team: Maria Harkiolaki, Illias Kounatidis and Chindinma Okolo, Harwell Science and Innovation Campus, Diamond Light Source. *C. difficile* spores were deposited on electron microscopy 3 mm flat grids along with 250nm gold fiducials prior to vitrification by plunge freezing in liquid ethane and liquid nitrogen. Cryo-structured illumination microscopy (cryoSIM) was utilised to map grids and identify areas of interest for cryo-SXT. The UltraXRM-S/L220c X-ray microscope and Pixis 1024 B CCD camera, Beamline 24, Diamond light source, was used to collect X-ray mosaic and tilt series images with a 25 zone plate, X-rays of 500 eV with increments of 0.2-1.0°.

4.3 Results.

4.3.1 Scanning electron microscopy.

SEM imaging was utilised to examine the external morphology of ungerminated *C. difficile* spores and *C. difficile* spores exposed to BHIS supplement with bile salt germinant sodium taurocholate to trigger germination. Ungerminated *C. difficile* NCTC 11204 spores were observed as ovoid in shape with variable length and width (figure 4.1). The outermost structure of the spores visible in the SEM images, the exposorium, encloses the spore and demonstrated a bumpy or 'bubble-wrap' appearance. Spores of *C. difficile* NCTC 11204 where germination had been initiated following 60 minutes exposure to BHIS supplement with 6.9 mM sodium taurocholate also demonstrated the typical ovoid appearance with variation in the size of individual spores (figure 4.2). These spores also demonstrated the 'bubble-wrap' morphology of the exosporium. There was no observable difference in the size and shape or appearance of the exosporium of *C. difficile* spores exposed to germinant and co-germinants present in BHIS supplement with 6.9 mM sodium taurocholate and ungerminated spores of *C. difficile* NCTC 11204.

4.3.2 Transmission electron microscopy.

TEM imaging was used to investigate the internal structure *C. difficile* spores. *C. difficile* spore samples of strains NCTC 11204 and 630 were prepared for TEM but due to the limited availability of the facilities at Centre for Electron Microscopy, University of Birmingham, only *C. difficile* 630 spores were examined. Ungerminated and *C. difficile* 630 spores undergoing the initial phase of germination following 5 and 20 minutes exposure to BHIS supplemented with 6.9 mM sodium taurocholate were examined. Transverse sectioning of ungerminated *C. difficile* 630 spores revealed the spore core in the centre of the spore but other internal structures including the cortex were not clearly defined (figure 4.3). TEM imaging of *C. difficile* 630 spores following 5 minutes

exposure to BHIS supplement with 6.9 mM sodium taurocholate to initiate germination demonstrated the internal structures. TEM images show the spore core at the centre of the spore surrounded by the spore cortex and other layers of the spore structure (figure 4.4). TEM images at a higher magnification of *C. difficile* 630 spores at this initial phase of germination demonstrated the internal structure in more detail (figure 4.5). The central darkly stained spore core is surrounded by a lighter thin band thought to be the inner membrane, this is surrounded concentrically by an outer band of the germ cell wall and a wider layer of the spore cortex. A darker band, likely to be the outer membrane can be seen between the layers of the cortex and multiple layers of the spore coat. The outermost of layer, the exosporium appears to be fragmented and detached from the spore. The structure of the internal layers of *C. difficile* 630 spores are also clearly observable in the TEM images of the spores exposed to BHIS supplemented with 6.9 mM sodium taurocholate for 20 minutes (figure 4.6). The spores imaged following the 20 minutes exposure to germinant and co-germinants also demonstrated a darkly stained spore core surrounded by a multi-layered structure presumed to be the inner membrane, germ cell wall, cortex and outer membrane. These are surrounded by the multi-layered spore coat and exosporium which appear to have become detached from the spores in images A and D of figure 4.6. The TEM images of *C. difficile* spores following 20 minutes exposure to BHIS supplemented with 6.9 mM sodium taurocholate at higher magnification also reveal the internal layers of the spore structure (figure 4.7). The layers identified in the TEM images as the spore cortex appear to be reduced in size for *C. difficile* 630 spores exposed to BHIS supplemented with 6.9 mM sodium taurocholate for 20 minutes compared to that of *C. difficile* 630 spores exposed to germinant and co-germinants for 5 minutes (figures 4.4 and 4.6). The reduction in size of the spore cortex can be seen more noticeably on the TEM images of higher magnification (figures 4.5 and 4.7).
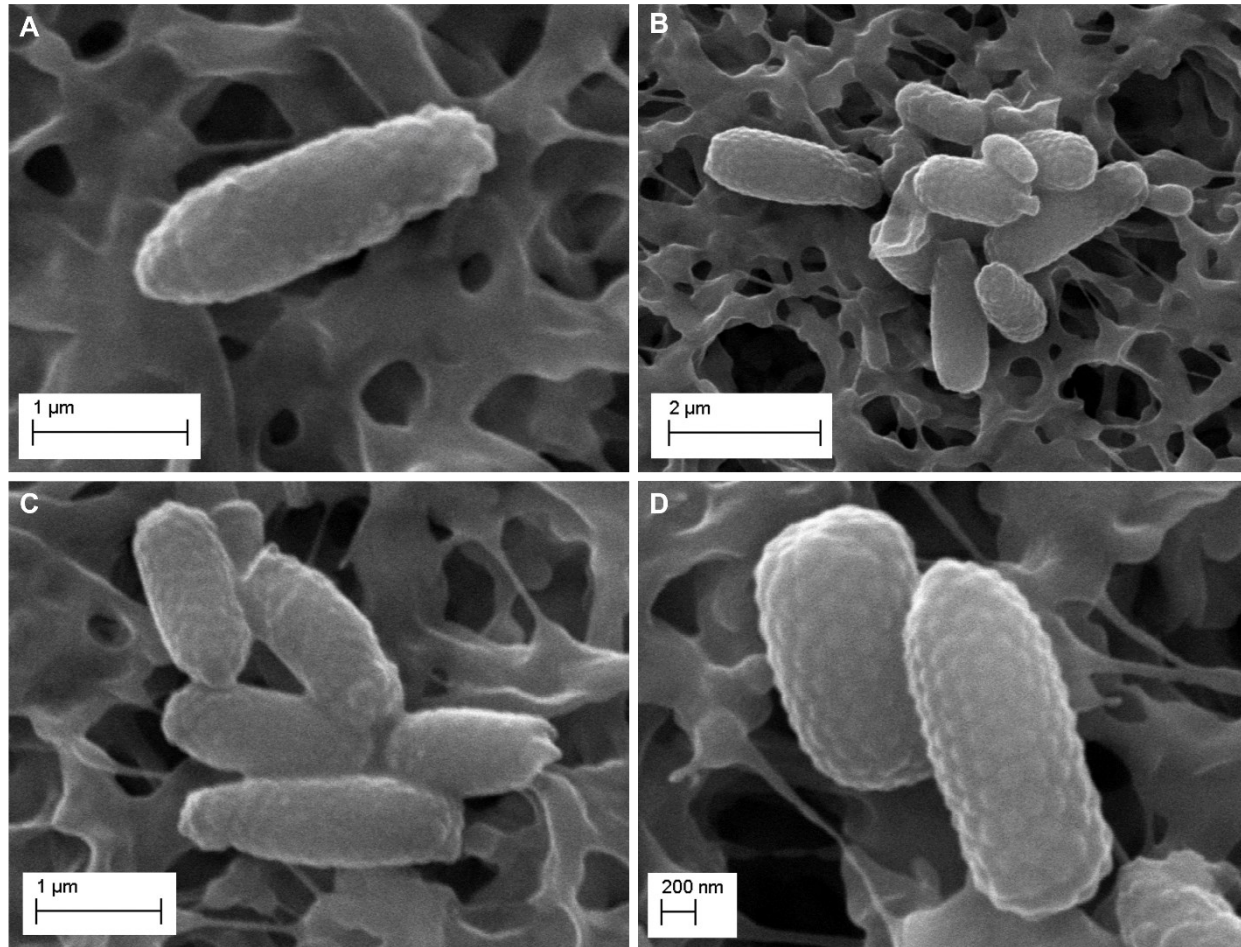
Figure 4.1 SEM imaging of ungerminated *C. difficile* NCTC 11204 spores (A, B, C and D). *C. difficile* spore samples were suspended in BHIS in the absence of bile salt germinant sodium taurocholate prior to fixing and preparation for imaging. Scale bars are provided for each image.
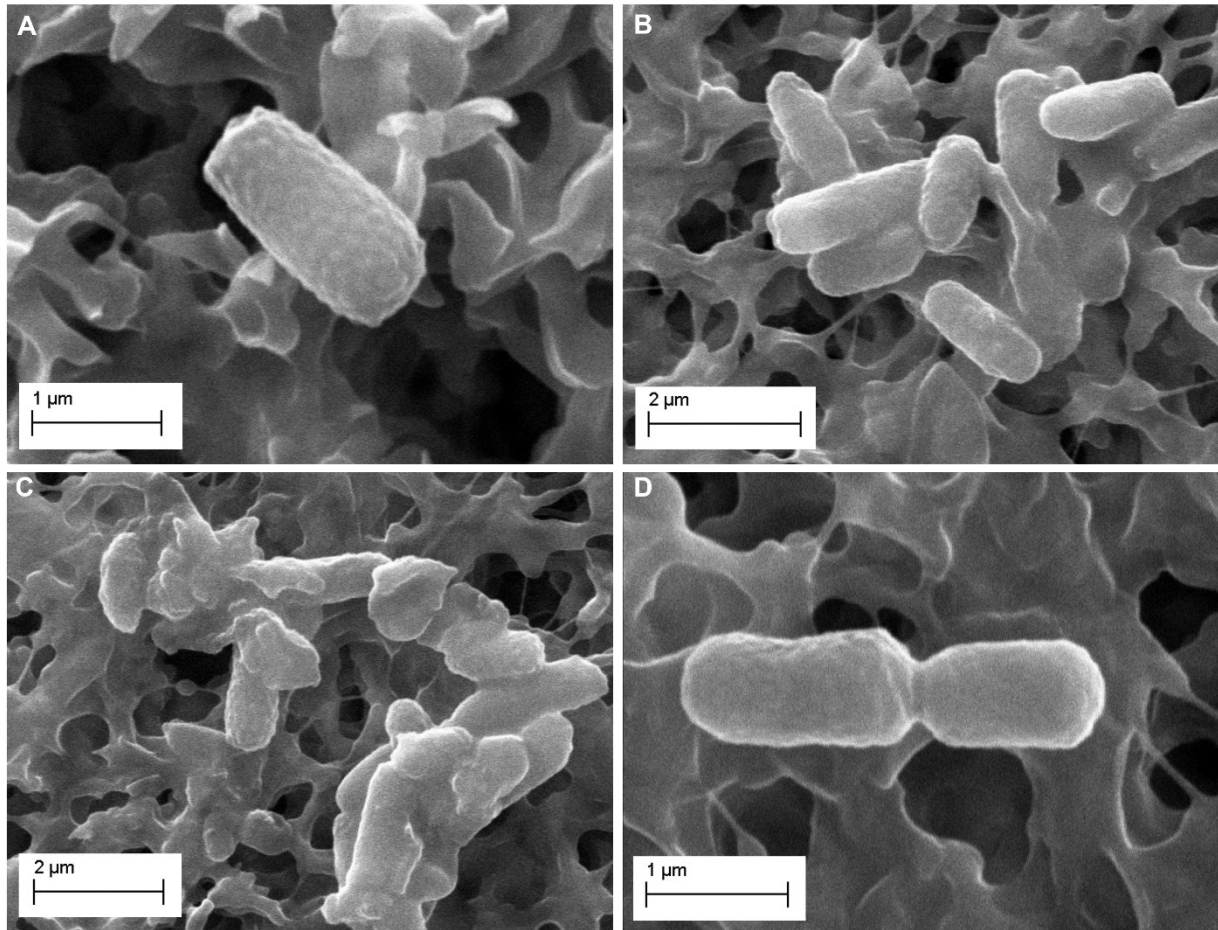
Figure 4.2 SEM imaging of *C. difficile* NCTC 11204 spores following 60 minutes exposure to BHIS supplement with bile salt germinant sodium taurocholate (A, B, C and D). *C. difficile* spore samples were suspended in BHIS with 6.9 mM sodium taurocholate prior to fixing and preparation for imaging. Scale bars are provided for each image.
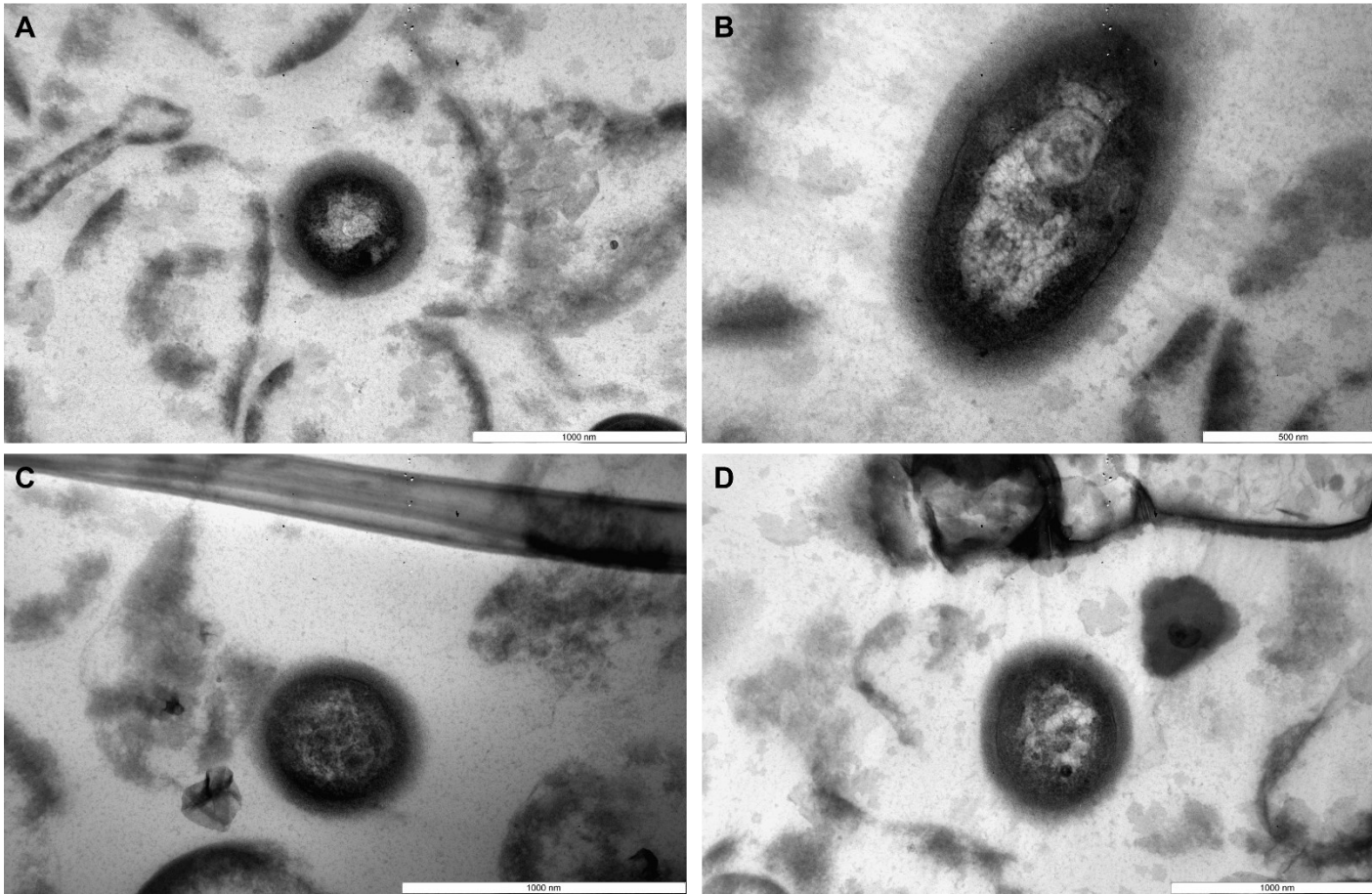
Figure 4.3 TEM imaging of sectioned ungerminated *C. difficile* **630 spores (A, B, C and D).** *C. difficile* **spore samples were suspended in BHIS in the absence of bile salt germinant sodium taurocholate prior to fixing and preparation for imaging. Scale bars are provided for each image.**
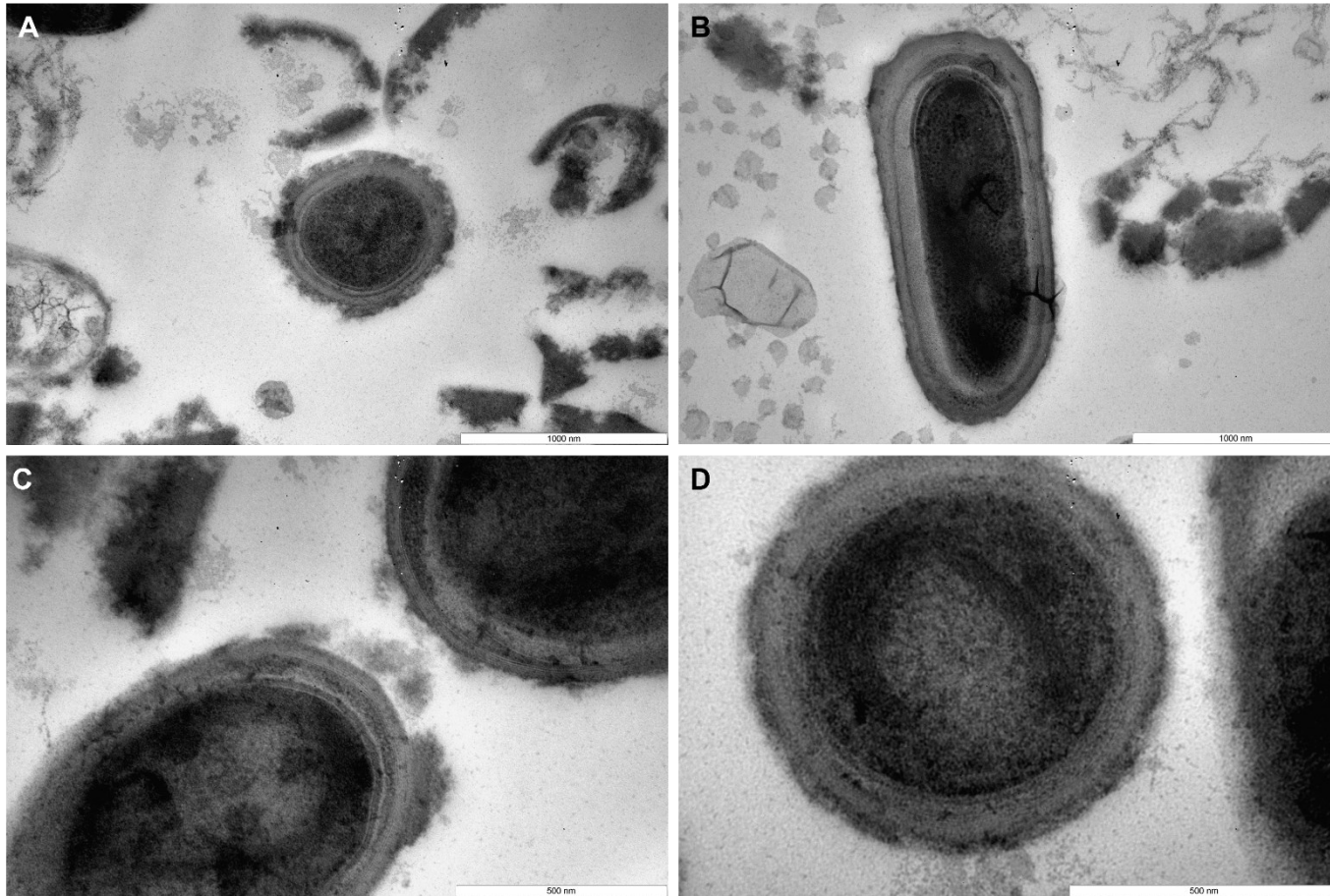
Figure 4.4 TEM imaging of sectioned *C. difficile* 630 spores following five minutes exposure to BHIS supplement with bile salt germinant sodium taurocholate (A, B, C and D). *C. difficile* spore samples were suspended in BHIS with 6.9 mM sodium taurocholate prior to fixing and preparation for imaging. Scale bars are provided for each image.
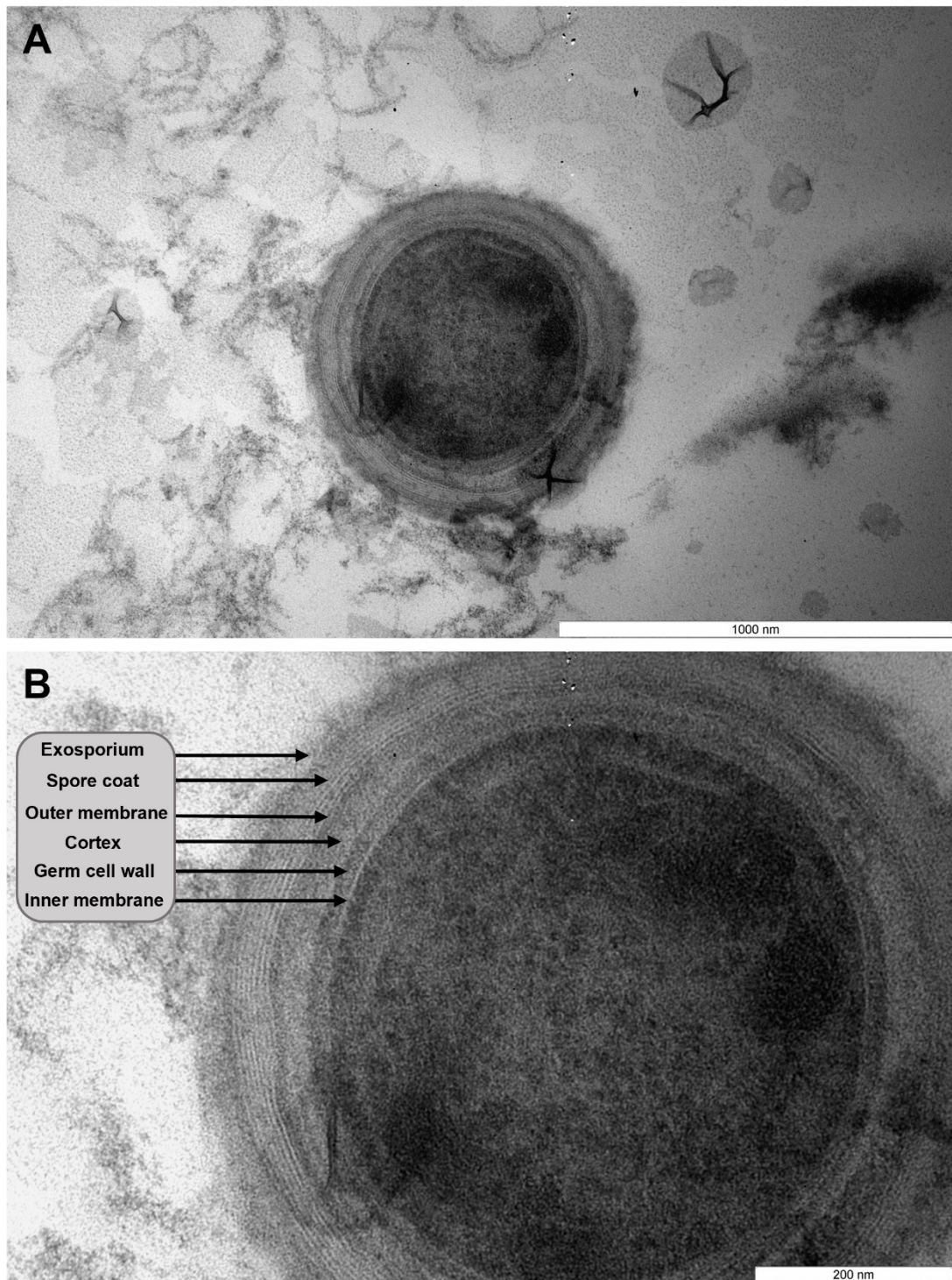
Figure 4.5 TEM imaging of sectioned *C. difficile* 630 spores following five minutes exposure to BHIS supplement with bile salt germinant sodium taurocholate (A and B). TEM of *C. difficile* 630 (A) with a magnified area of the image (B). *C. difficile* spore samples were suspended in BHIS with 6.9 mM sodium taurocholate prior to fixing and preparation for imaging. Scale bars are provided for each image.
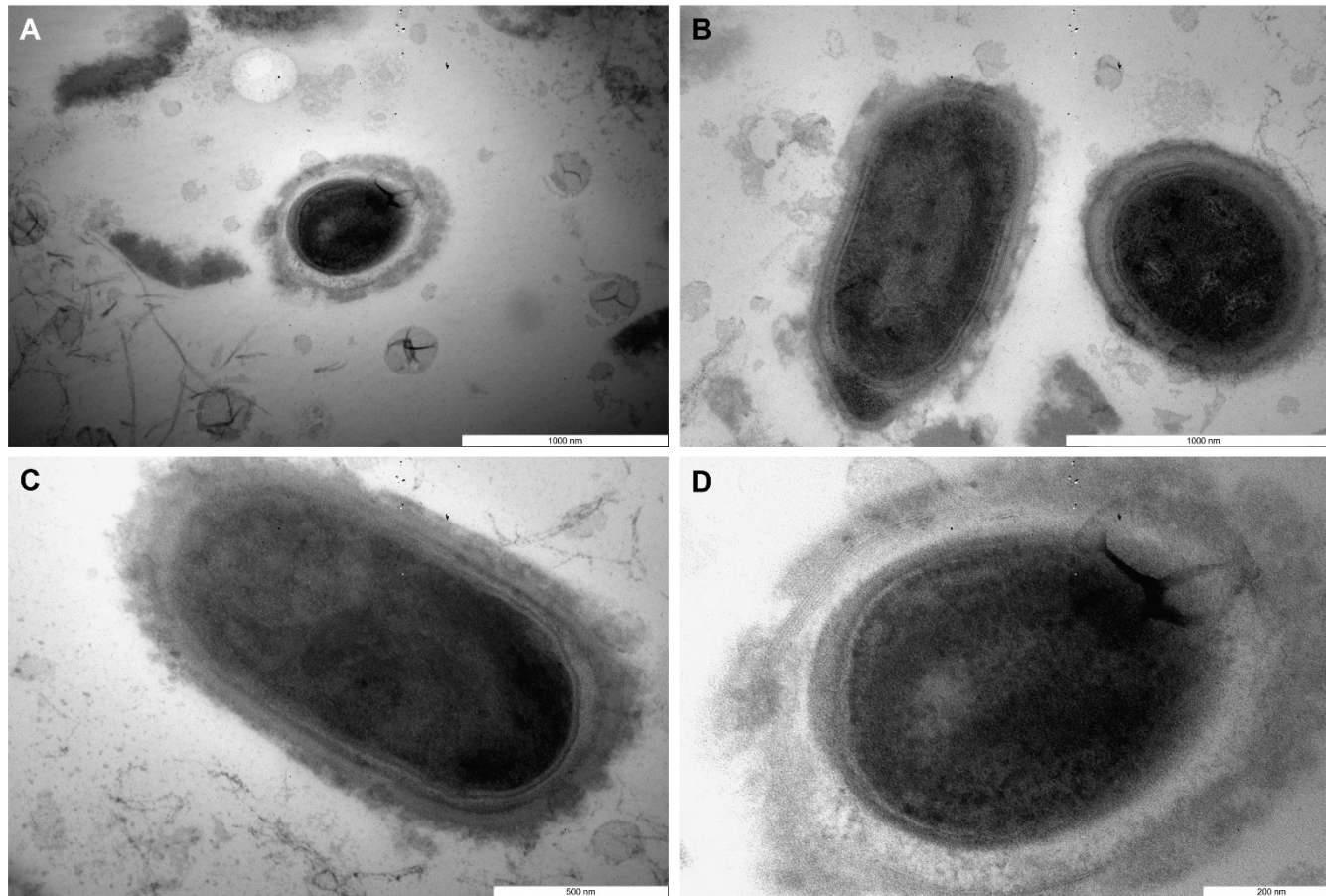
Figure 4.6 TEM imaging of sectioned *C. difficile* 630 spores following twenty minutes exposure to BHIS supplement with of bile salt germinant sodium taurocholate (A, B, C and D). *C. difficile* spore samples were suspended in BHIS with 6.9 mM sodium taurocholate prior to fixing and preparation for imaging. Scale bars are provided for each image.
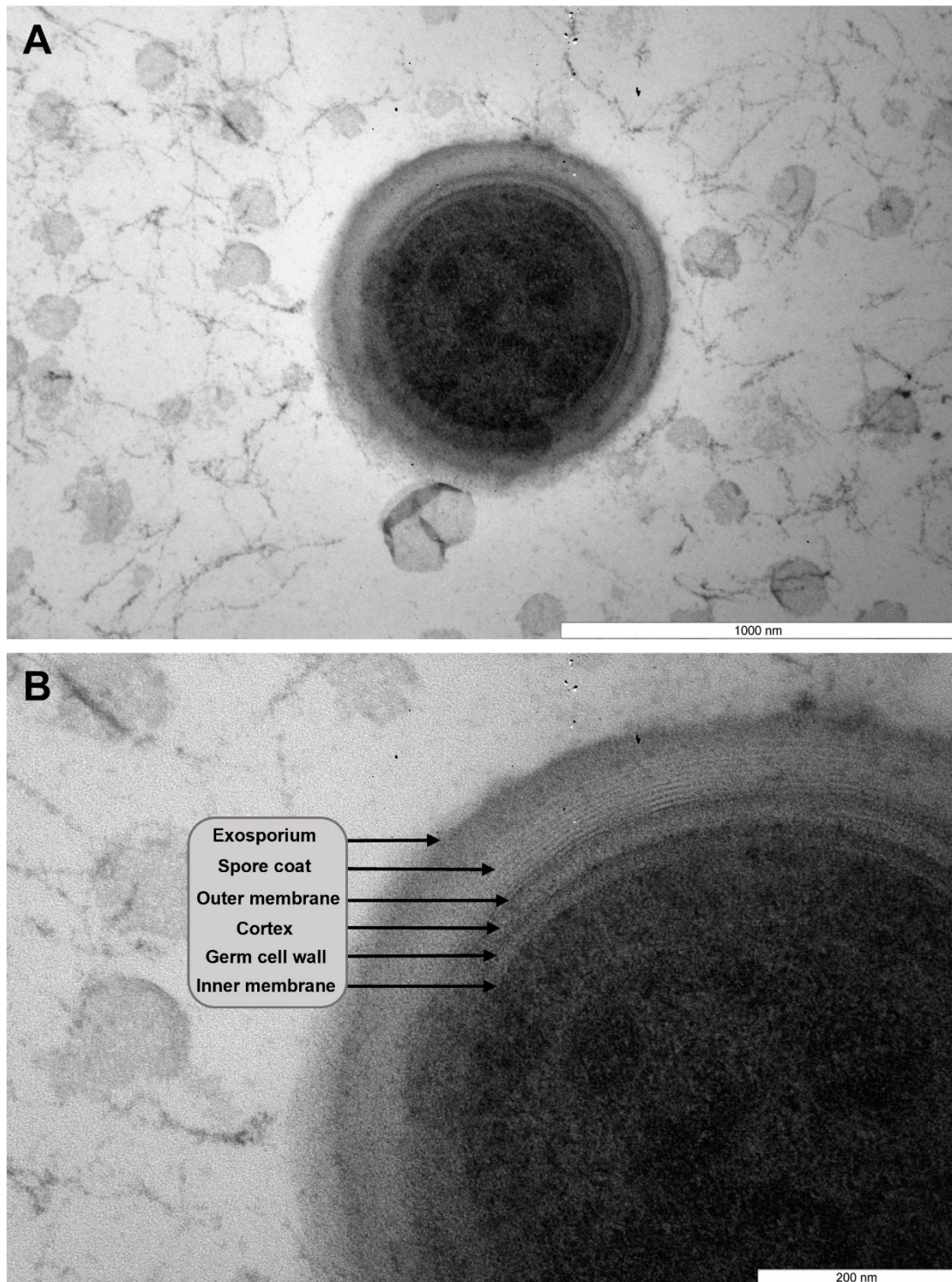
Figure 4.7 TEM imaging of sectioned *C. difficile* 630 spores following twenty minutes exposure to BHIS supplement with of bile salt germinant sodium taurocholate (A and B). TEM of C. difficile 630 (A) with a magnified area of the image (B). *C. difficile* spore samples were suspended in BHIS with 6.9 mM sodium taurocholate prior to fixing and preparation for imaging. Scale bars are provided for each image.

4.3.3 Cryo-Soft X-ray Tomography.

The viability of *C. difficile* spores prepared in the presence of Laurdan was investigated to determine if the fluorescent probe altered the ability of spores to germinate and proliferate. No difference in the calculated CFU/ml of Laurdan-labelled and unlabelled *C. difficile* spores was observed indicating there was no inhibitory effect on the germination response of spores or vegetative cell outgrowth. The fluorescent signal of Laurdan-labelled of *C. difficile* spores, prior to glutaraldehyde fixing, was confirmed with a fluorescent plate reader with excitation: 360 nm, emission: 440/490. Due to the restricted availability of access to the facilities at Diamond Light Source, Beamline 24 and the occurrence of a beamline malfunction during the allocated imaging period the cryo-SXT imaging the number of samples examined was limited. Cryo-SXT imaging was completed with Laurdan-labelled, ungerminated *C. difficile* 630 spores and *C. difficile* 630 spores following 5 minutes exposure to BHIS supplement with 6.9 mM sodium taurocholate only. Spores labelled with the fluorescent probe Laurdan were examined by fluorescent cryoSIM in preparation for cryo-SXT but were not found to be fluorescent when excited at a wavelength of 360nm. The use of Laurdan to label *C. difficile* spores was not found to be an appropriate methodology to identify the structures of the inner and outer membranes of the spores. Cryo-SXT imaging of ungerminated *C. difficile* 630 spores demonstrated the typical ovoid morphology with the central structure of the spore core surrounded by outer layers of the spore structure (figure 4.8). Identification of distinct layers of the internal spore structure could not be determined in the images. *C. difficile* 630 spores exposed to BHIS supplemented with 6.9 mM sodium taurocholate for 5 minutes demonstrated a very similar morphology to ungerminated *C. difficile* 630 spores in the cryo-SXT images (figure 4.9). Ovoid *C. difficile* 630 spores could be observed with a central structure of the spore core visible in some of the spores. As previously, the identification of distinct layers of the internal spore structure was not possible. Based on the available cryo-SXT images there was no observable difference in the morphology of ungerminated *C. difficile* 630 spores and *C. difficile* 630 spores exposed to germinant and co-germinants present in BHIS supplement with 6.9 mM sodium taurocholate.
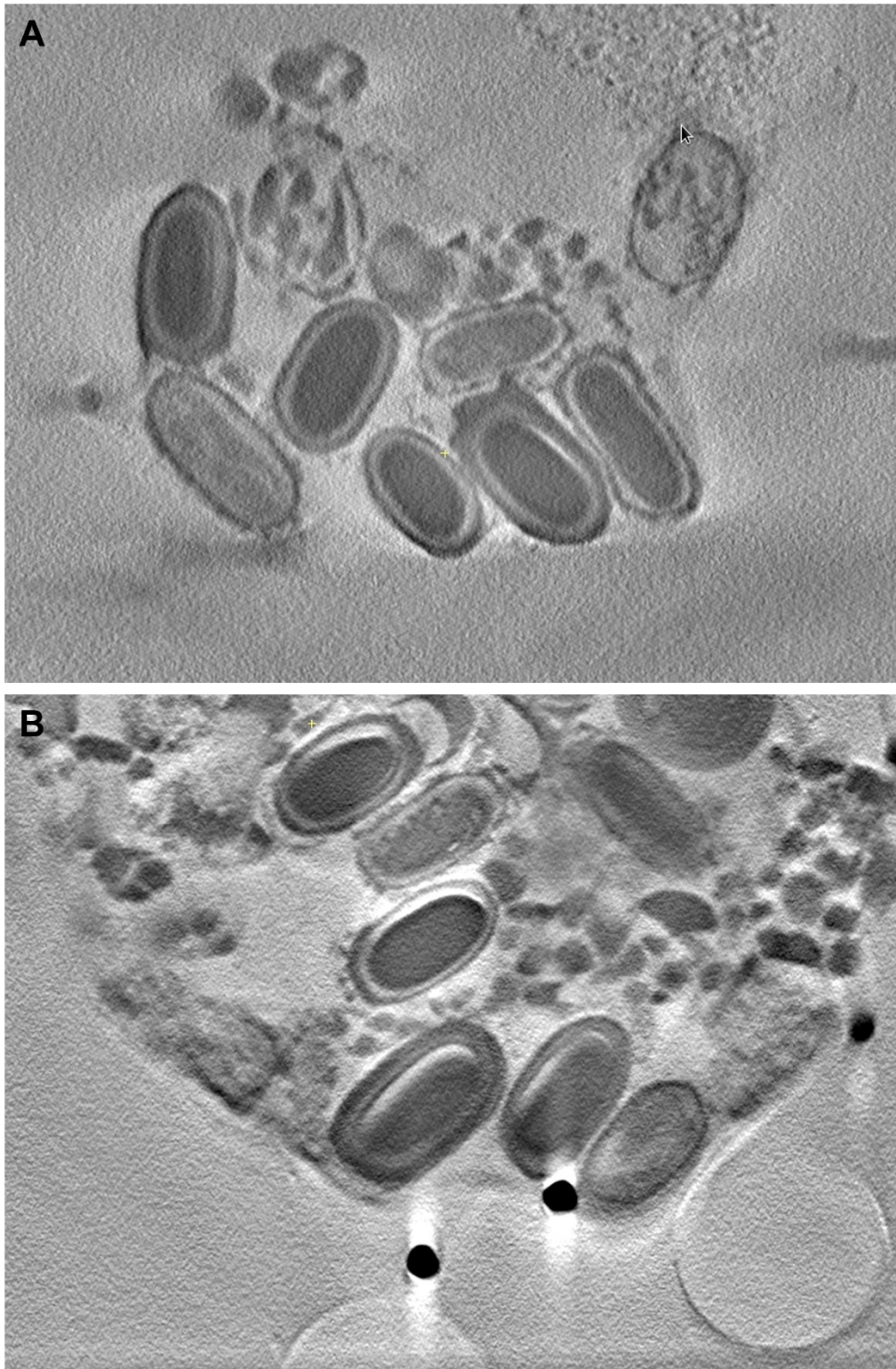
Figure 4.8 Cryo-SXT imaging of ungerminated *C. difficile* 630 spores (A and B). *C. difficile* spore samples were suspended in BHIS in the absence of bile salt germinant prior to fixing and preparation for imaging. Gold fiducials are visible in the images as black spheres.
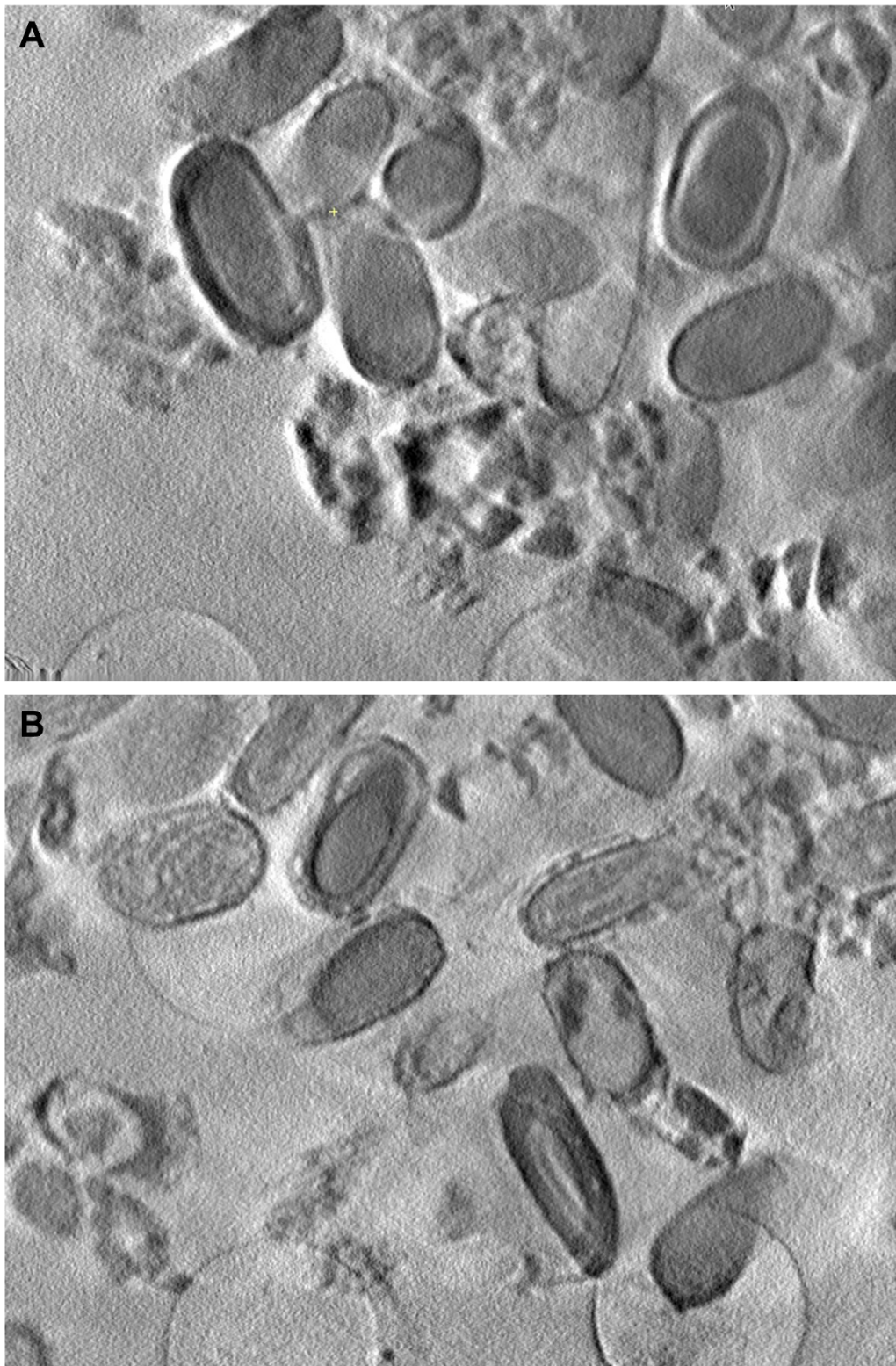
Figure 4.9 Cryo-SXT imaging of *C. difficile* 630 spores following five minutes exposure to BHIS supplement with of bile salt germinant sodium taurocholate (A and B). *C. difficile* spore samples were suspended in BHIS with 6.9 mM sodium taurocholate prior to fixing and preparation for imaging.

4.4 Discussion.

Knowledge of *C. difficile* spore structure and any transformation of the spore structure upon the initiation of germination may contribute to the understanding of the regulation of *C. difficile* spore germination. The identification of spore membranes with the *C. difficile* spore, where germinant and co-germinant receptors, along with other key germination regulators are thought to be located, may help to explain how these proteins interact to transduce the germinant signal and control *C. difficile* germination. A better understanding of the mechanism of germination would be applicable to sporicidal decontamination approaches to prevent CDI and specifically to the development of *C. difficile* pro-germination elimination strategies.

In this study utilisation of cryo-STX imaging of ungerminated *C. difficile* spore and spores exposed to germinants and co-germinants was unsuccessful in contributing to the wider understanding of *C. difficile* spore germination. SEM and TEM imaging of *C. difficile* spores revealed the internal and outermost structures of *C. difficile* spores in detail, confirming the presence of the multi-layered structures observed by other researchers. The SEM and TEM images presented of *C. difficile* spores following exposure to germinants and co-germinants in this chapter did not reveal significant changes to the external or internal structure upon the initial phase of germination.

The SEM images of *C. difficile* NCTC 11204 spores demonstrated spore morphology that were of a consistent size and shape reported previously for *C. difficile* spores (Lawson *et al.*, 2016; Snelling *et al.*, 2010). Clearly visible on the exterior of the majority of *C. difficile* spores in the SEM images was the bumpy or bubbly phenotype of the exosporium. This bumpy morphology concurs with the findings of Rabi *et al.* (2017) and that of Malyshev and Baillie (2020) who described the protruding ridges of the exosporium as a pineapple-like morphology. There was no observable difference in the size, shape or appearance of the exosporium of *C. difficile* exposed to germinant and co-germinants present in BHIS supplement with 6.9 mM sodium taurocholate and ungerminated spores of *C. difficile* NCTC 11204. This suggests any transformation changes to the structure of the *C. difficile* spore during the

initial phase of germination may occur internally only. This is in agreement with the recent findings of Baloh *et al.* (2022) who identified no visible difference in the appearance of ungerminated *C. difficile* spores and *C. difficile* spores where germination had been triggered following exposure to germinant and co-germinants for 5 and 20 minutes.

The spores of *C. difficile* NCTC 11204 and *C. difficile* 630 were prepared for TEM but only images were of *C. difficile* 630 were obtained. *C. difficile* 630 was included in this study as it has been more widely characterised than *C. difficile* NCTC 11204 and provided the opportunity to examine the morphology of another relevant *C. difficile* laboratory strain. TEM images of ungerminated *C. difficile* spores did not demonstrate clearly defined internal structures, while the central spore core was visible it was not possible to distinguish other features of the spore structure. The TEM imaging of spores following 5 or 20 minutes exposure to germinants and co-germinants revealed the internal structure of the *C. difficile* spores allowing the identification of the central spore core, inner membrane, germ cell wall, cortex, outer membrane, spore coat and exosporium. The TEM images obtained and examined in this study did not reveal substantial observable changes to the spore structure following the initiation of *C. difficile* spore germination.

While there are differences in the reported appearance of *C. difficile* spores imaged by TEM, the presence of internal spore structures observed in this study was consistent with TEM images obtained by others (Joshi *et al.*, 2012; Paredes-Sabja *et al.*, 2014; Rabi *et al.*, 2017). The differentially contrasting layers of the *C. difficile* spore in TEM images were identified as the key structural features of the *C. difficile* spores in figures 4.5 and 4.7 were based on spore structural layers identified in previous studies and existing knowledge of bacterial spore structure (Permpoonpattana *et al.*, 2011; Paredes-Sabja *et al.*, 2014; Pizarro-Guajardo *et al.*, 2016; Rabi *et al.*, 2017; Kochan *et al.*, 2018a; Baloh *et al.*, 2022; Setlow, 2003; 2007). Distinct components of the multilayer spore coat (inner and outer coat layers) identified by other researchers in TEM images of *C. difficile* were not observed in this study, therefore the differently contrasting structure internal to the laminations of the spore coat was

identified as the probable *C. difficile* inner membrane. However, there is no direct evidence presented here to determine the presence of the inner membrane or confirm the other key structural features of the *C. difficile* spore (Baloh *et al.*, 2022; Rabi *et al.*, 2017; Barra-Carrasco *et al.*, 2013)

Close examination of the internal structure of *C. difficile* spores exposed to BHIS supplemented with 6.9 mM sodium taurocholate for 5 and 20 minutes to initiate germination, at higher magnification, was indicative of a narrowed spore cortex in the spores exposed to germinants and co-germinants for the longer time period. Measurements of the relative width of the spore cortex of individual spores is needed to quantify and determine any significant difference but this change would be consistent with the initial rehydration of the spore core and subsequent degradation of the peptidoglycan of cortex during the second stage of germination (Burns *et al.*, 2010; Adams *et al.*, 2013; Francis *et al.*, 2015; Francis and Sorg, 2016; Setlow, 2003). This strategy was recently utilised by Baloh *et al.* (2022) who quantified the reduction of the thickness of the cortex of *C. difficile* spores after 5 minutes exposure to BHIS supplement with sodium taurocholate and glycine. The cortex thickness of *C. difficile* spores was reduced by 67% after 5 minutes exposure to germinants and co-germinants compared to ungerminated spores (Baloh *et al.*, 2022)

The bumpy morphology of the exosporium which was identifiable on both SEM and TEM images by Rabi *et al.* (2017) was not clearly observed on the TEM images in this study. Indeed, a smooth exosporium morphology, that was diffuse and detached (or even absent) was seen in the images of ungerminated and *C. difficile* 630 spores exposed to germinants and co-germinants. The detached or absent exosporium morphology observed was more apparent in images of spores exposed to germinant and co-germinants but was not considered to be linked to spore germination but is mostly likely a consequence of the purification of spore suspensions and preparation of spore samples for TEM. Malyshev and Baillie (2020) and Permpoonpattana *et al.* (2011) have demonstrated the significance of spore preparation methods on the resulting morphology of exosporium of *C. difficile* spores, sonication and protease treatment in particular were found to remove the exosporium layer

(Escobar-Cortés *et al.*, 2013). The integrity of the exosporium has also been shown to be altered during long-term storage of *C. difficile* spores and was seen to detach from some strains but this was variable amongst strains studied (Pizarro-Guajardo *et al.*, 2016).

The novel application of cryo-SXT with *C. difficile* 630 spores produced images that were not suitable for the identification of the key internal features of *C. difficile* spores, as the distinct layers of the spore internal structure were not evident. Furthermore, the quantity of cryo-SXT images obtained of ungerminated and *C. difficile* spores exposed to germinants and co-germinants was insufficient to observe or quantify any potential transformation as result of germination. Prior cryoSIM with *C. difficile* spores labelled the fluorescent probe Laurdan as part of correlative imaging with cryo-SXT did not demonstrate fluorescence when excited at the appropriate wavelength. It is likely that crosslinking of proteins caused by the glutaraldehyde treatment altered the fluorescence signal of Laurdan-labelled *C. difficile* spores. It was therefore not possible to identify the location of the inner and outer membrane of *C. difficile* spores using these imaging techniques.

The observations of ungerminated and *C. difficile* spores exposed to germinants and co-germinants by SEM and TEM imaging are limited to the two strains used in experiments presented in this chapter. SEM was completed only with *C. difficile* NCTC 11204 spores while TEM was completed with *C. difficile* 630 spores preventing the comparison of morphology and internal spore structure across the two *C. difficile* laboratory strains. The observations of an absence of significant transformation of the external or internal spore structure upon the initial phase of germination is therefore restricted to only the strains utilised. Further SEM and TEM imaging of ungerminated *C. difficile* spores and *C. difficile* spores at additional time points post exposure to germinants and co-germinants, utilising multiple laboratory strains is needed to draw more detailed conclusions on the potential structural changes that occurs within *C. difficile* spores during the initial phase of germination. Relative quantification of the spore cortex size in germinating *C. difficile* spores is needed to confirm the reduction in mass as per the findings of Baloh *et al.* (2022).

In this study, high resolution imaging techniques were utilised to investigate potential transformational changes to the external and internal structures spore of *C. difficile* spores following the initiation of germination. SEM and TEM imaging of *C. difficile* spores facilitated the identification of the distinct morphology of the exosporium and the key internal spore structures. A substantial transformation of *C. difficile* spore structures was not observed during the initial phase of germination. cryo-STX imaging was not found to be a suitable methodology to examine the external and internal spore structures of *C. difficile.* While the research presented in this chapter contributes to the existing knowledge of the initial phase of *C. difficile* germination, there was limited insight provided on the position of the spore membranes within the *C. difficile* spore structure and therefore potential location of key regulators of *C. difficile* germination.

Chapter 5

5.0 Investigation of novel compound C109 with dual germination and antimicrobial activity for the elimination of *Clostridioides difficile* spores.

5.1 Introduction.

Utilising knowledge of the environmental signals that initiate germination of *C. difficile* spores, Wheeldon *et al.* (2010) proposed the induction of germination as a mechanism to enhance the inactivation of *C. difficile* spores with traditional biocides. The 'germinate to exterminate' strategy comprises exposure *of C. difficile* spores to known bile salt germinants and amino acid co-germinants to initiate germination in the environment (Wheeldon, 2008; Wheeldon *et al.*, 2010). Upon germination, the release of Ca-DPA and rehydration of the spore core causes a rapid loss in the spore's resistant characteristics, rendering the germinating spore susceptible to thermal and chemical inactivation (Setlow, 2003; Pol *et al.*, 2001; Hornstra *et al.*, 2007; Omotade *et al.*, 2014).

The induction of germination as a method to enhance the killing of bacterial spores was first demonstrated by Stuy (1956) and was proposed for use in the food industry to enhance the elimination of bacterial spores that cause food spoilage and food-borne disease (Gould *et al.*, 1968). Hornstra *et al.* (2007) demonstrated that exposure of spores to germinants prior to chemical disinfection, significantly enhanced the elimination of *Bacillus cereus* spores that adhere to the surface of food processing equipment. More recently, the inclusion of a germination pre-treatment step was demonstrated to significantly improve the decontamination of *Bacillus* spores from a variety of surfaces (Mott *et al.*, 2017). The utilisation of an initial step to trigger germination is highly advantageous to enhance the eradication of bacterial spores during food manufacturing process, offering an improved mechanism to prevent contamination of foods, extending shelf life and the safety of food products (Hornstra *et al.*, 2007). A strategy to utilise an initial germinant treatment to

potentiate the susceptibility of spores to sporicidal agents has also been employed to enhance the elimination of *Bacillus anthracis* spores from contaminated soil (Celebi *et al.*, 2016).

The presence of germinants and co-germinants is crucial for the triggering of *C. difficile* germination but the extent and rate of germination is influenced by the temperature and pH of the environment, with optimum germination occurring at 37°C and in neutral ∕alkaline conditions of pH 6.3 -7.5 (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2008a). Despite the necessity of an anaerobic atmosphere for *C. difficile* outgrowth and replication, Wheeldon *et al.* (2008a) demonstrated that an anaerobic atmosphere is not a requirement of *C. difficile* germination and that there was no significant difference in the rate of germination of *C. difficile* spores in aerobic and anaerobic conditions. In fact, in the duodenum, where *C. difficile* spores are first exposed to bile salt germinants and co-germinants is aerobic, with the environment becoming increasingly anaerobic towards the cecum of the large intestine (Ridlon *et al.*, 2006; Friedman *et al.*, 2018). *C. difficile* germination can therefore be triggered by providing the specific germinant and co-germinant signals and does not require the direct replication of the conditions of passage through the host environment (Wheeldon *et al.*, 2008a). On this basis, Wheeldon (2008) first developed a germinant medium of thioglycollate broth supplemented with 1% (w/v) sodium taurocholate, thereby containing both germinant and co-germinants, to trigger the germination of *C. difficile* spores as part of a two-stage disinfection procedure to eliminate spores from hard surfaces. Preincubation of *C. difficile* spores with the germinant medium for 10 minutes and subsequent exposure to 70% (v/v) ethanol resulted in a 0.36-log reduction of *C. difficile* strain NCTC 11204 and 1.51-log reduction of ribotype 027 strain R20291 after 90 minutes. When preincubation of spores with the germinant medium was increased to 30 minutes, log reductions of 1.85 and 2.15 were achieved for the NCTC 11204 and 027 strains, within 30 minutes respectively. This significant reduction in spores was not observed when SDW was used in the place of 70% (v/v) ethanol, demonstrating an antimicrobial was required for effective elimination of *C. difficile* spores (Wheeldon, 2008). Utilising this two-stage disinfection procedure, Wheeldon (2008) demonstrated that a contact time of 20-30 minutes with the germinant medium, followed by 70%

(v/v) ethanol exposure was required for maximum elimination of *C. difficile* spores from a stainless-steel surface. The germinant medium, as part of the two-stage disinfection procedure was further utilised by Wheeldon *et al.* (2008c) in combination with the antimicrobial activity of copper. *C. difficile* spores inoculated onto the surface of copper discs and exposed to germinant medium produced a 2.67 and 2.96-log reduction of germinating *C. difficile* NCTC 11204 and 027 spores respectively, after 3 hours. The viability of dormant *C. difficile* spores was not affected by the antimicrobial activity of copper alone, in contrast to vegetative cells of *C. difficile* which were eliminated beyond the limits of detection within 30 minutes (Wheeldon *et al.*, 2008c). The germinant medium was further optimised by replacing the complex nutrients supplied in the thioglycollate medium with a combination of specific amino acids, which initiated maximum germination with taurocholate (Wheeldon *et al.*, 2010; 2011). To remove the requirement for a secondary antimicrobial agent in the two stage disinfectant procedure, the biocides benzyl alcohol and benzalkonium chloride were added to create a germinant solution (Wheeldon *et al.*, 2010). Application of this germinant solution achieved a 3-log reduction in *C. difficile* spores in one hour (Wheeldon *et al.*, 2010). Following the demonstration of the efficacy of the germinant solution to eliminate *C. difficile spores* in a single step, a patent (WO2011101661) for its application as an antiseptic composition for the decontamination of surfaces was filed and granted for use internationally (Worthington and Wheeldon, 2011).

The application of the 'germinate to exterminate' strategy to reduce the environmental contamination of *C. difficile* spores was further explored with the proposal to use *C. difficile* spore germinants incorporated into a smart surface to sensitise *C. difficile* spores to the bactericidal activity of traditional biocides. This led to the concept of combining the germination and antimicrobial activity into a single compound by molecularly coupling bile salt compounds with an antimicrobial agent for the elimination of *C. difficile* spores (Hird, 2014; Rathbone *et al.*, 2019). Research completed by Hird (2014) at Aston University investigated the synthesis of bile acid amides and subsequent covalent linking of the tertiary amine group of these bile acid amides with quaternary ammonium compounds to introduce antimicrobial activity. The project aimed to generate novel compounds that function

specifically as dual germinant and antimicrobial compounds that could be incorporated into a polymeric biomaterial for the development of targeted *C. difficile* sporicidal smart surface. Deoxycholic acid, lithocholic acid and cholic acid were chemically manipulated to synthesise bile amides but the efficient, well-documented *C. difficile* spore germinant, sodium taurocholate was not compatible with the synthesis techniques adopted (Hird, 2014; Sorg and Sonenshein, 2008). Chemical modification of lithocholate was found to produce analogues with potential germination and antimicrobial activity during preliminary testing, however the activity of the bile salt-derived novel compounds to elicit germination and antimicrobial activity when incorporated into a polymeric material as originally intended, was not assessed (Hird, 2014).

The germination and antimicrobial activity of the bile salt-derived novel compounds was assessed by exposing spores of *C. difficile* NCTC 11204 and *C. difficile* ribotype 027 to a solution comprising double strength thioglycollate media supplemented with 2% (w/v) of the novel compounds for 1 hour. Samples were then exposed to a heat challenge of 75°C or incubated on ice for 20 minutes. *C. difficile* CFU/ml was determined by serially diluting test samples and inoculating fastidious anaerobic agar plates supplemented with 5% (v/v) defibrinated horse blood and 0.1% (w/v) sodium taurocholate, which were incubated under anaerobic conditions for 48 hours. From these assays, compound C109, a lithocholate derived bile amide molecularly tethered to a quaternary ammonium cation, was identified as a novel bile salt-derived compound with promising dual germination and antimicrobial
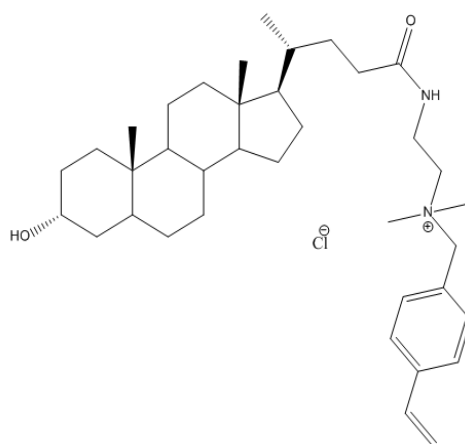
Figure 5.1 Chemical structure of compound C109.

activity. The exposure of *C. difficile* spores to C109 followed by the heat challenge generated a 2 and 1.5-log reduction of *C. difficile* NCTC 11204 and *C. difficile* ribotype 027 spores respectively and was considered an indirect measurement of germination activity. A 1.32 and 1.27-log reduction of *C. difficile* NCTC 11204 and *C. difficile* ribotype 027 achieved following exposure to C109 and incubation on ice was interpreted as probable antimicrobial or direct sporicidal activity. Whilst promising data were achieved in the research described, the methodology utilised and results obtained by Hird (2014) did not fully determine the germination and antimicrobial activity of C109.

The aim of the research presented in this chapter was to investigate the germination and antimicrobial activity of the lithocholate derived novel compound C109 using established methodology to measure spore germination and antimicrobial activity. Knowledge gained from these studies will be used to inform the development of sporicidal smart surfaces with the capacity to trigger germination of *C. difficile* spores and eliminate susceptible germinating spores through inherent antimicrobial activity.

5.2 Methods.

5.2.1 Bacterial strains and growth conditions.

C. difficile NCTC 11204 was sourced from Jon Brazier (HPA Anaerobic Reference Laboratory, Cardiff). *E. coli* NCTC 10538, *Pseudomonas aeruginosa* NCTC 11954, *Staphylococcus aureus* NCTC 10788 were obtained from the National Collection of Type Cultures, Public Health England. Frozen stocks of bacterial strains were stored at -80°C on Microbank™ beads (Pro Lab Diagnostics, Canada). *C. difficile* was resuscitated by inoculating a bead onto Wilkins-Chalgren agar (Oxoid, UK) supplemented with 0.1% (w/v) sodium taurocholate hydrate (Alfa Aesar, UK) and incubating anaerobically (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK) at 37°C for 48 hours. Bacterial strains *E. coli* NCTC 10538, *P. aeruginosa* NCTC 11954, *S. aureus* NCTC 10788 were resuscitated by inoculating a bead onto Nutrient agar (Oxoid, UK) and incubating aerobically at 37°C for 18-24 hours.

5.2.2 Preparation of spore suspensions of *C. difficile*.

Spore suspensions of *C. difficile* to measure germination by loss of resistance to ethanol and membrane integrity staining were prepared using a modified version of the method described by Shetty *et al.* (1999) and Wheeldon *et al.* (2008a). Frozen stocks of *C. difficile* NCTC 11204 stored at -80°C were used to inoculate Columbia blood agar (Oxoid, UK) supplemented with 5% defibrinated horse blood (E&O Laboratories Ltd, UK). The inoculated plates were incubated at 37°C under anaerobic conditions (MiniMACS Anaerobic Workstation, Don Whitley, UK) for 48-72 hours before being incubated aerobically at room temperature for 72-120 hours. Spores were harvested by removing the *C. difficile* colonies with sterile cotton swabs and suspending in 50% (v/v) sterile distilled water 50% (v/v) absolute ethanol. Spore suspension were stained with 5% (w/v) malachite green solution and counterstained with 0.5% (v/v) safranin to confirm the presence of spores as described by Schaeffer and Fulton (1933) and stored at 4°C until required. Prior to experimental use the spore

suspensions were diluted and/or re-suspended in 100% sterile distilled water and enumerated via serial dilution to determine the CFU/ml of *C. difficile* spore suspension. Spore suspensions of *C. difficile* NCTC 11204 used for the optical density germination assay were prepared using a modified version of the methodology described by Heeg *et al.* (2012) as described in chapter 2, section 2.2.2 of this thesis.

5.2.3 Determination of germination activity of novel compound C109 by loss of spore resistance to ethanol.

The methodology utilised to measure germination by loss of resistance to ethanol of *C. difficile* spores in response to the novel compound C109 was based on that described by Wheeldon *et al.* (2008a). Briefly, 250 µl of *C. difficile* spore suspension (10^6 CFU/ml) were mixed with 250 µl double strength thioglycollate medium (Oxoid, UK), and double strength thioglycollate medium containing sodium taurocholate or C109, resulting in a final concentration of 6.9 mM. After 30 and 60 minutes the solution was mixed with 1.17 ml 100% ethanol. Following a one-hour incubation, each solution was transferred into 8.33ml of Wilkins-Chalgren broth. Appropriate serial dilutions of ethanol challenged spores were used to inoculate Wilkins Chalgren agar plates supplemented with 0.1% (w/v) sodium taurocholate which were incubated under anaerobic conditions for 48 hours before the CFU/ml were determined.

5.2.4 Visualisation of germination of *C. difficile* spore by membrane integrity staining

The LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) was used to visualise the germination of *C. difficile* spores in response to C109. Aliquots of 100 µl of *C. difficile* spore suspensions (10^8 CFU/ml) were mixed with 100 µl C109 to achieving a final concentration of 6.9 mM or 100 µl SDW and incubated for 2 hours. After the appropriate time period a 20 µl aliquot was mixed and incubated for 15 minutes with 2 µl of the LIVE/DEAD® BacLight™ stains; SYTO 9 (3.34 mM) and propidium iodide (20

mM). Aliquots of 5 µl of the stained spores were mounted on glass slides before being examination using a Leica TCS SP5 II microscope.

5.2.5 Determination of germination of *C. difficile* spores by measurement of optical density.

The methodology for the measurement of germination by the optical density assay was based on that described by Sorg and Sonenshein (2008) and Heeg *et al.* (2012) and detailed in chapter 2, section 2.2.3 of this thesis. To investigate the germination activity of novel compound C109, BHIS broth or Tris-HCl, pH 7.3-7.4 was supplemented with 6.9 mM of C109.

5.2.6 Determination of bactericidal activity by quantitative suspension test (BS EN: 1040: 2005)

The antimicrobial activity of novel compound C109 was assessed in line with the BSEN 1040: 2005 guidelines (BS EN). *C. difficile* NCTC 11204 was cultured by inoculating a single colony in BHI broth and incubating anaerobically (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK) at 37°C for 18 hours and the viable count (CFU/ml) was subsequently determined. To investigate antimicrobial activity 1 ml aliquots of the overnight culture were mixed with 9 ml 6.9mM C109 solution and incubated for 30 and 60 minutes. A solution containing 5% ethanol 95% (v/v) SDW was used as the control. After the appropriate incubation period, 1 ml aliquots were mixed with 9 ml Dey-Engley neutralising broth (BD Biosciences, USA). Appropriate serial dilutions were prepared and used to inoculate Wilkins Chalgren agar plates which were incubated under anaerobic conditions for 48 hours before the CFU/ml was determined.

5.2.7 Determination of the antimicrobial activity of C109 by standard agar diffusion assay

Wilkins Chalgren and nutrient agar plates were prepared according to manufacturer's instructions before a 7mm diameter well was bored in the centre of the agar plate. *C. difficile* NCTC 11204 was cultured by inoculating a single colony in BHI broth and incubating anaerobically at 37°C for 18 hours. Cultures of *E. coli* NCTC 10538, *P. aeruginosa* NCTC 11954, *S. aureus* NCTC 10788 were prepared by inoculating a single colony of each strain in 5 ml nutrient broth (Oxoid, UK) and incubating aerobically at 37°C for 18 hours. The agar plates were inoculated with overnight cultures of the test organisms diluted to a 0.5 McFarland standard using a sterile cotton swab to achieve a confluent bacterial lawn. A volume of 50 µl of a 6.9 mM solution of C109 was placed directly into the wells of the inoculated agar plates. Similarly, controls were set up with 50 µl of the solvent used to dissolve C109 (50% absolute ethanol 50% (v/v) SDW). Handling of *C. difficile* cultures was conducted inside the anaerobic cabinet. The nutrient agar plates inoculated with *E. coli*, *P. aeruginosa* and *S. aureus* were incubated aerobically at 37°C for 24 hours. Plates inoculated with *C. difficile* were incubated under anaerobic conditions at 37°C for 24 hours. The zone of inhibition was determined by measuring the diameter of the inhibition of growth across the centre of the well. Values for the zone of inhibition including the diameter of the well (7mm) are presented as mean ± standard deviation, each value is derived from 3 biological replicates and 2 technical replicates.

5.2.8 Statistical analysis.

A two-way analysis of variance (ANOVA) was used to determine germination activity by loss of resistance to ethanol and bactericidal activity by quantitative suspension test of novel compound C109. The *post-hoc* Bonferroni's multiple comparisons post-hoc test was used to determine the significance of any reduction in *C. difficile* CFU/ml compared to the control in various test conditions. Both statistical tests were completed using the GraphPad Prism software (GraphPad, USA).

5.3 Results.

5.3.1 The germination activity of novel compound C109 measured by loss of resistance to ethanol

To investigate activity of C109 to initiate *C. difficile* spore germination the loss of resistance to ethanol method was utilised. *C. difficile* spore were exposed to thioglycollate medium supplemented with 6.9 mM of sodium taurocholate or C109 and challenged with ethanol. This methodology quantifies germination by exploiting the increased susceptibility of germinating spores to ethanol. *C. difficile* spores that survive the ethanol challenge are enumerated by subsequent germination and outgrowth on sodium taurocholate containing agar. The results are shown in figure 5.2. Exposure of *C. difficile* spores to 6.9 mM sodium taurocholate caused a decrease in *C. difficile* CFU/ml after 0.5, 1 and 2 hours demonstrating the germination of *C. difficile* spores. The respective log reductions in CFU/ml of 2.3, 2.9 and 1.7 were significant ($P = 0.0146$, $P = 0.0025$, $P = 0.0032$). Statistically significant 0.8 ($P = 0.0025$) and 1.2-log reductions ($P = 0.0032$) in CFU/ml of *C. difficile* were achieved after 1 and 2 hours exposure to thioglycollate medium supplemented with 6.9 mM C109. C109 used at 6.9 mM therefore caused a decrease in *C. difficile* CFU/ml but this was to a lesser extent than when sodium taurocholate was present and occurred only after 1 and 2 hours exposure.

5.3.2 The *C. difficile* spore germination response of novel compound C109 measured by membrane integrity straining

Membrane integrity straining of *C. difficile* spores was investigated with the LIVE/DEAD® BacLight™ Bacterial Viability kit to determine the effect of C109. A control *C. difficile* spore suspension in the absence of germinant and *C. difficile* spore suspension treated with 6.9 mM C109 for 2 hours were strained with SYTO 9 and propidium iodide and imaged by fluorescence microscopy. The control ungerminated spore suspension demonstrated visible spores and some remaining vegetative cells of
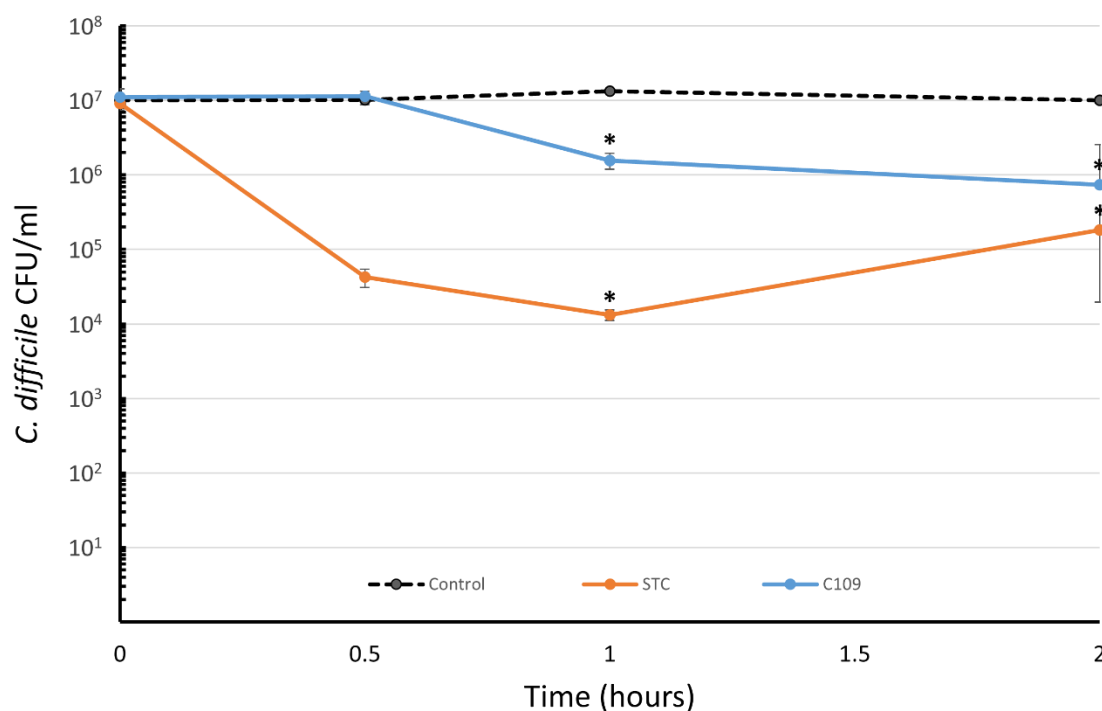
Figure 5.2. The activity of novel compound C109 to induce *C. difficile* NCTC 11204 spore germination measured by loss of resistance to ethanol. Germination of *C. difficile* spores exposed to thioglycollate medium only (dashed line) or thioglycollate medium supplemented with 6.9 mM sodium taurocholate (orange line) or C109 (blue line) was measured after 0.5, 1 and 2 hours by ethanol challenge. Error bars represent standard deviation. Statistically significant reductions in *C. difficile* CFU/ml compared to the control are indicated by an asterisk ($P < 0.01$).

C. difficile that were sparsely distributed in the field of view and were predominately stained red by propidium iodide, indicating loss of membrane integrity (figure 5.3 A). A very small number of spores were visible as stained green by SYTO 9, indicating intact cell membranes. Exposure of the *C. difficile* spore suspension to 6.9 mM C109 for 2 hours demonstrated spores and vegetative cells that were stained green by SYTO 9, consistent with the maintenance of membrane integrity (figure 5.3 B). No spores or vegetative cells stained red by propidium iodide were visible in the image. The visualisation of SYTO 9 stained spores indicates that the *C. difficile* spores had germinated in response to exposure to C109.
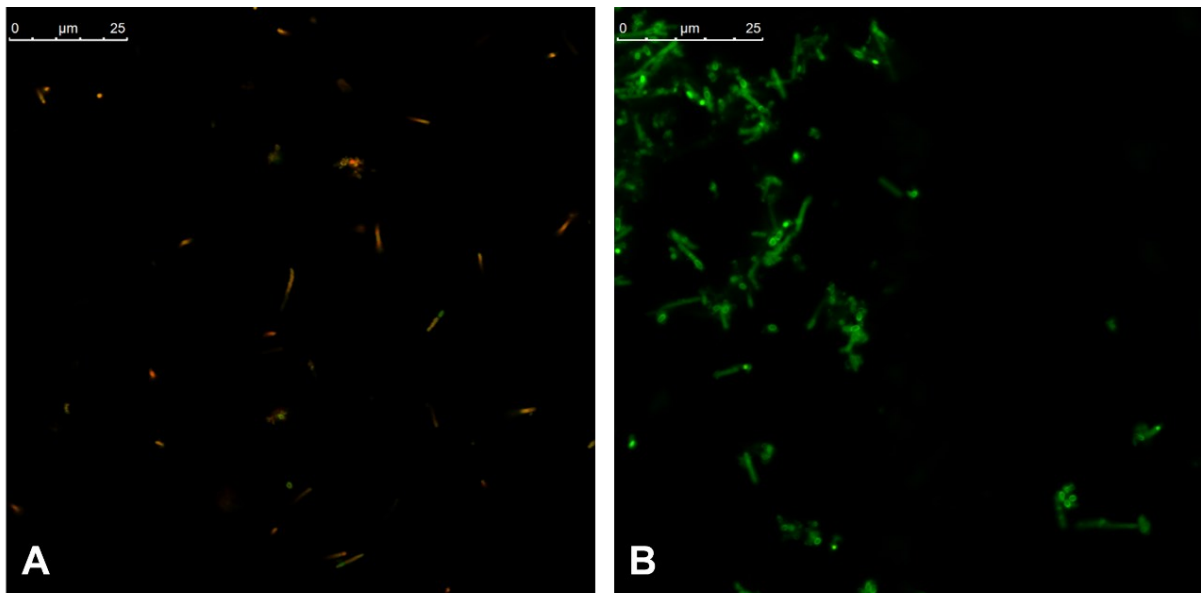
Figure 5.3 LIVE/DEAD staining of *C. difficile* NCTC 11204 spores in the absence and presence of novel compound C109. *C. difficile* **NCTC 11204 spores were visualised via fluorescence microscopy after 2 hours incubation with the control (A) or 6.9 mM C109 (B). Cells and spores with intact membranes are stained with SYTO 9 (green), those with damaged membranes are stained with propidium iodide (red).**

5.3.3 The germination response of *C. difficile* spores to novel compound C109 by measurement of optical density.

C109 demonstrated solubility incompatibility in both BHIS and Tris-HCl used in the optical density assay germination. The poor solubility of the novel compound severely affected the optical density measurement. C109 precipitated out of solution during the time course of the assay causing extremely variable and unreliable optical density measurements. This method was therefore unsuccessful in measuring the germination response of *C. difficile* spores to C109.

5.3.4 The antimicrobial activity of novel compound C109 measured by quantitative suspension test (European Standard EN: 1040: 2005).

The quantitative suspension test (European Standard EN: 1040: 2005) utilised for the evaluation of chemical disinfectants and antiseptics was selected to investigate the bactericidal activity of C109 against vegetative cells of *C. difficile* NCTC 11204*.* Exposure of *C. difficile* spores to 6.9 mM C109 resulted in a decrease in *C. difficile* CFU/ml compared to that of the control of 5% ethanol. Statistically significant 6 and 4-log reductions of *C. difficile* CFU/ml were achieved after 30 (<0.0001) and 60 minutes (<0.0001) exposure to 6.9 mM C109m, indicating bactericidal activity.
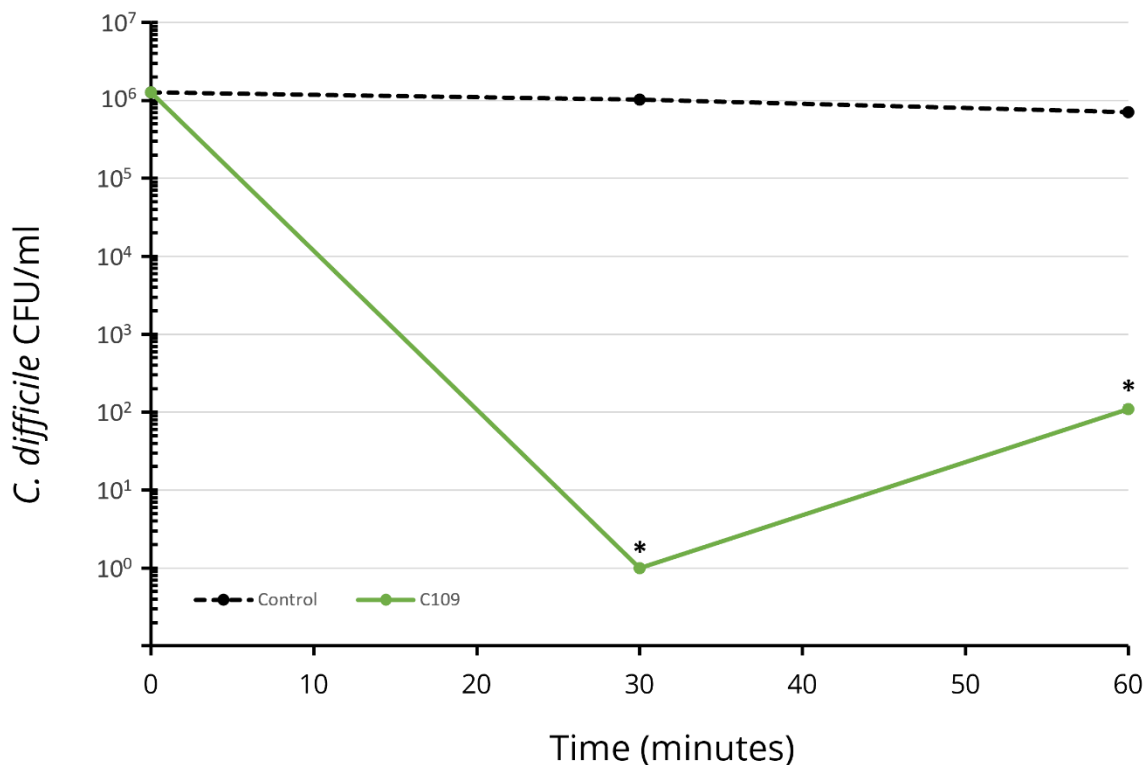
Figure 5.4 The determination of C109 bactericidal activity by quantitative suspension test (European Standard EN: 1040: 2005). The bactericidal activity of 6.9mM C109 was tested against *C. difficile* NCTC 11204 after 30 and 60 minutes. Statistically significant reductions in *C. difficile* CFU/ml compared to the control are indicated by an asterisk (*P* < 0.001).

5.3.5 The antibacterial activity of novel compound C109 measured by agar diffusion.

The susceptibility *C. difficile* vegetative cells and other bacterial strains to C109 was evaluated by agar diffusion. No inhibition of growth was recorded for *E. coli* NCTC 10538 or *P. aeruginosa* NCTC 11954 indicating C109 was not antimicrobial at the concentrations tested with these representative laboratory strains. Minor zones of growth inhibition, averaging 13.7 mm and 12.5 mm were recorded for *C. difficile* NCTC 11204 and *S. aureus* NCTC 10788 indicating antimicrobial activity. No zone of inhibition was recorded for the control, composed of the solution used to dissolve C109 (50% absolute ethanol 50% SDW).

Table 5.1. Agar diffusion assay to evaluate the antimicrobial activity of novel compound C109 against *C. difficile* and other bacterial strains. Zone of inhibition values including the diameter of the well (7mm) are presented as mean ± standard deviation. NA indicates no inhibition of bacterial growth.

Test Organism	Zone of inhibition (mm)
Clostridium difficile NCTC 11204	13.7 ± 1.4
Escherichia coli NCTC 10538	NA
Pseudomonas aeruginosa NCTC 11954	NA
Staphylococcus aureus NCTC 10788	12.5 ± 1.0

5.4 Discussion.

As part of a pro-spore germination strategy to eliminate the spores of *C. difficile,* novel bile salt-derived compounds were synthesized to have dual germination and antimicrobial activity against *C. difficile* spores. Preliminary testing by Hird (2014) indicated promising germination activity of lithocholate derived compound C109 but further analysis was necessary to determine if the activity of C109 to trigger *C. difficile* spore germination and antimicrobial activity against *C. difficile* could be demonstrated independently.

The successful initiation of germination of *C. difficile* spores by C109 was demonstrated by loss of resistance to ethanol. A resumption of metabolic activity and apparent germination of *C. difficile* spores was also seen by the staining of intact spore membranes in response to exposure to C109. The antimicrobial activity of C109 was demonstrated using the quantitative suspension test, causing a 6-log reduction of *C. difficile* vegetive cells after 30 minutes. The antimicrobial activity of C109 was also demonstrated by agar diffusion.

Novel compound C109 successfully induced the germination of *C. difficile* NCTC 11204 spores, demonstrating its activity as a *C. difficile* spore germinant. A 1.2-log reduction in CFU/ml of *C. difficile* was observed after 2 hours exposure to 6.9 mM C109 determined by loss of resistance to ethanol. While germination was successfully induced by C109, the extent of the germination response was lower than that demonstrated by the potent *C. difficile* germinant, sodium taurocholate which caused a 1.7-log reduction in CFU/ml of *C. difficile* after the same exposure period (Sorg and Sonenshein, 2008). A greater reduction in CFU/ml of *C. difficile* and therefore germination response to sodium taurocholate was also observed after exposure times of 0.5 and 1 hours. The *C. difficile* spore germination activity of C109 supports the results of the preliminary germination testing reported by Hird (2014).

The reduced activity of C109 to trigger germination of *C. difficile* spores compared to that of sodium taurocholate may be explained by a lower efficiency of C109 to induce *C. difficile* spore germination

in a similar way to lower reported germination activity of other bile salts (Sorg and Sonenshein, 2008; Bhattacharjee *et al.*, 2016a). It is also possible that the poor solubility C109 may have limited the potential germination response when using this methodology of measuring germination. Issues with reliability of C109 remaining in solution prohibited the use of the germination optical density assay to accurately measure the loss of the spore refractive index upon initiation of *C. difficile* spore germination in response to C109.

Membrane integrity staining of spores exposed to C109 also provided possible indirect evidence of *C. difficile* spore germination, although this methodology has not been widely used to assess the germination of *C. difficile* spores before. Visible untreated ungerminated *C. difficile* spores and vegetative cells were observed to be predominantly stained red indicating spore cell membranes that were permeable to propidium iodide. C109 treated *C. difficile* spores were stained green, demonstrating exclusion of propidium iodide (i.e. membrane integrity) following the induction of spore germination. BacLight™ staining has been utilised previously to examine germination and resistance of *Bacillus* species to sporicidal treatments (Laflamme *et al.*, 2004; Setlow *et al.*, 2016; Ghosh *et al.*, 2017). In agreement with this research, germinating spores were found to be strongly stained green by SYTO 9 but intact untreated and ungerminated spores did not efficiently take up either the SYTO 9 or propidium iodide BacLight™ stains (Setlow *et al.*, 2016). In contrast BacLight™ staining of untreated ungerminated *Bacillus cereus* spores were observed to be green when imaged by fluorescent microscopy (Laflamme *et al.*, 2004). This may suggest differential staining between bacterial species or issues with the experimental approach, where the penetration and staining with BacLight™ stains was limited with *C. difficile* spores.

The antibacterial activity and sensitivity of vegetative cells of *C. difficile* to C109 was assessed by the BSEN quantitative suspension test and agar diffusion. C109 was found to be highly bactericidal against *C. difficile* vegetative cells, causing a 6-log reduction in *C. difficile* CFU/ml after 30 minutes and a 4-log reduction at 1 hour. The antimicrobial activity of C109 was further demonstrated in the susceptibility

of *C. difficile* vegetative cells by agar diffusion. C109 caused the inhibition of growth of *C. difficile* and *S. aureus* NCTC 10788, although zones of inhibition were small. No inhibition of growth was observed when C109 was tested against *E. coli* NCTC 10538 and *P. aeruginosa* NCTC, suggesting the antimicrobial activity of C109 may be limited to Gram-positive bacteria. The results of the quantitative suspension test and agar diffusion susceptibility confirm the antimicrobial activity of C109 in addition to its germination activity.

As C109 has been established to have dual germination and antimicrobial activity, a mixed population of spores stained both red by propidium iodide and green by SYTO 9 might have been predicted during membrane integrity staining, as reported by other researchers (Ghosh *et al.*, 2017). The triggering of *C. difficile* spore germination by C109 is thought to cause the partial core rehydration and loss of spore resistance properties (Setlow, 2003; 2014). Germinated *C. difficile* spores therefore have increased susceptibility to the antimicrobial activity of C109, derived from its quaternary ammonium cation. The antimicrobial activity of quaternary ammonium compounds is attributed to the damage caused to cell membranes among other targets (Kwaśniewska *et al.*, 2020). The absence of observable propidium iodide stained *C. difficile* spores may be due to issues with adequate spore staining methodology or could suggest the antimicrobial activity of C109 against germinating *C. difficile* spores is derived from another mechanism of action other than adversely affecting the integrity of the spore membranes.

The reported germination and antimicrobial activity of C109 in this study is limited by the use of the single *C. difficile* laboratory strain 11204. Evaluation of the germination and antimicrobial activity of C109 by measurement of loss of resistance to heat and the quantitative suspension test with multiple laboratory and clinical strains of *C. difficile* would be beneficial to understand the spectrum activity of C109 with *C. difficile* strains. Investigation of antimicrobial activity derived from the quaternary ammonium cation should also be expanded to other clinically relevant microorganisms. This is particularly relevant as the incorporation of C109 in a polymeric material to create a sporicidal smart surface is intended for use in clinical settings where *C. difficile* spores and other pathogens, which are

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the cause of healthcare associated infections, contaminate the environment (Samore *et al.*, 1996; Sethi *et al.*, 2010; Department of Health, 2007b).

As demonstrated by the inability to measure the germination of *C. difficile* spores in response to C109 using the optical density assay, the poor solubility of C109 should be consider when evaluating the reliability of experimental results. While care was taken to ensure that C109 remained in solution throughout experiments presented in this chapter, it was observed that C109 could precipitate from solution when stored, particularly at lower temperatures. Experimental findings are therefore considered in the knowledge that the final concentration of 6.9 mM used may be not have been achieved due to precipitation of C109. While C109 was demonstrated to be effective at inducing the germination of *C. difficile* spores and have antimicrobial activity against *C. difficile* and other bacteria the extent of germination and antimicrobial activity may be underrepresented in liquid media due to the poor solubility of C109. To adequately assess the potential activity of C109 to germinate and consequently eliminate *C. difficile* spores, the novel compound should be tested in a polymeric material to form a sporicidal smart surface.

In conclusion, the novel compound C109, a lithocholate derived bile acid amide molecularly tethered to a quaternary ammonium cation was demonstrated to successfully trigger the germination of *C. difficile* spores by measurement of loss of resistance to ethanol and membrane integrity staining. Measurement of spore germination by the optical density assay was not compatible due to issues associated with the solubility of C109 in aqueous media. The use of a standard agar diffusion assay and the BSEN quantitative suspension test established the sensitivity of *C. difficile* vegetive cells to C109 and confirmed its antimicrobial activity against *C. difficile.* This demonstrates C109 has dual germination and antimicrobial activity against *C. difficile.* Antimicrobial activity of C109 against other clinically relevant microorganism was also indicated however an extended panel of test microorganisms is required to establish this in further work.

Chapter 6

6.0 Development of a prototype sporicidal smart surface incorporating novel compounds with dual germination and antimicrobial activity for the elimination of *Clostridioides difficile* spores.

6.1 Introduction.

The spores of *C. difficile* are the vector of transmission of CDI and ensure survival and persistence in the environment (Deakin *et al.*, 2012; Paredes-Sabja *et al.*, 2014). The spore structure is highly resistant to adverse environmental conditions, including disinfectants and biocides permitting dissemination in clinical environments (Driks, 2003; Fawley *et al.*, 2007; Samore *et al.*, 1996). Routine detergent-based cleaning is ineffective at eliminating contaminating spores while sporicidal agents intended to target spores of *C. difficile* have been found to deliver inadequate decontamination and even enhance the rate of *C. difficile* sporulation (Wilcox *et al.*, 2003; Speight *et al.*, 2011; Fawley *et al.*, 2007). To prevent the transmission of CDI and exposure of vulnerable patients to *C. difficile* alternative strategies to eliminate *C. difficile* spores in the clinical environment are necessary.

Exploiting the initiation of *C. difficile* germination as a mechanism to enhance the elimination of *C. difficile* spores with traditional biocides was first proposed by Wheeldon *et al.* (2010). The exposure of *C. difficile* spores to a germinant solution containing bile salt germinants and amino acid co-germinants causes the activation of the initial phase of germination where the spore core releases Ca-DPA and is partially rehydrated. This transformation results in the loss of spore resistant properties and increased susceptibility to biocides (Wheeldon, 2008; Wheeldon *et al.*, 2010; Setlow, 2014; 2003). Wheeldon demonstrated 30 minutes preincubation of *C. difficile* spores with a germination solution comprising thioglycollate broth supplement with 1% (w/v) sodium taurocholate and subsequent 70% ethanol challenge resulted in 1.85 and 2.15 -Log reduction for *C. difficile* NCTC 11204 and 027 strains

respectively (Wheeldon, 2008). The use of a germination solution to sensitise *C. difficile* spores was further developed with the use of a specific combination of amino acid co-germinants with sodium taurocholate and the biocides, benzyl alcohol and benzalkonium chloride, reducing the two-stage germination and disinfection procedure to a single application. The use of germinant and antimicrobial solution resulted in a 3-log reduction in *C. difficile* spores in one hour (Wheeldon *et al.*, 2010).

Other researchers have also utilised the 'germinate to exterminate' principle as a strategy to increase the susceptibility of *C. difficile* spores to chemical disinfection and adverse environmental conditions. Nerandzic and Donskey (2010) combined a germinant formulation, consisting of the bile salt sodium taurocholate, multiple amino acids and minerals with the secondary UV-C radiation or heat. The triggering of germination following exposure to the germinant formulation enhanced the killing of *C. difficile* spores by UV-C radiation, delivered by an automated room disinfection device or heat treatment at 80°C for 5 minutes. *C. difficile* spores that were germinated in response to the germinant formulation but not exposed to UV-C radiation or heat treatment also demonstrated reduced survival, thought to be as a result of exposure to environmental stressors such as atmospheric oxygen (Nerandzic and Donskey, 2010). The same authors utilised the germinant formulation with the addition of nisin, a preparation suitable for application onto the skin to demonstrate its potential to decontaminate hands (Nerandzic and Donskey, 2013). The germinant formulation was also combined with a quaternary ammonium compound to produce a disinfectant targeting *C. difficile* spores, emulating the approach taken by Wheeldon *et al.* (2010) (Nerandzic and Donskey, 2016). The dual germinant and biocide formulation produced a 1.2-2.7-log reduction of spores of *C. difficile* strains after 1 hours and a further 2-3.2 log reduction after 24 hours on the surface of stainless-steel carriers. The elimination of dormant spores was also demonstrated when the formulation was utilised on hospital surfaces inoculated with *C. difficile* spores. The use of the dual germinant and biocide formulation was also found to enhance the susceptibility of *C. difficile* spores to killing by acidic conditions, high intensity blue light and UV-C radiation (Nerandzic and Donskey, 2016). The application and successful use of germinant solutions and formulations containing germinants, co-germinants and

biocides to trigger germination and sensitise *C. difficile* spores to enhance elimination on a variety of surfaces demonstrates a promising strategy to target contaminating spores in the clinical environment.

Using the 'germinate to exterminate' concept as a basis for the elimination of *C. difficile* spores from surfaces, a series of bile salt-derived novel compounds with dual function were developed at Aston University (Hird, 2014). The novel compounds were proposed to function both as bile salt germinant and as antimicrobial agent, in the form of a covalently linked quaternary ammonium compound. The aim of the project was to synthesise a germinant and novel antimicrobial compound that could be incorporated into a polymeric biomaterial in order to germinate and eliminate *C. difficile* spores *in situ*. Initially several bile amides were produced from the chemical manipulation of deoxycholic acid, cholic acid and lithocholic acid. These bile amides were screened for germinant activity, before the tertiary amine group of promising candidates was quaternized to introduce antimicrobial functionality via a quaternary ammonium cation. Several candidates were considered to demonstrate dual germination and antimicrobial activity during initial testing in liquid media, indicating suitability for inclusion in a polymeric biomaterial such as a smart surface, however the efficacy of the dual germinant and antimicrobial novel compounds were not evaluated as part of a polymeric material as proposed (Hird, 2014).

Antimicrobial polymers specifically targeting *C. difficile* have previously been developed, Liu *et al.* (2014) demonstrated synthetic polymers incorporating compounds that mimic host-defence peptides were inhibitory against *C. difficile* vegetative cells. The synthetic polymers were also found to prevent the outgrowth of germinated *C. difficile* spores but did not have any germination inhibitory activity. There was no change in the rate of germination following pre-incubation with the synthetic polymer or antimicrobial peptides compare to the control (Liu *et al.*, 2014). While these synthetic antimicrobial peptides may have an *in vivo* application to prevent the outgrowth of ingested *C. difficile* spores, the lack of germination inhibitory activity excludes functionality as polymeric smart surface to target

contaminating *C. difficile* spores in the clinical environment. Antimicrobial smart surfaces however have been demonstrated to be effective against a number of other clinically relevant microorganism, including the antibiotic resistant and highly virulent ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens (Muñoz-Bonilla and Fernández-García, 2012; Kamaruzzaman *et al.*, 2019). Providing bactericidal or bacteriostatic activity upon contact, antimicrobial polymeric materials contain antimicrobials covalently bonded to a polymer matrix, producing reusable, chemically stable materials with long-term activity that offer an alternative to the use of antibiotics and biocides (Xue *et al.*, 2015; Kamaruzzaman *et al.*, 2019).
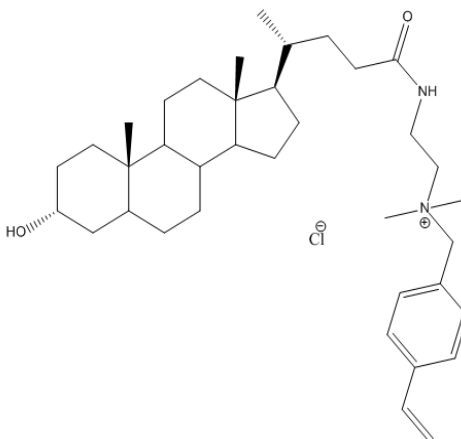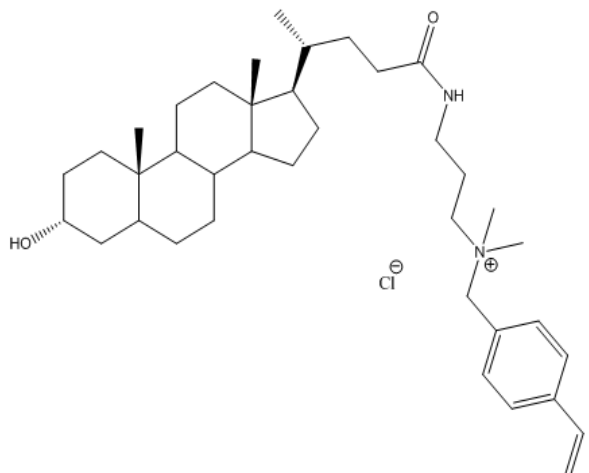
The aim of this chapter was to evaluate a series of polymer formulations incorporating bile salt-derived novel compounds developed at Aston University to deliver dual germination and antimicrobial activity, employing the principle of 'germinate to exterminate' as a strategy to eliminate *C. difficile* spores. A *C. difficile* targeting polymeric biomaterial has potential for use as a smart surface within clinical settings, providing passive decontamination of spores to reduce and prevent the transmission of CDI. The impact of polymer composition, processing conditions and post-processing purification of the polymer formulations incorporating bile salt-derived novel compounds on *C. difficile* spore germination activity of the polymer formulations was determined. Following the establishment of effective germination activity of the polymer formulations, the antimicrobial activity against *C. difficile* was investigated.

6.2 Methods.

6.2.1 Synthesis of novel compounds with dual germination and antimicrobial activity.

The synthesis of novel compounds designated C109, C114 and C119 was kindly completed by Dr Selma Riasat at the Polymer Processing and Performance Research Unit, Chemical Engineering and Applied Chemistry, Aston University as described by Dr Matthew Hird (Hird, 2014; Riasat, 2016). Full details of the synthetic routes are described in Appendix 1.

Table 6.1 Lithocholic acid derived novel compound chemical structures

Compound number	Chemical structure
C109	
C114	

C119

6.2.2 Production, processing and purification of polymers incorporating novel compounds with dual germination and antimicrobial activity.

Polymers incorporating novel compounds designated C109, C114 and C119 were kindly produced, processed and purified by Dr Selma Riasat at the Polymer Processing and Performance Research Unit, Chemical Engineering and Applied Chemistry, Aston University (Riasat, 2016).
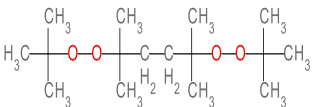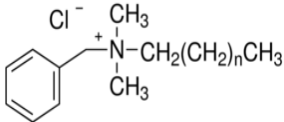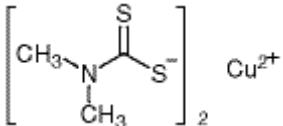
6.2.3. Polymer production.

Unstabilised high density polyethylene (HDPE) powder with no stabiliser (Lupolen 5261ZQ456) and stabilised HDPE pellets (BorPex HE1878E) containing 700 ppm Irganox 1076 were used throughout for preparation of the polymer formulations. Monomeric units of unstabilised or stabilised high-density polyethylene were polymerised by free radical polymerisation in the presence of novel compounds C109, C114 and C119 (3% v/v) and/or other additives (benzalkonium chloride and copper nanoparticles) and converted into thin films via compression moulding.

Processing of high-density polyethylene in the presence and absence of additives.

HDPE and the peroxide initiator (2,5-dimethyl-2,5-bis(t-butylperoxy)hexane, (Trigonox 101) were mixed using a small amount of hexane into a slurry to achieve good distribution, before being dried in a fume hood to remove traces of solvent. The slurry was stirred every 15 minutes during the drying process. After appropriate drying, the required amount of the chosen novel compound to achieve 3% (w/v) was weighed and transferred to the polyethylene/peroxide mixture which was homogenised on a roller for 48 hours in order to obtain an even distribution of the formulations.

Table 6.2 Commercial compounds used in the production of polymers

Compound	Commercial Name	Chemical structure	Supplier
Unstabilised high density polyethylene	Lupolen 5261ZQ456		Basell PO (Provided by Uponor Sweden)
Stabilised high density polyethylene	BorPex HE1878E		Borealis (Provided by Uponor Sweden)
2,5-dimethyl-2,5-bis(t-butylperoxy) hexane	Trigonox 101 (T101)		Akzo Nobel
Benzalkonium chloride (BKC)	Benzalkonium chloride		Sigma Aldrich
Copper nanopowder (Cu)	Copper nanopowder (25 nm particle)		Sigma Aldrich

6.2.4 Thermal processing of polymers formulations.

Compression moulding.

The selected polymer formulation (in powder or pellet form) was placed between Teflon sheets, inserted between two stainless steel sheets and placed between platens of a compression moulding machine (Turton & Bradley) pre-heated to the appropriate temperature. The heated platens were closed together for an initial 2 minutes at zero pressure, followed by a further 5 minutes under maximum pressure of 22 kg/cm unless otherwise stated. The film samples were then cooled inside the press platens immediately (by circulating cold water inside the platens) until the temperature dropped down to 50°C after which the polymer films were removed and stored in the dark for subsequent use.

Mini-Lab Haake extrusion.

The mixing chamber of a MiniLab (Haake) extruder was initially preheated and flushed with nitrogen for more than 2 minutes to remove oxygen from the chamber (to minimise polymer oxidation) before loading the dry formulation. The processing temperature and the concentration of the additives were varied but the conical screw speed was fixed at 65 rpm for 5 minutes. The melt temperature and the processing Torque were continuously monitored using dedicated software "PolyLab Monitor Version 4.16". After completion of the compounding process, the processed polymer was removed from the mixer and cooled down (in cold water) to avoid thermal oxidation. Polymer films were subsequently prepared by compression moulding as described above.

6.2.5 Purification of polymer films.

In order to establish if the novel compounds had been successfully integrated in the polymer matrix, the polymer film samples were subjected to one of the 3 following purification processes:

Methanol accelerated solvent extraction.

Purification of film samples was carried out in a Dionex accelerated Solvent Extractor 200. Polymer film pieces 4 x 2 cm were placed in stainless steel cells and extracted using the accelerated solvent extractor equipment. Extraction with methanol was achieved at optimised oven temperature of 80°C and pressure of 2000 psi for 5 cycles, each cycle being of 30 minutes duration. 100% methanol was used to remove any unreacted and free additive from the samples.

Soxhlet extraction with methanol.

Polymer films were exhaustively Soxhlet extracted in methanol under nitrogen for 48 hours, in order to remove any unbound homopolymerised additives and any low molecular mass material. The extracted films were dried at room temperature in a vacuum oven overnight.

Methanol wipe.

The surface of polymer films were wiped with paper towel soaked in methanol to remove any surface-loose additives.

Table 6.3 Summary of polymer formulations, processing, and purification methods.

The composition of polymer formulations incorporating novel compounds and the processing and purification methods utilised prior to microbiological testing. Unstabilised HDPE Lupolen (L), stabilised HDPE Borpex (B), trigonox 101 (T101), benzalkonium chloride (BKC), accelerated solvent extraction (ASE)

Polymer code	Composition				Processing conditions			Purification method
	HDPE	Trigonox 101 (% w/v)	Novel compound (% w/v)	Antimicrobial compound (% w/v)	Temperature	Compression time (minutes)	Mini-Lab Haake	
HDPE (L) 160°C	Unstabilised (Lupolen 5261ZQ456)	0.005			160°C	5		
HDPE (B) 160°C	Stabilised (BorPex HE1878E)	0.005			160°C	5		
3% C109 (L) 160°C	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)		160°C	5		
3% C109 (L) 180°C	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)		180°C	5		
3% C114 (L) 160°C	Unstabilised (Lupolen 5261ZQ456)	0.005	C114 (3%)		160°C	5		
3% C119 (L) 160°C	Unstabilised (Lupolen 5261ZQ456)	0.005	C119 (3%)		160°C	5		
3% C109 (B) 160°C	Stabilised (BorPex HE1878E)	0.005	C109 (3%)		160°C	5		
3% C109 (B) 180°C	Stabilised (BorPex HE1878E)	0.005	C109 (3%)		180°C	5		
3% C119 (B) 160°C	Stabilised (BorPex HE1878E)	0.005	C119 (3%)		160°C	5		

| Polymer code | Composition | | | | Processing conditions | | | Purification method |
	HDPE	Trigonox 101 (% w/v)	Novel compound (% w/v)	Antimicrobial compound (% w/v)	Temperature	Compression time (minutes)	Mini-Lab Haake	
6% C109 (B) 160°C	Stabilised (BorPex HE1878E)	0.005	C109 (3%)		160°C	5		
3% C109 (L) -T101 160°C	Unstabilised (Lupolen 5261ZQ456)	0	C109 (3%)		160°C	5		
3% C109 (L) 170°C	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)		170°C	5		
3% C109 (L) 160°C (10 CM)	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)		160°C	10		
3% C109 (L) 160°C Cu II	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)	Copper nanoparticles (15%)	160°C	5		
3% C109 (L) -T101 160°C Cu II	Unstabilised (Lupolen 5261ZQ456)	0	C109 (3%)	Copper nanoparticles (15%)	160°C	5		
3% C109 (L) 160°C BKC	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)	BKC (3%)	160°C	5		
3% C109 (L) -T101 160°C BKC	Unstabilised (Lupolen 5261ZQ456)	0	C109 (3%)	BKC (3%)	160°C	5		
3% C109 (L) 160°C BKC (5H)	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)	BKC (3%)	160°C	5	5 minutes at 160°C	

Table 6.3 continued. Summary of polymer formulations, processing, and purification methods

The composition of polymer formulations incorporating novel compounds and the processing and purification methods utilised prior to microbiological testing. Unstabilised HDPE Lupolen (L), stabilised HDPE Borpex (B), trigonox 101 (T101), benzalkonium chloride (BKC), accelerated solvent extraction (ASE).

Polymer code	Composition				Processing conditions			Purification method
	HDPE	Trigonox 101 (% w/v)	Novel compound (% w/v)	Antimicrobial compound (% w/v)	Temperature	Compression time (minutes)	Mini-Lab Haake	
3% C109 (L) -T101 160°C BKC (5H)	Unstabilised (Lupolen 5261ZQ456)	0	C109 (3%)	BKC (3%)	160°C	5	5 minutes at 160°C	
3% C109 (L) -T101 160°C Soxhlet extraction	Unstabilised (Lupolen 5261ZQ456)	0	C109 (3%)		160°C	5		Soxhlet extraction
3% C109 (L) -T101 160°C Methanol ASE	Unstabilised (Lupolen 5261ZQ456)	0	C109 (3%)		160°C	5		Methanol ASE
3% C109 (L) 160°C Methanol ASE	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)		160°C	5		Methanol ASE
3% C109 (B) 160°C Methanol ASE	Stabilised (BorPex HE1878E)	0.005	C109 (3%)		160°C	5		Methanol ASE
3% C109 (L) 160°C Soxhlet extraction	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)		160°C	5		Soxhlet extraction
3% C109 (L) 160°C methanol wipe	Unstabilised (Lupolen 5261ZQ456)	0.005	C109 (3%)		160°C	5		Methanol wipe

6.2.6 Microbiological testing of polymers incorporating novel compounds with dual germination and antimicrobial activity.

The microbiological testing of the various different polymers incorporating C109, C114 and C119, BKC and copper nanoparticles was carried out by the author as part of the research study described in this thesis.

6.2.6.1 Preparation of spore suspensions of *C. difficile* NCTC 11204.

Spore suspensions of *C. difficile* were prepared using a modified version of the method described by Shetty *et al.* (1999) and (Wheeldon *et al.*, 2008a). Frozen stocks of *C. difficile* NCTC 11204 stored at -80°C were used to inoculate Columbia blood agar plates (Oxoid, UK) supplemented with 5% (v/v) defibrinated horse blood (E&O Laboratories Ltd, UK). The inoculated plates were incubated at 37°C under anaerobic conditions (MiniMACS Anaerobic Workstation, Don Whitley, UK) for 48-72 hours before being incubated aerobically at room temperature for 72-120 hours. Spores were harvested by removing the *C. difficile* colonies with sterile cotton swabs and suspending in 50% (v/v) sterile distilled water 50% (v/v) absolute ethanol. The presence of a high concentration of spores was confirmed by the Schaeffer-Fulton staining technique and stored at 4°C until required (Schaeffer and Fulton, 1933). Prior to experimental use the spore suspensions were diluted and/or re-suspended in 100% sterile distilled water and enumerated via serial dilution to determine the CFU/ml of *C. difficile* spore suspension.

6.2.6.2 Determination of *C. difficile* spore germination activity of polymer samples by measurement of loss of resistance to ethanol.

The measurement of germination by loss of resistance to ethanol was based on the methods described by Levinson and Hyatt (1966) and utilised by Wheeldon *et al.* (2008a). This method exploits the loss of spore resistant properties upon initiation of germination. Germinating spores are consequently sensitive to heat and chemical inactivation. Spores that do not initiate germination during the experimental challenge are subsequently germinated under established germination and cultivation conditions in order to be enumerated (Gould *et al.*, 1968; Wheeldon *et al.*, 2008a).

Polymer samples were cut aseptically into 2 cm x 2 cm square coupons using sterile scissors and stored in a sterile petri dish. In aerobic conditions, the polymer coupons were inoculated with a 10 μl aliquot of a *C. difficile* spore suspension (10^7 CFU/ml) thus providing 10^5 CFU per coupon directly on the polymer surface. Inoculated polymers were stored at room temperature in sterile petri dishes. Each polymer sample was tested in triplicate (3 coupons) at 0, 2, 24 and 96 hours post inoculation.

At the appropriate time post inoculation, 0.3 ml of 100% ethanol was added directly to the surface of each polymer coupon and incubated at room temperature for 15 minutes to eliminate germinating *C. difficile* spores. Using sterile forceps, the polymer coupons were transferred to individual sterile universal tubes containing 10 ml of sterile distilled water (SDW). The universal tubes containing the polymer coupons were mixed vigorously by vortex for 30 seconds before a serial dilution was completed using SDW to enumerate the bacteria. For each of the relevant dilutions, an aliquot of 100 μl was spread over the surface of a Wilkins Chalgren agar plates, supplemented with 0.1% (w/v) sodium taurocholate. Wilkins Chalgren agar plates were incubated at 37°C under anaerobic conditions for 48 hours. Following incubation, the number of colonies on each agar plate was counted to determine the CFU/ml of *C. difficile*.

6.2.6.3 Determination of the antimicrobial activity of polymer samples against germinated *C. difficile* spores.

The method used to determine the *C. difficile* spore germination activity of polymer samples by measurement of loss of resistance to ethanol was modified to investigate the antimicrobial activity of polymer samples against germinated *C. difficile* cells. *C. difficile* spores are not exposed to ethanol, instead the intrinsic antimicrobial properties of the polymer formulations are responsible for the elimination of germinating spores. As previously spores that do not initiate germination during the experimental challenge are subsequently germinated by established germination conditions in order to be enumerated (Gould *et al.*, 1968; Wheeldon *et al.*, 2008a).

At the appropriate time, post inoculation, sterile forceps were used to transfer the polymer coupons directly to sterile universals containing 2 ml of Dey-Engley neutralising broth (BD Biosciences, USA). The universal tubes containing the polymer coupons and DE neutralising broth were mixed vigorously by vortex for 30 seconds and incubated for 5 minutes at room temperature. To complete serial dilutions, 8 ml of SDW was added to each universal tube. The method was then completed as described for the determination of *C. difficile* spore germination activity of polymer samples by measurement of loss of resistance to ethanol. Each polymer sample was tested in triplicate (3 coupons) at 0, 2, 24 and 96 hours post inoculation.

6.2.6.4 Statistical analysis.

A two-way analysis of variance (ANOVA) was used to determine the effect of polymer formulations and time point on *C. difficile* CFU/ml, as a measure of germination and antimicrobial activity. *Post hoc* Bonferroni's multiple comparisons tests were used to statistically analyse the results. Both statistical tests were completed using the GraphPad Prism software (GraphPad, USA).

6.3 Results.

6.3.1 The germination activity of prototype polymer formulations incorporating novel compounds.

Various prototype polymer formulations incorporating novel compounds C109, C114 and C119 were produced by altering the base HDPE, polymer additives and processing temperature in order to investigate the ability to trigger spore germination as a strategy to eliminate *C. difficile*. The germination activity of polymer formulations was determined by quantifying the loss of *C. difficile* spore resistance to ethanol, whereby germinated spores regain sensitivity to adverse environmental conditions (Levinson and Hyatt, 1966; Setlow, 2003). Germinating spores are therefore susceptible to the antimicrobial activity of ethanol and the reduction in viable cells can be determined. In order to first establish that polymer formulations incorporating the novel compounds were able to reduce the number of recoverable *C. difficile* spores through the initiation of germination, and that this reduction was substantially more effective than the reduction of recoverable *C. difficile* spores from control polymers containing no known germinants, spores of *C. difficile* NCTC 11204 were inoculated onto the surface of control and prototype polymer formulations coupons incorporating novel compounds C109, C114 and C119 as described in the methods. The results were analysed by comparing the *C. difficile* CFU/ml from polymer formulations incorporating the novel compounds and control polymers at the equivalent time points.

Investigations revealed diverse germination activity across the polymer formulations incorporating novel compounds at the different time points tested and a reduction of recoverable *C. difficile* spores from the control polymers. Of the polymer formulation produced from unstabilised HDPE (Lupolen 5261ZQ456), only the formulation of 3% C114 (L) 160°C produced a statistically significant 0.99 log reduction ($P < 0.0001$) in *C. difficile* CFU/ml after 24-hours compared to the control polymer at the same time point (figure 6.1A). Log reductions of 1.29 and 1.2 were observed at 2- and 96-hours but were not significant when compared with reductions of the control polymer with no

additive. The other unstabilised polymer formulations tested; 3% C109 (L) 160°C, 3% C109 (L) 180°C and 3% C119 (L) 160°C did not produce significant ($P > 0.05$) log reductions at 2-, 24- or 96-hours when compared to the control polymer at the same time point but demonstrated promising results for further investigation. Polymer formulation 3% C109 (L) 160°C and 3% C119 (L) 160°C produced a non-significant 0.78 and 0.81 log reduction of *C. difficile* spores respectively, at 2 hours.

For the polymer formulation produced from stabilised HDPE (BorPex HE1878E), the formulation containing 3% C109 (B) 160°C produced a significant ($P = 0.0004$) reduction in *C. difficile* CFU/ml after 2 hours, demonstrating a 2.10 log reduction compared to the control (HDPE (B) 160°C) at the same time point (figure 6.1B). The same formulation also demonstrated a non-significant ($P > 0.05$) log reduction of 1.86 at 24 hours and 1.53 at 96 hours compared to the control at the same time point. A significant reduction was not observed ($P > 0.05$) for any of the other stabilised polymer formulations tested compared to the control polymer at the same time point. As for the unstabilised polymer formulation, other stabilised polymer formulations while non-significant, also demonstrated promising results. Formulation 3% C119 (B) 160°C produced a non-significant 0.83 log reduction of *C. difficile* spores at 2 hours.

A reduction in the recovery of *C. difficile* spores from the unstabilised and stabilised control polymer formulations HDPE (L) 160°C and HDPE (B) 160°C was observed over the time points of the experiment but this was not determined to be a statistically significant reduction from time point zero. Polymer formulations incorporating novel compounds produced with stabilised and unstabilised high density polyethylene both demonstrated germination activity as measured by the reduction of recovered *C. difficile* spores. As a result of these findings, subsequent polymer formulations were produced to establish the optimal composition and processing conditions for the germination of *C. difficile* spores.

A

B

Figure 6.1 Determination of *C. difficile* spore germination activity by prototype polymer formulations containing novel compounds C109, C114, C119 produced from stabilized and unstabilised high density polyethylene by measurement of loss of resistance to ethanol. Germination activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. A) Unstabilised HDPE (Lupolen 5261ZQ456) polymer formulations. B) Stabilised (BorPex HE1878E) polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control at the same time point are marked with asterisks ($P < 0.05$).

6.3.2 The role of novel compound concentration on the germination activity of polymer formulations incorporating novel compound C109.

The initial experiments established that polymer formulations incorporating the novel compounds were effective at reducing the recoverable *C. difficile* spores compared to control polymers at the equivalent time point. Subsequent investigations of the polymer formulations incorporating the novel compounds quantified and analysed the reduction of recovered *C. difficile* (CFU/ml) at the specified time points compared to the recovered *C. difficile* (CFU/ml) at time zero. Unstabilised HDPE (Lupolen 5261ZQ456) was chosen as the base material for the production of polymer formulation used to investigate the role of novel compound concentration and peroxide initiator on the germination activity.

In order to investigate the dose dependant effect of novel compound C109 on germination activity, a polymer formulation with an increased concentration (6%) was produced. Increasing the concentration of C109 incorporated into the polymer formulation was not found to enhance germination activity. The unstabilised polymer formulation 6% C109 (L) 160°C produced a significant reduction ($P < 0.0001$) of *C. difficile* CFU/ml following 2, 24 and 96 hours exposure, demonstrating a 0.69, 0.49 and 0.52 log reduction respectively (figure 6.2). However, the polymer formation incorporating a lower concentration of the novel compound, 3% C109 (L) 160°C was found to induce a greater log reduction at the same time points, demonstrating significant 2.11 (P 0.0104), 1.52 ($P < 0.0001$) and 1.10 log reduction ($P < 0.0001$) at 2, 24 and 96 hours respectively.

Figure 6.2 Determination of *C. difficile* spore germination by polymer formulations containing novel compound C109 by measurement of loss of resistance to ethanol. Germination activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control are marked with asterisks ($P < 0.05$).

6.3.3 The role of peroxide initiator T101, on the germination activity of polymer formulations incorporating novel compound C109.

Polymer formulations produced without the peroxide initiator trigonox, demonstrated significant germination activity, but this was to a lesser extent compared to the polymer formations produced with trigonox. Unstabilised polymer formulation 3% C109 (L) -T101 160°C produced a significant reduction in *C. difficile* CFU/ml after 2 and 96 hours exposure, demonstrating 0.79 ($P < 0.0001$) and 0.72 log reductions respectively (figure 6.3A). A non-significant ($P > 0.05$) 0.70 log reduction was achieved after 24 hours. The equivalent polymer formulation produced with trigonox, 3% C109 (L) 160°C demonstrated a significant reduction ($P < 0.001$) of *C. difficile* CFU/ml of 2.11, 1.54 and 1.10 log at 2, 24 and 96 hours respectively. The completion of post processing purification via Soxhlet methanol extraction and methanol ASE reduced the germination activity of polymer formulations but the reduction of *C. difficile* spores remained statistically significant with the exception of one time point. Polymer formulation 3% C109 (L) -T101 160°C Soxhlet extraction produced a significant reduction of *C. difficile* CFU/ml at 2 and 24 hours exposure, demonstrating a 0.58 ($P = 0.0023$) and 0.67 log reduction ($P = 0.0147$) respectively (figure 6.3B). The 0.60 log reduction achieved at the 96-hour time point was not significant ($P > 0.05$). Polymer 3% C109 (L) -T101 160°C methanol ASE produced a significant reduction ($P < 0.0001$) of *C. difficile* CFU/ml at 2, 24 and 96 hours exposure, demonstrating a 0.30, 0.11 and 0.15 log reduction respectively (figure 6.3B).

Figure 6.3 Determination of *C. difficile* spore germination by polymer formulations containing novel compound C109 produced with and without the peroxide initiator Trigonox 101 (T101) by measurement of loss of resistance to ethanol. Germination activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. A) Unstabilised polymer formulations. B) Post processing purified unstabilised polymer formulations produced without the peroxide initiator T101. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control are marked with asterisks (*P* < 0.05).

6.3.4 The role of stabilised and unstabilised HDPE on the germination activity of polymer formulations incorporating novel compound C109.

The impact of the base material of the polymer formulation was re-examined by incorporating novel compound C109 into polymers comprised of unstabilised (Lupolen 5261ZQ456) and stabilised (BorPex HE1878E) high density polyethylene. Unstabilised and stabilised polymer formulation incorporating 3% C109 produced at 160°C both demonstrated statistically significant reductions in *C. difficile* CFU/ml during testing. The unstabilised (Lupolen 5261ZQ456) polymer formulation 3% C109 (L) 160°C produced a significant reduction of *C. difficile* CFU/ml at 2, 24 and 96 hours exposure, demonstrating a 1.88 ($P < 0.0001$), 1.52 ($P = 0.0021$) and 1.34 log reduction ($P = 0.0013$) respectively (figure 6.4A). The stabilised (BorPex HE1878E) polymer formulation 3% C109 (B) 160°C produced a significant ($P < 0.0001$) reduction in *C. difficile* CFU/ml of 1.83 and 1.63 log at 24 and 96 hours respectively (figure 6.4B). A 0.53 log reduction was achieved after 2 hours but was not statistically significant ($P > 0.05$).

The addition of post processing purification via methanol-accelerated solvent extraction reduced the germination activity of unstabilised and stabilised polymer formulations. Unstabilised (Lupolen 5261ZQ456) polymer formulation 3% C109 (L) 160°C methanol ASE produced a significant reduction in *C. difficile* CFU/ml of 0.29 ($P < 0.0001$) and 0.36 log reduction ($P < 0.0019$) at 2 and 24 hours respectively (figure 6.4A). Stabilised (BorPex HE1878E) polymer formulation 3% C109 (B) 160°C methanol ASE produced a significant ($P < 0.001$) reduction in *C. difficile* CFU/ml of 0.50 and 0.65 log reduction at 24 and 96 hours respectively (figure 6.4B)
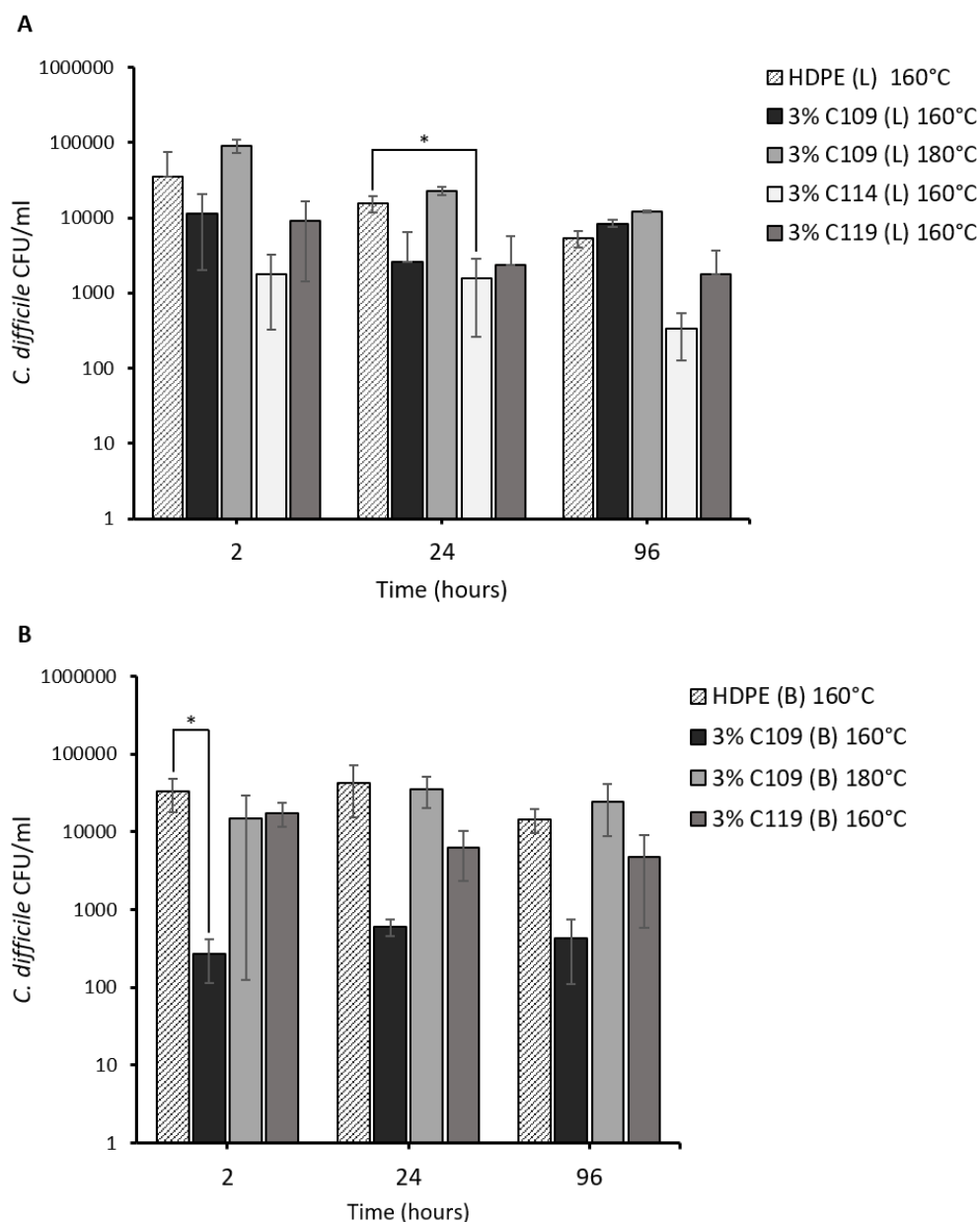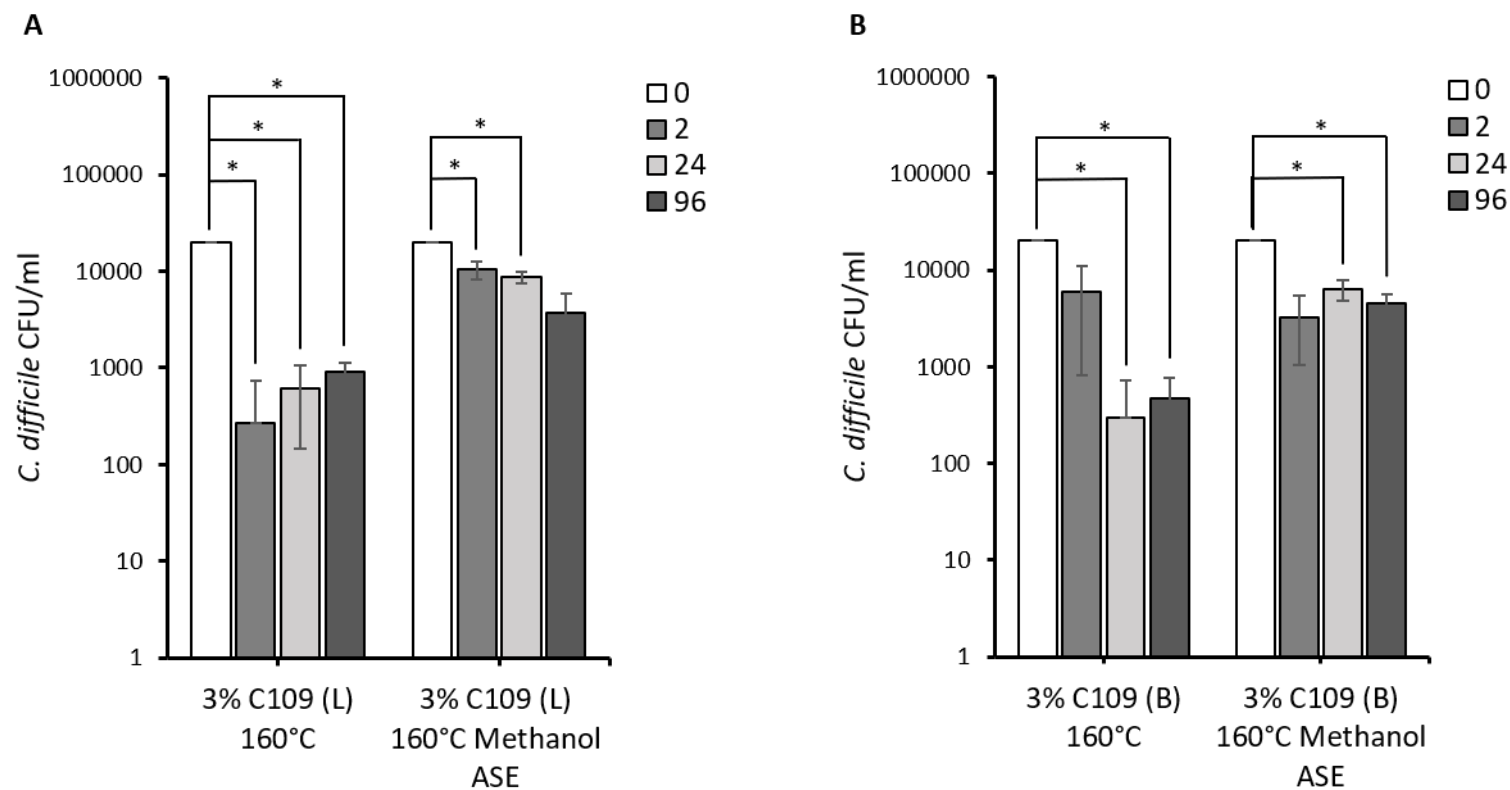
Figure 6.4 Determination of *C. difficile* spore germination by polymer formulations containing novel compound C109 produced with unstabilised (Lupolen 5261ZQ456) and stabilised (BorPex HE1878E) HDPE by measurement of loss of resistance to ethanol. Germination activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. A) Unstabilised polymer formulations. B) Stabilised polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control are marked with asterisks ($P < 0.05$).

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6.3.5 The role of thermal processing of polymer formulations on the germination activity of polymer formulations incorporating novel compound C109.

Unstabilised polymer formulation 3% C109 (L) 170°C demonstrated a significant reduction in *C. difficile* CFU/ml of 0.91 ($P = 0.0004$) and 0.74-log ($P < 0.0001$) at 24 and 96 hours respectively (figure 6.5A). A 2.11 log reduction was achieved at 2 hours, comparable to that achieved by 3% C109 (L) 160°C but was not statistically significant ($P > 0.05$). A significant reduction ($P > 0.05$) was not observed at any time point tested for the polymer formulation 3% C109 (L) 180°C compared to the control at time point zero. The unstabilised polymer formulation 3% C109 (L) 160°C (10 CM), which was compression moulded at maximum pressure for an additional 5 minutes demonstrated reductions of 1.67, 1.78, 1.25 log at 2, 24 and 96 hours, but were not found to be statistically significant ($P > 0.05$).

Post processing purification by Soxhlet extraction and methanol accelerated solvent extraction of polymer formulation 3% C109 (L) 160°C resulted in a reduction of germination activity compared to previously presented results (figure 6.4A). The unstabilised polymer formulation 3% C109 (L) 160°C purified via methanol ASE produced a significant reduction in *C. difficile* CFU/ml of 0.36 ($P = 0.0098$) and 0.73 log reduction ($P < 0.0001$) at 24 and 96 hours respectively (figure 6.5B). A significant reduction ($P > 0.05$) however was not observed at any time point tested for the polymer formulation 3% C109 (L) 160°C purified via Soxhlet extraction compared to the control at time point zero. Unstabilised polymer formulation 3% C109 (L) 160°C (10 min) post methanol ASE produced a 0.97, 0.48 and 0.86 log reduction at 2, 24 and 96 hours respectively but was not determined to be statistically significant ($P > 0.05$).
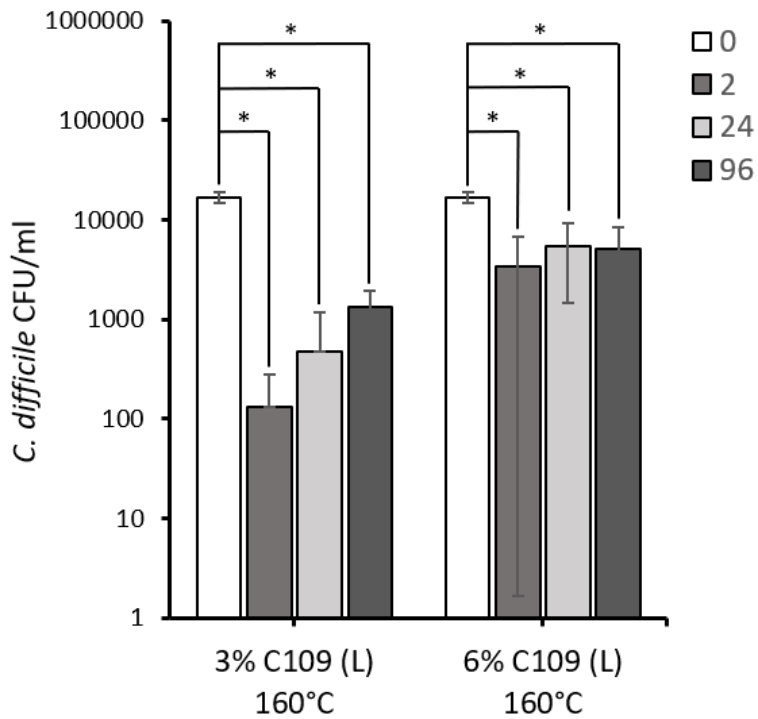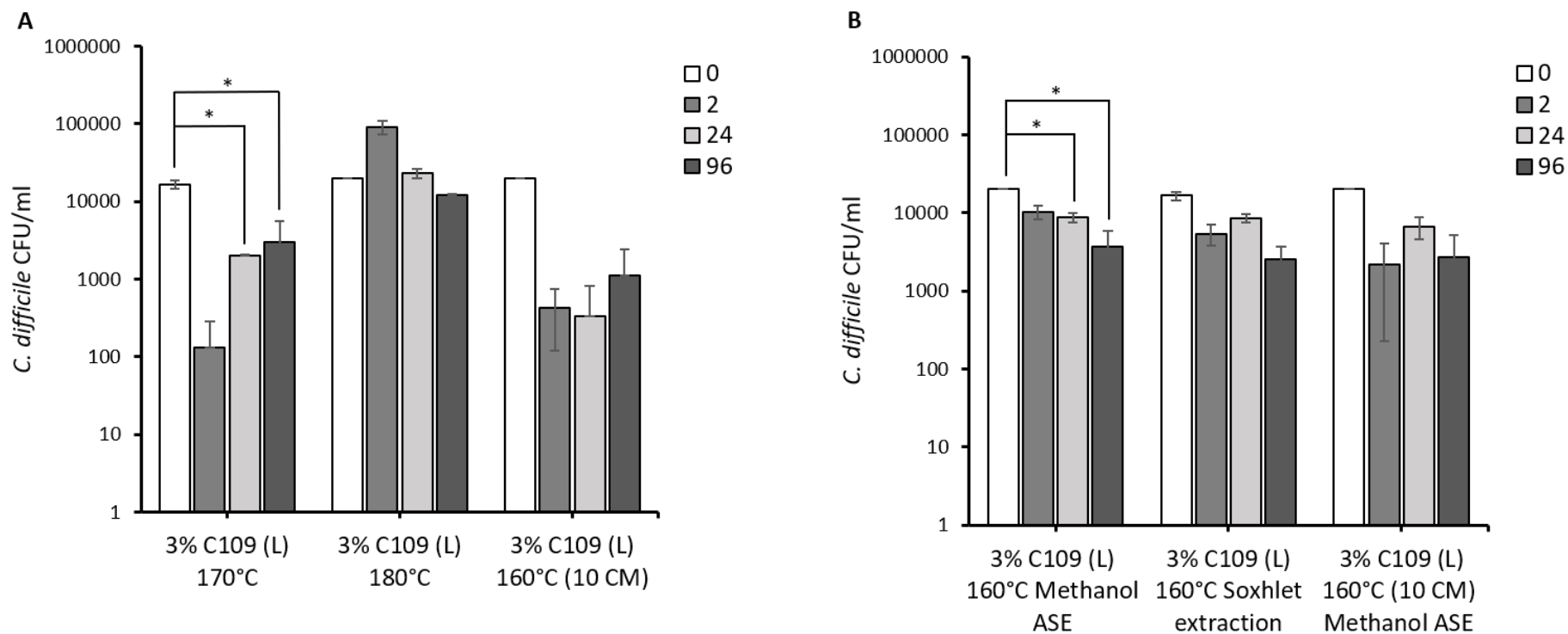
Figure 6.5 Determination of *C. difficile* spore germination by polymer formulations containing novel compound C109 produced under different thermal processing conditions by measurement of loss of resistance to ethanol. Germination activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. A) Unstabilised polymer formulations. B) Post processing purified unstabilised polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control are marked with asterisks ($P < 0.05$).

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6.3.6 The germination activity of polymer formulations incorporating novel compound C109 and copper nanoparticles.

Polymer formulations produced with copper nanoparticles did not demonstrate germination activity. A significant reduction ($P > 0.05$) of *C. difficile* CFU/ml was not observed at any time point tested for the polymer formulations 3% C109 (L) 160°C Cu II and 3% C109 (L) T101 160°C Cu II compared to the control at time point zero.
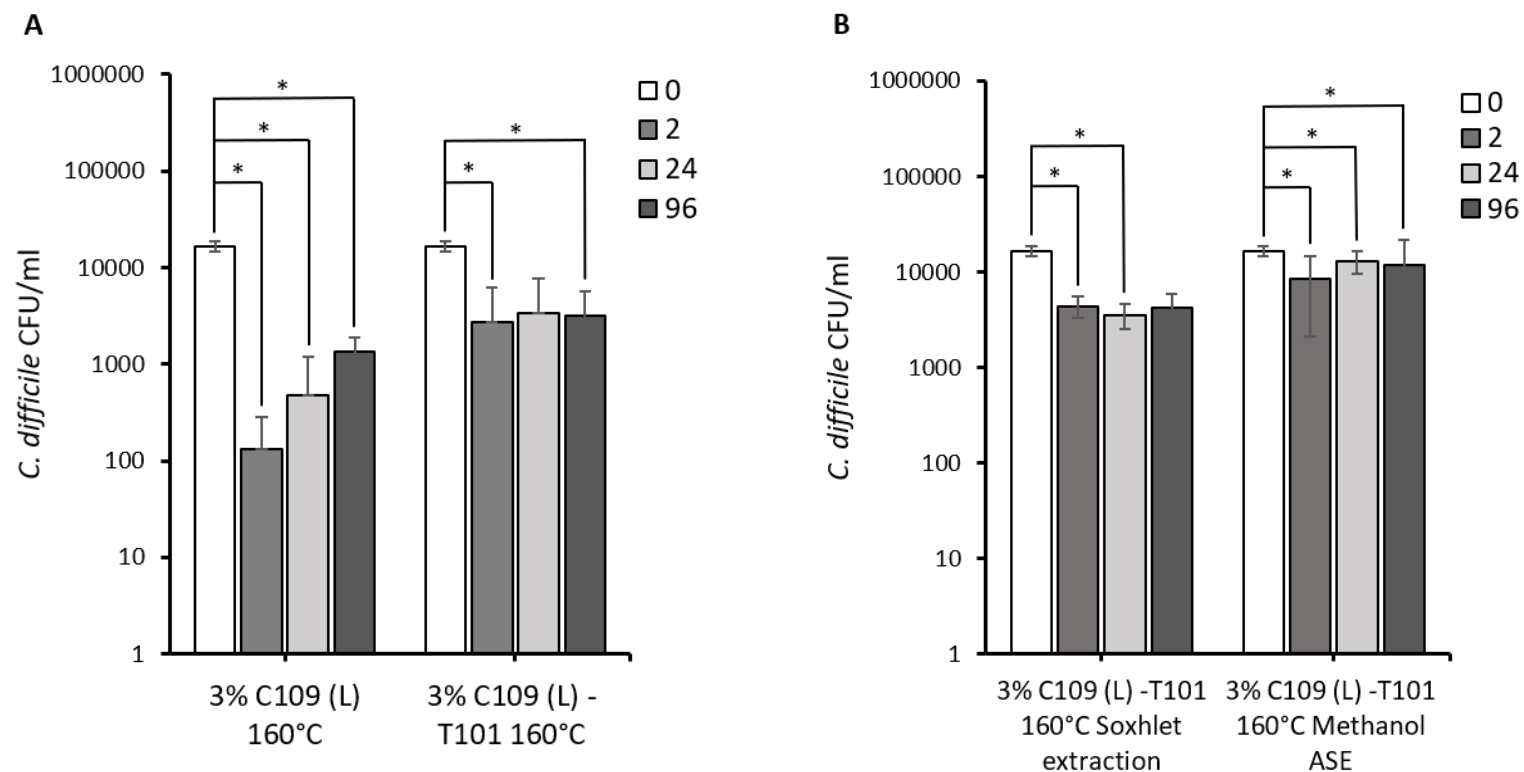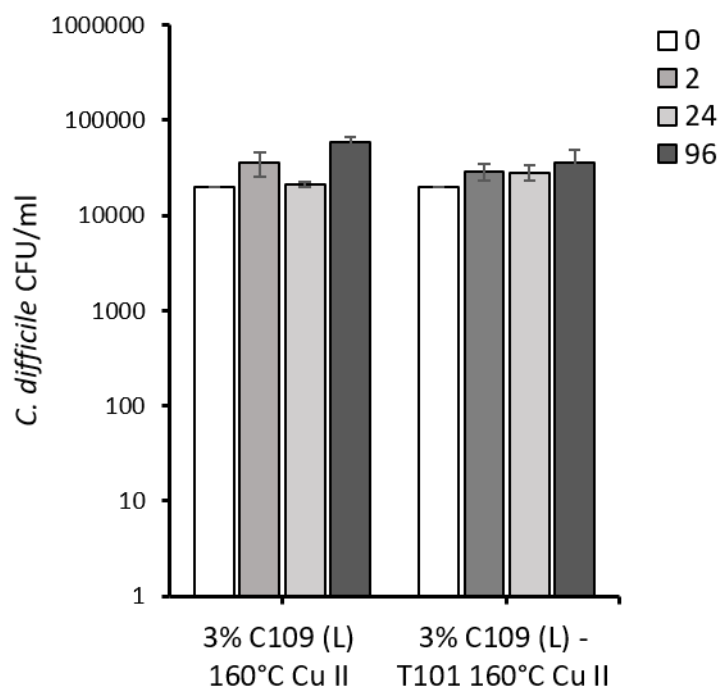

Figure 6.6 Determination of *C. difficile* spore germination by polymer formulations containing novel compound C109 produced with copper nanoparticles by measurement of loss of resistance to ethanol. Germination activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control are marked with asterisks ($P < 0.05$).

6.3.7 The germination activity of polymer formulations incorporating novel compound C109 and benzalkonium chloride.

Polymer formulation containing novel compound C109 and benzalkonium chloride were found to cause germination of *C. difficile* spores, but this was dependent upon the presence of peroxide initiator T101 and the processing temperature utilised to produce the polymer formulations. Polymer formulation 3% C109 (L) 160°C BKC demonstrated a significant reduction in *C. difficile* CFU/ml of 1.48 (P = 0.0007), 1.48 (P = 0.0016) and 1.14 log (P = 0.0026) at 2, 24 and 96 hours respectively (figure 6.5A). In the absence of peroxide initiator T101 germination activity was eliminated, a significant reduction (P > 0.05) of *C. difficile* CFU/ml was not observed at any time point tested for the polymer formulation 3% C109 (L) -T101 160°C BKC compared to the control at time point zero. The introduction of Mini-Lab Haake processing of polymer formulations also eliminated the germination activity. Polymer formulations 3% C109 (L) 160°C BKC (5H) and 3% C109 (L) -T101 160°C BKC (5H) did not produce a significant reduction (P > 0.05) of *C. difficile* CFU/ml compared to the control at any time point tested.
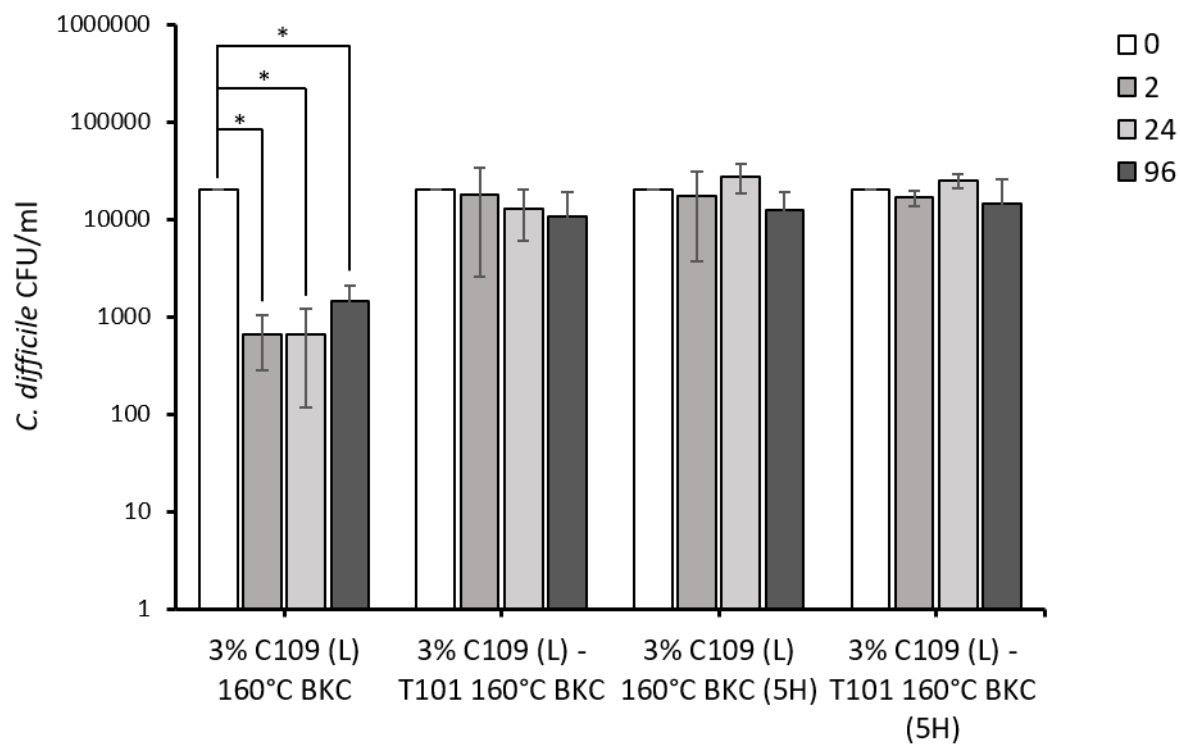
Figure 6.7 Determination of *C. difficile* spore germination by polymer formulations containing novel compound C109 produced with benzalkonium chloride by measurement of loss of resistance to ethanol. Germination activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control are marked with asterisks (*P* < 0.05).

6.3.8 The antimicrobial activity of polymers incorporating novel compound C109.

The antimicrobial activity of polymer formulations was investigated by modifying the method utilised to determine the *C. difficile* spore germination activity of polymer formulations. The elimination of germinating *C. difficile* spores sensitive to chemical inactivation is derived from the antimicrobial properties of the quaternary ammonium cation of novel compound C109 incorporated into polymer formulations, which also initiate germination. Polymer formulations demonstrated antimicrobial activity against germinating *C. difficile* spores but the reduction in CFU/ml was to a lesser extent than that of germinating spores exposed to ethanol. Unstabilised polymer formulation 3% C109 (L) 160°C produced a 1.51, 0.88 and 0.66 log reduction of *C. difficile* CFU/ml after 2, 24 and 96 hours exposure respectively but these were not determined to be statistically significant ($P > 0.05$) (figure 6.8A). A significant reduction ($P > 0.05$) of *C. difficile* was not observed at any time point tested for the polymer formulation 3% C109 (L) 170°C compared to the control.

Post processing purification via methanol ASE and Soxhlet extraction of the polymer formulations reduced the antimicrobial activity of the polymer formulations although some statistically significant results were obtained. Unstabilised (Lupolen 5261ZQ456) polymer formulations 3% C109 (L) 160°C Soxhlet extraction did not produce a significant reduction ($P > 0.05$) of *C. difficile* at any of the time points tested. Polymer formulation 3% C109 (L) 160°C methanol wipe produced a significant reduction of *C. difficile* CFU/ml after 2 and 96 hours exposure, demonstrating 0.33 ($P = 0.0094$) and 0.65-log reductions ($P = 0.0003$) respectively (figure 6.8B).
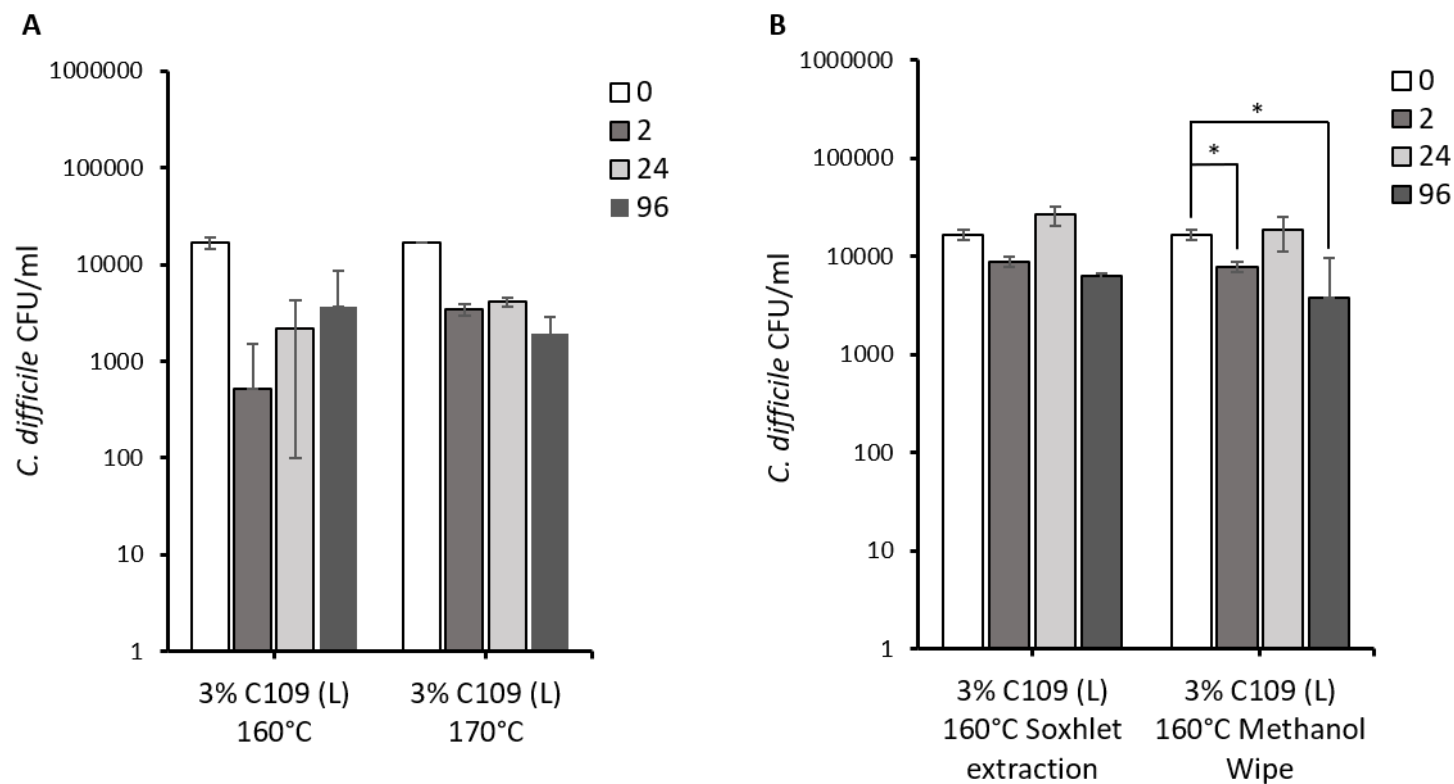
Figure 6.8 Determination of antimicrobial activity of polymer formulations incorporating novel compound C109 against germinating _C. difficile_ spores.

Antimicrobial activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. A) Unstabilised polymer formulations. B) Post processing purified unstabilised polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison _post-hoc_ test. Statistically significant reductions of survival compared to the control are marked with asterisks ($P < 0.05$).

6.3.9 The antimicrobial activity of polymers incorporating novel compound C109 and benzalkonium chloride.

Polymer formulations incorporating novel compound C109 and benzalkonium chloride demonstrated statistically significant antimicrobial activity against germinating *C. difficile* spores. Unstabilised polymer formulation 3% C109 (L) 160°C BKC produced a 0.43 (P = 0.001), 0.36 (P = 0.0026) and 0.32-log reduction (P = 0.0049) of *C. difficile* CFU/ml after 2, 24 and 96 hours exposure respectively (figure 6.9).
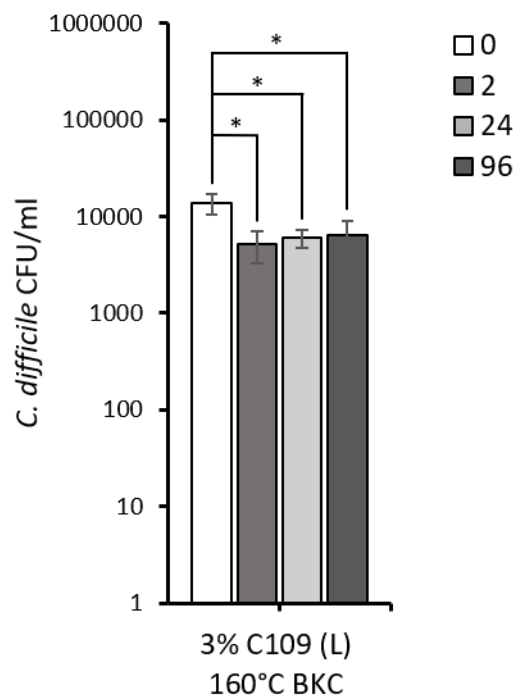
Figure 6.9 Determination of antimicrobial activity of polymer formulations incorporating novel compound C109 against germinating *C. difficile* spores. Antimicrobial activity was measured at 2, 24 and 96 hours post inoculation of polymer formulations. Statistical analysis was carried out via a two-way ANOVA and Bonferroni multiple comparison *post-hoc* test. Statistically significant reductions of survival compared to the control are marked with asterisks (*P* < 0.05).

6.4 Discussion.

Existing strategies to eliminate *C. difficile* spores from clinical surfaces utilise sporicidal biocides which are reliant effective application have been demonstrated to deliver inadequate decontamination (McDonald *et al.*, 2018; Speight *et al.*, 2011). The use of advanced UV and hydrogen peroxide vapour no touch room decontamination methods are time consuming and require patient rooms to be empty (Boyce *et al.*, 2008; Levin *et al.*, 2013). Employing the pro-germination elimination strategy of 'germinate to exterminate' a prototype sporicidal polymeric smart surface was designed and developed to specifically target *C. difficile* spores, providing reusable, passive decontamination to complement and enhance the routine use of disinfectants and sporicidal biocides in clinical environments (Wheeldon *et al.*, 2010). Bile salt-derived novel compounds incorporated in the polymeric biomaterial function as *C. difficile* spore germinants and antimicrobial agent, initiating germination and causing the loss of spore resistant properties (Setlow, 2003). The germinating spores become susceptible to the antimicrobial agents incorporated in the smart surface and are subsequently eliminated. This work characterises polymer formulations incorporating novel bile salt-derived compounds possessing both germination and antimicrobial properties, demonstrating the prototype polymeric biomaterial caused significant elimination of *C. difficile* spores from the surface of the smart surface. Germination activity of the polymer formulations was determined by the loss of resistance of germinating spores to ethanol while antimicrobial activity was measured by determining the sensitivity of the germinating spores to the antimicrobial compounds incorporated into the polymer formulations. The data presented provides evidence of the efficacy of a prototype sporicidal polymeric biomaterial targeting *C. difficile* spores and demonstrates the potential for the use of a pro-germination decontaminating smart surface for high-touch clinical surfaces to reduce the transmission of CDI.

Initial testing of prototype polymer formulations provided proof of concept that a pro-germination smart surface targeting *C. difficile* spores was successful, demonstrating significant germination activity compared to the control at the same time point. However the polymer formulations produced for the initial experiments did not provide comprehensive variation of the novel compounds incorporated, base HDPE and processing temperature, in order for independent variables to be evaluated individually. Only polymer formulations incorporating novel compound C109 were produced with both stabilized and unstabilised HDPE and at both initial testing temperatures. These polymer formulations demonstrated significant germination activity at 2 hours and non-significant results at 24 and 96 hours but failed to provide specific information on the appropriate formulation and processing conditions for optimal germination activity. Of the series of novel compounds developed and synthesised by Hird (2014) only a limited number were evaluated for germination activity. Candidate C109 was determined to have suspected sporicidal activity, with the initiation of germination and antimicrobial activity of the quaternary ammonium compound determined as the mechanism of action. The assessment of the germination and antimicrobial activity of these novel compounds was completed by attempting to dissolve the candidate novel compounds in liquid media, despite poor solubility in aqueous solutions and limiting the validity of conclusions. In the Hird (2014) preliminary tests, C109 achieved a 2 log reduction of *C. difficile* 11204 spores following 1 hour exposure, measured via loss of resistance to heat, suggesting promising germination activity. Considering this data and the characterisation of the germination and antimicrobial activity of C109 presented in chapter 5 of this thesis and the results of initial testing of prototype polymer formulations, C109 was chosen as the novel compound for inclusion in polymer formulations to investigate the optimisation of polymeric smart surface targeting *C. difficile*. A 3% (w/w) concentration of novel compound was selected to reflect the concentration of bile salt germinant used in culture media to maximise the recovery of *C. difficile* from patient samples and the efficient initiation of germination (0.1%) (Wilson, 1983; Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2011). While this concentration exceeds the maximum

level of taurocholate achieved in a healthy adult duodenum, a high concentration was considered necessary as only novel compound on the surface of the polymer material would be available to interact with *C. difficile* spores and initiate germination (Leverrier *et al.*, 2003; Mallory *et al.*, 1973). Further polymer formulations incorporating C109 were produced to evaluate the effects of polymer formulation composition, processing conditions and post-processing purification on *C. difficile* spore germination activity. Increasing the concentration of C109 in polymer formulations to 6% was found to negatively influence germination activity, Greater log reductions of *C. difficile* CFU/ml were achieved by the polymer formulation containing 3% C109 at all time points tested. The higher concentration of C109 is far beyond the typical industry standard of biocide additives of 0.001-1% (w/w) and may have an adverse effect on the structural order of the polymer, inhibiting the process of crosslinking of the novel compound to the base HDPE (Hahladakis *et al.*, 2018; Canevarol, 2020).

Polymer formulations incorporating C109 produced with stabilised or unstabilised HDPE both demonstrated *C. difficile* germination activity that was statistically significant. Incorporation of C109 in the unstabilised HDPE polymer formulation demonstrated significant germination at all 3 time points tested while the equivalent stabilised HDPE polymer formulation produced significant log reductions at only 24 and 96 hours. The use of unstabilised HDPE also provides an economical benefit of lower manufacturing cost. The inclusion of the peroxide initiator T101 in polymer formulations was found to be beneficial for the germination activity against *C. difficile*. This finding was as expected, as peroxide initiators are utilised to generate free radicals under thermal decomposition, promoting polymerisation through the initiation of crosslinking of monomeric units, including the copolymerisation of the base HDPE and the bile salt-derived novel compounds (Guerrero-Santos *et al.*, 2013).

The processing conditions of the polymer formulation proved to be highly influential on *C. difficile* germination activity. Compression moulding at temperatures over 170°C was determined to be detrimental to the germination activity of polymer formulations. This reduction in germination

activity was also observed when the duration of the polymer processing was increased during extended compression moulding to promote crosslinking or as a result of Mini-Lab Haake processing, utilised to improve the uniformity of additives throughout the polymer matrix. HDPE is known to be stable at high temperatures, indicating the likely degradation of the novel compound C109 at high temperature and during prolonged exposure to the optimal temperature used during compression moulding (Giles *et al.*, 2005; Sustaita-Rodríguez *et al.*, 2019).

Post processing purification of multiple polymer formulations incorporating C109 was examined to determine if the novel compound providing germination and antimicrobial activity could be extracted or removed from the surface of the polymer formulations. In every case examined, post processing purification via methanol-accelerated solvent extraction, Soxhlet extraction or methanol wipe, resulted in a reduction of the germination or antimicrobial activity of the polymer formulations but some activity was retained, producing statistically significant reductions of *C. difficile spores*. As germination or antimicrobial activity was not completely eliminated from the polymer formulations this demonstrates a proportion of C109 was successfully and permanently integrated into the base HDPE via non-covalent trapping within the polymer matrix while some remained unbound and was deposited on the surface or within the matrix of the polymer formulation. This inefficient grafting of the novel compound signifies the desired 3% concentration of incorporated C109 was not achieved and that the free novel compound could be lost into the environment. This limits the usefulness of the polymeric smart surface as a reusable method for passive decontamination to target *C. difficile* spores. It also has implication for migration and unintended exposure of humans to the bile derived novel compounds and the risk of the development of cross-resistance between quaternary ammonium compounds and therapeutic antibiotics by clinically important pathogens (Hahladakis *et al.*, 2018; Hegstad *et al.*, 2010).

The inclusion of antimicrobial agents had a varied effect on the germination activity of polymer formulations incorporating C109. Incorporation of copper nanoparticles eliminated the C109

derived germination activity while polymer formulations incorporating C109 and BKC demonstrated significant germination activity similar to that of the polymer formulation with C109 alone. Further investigation of the antimicrobial activity of polymer formulations incorporating C109 established *C. difficile* spores germinated and were eliminated from the surface of the polymer but the resultant reduction in viable spores showed considerable variation and was not statistically significant. Polymer formulations incorporating C109 and BKC however demonstrated statistically significant reductions in *C. difficile* at all time points tested. This finding validates the sporicidal polymeric smart surface, utilising the 'germinate to exterminate' strategy, to successfully eliminate *C. difficile* spores (Wheeldon *et al.*, 2010). While the current polymer formulations incorporating novel compounds provide modest but significant elimination of *C. difficile* spores, the capacity to trigger germination offers an opportunity to substantially enhance the efficacy of current routine decontamination of clinical surfaces with chlorine-based cleaning agents and terminal cleaning with hydrogen peroxide vapour (Department of Health and Health Protection Agency, 2008).

The exhibited germination and antimicrobial activity of the bile salt-derived novel compound synthesised at Aston University expands the knowledge of known *C. difficile* spore germinants and agents with antimicrobial activity against *C. difficile* (Hird, 2014). Incorporation of these compounds in a polymeric smart surface, to our knowledge, is the first description of a passive decontamination strategy to target *C. difficile* spores that contaminate the environment. The results achieved with the current polymer formulation incorporating novel bile salt-derived compounds and biocides demonstrate the capability of a pro-germination polymeric biomaterial to eliminate *C. difficile* spores and warrants further research to optimise the germination and antimicrobial activity against *C. difficile.* The application of a *C. difficile* targeting smart surface provides an opportunity to prevent the transmission and dissemination of *C. difficile* spores to reduce CDI in vulnerable patients. The use of the polymer formulation and incorporated novel bile salt-derived compound do not provide the environmental conditions to support *C. difficile* replication and consequently do not pose a risk of spore outgrowth or sporulation (Wheeldon *et al.,* 2008a; Kohler *et al.,* 2018). By

initiating the irreversible process of germination upon contact, the 'chain of infection' is broken (Setlow, 2003). Utilising a pro-germination smart surface in a clinical setting could reduce the currently required frequent environmental cleaning and the need for harsh chemical disinfectants which pose a risk of harmful respiratory exposure and can cause damage to surfaces (Department of Health and Health Protection Agency, 2008; Wilcox and Fawley, 2000; Speight *et al.*, 2011).

It was anticipated that increased exposure time of *C. difficile* spores with the polymer formulation incorporating the bile salt-derived novel compounds would lead to greater reduction in viable *C. difficile* spores, but this was not the case. In multiple circumstance we observed the opposite: a lower log reduction of *C. difficile* CFU/ml at 96 and 24 hours compared to that at 2 hours exposure. This observation may be a consequence of the heterogenous germination response of the spore population, particularly a 'superdormant' subpopulation which do not rapidly respond to the presence of germinants and co-germinants (Gould, 1970; Gould *et al.*, 1968). These superdormant spores are thought to require a higher level of germinant stimulus to triggering germination which is linked to the availability of germinant receptors (Ghosh and Setlow, 2009). Superdormant *Bacillus* sp*.* spores have been demonstrated to germinate in response to alternative germinants and have elevated heat resistance properties (Ghosh *et al.*, 2009; Ghosh and Setlow, 2009). An investigation of the stability of superdormant *Bacillus* spores by Ghosh and Setlow (2010) demonstrated isolated superdormant *Bacillus* species spores stored under a number of conditions became increasingly responsive to germinants over a 2 month period, suggesting that the 'germinability' of superdormant spores may be influenced by the environmental or storage conditions (Gould 1968). There are however distinct differences in the process and kinetics of germination in *C. difficile* and *Bacillus* species spores and superdormant spores of *C. difficile* have yet to be isolated and characterised (Wang *et al.*, 2015; Zhang and Mathys, 2019). Freeman and Wilcox (2003) demonstrated aerobic storage of *C. difficile* spores in faecal samples at 4°C or -20°C had no significant impact on the recovery of *C. difficile* spores, via germinant supplement media, up to 56 days. This finding was in disagreement with earlier research which reported the inability to recover

C. difficile from faecal samples by 10 days when stored aerobically at 5°C or by 4 days at 25°C (Bowman and Riley, 1986). Weese *et al.* (2000) also reported a decline in the recovery of *C. difficile* from inoculated equine faecal samples stored aerobically at 4°C over 11 days. Most recently and further adding to the conflicting evidence, Deng *et al.* (2015) demonstrated the divergent germination response of spores of 2 *C. difficile* strains to storage at room temperature. Spores of *C. difficile* strain M120 demonstrated a significant decrease in viability and germination capacity over the 4 months of the study period, while for spores of R20291 this significantly increased (Deng *et al.*, 2015). Very little data is available on the impact of storage at room temperature on viability and germination response and specifically the desiccated conditions of the *C. difficile* spores used during the assessment of the germination activity of the polymer formulation. The data presented by Deng *et al.* (2015), indicates spores are able to transition from a superdormant state to become responsive to germinants and possibly *vice versa*. The experimental conditions utilised to determine the germination activity of the polymer formulation may influence the germination response and viability of the *C. difficile* spores, leading to a reduction in germinability over time.

The heterogeneity of the germination response in this study over time could also be linked to the method used to prepare the *C. difficile* spores. The spore suspension was not purified and so the crude spore preparation contained cell debris, non-viable vegetative cells and spores at various stages of sporulation. To reflect the authentic conditions where patients shed *C. difficile* spores into the clinical environment the spore suspension was not subjected to sub-lethal heat activation, a method frequently used to stimulate a consistent germination response (Sethi *et al.*, 2010; Ghosh and Setlow, 2009; Ghosh *et al.*, 2009). A disparity in the germination response of spores has been linked with the methodology used to prepare *C. difficile* spores for experimental investigations (Malyshev and Baillie, 2020; Sorg and Sonenshein, 2010; Heeg *et al.*, 2012). The laboratory techniques used to produce standardised spore preparations ensure a reliable germination response but might not reflect the germination capacity characteristic of *C. difficile* spores shed by patients (Shen, 2020). The discrepancy of the germination response and subsequent elimination of

C. difficile spores during the evaluation of the polymer formulations incorporating novel bile salt-derived novel compounds may be related to spore maturity and the variety of morphotypes found in the crude spore preparation used in this study. A better understanding of the mechanisms and characteristics of spore super dormancy and germination capcity is essential to identify the required conditions to germinate the population of *C. difficile* spores in order to eliminate them via the germinate to exterminate strategy.

The observations of germination and antimicrobial activity in this study are limited by the use of the single *C. difficile* strain NCTC 11204. The variation in germination response by clinical and laboratory strains of *C. difficile* described by other researchers clearly demonstrates the need to utilise multiple *C. difficile* strains to evaluate the efficacy of polymeric smart surface incorporating bile salt-derived compounds to germinate and eliminate *C. difficile* spores (Heeg *et al.*, 2012; Francis *et al.*, 2013; Thanissery *et al.*, 2017; Rohlfing *et al.*, 2019). Experimental conditions, such as the use of the crude *C. difficile* spore suspension and the ambient testing temperature were selected to replicate the conditions in which the targeted smart surface is intended to be used. Further investigations should also address the efficacy of the polymer formulations incorporating bile salt-derived novel compounds in the presence of organic debris that frequently contaminates clinical environments and can inhibit the antimicrobial activity of quaternary ammonium compounds, with multiple representative strains of *C. difficile*, including those that are clinically relevant (Vohra and Poxton, 2011).

It is probable that insufficient novel compounds germinants were incorporated into the current polymer formulations to trigger efficient germination. The reduced activity of polymer formulations post purification suggests this is the case. The successful incorporation of a higher concentration of bile salt-derived novel compound could impact germination activity significantly, delivering a reliable and reusable smart surface for the elimination of *C. difficile* spores, appropriate for use in a clinical environment. Since clinical isolates *C. difficile* spores have demonstrated differential

germination responses to bile salt germinants and inhibitors, it could be advantageous to incorporate multiple bile salt-derived novel compounds, not fully evaluated in this study, to target strains which have altered sensitivity to bile salt germinants (Heeg *et al.*, 2012; Thanissery *et al.*, 2017; Rohlfing *et al.*, 2019). For this purpose, the germination and antimicrobial activity of other bile salt-derived novel compounds designed by Hird (2014) should also be investigated in a polymeric formulation, including compounds C114 and C119 which demonstrated germination activity in initial experiments.

To further enhance germination activity of the polymeric smart surface, co-germinants, including amino acids and calcium could be incorporated into the polymer formulations (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2011; Kochan *et al.*, 2017). Calcium in particular has an additional advantage as it is able to synergise with amino acid co-germinants, compensating for sub-optimal concentrations, to trigger germination in combination with bile salt germinants (Kochan *et al.*, 2018b). Further study of the novel bile salt-derived compounds, polymer composition and processing conditions is needed to determine optimal methods to maximise grafting of the novel compounds and enhance germination activity.

Chapter 7

7.0 Final discussion.

The research presented in this thesis aimed to investigate *C. difficile* spore germination and to utilise this knowledge to develop a pro-spore germination strategy to eliminate *C. difficile* spores in the environment. *C. difficile* is a major healthcare associated pathogen and a significant cause of morbidity and mortality (Balsells *et al.*, 2019). Key to the transmission of CDI is the formation of highly resistant *C. difficile* spores (Driks, 2003; Setlow, 2014; McFarland *et al.*, 1989; Samore *et al.*, 1996). Disruption of the intestinal microbiota, typically by broad spectrum antibiotics is the most common risk factor for the colonisation of patients with *C. difficile* (Poutanen and Simor, 2004; Vedantam *et al.*, 2012). The ingested spores of *C. difficile* germinate in response to specific environmental signals within the small intestine, transforming the metabolically dormant spore to an outgrowing vegetative cell (Shen, 2020; Viswanathan *et al.*, 2010). The clinical presentation of CDI, which ranges from mild diarrhoea to life-threatening pseudomembranous colitis and fulminant colitis, is a consequence of the production of the highly potent proteinaceous exocellular toxins TcdA and TcdB which cause intestinal damage, acute activation of innate immune response and fluid accumulation (Leffler and Lamont, 2015; Chandrasekaran and Lacy, 2017; Voth and Ballard, 2005). Despite antibiotic therapy to treat CDI, recurrence of CDI occurs in approximately 25% of cases, often due to the continued disruption to the gut microbiota (Kelly, 2012; Debast *et al.*, 2014).

The spores of *C. difficile* are highly resistant to the adverse environmental conditions facilitating their dissemination and persistence in clinical environments (Paredes-Sabja *et al.*, 2014; McFarland *et al.*, 1989; Samore *et al.*, 1996). Dormancy and durability of the spore is maintained by the low water content of the spore core and the protective spore structures which prevent damage from desiccation, UV radiation, high temperatures, enzymatic attack and chemicals or biocides (Nicholson *et al.*, 2000; Popham, 2002; Paredes-Sabja *et al.*, 2008; Setlow, 2014). Symptomatic and asymptotic CDI patients shed spores which contaminate the clinical environmental and acts as a

major reservoir for the transmission of CDI (Sethi *et al.*, 2010; Riggs *et al.*, 2007; McFarland *et al.*, 1989; Samore *et al.*, 1996).

The elimination of spores through adequate decontamination of surfaces is essential to prevent CDI in vulnerable patients but the use of detergents and some hospital cleaning and disinfection agents have been demonstrated as ineffective against the resistant *C. difficile* spore structure (Wilcox *et al.*, 2003; Fawley *et al.*, 2007). In addition, the presence of organic material deposited on the surfaces of clinical environment along with *C. difficile* spores, can inhibit or decrease the activity of some disinfectants and biocides (Vohra and Poxton, 2011). Sporicidal agents that specifically target *C. difficile* are needed to eliminate the source of transmission, but the exposure time required to effectively eradicate contaminating *C. difficile* spores is often beyond what can be achieved in a dynamic hospital environment (Speight *et al.*, 2011). Chlorine-releasing disinfectants and biocides are currently recommended to decrease the contamination of *C. difficile* due to their demonstrated effectiveness but are susceptible to inactivation by organic matter (Department of Health and Health Protection Agency, 2008; Perez *et al.*, 2005; Ungurs *et al.*, 2011).

Germination of *C. difficile* spores occurs in response to the presence of bile salt germinants and amino acids or calcium as co-germinants, ensuring that germination occurs in the small intestine of the host (Sorg and Sonenshein, 2008; Kochan *et al.*, 2017; Koenigsknecht *et al.*, 2015). The elimination of selective intestinal microbiota species by broad spectrum antibiotics inhibits the conversion of primary bile salts to secondary bile acids, thus increasing the concentration of *C. difficile* germinants and promoting spore germination (Ridlon *et al.*, 2006; Theriot *et al.*, 2016). While the bile salts cholate, taurocholate and glycocholate are *C. difficile* spore germinants, the presence of amino acids, principally glycine and/or calcium, as co-germinants is necessary to initiate *C. difficile* spore germination (Sorg and Sonenshein, 2008; Kochan *et al.*, 2017). Based on current models of the regulation of *C. difficile* germination, the activation of the proposed bile salt germinant and co-germinant receptors, CspC and CspA, transduce the germination signal to CspB

which causes the degradation of the cortex peptidoglygan via the activation of SleC, triggering the rehydration of the spore core and partial loss of the spore resistant characteristics (Francis *et al.*, 2013; Bhattacharjee *et al.*, 2016a; Shrestha *et al.*, 2019; Adams *et al.*, 2013; Francis *et al.*, 2015; Donnelly *et al.*, 2017; Francis and Sorg, 2016; Burns *et al.*, 2010).

Study of the factors that influence the initiation of *C. difficile* germination revealed an anaerobic atmosphere is not essential, permitting the initiation of germination of *C. difficile* spores in the environment (Wheeldon *et al.*, 2008a). As part of 'germinate to exterminate strategy' to target *C. difficile* spores, the specific germinant and co-germinant signals are provided to trigger germination, resulting in the loss of spore resistant properties and the increased susceptibility of germinating spores to traditional biocides. The preincubation of *C. difficile* spores with a germinant solution followed by exposure to 70% ethanol, or combining the germination solution with a biocide, significantly improved the killing of *C. difficile* spores (Wheeldon, 2008; Wheeldon *et al.*, 2010). The application of this alternative decontamination strategy offers a targeted and effective method to enhance the elimination *C. difficile* spores from the surfaces of clinical environments, with the potential to reduce the transmission of CDI.

To provide a greater understanding as to how *C. difficile* spore germination may be exploited to eliminate contaminating *C. difficile* spores in the environment, the research presented in this thesis sought to investigate germinant activity of bile salts and co-germinant activity of amino acids and calcium to initiate the germination of *C. difficile* spores. The optical density germination assay was successfully utilised to study the germination response of *C. difficile* NCTC 11204 spores to the bile salts cholate, taurocholate, glycocholate and deoxycholate. This method of measuring germination allowed the rate and extent of the germination response to be examined, identifying *C. difficile* NCTC 11204 spore germinants and inhibitors of germination. The efficacy of taurocholate as a potent inducer of *C. difficile* germination was demonstrated, which concurred with the findings of other researchers (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2008a). Bile salts cholate,

glycocholate and deoxycholate failed to exhibit germination activity in the optical density assay with *C. difficile* NCTC 11204 spores. These findings were also in agreement with published research where cholate and glycocholate did not demonstrate germination activity over a short time frame but were able to induce germination when incorporated into solid culture media (Sorg and Sonenshein, 2008; Bhattacharjee *et al.*, 2016a). No germination activity was observed for deoxycholate in this study, which is reported to induce *C. difficile* spore germination but inhibit the proliferation of outgrowing vegetative cells (Sorg and Sonenshein, 2008). Chenodeoxycholate did not induce *C. difficile* NCTC 11204 spore germination and was demonstrated to act as an inhibitor of taurocholate-induced spore germination, confirming the findings of other researchers (Sorg and Sonenshein, 2008; Heeg *et al.*, 2012). The germination activity of cholate, taurocholate, glycocholate and deoxycholate was also investigated with *C. difficile* reference strains representing ribotypes 001, 002 and 015. Taurocholate was demonstrated to trigger the germination of spores of *C. difficile* ribotypes 001, 002 and to a limited extent ribotype 015. Interestingly the bile salts cholate and deoxycholate initiated the germination of *C. difficile* ribotype 002 spores but not of ribotypes 002 and 015, while glycocholate failed to initiate the germination of spores of any of the *C. difficile* strains tested. The influence of butyrate and pH of germination media on taurocholate-induced *C. difficile* NCTC 11204 spore germination was also investigated. Butyrate was shown to have a limited impact on *C. difficile* spore germination, only higher concentrations tested generated a small reduction in the extent of the germination response, but this was not determined to be significant. The pH of the germination media had a significant impact on the germination of *C. difficile* spores. Taurocholate-induced germination occurred only at pH 6 and at pH 6.6 of unmodified BHIS. The pH of germination media has previously been demonstrated to be an important environmental factor that inhibits the germination of *C. difficile* spores. In this study germination occurred in a comparable but narrower pH range than observed by other researchers but indicates the pH of the environment may act as another signal that ensures *C. difficile* spores

germinate in the small intestine (Paredes-Sabja *et al.*, 2008; Wheeldon *et al.*, 2008a; Kochan *et al.*, 2017).

The optical density assay was also utilised to study the co-germinat activity of amino acids and calcium with *C. difficile* NCTC 11204 spores. Glycine was demonstrated to be an effective co-germinant as previously reported, however it was the only amino acid to act as an independent co-germinant in this study (Sorg and Sonenshein, 2008; Howerton *et al.*, 2011; Kochan *et al.*, 2018b; Shrestha and Sorg, 2017). This is in contrast to the co-germinat activity of amino acids observed by other researchers but is in agreement with the findings of Wheeldon *et al.* (2011) who also studied the germination response of *C. difficile* NCTC 11204 spores. The successful application of a germinant solution containing 5 specific amino acid co-germinants to induce *C. difficile* spore germination suggests that the combination of amino acids with glycine may augment co-germinant activity (Wheeldon 2010, 2011). In this study, no combination of additional amino acids with glycine was found to enhance the co-germinant activity to initiate *C. difficile* spore germination. In fact, the combination of glycine and arginine or valine was shown to suppress the germination response while aspartic acid with glycine inhibited spore germination completely. Only the combination of glycine with histidine was demonstrated to induce a germination response comparable to glycine as the exclusive *C. difficile* spore co-germinant, suggesting some amino acids are weak co-germinants that may act to inhibit the strong co-germinant activity exhibited by selected amino acids, principally glycine, as proposed by Howerton *et al.* (2011).

Calcium was demonstrated to be an effective co-germinant of *C. difficile* NCTC 11204 spore germination, confirming the important role of calcium in *C. difficile* germination identified by Kochan *et al.* (2017). Combining calcium with other amino acids was not found to enhance the co-germinant activity. The combination of either glycine, valine, arginine or histidine with calcium produced a germination response that was comparable to that observed with calcium as the exclusive co-germinant. It is unclear from these data if calcium is able to interact synergistically with

specific amino acids to enhance germination or if the germination response observed was due to the efficient co-germinant activity of calcium independently (Kochan *et al.*, 2017; Kochan *et al.*, 2018b). The addition of alanine, methionine or serine reduced the extent of the germination response of *C. difficile* spores compared to that of calcium as the exclusive co-germinant. Furthermore, calcium with aspartic acid failed to initiate *C. difficile* spore germination. These findings suggest, similar to the interaction with glycine, a weak or poor amino acid co-germinant is able to inhibit the strong co-germinant activity calcium. This happens in a competitive manner as both amino acids and calcium are believed to induce germination through the activation of the co-germinant receptor, CspA (Shrestha *et al.*, 2019).

Characterisation of the germinants and co-germinant of *C. difficile* spores, as presented in this thesis, demonstrates the varied germination response of *C. difficile* strains to bile salts germinants and amino acid co-germinants, thus adding to the existing literature of reported compounds with the capacity to initiate *C. difficile* spore germination (Sorg and Sonenshein, 2008; Paredes-Sabja *et al.*, 2008; Wheeldon *et al.*, 2011; Howerton *et al.*, 2011; Heeg *et al.*, 2012; Kochan *et al.*, 2017). Taurocholate has been identified consistently as an effective bile salt germinant while glycine and calcium function as potent co-germinants of *C. difficile* spores (Sorg and Sonenshein, 2008; Kochan *et al.*, 2017; Kochan *et al.*, 2018b). The germination response of *C. difficile* spores to reported germinants and co-germinants is reliant upon the *C. difficile* strain examined and the experimental approach taken, including the methodology to prepare and purify *C. difficile* spores. This knowledge has implications for the development and application of a pro-germination strategy to eliminate *C. difficile* spores. Inclusion of specific germinants and co-germinants may fail to trigger the germination of spores of clinical *C. difficile* strains or competitively inhibit the germination activity of strong germinant or co-germinant. Environmental factors are also important for spore germination, the activity of some amino acids as co-germinants is apparent only at 37°C while ensuring the pH of the germination media replicates the host environment, is also essential for

efficient *C. difficile* germination (Shrestha and Sorg, 2017; Paredes-Sabja *et al.*, 2008; Kochan *et al.*, 2018b).

In this research the use of high-resolution imaging to examine germinating *C. difficile* spores demonstrated the spore structure but did not allow the definitive identification of the *C. difficile* spore inner and outer membrane or provide an indication of the location of key regulators of *C. difficile* germination. SEM imaging of *C. difficile* NCTC 11204 spores demonstrated the 'bubble wrap' morphology of the *C. difficile* exosporium, previously reported by other researchers and which remained unaltered after the initiation of spore germination (Rabi *et al.*, 2017; Malyshev and Baillie, 2020; Baloh *et al.*, 2022). TEM imaging of ungerminated *C. difficile* 630 spores was unsuccessful in demonstrating the defined internal structures of the spore. However, examination of TEM images of *C. difficile* 630 spores 5 and 20 minutes post exposure to germinants and co-germinants to trigger germination, permitted the identification of the key structural features of the central spore core, inner membrane, germ cell wall, cortex, outer membrane, spore coat and exosporium of *C. difficile* spores. Spore structures were distinguished based on existing knowledge of bacterial spore structures and previous TEM imaging of *C. difficile* spores (Setlow, 2003; 2007; Permpoonpattana *et al.*, 2011; Pizarro-Guajardo *et al.*, 2016; Rabi *et al.*, 2017; Baloh *et al.*, 2022). The width of the *C. difficile* spore cortex was observed to decrease as a result of the initiation of germination. Spores exposed to germinants and co-germinants for 20 minutes demonstrated a visibly thinner spore cortex compared to those where germination had been initiated in a shorter time period of 5 minutes. This is consistent with the degradation of the spore cortex peptidoglycan during the initial phase of germination and was also identified by Baloh *et al.* (2022) who recorded the thickness of the cortex of *C. difficile* spores was reduced by 67% 5 minutes post the initiation of germination (Burns *et al.*, 2010; Adams *et al.*, 2013; Francis *et al.*, 2015).

As part of the advancement of the 'germinate to exterminate' disinfection strategy to enhance the elimination of *C. difficile* spores, a series of bile salt-derived novel compounds designed to function

as dual germinant and antimicrobial compounds were developed for the incorporation in a polymeric material to create a *C. difficile* sporicidal smart surface (Hird, 2014). Initial screening and testing identified the potential *C. difficile* germination and antimicrobial activity of the compound C109. In this study, the germination activity of C109 was successfully confirmed by loss of spore resistance to ethanol with a 1.2-log reduction in CFU/ml of *C. difficile* after 2 hours exposure to 6.9mM C109. Germination activity was also indirectly shown through membrane integrity staining of *C. difficile* spores. Ungerminated *C. difficile* spores demonstrated limited staining with propidium iodide while C109 treated spores were stained by STYO 9 and excluded propidium iodide, indicating membrane integrity. The efficient antimicrobial activity of C109 against vegetative cells of *C. difficile* was demonstrated independently utilising the quantitative suspension test. C109 demonstrated antimicrobial activity, producing a 6-log reduction in *C. difficile* CFU/ml after 30 minutes exposure. The susceptibility of vegetative cells of *C. difficile* to C109 was also demonstrated by small zones of inhibition in an agar diffusion assay.

The design of the chemical composition of C109 for its incorporation in a polymeric material presented limitations for its solubility in aqueous media. As a consequence, C109, was not compatible for use in the optical density germination assay and was observed to precipitate from solution under certain conditions. The extent of the germination and antimicrobial of C109 therefore may have been underrepresented where the experimental approach required its solubilisation in liquid media.

Accordingly, a series of polymeric formulations incorporating novel bile salt-derived compounds with dual germinant and antimicrobial activity, including C109, were evaluated with the aim of developing a *C. difficile* sporicidal smart surface. Initial polymeric formulations were varied in their constituents and processing conditions to identify factors that influenced the germination activity of *C. difficile* spores. Polymer formulation incorporating bile salt-derived compounds, C109, C114 and C119 demonstrated germination activity but this was variable based on the base polymer

material and processing temperature. During preliminary testing of the prototype polymer formulations, a formulation incorporating C109 demonstrated the greatest germination activity, producing a 2.10-log reduction in *C. difficile* CFU/ml after 2 hours and providing proof of concept that a pro-germination smart surface could successfully germinate *C. difficile* spores.

Further testing of the germination activity of polymer formulations incorporating C109 revealed the inclusion of peroxide initiator was advantageous while efforts to increase the concentration of C109 incorporated into the formulations beyond 3% (w/v) was detrimental to germination activity. Extended thermal processing or at a temperature above 160°C reduced the activity of the polymer formulations to induce *C. difficile* germination. In an effort to enhance the antimicrobial capacity of the polymer formulations, copper nanoparticles and benzalkonium chloride were incorporated in addition to compound C109. Copper nanoparticles had a negative impact on germination activity while polymer formulations incorporating C109 and benzalkonium chloride were found to successfully induce *C. difficile* spore germination, demonstrating a significant 1.48 log-reduction of *C. difficile* CFU/ml at 2 hours. Evaluation of the antimicrobial activity of polymer formulations incorporating C109 established *C. difficile* spores were first germinated and then eliminated, although the achieved reduction of viable spores was not significant. Polymer formulations incorporating C109 and benzalkonium chloride demonstrated statistically significant reductions in the recovery of *C. difficile* spores at all time points tested. This is the first description of the development a pro-germination sporicidal smart surface to target and successfully eliminate *C. difficile* spores. The experimental results obtained demonstrate the successful dual germination and antimicrobial activity of the polymer formulation incorporating C109, although the extent of antimicrobial activity against germinating *C. difficile* spores was limited. This dual activity may extend to other clinically relevant nosocomial pathogens. These research findings add to the existing evidence for the effective application of the 'germinate to exterminate' strategy to eradicate *C. difficile* spores through the inappropriate triggering of germination, rendering germinating spore susceptible to biocides. Further development of the existing polymer

formulations to improve the capacity to germinate and eliminate *C. difficile* spores offers a promising opportunity to generate targeted decontamination of *C. difficile* spores that may provide a novel adjunct to the current chlorine-based disinfectants and terminal cleaning approaches. A functional *C. difficile* sporicidal smart surface deployed on high-touch surfaces in clinical environments would provide passive disinfection of *C. difficile* spores that could reduce transmission of CDI.

The demonstrated germination and antimicrobial activity of C109 with spores of *C. difficile* NCTC 11204, particularly when incorporated into a polymeric material warrants further investigation as a smart surface for the eradication of *C. difficile* spores in the environment. Additional analysis of polymer formulation and processing conditions may improve grafting and stability of a reusable product to maximise germination and antimicrobial activity of polymers incorporating C109, improving the efficacy of *C. difficile* spore elimination. The germination and antimicrobial activity of other novel compounds C114 and C119, in addition to C109 should also be explored in a polymeric smart surface. This should include evaluation with spores of multiple laboratory strains and clinical isolates of *C. difficile* to understand possible variability of the germination and antimicrobial efficacy with *C. difficile* strains. Further testing of the polymeric smart surface should also determine the impact of biological debris on the capacity to germinate and eliminate spores by incorporating a soil load to the testing parameters, reflecting the conditions when patients shed spores of *C. difficile* into the environment. The antimicrobial activity of C109 and novel compounds C114 and C119 with nosocomial microorganisms should also be explored to determine if the smart surface may be effective against other clinically relevant pathogens.

Further work to explore combining the *C. difficile* targeting smart surface and the direct application of a germination solution may increase the capacity to eliminate *C. difficile* spores and overcome some of the limitations of the existing polymer formulations incorporating novel compound C109.

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Appendices

Appendix 1: Synthesis of novel compounds with dual germination and antimicrobial activity.

Synthesis of C109

Compound 2-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride designated C109.

Bile amide synthesis

A mixture of lithocholic acid (2.0 g, 0.005 mol) and N,N-dimethylethyldiamine (3.25 ml, 0.001 mol) was dissolved in toluene (200 ml). The solution was heated at reflux for 24 hours. Analysis by thin layer chromatography (TLC) indicated that reaction had not gone to completion so a further (6.4 ml, 0.003 mol) of N,N-dimethylethylenediamine was added. The solution was refluxed for further 24 hours and water was added to the solution, causing material to precipitate out. The precipitate was collected by vacuum filtration and the product was recrystallized from ethyl acetate to produce a white precipitate.

Quaternization of tertiary amines

The product of the above bile amide synthesis (0.2 g, 0.004 mol) was dissolved in chloroform (10ml). Vinyl benzyl chloride (0.64 ml, 0.004 mol) was added and the solution and stirred at ambient temperature for 76 hours. After this time a precipitate was formed, indicating the reaction has gone to completion. All of the chloroform was then evaporated using a rotary evaporator and the viscous material was dissolved in hexane leaving C109 as a precipitate. The precipitate was collected by vacuum filtration and dried under vacuum. The product formed was an off white solid.

Synthesis of C114

Compound 3-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13 dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride designated C114.

Methyl lithocholate formation

Lithocholic acid (5.0 g, 0.01329 mol) was added to methanol (90 ml) to produce a suspension. Acetyl chloride (0.5 ml, 0.006 mol) was then added. The solution was heated and stirred at 80°C for 40 minutes as a homogenous solution and then allowed to cool overnight in an ice bath. Water (150 ml) was added and the resulting precipitate was collected by filtration, washed with water (6 x 20 ml) and dried under vacuum.

Bile amide synthesis

A mixture of methyl lithocholate (0.5 g 0.001 mol) and 3-dimethylamino-propylamine 93 ml, 0.02 mol) was heated and stirred at 140°C for 24 hours in an argon environment. Analysis by TLC indicated that the starting material has been consumed. Ice water (3 ml) was added to the material and left to stir for two hours at room temperature. The resulting solid was collected by filtration, washed with water (3 x 20 ml) and left to dry overnight under vacuum to produce off brown crystals.

Quaternization of tertiary amines

The product of the above bile amide synthesis (1.0 g, 0.001 mol) was dissolved in dichloromethane (20 ml). Vinyl benzyl chloride (0.89 ml, 0.005 mol) was added and the solution stirred for 76 hours at ambient temperature. After 76 hours TLC indicated starting material was consumed, no precipitate was formed. All of the dichloromethane was then evaporated using rotary evaporator and hexane was added to the viscous solution to form solid. The resulting solid was collected by filtration and washed with petroleum ether 60/80 (3 x 20 ml), dissolved in methanol and washed a

second time with petroleum ether 60/80 (3 x20 ml). The solvent was removed under pressure to produce C114 as an off white solid.

Synthesis of C119

Compound (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17 tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]pentanamide chloride designated C119.

Bile amide synthesis

Lithocholic acid (0.5g, 0.001 mol) was dissolved in tetrahydrofuran (20 ml) with triethylamine (0.20 ml, 0.001 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.20 ml, 0.001 mol) was then added dropwise over a ten-minute period. Once added, the cold water was removed, and the solution was stirred for 2 hours at room temperature. After 2 hours 1-(2-aminoethy) (0.25 ml, 0.002 mol) was added and the solution was stirred for 3 hours. Water (50 ml) was added and the solution was extracted with ethyl acetate (3 x 50 ml). The organic layer was combined and dried over magnesium sulphate. The solvent was evaporated under reduced pressure to produce C119 as an off white powder.

Quaternization of tertiary amines

The product of the above bile amide synthesis (0.5 g, 0.001 mol) was dissolved in dichloromethne (20 ml). Vinyl benzyl chloride (1.74 ml, 0.01 mol) was then added and the solution stirred for 76 hours at ambient temperature. After 76 hours TLC indicated starting material was consumed, no precipitate was formed. All of the dichloromethane was then evaporated using rotary evaporator and hexane was added to the viscous solution to form solid. The resulting solid was collected by filtration and washed with petroleum ether 60/80 (3 x 20 ml), dissolved in methanol and again washed a second time with petroleum ether 60/80 (3 x 20 ml). The solvent was removed under pressure to produce off white solid.

Appendix 2: Presentations and Publication

Poster presentations

27th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria. 22nd -25th April 2017.

C109: a novel bile salt derivative with dual germination and antimicrobial activity against spores of *Clostridium difficile* NCTC 11204

Amelia Lawler, Dan Rathbone, Selma Riasat, Sahar Al-Malaika, Husam Sheena, Tony Worthington.

29th European Congress of Clinical Microbiology and Infectious Diseases, Amsterdam, Netherlands. 13th -16th April 2019.

Development of a novel antimicrobial germination surface to eliminate *Clostridium difficile* spores.

Amelia Lawler, Dan Rathbone, Selma Riasat, Sahar Al-Malaika, Husam Sheena, Tony Worthington

Presentations

Aston University Virtual Postgraduate Research Day, 7th July 2020

The role of *Clostridioides difficile* co-germinants during germination

Publications

Lawler, A. J., Lambert, P. A., Worthington, T. (2020). "A Revised Understanding of *Clostridioides difficile* Spore Germination." *Trends in Microbiology* 28(9): 744-752.

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