Optimising Gut Colonisation Resistance against *Clostridium difficile* Infection

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ABSTRACT

Clostridium difficile is the dominant cause of pseudomembranous colitis in nosocomial environments. *C. difficile* infection (CDI) generally affects elderly (≥ 65) hospital in-patients who have received broad spectrum antimicrobial treatment. CDI has a 30% risk of reinfection and subsequent 60% risk of relapse thereafter, leading to a high economic burden of over 7 billion pounds sterling and over 900,000 cases in the USA and Europe per annum. With the long-term consequences of faecal transplantation currently unknown, and limited spectrum of effective antibiotics, there is an urgent requirement for alternative means of preventing and treating CDI in high risk individuals. Metagenomics has recently improved our understanding of the colonisation resistance barrier and how this could be optimised. pH, oxidation-reduction potentials and short chain fatty acids have been suggested to inhibit *C. difficile* growth and toxin production *in vitro* and *in vivo* studies. This review aims to pull together the evidence in support of a colonisation resistance barrier against CDI.

INTRODUCTION

Clostridium difficile is an obligate anaerobic, heterotrophic, rod-shaped, ‘drumstick’ bacillus. (1 - 2) *C. difficile* is a toxigenic and proteolytic organism that was originally entitled ‘difficile’ due to its difficulty to isolate and cultivate (1). Several refined mechanisms contribute to the ability of *C. difficile* to survive within the environment, transmit and colonise within the host. *C. difficile* colonisation has been attributed to its ability to germinate from a dormant, highly-resistant spore form and to produce toxins (TcdA, TcdB and binary toxin) which has been suggested to hamper the adaptive immune response and influence the surrounding colonic environment (3).

C. DIFFICILE PATHOGENICITY

C. difficile spores have the ability to survive on a wide variety of surfaces and are ethanol resistant (4). These spores are transmitted to the host via the faecal/oral route where they begin to germinate within the small intestinal area into a vegetative cell form (1). Virulence factors such as adherence, achieved by S-layer high molecular weight protein, chemotaxis and motility via flagella (5 - 6) may further aid *C. difficile* colonisation of the colon. CDI is generally observed in elderly individuals with low microbial diversity, and those enduring antimicrobial treatments (1). The colonisation resistance barrier in the normal healthy colon is the result of high microbial diversity, substrate/area competition, immune response modulation and short chain fatty acid production (6 - 7).

Following colonisation of the large intestine, *C. difficile* initiates exponential growth, during which hydrolytic enzymes such as collagenase, chodrointin-4-sulphatase and hyaluronidase are produced, which results in epithelial cell inflammation, cell cytotoxicity and may

stimulate the release of further nutrients (8). At the stationary phase of growth, the production of toxins from *C. difficile,* toxin A (TcdA) and B (TcdB), peak (3). Both are encoded on the pathogenicity locus; TcdA is an enterotoxin which enters the cell through endocytosis and is activated through subsequent acidification, and TcdB is a cytotoxin activated by autocatalytic cleavage within the endolysosomal compartment. Both TcdA and TcdB inactivate the Rho-GTPases which regulate actin production within the epithelial cytoskeleton, which leads to cell rounding, cell shrinking and apoptosis within 24 hours, ultimately resulting in increased permeability and loss of barrier function (3, 5, 9 - 10). Certain strains of *C. difficile,* particularly hyper-virulent ribotypes, produce binary toxin which catalyses glucosylation to induce disorganisation of the actin in the cytoskeleton (10). Hyper-virulent strains of *C. difficile*, such as B1-NAP1-027 and 078 have been identified as toxin overproducers, which may account for their emergence as major pathogens with a mortality rate of 37% (1).

With approximate figures of around 900,000 cases per year, resulting in an annual economic burden of £7 billion in Europe and the USA (11), there is an urgent requirement for effective novel treatments for *C. difficile* infection (CDI). Recent advances have involved characterising and transplanting a "healthy" microbial flora into infected patients in a bid to restore colonisation resistance from apparently healthy subjects. Current preventative measures and treatment recommendations for CDI involve stricter broad spectrum antibiotic stewardship, discontinuation of antibiotic treatment and an arduous regimen of metronidazole, or vancomycin in more severe cases (12). Both antimicrobials are associated with as high as a 35% risk of recurrence or reinfection after initial infection (13). These relatively high recurrence rates suggest a requirement to switch the focus to other treatments that preserve susceptible intestinal bacteria required for a healthy colonic environment (12). This review looks at the current status of *C. difficile* research and the requirement for new, novel treatments and preventative methods. Potential intervention methods that restore the

inter-regulatory mechanisms involved in shaping the colonisation resistance barrier and optimal environment for growth of indigenous bacteria are reviewed to highlight physiologically relevant methods which could be implemented in human interventions.

C. DIFFICILE EPIDEMIOLOGY

The epidemiological profile and severity of CDI has changed significantly over the last decade. An increased incidence of outbreaks caused by closely-related strains was reported in the USA, Canada and Europe with the increased emergence of hypervirulent strains (6, 13 - 18). The rates of CDI vary between countries worldwide. CDI infection rates are lower in some areas of Europe (14 - 15), such as the Netherlands (14), due to the improvement of prevention, antibiotic stewardship, monitoring and reporting methods when dealing with the infection (16). Areas such as Chile have noted an increase and rapid spread of *C. difficile* ribotype NAP1/BI/027, which has accounted for 79% of cases (19). In North America in 2000, however, an increase in the incidence of CDI was noted from 0.68% to 1.2% of hospitalised patients, with 3.2% of these developing life-threatening symptoms (17). Patients in North America who have received antimicrobial treatment and have recently visited hospital, either as an outpatient or otherwise, have high CDI risk; only 20% of all CDI cases are community-associated (17). Queensland has noted an emergence of binary toxin ribotype UK 244, a genetic relative of 027 (20) whilst, within the UK, a decline in the incidence of ribotype 027 has been noted while ribotype 078 has emerged as a prominent cause for concern (21). Significant outbreaks in the UK from 2003-2005 involved the hypervirulent ribotype 027 resulted in an 11-12% mortality rate from both outbreaks (22). Davies *et al.,* (2014) also reported that there are approximately 40,000 cases of CDI that are undiagnosed amongst European inpatients (23). Consequently, under-diagnosis impacts upon monitoring the epidemiology of CDI and successful treatment of the infection (23).

DIAGNOSIS OF *C. DIFFICILE* INFECTION

Diagnostic guidelines dictate that only samples of diarrhoea or unformed stools are to be used for testing, with the exception of ileal fluid that is suspected of CDI (12). Positive diagnosis of CDI is defined as the presence of symptoms, generally diarrhoea and a positive stool sample, with identification of *C. difficile* toxins or toxigenic bacteria with the use of cytotoxin assays, enzyme immunoassay or polymaerase chain reaction (PCR) (12, 24). In other cases, diagnosis can be made by histopathological or colonoscopic findings revealing pseudomembranous colitis (12). The method with the highest sensitivity for the diagnosis of *C. difficile* infection is stool culture (12 - 24). However this is not practical for use as the standard diagnostic method as it has a long turnaround time and is not sufficiently specific due to the possibility of isolating non-toxigenic strains. Stool culture is recommended during epidemiological studies (12) and should be coupled with toxigenic culture to identify a standard for comparison to other clinical testing methods (12, 24).

Enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) are considered insufficient as the sole means of CDI diagnosis due to low sensitivity compared with other methods (12). These assay methods are, however, rapid and inexpensive techniques (6) and thus have been suggested for use if coupled with other more sensitive methods (12). Cytotoxin assays are presently considered to be the gold standard diagnostic methods used (12, 20, 24). This technique should be used in combination with an EIA, which screens for glutamate dehydrogenase, due to cytotoxin assays being time-consuming and having relatively low sensitivity in comparison with stool culture (12).

Genotyping methods have been of particular importance in the rapid detection, with good sensitivity and specificity (6, 12), these methods are increasingly utilised throughout Europe

132 although more data is required before they can be recommended as a routine method for
133 diagnosis (6, 12). Several genotyping methods, such as pulse field gel electrophoresis, widely
134 used in the US (6), and multi-locus variable number tandem repeat analysis (MLVA) has
135 been used in epidemiological studies but is not considered an efficient method of diagnosis
136 (25). MLVA is a widely used method for epidemiology, transmission and genotype studies
137 and has been used to assess toxigenic *C. difficile* isolates within a hospital environment and to
138 assess the role of asymptomatic *C. difficile* carriers in infection transmission (25).
139
140 **TREATMENTS *FOR C. DIFFICILE* INFECTION**

141 The recommended first-line treatment after CDI diagnosis is discontinuation of current
142 antibiotic therapy followed by administration of metronidazole or vancomycin, dependent on
143 infection severity or instance of recurrence (12). CDI bares a 35% risk of re-infection or
144 relapse when treated with metronidazole or vancomycin (13), bringing the effectiveness of
145 these antibiotics into question.

146 Other treatments such as Fidaxomicin, a macrocylic antibiotic which targets the protein
147 sheath (26) and SMT 19969, a heterocyclic non-absorbable agent, which has a high
148 selectivity for *C. difficile* over other members of the microbiota (27), have recently been
149 reviewed and suggested as potential 'new antibiotics' for CDI. Studies have shown that both
150 Fidaxomicin (currently in Phase III development) and SMT 19969 (in Phase II development)
151 are more selective for *C. difficile* than metronidazole and vancomycin and thus would be less
152 damaging to the intestinal flora (26 - 28), but these require further trials and elucidation of
153 cost before they can replace the current antibiotics of choice. As the current treatments for
154 CDI involve further administration of antibiotics, attempts have been made to identify other
155 means of therapy.

Probiotic interventions have been suggested in previous studies, specifically involving the use of Lactobacilli and Bifidobacteria, common bacterial groups that comprise part of the normal microbiota (29). Although some success has been reported, evidence remains inconsistent and no overall therapeutic efficacy can be drawn from basic probiotic treatments consisting of single bacterial strains (30). Restoration of the faecal microbiome has been investigated in the form of faecal transplantation. In the case of recurrent CDI and antibiotic refractory diseases, faecal transplantation of healthy donor faeces has boasted high success rates (31). Single methods of preparation of donor faeces and faecal microbial transplantation administration have demonstrated inconsistent success and it has been suggested that in future faecal transplantations are tailored to the individual patients (30). The full consequences of faecal transplantation of a donor's indigenous flora are not fully understood but may involve risk of infection from opportunistic pathogens harmless to the donor, or the effect of the flora on the recipient's mental wellbeing and potential consequences to weight loss or gain (12, 32).

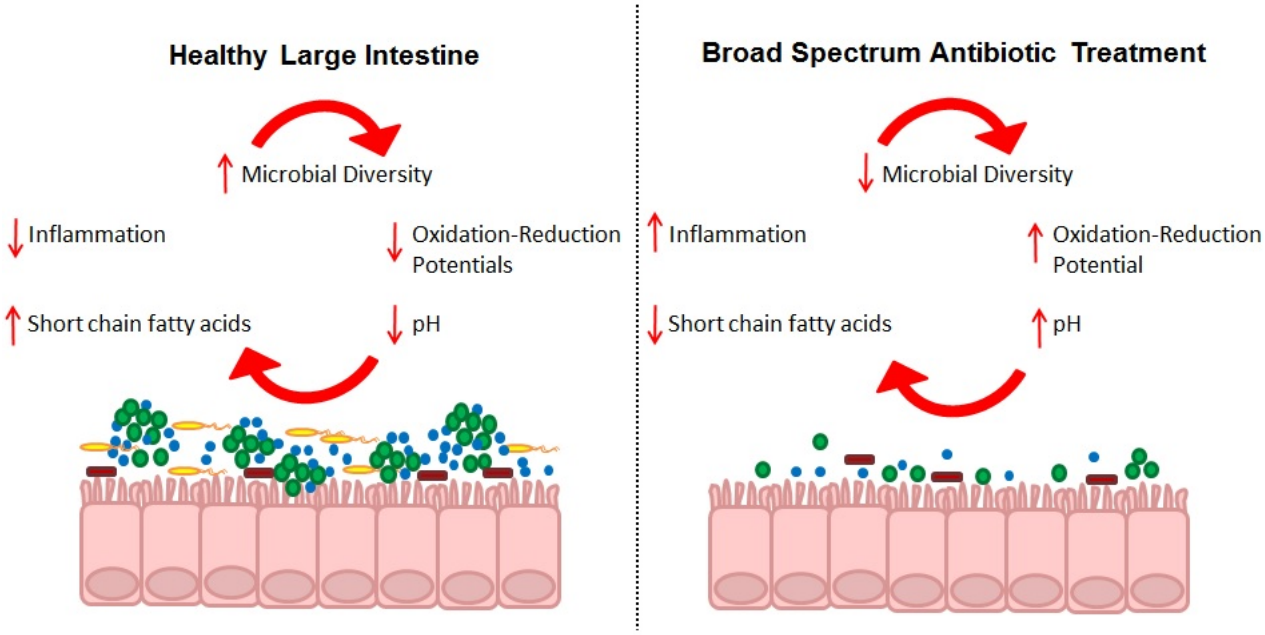
An effective treatment for CDI would prevent or inhibit the growth and/or toxin production of *C. difficile* whilst promoting the re-colonisation of the host's own microbiome. An ideal treatment would not involve arduous administration of antibiotics, would eliminate the challenge for patient of accepting donor faeces and would minimise the risk of potential long term consequences.

THE HEALTHY MICROBIOTA AND THE COLONIC ENVIRONMENT AND THE EFFECT OF BROAD SPECTRUM ANTIBIOTICS

An individual's gut microbiome is possibly unique and its diversity depends on the method of birth, whether breast fed or bottle fed in infancy and the individual's diet and lifestyle thereafter (33). The adult bacterial population of the colon contains approximately 10^{11}-10^{12} bacteria per gram of faecal content (34). Recent advances in metagenomic techniques have improved our understanding of the indigenous flora and the environmental niches which specific bacterial groups occupy (35). Although many members of the diverse microbiome are not yet cultivable, it is understood that there are groups of phylogenetically diverse bacteria which coexist harmoniously within the gut of each individual. The gut microbiome is dominated at the phylum level by Bacteroidetes and Firmicutes, with abundant taxa represented by some of the more dominant genera such as Bacteroides and Prevotella alongside a host of less abundant taxa such as Bifidobacteria and Lactobacilli (36). This microbial ecosystem is known to inter-regulate the intestinal environment alongside environmental mediators such as pH and oxidation-reduction potentials (ORP) and nutrient availability (7). The microbes present within the colon are known to contribute to environmental ORP through H_2S production, and conversely ORP influences bacterial colonisation by controlling areas which bacteria grow in to within a particular niche (37). Microaerophillic bacteria, for instance, will grow alongside epithelial cells whereas more anaerobic bacteria will occupy niches further into the lumen, at a more negative ORP (37). ORP is also influenced by a series of redox couples; the glutathione and thiol redox couple (GSSG/GSH), the cysteine redox couple (CyS/CySS) and thioredoxin (Trx) (38). Inflammation is likely to increase ORP due to the mucosal immune response and efflux of O_2 into the epithelial environment (37) (Figure 1).

The microbiota also produce the short chain fatty acids (SCFA) acetate, propionate, and butyrate as the main fermentation products (7). They perform a variety of important roles within the gut including resistance to pathogens (7). SCFA along with lactate are the major drivers towards acidic pH in the colon.. The pH within the intestine controls bacterial survival via disruption of the cell or provision of a niche. The pH of the colon can range between 5 – 7 dependent on whether the individual is fasted or fed and also on the amount of dietary fibre consumed in their diet and some gut microbes are sensitive to pH within the physiological range of the intestine (39).

Broad spectrum antibiotics such as cephalosporins, ciprofloxacin, clindamycin, amoxicillin and clavulanic acid have been found to decrease Bifidobacterium spp., Enterobacteriacea, Lactobacilli and Bacteroidetes as well as other gram positive anaerobes (40). Any disruption to the fragile balance between bacteria and environment via introduction of antibiotics decreases the diversity of bacteria and therefore is likely to adversely affect the colonisation resistance barrier as shown in Figure 1. In a study on the effect of neomycin on the luminal pH of rabbits, a significant increase was observed in pH from 6.07 to 6.66 (41). In elderly patients, with already diminished microbial diversity due to decreased bowel motility, nutrient production and constipation (42), and prescribed broad spectrum antimicrobial treatments, the collapse of the colonisation resistance barrier makes way for opportunistic pathogens such as *C. difficile* (1). Understanding the mechanisms that regulate *C. difficile* colonisation in the high risk cohort would allow for the development of targeted therapies.



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226 **Figure 1: The proposed effect of antimicrobials on the colonisation resistance barrier. As the microbial**
227 **diversity drops short chain fatty acid concentrations may also decrease in the colon which would cause a**
228 **rise in pH (43). Dysbiosis also leads to a mucosal immune response and subsequent inflammation which**
229 **increases ORP (37).**

230

231 **PROSPECTIVE AVENUES FOR OPTIMISATION OF THE COLONISATION**
232 **RESISTANCE BARRIER AND MODES OF INTERVENTION**

233 Recent metagenomic techniques have allowed greater insight into the gut microbiome and
234 have advanced our understanding of the role of the microbiome in human health (35).
235 Probiotics such as *Bifidobacterium longum*, *Lactobacillus acidophilus* and *Bacteroides*
236 *fragilis* may serve to inter-regulate the environment of the colon and loss of diversity.
237 Bifidobacteria and Lactobacilli have been linked to suppression of opportunistic pathogens
238 such as *C. difficile* (1, 29). Restoration of lost bacterial quantity and diversity through FMT
239 has also shown great promise as a technique for restoring the colonisation resistance barrier
240 and preventing *C. difficile* recurrence (31).

241 *In vitro*, pH and SCFA regulate *C. difficile* where decreased pH and physiological
242 concentrations of butyrate are inhibitory to *C. difficile* growth (7, 43). However, few clinical
243 studies exist where a systematic approach to restoring the colonisation resistance barrier has
244 been undertaken. Vegetative cells are sensitive to SCFA, most notably butyrate, at
245 physiological concentrations of approximately 160 mM (43). Acetate also produced a
246 protective epithelial cell response in a murine model from *Escherichia coli* 0157:H7 (44)
247 Vegetative cells are sensitive to acidic pH and fully germinate by the time they reach the
248 colon. Evidence suggests that the physiological pH within the colon can drop as low as 5
249 (39), which is shown to be well within the inhibitory range for *C. difficile* vegetative cells (7,
250 43), thus indicating a potential route for intervention by manipulating the pH of the intestine.
251 Fermentable fibres, such as inulin, act as substrates for the colonic microbiota and can
252 selectively promote the growth of probiotic bacteria (43). Fermentation of dietary fibre
253 results in a decrease in luminal pH through increased SCFA production, both of which inhibit
254 *C. difficile* growth (7, 43 - 44). Increased SCFA production is also associated with an
255 improved innate immune response, reducing inflammation and alleviation of symptoms in
256 IBD patients (7, 45 - 46). There is also the potential to introduce physiological concentrations
257 of SCFA directly into the large intestine, through direct instillation (although realistically
258 limited to the distal colon in patients), through encapsulation strategies or targeted delivery.

259

260 Oxidation-reduction potentials have also been found in to impact on toxin production of *C.*
261 *difficile* (47). Altering the ORP from -360 mV to +100 mV was found to increase toxin
262 production 100-fold, which has significant relevance to understanding the mechanisms which
263 promote increased toxin production within the colon of a CDI patient (47). Therefore, if ORP
264 can be modulated by GSH supplementation, this presents a further method for optimising the
265 colonisation resistance barrier against *C. difficile* colonisation (38, 48). *C. difficile* growth and

toxin production has been shown to be affected by pH, SCFA and ORP within physiological ranges. Manipulation of the colonic environment has been suggested as a clinical avenue for prevention and treatment, but it has never been systematically investigated (7). If an optimum inhibitory range for ORP, pH and SCFA can be established *in vitro*, then a strategy for translating this to the human intestine (the colonisation resistance barrier) could be explored.

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CONCLUSION

C. difficile infection is a worldwide problem, and although there have been advances in treatments, it still remains a significant economic burden which cannot be ignored. There is rationale for further understanding and taking advantage of the effect of the colonisation resistance barrier within the colon. Re-establishing normal physiological parameters in the colon, harmless to indigenous flora and colonisation but inhibitory to either *C. difficile* growth or production of virulence factors would prove a useful tool in the battle against CDI as a worldwide burden. Dietary and targeted delivery approaches that manipulate the colonisation resistance barrier are worthy of further investigation because they represent a low risk option for treatment but more importantly may offer solution in prevention of CDI.

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