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Clostridium difficile Infection: Pathogenesis, Diagnosis and Treatment

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Abstract

Clostridium difficile is a Gram-positive bacterium with the capacity of spore generation. The *C. difficile* infections, related to antibiotic treatment, have increased in number and severity during the last few years; increasing the health problems caused by this bacterium. One of the most important problems of the *C. difficile* infection is the recurrence. Due to all of these facts, researchers have been searching for new treatments such as faecal microbiota transplantation or bacteriocins development.

Keywords: clostridium, clinical, pathogenesis, persistence, resistance, treatment

1. Introduction

Clostridium difficile is a Gram-positive, spore-forming anaerobic bacterium discovered in 1935 by Hall and O'Toole [1]. In 1978, Barlett et al. identified *C. difficile* as an important cause of pseudomembranous colitis (PMC) associated with antibiotic use [2]. The manifestations of *C. difficile* infection (CDI) range from asymptomatic carriage to fulminant disease. Nonetheless, the commonest manifestations are diarrhoea and PMC [3]. One of the most serious problems associated with CDI is recurrence of the disease. *Clostridium difficile* infection can be acquired by person to person transmission, especially by the faecal-oral route, and it can also be acquired by environmental contamination [4]. *Clostridium difficile* is widely distributed in the soil and in the intestinal tracts of animals, both of which are considered as reservoirs of the bacterium [3].

Historically, CDI was not considered a severe disease. However, the number of cases and the severity of these have increased in the last 20 years [5]. One of the reasons for the increase in the incidence of CDI is that *C. difficile* produces spores that are capable of resisting heat, desiccation, and chemical agents. The appearance of *C. difficile* in the hospital environment has become problematical.

The incidence of CDI in hospitals depends on the type of unit, and the rates are highest in haematology, gastroenterology and nephrology units. The incidence also depends on the country considered, and within Europe, the rates are highest in Finland and Poland and lowest in Turkey, Bulgaria and in East European countries [6].

2. Clinical features

The most common symptom of CDI is watery (not bloody) diarrhoea accompanied by abdominal pain. Doctors should suspect CDI when the patient has three unformed or watery stools daily for 1 or 2 days. In case of more severe symptoms, the patient may present with fever, shock or hypotension and severe ileus with cessation of diarrhoea. The most severe symptoms are leucocytosis and elevated serum creatinine levels [7]. CDI may also lead to complications such as dehydration, electrolyte disturbance, hypoalbuminemia, toxic megacolon, bowel perforation, hypotension, renal failure, systemic inflammatory response syndrome, sepsis and death [4].

Colitis can affect any part of the colon but is commonly severest in the distal colon and rectum. Patients with CDI in these locations always present fever, abdominal pain, leucocytosis and a decrease in intestinal motility [3].

The most important risk factor for CDI is age, although the duration of hospitalization is also important, along with exposure to anti-microbial agents. Olson et al. showed that 96% of CDI cases had been exposed to anti-microbials about 14 days before the manifestation of diarrhoea, and all patients had received anti-microbial treatment about 3 months before [8]. Other investigators extend the time of influence of antibiotic treatment to 12 months and also include administration of proton pump inhibitors as a risk factor. Interestingly, the presence of diabetes mellitus has been associated with a decreased risk of CDI [6].

3. Pathogenic factors

3.1. Toxins

Clostridium difficile can produce three toxins: A, B and binary toxin. Toxins A and B were the first identified in this bacterium; both are encoded by genes in the pathogenicity locus (PaLoc) and are included in the large clostridial toxins (LCT), a family known to modify small GTPases [9]. These toxins act as glycosyltransferases that modify Rho and Ras proteins within the intestinal epithelial cells and disrupt the actin cytoskeleton, causing loss of intercellular junctions and the severe secretory diarrhoea associated with CDI [10, 11]. Hundsberger and

collaborators reported that two of the LCT genes, *tcdD* and *tcdC*, act as positive and negative regulators, respectively. These researchers have classified the *tcd* genes in two groups, one comprising A, B, D and E, and the other comprising C (as *tcdC* has the opposite orientation). It has also been found that *tcdC* is expressed in the exponential growth phase of the bacterium, while the other genes are expressed in the stationary phase [12, 13].

Some 10% of *C. difficile* strains are capable of producing binary toxin. This toxin is classified as an ADP-ribosyltransferase and is encoded by the *cdtA* gene (the enzymatic component) and the *cdtB* gene (the binding component) [3, 9]. Binary toxin acts on the actin cytoskeleton, producing microtubule-based protrusions on the surface of epithelial cells [5]. A number of studies have indicated that strains that produce binary toxins usually cause severe CDI [14]. Geric et al. used a rabbit ileal loop model to investigate the binary toxin and concluded that the toxin contributes significantly to eliciting a non-haemorrhagic fluid response [15]. A higher mortality rate was observed in patients infected with strains that produce all three toxins [5].

Regulation of toxins A and B has been widely studied for many years, and it is known that the *tcdR* regulator gene, present in the PaLoc, activates *tcdA* and *tcdB* transcriptionally and also activates its own two promoters [16]. The four upstream genes of the PaLoc (*tcdA, B, E, R*) can be co-transcribed by the *tcdR* promoter, and each of the toxin genes has its own promoters. A global regulator of gene expression commonly found in low G+C Gram-positive bacteria, known as *CodY*, has been identified in *C. difficile*. Inactivation of *CodY* in a *C. difficile* strain has led to expression of the PaLoc genes during both exponential growth of the bacterium and in the stationary phase, demonstrating that *CodY* regulates toxin production. *CodY* has been shown to bind to the *tcdR* promoter with high affinity, especially when GTP and branched-chain amino acids are present; *CodY* also binds to toxin gene promoters, but with low affinity, which suggests that the primary regulation affects *tcdR* (**Figure 1**). *CodY* works by repressing
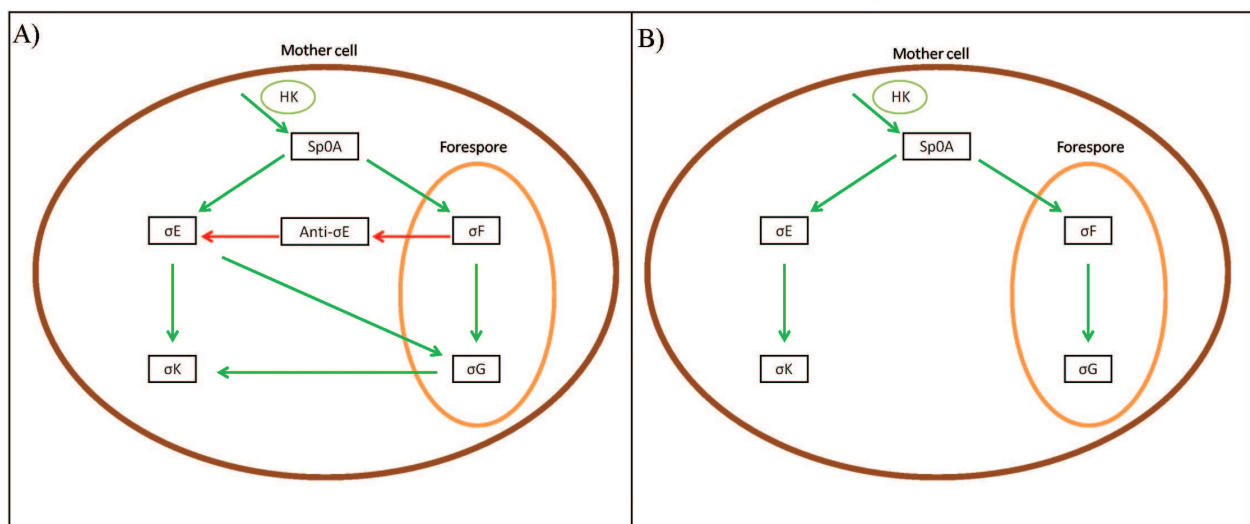
Figure 1. Regulation of sigma factors: (a) Regulation of the sigma factors in *Bacillus subtilis*, which has a criss-cross regulation between mother cell (where express factor E and K) and the forespore (where factors F and G have their function); factor F inhibits anti-σE factor which inhibits factor E. (b) Regulation of the sigma factors in *Clostridium difficile*, with its forespore inside the mother cell (like *Bacillus subtilis*).

some genes when nutrients are sufficient and de-repressing those genes when nutrients are limited. Researchers have discovered that *CodY* not only represses toxin genes but also represses *tcdC*; it is not known why *CodY* represses both toxins and its antagonist. It has been suggested that in the intestinal tract, where nutrients are not abundant, *CodY* may relax its repression of toxin genes so that the toxins will lyse epithelial cells in the intestinal tract, thus releasing nutrients [17].

Toxin production is affected both by the bacterial growth phase and by environmental factors. Researchers observed that the presence of glucose in the medium inhibits toxins, which implies catabolic repression. In addition, toxin production in laboratory cultures has also been found to be affected by the presence of biotin and some amino acids (cysteine and proline) and by environmental stress [16].

3.2. Persistence of spores

Spore production in bacteria is a mechanism of persistence, as it confers resistance to antibiotics and to the host immune system. Akerlund and colleagues observed an inverse relationship between toxin production and spore counts, which suggests that premature sporulation in the stationary phase shortens the time required for toxin production [18]. Merrigan et al. observed that some hypervirulent strains underwent early sporulation and produced large amounts of toxin, with greater efficiency than other strains. These researchers concluded that sporulation could contribute to the dissemination of infectious particles in the environment, thus helping toxins to confer adaptive advantages in the pathogenesis of hypervirulent strains of *C. difficile* [13].

The sporulation process has been widely studied in *Bacillus subtilis*. The sporulation decision in the genus Bacillus is regulated by some orphan histidine kinases whose function is to phosphorylate the master transcriptional regulator Spo0A [19]. Underwood et al. discovered that *C. difficile* also has five orphan histidine kinases and that inactivation of these significantly reduces spore formation related to wild-type. However, the Spo0A phosphorylation mechanism remains unclear [20].

Once a strain of *B. subtilis* has committed to sporulation (i.e. Spo0A has been phosphorylated), a cascade of activation of RNA polymerase sporulation-specific sigma factor occurs [21]. Recent genomic studies of *C. difficile* have shown that this bacterium does not have the characteristic criss-cross regulation of *B. subtilis* [22]. It is known that *B. subtilis* has four sigma factors (E, F, G and K) and that the active factor F has the capacity of inactivate anti-σ^E factor, thus enabling activation of factor E. Once factor E is activated, it can activate factor G, while at the same time factor G activates factor K. In addition, factor E is necessary for activation of factor K, while factor F is required for the activation of factor G. However, unlike *B. subtilis*, *C. difficile* uses factor F to activate post-translational factor G, and factor E activate factor F; but factor E is not necessary for activation of factor G, and factor G is also not necessary for activation factor K, as proteolytic activation is not useful in factor K [22]. The mechanism of regulation of all these factors is not known and is currently under study. However, Pereira et al. observed that as in *B. subtilis*, the activity of factors F and G in *C. difficile* is focused on forespores and that of factors E and K on the mother cell (**Figure 2**) [23].
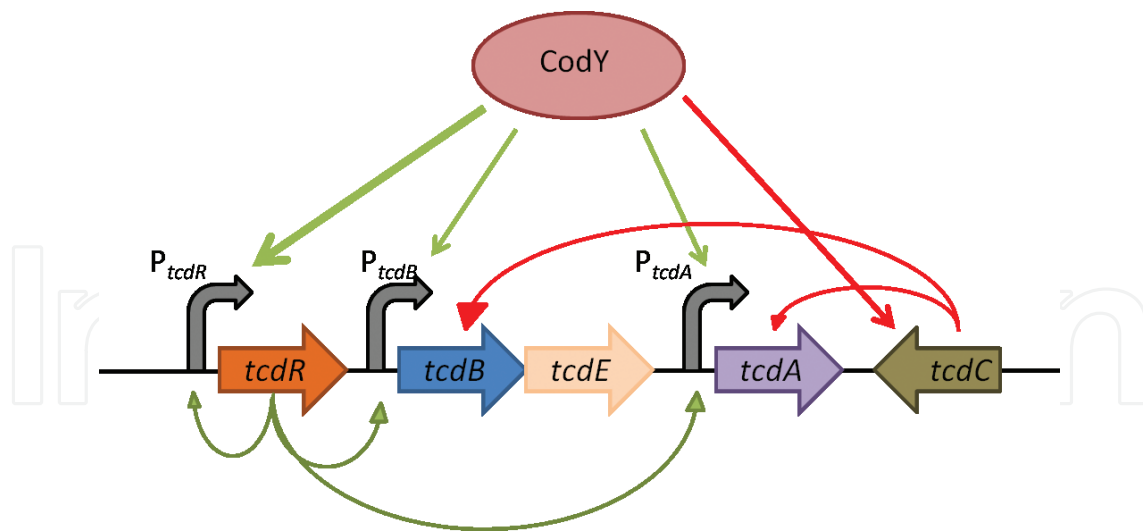
Figure 2. Regulation of toxins A and B in the PaLoc cluster. *tcdR* actives the promoters of *tcdB* and *tcdA*, meanwhile *tcdC* inhibits *tcdB* and *tcdA* genes; *tcdC* is also inhibit by CodY, till it active the tree promoters of the cluster.

Studies of the spore surface have identified surface receptors that interact with intestinal epithelial cells. *In vitro* experiments have shown that spores can become cytotoxic to macrophages and that spores disrupt the phagosomal membrane with the aid of their surface receptors [24].

Bacteria usually germinate when specific germinant receptors detect specific small molecules in the environment. However, *C. difficile* germinates after detecting some bile salts and L-glycine in the environment [25]. It is thought that CspC, a serine protease involved in germination in Clostridium perfringens, is a bile salt specific germinant receptor. It is known that CspC is required for Ca-DPA activation in spore germination in response to glycine and taurocholate [26].

Growth of *C. difficile* vegetative cells has been shown to be inhibited by cleaning agents and germicides, although the bacterium is not killed and undergoes sporulation. Spore formation makes it difficult to eliminate *C. difficile* and is one of the most common problems in the hospital environment. Several researchers are therefore investigating the use of cleaning agents and germicides to eliminate *C. difficile*. Fawley et al. found that neutral and hydrogen peroxide detergents do not reduce germination of spores, whereas chlorine-containing agents do reduce the rates of germination [27]. Destruction of *C. difficile* spores is very important, especially in healthcare environments, because if not destroyed, spores tend to accumulate and thus represent a potential health risk. Vapourized hydrogen peroxide has been shown to have an important sporicidal effect [28].

3.3. Resistance

Antibiotic resistance is a huge problem nowadays, especially with the appearance of new *C. difficile* ribotypes. The most dangerous of these is RT027, which is associated with excessive

use of fluoroquinolones (FQs). The findings of the numerous studies concerning resistance of *C. difficile* have shown that resistance to erythromycin, fluoroquinolones and ciprofloxacin is very common in clinical strains. *C. difficile* also generally displays resistance to second-generation cephalosporins but shows less resistance to third-generation cephalosporins. However, resistance to moxifloxacin and gatifloxacin has been detected in 34% of strains analysed [29]. Tenover and colleagues analysed the resistances of different ribotypes to clindamycin, metronidazole, moxifloxacin and rifampin. They observed that resistance rates are changed between strain types as well as in Europe and Far East [30].

Clostridium difficile has a number of putative β-lactamase genes that are probably involved in resistance to β-lactam antibiotics [29]. A ribosomal methylation mechanism related to macrolide resistance in *C. difficile* has been described (Ref). Erythromycin ribosomal methylases (ERM) confer resistance to macrolides, and some of these genes have been described in *C. difficile* [31]. These genes are widespread in clinical strains of *C. difficile*, despite the fitness cost associated with maintaining them.

Alterations in the quinolone-resistance determining region (QRDR), which confers resistance to fluoroquinolones, have been identified in *C. difficile* [32]. Resistance of *C. difficile* to tetracycline varies in different countries, ranging from 2.4 to 41.67% [32]. Fry et al. reported that although the tetracycline resistance gene *tetM* is predominant in *C. difficile*, others such as *tetW* are found in human and animal strains [33]. The presence of other mobile elements in the genome involved in resistance to tetracycline is also possible [33]. Freeman and colleagues investigated chloramphenicol resistance in clinical isolates of *C. difficile* and attributed it to the presence of *catD* gene, which encodes a chloramphenicol acetyltransferase that can be found in transposons [34].

3.4. Hypervirulent strains

Some hypervirulent strains of *C. difficile* have appeared in recent years, representing a huge health problem. All strains of *C. difficile* can be classified into 150 ribotypes and 24 toxinotypes. The toxinotypes are classified on the basis of different poly-morphisms in the PaLoc. All members of toxinotype III, to which ribotypes 027, 034, 075, 080 belong, produce binary toxin [3].

Merrigan and collaborators have shown that early sporulation in hypervirulent strains enables accumulation of more spores than in non-hypervirulent strains and thus explains the incidence of recurrent infection associated with hypervirulent strains [13]. Furthermore, the high rate of CDI has been related to the higher rate of toxin production in these strains than in non-hypervirulent strains. Yakob et al. made an epidemiological model of *C. difficile* transmission, in this model they could observe that hypervirulent strains seem to be more infectious, more likely to become established, extend faster and have a higher presence in the community, thus displacing endemic strains. These statements are based on the fact that in the last 15 years hypervirulent strains (especially ribotype 027) not only have appeared but also have become in the dominant strain worldwide [35].

Ribotype 027 is one of the most dangerous and best studied strains of *C. difficile*. This strain produces more A and B toxins than 'normal' strains and also produces binary toxin. Although ribotype 027 was first identified in Canada, it has extended throughout the world and is now endemic in the United States [7, 36]. The virulence of *C.difficile* ribotype 027 has been suggested to be due to delection in position 117 of the *tcdC* gene which produces an increase in toxin production and enables binary toxin production [37]. This strain is commonly associated with severe cases of CDI and high rates of recurrence, with elevated mortality.

Another hypervirulent strain has recently become problematical in Europe, especially in healthcare environments. Ribotype 078, which has been identified in many cases of CDI throughout Europe, shows similar hyperproduction of toxins as in ribotype 027 [37].

3.5. Diagnosis

CDI can be diagnosed by detection of genes or products or by bacterial culture. Culture of *C. difficile* takes at least 4 days to be detected on *C. difficile* plates. Culture strains are useful for typifying *C. difficile* and allow to store it for future research.

Different tests can be used to detect products. The cell cytotoxicity assay (CCA) is the gold standard assay for detecting CDI. There are also other more sensitive methods, such as the toxigenic culture method. This method involves culturing *C. difficile* in selective media and subsequent demonstration of toxin production by ELISA or CCA. However, the most commonly used test is detection of *C. difficile* toxins by enzyme immunoassay (EIA), either directly or with glutamate dehydrogenase antigen. This assay displays sensitivity of 63–94% and specificity of 75–100%. There is another EIA test against a common *C. difficile* antigen, glutamate dehydrogenase (GDH), which can also be used. This test displays 58–68% sensitivity and 94–98% specificity. Although this method is not sufficiently sensitive for routine laboratory use, it is useful for epidemiological research [4].

Real-time PCR is currently the fastest available test for CDI and is usually used to detect genes regulating synthesis of toxins A and B. The tests used must be capable of distinguishing between colonization and disease [3].

CDI can also be diagnosed by direct visualization, especially in cases of PMC. Nonetheless, direct visualization only detects CDI in 51–55% of cases and laboratory tests must be conducted to confirm the diagnosis [4].

4. Classical treatments

Treatment of CDI depends on whether the disease is classified as first episode, recurrent, or severe or complicated CDI. The treatment of choice for first episode CDI is usually metronidazole. However, other agents such as rifaximin and teicoplanin can be used. Rifaximin, a non-absorbable oral antibiotic, is effective against first and recurrent episodes of CDI. Teicoplanin, which is similar to vancomycin, is not approved for use in the US [10].

Administration of vancomycin is recommended in cases of recurrent CDI. Metronidazole is not used in the recurrent episodes or as long-term therapy because of its potential neurotoxicity. Fidaxomicin, a novel macrocyclic antibiotic, has appeared in recent years and doctors are being considered it as a substitute for vancomycin [4, 10].

For patients with severe or complicated CDI, vancomycin can be administered into the colon, or metronidazole can be administered intravenously. However, oral or rectal administration of vancomycin is recommended. In the most serious cases, or when there is no antibody response to antibodies, colectomy may be the best solution to save patients' lives. The surgery is only performed on patients with megacolon, colonic perforation, acute abdomen or septic-shock [4]. An alternative to colectomy is diverting loop ileostomy followed by an intraoperative lavage with 8 L of poly-ethylene glycol and 500 mg vancomycin every 8 hours [7].

5. New treatments

5.1. Monoclonal antibodies

Some researchers have tested the efficacy of intravenous monoclonal antibodies in preventing recurrence of CDI. Patients who were first treated with metronidazole or vancomycin received treatment with antibodies against toxin A (CDA1) and toxin B (CDB1) in a double-blind experiment. The results of the study showed that relative to control patients, those receiving the antibody treatment showed a lower rate of recurrence of CDI in the 12 weeks of the study [7].

5.2. Faecal microbiota transplantation (FMT)

This method consists of transferring a suspension of faecal matter from a healthy donor to a patient with the aim of recovering the 'normal' microbiota. The donor material is introduced into the patient via rectal enema, a nasoduodenal tube or colonoscopy. An FMT is recommended after a third CDI episode [38]. Kelly et al. conducted a randomized trial, in which they selected adults with three or more documented CDI recurrences. The faecal microbiota from patients and donors was analysed 5 days before and some weeks after the treatment. The first analysis revealed that the patients had more gammaproteobacteria and betaproteobacteria and fewer firmicutes and Bacteroidetes than donors. They concluded that administration of fresh FMT from a donor via colonoscopy to patients who were first administered a course of vancomycin was successful in preventing further CDI episodes. The researchers also observed that the efficacy of the treatment varied depending on the part of the intestine where the infection occurred, and therefore some patients may not benefit from FMT [39].

Although this treatment has been shown to be successful in some cases, some adverse events also occurred due to the impracticability of screening all possible pathogens carried by the donor. The long-term consequences of the treatment are also unpredictable. The US Food and Drug Administration (FDA) has classified FMT as a drug and biological product, so that the procedure is subjected to the same regulations as traditional pharmaceutical drugs [38].

5.3. Spore formulation

This treatment is similar to FMT, although in this case, a specific number of strains compete with *C. difficile* [7]. Khanna et al. produced a mixture of spores of 50 species of Firmicutes obtained from healthy donors. After some success in preclinical studies, the mixture, known as ser-109, was formulated for oral administration to patients between 18 and 90 years with recurrent CDI. The researchers observed a reduction at recurrence of the CDI of 87.7% over 8 weeks of treatment [40]. Gerding et al. recently began a phase 2 trial of administration of spores from a single non-toxigenic *C. difficile*, in patients who had responded to antibiotics in the first episode or the first recurrence [41]. Positive results of the studies support the use of spores as a treatment against recurrent CDI, although further detailed studies are required.

5.4. Whole genome sequencing (WGS)

Whole genome sequencing (WGS) provides high-resolution data, enabling researchers to identify the strains of *C. difficile* isolated from patients and thus to distinguish between reinfection and relapse and to help to understand the complex transmission epidemiology of CDI. WGS can also be used to construct a transmission map. Researchers have used WGS and other similar techniques to elucidate CDI transmission events based on culture of isolates from patients with CDI [42].

WGS has been used to map the transmission of strains through patients and across countries [43]. For example, various genetically closely related strains have been identified in Australia. The researchers hypothesized that an animal vector is the cause of this expansion. An Australian strain has been identified in England, in a patient who had was previously visited Australia [44].

A new sequencing method known as Oxford Nanopore's MinION sequencer (http://www. nanoporetech.com/) has been shown to be potentially useful for CDI fast diagnosis [7].

5.5. Microbiome-wide association studies (MWAS)

MWAS are carried out to help in understanding the interactions between bacteria within their communities and to discover 'which produce infections and why' [45]. Koenigsknecht et al. showed that the microbiome modifies bile-acid metabolite profiles during establishment of *C. difficile* in mice [46]. The study involved the use of metagenomics, metatranscriptomics, metaproteomics and metabolomics to determine how microbiota helps the host fight against CDI. Clostridium scindens was identified by MWAS as a candidate for fighting against *C. difficile* [45]. Allegretti et al. found that some bile acids are involved in resistance to *C. difficile* during treatment with antibiotics [47]. MWAS is proved useful in predicting responses to treatment or the development of disease [45].

5.6. Probiotics

Probiotics are defined as live microorganisms, which, when administered in adequate amounts, confer a health benefit to the host [48]. Probiotics have three modes of action: (i) modulation of

host defences; (ii) effects on other microorganisms. Probiotic bacteria adhere to epithelial cells, which block adherence of pathogens. Thus, if pathogens cannot adhere to epithelium they cannot invade the cells and (iii) effects on microbial products such as toxins or host products. Some microorganisms can inhibit toxin production by producing other toxins. For example, the presence of *Saccharomyces boulardii* provides some protection against *C. difficile* toxin A [49].

Tung et al. conducted a review of the use of S. boulardii to treat diarrhoea. These researchers showed that *S. boulardii* plays an important role in preventing both primary and recurrent CDI [50]. They conducted an observational study of the efficacy of a probiotic mixture (containing Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus rhamnosus); the probiotic was administered about 2–12 hours after antibiotic for 30 days or until the end of the treatment. A reduction of 39% of CDI cases was observed over the following 10 years [51].

Bioengineering of microorganisms to target specific pathogens has gained popularity in recent years [38]. Up to date, no research has been carried out in this field in relation to *C. difficile*.

5.7. Small molecule inhibitors

Many small molecules in the human body are capable of interfering with cellular processes by inhibiting or enhancing them. Researchers have found that both types of *C. difficile* toxins have a putative binding domain, which is a cysteine protease domain (CPD) and a glucosyltransferase domain (GTD). When bacteria find IP6 (1D-myo-inositol hexakisphosphate), the CPD activates GTD and produces toxicity [52].

In a study carried out with the aim of finding an inhibitor of TcdB CPD activator, Bender and colleagues discovered 44 inhibitors, the most promising of which was ebselen (phase 2 clinical trials). Ebselen is a synthetic low weight compound that is capable of reducing oxidative stress. Ebselen has been shown to inhibit CPD by blocking binding to IP6, which implies inhibition of toxic effects of TcdB *in vivo* [53]. Both *TcdA* and *TcdB* are regulated by thiolactone molecule, so that if the inhibitor of thiolactone can be identified, it should be possible to create a non-antibiotic treatment for CDI [54].

5.8. Bacteriocins

Bacteriocins are anti-microbial peptides produced by bacteria. Although a number of bacteriocins against *C. difficile* have been identified, three in particular appear to be the most effective: lacticin 3147, nisin and thuricin D.

Lacticin 3147 is a two component antibiotic produced by Lactobacillus lactis [55]. This compound is active at physiological pH, unlike nisin. Rea et al. demonstrated that lacticin can clear a broth of *C. difficile* when added during exponential growth of the bacterium. They also demonstrated that lacticin does not affect non-spore-forming Gram-negative bacteria, but it can reduce the presence of Gram-positive bacteria such as enterococci, lactobacilli and bifidobacteria. These researchers also showed that lacticin can kill *C. difficile* in a model faecal environment. As lacticin cannot resist gastric transit, administration via enema is recommended [56].

Thuricin CD is a two component agent belonging to the sactibiotic subclass of bacteriocins. It is produced by Bacillus thuringiensis and has been demonstrated to be more effective than vancomycin against *C. difficile*. Thuricin CD displays a potent activity against *C. difficile* and its activity against ribotype 027 has been highlighted. The capacity of thuricin CD to effectively kill *C. difficile* in a model of the distal human colon has been demonstrated [57]. However, like other bacteriocins, thuricin CD may not survive gastric transit, especially because one of its compounds is particularly susceptible to degradation. However, Rea et al. have demonstrated that thuricin CD administrated via the rectal route was effective in reducing CDI symptoms [58].

Nisin is a polypeptide of 34 aminoacid residues produced by Lactococcus lactis subspecies and inhibits a wide range of pathogens. Unlike lacticin and thuricin CD, nisin is classified as GRAS (generally recognized as safe) and can therefore be used as a food additive. Lay et al. determined the MICs of nisin A and showed that this bacteriocin is at least as effective against *C. difficile* as vancomycin. These researchers also demonstrated that nisin can inhibit the growth of *C. difficile* after germination, but that is not able to inhibit spores [59].

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